Chapter 13

Targeted Manipulation/Repositioning of Subcellular Structures and Molecules

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Abstract

Technical advances in live-cell imaging have made cell biology into a highly dynamic field, allowing the visualization and quantification of complex processes in individual cells and in real time. To follow changes and to specifically manipulate factors potentially involved in processes like DNA replication, transcription or repair, we set up a universal targeting approach, allowing directed manipulation of subcellular structures and molecules therein. This strategy is based on the very strong and specific interaction of GFP and GFP-binding nanobody. We describe in detail how to set up the targeting approach with appropriate controls, as well as how to improve and validate its efficiency and finally provide exemplary applications.

Key words Green fluorescent protein, GFP-binding nanobody, Live-cell microscopy, Protein–protein interaction, Targeted manipulation, Targeted repositioning

1 Introduction

The visualization and quantification of processes in individual cells and in real time enables the tracking of spatiotemporal changes and evaluating their potential impact and function. To answer fundamental questions and understand the function of individual factors on multiplex processes it is important to be able to study the role of any factor X on any process Y. Thus, it is of major advantage to specifically manipulate DNA, RNA, proteins, or even subcellular structures in a controlled and targeted manner. The manipulation of proteins localization via bait and prey type of approaches, such as fluorescence two-hybrid/tri-hybrid assays [1, 2] among others, is a very useful tool to study protein–protein interactions. It would be of major advantage to use the same principles to manipulate the localization of entire subcellular structures and study complex questions like the effect of location on function.

Here, we describe a strategy that is based on the strong and highly specific interaction of GFP and GFP-binding protein (GBP). GBP is a recombinant 13-kDa GFP binding fragment derived from

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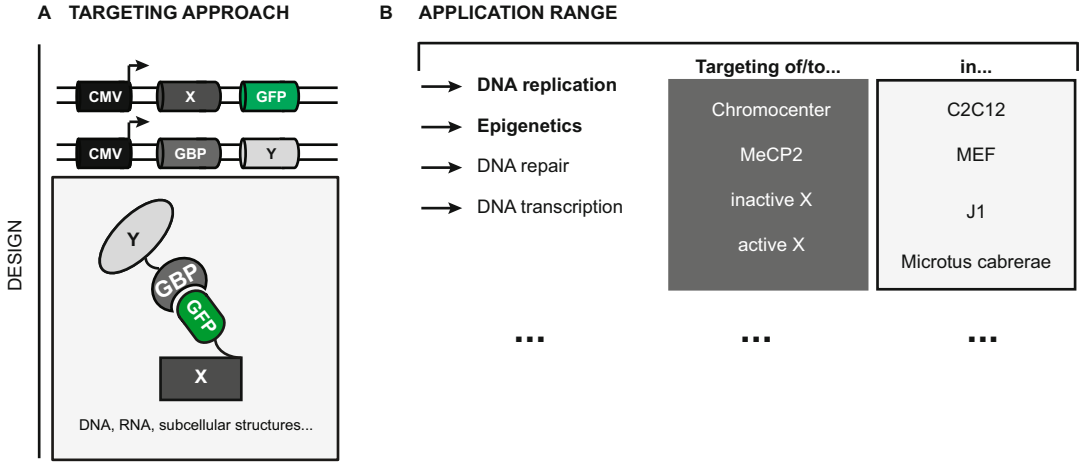

Fig. 1 (**a**) Schematic representation of the targeted manipulation approach. The strategy is based on the strong interaction of GFP and GFP binding protein (GBP). Both counterparts can be designed and cloned to any desired factor X and Y to target DNA, RNA, or even subcellular structures. Upon coexpression of both constructs, the interaction of GFP with GBP is strong enough to even reposition large genomic regions. (**b**) The application range of the targeting approach is broad and potential processes include DNA replication, epigenetics, DNA repair, and DNA transcription. Targeting of structures to reposition them is one application but also targeting of factors to a desired locus. Successful targeting can be achieved in different cell lines, depending on the factors used and processes under study

a llama single chain V_HH antibody [3, 4] (Fig. 1). Because of the high affinity of GFP and GBP with a K_d in the subnanomolar range, it is possible to target any desired factor and even reposition large genomic regions when both factors are coexpressed in cells. The efficiency and strength of this targeting approach becomes obvious, since it is possible to even reposition constitutive heterochromatin in murine cells to the nuclear periphery to study the effect of nuclear position on basic cellular processes such as DNA replication and epigenetic composition [5].

In this chapter, we explain in detail how to design an individual and successful targeting assay and how to develop the appropriate controls. Depending on the question to be addressed, different control setups are possible (Fig. 2a). A control setup with no targeting or manipulation is achieved by transfection of X-GFP alone without the additional transfection of the GBP-carrying counterpart (Fig. 2a setup 1). To rule out any effect caused by the overexpression of factor Y one could remove the GBP part and simply transfect factor Y with X-GFP, leading to no interaction (Fig. 2a setup 2). Furthermore, the disruption of the targeting for a control setup can be achieved by replacing GFP by RFP, which does not interact with GBP (Fig. 2a setup 3). Lastly, it is possible to cotransfect X-GFP with a GBP—carrying an inactive form of protein Y (Fig. 2a setup 4). In the case of enzymes one could inactivate the catalytic activity by site-directed mutagenesis and thus target an

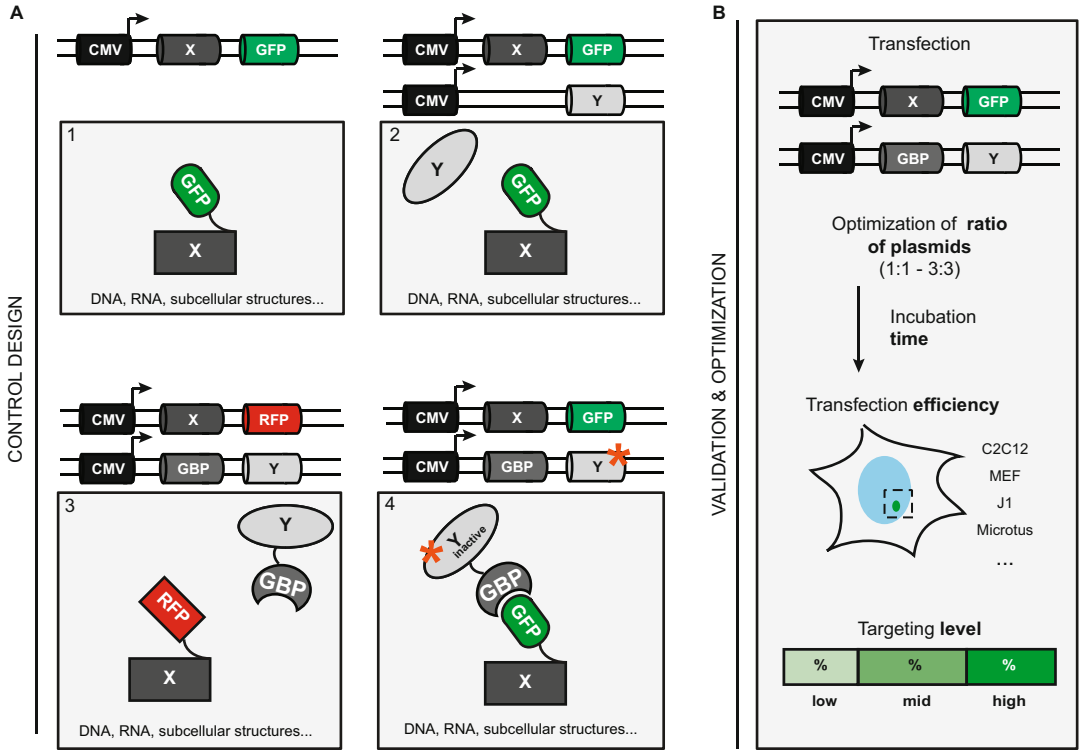

Fig. 2 Schematic representation of the design of appropriate controls and the validation thereof. (**a**) The design of appropriate controls is flexible and dependent on the question to be answered. Here, we show different strategies to disrupt targeting, leading to a control setup. First, an untargeted state is achieved by transfecting only the X-GFP part without any interaction partner (setup 1). Furthermore, the X-GFP is transfected with Y alone, without the GBP part, which is required for interaction of both constructs. One advantage of this method is to elucidate, whether the overexpression of Y alone already leads to an effect on the investigated process (setup 2). Another strategy to achieve an untargeted control state, especially when targeting a factor to a desired locus, is the inactivation of factor Y. If Y is an enzyme, one could inactivate the catalytic activity by point mutation (setup 4). As GBP interacts with GFP but not with RFP, another strategy includes the design of an RFP-tagged X, which no longer interacts with the GBP-Y counterpart (setup 3). (**b**) After successful design of controls, the validation thereof as well as of the targeting is of upmost importance. The first step is to transfect cells with the appropriate constructs and validate the perfect ratio of plasmids to achieve a targeting signal. The incubation time after transfection needs also to be optimized. When cells show a targeted signal, the transfection efficiency can be determined, meaning the percentage of cells expressing the constructs and showing the desired targeted signal. The power of targeting could be categorized into different levels (e.g., low, mid, and high targeting) to check which level is the most desired one and depicts no toxic effects. Control and targeted cells should show their characteristic morphology to rule out toxic effects, which can lead to cell death

inactive version of the factor to the desired location. Furthermore, we present in detail how the validation of the targeting and control setup is accomplished (Fig. 2b). Different parameters need to be considered when optimizing the targeting approach, from the best ratio of both plasmid constructs during transfection to incubation time after transfection up to validation of transfection efficiency.

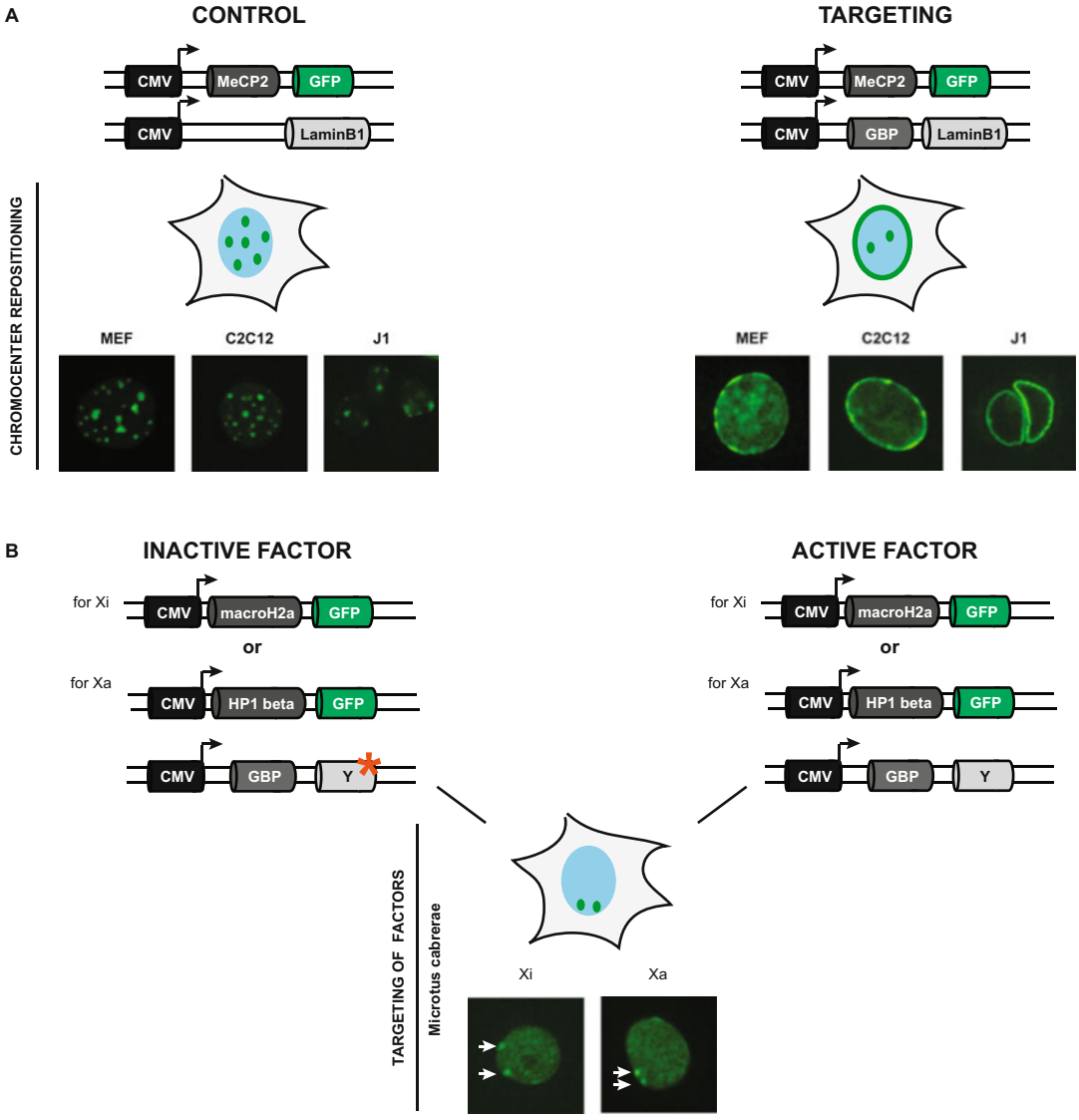

Fig. 3 Application of the targeting approach to constitutive and facultative in different cell lines and species. (**a**) Repositioning of constitutive heterochromatin in various cell lines. MeCP2-GFP was coexpressed with LaminB1 without the GBP. No interaction of both targeting parts took place and thus constitutive heterochromatin was distributed throughout the whole nucleus. Upon coexpression of MeCP2-GFP and GBP-LaminB1, constitutive heterochromatin was repositioned to the nuclear lamina, resulting in a clear peripheral targeting ring. The targeting strategy was performed in different somatic and embryonic mouse cell lines: MEF, C2C12, and J1. (**b**) Macro-H2a and HP1-beta were tagged to GFP to either recognize the heterochromatin of the inactive X chromosome (Xi) or the active X chromosome (Xa) in *Microtus cabrerae* female fibroblasts. Upon coexpression with GBP-Y, where Y is any desired factor, Y is transported to the Xi or Xa. For the control setup, factor Y could be inactivated by, for example, point mutation

This targeting strategy was successfully used to study the effect of nuclear position of DNA on its DNA replication timing. For this study constitutive heterochromatin was repositioned to the nuclear periphery into an environment of mid-replicating facultative heterochromatin [6]. MeCP2 as a methyl-cytosine binding protein [7] was used as X and tagged to GFP. MeCP2 binds methylated cytosines, which are highly abundant in constitutive heterochromatin. The latter forms micrometer sized aggregates in mouse cells termed chromocenters [8]. LaminB1, as a component of the nuclear lamina, was used as Y and was transfected without the GBP-counterpart to give normally localized GFP-labeled chromocenters (Fig. 3a). To reposition constitutive heterochromatin, MeCP2-GFP and GBP-LaminB1 were coexpressed, leading to a clear green targeting ring at the nuclear periphery. With this approach constitutive heterochromatin was recognized, labeled, and repositioned in different cells, including mouse embryonic fibroblasts (MEF [9]), embryonic stem cells (J1 [10]), and myoblasts (C2C12 [11]). Additionally, we were able to transport specific factors to a desired location. We transported factor Y (a histone acetylating enzyme) to the heterochromatin of the inactive X chromosome (Xi) with the help of the macroH2a histone variant [12] and to the active X chromosome (Xa) with the help of HP1 beta [13, 14] in *Microtus cabrerae* [15] cells (Fig. 3b). For this purpose, an additional control should be added including the inactive form of the enzyme.

All in all, this experimental strategy enables specific tethering of any DNA, RNA, protein, or even subcellular structures of interest to any desired location and lays the ground for controlled manipulation of factors such as epigenetic regulators, transcription factors, or ultimately subcellular localization of structures.

2 Materials

All materials and solutions used for cell culture, fixed cell studies, and live-cell microscopy must be sterile.

1. Cell lines used for live-cell microscopy should be adherent cell cultures or be spun down on a support, such as glass coverslip. While the cell line to be used depends on the interest of the scientist, there are various considerations simplifying the data acquisition (*see* **Note 1**).
2. Culture medium: use the standard medium required for the particular cell line.
3. Prewarmed PBS containing 0.5 mM EDTA and 0.25% trypsin in PBS.
4. 0.2% gelatin for coating the microscopy dishes or coverslips.

5. Plasmids: mammalian expression vectors coding for the setup of the targeting strategy of interest (one carrying the GBP counterpart and one carrying the GFP; *see* **Notes 2** and **3**). Additional fluorescent markers should be chosen according to the wavelengths that can be imaged using the microscope available.
6. Transfection reagents: nucleofection system from Amaxa (Lonza) or Neon nucleofection system (Thermo Fisher Scientific) [16], nucleofection solutions, cuvettes/tubes, and pipettes (*see* **Notes 4** and **5**).
7. Microscopy dishes: form and size depends on the optical table inset available for the microscope. The bottom of the dish needs to be thin enough for higher magnification immersion lenses to be able to image through the sample. Material can be either glass or optical plastic (*see* **Note 6**). Glass lids are recommended for optimal contrast images.
8. Microscope: for imaging of live cells and fixed cells, we recommend the use of a spinning disc confocal microscope, characterized by high-speed acquisition and low level of phototoxicity to cells. The stage should be motorized to allow the acquisition of 3D stacks at several time points and several stage positions in one experiment.

3 Methods

Here, we present a detailed protocol to target, manipulate or reposition specific factors and genomic regions to any desired location. The protocol is adapted to image fixed cells on glass coverslips or to image living cells in a 35 mm diameter glass-bottomed dish:

1. Prewarm growing medium and PBS–EDTA to 37 °C and trypsin as well as transfection solutions to room temperature.
2. Prepare the dish where transfected cells will be seeded by coating for 20 min with 0.2% gelatin. Wash twice with prewarmed medium to fully remove gelatin and add the final volume of growing medium (2 mL for a 35 mm diameter dish) and keep it in an incubator so that the medium reaches 37 °C and CO_2 diffuses into it.
3. Use 3×10^5 adherently growing cells of a 10 cm diameter plate (*see* **Note 7**). Remove growing medium and wash carefully with 5 mL PBS–EDTA, avoiding detaching cells from the surface. Add 0.5 mL trypsin and incubate at 37 °C for 2–5 min. Check cell detachment under a microscope. When most of the cells have detached from the substrate and are separated from each other, stop the enzymatic reaction by adding 4.5 mL growing medium. If cells are clumped together, carefully break up the

cell clumps by pipetting up and down a couple of times. Centrifuge the cells for 6 min at 300 × *g*.

4. Prepare 100 μL of transfection solution with the appropriate and optimized amount of plasmid DNA (*see* **Notes 8** and **9**).
5. Once the cells are pelleted, discard the supernatant and carefully resuspend in transfection solution. Transfer the cell suspension into an electroporation cuvette/tube. Make sure to avoid bubbles and that the solution is clear without phenol red, because this can cause error messages during the electroporation process. Immediately perform the electroporation using the appropriate program for your cells (*see* **Note 5**). Use the previously prepared dish from the incubator and seed cells into the dish. Carefully shake the plate to homogenously distribute the cell suspension and return it into the incubator. Incubate overnight (*see* **Notes 10** and **11**).
6. On the next day, remove the medium and wash twice with prewarmed medium to remove dead cells, debris and add fresh medium.
7. For fixed cell studies, cells are fixed with 4% formaldehyde for 10 min, permeabilized for 20 min with 0.5% Triton X-100–PBS prior to immunostainings with the desired antibodies (*see* **Note 12**).
8. In case of live-cell imaging make sure that your incubation chamber is ready: 37 °C, 5% CO_2 and over 40% humidity level.
9. Place the dish with the transfected cells on the microscope. Allow the dish to acclimatize to new conditions prior to starting imaging. Slight changes in temperature may affect the material in such a way that the focal plane can change dramatically during the first 10–20 min.
10. Look for transfected cells by using short exposure times to minimize phototoxicity. Select cells that express the minimal amount of the fluorescent proteins that can be imaged properly. Too high expression levels may lower the chances that transfected cells will pass normally through S-phase and/or increases the chances that the cells undergo apoptosis.
11. Set up the imaging conditions finding a compromise between phototoxicity and undersampling. The ideal conditions depend strongly on the cell line, since some cells are more sensitive to transfection and phototoxicity, as well as of course on the transfection strategy you have set up. In general, acquiring z-stacks at a time interval of 20 min is usually enough to follow changes of, for example, S-phase. The minimal amount to acquire an entire cell cycle depends on how fast the cells divide (*see* **Note 3**). Under normal conditions cells can be kept on the microscope stage and be imaged over 2 days.

4 Notes

1. Factors to consider when choosing a cell line to perform targeted manipulation and live-cell imaging of the targeting approach:
   - How well the cells can be transfected (transfection and expression rate).
   - How the cells can tolerate imaging-derived phototoxicity.
   - How much the cells move, which makes long term imaging difficult.
   - How fast cells divide and how they handle targeting in the subsequent cell cycles.
2. Factors to consider when setting up your targeting approach:
   - Which factor is targeted?
   - Where is the factor targeted?
   - Are the target and/or the location specific enough for a manipulation?
3. We always suggest linking the GBP to the leading part of the targeting assay and the targeted factor to the GFP. With this setup you are directly able to check by eye whether your factor is targeted by visual inspection of the GFP signal.
4. While a good transfection rate is a factor to consider when choosing your transfection method, when following cell cycle progression at a single cell level, it is more important to achieve a moderate expression level. We recommend the nucleofection system from Amaxa (Lonza) for somatic cells and Neon Nucleofection (Thermo Fisher) for embryonic stem cells, although other methods can be used.
5. We suggest for myoblasts and fibroblasts the B-032 program (e.g., C2C12, *Microtus cabrerae*) and for mouse embryonic fibroblasts the A-024 program (e.g., MEF) of the Amaxa machine. For mouse embryonic stem cells like J1 we suggest to use the Neon nucleofection system with a transfection setup of 1250 V, 20 ms width, and two pulses.
6. Irrespective of whether you use your targeting approach for fixed cell studies or live-cell imaging we highly suggest to coat glass surfaces with gelatin as this improved the fast attachment of transfected cells and increased the general transfection efficiency.
7. Cell density is a key factor for live-cell imaging and to provide enough cells prior to transfection as a higher amount of cells is dying during the process of electroporation. While a too high density can result in cell contact inhibition, preventing cells from cycling, a too low density can result in cells moving

more freely along growing surface, making it extremely hard to keep them in frame over several hours. In case of low transfection efficiency the likelihood of having enough cells for your studies is further decreased. The optimal cell density depends on the cell line used: mouse myoblasts and fibroblasts tend to move and a rather high density is recommended. In case of embryonic stem cells like for J1 for instance, a lower density makes more sense to avoid large colonies of cells, further complicate the imaging and validation of targeting in single cells.

8. The optimal ratio of both plasmids is of upmost importance. Therefore we suggest ratio measurements before starting experiments. We always used combinations (in μg) from 1:1 up to 3:3, changing for every plasmid to make sure to achieve the perfect combination, resulting in healthy cells with typical morphology but an efficient targeting with high transfection efficiency.
9. Further optimization is needed when you want to combine your targeting approach with, for example, a PCNA transfection to label active replication sites. We checked triple transfections as well as quadruple transfections, both is suitable, but requires further optimization step. As already indicated, use as less plasmid as possible to not harm the cells too much but still enough to have an appropriate targeting efficiency.
10. During optimization we recommend to check for the perfect incubation time after transfection. Too high expression levels may lower the chances that transfected will pass normally through S-phase and/or increases the chances that the cells undergo apoptosis. High targeted cells often appear fast after nucleofection but also die quite fast, so make sure to estimate the right level of targeting for your experiments. For imaging and reliable cell cycle studies, cells with low or mid expression rate are preferable. These cells appear slightly later after transfection process.
11. During the establishment of different targeting strategies in different cell lines we always incubated in a range in between 20 and 24 h for first cell cycle studies and up to 49 h for second cell cycle studies. But these numbers strongly depend on the cell line you are using and its doubling time.
12. When targeted cells are used for fixed cell studies like FISH, pulse labeling setups, or immunostainings in general, we suggest to boost the signal with an additional GBP (1 mg/mL) incubation step prior to DAPI staining when the GFP signal is very weak. We suggest a step of 1 hour at room temperature.

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References

1. Zolghadr K, Rothbauer U, Leonhardt H (2012) The fluorescent two-hybrid (F2H) assay for direct analysis of protein-protein interactions in living cells. Methods Mol Biol 812:275–282
2. Herce HD, Deng W, Helma J, Leonhardt H, Cardoso MC (2013) Visualization and targeted disruption of protein interactions in living cells. Nat Commun 4:2660
3. Rothbauer U, Zolghadr K, Tillib S, Nowak D, Schermelleh L, Gahl A, Backmann N, Conrath K, Muyldermans S, Cardoso MC et al (2006) Targeting and tracing antigens in live cells with fluorescent nanobodies. Nat Methods 3:887–889
4. Rothbauer U, Zolghadr K, Muyldermans S, Schepers A, Cardoso MC, Leonhardt H (2008) A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. Mol Cell Proteomics 7:282–289
5. Heinz KS, Casas-Delucchi CS, Torok T, Cmarko D, Rapp A, Raska I, Cardoso MC (2018) Peripheral re-localization of constitutive heterochromatin advances its replication timing and impairs maintenance of silencing marks. Nucleic Acids Res 46:6112–6128
6. Casas-Delucchi CS, Cardoso MC (2011) Epigenetic control of DNA replication dynamics in mammals. Nucleus 2:370–382
7. Brero A, Easwaran HP, Nowak D, Grunewald I, Cremer T, Leonhardt H, Cardoso MC (2005) Methyl CpG-binding proteins induce large-scale chromatin reorganization during terminal differentiation. J Cell Biol 169:733–743
8. Vissel B, Choo KH (1989) Mouse major (gamma) satellite DNA is highly conserved and organized into extremely long tandem arrays: implications for recombination between nonhomologous chromosomes. Genomics 5:407–414
9. Peters AH, O'Carroll D, Scherthan H, Mechtler K, Sauer S, Schofer C, Weipoltshammer K, Pagani M, Lachner M, Kohlmaier A et al (2001) Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. Cell 107:323–337
10. Li E, Bestor TH, Jaenisch R (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell 69:915–926
11. Yaffe D, Saxel O (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 270:725–727
12. Abbott DW, Chadwick BP, Thambirajah AA, Ausio J (2005) Beyond the Xi: macroH2A chromatin distribution and post-translational modification in an avian system. J Biol Chem 280:16437–16445
13. Nielsen AL, Oulad-Abdelghani M, Ortiz JA, Remboutsika E, Chambon P, Losson R (2001) Heterochromatin formation in mammalian cells: interaction between histones and HP1 proteins. Mol Cell 7:729–739
14. Cheutin T, McNairn AJ, Jenuwein T, Gilbert DM, Singh PB, Misteli T (2003) Maintenance of stable heterochromatin domains by dynamic HP1 binding. Science 299:721–725
15. Fernandez R, Barragan MJ, Bullejos M, Marchal JA, Martinez S, Diaz de la Guardia R, Sanchez A (2001) Molecular and cytogenetic characterization of highly repeated DNA sequences in the vole *Microtus cabrerae*. Heredity (Edinb) 87:637–646
16. Kim JA, Cho K, Shin MS, Lee WG, Jung N, Chung C, Chang JK (2008) A novel electroporation method using a capillary and wire-type electrode. Biosens Bioelectron 23:1353–1360