Visualization of specific DNA sequences in living mouse embryonic stem cells with a programmable fluorescent CRISPR/Cas system

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Abbreviations: CRISPR, Clustered Regulatory Interspaced Short Palindromic Repeats; Cas, CRISPR-associated; eGFP, enhanced-green fluorescent protein; 3D-FISH, 3D-fluorescent in situ hybridization; gRNA, guide RNA; bp, base pair; MaS, major satellite repeats; MiS, minor satellite repeats; CC, chromocenter; 3D-SIM, 3D-structured illumination microscopy

Labeling and tracing of specific sequences in living cells has been a major challenge in studying the spatiotemporal dynamics of native chromatin. Here we repurposed the prokaryotic CRISPR/Cas adaptive immunity system to specifically detect endogenous genomic loci in mouse embryonic stem cells. We constructed a catalytically inactive version of the Cas9 endonuclease, fused it with eGFP (dCas9-eGFP) and co-expressed small guide RNAs (gRNAs) to target pericentric, centric, and telomeric repeats, which are enriched in distinct nuclear structures. With major satellite specific gRNAs we obtained a characteristic chromocenter (CC) pattern, while gRNAs targeting minor satellites and telomeres highlighted smaller foci coinciding with centromere protein B (CENP-B) and telomeric repeat-binding factor 2 (TRF2), respectively. DNA sequence specific labeling by gRNA/dCas9-eGFP complexes was directly shown with 3D-fluorescent in situ hybridization (3D-FISH). Structured illumination microscopy (3D-SIM) of gRNA/dCas9-eGFP expressing cells revealed chromatin ultrastructures and demonstrated the potential of this approach for chromatin conformation studies by super resolution microscopy. This programmable dCas9 labeling system opens new perspectives to study functional nuclear architecture.

Introduction

Almost one hundred and fifty years since the original description of chromosomes, many genomes have been fully sequenced. While our knowledge of DNA sequences has reached base pair (bp) resolution, detailed information on the positioning, nuclear arrangement and interactions of specific gene loci in living cells is still limited.

Despite the absence of internal membranes, the nucleus is a highly organized organelle. With fluorescent in situ hybridization (FISH) all chromosomes in an interphase nucleus were mapped and shown to occupy distinct territories.1 Additionally, gene rich chromosomes were found to be preferably located in the center of the nucleus, whereas gene poor chromosomes reside mostly in proximity to the nuclear periphery reflecting a functional nuclear organization.2,3 Clearly, FISH represents an important tool to label specific DNA sequences and to study nuclear architecture, but it is restricted to fixed specimens.

By now, several methods have been employed to label DNA in vivo4 and to investigate chromatin spatiotemporal dynamics, for instance by the incorporation of fluorescently tagged chromatin proteins, like H2B-GFP.5 However, these methods do not distinguish specific genomic sequences. To overcome these limitations, the lac operator and/or repressor recognition system has been developed.6 This system, however, relies on artificially introduced sequences and does not provide information on endogenous genomic loci.

To date, targeting of specific endogenous genomic loci has been based on the sequence specific binding of Cys_His zinc finger modules (ZF),7-9 where individual ZFs bind to a distinct trinucleotide sequence and are combined into polydactyl zinc finger proteins (PZF). It has been shown, however, that the target specificity of ZFs can be affected by their neighboring modules, which requires evaluation of every newly designed PZF.10,11 PZFs have been widely replaced by designer transcription activator-like effectors (dTALEs), which have proven to be a powerful tool for genome engineering,12,13 influencing gene transcription14-16 and were recently applied for labeling genomic sequences in vivo.17,19 Yet, the fact that DNA binding of dTALEs is mediated by tandemly arranged repeats, whereby every repeat only differs in two residues, necessitates the use of elaborate cloning techniques.20,21

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New experimental options became available with the discovery of the type II CRISPR/Cas system that is composed of clustered regularly interspaced short palindromic repeats (CRISPR) as well as CRISPR-associated (Cas) proteins and plays a vital role in prokaryotic adaptive immunity. Upon viral infection or plasmid uptake, short stretches (~30 bp) of foreign DNA (termed spacers) are incorporated between identical direct repeats into CRISPR arrays. Transcription of these arrays results in pre-CRISPR RNA (pre-crRNA), which subsequently interacts with a trans-activating crRNA (tracrRNA). This pre-crRNA/tracrRNA duplex forms a complex with the endonuclease Cas9, followed by further processing of pre-crRNA into crRNA. Endonuclease target-specificity is determined by the complementarity between spacer (crRNA) and protospacer (viral or plasmid) sequences. As an important step toward the applicability of this system, a crRNA:tracrRNA chimera, named guide RNA (gRNA), has been shown to be able to replace the two RNA components and to specifically target Cas9 to user-defined DNA sequences. The CRISPR/Cas system has recently been applied as a versatile tool for genome editing in a wide range of organisms.

Here, we present an approach for labeling specific endogenous genomic loci in living murine embryonic stem cells based on a modified CRISPR/Cas system.

**Results**

Adapting the CRISPR/Cas system for tracing specific DNA sequences in living cells

The CRISPR/Cas system features easily programmable sequence recognition, but combines it with an endonuclease activity. We, therefore, introduced mutations to inactivate the endonuclease activity of Cas9 and fused it to the enhanced variant of GFP (eGFP) (Fig. 1A). By co-transfecting a plasmid encoding this eGFP-tagged nuclease deficient Cas9 (dCas9-eGFP) together with a gRNA expression vector, we aimed to target specific genomic loci in mouse embryonic stem cells. In this way, we expected to achieve specific targeting of dCas9-eGFP without cleavage of the underlying sequences (Fig. 1B).

To test the feasibility of our method, we chose tandemly arranged repetitive DNA sequences, which enabled us to target dCas9-eGFP to extended genomic loci with a single gRNA construct. To this end, gRNAs directed to major (MaSgRNA) and minor satellite (MiSgRNA) repeats, as well as telomeres (TelgRNA) were designed. In mice, major satellite repeats consist of 234 bp repeat units that span within a 6 Mb region, whereas minor satellite repeats range between 0.6 to 1.2 Mb and consist of 120 bp repeat units. Telomeric repeats vary in length reflecting the cell’s replicative potential and in mouse amount to approximately 20–30 kb with the 6 bp repeat sequence TTAGGG (Fig. 1C).

**Effective tracing of repetitive DNA sequences using the CRISPR/Cas system**

Mouse chromosomes are acrocentric as depicted in Figure 1C. Fluorescent in situ hybridization (FISH) experiments on metaphase chromosomes have shown that minor satellites are centromeric, whereas major satellite repeats occupy the subcentromeric part of the chromosome and are implicated in heterochromatin formation and sister chromatid cohesion. In interphase nuclei, centromeres cluster and form distinct chromocenters (CCs), which can be readily distinguished by enhanced DAPI-staining intensity due to their AT-richness.

J1 mouse embryonic stem cells were co-transfected with dCas9-eGFP and MaSgRNA encoding plasmids and imaged 48 h post-transfection. As depicted in Figure 1D, the CCs of MaSgRNA/dCas9-eGFP expressing cells show a bright eGFP signal, verifying the successful targeting of genomic DNA. The distribution exhibits remarkable specificity with very low background signals from freely diffusing dCas9-eGFP.

MiS repeats have been observed as individual foci that are not detectable in the periphery of CCs. We were able to observe this characteristic distribution as eGFP fluorescent foci in the corresponding regions of MiSgRNA/dCas9-eGFP expressing cells (Fig. 1E).

Telomeres are the ends of chromosomes and have been found localized throughout the nucleus. Due to the acrocentric nature of mouse chromosomes, telomeres located at the acrocentric end can also be detected in the direct vicinity of CCs apart from remote nuclear sites. By expressing TelgRNA/dCas9-eGFP in J1 cells, small foci, often in the vicinity of CCs, were visible. The observed variable size of labeled telomeres is consistent with the fact that telomeres may form clusters (Fig. 1F).

In some TelgRNA/dCas9-eGFP expressing cells, an elongated and fiber-like telomere-related eGFP signal was apparent, which was not observed in cells expressing MaS- or MiSgRNA/dCas9-eGFP. Taken together our results show that for all three targeted DNA sequences distinct labeling patterns were observed.

**Combined gRNA/dCas9-eGFP labeling and 3D DNA-FISH**

To validate the sequence specificity of the dCas9 labeling system, we verified the accurate targeting of all live cell-introduced fluorescent genomic tags by 3D DNA-FISH experiments. For this purpose, FISH probes specific for MaS, MiS and telomeric repeats
were generated and immuno-FISH experiments were performed. We used a protocol optimized for eGFP epitope preservation and efficient probe hybridization, yielding robust and homogeneous signals (Fig. 2). As it can readily be seen in Figure 2A, the probe

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**Figure 1.** For figure legend, see page 164.
for MaS repeats shows confined localization at the CCs, while MaSgRNA/dCas9-eGFP complexes exhibit strict co-localization with the probe. Accordingly, immuno-FISH experiments showed correct localization of MiSgRNA/dCas9-eGFP at MiS repeats (Fig. 2B). Moreover, simultaneous hybridization with MaS and MiS probes showed that the gRNA/dCas9-eGFP complexes specifically labeled their respective target structure and did not affect the spatial integrity of neighboring structures (Fig. 2C). In immuno-FISH of TelgRNA/dCas9-eGFP expressing cells, FISH probe and dCas9-eGFP were fully co-localized and exhibited the same variety of signal intensities with brighter foci possibly representing longer telomeres or telomere clusters (Fig. 2D).

In each case, neighboring non-transfected cells in our specimens served as an internal control for the visualization of normal distribution of the FISH probes and comparison of signal strength. It must be noted here that on some occasions FISH probes did not fully penetrate to the core of well-associated stem cell colonies, while dCas9-eGFP signal was detected. Our observations confirm the specific and restricted localization of dCas9-eGFP to their targeted DNA sequences.

gRNAs/dCas9-eGFP complexes remain associated with mitotic chromosomes

During cell division, chromatin undergoes dramatic structural changes in order for chromosomes to compact and segregate properly. This chromatin rearrangement could possibly affect binding of recombinant macromolecular complexes. In addition, the targeted MiS repeats, forming the centromeres, are the sites of kinetochore formation and ensure normal chromosome segregation. Likewise, pericentromeric regions, containing MaS repeats, play an important role in mitotic progression, as they have been linked to sister-chromatid cohesion. Furthermore, telomeric dysfunction has been shown to promote chromosome fusions, anaphase bridges and genome reorganization. Therefore, targeting of dCas9-eGFP at these essential regulatory sequences could potentially perturb chromosome arrangements and mitotic progression. Hence, we investigated, whether dCas9-eGFP stably maintained its association to targeted DNA sequences during mitosis. We were able to observe MaS, MiS, and TelgRNA/dCas9-eGFP expressing cells in different mitotic stages. A metaphase for the MaSgRNA/dCas9-eGFP targeting is depicted in Figure 3A, showing the robust labeling of the MaS domains and its restricted localization to the highly condensed CC regions. Similarly, two optical sections of a metaphase plate (z1, z2) showed that TelgRNA/dCas9-eGFP labeled both ends of mitotic chromosomes and multicolor FISH confirmed the pericentromeric and centromeric arrangement of MaS and MiS repeats, respectively (Fig. 3B).

For a more detailed investigation of the MiS signals, we applied 3D-SIM microscopy and examined metaphases in MiSgRNA/dCas9-eGFP expressing cells (Fig. 3C). We were able to distinguish distinct dCas9-eGFP foci in every chromosome and strict co-localization with the MiS probe. Using conventional microscopy, MiS clusters appear as diffraction-limited foci. With 3D-SIM, these clusters displayed ultrastructural organization, visible by FISH probe, as well as dCas9-eGFP labeling (Fig. 3C, magnification insets).

Application of CRISPR/Cas labeling in super resolution studies

Next, we investigated whether the coverage of dCas9-eGFP labeling correlated with known proteins bound at these genomic loci, perturbed the canonical distribution of these proteins or prevented antibody binding. For this purpose, J1 cells expressing MaSgRNA/dCas9-eGFP were immunolabeled with anti-centromere protein B (CENP-B) antibodies. CENP-B is a constitutive centromere protein located at the primary constriction by direct binding to MiS repeats at the CENP-B box.58,59 In mitosis it forms a link between the kinetochore and the underlying centromeric repeats56,57 and regulates centromere formation.58,59 In interphase it has been visualized in association with MiS repeat clusters at the periphery of the CCs.41

In MiSgRNA/dCas9-eGFP expressing cells, distinct CENP-B foci were consistently associated with dCas9-eGFP clusters (Fig. 4A), suggesting that incorporation of the gRNA/dCas9-eGFP does neither interfere with the recruitment of CENP-B to MiS repeats, nor antibody binding upon fixation. Compared with wide-field deconvolution (wf), where MiS domains appeared as diffraction-limited foci, 3D-SIM revealed a sub-structural organization of the targeted repeats (Fig. 4A, blowups 3D-SIM compared with wf).

Consistent with the above findings, in TelgRNA/dCas9-eGFP expressing cells, the incorporation of the complexes to telomeric repeats allowed binding of anti-telomeric repeat-binding factor 2 (TRF2) antibodies (Fig. 4B). Telomeric DNA sequences have been shown to form a T-loop structure, which is thought to protect the 3’-overhangs and regulate telomerase activity.60,61 By conventional fluorescence microscopy, telomeres are detected as diffraction-limited foci. In a recent study applying 3D-Storm, FISH detected telomeres appeared as ovoid clusters with an average diameter of 180 nm.62 Although SIM does not offer the localization accuracy of STORM,63 high-resolution microscopy of telomeres using the dCas9 labeling system revealed a consistent morphology to the above-mentioned study (Fig. 4B).

With dCas9 labeling, we were able to visualize repetitive DNA sequences on fixed cells without the need of FISH probes and thus avoided flattening or destruction of chromatin due to sample denaturation. Furthermore, combination of 3D-SIM with immunostaining and dCas9 labeling highlighted ultrastructural properties of targeted MiS and Tel repeats.

Discussion

For modern cytogenetics and diagnostics, fluorescent in situ hybridization (FISH) has proven to be an indispensable method, but its application is still limited, due to its complexity and variability. Since its original introduction, numerous scientific publications have dealt with the optimization of probe and sample preparation. Although improvements have been made, harsh treatments, such as heat denaturation are required for probe hybridization that may compromise sample integrity.64,65 Furthermore, combining 3D-FISH with protein
immunofluorescent detection remains a challenge and on occasion is not feasible due to detrimental effects on epitopes. Therefore, even at the fixed cell level, live-cell genomic labeling systems offer superior sample preservation and their use is far less laborious compared with FISH probe creation and long hybridization and/or detection procedures. Noteworthy is the fact that at the dense stem cell colony level, we observed on occasion that FISH probes did not penetrate efficiently the core of the colony. In these cells, dCas9 labeling was uniform thereby rendering this method more efficient than FISH.

Here we report the successful labeling of endogenous centric, pericentric and telomeric chromatin loci in living mouse embryonic stem cells by repurposing the prokaryotic CRISPR/Cas system. During the preparation of this manuscript, a similar study has been published, which showed that, besides repetitive sequences, also individual genes can be labeled by a catalytically inactive Cas9 endonuclease, confirming the potential of the dCas9 system to label genomic DNA in vivo. While their study focused on detection sensitivity, we performed a thorough comparison with 3D-FISH and immunolabeling methods.

Figure 2. 3D-FISH shows precise targeting of dCas9-eGFP. (A, B, C, D) Immuno-FISH experiments in gRNA/dCas9-eGFP expressing cells. Hybridization of probes designed to target MaS (A, red and C, blue), MiS (B-C, red), Tel (D, red) demonstrate that dCas9-eGFP co-localizes with the respective sequences. Multicolor immuno-FISH in C (MiS, red; MaS, blue) highlights the restricted targeting of MiSgRNA/dCas9-eGFP and demonstrates that non-targeted neighboring nuclear structures maintain their integrity. Bars, 5 µm; insets, 1 µm.
In addition we demonstrated potential applications in high resolution microscopy studies.

So far, dTALE based approaches are commonly applied as powerful tools for genome manipulation and live-cell tracing of chromatin. However, large-scale studies of nuclear organization require the design of dTALEs specific for many different target sequences including single copy genes. Due to laborious cloning procedures, large-scale production of these DNA binding proteins still remains a challenge. In contrast, the target specificity of the dCas9 labeling system relies on small and easily exchangeable gRNAs, which greatly expands the range of possible targets and is the basis for the convenience of dCas9 labeling. Moreover, combining dCas9 labeling with 3D-SIM microscopy enabled us to visualize the ultrastructure of diffraction limited chromatin clusters and their spatial relationship with known associated proteins.

Clearly, this programmable dCas9 DNA labeling system represents a powerful tool to monitor the spatiotemporal dynamics of endogenous genomic loci during cell cycle progression and differentiation and opens new perspectives to study functional nuclear architecture.

Figure 3. Association of gRNA/dCas9-eGFP to chromatin in mitotic cells. (A) Confocal optical section of a metaphase plate shows successful targeting of MaSgRNA/dCas9-eGFP. Note the robust eGFP signals at the CCs (DAPI bright regions, middle panel). Bar, 5 µm. (B) Two confocal optical sections (z1, z2) of a multicolor immuno-FISH stained metaphase show TelgRNA/dCas9-eGFP signals at the ends of chromosomes. The Integrity of (peri-) centromeric chromatin (MaS, MiS) is not compromised. Bar, 5 µm; insets, 1 µm. (C) Metaphase plate of a MiSgRNA/dCas9-eGFP expressing cell after immuno-FISH with MiS probe (red) acquired via 3D-SIM. Note the overlap between MiS probe and dCas9-eGFP. Bar, 5 µm; inset, 1 µm.
**Materials and Methods**

**Cell culture and transfection**

J1 embryonic stem cells (ESCs) were cultivated at 37 °C and 5% CO₂ on gelatin-coated petri dishes in Dulbecco’s modified Eagle’s medium supplemented with 16% fetal bovine serum (Biochrom), 0.1 mM β-mercaptoethanol (Invitrogen), 2 mM L-glutamine, 1x MEM non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin (PAA Laboratories GmbH), 1000 U/ml recombinant mouse LIF (Millipore), 1 µM PD032501, and 3 µM CHIR99021 (Axon Medchem). Transfection of J1 cells was performed using Lipofectamin 2000 (Invitrogen) according to the manufacturer’s instructions. For FISH experiments 2.5 × 10⁵, for all other experiments 5 × 10⁵ cells were transfected. Cells were analyzed 48 h post-transfection.

**Plasmid generation**

For generating the dCas9-eGFP construct, plasmid hCas9_D10A was purchased from Addgene (addgene ID: 41816). Inactivation of the second nuclease domain (H840A) was performed by site-directed mutagenesis using primers dCas1-F, dCas2-R, dCas2-F, and dCas3-R. The resulting PCR-product (dCas9) was digested with BsrGI and XbaI and ligated into pCAG. The NLS-eGFP-sequence was amplified by ligating downstream of dCas9. eGFP1-expression vectors were generated by amplifying pEX-A-U6-gRNA with forward and reverse primers, which introduced the protoscaler sequence for minor satellites repeats (MiS), major satellites repeats (MaS) and telomeres (Tel), respectively.

Nucleotide sequences:

- dCas1-F: 5′-aaagcgatcg ctctagaatg gacaagaagt actccattgg g-3′
- dCas2-R: 5′-ctggggcacg atagcatcca cgtcg-3′
- dCas2-F: 5′-cgacgtggat gctatcgtgc cccag-3′
- dCas3-R: 5′-tttgcggccg ctcattgtac aatcaccttc ctcttcttct tggggtc-3′
- eGFP1: 5′-aaagcgatcg catccaaaga agaagagaaa ggtcatggtg agcaagggcg agg-3′
- eGFP2: 5′-tttgcggccg cttacttgta cagctcgtcc atgcc-3′
- MaSgRNA-F: 5′-ggcaagaaaa ctgaaaatca gttttagagc tagaaatagc aag-3′
- MaSgRNA-R: 5′-tgattttcag ttttcttgcc cggtgtttcg tcctttccac-3′
- TelgRNA-F: 5′-tagggttagg gttagggtta gttttagagc tagaaatagc aag-3′
- TelgRNA-R: 5′-tgattttcag ttttcttgcc cggtgtttcg tcctttccac-3′
- MiSgRNA-R: 5′-acgaatggtg ttttcagtgt cggtgtttcg tcctttccac-3′

pEX-A-U6-gRNA: Sequence is available upon request.

**Immunofluorescence staining (IF) and fluorescent in situ hybridization (FISH)**

Immunostaining and FISH experiments were performed as described previously. Briefly, J1 ESCs grown on coverslips were washed 48 h post-transfection with phosphate buffered saline.
saline (PBS) and fixed with 4% formaldehyde for 10 min. After permeabilization with 0.5% Triton X-100 in PBS, cells were incubated with PBST (PBS, 0.02% Tween) supplemented with 2% BSA and 0.5% fish skin gelatin (blocking buffer) for 1 h. Both primary and secondary antibodies were diluted in blocking buffer and cells were incubated with the respective antibodies for 1 h in a dark humidified chamber at room temperature. Washings were performed with PBST. For immuno-FISH detection, cells were first incubated with primary (anti-GFP, Roche) and secondary antibodies, followed by postfixation and pre-treatment for hybridization. Hybridization was performed overnight at 37 °C. Following post-hybridization washings were performed with 2xSaline Sodium Citrate (SSC) at 37 °C and 0.1xSSC at 61 °C. FISH probes for MiS and MaS were generated by PCR using mouse Cdt1 DNA (Invitrogen) as a template (MaS-primers: 5’-GGG AGA CTG AAA ATC AC-3’ and 5’-TCA AGT CGT CAA GTG GAT G-3’; MiS-primers: 5’-CAT GGA AAA TGA AAA CC-3’ and 5’-CAT ATA TGT TCT ACA GTG TGG-3’). The probe for telomeric repeats was produced by using self-annealing primers in the PCR (5’-TTA GGG TTA GGG TTA GGG-3’ and 5’-CCC TAA CCC TAA CCC TAA CCC TAA-3’). All probes were directly labeled by nick translation with Texas Red-dUTP or Cy3-dUTP and dissolved in hybridization mixture (50% formamide, 10% dextran sulfate, 1xSSC) at a concentration of 10–20 ng/ml. Cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI), mounted with antifade medium (Vectashield, Vector Laboratories) and sealed with Covergrip sealant (Biotium). Primary antibodies used in this study were: anti-GFP (1:400; Roche 1181446001; PMID:4031anza); anti-TRF2 (1:250, Abcam ab3579), anti-CENP-B (1:500, Abcam ab84489). The secondary antibodies were anti-rabbit conjugated to DyLight 594 (Jackson ImmunoResearch, 711-505-152), anti-mouse conjugated to Alexa 488 (Invitrogen, A21202) and anti-mouse conjugated to Alexa 594 (Invitrogen, A11032). Cells for 3D-SIM were grown on precision cover glass, thickness no. 1.5H (170 µm ± 5 µm; Marienfeld Superior) using immersion oil with a refractive index of 1.514 to minimize spherical aberration.

Microscopy and image acquisition

Optical sections for Figure 1D were acquired with an Ultraview VoX spinning disk confocal microscope (PerkinElmer), which was operated with the Velocity® software. For Figure 1E–F, 2, and 3A-B, single optical sections or stacks of optical sections were collected with a Leica TCS SP5 confocal microscope using a Plan Apo 63×/1.4 NA oil immersion objective. High-resolution images (Fig. 3C and 4) were obtained with a DeltaVision OMX V3 3D-SIM microscope (Applied Precision Imaging, GE Healthcare), equipped with a 60×/1.42 NA PlanApo objective and sCMOS cameras (Olympus). Images were acquired with a z-step size of 125 nm. Reconstruction and image deconvolution was applied to the raw data using the SoftWoX 4.0 software package (Applied Precision). Image processing and assembly was performed with Fiji and Photoshop CS5.1 (Adobe), respectively. Stacks of confocal optical sections were corrected for chromatic shifts with ImageJ plugins.71

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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