Efficient CRISPR/Cas9-assisted gene targeting enables rapid and precise genetic manipulation of mammalian neural stem cells

Raul Bardini Bressan¹, Pooran Singh Dewari¹, Maria Kalantzaki¹, Ester Gangoso¹, Mantas Matjusaitis¹, Claudia Garcia-Diaz¹, Carla Blin¹, Vivien Grant¹, Harry Bulstrode¹, Sabine Gogolok¹, William C. Skarnes² and Steven M. Pollard¹

Authors Affiliations

¹ MRC Centre for Regenerative Medicine, University of Edinburgh, Edinburgh, UK
² Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, UK

* Corresponding authors contact:

Steven Pollard: steven.pollard@ed.ac.uk  Tel: +44 (0)131 6519544

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Abstract

Mammalian neural stem (NS) cell lines provide a tractable model for discovery across stem cell and developmental biology, regenerative medicine and neuroscience. They can be derived from foetal or adult germinal tissues and continuously propagated in vitro as adherent monolayers. NS cells are clonally expandable, genetically stable, and easily transfectable – experimental attributes compatible with targeted genetic manipulations. However, gene targeting – so critical for functional studies of embryonic stem cells – has not been exploited to date in NS cells. Here we deploy CRISPR/Cas technology to demonstrate a variety of sophisticated genetic modifications via gene targeting in both mouse and human NS cell lines, including: 1) efficient targeted transgene insertion at safe harbor loci (Rosa26 and AAVS1); 2) biallelic knockout of neurodevelopmental transcription factor genes; 3) simple knockin of epitope tags and fluorescent reporters (e.g. Sox2-V5 and Sox2-mCherry); and 4) engineering of glioma mutations (TP53 deletion; H3F3A point mutations). These resources and optimized methods enable facile and scalable genome editing in mammalian NS cells, providing significant new opportunities for functional genetic analysis.
Introduction

Targeted editing of endogenous genes via homologous recombination (HR) – termed gene targeting – has been widely deployed in studies of pluripotent stem cells, most notably mouse embryonic stem (ES) cell (Capecchi, 2005). Complex genetic manipulations in these cells is possible due to their inherent experimental attributes: they can be expanded to large numbers without genetic transformation; are easily transfected, enabling efficient delivery of exogenous DNA; can undergo homologous recombination (HR); and perhaps most critically, can be selectively propagated by clonal expansion in order to isolate defined genetic variants (Glaser et al., 2005). Such properties have underpinned a repertoire of genetic engineering technologies, including gene knockouts, conditional mutagenesis, and endogenous protein tagging. This has transformed our ability to explore mammalian gene function.

Similarly, the in vitro culture of neural stem (NS) and progenitor cells – of various distinct classes – has proven a valuable experimental approach for exploring molecular processes controlling self-renewal and differentiation across development, tissue homeostasis, and in disease models of the central nervous system (CNS) (Gage and Temple, 2013). NS cell lines display molecular hallmarks of forebrain radial glia and can be readily established and expanded as adherent monolayers, either following in vitro differentiation of pluripotent cells, or more directly via primary culture of germinal tissues from the developing and adult mammalian CNS (Conti and Cattaneo, 2010; Conti et al., 2005; Pollard et al., 2006). These tissue stem cells can be routinely propagated and clonally expanded as primary stem cell lines in the absence of spontaneous differentiation and/or genetic transformation, therefore providing an experimentally tractable somatic stem cell model.

Genetically normal NS cell lines have proven particularly useful in studies where large numbers of tissue-restricted stem cells are needed, such as biochemical analyses (Engelen et al., 2011), transcriptome profiling (Johnson et al., 2008; Webb et al., 2013), genome-wide
mapping of chromatin modifications, DNA methylation and transcription factor binding (Bernstein et al., 2006; Caren et al., 2015; Meissner et al., 2008; Mikkelsen et al., 2007), and chemical or genetic screening (Diamandis et al., 2007; Hubert et al., 2013). Importantly, malignant cells displaying phenotypic and functional properties analogous to NS cells can also be isolated from glioblastoma (GBM) patient samples using similar culture conditions (Pollard et al., 2009; Singh et al., 2004). This enables comparison of genetically normal NS cells to their malignant GBM counterparts, which has been helpful in defining tumour-specific vulnerabilities (Danovi et al., 2013; Ding et al., 2013; Hubert et al., 2013; Pollard et al., 2009).

Despite experimental attributes that are analogous to ES cell cultures, targeted genetic manipulations directly in mammalian NS cell lines have not yet been reported. Gene targeting has been notoriously difficult in most somatic cell types due to the inefficiency of HR. However, recent improvements in design and production of customizable nucleases capable of introducing site-specific DNA double-strand breaks (DSBs) have provided increasingly refined and reliable ways to manipulate the mammalian genome (Mali et al., 2013). Once introduced, DSBs are able to trigger endogenous homology-directed repair (HDR) mechanisms, thereby increasing gene targeting efficiencies at a locus of interest when an exogenously introduced repair template is delivered. Alternatively, DSBs can be repaired through the error-prone non-homologous end-joining (NHEJ) pathway, which normally results in random insertion or deletion (indel) mutations (Hsu et al., 2016). Therefore, in addition to facilitating gene targeting by HR, DSBs result in site-specific mutagenesis – one of the most common applications of CRISPR/Cas9.

Among the different platforms described for introduction of site-specific DSBs in eukaryotic cells, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system has emerged as the favored technology (Hsu et al., 2014). Derived from the prokaryotic adaptive immune system, this consists of an RNA-guided endonuclease (Cas9) able to efficiently generate DSBs using Watson-Crick base pairing to identify target DNA (Jinek et
al., 2012). CRISPR/Cas has been adapted for mammalian genome editing purposes through human codon optimization of Cas9 and generation of a chimeric single guide RNAs (sgRNA) (Mali et al., 2013). The Cas9 has also been further modified by mutation of one of the two independent nuclease domains in order to generate a nickase variant (Cas9n) that provides greater target specificity when used with a pair of strand specific sgRNAs (Cong et al. 2013; Ran et al, 2013).

Here we exploited the CRISPR/Cas9 technology to demonstrate complex and precise genetic manipulations in both mouse and human NS cell lines. We find that CRISPR/Cas9-assisted gene targeting in NS cells is highly efficient, easy to implement, and scalable. Optimized strategies and protocols were developed to support a range of targeted genetic manipulations, such as gene knockouts, knockins of epitope tags and fluorescent reporters, and delivery of disease-relevant mutations. As an example of the potential of this new technology, we focused our efforts on neurodevelopmental transcription factors, given the wide interest in these across stem cell biology, reprogramming, regenerative medicine and neurooncology.
Results

Mouse and human NS cell cultures can undergo gene targeting via Cas9-assisted homologous recombination at ‘safe-harbour’ loci

Genomic safe harbours are known to undergo efficient HR in ES/iPS cells and enable predictable expression of exogenous DNA elements across various cell types. To first determine whether mouse and human NS cells were amenable to Cas9-assisted gene targeting we focused on the widely used safe harbor loci mouse Rosa26 and human AAVS1. Targeting vectors with 1kb homology arms flanking a constitutive Luciferase-2A-GFP-IRES-BSD expression cassette were produced and tested in an adult mouse NS cell line (ANS4) and human foetal NS cell line (U3) (Figure 1A-B; Fig S1). Two matching CRISPR sgRNAs were designed to lie in close proximity and target opposite strands of each locus and could therefore be used as a pair with the Cas9n (Ran et al., 2013).

Transient plasmid transfection of Cas9n and sgRNA pairs was performed and GFP expressing cells were isolated using fluorescent activated cell sorting (FACS) 10-15 days after transfection and drug-selection (Figure 1B-C). PCR-based genotyping of the sorted population indicated targeted insertion of the expression cassette into Rosa26 and AAVS1 loci, and the majority of cells stably expressed high levels of GFP (>80%, visualized by microscopy) and luciferase (Figure 1D–F). To determine the precise efficiency of correct insertion, clonal lines were generated from the sorted population using improved colony formation conditions (see methods) and PCR-genotyped (Figure S2A). We achieved targeting efficiencies of ~23% (11/48) and ~16% (3/19) in mouse and human NS cells, respectively. Similar efficiencies were achieved in freshly derived foetal E11.5 mouse NS cell lines (FNS2; Figure S2B). Further PCR screening did not detect vector backbone sequences and qPCR copy number analysis indicated single copy Rosa26 integration in the majority of correctly targeted clones (Figure S2C-D). Genome-edited clonal lines proliferated normally, and uniformly expressed GFP and the known NS cell markers Nestin and Sox2.
Diploid karyotype as well as glial and neuronal potential was maintained after continuous in vitro expansion (Figure S2E-F).

Both Rosa26-LUC-GFP and AAVS1-LUC-GFP targeting vectors were constructed using an intermediate vector compatible with the Gateway cloning system (Figure S3A). This strategy provides flexibility as it enables straightforward exchange of alternative cargos, which was exemplified by the generation a CAG promoter-driven Cas9-2A-GFP expression cassette for knockin to Rosa26 (Figure 1G). Delivery of this construct into mouse NS cells enabled facile isolation of constitutively expressing Cas9 NS cells (termed CAS9-NS cells; used later in this study) using FACS for GFP (Figure 1H-I). A similar strategy for targeting AAVS1 also enabled rapid generation of Tet-inducible-GFP expressing human NS cells (Figure S3B-D).

Altogether, these results demonstrate that both mouse and human NS cells can undergo Cas9-assisted gene targeting and that promoter-driven expression cassettes can be efficiently knocked into safe harbor loci via HR. This has practical value, as it circumvents two issues – insertional mutagenesis and transgene silencing – that are often associated with conventional transgenic approaches (e.g. viral vectors or transposase delivery system).

Efficient biallelic disruption of Olig2 in mouse NS cells using Cas9-induced NHEJ

Targeted inactivation of endogenous genes via deletion or mutation of coding sequences is the standard reverse genetic strategy to define gene function. Unlike RNAi technologies, loss of the gene product is unequivocal and permanent, while risks of undesirable non-specific effects are minimized. We therefore tested whether the CRISPR/Cas9 system could be used to efficiently disrupt gene expression in mouse NS cell lines via site-specific mutagenesis.

For this purpose, we focused on Olig2, a known NS cell transcriptional regulator that is highly expressed in mouse NS cell lines (Ligon et al., 2007). A pair of sgRNAs targeting
Olig2 coding sequence and a Cas9n-2A-GFP expression plasmid were transiently delivered into mouse NS cells. Successful transfectants were harvested by FACS on the basis of GFP expression (Figure 2A). Formation of indel mutations around the sgRNA target sites was confirmed using the T7EI assay (Figure 2B). The frequency of biallelic mutations that result in loss of the Olig2 protein product was quantified using immunocytochemistry (ICC) with a specific Olig2 antibody. Using this strategy, we observed biallelic knockout efficiencies of ~7% in unsorted cells, and up to ~25% in the sorted fraction (Figure 2B-C). A similar frequency of Olig2-negative colonies was observed when the transfected cells were clonally expanded (32%, n=25; not shown). Similar efficiencies could be achieved using the independent foetal forebrain NS cell line FNS2 (Figure S4A).

Recent evidence suggests that Cas9 off-target cleavage is not as pervasive as initially feared and can be minimized using appropriate sgRNA design rules (Kim et al., 2016). We therefore tested the wild-type Cas9 (WTCas9-2A-GFP), rather than the nickase, as this provides a more convenient strategy. The two sgRNAs (predicted to target unique genomic sites; see Methods) were delivered separately and, following FACS enrichment, we noted significantly higher efficiencies could be achieved to the Cas9n (Figure S4B). Thus, both Cas9 and Cas9n can work effectively, with the latter being useful if unique gRNAs are unavailable.

We next delivered the individual sgRNA plasmids into our newly generated CAS9-NS cell line to test if even higher levels of mutations were possible (Figure 2D). The T7EI assay indicated indel formation around the predicted cutting site, thereby confirming the activity of the constitutively expressed Cas9 (Figure 2E). Remarkably, using this approach we achieve knockout efficiencies of around >50% for each sgRNAs (Figure 2E-F). Altogether, these results demonstrate the power of CRISPR/Cas9 to enable efficient biallelic disruption of Olig2 via NHEJ in mouse NS cells. This can be achieved either by transient plasmid delivery of Cas9 or with increased efficiency using a constitutively expressing Cas9 NS cell line.
Efficient generation of \textit{Olig2} knockout NS cell lines using CRISPR/Cas9-assisted gene targeting.

Despite the value in generating random indels by NHEJ, a more precise and flexible approach to manipulate endogenous genes is provided by HR-based gene targeting. This offers complete control over the type of allele to be generated and enables removal or replacement of any desired sequence. To test whether this was possible in mouse NS cells, we employed a gene targeting strategy recently developed for human iPS cells (Skarnes lab, pers comm). A selectable marker, in this case PuroR, is used to replace a target exon of interest, thereby enabling enrichment for correctly targeted cells; these then emerge as discrete resistant colonies that can be selectively propagated and genotyped. Provided that biallelic cleavage is possible and DSBs are likely to be repaired more frequently by NHEJ than HDR mechanisms, we anticipated that the most frequent type of editing event would be targeted replacement of one allele with the selectable marker, and generation of indels at the other. Thus, biallelic loss-of-function mutations might emerge from a single round of transfection and selection. Importantly, this approach does not require cell sorting or Cas9-expressing transgenic lines, and genotyping of clones is simplified by the fact that the indel containing allele can be directly sequenced from the PCR reaction.

We first focused on the mouse NS regulator \textit{Olig2}, attempting to replace its single coding exon with an Ef1α-Puromycin selection cassette and assessing the presence of indel mutations on the second allele (Figure 3A-B). The sgRNA pair described above was used together with a newly generated targeting vector with 1kb long homology arms flanking the Ef1α-Puromycin cassette. This was generated via production of a Gateway cloning compatible intermediate vector, as described for \textit{Rosa26} and \textit{AAVS1} vectors.

Mouse NS cells were transfected with the circularized targeting vector alone or in combination with Cas9n/sgRNAs plasmids, and treated with puromycin. Resistant NS cell
colonies emerged within 7 days (Figure 3C), and PCR genotyping of pooled colonies was used to verify correct targeting at Olig2 locus (Figure 3D). Importantly, correct replacement of the Olig2 coding sequence by the Ef1α-Puro cassette was only achieved when both the Cas9n and sgRNAs were co-transfected, thus confirming the need for the CRISPR/Cas9 system to achieve efficient HR in NS cells (Figure 3D). As anticipated, using a T7EI assay we also observed formation of indels around the sgRNA cutting site, which are likely associated with NHEJ events within the non-targeted allele (Figure 3D).

To determine targeting efficiencies and the precise status of each allele, puromycin resistant colonies were picked and expanded as clonal NS cell lines. PCR-based genotyping confirmed correct targeting at the Olig2 locus in ~26% of the screened clones (n=112, Figure 3E). Biallelic replacement of Olig2 coding sequence was not observed, as PCR with internal primers was in all cases able to amplify the non-targeted allele (Figure 3E). Nevertheless, Sanger sequencing of the PCR products revealed a remarkably high frequency of indel mutations within the remaining allele of correctly targeted clones (n=16/29; Figure 3F-G), thereby demonstrating the value of the strategy for efficient generation of biallelic mutants. Complete ablation of Olig2 protein levels was confirmed by ICC and WB in the clones harbouring frame-shifting indels (i.e. 3N+1 or 3N+2) (Figure 3G-H and S5A). The edited clonal lines were diploid, maintained NS cell morphology, Nestin expression, and proliferated similarly to parental controls (Figure 5B). qPCR copy number analysis indicated single insertion of the EF1a-Puromycin cassette in the majority of correctly targeted clones (Figure S6A).

We also tested the Olig2 targeting in a PDGFRα-H2B-GFP reporter NS cell line (PG1-1) derived from a previously generated mouse strain (Hamilton et al., 2003). PDGFRα is a marker of oligodendrocyte progenitor cells and is activated as NS cells undergo differentiation. Puromocyn-resistant colonies were generated and, consistent with its known role, we found that Olig2-negative NS cell colonies failed to give rise to GFP-positive oligodendrocyte precursor cells when triggered to differentiate (Figure S7).
Together, these data demonstrate that generation of NS cell lines with biallelic loss of function via gene targeting is highly efficient in mouse NS cell lines. Remarkably, this can be achieved with only a single round of transfection and screening of a handful of clones, thereby greatly simplifying reverse genetics in NS cells.

A scalable strategy to generate mutant alleles in mouse NS cells using CRISPR/Cas9-assisted gene targeting.

To determine whether the observed high knockout efficiency was unique to Olig2 we next assessed gene targeting for a further three neurodevelopmental transcription factor genes: Cebpb, Ascl1 (also known as Mash1) and Sox2 (Carro et al., 2010; Castro et al., 2011; Gómez-López et al., 2011). The coding sequences of these genes also lie within a single exon, thereby enabling their complete ablation. Utilizing the same gene targeting strategy and delivery methods as for Olig2 (Cas9n, plus Ef1a-PuroR exon replacement), an even higher targeting efficiency was achieved for Cebpb (~57%; n=88). The majority of correctly targeted clones also harbored indel mutations at the non-targeted allele (36/50; biallelic mutation efficiency 72%). By comparison, for Ascl1 we observed relatively lower targeting efficiency (13.6%) and frequency of biallelic mutations (1.5%; n=66). However, simply switching to wild-type Cas9 significantly increased the frequency of Ascl1 targeted clones and presence of indels within their second alleles (~35 and ~30%, respectively; n=86) (Table1, Figure 3I). Approximately 70% of correctly targeted CebpB and Ascl1 clones showed single copy transgene integration as confirmed by qPCR copy number analysis (Figure S6A).

Sox2 is critical for in vitro NS cell self-renewal (Gómez-López et al., 2011), and therefore recovery of expandable NS clones with biallelic mutations and consequent loss-of-function should not be possible. To achieve targeting at Sox2 locus, we tested sgRNA pairs lying near to either the start or the stop codon (sgRNA pair 1-2 and 3-4, respectively) in
combination with Cas9n or wild-type Cas9. As anticipated only a limited number of puromycin-resistant NS colonies emerged, and low targeting efficiencies ranging from \(~1.3\%\) to \(~9.0\%\) (\(n=79\) and \(n=22\), respectively) were achieved (Table1). Importantly, biallelic knockout was not observed. The recovered targeted clones contained no damage or only small in-frame indels on the second allele (Figure 3I and S8), confirming the essential role of Sox2 in NS cell colony formation. Failure to recover loss of function mutations on the NHEJ damaged allele thereby provides unbiased functional genetic evidence that a gene is necessary for NS cell self-renewal.

To enable medium throughput experiments, we optimized conditions to allow parallel transfection of 16 samples using reduced cell numbers and DNA amount with the Amaxa 4D system (Lonza) (see methods). We designed and built targeting vectors and matched sgRNA pairs to knockout a set of 16 candidate regulators of NS cell self-renewal, chosen as potential Sox2 downstream transcriptional targets (Lodato et al., 2013; Pollard lab, unpublished). Wild-type Cas9 was used in these experiments to maximize recovery of targeted clones. We found that efficient targeting can be achieved using only 400k cells and \(<1\ \mu\text{g}\) of plasmid DNA (Figure S9).

Using these optimized conditions, we targeted 16 genes in a single experiment, with mutant clones obtained, expanded and genotyped within 3 weeks. Eleven of these were successfully targeted (\textit{Olig2}, \textit{Nfe2l2}, \textit{Klf7}, \textit{Rorc}, \textit{Foxj3}, \textit{Fox}, \textit{Hoxa5}, \textit{Lhx2}, \textit{Trp73}, \textit{Klf6}, \textit{Hes6}), with efficiencies ranging from 5–56\% and loss-of-function mutants recovered in most of the cases (Table1, Figures 3I and S8). Thus, biallelic knockouts can be routinely generated via Cas9-assisted gene targeting in an efficient and scalable manner in mouse NS cells.
**Knock in of a fluorescent reporter to generate Sox2-mCherry reporter NS cells**

Knockin of fluorescent reporters to generate in-frame fusion proteins provides a useful experimental approach to monitor levels and localization of a specific gene product. Such a strategy has been widely deployed in ES/iPS cells to allow real-time observation of gene-expression dynamics, cell-lineage tracing, and isolation of a specific cell population of interest from differentiating cultures or embryos (Goulburn et al., 2011; Hendriks et al., 2016; Ying et al., 2003).

To test whether fluorescent reporters could be knocked in using CRISPR/Cas-assisted gene targeting in mouse NS cells, we focused on Sox2. We designed an antibiotic-selection free strategy in which a mCherry fluorescent reporter cassette, plus small flexible linker, is introduced at the Sox2 C-terminus, creating a new Sox2-mCherry fusion protein product (Figure 4A). The targeting vector was promoterless, and consequently mCherry signal can only arise from the correct, in-frame insertion at the endogenous Sox2 locus.

Cells were transfected with the targeting vector together with the Cas9n and a pair of sgRNAs that cut in the 3'UTR sequence adjacent to the stop codon (sgRNAs 5-6). After 10 days, ~1% of cells were identified as mCherry-positive and isolated by cell sorting (Figure S10A). Wide-field immunofluorescence microscopy confirmed nuclear localization of the mCherry in live cells; this co-localized with Sox2 antibody staining by ICC (Figure 4C-D and S10B). Clonal lines were subsequently derived from the sorted mCherry-positive subpopulation and PCR genotyped (Figure 4E). From 23 clones screened, 20 were correctly targeted at the Sox2 locus (~87% targeting efficiency). Notably, biallelic targeting to create homozygous reporters was achieved with high efficiency (~26%; n=6). Flow cytometry analysis confirmed uniform mCherry expression in homozygous targeted Sox2-mCherry NS clonal line (Figure 4F). This proliferated normally, displayed NS morphology and nestin expression and diploid karyotype (Figure S10C-D). Together the results demonstrate the power of Cas9-assisted HR for facile knockin of fluorescent protein reporters at endogenous loci.
genes in NS cells, and highlight the value of promoterless targeting vectors to isolate biallelic targeted cells.

**Efficient knock-in of epitope tags using single-stranded oligonucleotide donor templates**

Epitope tagging involves fusion of a small peptide (e.g. V5, FLAG, HA or MYC) to the protein of interest. This simplifies immunoprecipitation, immunoblotting, and immunocytochemistry, and is highly desirable when good quality antibodies are unavailable. The approach, however, has been mainly limited to ectopically expressed transgenes, with the attendant limitations of their non-physiological levels of expression. Knock-in of epitope tags to endogenous genes therefore represents a more attractive experimental approach and has been widely employed in model organisms, such as *S. cerevisiae*, where HR is highly efficient (Ghaemmaghami et al., 2003). Recently this has also been reported in mammalian ES cell and cancer cell lines through CRISPR/Cas9-assisted gene editing (Savic et al., 2015; Yang et al., 2013). We therefore assessed whether knock-in of epitope tags to endogenous genes could be achieved in mouse NS cell lines and developed optimized protocols for this purpose.

Because of the reduced length of coding sequences for epitope tags, we set to use short oligodeoxynucleotides as a donor template, thus avoiding the need for targeting vector production. Initially, we attempted to insert a V5 tag into *Olig2* and *Sox2* using a strategy previously described in human ES cell cultures (Ran et al., 2013). This relies on NHEJ-based insertion of double-stranded oligodeoxynucleotide (dsODN) donors containing overhangs compatible with Cas9n-induced double nicking. While ICC identified clear V5 nuclear staining in ~1.5% of cells, unfortunately this strategy was undermined by the persistent emergence of undesirable flanking indels in the resulting clonal lines (data not shown).
A simpler alternative strategy was therefore pursued, wherein single-stranded ~185-nt long oligodeoxynucleotides (ssODN) were used as repair templates. These comprised 45 nucleotides of a V5 tag sequence flanked by 70-nt long homology arms. To avoid disruption of endogenous gene coding sequences, gRNAs were designed to target the 3’UTR region proximal to the stop codons (Figure 5A). The PAM sequences contained in the ssODN repair templates were removed. The knockin efficiencies were quantified through ICC with a V5-tag antibody and three distinct delivery strategies were assessed: 1) transient Cas9- and sgRNA-coding plasmids; 2) in vitro transcribed (IVT) sgRNA complexed with recombinant Cas9 protein (rCas9-sgRNA complex); 3) delivery of IVT sgRNA into the Cas9-expressing NS cells (Figure 5B). For the delivery of recombinant Cas9 and IVT sgRNA, we optimized the transfection protocol with the Amaxa 4D system (Figure S11A-B).

Highest levels of knockin (~10%) were achieved using the CAS9-NS cells by transfection of only IVT gRNAs and ssODNs (Figure 5C-D), whereas plasmid delivery of all components was much lower. However, ~5% Olig2 V5-positive cells were achieved when using the pre-assembled rCas9/sgRNA complex (> 5-fold greater than plasmid delivery). This is efficient enough that clonal lines could be established, and offers greater flexibility then using the CAS9-NS cells, as any existing NS cell line can be engineered with this approach.

To determine the precise status of each allele, clonal lines were derived from the bulk population and PCR genotyped (Figure 5E). Two out of five V5-positive clones were found to be tagged at each allele, demonstrating the remarkable power of this approach to quickly isolate clones with biallelic tagged proteins. Sanger sequencing of the PCR products confirmed correct, in-frame insertion of the V5 tag sequence into Olig2 C-terminus (Figure S11C), while ICC indicated complete co-localization of V5 tag and Olig2 signals in the established clonal lines (Figure 5F).

Successful knockin of the V5 tag was also achieved at Sox2 locus using both rCas/sgRNA complex and CAS9-NS cells (~1.5 and 5.5%, respectively) (Figure S11D). Similar tagging
efficiencies were also observed in the primary mouse foetal FNS2 line and in a glioma-initiating line harbouring Ink4a/ARF and EGFR overexpression (Bruggeman et al., 2007) (Figure S11E). Altogether, these results demonstrate that epitope tags can be effectively knocked into mouse NS cell lines without recourse to any selectable markers, targeting vectors or cell sorting.

**Engineering of glioma driver mutations into human NS cells**

Glioblastoma stem cells display molecular and phenotypic similarities to NS cells and likely arise from endogenous CNS stem or progenitor cells (Stiles et al., 2008). Engineering glioma mutations stepwise or in combination into genetically normal human NS cells is an attractive possibility that could open up significant new opportunities for studying mechanisms involved in brain tumour initiation, growth and evolution. Building upon the successful demonstration of gene targeting at *AAVS1* locus in human NS cell lines (Figure 1), we next explored whether disease-relevant knockouts and introduction of point mutations were possible in human foetal NS cells, similarly to their mouse counterparts (Figure 6).

To demonstrate possibilities for loss-of-function mutations, we focused on *TP53*, one of the most frequently mutated tumour suppressors in human cancers, including the majority of GBMs (Brennan et al., 2013). We used the same exon-replacement strategy as described the mouse NS cells lines, in which a vector containing 1kb long homology arms flanking the Ef1α-Puromycin cassette was used to target *TP53* exon 5 (encodes the DNA-binding domain of p53) (Figure 6A). Human NS cells (U3 line) were transfected with the targeting vector, a pair of sgRNAs and Cas9n plasmids, and selected five days post-transfection. Human NS cells proliferate more slowly than mouse NS cells, which makes genetic manipulations more time-consuming. Nevertheless, puromycin-resistant colonies emerged (Figure 6B) and clonal lines could be established over a period of 4-6 weeks. PCR genotyping of the clonal lines confirmed successful targeting of one *TP53* allele by the
selection cassette in ~67% of the case (n=8/12; Figure 6C). Importantly, Sanger sequencing showed the presence of frame-shifting indels in the non-targeted TP53 allele in the majority of correctly targeted clones (an exemplar clone is shown; Figure 6D). Similar high knockout efficiencies were also achieved for two independent human foetal NS cell lines (U5 and U3 Hind, not shown). All correctly targeted clones lacked detectable p53 protein levels by WB and ICC analysis (Figure 6C and S12). As anticipated, increased cell proliferation was observed following TP53 knockout (Figure 6E).

To illustrate engineering of candidate gain-of-function oncogenic mutations, we pursued the delivery of somatic point mutations affecting H3F3A, which encodes the histone variant H3.3 and is frequently mutated in childhood gliomas. Such mutations occur within the N-terminal tail of the histone H3.3 and result in single amino acid substitutions (either K27M or G34R/V) (Schwartzentruber et al., 2012; Wu et al., 2012). From a practical standpoint, the proximity of the mutations to the N-terminus proved useful, as we could test if the mutant alleles could be introduced to the endogenous gene, using V5 tag as a reporter of successful knockin. We thus used a pair of sgRNAs targeting the 5’UTR region directly upstream of the start codon together with a targeting vector containing 1kb long homology arms flanking the N-terminal tagged versions of the first coding exon (WT, K27M or G34R) (Figure 6F). Targeting efficiencies varied from 0.2 to 0.5% as determined by ICC for V5 tag (Figure 6G), with similar efficiencies being achieved for the other two independent human cell lines (U5 and U3 Hind, not shown). WB using antibodies against the V5 tag and Histone H3 specific antibodies confirmed the expected size of the tagged protein (around 17kDa) (Figure 6H). Correct targeting at H3F3A was demonstrated by PCR-based genotyping using primers lying within the V5 tag sequence and downstream of the right homology arm (Figure 6H). Additionally, Sanger sequencing of the PCR product confirmed the presence of the expected point mutations in the targeted cells (Figure 6I).
Discussion

New tools and strategies that enable precise genetic manipulations in NS cells are highly desirable and would open up considerable new opportunities for discovery across stem cell biology, regenerative medicine, neuroscience and related fields. However, targeted genetic manipulations using HR – the mainstay of functional genetic analysis of pluripotent cells – has been technically challenging in most somatic stem cells, largely due to practical constraints limiting the ability to deliver, isolate and clonally expand cells with the desired genetic change. We reasoned that NS cell lines might be particularly well suited to targeted genetic manipulations, as they are readily transfectable and can be clonally expanded in adherent and feeder-free culture conditions. Here we have demonstrated that mouse and human NS cell lines are highly amenable to a range of precise genetic manipulations facilitated by the CRISPR/Cas9 technology.

To date, the simplest application of CRISPR/Cas9 in mammalian cells, including mouse neural precursors in vivo, has been to disrupt gene function through the generation of random indel mutations (Chen et al., 2015; Kalebic et al., 2016). This is clearly an important application and is particularly well suited to genome-wide screening, as demonstrated recently in human NS cell cultures (Toledo et al., 2015). We confirmed that this works well for mouse NS cell lines using transient plasmid delivery of CRISPR/Cas9 components followed by FACS-based enrichment of transfected cells. NHEJ-mediated gene disruption was also highly efficient when using the mouse Rosa26-Cas9 NS cell line (CAS9-NS), which avoids the need for FACS and has particular value for genetic screenings using pooled libraries of CRISPR sgRNAs, as previously achieved in mouse ES cells (Koike-Yusa et al., 2013).

However, the obvious limitations of gene disruption via indel formation is the lack of control over the types of mutations that emerge and a requirement for screening large numbers of clones to identify the desired loss-of-function mutants. Following the demonstration of
transgene insertion via HR at safe harbour loci, we deemed it important to explore whether
gene disruption in NS cell lines could be achieved via gene targeting, as originally developed
in mouse ES cells (Capecchi 2005). Using an exon-replacement approach recently devised
for human iPS cells (Skarnes lab, pers comm), we demonstrate that mouse NS cell lines are
highly amenable to gene knockout via HR, with truly remarkable efficiencies of biallelic
knockout possible.

Although we did not observe biallelic targeting – i.e. both alleles undergoing successful
replacement by HR – we did consistently observe monoallelically targeted clones harbouring
indel mutations on the non-targeted allele. While deemed disadvantageous under certain
circumstances (e.g. knockin of fluorescent reporters; Merkle et al. 2005), the presence of
these indel mutations has considerable practical value as it enables a single round of
targeting and selection to virtually guarantee isolation of loss-of-function mutant clones.
Moreover, due to the random nature of the indels, this offers a platform for simple generation
of partial loss-of-function (hypomorphic), or, less frequently, gain-of-function (e.g., dominant-
negative) alleles in a controllable manner. As only one allele is left after the HR event (i.e.
cells are hemizygous) studying the resulting allelic series of distinct clonal lines can offer
valuable insights into specific residues or domains of interest. Indeed, sgRNAs might be
specifically designed with this purpose in mind - e.g. to probe protein or DNA interaction
domains, phosphorylation sites, etc.

To open up possibilities for higher throughput production of mutants, we successfully
optimized the transfection protocols, scaling down the amount of cells and DNA used.
Remarkably, mutation of 11 transcription factors in NS cells was achieved in only a few
weeks (plus ~2 weeks for targeting vector construction) using a single round of sixteen
transfections in parallel. From a practical standpoint, this means typically only a handful of
NS cell colonies need to be picked and screened to isolate biallelic mutants, thereby
significantly accelerating precise genetic modification of NS cells. These findings are
particularly timely, as the plummeting costs of DNA synthesis now enables rapid production
of bespoke and elaborate targeting vectors in weeks rather than months. Furthermore, direct engineering of NS cells is advantageous, as it sidesteps the need for ES or induced pluripotent stem (iPS) cell modification and subsequent differentiation, which can be laborious and problematic, particularly if the gene is required earlier in development.

Our optimized protocols for efficient gene targeting in mouse NS cell lines led us to attempt the more challenging production of knockin alleles. We focused on generation of fusions of either fluorescent proteins or peptide epitope tags. The knockin of the fluorescent report to Sox2 – a highly expressed gene in NS cells – was remarkably efficient, highlighting the value of flow cytometry and use of promoterless targeting vectors to enrich biallelic targeted cells. Monitoring protein levels and localisation in live cells using endogenous regulatory elements is therefore now possible and will greatly facilitate functional studies. Complementary, epitope tagging of endogenous genes can now simplify protein interaction studies and mapping of genome-bound sites using IP-MS or Chip-Seq, respectively. This will be a massive boost for investigators who are frequently hampered by the limitations of poor quality, non-specific or unavailable antibodies. We found that such knockin of epitope tags can be rapidly implemented in NS cells, and our data suggest that delivery is most efficient using recombinant Cas9 protein/gRNA complex into NS cell cultures. Notably, efficiencies are high enough that cell sorting or drug selection strategies are not required.

The ability to precisely manipulate the NS cell genome has also great potential in neurooncology. There is now a clear opportunity to engineer precisely candidate driver mutations or epimutations uncovered in genome sequencing projects. We illustrated this by successful mutation of the endogenous TP53 and H3F3A loci in genetically normal human NS cell lines. It should be now possible to test the effects of glioma mutations, step-wise and in combination, across a range of spatial and temporally diverse NS cells cultures; and vice versa, efficient gene targeting could now enable gene correction of putative driver mutations in patient derived GBM cell lines. Together, both approaches create useful isogenic panels of human cellular models for brain tumour research – an important requirement for effective
cell based phenotypic screening.

Risks of off-target mutations with CRISPR are often discussed (Tsai and Joung, 2016). However, for in vitro cell lines – as opposed to genome editing in embryos – there are less concerns over potential off-target mutation, as one can generate multiple clones using different sgRNAs and also readily perform genetic rescue to provide confidence in any newly identified cellular phenotype. When unique sgRNAs are unavailable, then use of Cas9 nickase reduces the risk of off-target mutations (Cho et al., 2014). Nonetheless, the most recent studies exploring CRISPR-Cas9 off-targets effects have not identified significant issues when sgRNAs with unique targets are used (Kim et al., 2016) and therefore initial fears of widespread off-target damage have now waned.

CRISPR/Cas9 technology has rapidly transformed possibilities for mammalian functional genetics. We can finally move beyond transformed cell lines such as HeLa (Hyman and Simons, 2011), into a new era of systematic functional gene annotation, with multiple genes or pathways being critically explored in parallel in relevant human cells. Genetic control of NS cell self-renewal can be investigated using elegant genetic strategies that have been so successful in understanding ES cells (Martello and Smith, 2014). In summary, our findings indicate that targeted, precise, and complex genetic manipulations can now be readily performed in NS cell lines. This now opens up a wealth of opportunities to explore gene function in CNS development, adult homeostasis and pathological processes.
Materials and Methods

Design and construction of CRISPR sgRNAs and targeting vectors

CRISPR sgRNAs were designed using the Optimized CRISPR Design tool (http://crispr.mit.edu). Predicted sgRNA off-target sites were retrospectively analyzed using the WTSI Genome Editing tool (http://www.sanger.ac.uk/htgt/wge). All sgRNAs were predicted to target unique genomic sites. For the majority of those, similar sequences contained mismatches of three or more nucleotides (with at least one occurring in the PAM proximal region), and therefore off-target cleavage is unlikely (Cho et al., 2014). Sequences are provided in Table S1.

For sgRNA encoding plasmids, single-stranded oligonucleotides (Integrated DNA Technologies) containing the guide sequence of the sgRNAs were annealed, phosphorylated and ligated into BsaI site of U6-BsaI-sgRNA backbone (kindly provided by S. Gerety, Sanger Institute). For in vitro transcription (IVT), dsDNA templates for T7-driven transcription were generated by annealing two oligonucleotides – one containing the T7 promoter and guide RNA target sequences, and the other containing the Cas9-binding tracrRNA sequence. The annealed oligo pair was gap-filled using T4 DNA polymerase, column-purified, and then used as a template for IVT (MEGAscript® T7 Transcription Kit). The RNA was purified using MEGAclear™ Transcription Clean-Up Kit.

Targeting vectors were constructed via Gibson assembly and Gateway cloning (Figure S3). Briefly, linearized backbone and a Zeo/PheS bacterial selection cassette were obtained through EcoRV digestion of existing plasmids. Homology arms of ~1kb were amplified from genomic DNA using PCR primers with 22bp overhangs compatible with both backbone and the Zeo/PheS double-selection cassette. Gibson reactions were performed using standard protocol with home-made enzyme mix to create the intermediate Gateway cloning compatible intermediate vector. The Zeo-PheS cassette was replaced via LR Gateway cloning using a FRT-Ef1a-Puromycin-FRT mammalian selection cassette. For AAVS1 and
Rosa26 targeting vectors, the LUC-2A-GFP, Cas9-2A-GFP or rtTA expression cassettes were PCR amplified from existing plasmids (gift from M. Pule, UCL) and cloned into Gateway pDONR™221 using BP cloning. The cassettes were then delivery into the intermediate targeting vectors via Gateway LR cloning. Construction of the AAVS1-TRE-GFP targeting vector involved restriction digestion followed by ligation of a custom gene vector (Life Technologies) containing the TRE-GFP-2A-Puromycin cassette into the digested AAVS1 intermediate targeting vector (Figure S3). For mouse Sox2-mCherry targeting vector, 1kb long arms were PCR amplified and tethered to mCherry sequence using Gibson reaction. The sgRNA targeting region was removed from the R-HA in targeting vector to avoid re-cutting of residual Cas9 after the homologous recombination event. For H3F3A targeting vectors, homology arms were amplified from genomic DNA and Gibson-assembled with synthetic DNA fragments (Life Technologies) containing the V5 tagged, mutant sequences of the first coding exon.

**Plasmid encoded- and recombinant Cas9**

Human codon-optimized *Streptococcus pyogenes* wild-type Cas9 (Cas9-2A-GFP), Cas9 nickase (Cas9n-2A-GFP) plasmids were obtained from Addgene (#44719 and #44720; G. Church lab). Recombinant wild-type Cas9 was purchased from PNA Bio.

**Cell culture**

Mouse adult NS line (ANS4) was derived from the subventricular zone as previously described (Pollard et al., 2006). Mouse foetal NS cell lines FNS2 and PG1-1 were derived from the telencephalon region of E11.5 and 17.5 embryos, respectively. Human lines were derived from the telencephalon of ~8-week human foetal material provided by the MRC-Wellcome Trust Human Development Biology Resource (ICH, London). Detailed protocol for derivation of mouse and human foetal cell lines is described elsewhere (Conti et al., 2005; Sun et al., 2008). RNA-seq profiling of human NS cells confirmed expression of NS affiliated lineage marker. Genomic profiling of these lines using GeneChip SNP 6.0 arrays
(Affymetrix) did not identify any structural genetic alterations (not shown).

Mouse and human lines were expanded for more than 15 passages before use in gene targeting experiments. Established lines were propagated in serum-free basal medium supplemented with N2 and B27 (Life Technologies), laminin (Sigma, 1μg/ml) and growth factors EGF and FGF2 (Peprotech, 10 ng/ml). Medium was changed every 2-3 days and cells split 1:3 or 1:5 once per week after dissociation with Accutase solution (Sigma). Laminin was added directly to the culture media, with no need for pre-coating of flasks. This improved colony formation and simplified screening. Colonies were picked by manual aspiration using a 20μl pipette. Confluence analysis and growth curves were determined using Incucyte (Essen Bioscience) live cell imaging system.

**Cell transfection**

Cells were transfected using either Amaxa 2B or 4D nucleofection systems (Lonza) according to manufacturer’s instructions. For the 2B system, 2 x 10^6 cells were pre-mixed with plasmid DNA; 2 μg of the indicated Cas9 vector, 1 μg of each sgRNA and 1μg of the targeting vector or 2 μg of ssODN in 100 μl of Neural Stem Cell Amaxa nucleofection buffer. Nucleofection program T-030 and X-005 was used for mouse and human NS cells, respectively. For the 4D system, 16-strip cuvettes were loaded with, unless otherwise stated, 4 x 10^5 cells and 0.8μg plasmid DNA (0.4μg Cas9, 0.1μg each sgRNA and 0.2μg targeting vector) in SG transfection solution. Program DN100 gave best survival and transfection efficiency for plasmid DNA delivery. For delivery of Cas9/sgRNA complex, 5 μg (unless otherwise stated) of recombinant Cas9 (PNA Bio) were mixed with 3μg of in-vitro transcribed sgRNA and allowed to form ribonucleoprotein complex at room temperature for 10 min. The Cas9/sgRNA complex together with 1.5 μg of ssODN was transfected into 2 x 10^5 cells using the Amaxa 4D 16-strip cuvettes in SG transfection buffer. Program EN138 gave the best results for rCas9/IVT sgRNA delivery. After transfection, cells were recovered in pre-warmed culture media, plated onto 10 cm culture dishes for 5 days prior to drug selection or
downstream assays.

**T7 endonuclease 1 assay**

Genomic regions flanking the CRISPR sgRNA target sites were PCR amplified using gene-specific primers (Supplementary Table 2). PCR products were purified with MinElute PCR Purification Kit (Qiagen) and hybridized in NEB buffer 2 (95°C, 5 min; 95-85°C at −2°C/s; 85-25°C at −0.1°C/s; hold at 4°C). After treatment with T7 endonuclease I (5U, NEB) at 37°C for 1 hour, the resulting fragments were subjected to electrophoresis in a 2.5% agarose gel and visualised by staining with ethidium bromide. Cleavage quantification was based on relative band intensities using ImageJ.

**Immunocytochemistry**

Cells were fixed in 4% paraformaldehyde, permeabilized and blocked in 0.1% BSA plus 3% goat serum solution. Samples were incubated overnight with primary antibodies followed by incubation with appropriate secondary antibodies and 4′,6-diamidino-2-phenylindole (DAPI). Immunopositive cells were quantified using ~5,000 cells (10 random fields). Total cell number was determined by DAPI nuclear staining. The following primary antibodies were used: mouse Nestin (DSHB, Rat-401, 1:10), human Nestin (1:500, R&D systems), mouse Sox2 (1:100, Abcam), human Sox2 (1:50, R&D systems), BLBP (1:200, Santa Cruz), GFAP (1:1000, Sigma), Tuj1 (1:1000, Biolegend), GFP (1:1000, Abcam), Olig2 (1:400, Millipore), mCherry (1:500, Abcam), V5 tag (1:1000, eBioscience), p53 (Cell Signaling, 1:400).

**Western immunoblotting**

Immunoblotting was performed using standard protocols. Antibodies were diluted in 5% milk powder in PBS-T, and protein detection was carried out with HRP-coupled secondary antibodies and exposed to X-ray film. The following primary antibodies were used: Olig2 (1:3000; Millipore), GAPDH (1:1000; GenTex), p53 (1:500; Santa Cruz), V5 tag (1:1000; eBioscience); Histone H3 (1:2000; Abcam).
**Drug selection and clonal expansion**

Cells with stable targeting vector integration were selected using 5 μg/ml blastidicin or 0.1 μg/ml puromycin. Selection commenced 5 days post-nucleofection to enable time for expression of Cas9, HR and expression of selection cassettes. After 7-10 days of blastidicin selection, Rosa26 and AAVS1 targeted cells were enriched by FACS (GFP expression) and plated at <5K cells/10cm dish for recovery of colonies. For the knockout experiments, cells were kept in puromycin for 7 days. Resistant colonies were individually picked into 2 x 24 or 96-well replica plates and expanded for cell banking and DNA extraction. Cells for genomic DNA extraction were cultured until >90% confluent.

**PCR-based genotyping of targeted clones**

Genomic DNA was isolated from each well of a confluent 24- or 96-well plate as follows: cells were incubated for 2hr at 55 °C in 20 or 40 μl of lysis buffer (0.45% NP40, 0.45% Tween20, 1x NEB LongAmp PCR buffer) and subsequently heated to 95 °C (10 min). 1–2 μl of this lysate was used in a 10μl PCR reaction. PCR reactions comprised 0.2 μl DMSO (100% v/v, Sigma), 0.3 μl dNTPs (10 mM, Thermo Fisher Scientific), 2.0 μl LongAMP buffer (5x NEB), 0.4 μl LongAMP Taq (NEB), and 12 pmol of each primer. Thermal cycling was performed using the following conditions: 1 cycle 94 °C for 3 min; 40 cycles 94 °C for 15 s, 60°C for 30 s, 65 °C for 2 min; followed by final extension at 65°C for 10 min.

For each targeted locus, two sets of genotyping primers spanning the junction of genomic sequences and targeting vector were used (left and right arms). Gene-specific primers were designed outside the 5’ and 3’ homology arms and were used in combination with primers in the knockin cassette (either CAG-LUC-2A-GFP-ires-BSD for targeting Rosa26 and AAVS1 loci, or Ef1α-Puromycin for the knockout experiments). To identify NHEJ-based indel formation on the second, non-targeted alleles, the region flanking the sgRNA target sites (500-600bp) was amplified using PCR with gene-specific primers and directly assessed by Sanger sequencing. Sequences of all primers are provided (Supplementary Table 2).
qPCR copy number analysis

Quantitative PCR with TaqMan Copy Number Assay (Applied Biosystems) was performed as previously described (Schick et al., 2016). A custom Puromycin-resistance gene (PuroR) probe was used with reference Tfrc (4458367). Genomic DNA from a mouse ES line harbouring a single genomic copy of GFP-IRES-PuroR (TNG; gift from Ian Chambers) was used as calibrator sample.
Author contributions

RB performed the majority of experiments. PD, MKa, EG led the experiments involving safe harbor knockin, Sox2-Cherry knockin, V5 tagging, respectively; VG provided technical support for the human NS cell line work. HB, SG produced key reagents for some of the experiments. MM and CG provided technical support for the scale up. WS provided expert advice and reagents for the targeting vector production. RB and SMP wrote the manuscript. RB, WS and SMP conceived and designed the overall study. SMP supervised the study.

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References


**Figure 1 | Mouse and Human NS cells are amenable to CRISPR/Cas-mediated gene targeting**

(A) Schematic diagram of the experimental strategy for generating constitutive Luciferase (Luc)-GFP expressing mouse and human NS cells by gene targeting at the safe harbor loci Rosa26 or AAVS1. Targeting vectors contained the CAG-Luc-GFP tethered by an IRES to a blasticidin resistance cassette (BSD); the expression cassettes were flanked by ~1kb long homology arms.

(B) Schematic depiction of the targeting strategy for the Rosa26 (left side) and AAVS1 loci (right side). Exons are depicted as dark grey-filled blocks. Light grey rectangles indicate the location of the homology arms flanking the expression cassette (L-HA, left homology arm; R-HA, right homology arm). CRISPR sgRNA target sites are indicated with yellow triangles. Horizontal arrows indicate genotyping PCR primers used to confirm on-target integration of the expression cassette.

(C) Using fluorescence-based cell sorting (FACS), targeted cells were enriched on the basis of GFP expression after blastidicin selection. Wild-type (WT) non-transfected mouse and human NS cells were used as a control to set gates for cell sorting. SSC, side scatter.

(D) PCR-based genotyping using primer sets 1 and 2 (depicted in panel A) confirmed correct targeted integration of the CAG-LUC-GFP cassette into the Rosa26 and AAVS1 loci. Non-transfected parental cells were used as negative control for the genotyping.

(E) Representative live phase contrast and wide-field fluorescent microscopy images of sorted GFP-positive mouse and human NS cells. Scale bar 100 um.

(F) Luciferase levels were determined using a microplate reader and confirmed functionality of the targeted cassette in both mouse and human cells.
(G) Schematic of the Gateway cloning-based strategy for repurposing targeting vectors with different cassettes of interest. In the example shown, the LUC-GFP in the Rosa26 targeting vector is replaced by a Cas9-GFP expression cassette via LR Gateway cloning.

(H) Mouse NS cells were transfected with the Rosa26 Cas9-GFP targeting vector and enriched by FACS on the basis of GFP expression.

(I) PCR-based genotyping (top panel) confirms correct insertion of Cas9-GFP expression cassette at Rosa26 locus; western immunoblotting (bottom panel) confirms high levels of Cas9 protein expression in a clonal NS cell line derived from the GFP-sorted cells.
**Figure 2 |** Olig2 knockout in mouse NS cells via CRISPR/Cas9-induced NHEJ repair

(A) Experimental strategy for Olig2 deletion in WT mouse NS cells using transient plasmid delivery. Cells were transiently transfected with the CRISPR sgRNA pair (target site shown as yellow triangles) together with a Cas9n-2A-GFP plasmid. Transfected cells were enriched by FACS on the basis of GFP expression.

(B) Generation of indel mutations in the transfected cells was assessed using a T7EI cleavage assay. Larger arrow indicates the predicted WT/uncleaved PCR product; smaller
arrows indicate T7E1-cleaved fragments used to estimate indel frequency.

(C) Immunocytochemistry (ICC) was used to determine the frequency of biallelic knockout, which should result from frame-shifting mutations and resulting Olig2-negative cells (white arrows). Graph shows % of Olig2-negative cells in relation to total DAPI-stained nuclei. Scale bar: 50 μm.

(D) Experimental strategy for Olig2 deletion in the CAS9 NS cell line. Cells were transfected with the two CRISPR sgRNAs individually and (E) cleavage at target site confirmed by the T7E1 assay. (F) Efficiency of biallelic knockout was quantified by ICC for Olig2. Scale bar 50 μm.
Figure 3 | CRISPR/Cas9-mediated homologous recombination enables facile knockout of transcription factor genes in mouse NS cells.

(A) Schematic of the experimental strategy to knockout Olig2 via CRISPR/Cas9-assisted
gene targeting. Targeted cells are enriched by puromycin selection and should emerge as discreet NS cell colonies. Biallelic knockout clones are expected to have one allele replaced by the Ef1α-Puro cassette and the second allele damaged by an indel mutation at the CRISPR sgRNA cutting site (yellow triangle).

(B) Representation of the mouse Olig2 locus (top), predicted targeted alleles following HDR (middle) or NHEJ (bottom). CRISPR sgRNA cutting site is shown as yellow triangle and predicted resulting indel as yellow rectangle within the non-targeted allele. Olig2 coding sequence is shown in dark grey. Adjacent white rectangles represent untranslated regions (5’ and 3’ UTR). PCR genotyping (PCR1 and 2, left and right arms, respectively) were designed with primers within the Ef1α-Puro cassette and outside of the homology arms (light grey rectangles). PCR3 product could be used in a T7EI assay or Sanger sequencing to confirm the presence of indels in the NHEJ allele.

(C) Representative phase contrast image of discreet puromycin-resistant NS cell colony emerged after transfection with Olig2 targeting vector. Scale bar: 200 µm.

(D) PCR genotyping of pooled puromycin resistant colonies after transfection with Olig2 targeting vector (Ef1α-Puro TV) alone or in combination with CRISPR Cas9 nickase and/or sgRNA pair. PCR1 and PCR2 were used to confirm correct HR event at Olig2 locus (top and middle panels); T7EI assay for checking NHEJ-mediated damaged at the sgRNA targeted site (bottom lane).

(E) Representative genotyping PCR results of 10 puro-resistant clones, picked and expanded as clonal lines following transfection with targeting vector and CRISPR-Cas components.

(F) Summary of frequency of different types of indel mutations identified by Sanger sequencing of PCR3 product (shown in B) on the second allele in correctly targeted Olig2 clones. WT, no indel; In-frame, 3N; frame-shift, 3N+1 or 3N+2.
(G) Representative Sanger sequencing trace of one Olig2 targeted clone showing 10bp insertion within the remaining Olig2 coding exon.

(H) ICC in WT parental cells and Olig2 clone harboring the 10bp insertion confirms complete ablation of the Olig2 protein. Scale bar: 50 µm.

(I) Scale up of the same strategy used for Olig2 in 14 other transcription factors and frequency of idels present on the second allele of correctly targeted clones. WT, no damaged; In frame, 3N indels; frame-shift, 3N+1 or 3N+2; mosaic, different indels present within the same clone. Mosaic clones were identified by mixed Sanger sequencing traces (see Figure S5B)
Figure 4 | Generation of homozygous Sox2-mCherry reporter NS cell line

(A) Schematic of the experimental strategy for knockin of mCherry reporter at the Sox2 C’ terminus. Cells were transfected with the targeting vector together with sgRNA pair and Cas9n plasmids. After 10 days, mCherry-positive cells were isolated by cell sorting.

(B) Representation of Sox2 targeted locus and PCR-based genotyping. No promoter sequence is contained within the targeting vector (promoterless construct) and therefore mCherry expression is expected only when the Sox2 locus is correctly targeted. sgRNA pair was designed to cut in the 3’ UTR close to the stop codon. mCherry was fused to the Sox2-coding sequence through a flexible peptide linker (white box).

(C) Live images of sorted cells showing nuclear localized mCherry signal. Scale bar: 50 µm
(D) ICC reveals overlap of mCherry and Sox2 in the nucleus. Scale bar: 50 µm

(E) PCR genotyping of clonal lines derived after cell sorting. Homozygous clone (C11) shows only an upper band (3.0 kb), while heterozygously targeted clones also show lower, WT band (2.3 kb)

(F) Flow cytometry histogram confirming consistent mCherry expression in homozygous Sox2-mCherry clonal line. Parental NS cells (grey line) were used to set the gates. Data are plotted as a percentage of the total number of cells.
Figure 5 | CRISPR/Cas-based gene targeting enables epitope tagging of endogenous transcription factors genes.

(A) Representation of strategy to knockin the V5 tag in frame into the Olig2 C-terminus using a single-stranded oligonucleotide DNA (ssODN) as a donor template. V5 tag sequence is shown in green, homology arms in grey and stop codon in black. Yellow triangle represents the sgRNA target site (sgRNA-3) at Olig2 3’UTR.

(B) Schematic depicting the three strategies employed for the knockin of V5 epitope tag: (a) delivery of plasmids encoding sgRNA and Cas9-2A-GFP followed by FACS enrichment; (b) delivery of a ribonucleoprotein complex made by conjugating in vitro transcribed (IVT) sgRNA and recombinant Cas9 protein (rCas9); (c) delivery of IVT sgRNA into the CAS9 NS
cells (constitute Cas9 expressing from Rosa26).

(C) Representative image of V5-tagged Olig2 cells identified using ICC against the V5 tag. DAPI was used for nuclear staining. Scale bar: 50 µm.

(D) Quantification of Olig2-V5-positive cells using the different delivery strategies (shown in B). Values represent the percentage of V5-positive cells in relation to total DAPI nuclear counting.

(E) PCR-based genotyping of representative v5-Olig2 clones derived from the bulk cells transfected with rCas9+IVT sgRNA complex. PCR1 used primers within the V5 sequence and outside the R-HA. Homozygosity was confirmed using primers flaking the V5 insertion site (PCR2). Homozygous targeted clones (A1 and A4) were identified by a single upper band as compared the control, WT cells. Clones displaying two bands were considered as heterozygous.

(E) ICC for the V5 tag and Olig2 in homozygously tagged clonal lines. As anticipated, V5 staining is nuclear localized and overlaps with native Olig2 staining. Scale bar: 50 µm.
Figure 6 | Delivery of glioma-relevant mutations into genetically normal human NS cells.

(A) Schematic depiction of the targeting strategy to knockout TP53 via gene targeting. CRISPR sgRNA pair targeting site is indicated with yellow triangle. Horizontal arrows indicate PCR genotyping primers for assaying TP53 locus targeting (PCR1 and PCR2) and presence of an indel within the second allele (PCR3).

(B) Representative phase contrast image of puromycin-resistant human NS cell colony after 10 days of selection. Scale bar: 100 μm.

(C) PCR-based genotyping (top panel) and WB analysis (bottom panel) of human TP53 targeted NS clonal lines. Parental, non-transfected cells were used as a control.

(D) Sanger sequencing trace of an exemplar correctly targeted clone harboring an 85bp deletion on the second allele.

(E) Growth curve analysis of the TP53 targeted clones harbouring the 85bp deletion confirming positive proliferative effect of p53 ablation.
(F) Illustration of the strategy for gene targeting the first H3F3A coding exon. Yellow triangle indicates sgRNA pair targeting site. Horizontal arrows indicate PCR genotyping primers.

(G) ICC using V5 tag specific antibody to identify targeted cells. DAPI was used for nuclear staining. Scale bar: 50 µm.

(H) Western blotting using V5 tag and Histone H3 specific antibodies and PCR genotyping of parental and transfected cells.

(I) Sanger sequencing traces of the genotyping PCR products confirm presence of the point mutations in the V5 tag-positive cells.
Table 1. Summary of targeting efficiencies in mouse NS cell gene knockout experiments

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<th>Transfection system</th>
<th>Cell number / DNA amount</th>
<th>Cas9 type</th>
<th>sgRNA pair</th>
<th>Colonies per transfection</th>
<th>Clones screened</th>
<th>Targeted clones</th>
<th>Targeting efficiency (%)</th>
<th>NHEJ-repair second allele</th>
<th>Biallelic mutation efficiency (%)</th>
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Figure S1 | Characterisation of parental mouse and human NS cell lines used in the study.

(A) Newly derived mouse and human NS cell lines (ANS4 and U3, respectively) displayed the characteristic NS cell morphology and uniformly expressed the defining NS markers Sox2 and Nestin as well as the radial glia marker BLBP. Analysis of metaphase spreads indicated normal chromosomal number after culture expansion.
of both lines (modal chromosomal number = 40 in mouse cells and = 46 in human cells). Mouse and human lines were analyzed at passage 25 and 14, respectively.

(C) In differentiation conditions (see methods) astrocytes and neurons emerge, as shown by ICC and qPCR for the lineage markers GFAP (astrocyte) and TuJ-1 (neurons).
Figure S2 | Characterisation of mouse Rosa26 and human AAVS1 edited NS clonal lines.

(A) General PCR-based genotyping strategy for determining correct gene targeting at Rosa26 and AAVS1, and representative genotyping results of clonal lines derived from the GFP sorted population. Biallelic targeting was not achieved, as PCR with primer set 3 was in all cases able to amplify the non-targeted allele.

(B) Targeting at Rosa26 in the primary foetal mouse NS cell (FNS2).

(C) PCR-based strategy to detect vector backbone sequences in correctly targeted clones, and exemplar results of Rosa26-Luc-GFP mouse clonal lines. Mouse ES cell line TNG (Nanog-GFP-IRES-Puromycin) was used as control.

(D) Summary of GFP copy number analysis using quantitative PCR in Rosa26-Luc-GFP mouse clonal lines. 9/11 clones were shown to contain a single copy integrated in the genome.
(E) Growth curves of mouse and human clonal NS lines targeted with LUC-GFP cassette at Rosa26 and AAVS1 loci demonstrate similarly doubling time to unedited controls.

(F) Edited clonal lines displayed typical NS cell morphology, uniformly expressed the NS markers Nestin and Sox2 (left) and maintained diploid karyotype (modal chromosome number of 40 and 46 for mouse and human, respectively) as well as glial and neuronal differentiation potential (right).
Figure S3 | General strategy for construction of Gateway compatible targeting vectors and generation of Dox-inducible GFP human NS cells by gene
targeting at AAVS1.

(A) Construction of intermediate targeting vectors (left panel) involved PCR amplification of homology arms, followed by Gibson assembly of a Gateway compatible vector containing the bacterial double-selection cassette CmR-CcdB. LR Gateway reaction (right panel) was then used to exchange the bacterial selection by a mammalian expression cassette of interest. Empty white boxes represent a generic expression cassette. For generation of targeting vectors for the knockout experiments, the bacterial Zeo-PheS double-selection cassette was used in the intermediate vectors and Ef1a-Puro cassette introduced by LR-Gateway into the final vector (see Material and Methods).

(B) Experimental strategy for biallelic knockin at AAVS1 in human NS cells. Cells were transfected with two targeting vectors together with sgRNA1 and Cas9 expression plasmids. Cells targeted with both constructs were selected with puromycin in the presence of Doxycyclin (DOX).

(C) Schematic representation of the AAVS1 targeting vectors containing the components of the Tet-On inducible expression system. rtTA was introduced in one intermediate vector through LR Gateway cloning, while a second vector containing the TRE driving expression of GFP-2A-Puro was generated through conventional cloning (see details in Materials and Methods).

(D) Live cell imaging of drug-selected cells in the presence and absence of DOX, confirming functionality of the Tet-on inducible system in human NS cells. Scale bar 100 um
**Figure S4 | Olig2 knockout in mouse NS cells using CRISPR/Cas9-induced NHEJ.**

(A) Efficient biallelic knockout of Olig2 using transient plasmid delivery of Cas9n-2A-GFP and sgRNA pair in primary foetal forebrain mouse NS cells (FNS2). White arrows indicate Olig2-negative cells. Plot shows percentage of negative cells in relation to the total DAPI-stained nuclei. Scale bar: 50 µm.

(B) Delivery of wild type Cas9 and individual sgRNAs resulted in relatively higher Olig2 knockout efficiencies in the mouse NS cell line ANS4 following GFP-sorting. White arrows indicate Olig2-negative cells. Plot shows percentage of negative cells in relation to the total DAPI-stained nuclei. Scale bar: 50 µm.
Figure S5 | Characterisation of Olig2 mutant mouse NS clonal lines generated via CRISPR/Cas9-assisted gene targeting

(A) qPCR and Western blotting confirmed complete ablation of Olig2 protein, but not mRNA levels, in clonal lines harboring frame shifting indels on the second allele. Parental cells and targeted clones with non-mutated second alleles were used for comparison.

(B) Olig2 mutant clonal lines maintained a normal karyotype as determined by metaphases spread (modal chromosomal number = 40; n = 20-30), typical NS morphology and expression of Nestin (middle lanes) and proliferated normally under optimal self-renewing conditions (bottom lane). Blue lines represent the confluence curves of the indicated clones. Growth of parental cells (grey line) is shown for comparison.
Figure S6 | Summary of copy number analysis of puromycin-resistance gene in mouse clonal lines.

(A) Quantitative PCR analysis using Taqman Copy number assay determining the number of genomic copies of puromycin-resistance cassette in mouse targeted clonal lines. Approximately seventy-five percent of clones showed a single copy insertion.

(B) Exemplar mixed sequencing trace of the non-targeted allele from a CebpB-targeted mosaic colony.
Figure S7 | Olig2 targeting in a mouse foetal PDGFRαH2B-GFP reporter NS cell line. Following transfection with Olig2 targeting vector and CRISPR sgRNAs, puromycin-resistant colonies were differentiated for 4 days in the absence of EGF. Olig2-negative colonies did not generate GFP-positive, oligodendrocyte precursor-like cells.
Figure S8 | Representative indels found in the second alleles of the 14 successfully targeted genes. Sanger sequencing traces identify the status of the genomic sequence around the target site of the sgRNA pairs used in the knockout experiments. Targeted gene exons can be identified by the Ensembl exon accession number shown at the top right corner of each box.
Figure S9 | Optimization of the 4D Amaxa nucleofection system for gene knockout via CRISPR/Cas9 assisted gene targeting.

(A) Optimisation of transfection buffer and nucleofection program using a GFP expression plasmid. Solution G and program DN100 produced the maximal results in terms of cell survival and transfection efficiency.

(B) Olig2 gene targeting in mouse NS cells using the optimized transfection protocol.
Left - Targeting and genotyping strategy used (same as in Fig.3). Right - puromycin-resistant colony counts and PCR genotyping results after transfection of different cell amounts and Cas9 type.

(C) Exemplar genotyping results of puromycin-resistant colonies following Amaza 4D transfection using WTCas9 and 400k cells.

(D) qPRC copy number analysis of puromycin-resistant gene in \textit{Olig}2 correctly targeted clonal lines obtained with Amaza 4D transfection system.
Figure S10 | Characterization of mouse Sox2-mCherry reporter NS cell line.

(A) FACS plot indicating the percentage of mCherry positive cells 10 days post-transfection with a promoterless Sox2-mCherry targeting vector. Parental, non-transfected cells were used to set the gates. SSC, side scatter.

(B) ICC analysis confirmed co-localization of mCherry and Sox2 staining in the sorted but not in the parental cells. DAPI counterstaining was used to highlight nuclear localization of Sox2-mCherry staining.

(C) Growth curves of parental ANS4 (grey line) and homozygously targeted Sox2-mCherry clonal line (red).

(D) Sox2-mCherry clonal line maintained normal NS morphology, uniform expression of nestin and diploid karyotype (modal chromosomal number = 40).
Figure S11 | Epitope tagging in mouse NS cells using single-strand DNA oligonucleotides as donor templates.

(A) Optimization of rCas9/IVT sgRNA delivery for Olig2 tagging using the Amaxa 4D system. 15 transfection programs were tested in SG cell line buffer and knock-in efficiencies quantified by V5 ICC. Program EN-138 shows maximum efficiency and was used for rCas9/IVT sgRNA delivery in all experiments.

(B) Varying amounts of rCas9 were compared for transfection and Olig2 V5 knock-in efficiency.

(C) Sanger sequencing confirms correct, in frame insertion of the V5 tag into Olig2 C’terminus in homozygously tagged clonal line. Different features are highlighted in the sequence shown; Olig2 coding region (grey), V5-tag sequence (green), stop

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condon (bold black), PAM (red) and sgRNA sequence (blue). Note that PAM sequence was disrupted to avoid re-cutting after the HR event.

(D) Efficiencies of V5 tagging of Sox2 in mouse NS cells using the different delivery methods. Tagged cells were identified by V5 staining (indicated by the white arrows).

(E) Efficiencies of V5 tagging for Olig2 and Sox2 using rCas9/sgRNA delivery in two independent mouse NS cell lines. FNS – primary foetal forebrain NS cell line; IENS – tumour initiating mouse NS cell line.
**Figure S12 | P53 expression in TP53-deleted human NS cell clonal line.** Phase contrast images and P53 staining of parental human NS cells and representative TP53 deleted clonal line. Plot shows levels of TP53 mRNA levels assayed by qPCR.

Scale bar: 100um
Table S1. List of sgRNAs used in this study.

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SUPPLEMENTARY METHODS

Metaphase spread and SNP profiling

For karyotyping, cells were treated with nocodazole (5 hours) and metaphase spreads prepared as previously described (Campos et al., 2009). Modal chromosomal number of the parental and clonal lines was determined by counting chromosomes of at least 20 mitotic cells.

Differentiation assay

Glial and neuronal differentiation was initiated by removing EGF from the culture media for three or seven days (mouse and human, respectively). Cells were then allowed to differentiate in the presence of 1% FCS for additional 3 or 14 days. For differentiation of mouse PG1-1 cells into oligodendrocyte precursors, EGF was removed from the media for four days.

qRT-PCR

RNA was extracted using RNeasy spin column kit (Qiagen), plus DNase treatment to eliminate gDNA. cDNA was generated with SuperScript III (Invitrogen), and quantitative RT-PCR was performed using Taqman Universal PCR Master Mix (Applied Biosystems). The following Taqman assays (Life Technologies) were used: mOlig2 (Mm01210556_m1), mGAPDH (Mm99999915_g1), hTP53 (Hs01034249_m1) and hGAPDH (Hs02758991_g1).

References