Direct Cloning of Isogenic Murine DNA in Yeast and Relevance of Isogenicity for Targeting in Embryonic Stem Cells

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Abstract
Efficient gene targeting in embryonic stem cells requires that modifying DNA sequences are identical to those in the targeted chromosomal locus. Yet, there is a paucity of isogenic genomic clones for human cell lines and PCR amplification cannot be used in many mutation-sensitive applications. Here, we describe a novel method for the direct cloning of genomic DNA into a targeting vector, pRTVIR, using oligonucleotide-directed homologous recombination in yeast. We demonstrate the applicability of the method by constructing functional targeting vectors for mammalian genes Uhrf1 and Glap. Whereas the isogenic targeting of the gene Uhrf1 showed a substantial increase in targeting efficiency compared to non-isogenic DNA in mouse E14 cells, E14-derived DNA performed better than the isogenic DNA in JM8 cells for both Uhrf1 and Glap. Analysis of 70 C57BL/6-derived targeting vectors electroporated in JM8 and E14 cell lines in parallel showed a clear dependence on isogenicity for targeting, but for three genes isogenic DNA was found to be inhibitory. In summary, this study provides a straightforward methodological approach for the direct generation of isogenic gene targeting vectors.

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Introduction
Since its first demonstration [1], gene targeting in embryonic stem (ES) cells has evolved to be a routine technique to modulate gene function in vivo. The sequential steps of genomic DNA isolation, vector construction, homologous recombination in ES cells and chimera production have seen increases in efficiency that have accelerated the number of new mutants each year such that more than 16,000 targeted mutations in mouse ES cells have already been made [2,3]. Indeed, the first encyclopedia of mutations in the mouse is near to completion, largely due to productivity gains in serial recombination techniques [4]. Although knockout and conditional ablation of genes are the first steps in understanding gene function in vivo, allelic variants such as disease-mimicking point mutations or whole gene replacement (e.g., with human disease variants) are quickly emerging as necessary tools to phenocopy human genetic disease in model organisms. With regard to species relevance or for clinical applications, these mutations are increasingly created in human ES or iPS cells [5–10], a process that is still somewhat tedious due to its use in niche applications [11]. Nonetheless, paired with new technologies to increase homologous recombination such as zinc-finger nucleases, TALEns, and CRISPR [12–15], targeting vectors are employed widely for genome modification.

Genomic DNA cloning is a key step in targeting vector construction. Inclusion of homologous isogenic targeting sequences is common practice to avoid low targeting frequencies caused by single nucleotide or larger polymorphisms such as insertions, deletions or inversions [16–18]. Yet, standard methods employed for the modification of genomic-derived DNA sequences are often not optimal for the construction of isogenic targeting constructs. For example, bacteriophage lambda-red based homologous recombination in bacteria has simplified genomic DNA modification [19], but has the prerequisite of a library of mapped BACs or plasmid vectors as a source of genomic DNA. The library requirement hinders its application in, for instance, targeting campaigns of unique human iPS cell lines or in patient-specific gene-therapy, although substantial improvements have been made here as well [20]. Moreover, the lambda-red methodology requires sequential steps in targeting vector construction. Alternative methods generally employ multiple-step PCR, which is challenging for longer sequences from genomic templates and introduces undesirable mutations at higher frequencies than traditional cloning.
Substantial progress has been made with simultaneous multi-
fragment cloning in the yeast *Saccharomyces cerevisiae*, in which short
homologous sequences are joined by the DNA double-strand
break repair machinery [21–24]. In particular, larger homologous
regions [25] or overlapping oligomers [26] have been successfully
used to retrieve genomic DNA from BACs for the introduction
into targeting constructs carried on yeast shuttle vectors. Import-
antly, yeast readily recombines multiple fragments with a
restricted plasmid vector that can replicate and be selected for
with positive or negative selection, eliminating sequential steps in
targeting vector construction and improving throughput.

In this work we sought to extend yeast homologous recombi-
nation cloning methodology to directly retrieve DNA fragments
from host cellular genomic DNA using oligonucleotides and
without the use of intermediate library preparation or PCR steps.
We have constructed a novel yeast vector for Retrieval of
Targeting sequences with Verbatim Isogenic Regions (pRTVIR,
or “pRetriever”) that efficiently retrieves genomic sequences when
co-transformed with isolated chromosomal DNA from embryonic
stem cells. In a series of experiments involving more than 70
targeting constructs, we find a significant association between
correct targeting and isogenic DNA. Therefore, direct DNA
cloning from purified genomic DNA in yeast should prove to be a
valuable method for sensitive targeting applications.

Results

A Yeast Plasmid for Retrieval of Targeting Sequences
(pRTVIR) Facilitates the Construction of Targeting Vectors
for Embryonic Stem Cells

Cloning overlapping DNA fragments in yeast has been
established for rapid cloning of mammalian targeting vectors
[23]. This methodology relies on the homologous recombination
between mammalian DNA sequences and a shuttle plasmid vector
that can replicate and be selected for in both yeast and *Escherichia
coli*. We set out to increase the frequency of successful recombi-
nation by attempting to minimize the background resulting from
unwanted recombination and ligation events. First, we designed a
vector for recombination cloning without any sequence homology
to the yeast genome. Briefly, an heterologous auxotrophic selection
marker *CaURA3* (orotidine-5-phosphate decarboxylase) [27] from
*Candida albicans* driven by the *TEF* promoter from *Ashbya
gosypii* was combined with replication sequences derived from the yeast
episomal 2 micrometer plasmid. *E. coli* replication sequences and a
bacterial ampicillin selection marker were introduced in the
plasmid resulting in the 4.5 kb plasmid pCA771. When pCA771 is
used to transform a *ura3* yeast strain devoid of its endogenous 2
micrometer plasmid ([Cir]) to *Ura*+, no sequence homology exists
between this vector and the host DNA, thus effectively eliminating
background arising from homologous recombination.

Second, we wanted to eliminate background arising from *in vivo*
ligation, which in yeast occurs with high frequency [21] by making
the functionality of the *CaURA3* selection marker in pCA771
conditionally dependent on a successful recombination event.
Briefly, we made small 3’ truncations of the *CaURA3* gene of our
pCA771 to define a minimal deletion that abolished function.
Deletion of the last two codons of *CaURA3* (ΔQL) did not affect
the capacity to transform *ura3* yeast cells to *Ura*+, while a four-
codon deletion ΔATGQL decreased colony formation frequency
and a six-codon deletion ΔKKTGQL completely abolished
transformation to *Ura*+ (Fig. 1A). Inspection of a structure model
of CaUra3 based on the structure of *S. cerevisiae* Ura3 [28] revealed
that the deletions had truncated an alpha-helix that is important
for the structural integrity of the orotidine-5-phosphate decarbox-
ylase enzyme. We named the new vector pRTVIR, plasmid for
Retrieval of Targeting sequences with Verbatim Isogenic Regions
(Fig. 1B).

Next, we tested if reintroduction of the four codons KKTG in
pRTVIR by homologous recombination restored the activity of
*CaURA3* thereby facilitating selection of correct clones as *Ura*+
transformants. The vector was linearized at unique
*HindIII* and *EcoRV* sites located restriction site of pRTVIR that
contains standard *E. coli* replication (pUC) and antibiotic selection
sequences (Amp) and replicates in yeast by means of a 2 micrometer plasmid
sequence (2 μm). The *Caura3*ΔKKTGQL marker is nonfunctional but can
be restored to function by homologous recombination that adds back
KKTG codons. *HindIII* and *EcoRV* sites facilitate restriction of pRTVIR that
enhances homologous recombination. (C). *HindIII* and *EcoRV* restricted
pRTVIR was used to co-transform CAY1179 to *Ura* in duplicates
with together with either a *Apr*−/lacZ*PC* PCR product flanked by homology to
the vector (Graphic) or with a similar PCR product without vector
homology but in the presence of bridging single-stranded oligonucle-
otides. *Ura*+ colony forming units (CFU) for each condition are
presented. Following plasmid rescue from the yeast transformants,
the fractions of correct LacZ-carrying recombinants were quantified
by X-Gal blue/white-screening (marked in blue). doi:10.1371/journal.pone.0074207.g001
homology to each of the free ends of the vector, respectively. While control transformations in duplicates using only HindIII and EcoRV restricted pRTVIR gave no Ura+ colonies, inclusion of the P_Acet-LacZ PCR in the transformation gave encouraging 1570 and 1970 Ura+ colonies, respectively (Fig. 1C). We pooled the yeast transformants, rescued the plasmids (see Methods) and quantified the number of P_Acet-LacZ carrying recombinants by using X-gal blue/white-screening of the ampicillin resistant E. coli colonies. 76% and 73%, respectively, of the transformants were blue and therefore carried a functional LacZ gene. In parallel experiments, we employed bridging single-stranded oligonucleotides instead of flanking homology regions to direct the homologous recombination. Briefly, each oligonucleotide was synthesized with 35 bp homology to both one end of the vector and the corresponding end of the P_Acet-LacZ PCR product. Although the yeast transformation efficiency was low in this particular setup due to the use of single and not double stranded oligonucleotides, plasmid rescue and X-gal blue/white-screening revealed 100% efficiency for obtaining correct recombinant plasmids (Fig. 1C).

**Multiple Fragment Targeting Vector Assembly using pRTVIR**

To directly test if pRTVIR functions in homologous recombination of multiple overlapping DNA fragments, a five-fragment mammalian targeting vector for targeting a Tau-EGFP and hygromycin fusion (with adjoining T2A sequences [30]) to Tubb3 was designed and products were amplified by PCR [31]. We also included a direct comparison with a standard yeast 2μJ vector that contains extensive homology to the yeast genome, pRS316 [32]. In striking contrast to the zero background of pRTVIR upon single-tube transformation, pRS316 gave extensive background of yeast transformants and only modest increase in the number of transformants when inserts were added. Colonies containing correctly recombined fragments were identified by junction PCR and verified by restriction digest. The frequency of correct clones was 20/96 (20.8%) for pRTVIR and 13/96 (13.5%) for pRS316 (Fig. 2). Apparently, plasmid rescue in E. coli results in selection of functional plasmids and thereby of correct clones, compensating to some extent for the poor performance of pRS316 during yeast recombination. The recombined pRTVIR-Tubb3-TauEGFPPhy vector was then electroporated into embryonic stem (ES) cells and cells were selected in G418-containing medium. Positively targeted clones (targeting frequency: 4/96) were identified by long-range PCR and analyzed by immunofluorescence (Fig. 2B, S1). As expected, the C-terminal fusion to TauEGFP is localized exclusively in neuronal processes derived from in vitro differentiated ES cells (Fig. 2C), demonstrating that ES targeting vector construction is possible and sequence fidelity is preserved using pRTVIR.

**Cloning of Sequences from BACs and Genomic DNA into pRTVIR**

Sensitive genomic applications preclude the use of PCR in mammalian targeting arm amplification due to the frequency of spontaneous mutations. Therefore, to analyze if pRTVIR would be able to retrieve flanking genomic DNA for a targeting vector directly from a murine bacterial artificial chromosome (BAC), bridging oligonucleotides containing overlap to the vector and genomic sequence of interest have previously been employed with success [26]. We used a modified protocol to transform a double-stranded primer containing a 40 bp overlap with genomic DNA together with linearized pRTVIR and 40 bp to the corresponding BAC for either Gfap, encoding a cell cycle regulated E3 ubiquitin ligase (Fig. 3A). Initial results performed in triplicate showed poor retrieval of the ~9 kb fragments, 2/96 (2.1%) for Gfap and 0/96 for Uhrf1, but when restriction of the BAC outside the region of interest was introduced, the frequency increased dramatically to 91/96 (94.6%) and 51/96 (53.1%), respectively (Fig. 3B). We also compared the efficiency of linear retrieval in the standard yeast vector pRS316, which gave 0/96 and 1/96 (1.0%), respectively, compared to correctly cloned fragments. This shows that the pRTVIR vector combined with linearization is an efficient tool for retrieval of genomic DNA fragments from linearized BAC DNA.

Genomic DNA isolated directly from cells for targeting experiments has the advantage of absolute isogenicity. Due to the substantial increase in efficiency of genomic DNA retrieval from a BAC over traditional cloning approaches, we attempted to retrieve targeting vector homology directly from genomic DNA derived from embryonic stem cell lines isolated from genetically distinct mouse strains. In experiments conducted in triplicate, following co-transfection of 100 μg of highly purified phenol-chloroform extracted and digested genomic DNA from ES cells together with pRTVIR and overlapping 80-mer double-stranded oligonucleotides corresponding to end sequences at flanking genomic XhoI and NotI restriction sites, it was possible to retrieve 8.4 kb of Uhrf1 genomic DNA directly from E14TG2a (129/Ola) and JM4 (C57BL/6) linearized genomic DNA, albeit at a modest frequency of 2.1% (Fig. 3). An additional retrieval gave 14.3% and 1.0% successful clones when isolating 8.4 kb of E14 and JM4 genomic Gfap DNA, respectively, digested with flanking EcoRI restriction sites. This demonstrates that selection with the pRTVIR is sufficient to retrieve restricted genomic DNA directly from purified cellular DNA without further amplification steps.

DNA engineering using homologous recombination in yeast does not rely on restriction sites for fixing the fragments and therefore allows for seamless fusion of DNA. To introduce an in-frame fusion of EGFP to Uhrf1 before the ultimate stop codon, the 8.4 kb Uhrf1 and Gfap homology vectors were introduced into yeast together with a PCR amplified EGFP-SV40 promoter-Neo-Neo cassette containing 40 bp of homology at the ends. Following transformation and selection on kanamycin, several of the clones screened for Gfap and Uhrf1 correctly contained the cassette (Fig. S2) and were used for targeting experiments described below.

**Targeting with Isogenic and Non-isogenic Yeast-derived Vectors**

Isogenic matching of targeting vector to target cell line is reported to be conducive for robust targeting [16–18]. To confirm this for yeast-derived vectors, we conducted a targeting experiment with vectors containing genomic DNA retrieved from either JM8 or E14 ES cell lines for the Uhrf1 and Gfap genes (Fig. 4A). Vectors were prepared in parallel and electroporated in triplicate in JM8 and E14 cell lines and 32 colonies were picked per 10 cm plate (96 total) and analyzed for targeting frequency by long range PCR as well as GFP expression in ES cells and under neural differentiation conditions (Figs. 4B, S2, S3). Surprisingly, isogenicity was not always favored, as the vector derived from the E14 cell line performed better for both genes in JM8 cells than the JM8 isogenic vector (74 versus 52 for Uhrf1, 48 versus 10 for Gfap). In the reciprocal experiment, the JM8 vector performed as expected better in the JM8 cell line for Uhrf1 (52 versus 42) but not for Gfap (48 versus 20). Perhaps most surprising, the Uhrf1 E14 vector did not yield positive colonies in E14 cells but targeted 50% of clones.
correctly in JM8 cells, suggesting that there is an incompatibility when targeting with the isogenic sequence. Sequence analysis of Uhrf1 cloned yeast sequences did not reveal changes other than sporadic nucleotide polymorphisms differing from the reference genome (GRCm38) that may affect targeting frequency. However, Gfap E14-derived DNA showed substantial polymorphisms in a 245-bp region in a heavily-repeated and alternatively spliced 3' UTR in exon 8a (Fig. S4). Yeast-cloned JM8 DNA for Gfap also showed variation over a neighboring 214-bp region. It is possible that these regions are involved in transcript stability that may lead to a preference of the non-isogenic vector when performing targeting experiments.

Given the disparity between published reports and our targeting outcome, we chose to examine if isogenic DNA improved targeting efficiency under defined circumstances for a larger set of genes. We therefore carried out a targeting campaign of 70 different genes from the DiGtoP resource [33] in E14 and JM8 cells in parallel using recombineering-derived tagging vectors from C57BL/6 genomic DNA. In each vector, the targeting sequences contain an EGFP-promoter-neomycin resistance cassette and are directed to the penultimate codon of each gene to generate an EGFP-fusion to the endogenous gene. By picking 32 colonies from each targeting plate for analysis, a consensus targeting frequency could be applied to a given vector (Fig. 4C). As expected, 66 of 70 vectors gave positive clones for isogenically matched C57BL/6 DNA in JM8 cells, whereas only 38 of 70 the same vectors gave positives in E14 cells. A significant preference for isogenicity was seen in the percentage of successful clones for each gene when performing targeting experiments.

Discussion

In this study we have developed the novel yeast cloning vector pRTVIR that simplifies the cloning of DNA from single or multiple PCR products, BACs and even facilitates cloning directly from complex genomic DNA preparations. Homologous recombination cloning based on pRTVIR can rely on sequence homology in the flanking ends of PCR products or on oligonucleotides that provides bridging sequence homology for recombination. The methodology outlined in this study can readily be implemented for example when cloning genomic DNA for sequence sensitive applications such as ES gene targeting. In the broader perspective, pRTVIR is a general-purpose vector for cloning by yeast homologous recombination and is by no means restricted to mammalian genomic DNA sequences. A few specific points regarding the use and design of pRTVIR are noteworthy. First, homologous recombination cloning is not...
Direct Cloning of Isogenic DNA in Yeast

Our driving force that led us to develop the pRTVIR technology stems from an interest in ES cell gene targeting [3]. Working with isogenic targeting vectors have long been the gold standard in designing gene-targeting campaigns and has become increasingly important in the functional study of DNA polymorphisms, often linked to disease. Indeed, pRTVIR can readily be implemented in the construction of isogenic targeting constructs, even directly from genomic DNA. Also in our hands we find superior targeting frequencies when employing isogenic vectors, but for individual genes there are exceptions to this rule. We cannot at this point offer an explanation why these specific combinations of DNA donor:recipient score better than isogenic DNA. The vectors we used in our study do not eliminate gene function and would not be expected to cause haploinsufficiency or changes in expression level that may preclude correct targeting. It has been reported that even a 2% strain difference between targeting constructs can lead to a >25 fold in targeting success [34], yet polymorphisms are not expected to act in favor of the non-isogenic construct. Targeting the 3’ UTRs of genes may also lead to a bias, as they are known to be more highly polymorphic than coding regions. Their role in mRNA structure and stability may also reflect a unique DNA structure that should be taken into consideration for its recombinogenic potential. Additionally, recombination hotspots, potential for random integration, repetitive regions and chromatin structure as well as other factors such as imprinting or copy number sensitivity may play a role in the correct integration of targeting construct DNA. In the absence of information defining these features, isogenicity is still the gold-standard for gene targeting campaigns.

With this study we aimed to establish a proof-of-principle for direct retrieval for genomic DNA into a vector system. Although optimization is clearly necessary for large-scale application, this method can be a valuable tool for targeting experiments where absolute genomic integrity is necessary.

Methods

Construction of Plasmid Vectors pCA771 and pRTVIR

Parental plasmid pCA771 was constructed by homologous recombination in yeast (Ura+), containing 2 microcentimeter sequences, ampicillin marker and E. coli origin of replication from template pRS423 [35] and a 1.2 kb PCR product encompassing the Ura+ selection marker and the genomic DNA of interest along with ampicillin marker and the vector is therefore not suitable for experiments in yeast cell biology.

Yeast Transformation and Plasmid Rescue

Yeast strain CAY1179 was used for all experiments and is a spontaneous Ade derivative of [Cir+] strain yAF7 [37] with the genotype (MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1). Yeast was transformed by an adapted protocol described by [38]. Briefly, 10 ml CAY1179 was grown over night in 2 ml YPD medium at 30°C. In the morning the strain was diluted in fresh 2 ml YPD medium (3 ml per transformation) and grown at 30°C from starting OD600 = 0.1. After ~5 h harvest, the cultures had reached OD600 = 1.0 and 3 ml of cell culture for each transformation was harvested by centrifugation (1.5 ml Eppendorf tubes) and washed by resuspension and centrifugation in 100 mM LiOAc (1 ml per transformation). 50 µl of carrier DNA (2 mg/ml heat-denatured salmon testis DNA, Sigma D1626 in 100 mM LiOAc) was added together with vector digest and source DNA to

Figure 3. Direct genomic retrieval from BAC and purified genomic DNA into pRTVIR. (A). Genomic DNA can be cloned efficiently into pRTVIR in a single step. Freshly purified BAC or genomic DNA was mixed with bridging oligonucleotides containing overlap to the Cauras- selection marker and the genomic DNA of interest along with linear pRTVIR. Following high efficiency transformation into yeast in triplicate, the frequency of retrieval of 9 kb of unmodified genomic DNA in pRTVIR was slightly increased compared to pRS316. However, introduction of restriction cleavage sites (BsrGI; or Swal, NotI) immediately outside the respective genomic region facilitated correct cloning for pRTVIR, but not for pRS316. Due to the improved cloning efficiency of pRTVIR, retrieval of the same region from 100 µg of freshly purified and digested genomic DNA by phenol/chloroform extraction was attempted, giving correctly recombined vectors for both E14- and JM8-derived genomic DNA.

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| Vector       | Positive colonies |
|--------------|-------------------|
| GFAP BAC, uncut | pRTVIR 2/96 (2.1%) |
| GFAP BAC, BsrGI | pRTVIR 91/96 (94.8%) |
| GFAP BAC, BsrGI | pRS316 0/96 (0%) |
| Uhrf1 BAC, uncut | pRTVIR 0/96 (0%) |
| Uhrf1 BAC, Swal/NotI | pRTVIR 51/96 (53.1%) |
| Uhrf1 BAC, Swal/NotI | pRS316 1/96 (1.0%) |
| gDNA         | Positive colonies |
| Uhrf1, Swal/NotI | E14 1/48 (2.1%) |
| Uhrf1, Swal/NotI | JM8 1/48 (2.1%) |
| GFAP, BsrGI  | E14 1/7 (14.3%) |
| GFAP, BsrGI  | JM8 1/6 (1.0%) |
the cell pellet. The suspension was briefly mixed by centrifugation followed by the addition of 300 µl 39% PEG 3350 100 mM LiOAc and intense vortexing for 15 s. After heat shock in a water bath at 42°C for 40 min cells were pelleted by centrifugation, resuspended in 150 µl Milli-Q water and plated on solid SC-ura.
medium. Colonies were observed after 2 days of incubation at 30°C.

For plasmid rescue, single or pooled yeast colonies were picked and standard silica miniprep kits (including Qiaprep, Qiagen) were after lyticase digestion employed on a cell pellet corresponding to ~20 μl. Lyticase digestion to weaken the yeast cell wall prior DNA extraction was performed by adding 3 μl Zymolase (Zymoresearch) to the cells resuspended in 200 μl 10 mM Tris-EDTA buffer (or similar cell suspension buffer belonging to the miniprep kit) and incubating for one hour. Tested commercial preparation of lyticase (including Zymolase) contains a plasmid that makes E. coli ampicillin resistant, which necessitate its removal by DNase I treatment. The plasmid is present in minute amounts but can be detected by transforming E. coli with the lyticase solution and selecting for ampicillin resistance. Following lysis, silica column binding, washing and elution, DNA was electroporated into E. coli cloning strain DH10B [39].

PAGP-LacZ Recombination Experiments

For experiments involving PCR products carrying flanking homology regions a 3.8 kb PAGP-lacZ product was PCR amplified with Phusion DNA polymerase from YCpAGP1-lacZ [29] using primers TATAGAGATGCTGGTTGGAATGCTTATT and primer were combined in 1 μl PCR crude product or unpurified PCR evidence on selective SC-ura medium and scored. The yeast transformation frequency is due to a well-established targeting pipeline was carried out as described [4]. The exceptionally high gene targeting frequency is due to a well-established targeting pipeline in projects contributing to the International Knockout Mouse Consortium [3]. In brief, media was changed the night before electroporation, 2.5 μg of targeting vector was linearized and resuspended in 50 μl sterile PBS and electroporated in triplicate into 1x10^6 ES cells (E14TG2a.A4 or JMBAL1.N3) in a BTX electroporator (700V, 400Ω, 25 μF, pulse count at 1) and transferred to plates and distributed evenly. The next day, cells were examined for growth and changed to media containing selection antibiotic G418 125 μg/mL. 10 days following electroporation, cell colonies were picked, trypsinized and expanded. Genomic DNA preparations and long-range PCR were performed as described [4] to characterize correct recombination events. Primer sequences are included in Table S1.

ES Cell Electroporation

Electroporation of E14TG2a (ATCC) and JMBAL1.N3 [43] cells was carried out as described [42]. Briefly, 5x10^5 cells were plated on 6 well dishes in N2B27 neural differentiation medium. Media was changed every day for 8 days and cells were fixed and stained with anti-GFP (Aves Labs) and anti-Tubb3 (Abcam) to confirm co-localization.

Supporting Information

**Figure S1** Multiple fragment targeting vector assembly of pRTVIR-Tubb3-TauEGFPhyg. A: Junction colony PCR on yeast clones demonstrating correctly recombined clones are shown with corresponding primers (Table S1) and expected sizes compared to DNA standards. B: Restriction digests of correctly assembled vectors are shown along with indicated enzymes and expected sizes. C: Targeting in E14 ES cells is demonstrated by long range PCR between two independent primers binding to the genomic region outside the targeting arms and an internal primer as shown together with expected sizes. (TIF)

**Figure S2** Genomic retrieval and integration of EGFP-SV40-Neomycin cassette into pRTVIR vectors. A: Colony junction PCR. Left side: Restricted pRTVIR was transformed together with genomic DNA into competent yeast and colonies were evaluated for correctly recombined clones using junction colony PCR. Right side: Undigested vectors containing Uhf1 and
Gfap genomic DNA were co-transformed with a PCR fragment containing EGFP-SV40-Neomycin into competent yeast and bacterial colonies were evaluated by junction colony PCR. Restriction digests of correctly recombined vectors are shown along with indicated enzymes and expected sizes. Targeting in ES cells is demonstrated by long range PCR between two independent primers binding on each genomic side (four total) outside the targeting arms and two respective internal primers as shown together with expected sizes. Order is LR5a, LR5b, LR3a, LR3b. (TIF)

Figure S3 EGFP fluorescence in Uhrfl- and Gfap-EGFP-SV40-Neomycin targeted ES cell lines. Brightfield and epifluorescent images of ES cells and partially differentiated neuronal monolayers (ND) for Uhrfl and Gfap targeted lines are shown. No fluorescence is observed in the Gfap image, due to the likely absence of astroglial cells. (TIF)

Figure S4 Multiple sequence alignment of Gfap. Sequencing of JMB and E14-derived yeast vectors revealed two polymorphic regions in the 3' UTR (brackets) of an alternatively spliced transcript of Gfap, terminating at exon 8a. (TIF)

Table S1 Primers used in vector construction and genotyping. (DOC)

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Author Contributions

Conceived and designed the experiments: CA AS SP WS WW JS. Performed the experiments: CA AS SP. Analyzed the data: CA AS SP. Contributed reagents/materials/analysis tools: CA MS. Wrote the paper: CA AS SP.

References

1. Capecci MR. (1989) Altering the genome by homologous recombination. Science 244: 1288–1292.
2. Eppig JT, Blake JA, Bult CJ, Kadin JA, Richardson JE, et al. (2012) The Mouse Genome Database (MGD); comprehensive resource for genetics and genomics of the laboratory mouse. Nucleic Acids Research 40: D881–D886.
3. Bradley A, Anastassiadis K, Ayadi A, Baytey JF, Bell C, et al. (2012) The mammalian gene function resource: The International Knockout Mouse Consortium. Mamm Genome 25: 580–586.
4. Skarnes WC, Rosen B, West AP, Koutourakis M, Bushell W, et al. (2011) A conditional knockout resource for the genome-wide study of mouse gene function. Nature 474: 337–342.
5. Zou J, Mali P, Huang X, Dowey SN, Cheng I. (2011) Site-specific gene correction of a point mutation in human iPSC cells derived from an adult patient with sickle cell disease. Blood 118: 4599–4609.
6. Howden SE, Goward A, Li Z, Fung HL, Nider BS, et al. (2011) Genetic correction and analysis of induced pluripotent stem cells from a patient with gyrate atrophy. Proc Natl Acad Sci U S A 108: 6537–6542.
7. Cheng LT, Nagata S, Hirano K, Yamaguchi S, Horie S, et al. (2012) Cure of ADPKD by selection for spontaneous genetic repair events in Pkd1-mutated iPSC cells. PLoS One 7: e32018.
8. Sebastiano V, Maeder ML, Angstman JF, Haddad B, Khayter C, et al. (2011) In vivo gene correction in mouse embryonic stem cells through homologous recombination with isogenic DNA constructs. Proc Natl Acad Sci U S A 108: 9048–9053.
9. Sarov M, Schneider S, Prowiackiowski A, Rogove A, Ernst S, et al. (2006) A recombineering pipeline for functional genomics applied to Caenorhabditis elegans. Nat Methods 3: 839–844.
10. Nekladova M, Maresca M, Fu J, Rostovskaya M, Chenra R, et al. (2011) Targeted isolation of cloned genomic regions by recombineering for haplotype phasing and isogenic targeting. Nucleic Acids Res 39: e157.
11. Suzuki K, Imai Y, Yamashita I, Fukushima S. (1983) In vivo ligation of linear DNA molecules to circular forms in the yeast Saccharomyces cerevisiae. J Bacteriol 155: 747–754.
12. Gibson DG, Benders GA, Axelrod KC, Zaveri J, Alipaz JA, et al. (2008) One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic Yeplasm answeral genomc. Proc Natl Acad Sci U S A 105: 20404–20409.
13. Storck T, Kruh U, Kolbhezer R, Sprengel R, Seeburg PH. (1996) Rapid construction in yeast of complex targeting vectors for gene manipulation in the mouse. Nucleic Acids Res 24: 4594–4596.
14. Gibson DG (2009) Synthesis of DNA fragments in yeast by one-step assembly of overlapping oligonucleotides. Nucleic Acids Res 37: 6984–6990.
15. Bhargava J, Shashikant CS, Carr JL, Juan H, Bentley KL, et al. (1999) Direct Cloning of Genomic DNA by Recombinogenic Targeting Method Using a Yeast-Bacterial Shuttle Vector, pGlasper. Genomics 62: 255–255.
16. Raymond CK, Sims EH, Olson MV (2002) Linker-mediated recombinatorial subcloning of large DNA fragments using yeast. Genomics Res 12: 190–197.
17. Gillum AM, Tsay EY, Kirsch DR (1984) Isolation of the Candida albicans gene for ornithine-5-phosphate decarboxylase by complementation of S. cerevisiae orn- and E. coli prf mutants. Mol Gen Genet 193: 179–182.
18. Miller BG, Hassell AM, Wolfenden R, Milburn MV, Short SA (2000) Anatomy of a proficient enzyme: the structure of ornithine-5-monophosphate decarboxylase in the presence and absence of a potential transition state analog. Proc Natl Acad Sci U S A 97: 2011–2016.
19. Inzani I, Visser S, Bernard F, de Craene JO, Boes E, et al. (1999) Amino acid signaling in Saccharomyces cerevisiae: a permissive-like sense of external amino acids and F-Box protein Grr1p are required for transcriptional induction of the AGP1 gene, which encodes a broad-specificity amino acid permease. Mol Cell Biol 19: 989–1001.
20. Donnelly ML, Hughes LE, Luke G, Mendoza H, tan Dam E, et al. (2001) The ‘cleavage’ activities of foot-and-mouth disease virus 2A site-directed mutants and naturally occurring 2A-like sequences. J Gen Virol 82: 1027–1041.
21. Oldenburg KR, Vo KT, Michaelis S, Padlon C (1997) Recombination-mediated PCR-directed plasmid construction in vivo in yeast. Nucleic Acids Res 25: 451–452.
22. Pikorski RS, Hinter P (1999) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. Genetics 122: 19–27.
23. DIIGoP Available: http://www.digtop.de/.
24. van den Hurk J, Wieringa B (1992) Targeting of the calcium kinase M gene in embryonic stem cells using isogenic and nonisogenic vectors. Nucleic Acids Res 20: 3815–3820.
25. Christianson TW, Pikorski RS, Danz M, Shero JJ, Hinter P (1999) Multifunctional yeast high-copy-number shuttle vectors. Gene 110: 119–122.
26. Goldstein AL, Pan X, McCusker JH (1999) Heterologous URA3MX cassettes for gene replacement in Saccharomyces cerevisiae. Yeast 15: 507–511.
27. Falcon AA, Roux N, Ari, JP (2005) 2-micron circular plasmids do not reduce yeast life span. FEMS Microbiol Lett 250: 245–251.
38. Gietz RD, Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol 350: 87–96.

39. Grant SG, Jessee J, Bloom FR, Hanahan D (1990) Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. Proc Natl Acad Sci U S A 87: 4645–4649.

40. Rodriguez I, Feinstein P, Mombaerts P (1999) Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system. Cell 97: 199–208.

41. Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, et al. (2004) Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. Nat Biotechnol 22: 589–594.

42. Yang QL, Stavridis M, Griffiths D, Li M, Smith A (2003) Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol 21: 183–186.

43. Pettitt SJ, Liang Q, Rairdan XY, Moran JL, Prosser HM, et al. (2009) Agouti C57BL/6N embryonic stem cells for mouse genetic resources. Nat Methods 6: 493–495.

44. Kelley LA, Sternberg MJ (2009) Protein structure prediction on the Web: a case study using the Phyre server. Nat Protoc 4: 363–371.