Methods 53 (2011) 437–452

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Recombineering, transfection, Western, IP and ChIP methods for protein tagging via gene targeting or BAC transgenesis

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ARTICLE INFO

Article history:
Available online 31 December 2010

Keywords:
Recombineering
BAC transgenesis
Gene targeting
Protein tagging
Native affinity purification
Western blotting
Chromatin immunoprecipitation
Genotyping by long range PCR

ABSTRACT

Protein tagging offers many advantages for proteomic and regulomic research. Ideally, protein tagging is equivalent to having a high affinity antibody for every chosen protein. However, these advantages are compromised if the tagged protein is overexpressed, which is usually the case from cDNA expression vectors. Physiological expression of tagged proteins can be achieved by gene targeting to knock-in the protein tag or by BAC transgenesis. BAC transgenes usually retain the native gene architecture including all cis-regulatory elements as well as the exon–intron configurations. Consequently most BAC transgenes are authentically regulated (e.g. by transcription factors, cell cycle, miRNA) and can be alternatively spliced. Recombineering has become the method of choice for generating targeting constructs or modifying BACs. Here we present methods with detailed protocols for protein tagging by recombineering for BAC transgenesis and/or gene targeting, including the evaluation of tagged protein expression, the retrieval of associated protein complexes for mass spectrometry and the use of the tags in ChIP (chromatin immunoprecipitation).

1. Introduction

A major problem with proteomic research involves the need to either find or generate a good specific antibody for a protein, allowing various protein-specific biochemical methods (e.g. immunoprecipitation in ChIP or for mass spectrometry or immunofluorescence). These problems can be circumvented by expression of a tagged version. Protein tagging has been very successful in yeast, particularly with the large TAP tag [13,15,28]. More than two-thirds of yeast open reading frames have been TAP tagged on the C-terminus by knock-in gene targeting. About 85% of them have been successfully immunoprecipitated using a generic protocol partly developed in our lab [25,23]. This success rate is notable because the average size of yeast proteins is about the same size as the TAP tag. The acquired protein interaction data has established the most comprehensive and accurate proteomic map for any cell [4,26]. Given the success of protein tagging for proteomic mapping in yeast, attention has been directed towards developing the same approaches for mammalian systems.

In the course of the development of tagging methods in yeast, it was realized that overexpression of tagged proteins generated noisy data whereas physiological expression was critical to the quality of the protein interaction data [35,26]. In yeast, physiological expression is easily achieved because tagging endogenous genes by gene targeting is straightforward.

However, gene targeting in mammalian systems is, except for mouse embryonic stem cells (ESCs), laborious or impractical. These disadvantages have been bypassed by the development of recombineering [41,18] and BAC transgenesis for tagged protein expression [18,24]. Unless the gene is too large to fit into one BAC, BAC transgenes carry all of the endogenous control elements in their natural configuration and therefore almost always recapitulate all endogenous controls such as cell cycle regulation of transcription, microRNA regulation of translation or alternative splicing. Consequently, protein expression from a BAC transgene usually occurs at physiological levels in a copy-dependent manner. For tagged protein expression, BAC transgenesis is almost as good as the insertion of the tag into the endogenous gene by gene targeting. Here we describe the generation of BAC transgenes by recombineering their introduction into mammalian cells in culture. We also describe targeting into mouse ESCs to knock in the same protein tag and accompanying methods to characterize the expression of the tagged protein. The overall content of this article is illustrated in Fig. 1.

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doi:10.1016/j.ymeth.2010.12.026

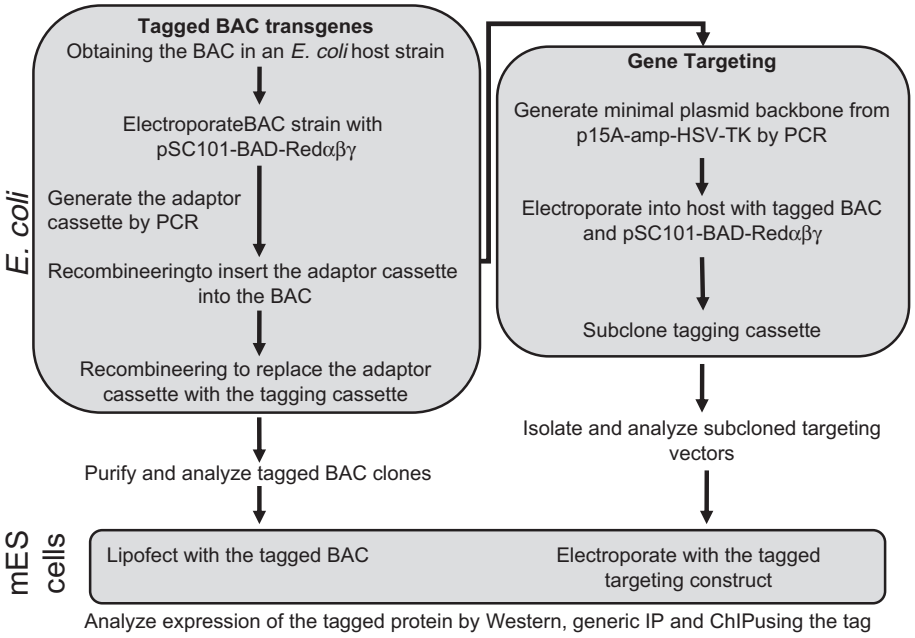

Fig. 1. General scheme of the methods presented in this article. The steps to introduce a tag into a BAC by recombineering are illustrated at the left. Subcloning to generate a targeting construct is shown at the right. Both methods largely take place in an *E. coli* host. Below are illustrated the further steps to purify the BAC transgene (or plasmid) and transfect mouse ES cells, as well as the analysis of gene targeting and protein expression by Western. Generic immunoprecipitation (IP) and chromatin immunoprecipitation (ChIP) methods using the tag are also included.

1.1. Recombineering

The original methods for recombinant DNA production utilize restriction enzymes and DNA ligase. Even though the advent of oligonucleotide synthesis and PCR greatly expanded technical capacities, all these methods suffer from a size limitation because it becomes increasingly difficult to achieve a precise product as the DNA engineering task becomes larger. A number of years ago we reasoned that methodology based on homologous recombination in *Escherichia coli* (*E. coli*) would overcome this limitation. This led to our discovery that phage recombinase systems from either the rac prophage or the closely related λ phage Red operon, mediate extremely efficient homologous recombination at any chosen position, requiring only short homology arms [41,18]. This DNA engineering methodology is now termed 'recombineering' [8,40,17,20,5,6] and includes a broad range of applications including subcloning by gap repair [42], point mutagenesis in BACs [19], oligonucleotide-directed mutagenesis [10], BAC engineering for gene targeting [32,34,39,38,12], high throughput DNA engineering [24,27,29] and a variety of other, often complex, applications. Here we concentrate on recombineering methods for the generation of tagged BAC transgenes [24,27] and targeting vectors to express tagged proteins.

1.2. Design of the tag

Here we only consider protein tagging using C-terminal tagging cassettes (Fig. 2). N-terminal and internal tagging strategies require different cassettes with additional features and will not be discussed in this article.

From 5′- to 3′-end, the PGK-em7-neo C-terminal tagging cassette consists of:

(a) 2xTy1, two copies of the Ty1 peptide, for which a good monoclonal antibody (Ty1/BB2, Diagenode) is available [9]. This DNA sequence also serves in sequential recombineering first as primer site for PCR amplification and second as the 5′ homology arm for the insertion of the tagging cassette;
(b) PreS, two copies of the recognition sequence for PreScission protease (GE Healthcare) enabling the specific elution of the purified protein from affinity-tag columns [36];
(c) mVenus, the coding region for codon optimized, CpG dinucleotide-reduced, Venus fluorescence protein [21];
(d) Biotin, the coding region for the biotin tag [7];
(e) rox, a rox site for the deletion of the PGK-neo gene upon Dre recombination [1];
(f) PGK, the human phosphoglycerate kinase promoter;
(g) em7, the *E. coli* em7 promoter, which is embedded in the 5′ non-coding region of the neo gene;
(h) neo, the coding region for neomycin/kanamycin resistance gene;
(i) polyA, the SV40 polyadenylation region;
(j) rox, a second rox site orientated to delete the PGK-neo gene upon Dre recombination;
(k) 3xFlag, three copies of the Flag tag [31]. This DNA sequence also serves in sequential recombineering first as a primer site for PCR amplification and second as the 3′ homology arm for the insertion of the tagging cassette. Initially, the three copies of Flag are outside of the ORF. However, Dre-recombination to delete the PGK neo gene attaches the Flag tags onto the 3′end of the protein tag.

The T2A-gb3-neo polycistronic tagging cassette is the same except from (f) to (k):

(f) T2A, the coding region for the 2A peptide from Picornavirus [30];
(g) gb3, the *E. coli* gb3 promoter, which is included within the open reading frame of the neomycin/kanamycin antibiotic resistance coding region;
(h) neo, the coding region for the neomycin/kanamycin resistance gene
(i) rox, a second rox site orientated to delete the T2A-neo gene upon Dre recombination.
(j) 3xFlag, three copies of the Flag tag [31]. This DNA sequence also serves in sequential recombineering first as a primer

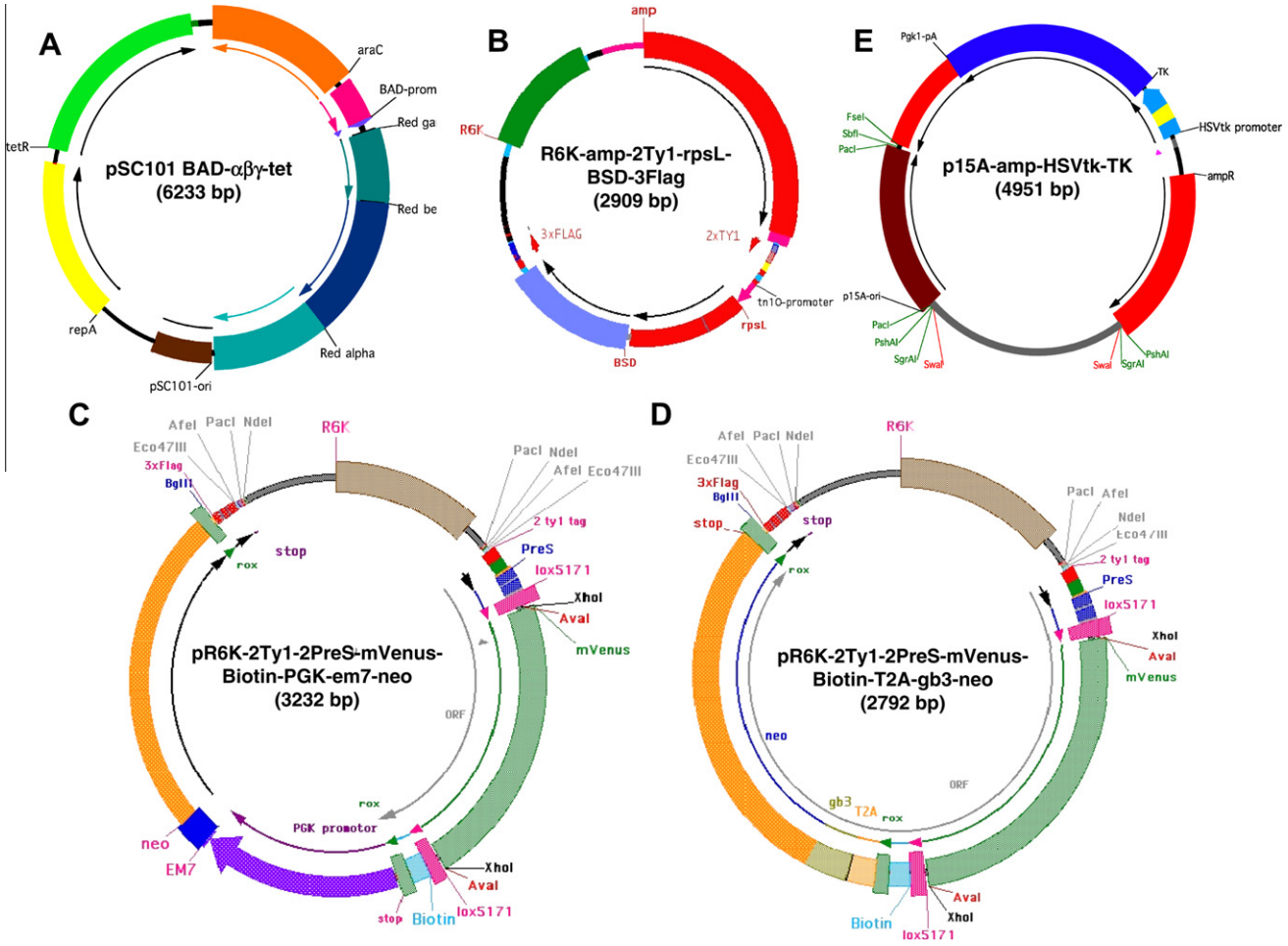

Fig. 2. Plasmids used for BAC tagging and Gene targeting. (A) All recombineering steps require the plasmid pSC101-BAD-Redαβγ (or pSC101-BAD-RedαβγA) for the L-arabinose inducible expression of the λ-Phage proteins Redαβγ. This plasmid conveys tetracycline resistance and its repA protein is temperature sensitive so the plasmid can be eliminated at 37 °C. (B) The adaptor plasmid pR6K-amp-2Ty1-rpsL-BSD-3Flag is used for the initial modification of the stop codon of the gene of interest. The plasmids serves as a template for PCR amplification using oligonucleotides that also contain the 50 nt homology arms to the gene of interest. The 2xTy1 and 3xFlag sequences function as internal homology/adaptor regions for the subsequent integration of the targeting cassette. The two R6K-plasmids that contain the tagging cassettes are shown in (C) pR6K-2Ty1-2PreS-mVenus-Biotin-PGK-em7-neo and (D) R6K-2Ty1-2PreS-mVenus-Biotin-T2A-gb3-neo. The tagging cassettes are isolated from the plasmids by digestion with either enzyme NdeI, PacI, AfeI or Eco47III. (E) For subcloning of the tagged C-terminal region to make a targeting construct, the plasmid p15A-amp-HSVtk-TK is used as PCR-template for the minimal plasmid backbone. The plasmid is amplified from very close to the Swa1/SgrA1/PshA1 sites on either end to include the p15A origin, the HSVtk TK gene and the ampicillin resistance gene. All R6K plasmids should be grown in a special host like GB05-pir.

site for PCR amplification and second as the 3′ homology arm for the insertion of the tagging cassette. Initially, the three copies of Flag are outside of the ORF. However, Dre-recombination to delete the PGK neo gene will attach the Flag tags onto the 3′end of the protein tag.

2. Methods

2.1. Strategy design

2.1.1. Obtaining a BAC

Most sequenced metazoan genomes are available as bacterial artificial chromosome (BAC) libraries. BACs are extremely useful because one BAC can cover 100–400 kb genomic DNA. Hence, most metazoan genes and their regulatory elements can be fully encompassed by a single BAC. Available BACs should be inspected using a genome browser such as Ensembl (http://www.ensembl.org/index.html). The Ensembl browser also allows detailed sourcing, comparison and analysis of genomic data, transcripts and proteins, as well as known gene regulatory domains (e.g. CpG islands, histone methylation sites) or DNA polymorphisms. Using the browser, genes or genomic sites of interest can be found by searching either by the gene name or sequence blast. If possible select genes from "Havana" or "Vega" (manually annotated genome) which is more reliable annotated than the default "Ensembl" annotation. Selected genes are optimally displayed in the "Location-based display" or "Gene-based display" mode with genomic annotations of the gene and transcripts. To search for BACs covering this region, the Ensemble browser needs to be configured via the menu tap "Configure this page". In the newly opened "Control Page" window, various BAC libraries (BACmap, CHORI-29 clones and M37-129AB22) are selectable as "External Data". After saving the settings, the browser renews and will display BACs covering the specific genomic location. By clicking on the individual BAC maps you get information about the genomic region covered (Start and Stop positions on the chromosome), Source and Identification number (Id) of the specific BAC. Selected BACs can then be obtained from the specific genome provider (e.g. C57BL/6J BAC from Childrens Hospital Oakland Research Institute (CHORI), http://bacpac.chori.org; bMQ BAC from Source Bioscience http://www.lifesciences.sourcebioscience.com/).

The genomic DNA sequence covered by the selected BAC should be exported in a suitable format, possible by the "Export data" menu tap of the Ensemble browser, into a DNA management

program like VectorNTI (Invitrogen) or Gene Constructor Kit (TEXTCO BioSoftware) for *in silico* design of the final tagged BAC transgene. The BAC sequence, covering the genomic DNA and BAC backbone (which is normally pBACe3.6), is essential for the design of the recombineering strategy (e.g. generation of constructs, oligonucleotides needed for recombineering and sequencing), as well as for the analysis to generate restriction maps for analytical BAC digestion. More information and examples can be found at the web page; http://www.biotec.tu-dresden.de/research/stewart/group-page/genetargeting/.

2.1.2. The protein tag and tagging strategy

The protein tag is inserted into the BAC in two sequential recombineering steps (Fig. 3).

In the first step, a short adaptor cassette generated by PCR is inserted. Two oligonucleotides are synthesized to include the homology arms to the BAC and are attached by PCR to the adaptor cassette, using the plasmid pR6K-amp-2xTy1-rpsL-BSD-3xFlag as template DNA (Figs. 2 and 3). The BAC specific homology arms are 50 nt each and the PCR primers are ~20 nt each, so the two oligonucleotides are ~70 nt each. After PCR, the adaptor cassette consists of (1) terminal homology arms flanking the cassette to target the cassette into the chosen site of the BAC, (2) a streptomycin counterselectable gene (rpsL), (3) blasticidin selectable gene (BSD) framed by (4) 2xTy1 and (5) 3xFlag. The template plasmids are based on the R6K-origin, which requires the Pi protein for their replication [11,14]. Most standard *E. coli* hosts lack Pi so R6K plasmids will not replicate. Consequently, (a) antibiotic resistant colonies caused by carryover of the PCR template (rather than the intended recombineering event) are completely eliminated; and (b) this R6 K plasmid needs to be grown in a special Pi + *E. coli* host. We use GB05-*pir*.

In the second recombineering step, the protein tagging cassette replaces the adaptor cassette. Thereby the tagging cassette is not PCR amplified but generated by restriction digestion of a plasmid. The tagging cassette consists of (1) standard homology regions; 2xTy1 at the 5′ end and 3xFlag at the 3′ end, (2) the protein tag (e.g. mVenus) and (3) the neomycin/kanamycin antibiotic resistance gene.

2.1.3. Design of the tagging cassette – the antibiotic resistance gene

The tagging cassettes 2Ty1-2PreS-mVenus-Biotin-PGK-em7-neo and 2Ty1-2PreS-mVenus-Biotin-T2A-gb3-neo are illustrated

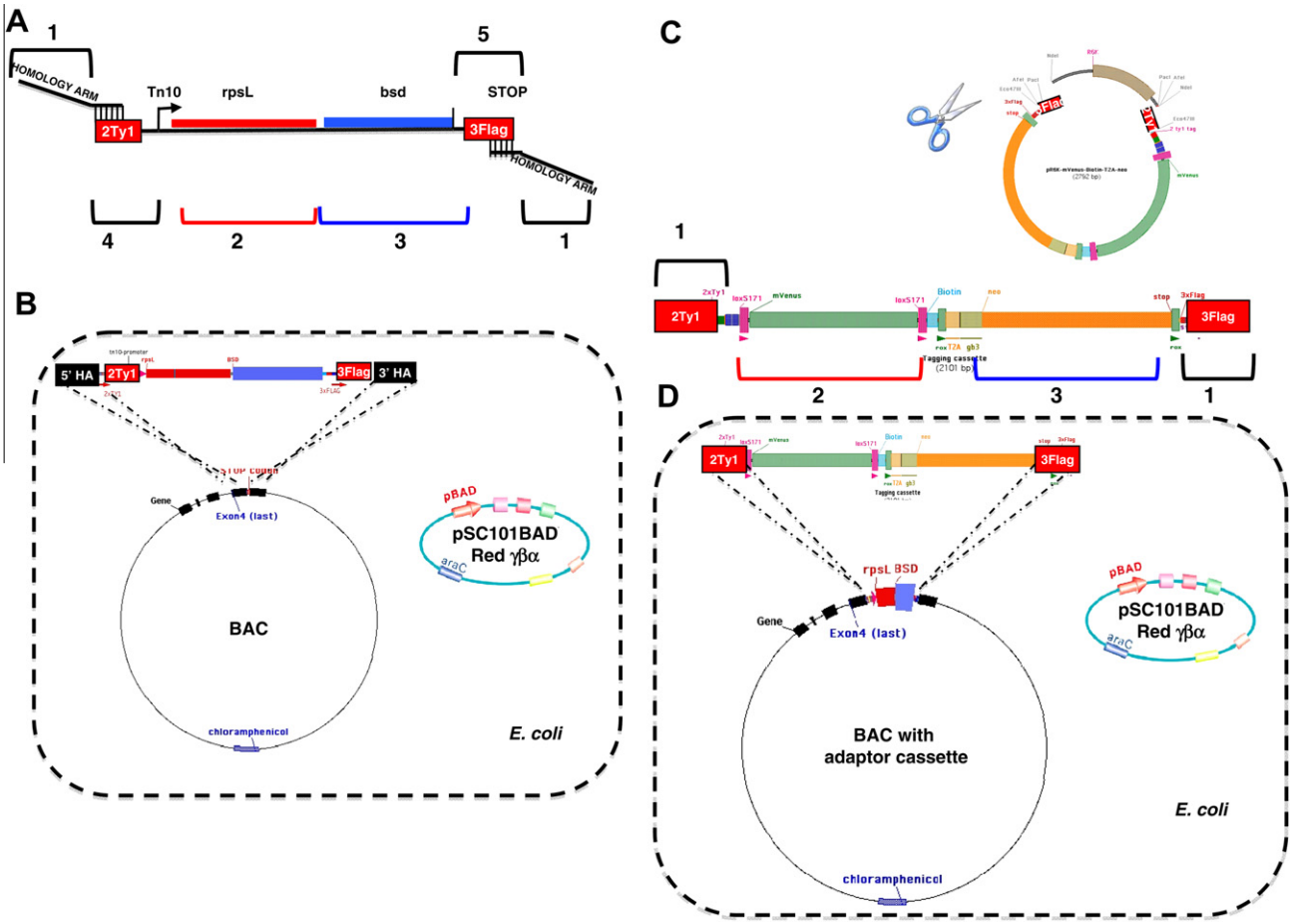

Fig. 3. Generating tagged BAC transgenes by recombineering. In this schematic all steps for sequential BAC tagging are shown. (A) In a first step a short adaptor cassette is generated by PCR. Here primers with the terminal homology arms (1) are attached to the adaptor cassette, using the plasmid pR6K-amp-2Ty1-rpsL-BSD-3Flag as template DNA. The two PCR oligos include the BAC specific homology arms (of 50 nt each) and short cassette specific primers (around 20 nt) for amplification from the plasmid. The PCR generated adaptor cassette consists than of (1) terminal homology arms flanking the cassette to target the cassette in-frame in the STOP codon of the gene on the BAC, (2) a streptomycin counterselectable gene (rpsL), (3) blasticidin selectable gene (bsd) framed by (4) 2xTy1 and (5) 3xFlag sequences. (B) After introduction of the recombineering expression plasmid, pSC101-BAD-Redαβγ, the adaptor PCR product is electroporated and inserted into the BAC by recombineering, replacing the STOP codon of the target gene. Expression of the Red αβγ proteins mediates homologous recombination at the BAC-target site. (C) The tagging cassette is generated by plasmid linearisation of pR6K-2Ty1-2PreS-mVenus-Biotin-PGK-em7-neo or R6K-2Ty1-2PreS-mVenus-Biotin-T2A-gb3-neo. The integrated adaptor cassette contains the homology regions, termed 2xTy1 and 3xFlag, for the tagging cassette thus avoiding error prone PCR amplification of the tagging cassette. The tagging cassette consists of (1) homology region 2xTy1 at the 5′ end and 3xFlag at the 3′ end (2) the protein tags (e.g. mVenus) and (3) the neomycin/kanamycin antibiotic resistance gene. (D) The tagging cassette replaces the adaptor cassette by recombineering. Selection for kanamycin resistant *E. coli* clones and counterselection with streptomycin against the adaptor cassette improves the selection for BAC clones with integrated tagging cassette only.

in Fig. 2C and D. The antibiotic resistance gene in the tagging cassette is configured to express in *E. coli* as well as in mammalian cells [2]. In *E. coli*, the gb3 or em7 promoter drives expression. In mammalian cells, expression is driven by an independent eukaryotic promoter (here PGK) or polycistronically by a T2A peptide [30]. For the T2A polycistronic strategy, expression of antibiotic resistance is directly dependent on the expression of the tagged gene. Thus, if the tagged gene is not expressed in the host cells during selection, the PGK version must be used. When possible, we recommend use of the polycistronic approach because the presence of the PGK promoter/antibiotic resistance gene can interfere with the expression of the tagged gene, either at the transcriptional level by promoter interference or at the post-transcriptional level by impaired RNA processing. Consequently the PGK promoter/antibiotic resistance gene is flanked by the rox target sites for Dre-recombinase, so that this region can be removed in a subsequent site specific recombination step. *Note:* Excision of the resistance gene at the rox sites will attach the 3xFlag sequence in frame to the 3′ end of the protein tag. Thus completion of the Dre recombination can be monitored by the addition and expression of the Flag-tag on the tagged protein.

The final BAC transgene should be first made *in silico*, using a DNA management program so that the sequence of the tagging cassette is inserted in frame at the stop codon, replacing the endogenous stop of the chosen gene. The homology arms to be included in the PCR oligonucleotides are the 50 nts upstream, and the reverse complement of the 50 nts downstream, of the stop codon.

2.2. Preparation of the adaptor cassette by PCR

2.2.1. Materials

You will need the template plasmid pR6K-2Ty1-rpsL-BSD-3Flag for the first step of the sequential recombineering strategy (Fig. 2B).

Design and order the 75mer PCR oligonucleotides for each gene to attach the homology arms. The 5′ oligonucleotide consists of 50 nt upstream of the stop codon followed by 25 nt of the 5′ PCR primer (see Table 1). Thereby the sequence of the protein tag is fused in-frame onto the coding region of the chosen gene. The 3′ oligonucleotide consists of the reverse compliment sequence of the first 50 nt downstream of the stop codon, followed by 25 nt the 3′ PCR primer (Table 1). The stop codon is not included in either homology arm. The quality of the synthesized oligonucleotides is critical. Any mismatch in the homology arms may cause the recombineering to fail or produce mutations in the recombineered BAC transgene.

Use a high fidelity proof reading polymerase, like Phusion or Phusion Flash (Finnzymes) for the PCR. Follow the instructions of the manufacturer, however the settings of the PCR should be optimized by variation of the standard parameters (annealing temperature, DMSO addition, hot start, etc.) until a good amplification (at least 400 ng/100 μl reaction) of the PCR product without secondary PCR contamination is achieved. The ratio of PCRproduct to unicorporated PCRoligo should be high because unincorporated oligonucleotides will impair the recombineering efficiency. The PCR product is concentrated and purified using a standard PCR purification kit (we recommend MSB Spin PCRapace, Invitek). For the elution of the PCR product use ddH_2O only, NOT buffer! The final concentration of PCR product should be $\geqslant$100 ng/μl. Use immediately or store at −20 °C.

2.3. Recombineering

2.3.1. General

BACs are usually shipped by the genome suppliers inside an *E. coli* host strain. Because about 5% of BACs are not the same as the annotated version on the genome browser, we usually check the BAC identity by restriction digestion or PCR of the chosen cassette insertion site. (NB; if the recombineering step does not work, sequencing this PCR product is a good idea.) For recombineering, the first step involves the introduction of an inducible Red/ET expression plasmid into the *E. coli* host harbouring the chosen BAC. Our Red/ET expression plasmid encodes the three genes from the λ phage Red operon, Redγ (an inhibitor of RecBCD), Redβ (a DNA annealing protein) and Redα (a 5′–3′ exonuclease; see [16] for a recent discussion).

Each step in the protocol is characterized by a resistance to a specific antibiotic. Selecting for one or the other facilitates the process of recombineering. Usually, the BAC carries chloramphenicol resistance; our Red/ET expression plasmid conveys tetracycline resistance. The adaptor cassette conveys blasticidin resistance and the tagging cassette conveys kanamycin resistance, so that the subsequent acquisition of blasticidin and thereafter of kanamycin resistance indicates the insertion of the cassettes into the BAC by homologous recombination.

2.3.2. Transformation with Red/ET expression plasmid

Materials: For recombineering, the host *E. coli* strain carrying the chosen BAC is transformed with the inducible Red/ET expression plasmid pSC101-BAD-Redαβγ or pSC101-BAD-Redαβγ*A* (Fig. 2A; [37]). These two plasmids are effectively the same for the applications described here. A benchtop centrifuge at 2 °C; an electroporator (e.g. Electroporator 2510, Eppendorf) and electroporation cuvettes (1 mm gap, BioRad). Prepare 10% L-arabinose (Sigma A-3256) in ddH_2O and freeze aliquots at −20 °C.

Method: Before starting with the experiment, you should have prepared the adaptor cassette by PCR as described above and have streaked out the glycerol stock of the *E. coli* BAC clone you obtained from the genome provider on LB plates plus 15 μg/ml chloramphenicol.

Day 1: Set up an overnight culture by picking 6–10 colonies of *E. coli* carrying the BAC from the plate and inoculate into microfuge tubes containing 1.0 ml LB medium plus 15 μg/ml chloramphenicol. Puncture a hole with a needle (25G) in the lid for aeration. Incubate at 37 °C overnight with shaking (we use Eppendorf Thermomixer).

Day 2: Before starting: Chill ddH_2O (or alternatively ddH_2O with 10% glycerol) and the electroporation cuvettes on ice. Precool the benchtop centrifuge to 2 °C.

(1) Set up two microfuge tubes containing fresh 1.4 ml LB medium with 15 μg/ml chloramphenicol and inoculate with 30 μl of fresh overnight culture.
(2) Culture for 2 h at 30 °C and 40 min at 37 °C by shaking at 1000 rpm.

Table 1
Primer sequences for PCR amplifications.

pR6K-2Ty1-rpsL-BSD-3Flag		
pR6K-2Ty1-2PreS-mVenus-Biotin-PGK-em7-neo	5′ primer	...GAAGTGCATACCAATCAGGACCCGC
R6K-2Ty1-2PreS-mVenus-Biotin-T2A-gb3-neo	3′ primer	...GTTCCTTCTTCACTGTCCCTTATTC
p15A-HSV-Tk-amp	5′ primer	...ATTTAAATCACCGGTGACCCGGGTC
	3′ primer	...ATTTAAATCGCCGGCGACTTAAGTC

Note, the following four steps should be done as quickly as possible

(3) Prepare the cells for electroporation. Centrifuge for 30 s at 9000 rpm in the cold benchtop centrifuge. Discard the supernatant by quickly tipping out the supernatant, and place the cell pellet on ice. Resuspend the pellet with 1 ml ice cold ddH_2O, pipetting up and down three times to mix the suspension. Repeat the centrifugation at 11,000 rpm, tip out the supernatant and resuspend the cells again. Centrifuge and tip out the supernatant once more to leave 20–30 μl of liquid in the tube with the pellet. Keep the tube on ice.
(4) Add 2 μl (about 10–100 ng) pSC101-BAD-Redαβγ or water as a negative control to your cell pellets. Mix briefly. Keep the tubes on ice. Transfer the cell suspensions from the tubes to the chilled electroporation cuvettes.
(5) Electroporate at 1250 V, 10 μF, 600 Ω = ~5 ms pulse time (Electroporator 2510, Eppendorf) using an 1 mm gap electroporation cuvette. Other devices can be used, but high voltage is recommended.
(6) Add 1 ml LB medium without antibiotics to the cuvettes and return the cells to the microfuge tubes.
(7) For recovery incubate at 30 °C for 70 min, shaking at 1000 rpm. (Note: the Red/ET expression plasmid pSC101Redαβγ replicates at 30 °C but not at 37 °C, because replication of the pSC101 plasmid is temperature sensitive.)
(8) Using a spreader, plate 50 μl cells on LB agar plates containing the antibiotics to select for the BAC and the Red expression plasmid (i.e. 15 μg/ml chloramphenicol plus 5 μg/ml tetracycline). Incubate the plates at 30 °C overnight (or for at least 15 h). Protect the plates from light because tetracycline is light-sensitive. Make sure the cells stay at 30 °C otherwise the pSC101-BAD-Redαβγ plasmid will be lost.

2.3.3. Inserting the adaptor cassette into a BAC

As described above in Section 2.1.2, the protein tag will be integrated by two sequential recombineering steps. Firstly a PCR generated adaptor cassette is inserted into the BAC to introduce homology arms for the tagging cassette. In the second recombineering step, described below in Section 2.3.4, the protein tagging cassette replaces the adaptor cassette using the homology arms (2xTy1 and 3xFlag) introduced with the adaptor cassette.

The protocol to insert the adaptor cassette is very similar to the protocol above (Section 2.3.2) for transformation with the Red/ET expression plasmid, but additionally involves the addition of L-arabinose shortly before preparing electrocompetent cells for electroporation of the PCR amplified adaptor cassette. Note, the BAD promoter is not only induced by L-arabinose but also repressed by glucose so make sure glucose is not included in the culture medium. Also note, this protocol continues on from the steps above. Hence the next step is day 3.

Day 3: Set up overnight cultures by picking 5–10 colonies from the tetracycline and chloramphenicol plate. Inoculate one microfuge tube containing 1.0 ml LB medium plus the antibiotics. Puncture a hole in the lid of the tubes for aeration. Incubate the cultures while shaking at 30 °C overnight.

Day 4: Before starting: Chill ddH_2O (or alternatively ddH_2O with 10% glycerol) and electroporation cuvettes on ice. Also precool the benchtop centrifuge to 2 °C.

(1) Set up two microfuge tubes labeled (+) and (−) containing fresh 1.4 ml LB medium with 15 μg/ml chloramphenicol plus 3 μg/ml tetracycline and inoculate with 40 μl from the overnight culture (i).
(2) Culture for about 2 h at 30 °C, shaking at 1100 rpm until OD_{600nm} = 0.3.
(3) Add 20 μl 10% L-arabinose to one of the tubes labeled (+) to induce the expression of the Red proteins (ii). Incubate at 37 °C shaking for 40 min (iii).
(4) Prepare the cells for electroporation. Centrifuge for 30 s at 9000 rpm in the cold benchtop centrifuge. Discard the supernatant by quickly tipping out the supernatant, and place the cell pellet on ice. Resuspend the pellet with 1 ml ice cold ddH_2O, pipetting up and down three times to mix the suspension. Repeat the centrifugation at 11,000 rpm, tip out the supernatant and resuspend the cells again. Centrifuge and tip out the supernatant once more to leave 20–30 μl of liquid in the tubes with the pellet. Keep the tubes on ice.
(5) Add 2 μl (~500 ng) of PCR amplified adaptor cassette to the pellet in each of the two microfuge tubes (+) induced and (−) uninduced, mix and pipette the cells suspension into the ice cold electroporation cuvettes.
(6) Electroporate at 1350 V, 10 μF, 600 Ω = ~5 ms pulse (Electroporator 2510, Eppendorf) using 1 mm gap electroporation cuvettes.
(7) Add 1 ml LB medium without antibiotics to the cuvette. Mix the cells carefully by pipetting up and down and pipette back into the microfuge tube. Incubate the cultures at 37 °C with shaking for 60 min for recovery.
(8) Streak 100 μl of the cultures with a spreader onto LB agar plates (pH 8.0) containing 15 μg/ml chloramphenicol (to select for the BAC) and 30 μg/ml blasticidin (to select for the integration of the adaptor cassette). Usually 100 μl is sufficient, but also spin down the remaining 900 μl, re-suspend to 100 μl and streak onto a second plate (iv).
(9) Incubate the plates at 37 °C overnight.

You should obtain around 100 colonies and the ratio of induced to uninduced colonies should exceed 100:1 (v).

Notes:

(i) the simplest control experiment involves a parallel tube where all things are the same except that L-arabinose is not added (uninduced control). You should not get resistant colonies from this control;
(ii) do not use D-arabinose;
(iii) pay attention to the temperature change from 30 to 37 °C. Recombination requires 37 °C, whereas the expression plasmid pSC101-BAD-Redαβγ requires 30 °C for replication and will not replicate at 37 °C. There are about five copies of this temperature-sensitive plasmid per cell. Any daughter cell will still have on average 2–3 copies left. The plasmid will be lost after longer incubation such as 37 °C overnight so that the BAC can be purified without having this plasmid contaminating the preparations.
(iv) when eliminating the pSC101-BAD-Redαβγ plasmid by incubation at 37 °C, do not continue tetracycline selection.
(v) the fidelity of recombineering is very high so that nearly all antibiotic resistant clones have the intended recombination event. However unwanted events such as deletions between internal repeats within the BAC, may have occurred. The frequency of these unwanted events varies greatly from one BAC to another.

2.3.4. Exchanging the adaptor cassette for the tagging cassette

Materials: pR6K-2Ty1-2PreS-mVenus-Biotin-PGK-em7-neo or pR6K-2Ty1-2PreS-mVenus-Biotin-T2A-gb3-neo plasmid.

Now the tagging cassette will be inserted into the BAC to replace the adaptor cassette. This protocol is virtually identical to the above two protocols, except the PCR amplified adaptor cassette

is replaced with the tagging cassette, which is prepared by restriction digestion.

Day 5:

(10) Repeat the protocol to transform pSC101-BAD-Redαβγ (Section 2.3.2) except also select for the adaptor modified BAC as well as the pSC101-BAD-Redαβγ plasmid (i.e. 30 μg/ml blasticidin plus 5 μg/ml tetracycline). Incubate the plates at 30 °C overnight (or for at least 15 h). Protect the plates against the light because tetracycline is light-sensitive. Make sure the cells stay at 30 °C otherwise the pSC101-BAD-Redαβγ plasmid will be lost.

In the meantime prepare the appropriate tagging cassette from plasmid pR6K-2Ty1-2PreS-mVenus-Biotin-T2A-gb3-neo or pR6K-2Ty1-2PreS-mVenus-Biotin-PGK-em7-neo (Fig. 2C,D). Therefore digest 10 μg of the plasmid with a restriction enzyme, i.e. NdeI, PacI, AfeI, or Eco47III (NEB), according the instructions of the manufacture. Check for complete digestion by loading an aliquot on an agarose gel and purify the tagging cassette using a DNA purification kit (we recommend MSB Spin PCRapace, Invitek). For the elution of the tagging cassette use 10 μl ddH_2O only, NOT buffer! The final concentration of DNA product should be $\geqslant$100 ng/μl. Use immediately or store at −20 °C.

(11) Prepare now the *E. coli* containing the BAC with adaptor cassette and the pSC101-BAD-Redαβγplasmid for the final recombineering step as described above in Section 2.3.3 starting with Day 3 up to step 8 using LB medium (pH 8.0) with 30 μg/ml blasticidin plus 4 μg/ml tetracycline as culture medium.
(12) After recovery streak 100 μl of the cultures with a spreader onto LB agar plates containing 10 μg/ml kanamycin (to select for the integration of the tagging cassette) and 200 μg/ml streptomycin (to counter select against unrecombined BACs still containing the adaptor cassette). Plating 100 μl is usually sufficient, but if necessary spin down the remaining 900 μl, re-suspend to 100 μl and streak onto a second plate. Protect the plates from light because streptomycin is light sensitive! Incubate the plates at 37 °C overnight.

You should obtain around 100 colonies and the ratio of induced to uninduced colonies should exceed 100:1.

2.4. Quality control of recombineering

To check for successful recombineering of the tagged BAC transgene, we routinely perform two tests. First, we examine the BAC transgene using restriction enzymes. To isolate the BAC DNA, pick five or more colonies for BAC mini-preps and for comparison the original unmodified BAC.

Second, the 5′ and 3′ recombineering junction is sequenced either after PCR amplification or directly using a BAC mini-prep as template. Sequencing across the 5′ homology arm and PCR primer region into the 5′ end of the tagging cassette ensures that the reading frame is intact and excludes the possibility that the 5′ synthetic oligonucleotide has introduced an unwanted mutation. Sequencing the 3′ recombineering junction is mainly to confirm the correctness of the 3xFlag. Usually we do not examine the 3′ recombineering junction because mutations here are unlikely to have any functional impact and the overall rate of junction mutagenesis is usually low.

2.5. BAC mini-prep protocol

Use the Plasmid Purification Buffers from Qiagen; Buffer P1 (resuspension buffer; 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, pH 8.0, 100 μg/ml RNase A), Buffer P2 (lysis buffer; 200 mM NaOH, 1% SDS); Buffer P3 (neutralization buffer; 3.0 M potassium acetate, pH 5.5); ThermoMixer (Eppendorf).

(1) Start an overnight culture from single colonies in 1.6 ml LB plus 15 μg/ml chloramphenicol and 15 μg/ml kanamycin in 2 ml microfuge tubes. Puncture the cap for aeration. Incubate in a ThermoMixer at 37 °C, 1000 rpm for 14–16 h.
(2) Transfer from each culture 200 μl into fresh microfuge tubes. These aliquots serve as backup cultures of the individual clones.
(3) Spin down the remaining over night cultures at 11,000 rpm for 1 min and discard the supernatant.
(4) Add 200 μl of buffer P1 and resuspend thoroughly by vortexing. Check for complete resuspension of the cell pellets.
(5) Add 200 μl of buffer P2 and mix by inverting the sealed tube 4–6 times. Incubate at room temperature for not more than 5 min.
(6) Add 200 μl of buffer P3 and mix immediately by inverting the sealed tube 4–6 times. Leave at RT for 10 min.
(7) Spin down the white lysate in a centrifuge at highest speed for 10–20 min at 4 °C.
(8) Set up new 1.5 ml microfuge tubes with 500 μl of 100% isopropanol in each tube.
(9) Transfer the clear supernatant into the new tubes.
(10) Cover the rack with the lid and mix vividly by shaking 5–10 times.
(11) Spin at highest speed for 20 min at 4 °C.
(12) Discard the supernatant.
(13) Gently add 1 ml of 70% ethanol.
(14) Carefully aspirate off the supernatant and invert the tubes on a piece of tissue to evaporate residual ethanol. Alternatively dry the DNA pellet in the ThermoMixer at 45 °C for up to 10–20 min.
(15) Dissolve the DNA pellet in 20 μl of H_2O or directly in digestion buffer for the chosen restriction enzyme.
(16) After restriction digestion, run the samples on a 0.7% agarose gel slowly at 60 V or less to resolve the BAC DNA cleavage pattern.

The usual yield from a mini-prep is sufficient for one restriction analysis or one sequence reaction. To purify larger or cleaner amounts of BAC-DNA use the maxi-prep protocol.

2.6. BAC maxi-prep protocol

Use the Plasmid Purification Buffers from Qiagen; Buffer P1 (resuspension buffer; 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, pH 8.0, 100 μg/ml Rnase A), Buffer P2 (lysis buffer; 200 mM NaOH, 1% SDS); Buffer P3 (neutralization buffer; 3.0 M potassium acetate, pH 5.5).

Note: BAC DNA can be prepared by using NucleoBond BAC 100 (Clontech) following the manufacturer instruction. This kit allows recovery of about 100 μg DNA. Otherwise proceed with the protocol below.

(1) Inoculate a starter culture of 1.6 ml LB plus 15 μg/ml chloramphenicol and 15 μg/ml kanamycin. Puncture the cap for aeration. Incubate for ∼8 h at 37 °C with vigorous shaking at 1000 rpm.
(2) Dilute 1 ml of the starter culture into 250 ml LB plus 15 μg/ml chloramphenicol and 15 μg/ml kanamycin. Grow at 37 °C overnight with vigorous shaking.
(3) Harvest the bacterial cells by centrifugation at 6000*g* for 15 min at 4 °C.
(4) Resuspend the bacterial pellet in 10 ml Qiagen Buffer P1.

(5) Add 10 ml Qiagen Buffer P2, mix gently but thoroughly by inverting 4–6 times, and incubate at room temperature for not more than 5 min.
(6) Add 10 ml chilled Buffer P3, mix immediately but gently by inverting 4–6 times, and incubate on ice for 10 min.
(7) Centrifuge at ⩾20,000*g* for 30 min at 4 °C.
(8) Transfer and filter the supernatant containing the BAC DNA through a folded filter paper (Whatman) pre-wetted with distilled water in a 50 ml conical tube. Repeat the filtering until the supernatant is clear.
(9) Precipitate DNA by adding 0.6 volumes (approximately 18 ml) isopropanol. Mix and centrifuge immediately at ⩾15,000*g* for 30 min at 4 °C (e.g. Beckman JLA 16250). Carefully decant the supernatant.
(10) Wash the DNA pellet with 5 ml 70% ethanol, and centrifuge at ⩾15,000*g* for 15 min. Carefully decant the supernatant without disturbing the pellet.
(11) Air-dry the pellet for 5–10 min, and redissolve the DNA by shaking in 500 μl ddH_2O. Transfer the DNA into a clean 1.5 ml microfuge tube.

Contaminating genomic DNA can be removed by overnight treatment with Plasmid-Safe™ ATP-Dependent DNase (Epicentre) according to the manufacturers instructions.

(12) Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the sample, vortex thoroughly and centrifuge at 10,000*g* for 5 min to separate the phases.
(13) Carefully transfer the top aqueous layer to a clean tube. Discard the lower inter- and phenol face.
(14) Repeat the extraction once or twice.
(15) Precipitate the DNA by adding 0.7 volumes isopropanol.
(16) Spin the tube in a microcentrifuge at full speed for 5 min at 4 °C.
(17) Carefully remove and discard the supernatant. Wash the pellet with 500 μl ice-cold 70% ethanol.
(18) Spin the tube in a microcentrifuge at full speed for 5 min at 4 °C. Carefully aspirate off all of the supernatant.
(19) Air dry the pellet for approximately 15 min at room temperature to evaporate residual ethanol.
(20) Dissolve the DNA pellet in 200–500 μl sterile H_2O and store at 4 °C. More than 25 μg should have been retrieved.

2.7. Transfection of mouse ES cells to establish stable BAC transgenic lines

Mouse embryonal stem cells (mESCs) are seeded 1 day before transfection with a density of 2.5×10^5 cells/well of 6-well plates in 2 ml mES medium (Dulbecco's Modified Eagles Medium, DMEM with 4.5 g/l glucose (GIBCO); 15% Fetal Calf Serum; 2 mM L-glutamine (GIBCO); 0.1 mM MEM non-essential aminoacids (GIBCO); 1 mM sodium pyruvate (GIBCO); 0.1 mM β-mercaptoethanol (Sigma), 1000 U/ml Leukemia Inhibitory Factor (ESGRO (LIF), Millipore); optional with 100 U/ml, streptomycin, 100 U/ml penicillin (GIBCO)). Cells should be not more than 70% confluent on the next day. To keep cells proliferating change the medium with 2 ml of fresh mES medium. Transfection is carried out using Lipofectamine LTX PLUS reagent (Invitrogen) and performed using the supplier's protocol. In brief, dilute 1 μg of BAC DNA in 500 μl Opti-MEM (GIBCO) and mix well. Add to the diluted DNA 2.5 μl of PLUS reagent, mix and incubate for 5 min at room temperature. Then add 6.25 μl of the Lipofectamine LTX solution, mix gently and incubate the mixture for at least 20 min at room temperature. Transfect the cells by distributing the entire DNA–lipid mixture dropwise to the 2 ml mES medium of one well. For stable transfection, the cells are subjected to 100–200 μg/ml G418 (neomycin) selection in mES medium 24 h after lipofection. The medium should be changed every day. Growing resistant ESC colonies should be detectable after 6–12 days.

2.7.1. Picking colonies for clonal analysis

To test for expression of the tagged protein, several ES cell clones should be evaluated. Because the number of integrated BACs can vary, expression can differ between individual clones.

(1) Replace the mES medium with 2 ml PBS or pure DMEM (GIBCO) without FCS.
(2) Use of an inverted phase contrast/fluorescence microscope at 2.5 to 5× magnification can help for better visualization. Alternatively, remove most medium and tip the plate so that some colonies can be seen interfering with the meniscus at the edge of the remaining medium. Scrape a single colony from the plastic with a sterile 20 μl pipette tip. Suck the loose colony into the tip. If a GFP or Venus tagged protein is strongly expressed, fluorescent colonies can be used to preselect for positive clones.
(3) Collect at least four colonies and place each into a separate well of a 96 well dish.
(4) Add 25 μl of 0.25% trypsin–EDTA to each well. Mix by gently tapping on the plate, not by pipetting!
(5) Incubate for 5 min at 37 °C.
(6) Add 125 μl ES medium and pipette up and down about 15 times to resuspend the cells as a single cell suspension. Try to limit the formation of foam.
(7) If working with a feeder-dependent ES cells, transfer the entire cell suspension to a 96 well plate that has been preseeded with MEFs.
(8) Change the medium daily. After 1 or 2 days, the clones should be transferred and expanded in wells/dishes of increasing size according to the growth of each clone.

Analyze the clones for expression of the tagged proteins following the Section 2.11.

2.8. Generation of gene targeting constructs by recombineering

2.8.1. General

Genetic alterations in mouse ES cells using homologous recombination requires the design and construction of targeting vectors to modify target genes. Recombineering simplifies targeting vector construction for the modification of mammalian genomes [2]. More information and examples can be found in [12], or at the web page; http://www.biotec.tu-dresden.de/research/stewart/group-page/genetargeting/.

The design of efficient targeting constructs is guided by three parameters. The more that these parameters can be incorporated in a targeting construct, the greater the chance of success.

(1) Isogenic is better. Homologous recombination relies on sequence identity between the targeting construct and the genome. The best way to secure identity is to use the target genome as the source of the homology arms in the targeting construct. Hence when using 129 ES cells (e.g. E14TG2α), it is advisable to use 129 genomic DNA (e.g. from M37-129AB22 BAC library). Or when using C57Bl6 ES cells (e.g. JM8; [22] use C57Bl6 genomic DNA (e.g. RP23 or RP24 BAC libraries).
(2) Longer homology arms are better. However in practice, homology arms longer than 5 kb create a problem for Southern blot analysis of the targeting event. So we recommend use of 4–5 kb homology arms. If either arm is shorter than 3 kb, targeting efficiency will probably be impaired.

(3) Promoterless selection is better. If the target gene is expressed in the host cells, then the targeting construct can be designed so that expression of the antibiotic resistance gene is driven by the endogenous promoter after homologous recombination. Consequently a greater proportion of resistant colonies will be due to correct targeting.

Earlier in this article we described the construction of BAC transgenes. Now we describe the generation of gene targeting constructs using Red/ET recombineering from these BAC transgenes. As illustrated in Fig. 4, the extra recombineering steps involve the same basic recombineering protocol. However the PCR product is a plasmid backbone, including origin of replication and selectable gene, flanked by homology arms. In this application, the homology arms define the region that is to be copied from the BAC into the plasmid.

2.8.2. Protocol for generating subcloning vectors by PCR – oligo design

In the subcloning step, the tagging cassette plus regions of 4–5 kb either side will be copied from the tagged BAC into a PCR amplified p15A vector (Fig. 2E) to create a plasmid subclone. The ends of the 4–5 kb regions are defined by 50 nt HAs, which are selected by ordering ~75 nt oligonucleotides (including 25 nt for PCR primers at their 3′ ends; Table 1) and attached to the p15A/ampicillin vector by PCR. Technically, this is very similar to the tagging recombineering step described above. However, conceptually it is different because the tagging step is based on insertion of a PCR product into a BAC whereas subcloning is based on copying a defined region from the BAC into a plasmid. This subcloning mechanism has also been called 'gap repair' because the p15A/ampicillin PCR product must circularize before it can replicate. In practice, the key difference between inserting a cassette and subcloning is the orientation of the homology arms. This is illustrated in Fig. 4 and requires your attention.

The two oligos you need for subcloning act as linkers between the genomic DNA fragment you want to clone from a BAC and the p15A-HSV-TK-amp backbone. The design of the oligos is best done by generating the construct first *in silico* using DNA management software. The 5′ 50 nt homology arm (HA), which defines the 5′ end of the sequence you want to subclone, starting around 4–5 kb upstream from the tagging cassette, plus 25 nt PCR primer to the p15A plasmid template. The 3′ oligonucleotide is similarly composed and directed to the other end of the p15A acceptor plasmid, plus the 3′ HA, which should be located around 4–5 kb downstream from the tagging cassette (Fig. 4). Before selecting the 5′ and 3′ HAs, look for sequence repeats shared between the candidate 50 nt HAs and the entire BAC, because any repeat >15 nt can produce unwanted recombineering products.

Note: With the p15A vector it is possible to counterselect ES cells after targeting for random integrated tagging cassettes. This is especially recommended when the PGK promoter drives antibiotic gene expression (e.g. tagging cassette-2Ty1-2PreS-mVenus-Biotin-PGK-em7-neo). Therefore include in one PCR primer a unique restriction enzyme cleavage site. Linearisation of the subclone cassette by this enzyme then generates a tagging cassette remaining associated with the thymidine kinase gene (TK) at the 3′- or 5′-end of the homology arm. Integration by homologous recombination in ES cells will exclude the integration of the TK gene outside of the homology arms. However not wanted random integration events allow the integration of the TK gene. These cells can be counterselected by FIAU or gancyclovir [3].

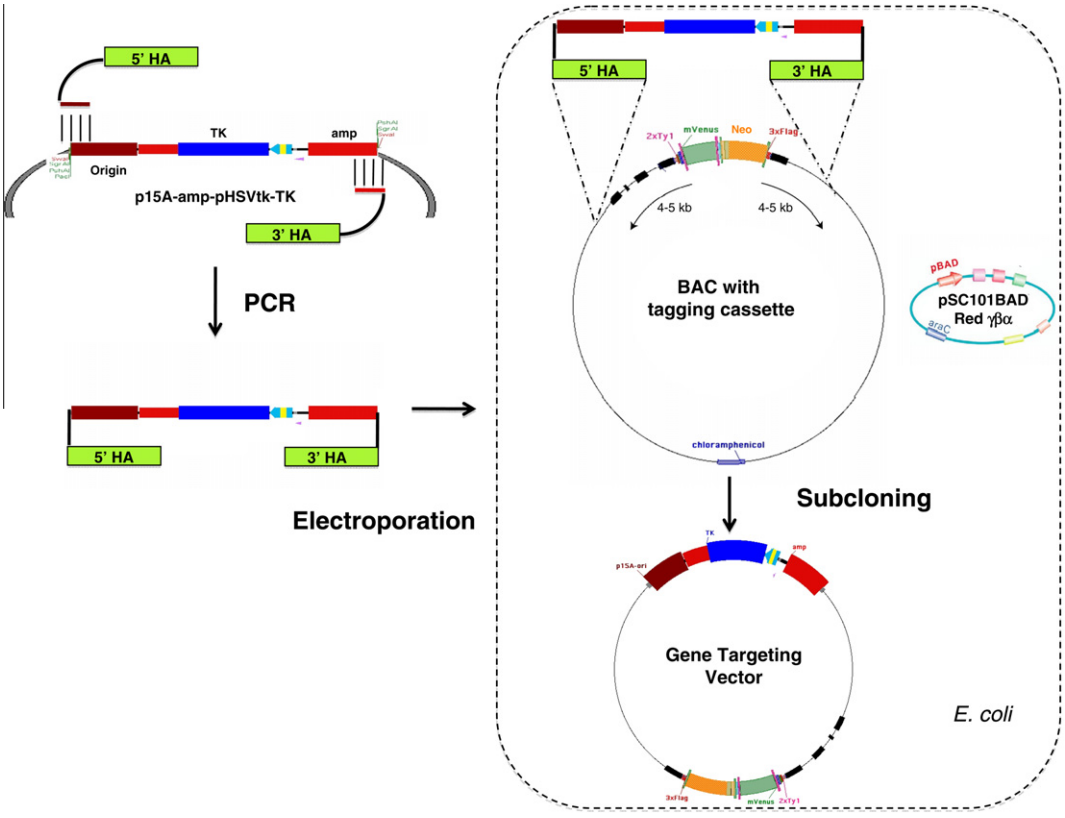

Fig. 4. Subcloning the gene targeting construct from the BAC transgene. The subcloning vector is PCR amplified from the plasmid p15A-amp-HSVtk-TK using oligonucleotides containing the homology arms (HA) at their 5′ end and PCR primers at their 3′ ends. Note the orientation of the homology arms with respect to the PCR primers. The PCR product is electroporated into an *E. coli* host harbouring the target BAC (illustrated) containing the region to subclone and recombineering expression plasmid, pSC101-BAD-Redαβγ. Selection for ampicillin resistance (from the linear vector) plus kanamycin resistance (from the tagging cassette) selects for recombinants.

2.8.3. Preparation of the vector cassette by PCR

Materials: You will need the template plasmid p15A-HSV-Tk-amp (Fig. 2E).

The high fidelity PCR is essentially the same as described earlier in the section "Preparation of the tagging cassette by PCR".

2.8.4. Subcloning of a specific region from a BAC by recombineering

Materials: You need the *E. coli* strain carrying the tagged BAC and the plasmid pSC101-BAD-Redαβγ. This is most easily obtained by culturing the cells at 30 °C after the tagging step described in step 8 of "Inserting the tagging cassette into a BAC" above. Instead of streaking colonies onto chloramphenicol + kanamycin plates and incubating at 37 °C, streak colonies onto 3 μg/ml tetracycline (to select for the pSC101 BAD αβγ plasmid) + 15 μg/ml kanamycin plates and incubate at 30 °C.

Alternatively transform the *E. coli* strain carrying the BAC with the Red/ET expression plasmid before staring with the protocol. For detailed information about the transformation with Red/ET expression plasmid see the Section 2.3.2 above.

Day 1:

To start overnight cultures, pick 5–10 colonies of the *E. coli* strain carrying the BAC and pSC101-BAD-Redαβγ from the plate and inoculate in a 2 ml microfuge tube containing 1.5 ml LB medium plus tetracycline (3 μg/ml), chloramphenicol (15 μg/ml) and kanamycin (15 μg/ml). Puncture a hole in the lid of the tubes for aeration. Incubate the cultures while shaking at 30 °C overnight.

Day 2:

Before starting: Chill ddH_2O (or alternatively ddH_2O with 10% glycerol) and 1 mm gap electroporation cuvettes on ice for at least for 2 h. Precool a benchtop centrifuge to 2 °C.

(1) Set up two microfuge tubes, lid-punctured for aeration (a) for the subcloning, (b) as uninduced control, containing 1.4 ml LB medium conditioned with the same antibiotics as used for the overnight culture. Inoculate them with 30 μl fresh overnight culture.
(2) Incubate the tubes at 30 °C for approximately 2 h, shaking at 1000 rpm until OD_{600nm} ~0.3.
(3) Add 20 μl 10% L-arabinose to tube (a), giving a final concentration of 0.1–0.2%. This will induce the expression of the Red/ET proteins. Leave the other tube without L-arabinose induction as non-induced negative control. Incubate the two tubes now at 37 °C, shaking for 45 min to 1 h.

Note: It is important that cells are incubated at 37 °C because Red-mediated homologous recombination does not occur at 30 °C (unpublished results).

(4) Prepare the cells for electroporation. Centrifuge for 30 s at 9000 rpm in the cold benchtop centrifuge. Discard the supernatant by quickly tipping out the supernatant, and place the cell pellet on ice. Resuspend the pellet with 1 ml ice cold ddH_2O, pipetting up and down three times to mix the suspension. Repeat the centrifugation at 11,000 rpm, tip out the supernatant and resuspend the cells again. Centrifuge and tip out the supernatant once more to leave 20–30 μl of liquid in the tubes with the pellet. Keep the tubes on ice.
(5) Add 1–2 μl (100–200 ng) of your prepared linear vector PCR product to the pellets of the two microfuge tubes (a) induced and (b) uninduced, and pipette the mixture into the chilled electroporation cuvettes.
(6) Electroporate at 1350 V, 10 μF, 600 Ω. This setting applies to an Electroporator 2510 (Eppendorf) using a 1 mm gap electroporation cuvette.
(7) Add 1 ml LB medium without antibiotics to the cuvette. Mix the cells carefully by pipetting up and down and pipette back into the microfuge tube. Incubate the cultures at 37 °C for recovery with shaking for 60 min.

Streak the cultures with a spreader onto LB agar plates (100 μl is usually sufficient, if necessary spread the other 900 μl on a second plate) containing ampicillin (100 μg/ml) and kanamycin (15 μg/ml). Incubate the plates at 37 °C overnight. The Red/ET expression plasmid (pSC101 BAD αβγ) will disappear at 37 °C. The plates should not contain tetracycline, otherwise pSC101 BAD γβα will persist or the cells will die. The ratio of bacterial colonies from culture (a) induced to (b) uninduced should exceed 100:1.

2.8.5. Verification and preparation of DNA for targeting

Use standard plasmid or BAC preparation protocols and analyze the purified DNAs by restriction digestion and agarose gel electrophoresis.

Linearise 20–40 μg plasmid by a suitable digestion enzyme, e.g. SwaI, SgrAI, or PshAI (NEB), which cleave at the junction between the tagging cassette and the plasmid backbone. Make sure that the selected enzyme does not cleave in the targeting cassette. Purify the linearised fragments by phenol/chloroform extraction, precipitate the DNA by ethanol precipitation and resuspend in 30 μl PBS.

2.9. Targeting in mouse ES cells

Materials: Electroporator (e.g. Gene Pulser, BioRad), 4 mm gap electroporation cuvettes (BioRad).

Seed mouse embryonic stem cells (mESCs) 1 day before electroporation at a density of 1–2 × 10^6 cells in 10 cm dishes in mES medium (Dulbecco's Modified Eagles Medium, DMEM with 4.5 g/l glucose (GIBCO); 15% Fetal Calf Serum; 2 mM L-glutamine (GIBCO); 0.1 mM MEM non-essential aminoacids (GIBCO); 1 mM sodium pyruvate (GIBCO); 0.1 mM β-mercaptoethanol (Sigma), 1000 U/ml Leukemia Inhibitory Factor (ESGRO (LIF), Millipore); optional with 100 U/ml, streptomycin, 100 U/ml penicillin (GIBCO). On the next day you should have about 5 × 10^6 cells for electroporation. Wash the cells once in PBS (GIBCO). Harvest the cells by incubation in 1 ml of 0.05% Trypsin/EDTA (GIBCO) for 3 min at 37 °C. Add 10 ml of mES medium and pipette up and down to become a single cell suspension. Spin down the cell suspension at 800g and resuspend the cell pellet in 900 μl PBS. The electroporation is carried out with a Gene Pulser (BioRad) using the exponential settings (4 mm Distance, 250 V, 500 μF) adding 20–40 μg linearised targeting construct to 5 × 10^6 cells. A good electroporation has a pulse of about 5.4 ms. After electroporation quickly plate the cells in five fresh 10 cm dishes with 10 ml mES medium. Exchange medium the next day using fresh mES medium containing 100–200 μg/ml G418. The mES medium with G418 should be changed every day. Resistant ESC colonies should be detectable after 6–12 days.

The expression of the tagged protein after successful gene targeting should be tested and compared by analyzing several distinct single cell colonies as described before in the Section 2.7.1.

2.10. Genotyping using long range PCR

The successful targeting of the genomic location is usually confirmed by Southern blot analysis. However long range PCR (LR-PCR) offers a fast alternative. In the case that the tagged protein is expressed in the tagged cells, the combination of LR-PCR and Western blotting can obviate the need for Southern analysis.

LR-PCR can amplify DNA fragments up to 20 kb long so it is possible to analyze each side of the targeting event from outside of the 5 kb homology arm to the tagging cassette. Order two 25 nt primer pairs, one primer pair for the amplification of the 5′ region and

another for the 3′ region (See Fig. 5). The 5′ distal primer should be taken from the genomic DNA sequence a little upstream of the 5′ end of the 5′ homology arm. Similarly the 3′ distal primer pair should be taken from the genomic DNA sequence a little further 3′ than the end of the 3′ homology arm. The two internal primers anneal to sequences at the 5′- and 3′-ends of the tagging cassette and need to be chosen to match the properties of the external, genomic primer partner. Each primer should consist of 25 nt with an annealing temperature of $\geqslant$65 °C. Often you will need to try more than one combination.

For the LR-PCR we use the LongAmp kit (NEB) according to the NEB protocol. In brief, in a 50 μl reaction use 50–500 ng genomic DNA (purified by phenol–chloroform extraction and EtOH precipitation) from targeted cells or DNA from non-targeted ES cells as a control, 10 μM primers, 10 mM dNTPs and 3% DMSO.

The LR-PCR cycling is initiated with a denaturation for 2 min at 94 °C. The first five cycles start with 15 s at 94 °C, annealing for 15 s at 65 °C and elongation for 7 min at 65 °C, by stepwise decreasing the elongation temperature by 1 °C after each cycle to 61 °C. The next 30 cycles are then 15 s at 94 °C, annealing for 15 s at 65 °C and elongation for 7 min at 65 °C. The last cycle is followed by a final elongation cycle for 10 min at 65 °C. Be aware that the elongation time is dependent on the product size, meaning 1 min per 1 kb.

2.11. Preparation of cells for Western blot analysis

For Western blot analysis, usually 1×10^5–10^6 cells (one 80% confluent well from a six well dish) are sufficient to analyze specific protein expression. The cells are scraped off the culture dish in ice-cold PBS and collected by centrifugation at 800*g* for 5 min at 4 °C. The cell pellets are then resuspended in 70–100 μl of one of the buffers and treated according to one of the following cell homogenisation methods.

(a) Sonication; use ice cold Homogenisation buffer (20 mM HEPES pH 8.0; 150 mM NaCl; 1.5 mM $MgCl_2$; 10% glycerol; 2 mM EDTA pH 8.0; 0,5% Tween-20; 1 mM DTT and 1x HALT protease inhibitor (Thermo Scientific) added immediately prior to use. Cell extracts are sonicated briefly to shear the chromatin. For the BioRuptor waterbath sonicator (Diagenode) we use the high power setting for 5 min with 30 s ON and 30 s OFF, in ice cold water. Proceed as described in below.

(b) Extraction of proteins; resuspend the cell pellets thoroughly in ice cold Extraction buffer (Homogenisation buffer supplemented with 420 mM NaCl). Incubate the samples for 5 min on ice. Proceed as described in below. Most soluble cytoplasmic and nuclear proteins including transcription factors or DNA modifying enzymes are extracted by this fast and easy method. However histones or proteins tightly associated in polymers/filaments will not be extracted. To analyze those proteins use either method (a) or (c).

After cell homogenisation by sonication or extraction, insoluble material should be precipitated by centrifugation at 14,000*g* for 10 min at 4 °C. Transfer the cleared supernatants into fresh reaction tubes. To achieve equal protein loading (20–100 μg) of multiple samples in the SDS–PAGE, measure the protein concentrations of the supernatants at 280 nm before mixing with 2xSDS sample buffer (Sigma) and denaturation at 95 °C for 5 min.

(c) Alternative whole cell protein lysis; resuspend the cell pellets thoroughly in 120 μl ice cold DNase buffer (Homogenisation buffer supplemented with 3 mM $MgCl_2$ and freshly added benzonase nuclease (125 U/ml; Novagen). Then add 40 μl of 2× SDS sample buffer (Sigma), vortex shortly and incubate the samples at 37 °C for 5–15 min to digest the DNA (benzonase rapidly looses activity in buffer exceeding 1% SDS concentration) and subsequently denature at 95 °C for 5 min.

Finally, the samples are directly loaded and analysed by standard protocols for SDS–PAGE and Western blotting or stored at −80 °C. The proteins tagged by GFP or Venus can be analyzed using anti-GFP antibodies. We use a goat polyclonal anti-GFP antibody,

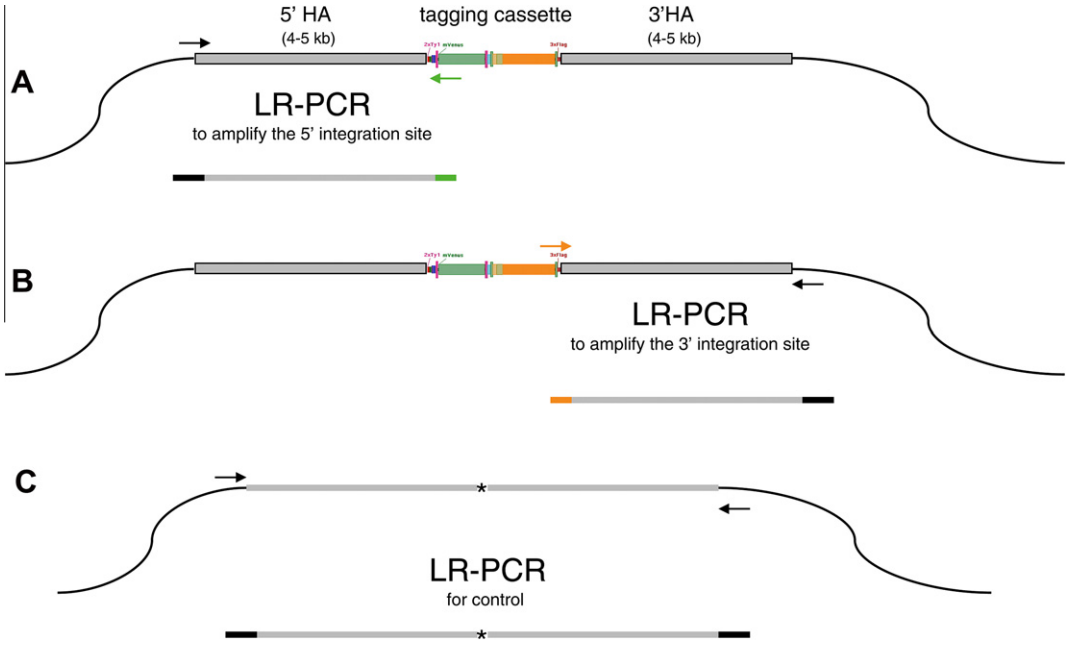

Fig. 5. Long range (LR)-PCR. Depicted are the primer positions to screen by LR-PCR for successful targeting. In black are primers with homology to the genomic DNA outside of the homology arms (HA). In green or orange are the reverse complement primers with homology to the tagging cassette. The integration is analysed by three independent LR-PCR reactions (A) amplifying the region of the 5′ HA, (B) amplifying the region of the 3′ HA, (C) control LR-PCR to also detect negative clones lacking the tagging cassette.

generated at the MPI-CBG Dresden, Germany, however any reasonable commercially available GFP antibody should be fine.

2.12. Immunoprecipitation of GFP/Venus-tagged proteins for mass spectrometry

For immunoprecipitation with GFP or Venus-tags, we use either a polyclonal goat anti-GFP antibody (anti-GFP, 15 mg/ml, generated at the MPI-CBG Dresden, Germany) together with proteinA-G agarose beads (Santa Cruz) or single domain GFP-binding protein coupled to beads (GFP-Trap, Chromotek).

Before starting prepare the beads for the immunoprecipitation Use 5 μl of pure beads (real bead volume) for 1 μg antibody used, if not stated differently. For GFP-Trap beads (Chromotek) we use 10–20 μl of bead slurry for 1×10^6 cells. Wash the beads three times in PBS at room temperature (RT). Then resuspend the beads in 0.5 ml ice-cold Homogenisation buffer after the last wash and store on ice.

Cells expressing GFP/Venus fusion proteins and control cells are cultured to yield 1×10^8, which for mESCs equals 4–5 near confluent 15 cm dishes. After rinsing with PBS, cells are scraped in ice-cold PBS and collected by centrifugation at 800 rpm for 5 min at 4 °C. The cell pellet is resuspended in 1 ml ice-cold Homogenisation buffer and transferred to a 1.5 ml microfuge tube. Keep the samples at 4 °C from now on. Cells are homogenised either by sonication or by nuclease treatment. (a) For sonication, cells are homogenised in a waterbath sonicator (e.g. for BioRuptor (Diagenode): 10 min, high power, alternating between 15 and 30 s ON and 30 s OFF) in the cold room placing the tube on icewater when not sonicating. (b) To avoid sonication, cells can be homogenised by nuclease treatment. Gently resuspend the cell pellet in 1 ml Homogenisation buffer supplemented with freshly added benzonase nuclease (125 U/ml; Novagen) and 3 mM $MgCl_2$ and incubate at 4 °C for 30 min to 1 h. Stop the nuclease treatment by adding 3 mM EDTA. After homogenisation samples are cleared by centrifugation at 12,000 g for 10 min at 4 °C. The cleared supernatants are transferred into new microfuge tubes and best immediately processed further, however samples can be frozen down at −80 °C.

For the immunoprecipitation (IP), transfer the cleared supernatants into new 15 ml conical screw cap tubes. Add either 40 μg of anti-GFP antibody or the prepared GFP-Trap beads (Chromotek) and bring samples to a volume of 3 ml. Note, co-precipitation of the tagged protein together with interacting proteins requires protein concentration of $\geqslant$5 mg/ml. The IP is performed for 1–3 h at 4 °C by gently mixing. To pull down the GFP-antibody complex, add the pretreated proteinA-G agarose beads after 1–2 h and incubate the samples further for a 1 h at 4 °C by gently mixing. After incubation, spin down the sample very gently at 100–500 g and subsequently wash the beads two times with 5 ml Homogenisation buffer for 5 min. If it is important to remove the detergents from the samples (e.g. for mass spectrometry), wash three times additionally with washing solution (20 mM HEPES pH 8.0, 150 mM KCl, 1.5 mM MgCl2, 10% glycerol, 2 mM EDTA pH 8.0). After the last washing step, elute bound proteins by resuspending the beads in 50 μl of 0.1 M Glycine pH 2.0 and incubate for 15 min at RT on a rotation platform. Collect the supernatant by centrifugation at 2000 rpm for 5 min at 4 °C and transfer to a new tube. Neutralize the pH by adding 7.5 μl of 225 mM Tris–HCl pH 9.0. Repeat the elution of the beads and pool the eluate. The IP is now ready for further analysis or stored by freezing down at −80 °C. Usually 5–10 μl of the eluate should be sufficient to analyze the performance of the IP by Western blotting. To control for the efficiency of elution, bead aliquots should be taken before and after elution and denatured directly in 1×SDS sample buffer (Sigma). Instead of the low-pH elution described here, other elution methods are possible. Because the protein tags described here contain a specific protease cleavage site, the tagged protein and associated proteins can be selectively eluted by incubation with PreScission protease (GE Healthcare), according to manufactures instructions. From the range of commercially available IP elution kits we have used the Gentle AG/Ab Elution Buffer (Thermo Scientific) in connection with Zeba Desalt Spin Columns (Thermo Scientific) successfully.

2.13. Chromatin immunoprecipitation

The quality of a good chromatin immunoprecipitation (ChIP) primarily depends on the specificity of the antibody to the protein or protein-tag. In ChIP formaldehyde (FA) is commonly used to fix and capture the DNA–protein interaction by cross linking nucleotides and basic and aromatic amino acids (Lys, Arg, His, Trp, Tyr; [33]. These amino acids in or adjacent to the epitope react with formaldehyde and lose their affinity for the antibody. Thus short protein tags with these amino acids in the epitope will be problematic to pull down efficiently by ChIP. We prefer tags based on the fluorescent proteins YFP or GFP because they maintain a stable conformation and many polyclonal antibodies are available that recognize multiple epitopes. Hence they are not sensitive to formaldehyde.

It is important to find and optimize conditions for a specific, quantitative pull-down of the protein–DNA complex. Each tagged protein can require special conditions and optimization of the subsequent steps (e.g. formaldehyde fixation, chromatin shearing, ChIP-buffer composition, detergent treatment including concentration, incubation times, washing steps and elution). However special effort should be put into optimizing the shearing method to generate chromatin fragments mostly 150–250 bp long. Fragments of this size are a critical prerequisite for further processing and identification of the co-precipitated DNA by most next-generation sequencing methods (ChIP-seq). This can either be done by sonication or by digestion with micrococcal nuclease treatment (see Fig. 6). Both techniques are described below.

2.13.1. Optimization of DNA shearing by sonication

Materials: 37% formaldehyde solution (Merck), 1 M glycine, 5 M NaCl; 20 mg/ml Proteinase K solution (Merck); ice-cold PBS, rubber scraper, Sonicator (e.g. BioRuptor, Diagenode).

Culture around $6–8 \times 10^7$ cells expressing the tagged protein. This is approximately three 15 cm dishes of cells.

1. Crosslink cells equally by adding formaldehyde dropwise directly to the cells in culture medium while gently mixing to a final concentration of 1% (optionally a range of 0.2–1% formaldehyde can be used) and incubate for 10 min at RT.
2. Quench formaldehyde reaction by adding glycine to 125 mM (2.5 ml 1 M/20 ml medium) and incubate for 5 min at room temperature.
3. Aspirate the medium and wash the cells with ice-cold PBS.
4. Harvest cells using a rubber scraper and 2×5 ml ice-cold PBS for each dish. Pool the cells together in one 50 ml conical tube.
5. Pellet cells by centrifugation for 4 min at 1500 rpm at 4 °C, than aspirate the supernatant.
6. Resuspend the cell pellet in 1 ml ChIP buffer (20 mM Hepes–KOH pH 8.0; 100 mM KCl; 0.5 mM DTT; 1 mM EDTA; 2% glycerine; 0,05% NP40; protease inhibitor (complete, GE Healthcare) and transfer into a fresh 15 ml conical tube or other tubes recommended for the sonicator.
7. Sonicate the sample. We use a water bath sonicator (BioRaptor, Diagenode) using high power, with 15 s ON then 30 s OFF by placing the tubes in an ice-cold water bath. Because sonication produces heat, ensure the water remains ice-cold. To monitor the shearing, take 50 μl aliquots into fresh tubes

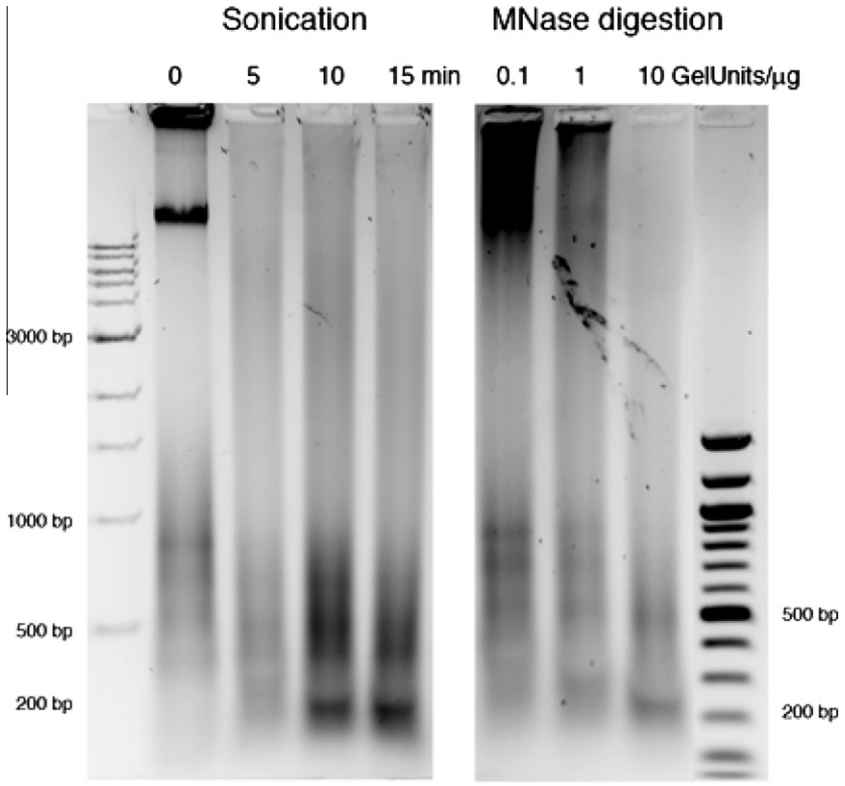

Fig. 6. Optimization of chromatin shearing by sonication or micrococcal nuclease digestion. Different sonication times from 5 to 15 min were tested using high power settings with 15 s ON and 30 s OFF (BioRuptor, Diagenode). Here, the 10 min setting appears optimal for the ChIP. Different micrococcal nuclease concentrations from 0.1 to 10 U were tested for digestion of nuclei equal to about 1 µg of DNA. Here 10 U per mg of DNA appears optimal for the ChIP.

at different time points and store on ice (e.g. after 0, 5, 10, 15 min total sonication time). If optimal conditions are already known, sonicate the whole sample.

8. Centrifuge aliquots and/or samples at 12,000 rpm for 5 min to precipitate remaining cell debris, then transfer the cleared supernatant into fresh tubes.
9. To check the DNA size of the sonicated aliquots, add 8 µl of 5 M NaCl (200 mM final) to the 50 µl aliquots and reverse the crosslink by incubation at 98 °C for 15 min.
10. After cooling down, add 2 µl of Proteinase K solution (10 mg/ml) and incubate at 55 °C for 30–60 min.
11. Check the size of sonicated DNA by gel electrophoresis using a 1.5–2% agarose gel. See Fig. 6. Select the shortest sonication time generating mainly the desired DNA-fragment length of about 200 bp. Because sonication not only will fragment genomic DNA but also large proteins or DNA–protein complexes, longer sonication times will increasingly disrupt the sample and cause decreased efficiency of the immunoprecipitation.

2.13.2. Optimization of DNA shearing by nuclease digestion

Materials: As above plus, Micrococcal nuclease (2,000,000 Gel U/ml, NEB); 1 ml cell douncer, ThermoMixer (Eppendorf).

Enzymatic shearing offers an alternative to sonication avoiding the difficulties of variance of DNA fragmentation, fragmentation of larger proteins (>150 kDa) or protein–DNA complexes and foam generation.

Follow steps 1–5 as described above in Section 2.13.1. Make sure to keep the samples and enzyme on ice all the time except when otherwise stated! As an alternative to this protocol, we have had good experience using the ChIP-IT Enzymatic Shearing kit (Active Motif).

(6) Resuspend the cell pellet in 1 ml MN buffer (0.32 M sucrose; 50 mM Hepes pH 8; 4 mM $MgCl_2$; 3 mM $CaCl_2$; with freshly added protease inhibitors (complete, GE Healthcare), 1 mM PMSF and NP40 to 0.2%. Lyse cells by douncing. Keep everything cold.
(7) Centrifuge the cell homogenate at 1000 rpm for 5 min and discard the supernatant
(8) Resuspend the nuclear pellet in 500 µl MN buffer without NP40 and aliquot 100 µl into five fresh 1.5 ml tubes and keep the samples on ice.
(9) Dilute the micrococcal nuclease 1:20 (4 µl in 800 µl in 10% glycerol) to 100 Gel units/µl.
(10) The DNA concentration of the sample should be approximately 2 µg/µl from this number of mES cells. Add different amounts of micrococcal nuclease ranging from 1 to 100 Gel units/µg DNA.
(11) Place the tubes in a preheated thermoblock at 37 °C for 10 min while shaking at 1000 rpm.
(12) Transfer the tubes to ice and stop reaction by adding 20 µl 500 mM EDTA solution, pH 8.0.

Centrifuge aliquots at 12,000 rpm for 5 min to precipitate remaining cell debris and transfer the cleared supernatant into fresh tubes.

Proceed to analyse the samples continuing with step 8 as described above in Section 2.13.1. Because the MNase digestion is affected by cell number or DNA concentration, enzyme concentration, time of digestion and digestion temperature, changes to any of these parameters will result in a different outcome.

MNase digestion generally produces a ladder of DNA fragments ranging between 2000 and 150 bp. Select the shortest digestion time generating mainly the desired DNA-fragment length of about 200 bp (See Fig. 6).

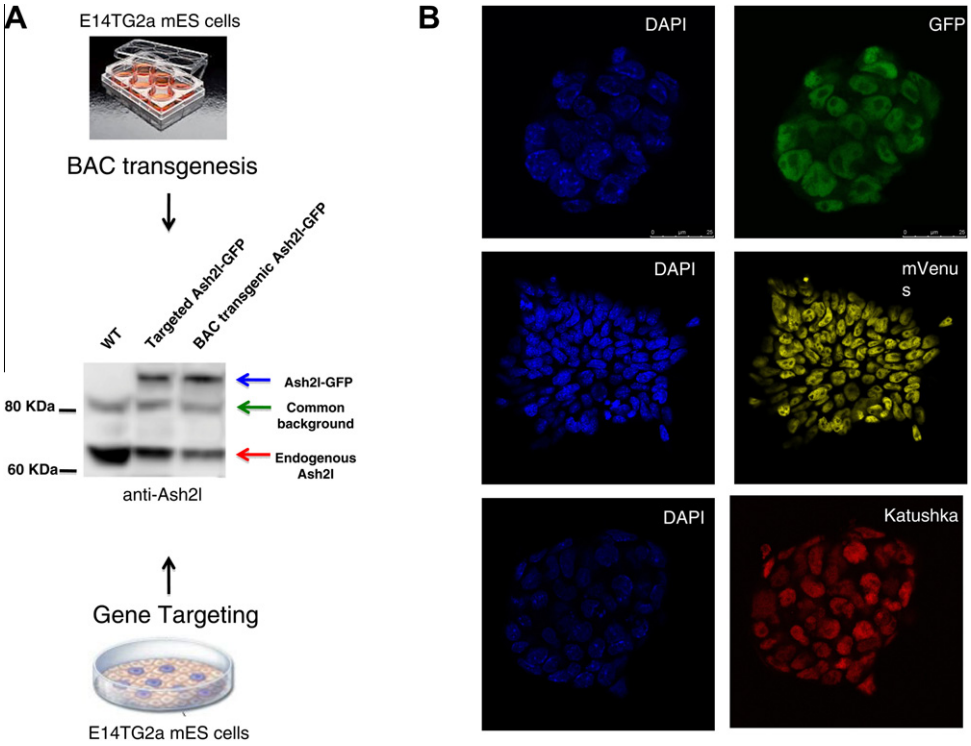

Fig. 7. Western blot and fluorescence analysis of BAC transgene versus gene targeting for expression of tagged proteins. BAC transgenes are delivered to mESCs by lipofection whereas gene targeting employs electroporation. (A) Total protein extracts of wild type (lane 1), Ash2l-GFP BAC transgene (lane 2) or Ash2l-GFP targeted (lane 3) mouse ES cell lines were separated by SDS–PAGE and transferred onto PVDF membrane. The membrane was probed with an anti-Ash2l antibody (Bethyl Laboratories) detecting endogenous Ash2l protein at 68 kDa and the tagged Ash2l-GFP at 110 kDa. Very similar levels of expression were achieved from either BAC transgene or targeted clone. As expected, after gene targeting half of Ash2l was expressed with the tag and half without. (B) Different Ash2l BAC transgenes were generated using cassettes with EGFP, mVenus or Katushka fluorescent proteins. Cells were stained with DAPI and analyzed by fluorescence microscopy.

2.13.3. Chromatin immunoprecipitation protocol

Materials: Protein A/G-agarose beads (Santa Cruz Biotechnology) or Dynabeads protein G (Invitrogen); 15 ml low DNA binding polystyrene tubes (BD Falcon); 1.5 ml LoBind tubes (Eppendorf); Salmon Sperm DNA (Stratagene); Cold water fish skin gelatin (Sigma).

Alternatively, most reagents can be obtained ready to use with the Chromatin Immunoprecipitation Assay kit (Millipore).

For a ChIP control, use the original non-transfected cells (EGFP/YFP negative).

Preparation of beads:

Beads for the Preclearing: Wash 250 μl magnetic Dynabeads or 100 μl protein A/G-agarose per sample in PBS, pellet the beads using a magnetic stand or spin at 1000*g* for 1 min. Repeat the washing another two times and then resuspend beads in 1 ml ChIP buffer.

Preparation of beads for immunoprecipitation: Wash 500 μl Dynbeads or 200 μl protein A/G-agarose per sample in PBS, pellet the beads using a magnetic stand or spin at 1000*g* for 1 min, wash again twice, and then resuspend beads in 1 ml PBS containing 200 μg sonicated Salmon Sperm DNA (Stratagene) and 1.5% cold water fish skin gelatin (Sigma). Incubate while mixing for at least 2 h at 4 °C. Wash the beads once with 1 ml ChIP buffer and store the beads in 0.5 ml ChIP buffer at 4 °C.

(1) Complete steps 1–7 for sonication or 1–13 for nuclease treatment according to the previously optimized shearing conditions. Unlike the optimization, the sample will now be treated as one. Then continue with the protocol below.
(2) After centrifugation at 12,000 rpm for 5 min at 4 °C, transfer the 1 ml supernatants of the sheared samples into fresh 15 ml low DNA binding polystyrene tubes (BD Falcon). Discard the pellet.
(3) *Preclearing:* Pre-clear the cell supernatant by adding 250 μl magnetic Dynabeads or 100 μl protein A/G-agarose beads. Adjust samples to 3 ml with ChIP buffer. Incubate for 30 min while gently rotating/mixing at 4 °C to reduce non-specific protein or DNA binding to the beads. Increasing the incubation time to 1 h at 4 °C may further reduce the nonspecific binding.
(4) Pellet the beads by centrifugation at 200*g* or using a magnetic stand and transfer the whole supernatant into fresh 15 ml low DNA binding polystyrene tubes (BD Falcon). Transfer two 50 μl aliquots of each sample into fresh tubes and freeze down at −20 °C. These are the INPUT samples. One will be used as total DNA Input of the ChIP and the second for Western-blot analysis of the IP performance.
(5) Add 40–70 μg polyclonal anti-GFP antibody. Incubate overnight while gently rotating/mixing at 4 °C.
(6) Add the 0.5 ml of before prepared pre-blocked beads for immunoprecipitation to the ChIP samples and incubate while gently rotating/mixing for 1–2 h at 4 °C.
(7) Pellet the beads by centrifugation at 200*g* or using a magnetic stand. Transfer a 50-μl aliquot of the supernatant of each sample into a fresh tube and freeze down at −20 °C. These are the unbound samples, used later for Western-blot analysis of the IP performance. Discard the remaining supernatant. Carefully transfer beads with 1 ml ChIP buffer into 1.5 ml LoBind tubes (Eppendorf). Wash the beads by rotating for 3–5 min at 4 °C. Pellet the beads by gentle centrifugation at 200*g* or using a magnetic stand. Discard the supernatant.

(8) Wash the beads for 5 min at 4 °C in 1 ml of each the buffers listed below as described before:
   (a) Twice with ice-cold Low Salt Immune Complex Wash Buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA pH 8.0; 20 mM Tris–HCl pH 8.0; 150 mM NaCl).
   (b) Twice with ice-cold High Salt Immune Complex Wash Buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA pH 8.0; 20 mM Tris–HCl pH 8.0; 500 mM NaCl).
   (c) Twice with ice-cold LiCl Immune Complex Wash Buffer (0.25 M LiCl; 1% IGEPAL CA630 (SIGMA); 1% deoxycholic acid (sodium salt); 1 mM EDTA pH 8.0; 10 mM Tris pH 8.0).
   (d) Twice with ice-cold TE buffer (10 mM Tris–HCl pH 8.0; 1 mM EDTA pH 8.0) Before spinning down after the last washing step transfer a 50 μl aliquot of beads suspension from each sample into a fresh tube for analyzing the IP pull down by Western blot.

(9) Elute the precipitated protein complex by adding 250 μl freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$) to the pelleted beads. Vortex the samples briefly, and incubate at room temperature for 15 min while rotating. Pellet the beads by centrifugation at 200*g* or using a magnetic stand. Carefully transfer the supernatants into fresh 1.5 ml LoBind tubes (Eppendorf) and repeat elution once again. Combine both eluates resulting in 500 μl total.

(10) Thaw the input sample taken at step 4 and bring to 500 μl with elution buffer buffer.

(11) Add 20 μl 5 M NaCl to the eluates and input sample (200 mM final) and reverse cross-link the protein–DNA complex by incubation at 65 °C for >4 h or overnight.

(12) Add 10 μl 0.5 M EDTA pH 8.0 (10 mM final), 20 μl 1 M Tris–HCl pH 6.5 (40 mM final) and 2 μl Proteinase K (10 mg/ml solution) to the eluates and input sample. Incubate for 1 h at 55 °C shaking at 800 rpm in a ThermoMixer.

(13) Cool the samples to room temperature (RT). Add 500 μl phenol/chloroform/isoamyl alcohol (25:24:1), vortex and spin at 12,000 rpm for 2 min at RT. Transfer the top aqueous phase into new 1.5 ml tubes. Add 500 ml chloroform/isoamyl alcohol (24:1), vortex and spin at 12,0008 rpm for 2 min at RT. Transfer the top aqueous phase into new 1.5 ml tubes.

(14) Precipitate the DNA by EtOH precipitation. To support the precipitation add 1 μl glycogen (20 μg/ml, Stratagene) before EtOH precipitation and leave. Leave the samples at −20 °C overnight. Wash the DNA pellet with 1 ml 70% ethanol and let the pellet dry at RT for 15–30 min.

(15) Resuspend the DNA pellets in 21 μl ddH_2O. Freeze the samples down at −20 °C. The ChIP samples are ready for further analysis.

2.13.4. Probing the efficiency of the immunoprecipitation during the ChIP

The 50 μl aliquots of the input, beads with the purified DNA–protein complex and the unbound fraction can be directly denatured by adding 50 μl 2× SDS sample buffer (Sigma) and boiling. Run 20 μl of each sample on a SDS–PAGE and analyse by Western blot. The tagged protein should be efficiently pulled down and thus detectable at the expected molecular size in the input and bead samples. A smaller amount of tagged protein may be left in the unbound fraction. However, if there is only a small amount in the bead sample, while most is in the unbound fraction, optimize the immunoprecipitation conditions:

(a) Optimize the binding of the antibody to the tagged proteins (e.g. change ChIP-buffer composition, detergent, e.g. 0.1% SDS; change incubation times or stringency of washing steps).
(b) Make sure the shearing worked optimally.
(c) Use more antibody or test a different one.

The quality and quantity of DNA specifically pulled down in the ChIP is best determined by qPCR. Use or generate a validated PCR primer pair for a genomic DNA sequence that is known to be a target site of the tagged protein. The primers should amplify 100–200 bp. The ChIP sample of the tagged protein should ideally have a >5-fold enrichment of the DNA sequence as compared to the control ChIP sample of non-transfected ES cells (EGFP/YFP negative).

For the qPCR dilute 1 μl of each ChIP sample 1:10 and input sample 1:500 in ddH_2O. Use 1 μl of the diluted DNA for a 20 μl PCR. Prepare and run the qPCR following the instructions of the supplier.

3. Discussion

A comparison of protein expression by BAC transgenesis and targeting using Ash2l as the protein of choice, is shown in Fig. 7. As can be seen, BAC transgenesis often delivers expression levels that are very close to the endogenous level. Fig. 7 also illustrates fluorescence images obtained by expression of Ash2l tagged with Venus (as described here; Fig. 1, 2), Katushka or EGFP. These tagging cassettes are available on request.

Acknowledgments

This work was supported by funding to AFS from the EU 6th Framework program EuTRACC (European Transcriptome, Regulome and Cellular Commitment Consortium) and the BundesMinisterium fuer Bildung und Forschung, National Genome Forschung Netzwerk program, DiGtoP (Disease Genes to Protein Pathways).

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