ONE-STEP tagging: a versatile method for rapid site-specific integration by simultaneous reagent delivery.

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Abstract

Site-specific integration of DNA sequences into the genome is an important tool in fundamental research, synthetic biology and cell therapeutic applications. It can be used for protein tagging to investigate expression, localisation, and interactions as well as for expression of transgenes either under endogenous regulatory elements or at consistent safe harbour loci. Here we develop and optimise a simple and effective method for site specific integration in a single step that combines CRISPR-Cas9 mediated homology directed repair using single stranded oligonucleotide templates with the site-specific recombinase Bxb1 to allow large cargos to be integrated at any location in the genome. Our technology requires off the shelf Cas9 and oligonucleotide reagents combined with a set of cargo plasmids that are universal to any integration site. We demonstrate the methods adaptability by tagging at multiple sites and in multiple cell types including induced pluripotent stem cells and primary T cells. We show that our method can integrate large (up to 14 kb) cargos and that it is possible to simultaneously tag two genes or edit two sites with combination of integration and Cas9-mediated knockouts or other HDR events.

Introduction

Recent advancements in genome-editing technologies have provided efficient tools for specific genome modifications across various cell types and organisms. An important component of genome engineering is site-specific integration of DNA sequences to genomically tag particular proteins to investigate their function and allow expression of transgenes under endogenous regulatory elements or at specific safe harbour loci. These are important for studying the localisation, temporal dynamics and protein interactions of genes for understanding cellular function and for controlling transgene expression in cell therapeutics such as CAR-T therapies (T. Li et al. 2023).

CRISPR-Cas9 enhanced homology-directed repair (HDR) has become a key technology for transgene integration (Pacesa, Pelea, and Jinek 2024). This involves creating a double-strand break (DSB), and supplying an excess of a homologous template DNA which is used to elicit a highly precise repair (Ran et al. 2013). Short synthetic single-stranded DNA (ssDNA) oligonucleotides with ~100 nt of homology have been highly effective in many cell types (Chen et al. 2011; Wang et al. 2014) but they are limited in cargo capacity to around 100 nt making insertion of large transgenes impossible. Longer plasmid DNA with around 500-1000 nt of homology can be used to integrate larger fragments (Friedel et al. 2005), but there have been observations of complex, multimeric integration events at the on target site (Norris et al. 2020). Others have used long ssDNA (Roth et al. 2018) or linear double-strand DNA (dsDNA) to circumvent these problems (F. Song and Stieger 2017), but the former is difficult to produce, especially with longer cargos, and the latter is prone to random integration at off-target sites in the genome (Zelensky et al. 2017; A. Song et al. 2017). Protection of linear dsDNA with DNA structures or chemical modifications such as biotin has been shown to reduce non-specific integration and achieve tagging in some cell types (Gutierrez-Triana et al. 2018; Shy et al. 2023). However, with all of these methods, it is still necessary to produce a HDR template DNA of thousands of bases that is different for every targeted site. Also, the efficiency of integration drops rapidly with increasing insertion size, making it difficult to insert inserts of more than 10 kb (K. Li et al. 2014).

To overcome the size limitation, some groups have successfully combined HDR-mediated integration of a landing pad at a specific genomic locus followed by a second step of site-specific recombination (Feng et al. 1999; Low et al. 2023; Xu et al. 2013; Mulholland et al. 2015). The serine integrase Bxb1 is particularly useful for this, as it efficiently and specifically recombines heterologous attB and attP sites without known pseudo-sites in the human genome (Russell et al. 2006). Its recombination is directional and irreversible (Singh, Ghosh, and Hatfull 2013). However, such integration typically involves a two-step process, with clonal selection after the HDR event, making it quite lengthy and difficult to scale to multiple sites.

An alternative method for integration employs the non-homologous end joining (NHEJ) repair mechanism that ligates two dsDNA ends together (Suzuki et al. 2016; Zeng et al. 2020). By simultaneously cutting the genome and a donor DNA within the cell, this can be exploited to insert the donor DNA into any desired genomic site. This allows common donor plasmids to be used, avoiding the need for cloning, and making scaling of this method possible. It also has less of a length limitation than HDR-based methods, and tens of kilobases can be integrated using these methods. However, efficiency is variable between cell types, there is no control over orientation of the insertion, in some cases the whole plasmid will be integrated, and the NHEJ repair mechanism can sometimes introduce small insertions and deletions around the genomic cut site.

Prime editing (PE) combines a modified single-guide RNA (pegRNA) containing the template for the desired edit, with a reverse transcriptase (RT) fusion to Cas9 (Anzalone et al. 2019). This makes a nick at a genomic locus and extends the genomic DNA by reverse transcription of the pegRNA to introduce the edit, and through manipulation of mismatch repair (MMR) pathways can be biassed towards incorporation of the newly edited strand (Ferreira da Silva et al. 2022). PE offers advantages over HDR

such as fewer mutagenic DSBs, but it also has limitations, including fewer targetable sites and limited insertion length (<50 bp) (Fichter, Setayesh, and Malik 2023). Recently, insertion of a site-specific recombinase site using PE, and simultaneous delivery of the cognate recombinase resulted in efficient integration of large cargos into specific genomic sites in a single-delivery reaction (PASTE technology) (Yarnall et al. 2023). Recombination efficiency has recently been improved using evolved Bxb1 integrase (eeBxb1 and evoBxb1) (Pandey et al. 2024). Similarly, template-jumping prime editing (TJ PE) has allowed insertions up to 800 bp (Zheng et al. 2023). However, despite progress with computational prediction tools, it is still difficult to design effective PE guides without testing several permutations (Wu et al. 2023). Also, the target sites are somewhat limited by directionality of the PE process and availability of Cas9 cut sites (Durrant et al. 2024).

All of these methodologies rely on the endogenous DNA repair pathways of HDR, NHEJ or MMR. The efficiency of these repair pathways varies significantly between cell types. Embryonic stem cells and induced pluripotent stem cells (iPSCs) tend to favour HDR pathways (Guo et al. 2018), but many cancer cell lines and terminally differentiated cells preferentially repair through NHEJ (Srivastava and Raghavan 2015; Dharanipragada et al. 2023). Thus, the choice of the method will depend on the cell type and respective repair pathways that are active.

We present ONE STEP tagging, a technology allowing simple, efficient, and directional integration of large transgenes at any genomic site using a single-step delivery protocol that employs single-stranded oligodeoxynucleotides (ssODN)-templated HDR combined with Bxb1-mediated integration. We show its utility in tagging at multiple genomic locations in multiple cell types including pluripotent stem cells, cancer cell lines and primary T cells. We further demonstrate that large cargos of up to 14 kb can be integrated. Tagging plus other Cas9-mediated knockout or HDR events can be performed simultaneously at two different sites. We also optimised the use of heterotypic Bxb1 recombinase sites to avoid the integration of plasmid backbones and enable directional dual tagging. Our system allows the use of completely off-the-shelf reagents, namely commercially available Cas9 protein, synthetic sgRNAs and ssODNs to define the genomic location and a common set of cargo vectors to define the inserted fragment which will make this methodology scalable to a large number of target sites.

Results

ONE STEP tagging combines CRISPR-Cas9 editing with Bxb1 site specific integration

We have devised a versatile integration system that combines the precision of CRISPR-Cas9-based editing with the efficient, and less size-dependent integration of DNA cargos by Bxb1 serine integrase. Bxb1 is functional in mammalian cells and efficiently catalyses unidirectional (non-reversible) recombination between dsDNA sequences containing an attP and their complementary attB attachment site. By utilising CRISPR-Cas9 to position the integrase attP sites at specific genomic locations, we can direct Bxb1 (delivered in trans) to act at the chosen sites. By simultaneously providing a circular double-stranded DNA template containing the attB attachment site, we aim to achieve direct integration in a single step (Fig 1a).

Due to its specificity for dsDNA over ssDNA, Bxb1 cannot recombine attP attachment sites in a ssODN donor template until it is inserted at the chosen genomic location and made double stranded, and thus recombination with the dsDNA cargo donor will only occur after attP integration. Since Bxb1 leaves residual sequences in the genome (termed attL and attR) after recombination, we utilise these genomic scars as protein linkers by strategically positioning the attB site on the cargo which we initially delivered on a minicircle. We designed an experiment aimed at endogenously tagging the N-terminus of the constitutively expressed ACTR10 protein with a fluorescent protein (mNeonGreen) in hiPSCs. We combined all necessary reagents and delivered them simultaneously in a single nucleofection. This included the CRISPR-Cas9 ribonucleoprotein complex which carried an sgRNA targeting our site of interest, along with a short (200 nt) single-stranded HDR template containing the attP site and

approximately 70 bp of homology arms each side of the specific site. Additionally, we included a plasmid for the mammalian expression of Bxb1 and a circular double-stranded DNA cargo containing the attB attachment site and the sequence encoding for mNeonGreen (Fig 1a). To avoid integration of plasmid backbone sequence, we generated this cargo as a minicircle by self-circularising a restriction fragment.
We assessed the outcome by flow cytometry analysis of mNeonGreen fluorescence (Fig S1a). By titrating the amount of each component, we found that delivering 24 pmol (4 ug) Cas9, 45 pmol sgRNA, 50 pmol (3.1 ug) of ssODN HDR template, 127 fmol (500 ng) of Bxb1 integrase plasmid and 0.8 pmol (380 ng) of circularised donor yielded optimal efficiency. Under these conditions, 11.3% of cells exhibited mNeonGreen fluorescence indicating in-frame tagging (Fig 1b). Correct and precise integration was further confirmed by PCR-based genotyping (Fig S1b) and Sanger sequencing of the products. In conclusion, our results demonstrate that we can achieve site-specific integration of mNeonGreen at the N-terminus of ACTR10 in a single step, hence the designation of ONE STEP tagging. By comparing integration rate of mNeonGreen in the presence or absence of the ssDNA donor containing the attP site, we observed that random integration of the tag was minimal. Tagging ACTR10 with increasing concentrations of mNeonGreen dsDNA cargo donor significantly enhanced tagging efficiency, thus demonstrating that an excess of cargo donor alone is adequate to improve results.

Optimisation of ONE STEP tagging in hiPSCs

NHEJ and HDR are the two major branches of DNA damage response pathways that process DSBs. NHEJ is characterised by the modification and ligation of blunt DNA ends, and acts throughout the cell cycle. It functions independently of sequence homology, is kinetically faster than HDR-related mechanisms and is the predominant DSB repair pathway in many cell types. By comparison, HDR is limited to the S- and G2-phases of the cell cycle, where the presence of a sister chromatid allows for faithful and potentially error-free repair.

Efforts to enhance repair by template-based pathways include regulation of key DNA repair factors, modulation of the CRISPR-Cas9 components and alterations of the intracellular environment around DSBs (Charpentier et al. 2018; Rees et al. 2019; Aird et al. 2018). Recent work by Wimberger et al. confirms that inhibition of DNA-PK, a key player in the NHEJ pathway, is most effective at improving CRISPR-mediated insertions(*). To that end, we tested four commercial DNA-PK inhibitors (NU7441, AZD-7648, M3814 and IDT HDR Enhancer) with the aim of promoting repair via HDR and ultimately improving tagging efficiency. Optimal concentrations of inhibitors were established using a hiPSC reporter line composed of a single copy of blue fluorescent protein (BFP) integrated at the hROSA26 locus of the Kolf2.1s cell line which can be specifically targeted at the fluorophore binding site by CRISPR-Cas9 generating a DSB. Subsequent repair by NHEJ will predominantly result in indel formation and loss of BFP fluorescence. However, if a template for HDR introducing a 2-nucleotide mutation is utilised in the repair process, fluorescence is altered from blue to green, indicating measurable levels of repair by HDR (BFP-GFP reporter assay). In this system, AZD-7648 proved to be the most effective at increasing rates of repair by HDR with 1.6 fold increase relative to controls (Fig S2).

Application of DNA-PK inhibitors in the ONE STEP tagging system produced similar results, with tagging of ACTR10 with mNeonGreen improving 5-fold and 8-fold in A1ATD and Kolf2.1s cells respectively (Fig 1c). Tagging of three further sites (MAP4, LMNA and FBL) and subsequent analysis of attP integration by ICE (Conant D, et al. CRISPR J. 2022 Feb;5(1):123-130) confirmed improved rates of HDR with the use of AZD-7648 (Fig S3). In all subsequent experiments, we added AZD-7648 (0.5μM) after nucleofection and changed the media 24 hours later.
Utilising the BFP reporter line, we additionally assessed the optimal concentration of ssODN oligo by titrating the amount of HDR template employed to introduce the 2-nucleotide mutation in the BFP

reporter gene, resulting in subsequent conversion to GFP. The addition of 100 pmol of HDR template yielded HDR efficiency above 60%, whereas 25 and 12.5 pmol of the same template still resulted in approximately 40% HDR efficiency (refer to Fig S4).
In all subsequent experiments, we kept the oligo amount at 25 pmol.
Tagging ACTR10 with increasing concentrations of mNeonGreen dsDNA cargo donor significantly enhanced tagging efficiency, even in the absence of AZD-7648, thus demonstrating that an excess of cargo donor alone is adequate to improve results (Fig1d). In all subsequent experiments, we maintained the donor concentration at 6 fold to maintain optimal efficiency without affecting cell viability.

Directional ONE STEP tagging with variant recombinase sites

The central dinucleotide within the attP and attB sites of Bxb1 plays a crucial role in the integration process by facilitating the association of these attachment sites (Ghosh, P., Kim, A. I. & Hatfull, G. F., 2003). Since one bottleneck of the technology is the efficient and reproducible generation of the minicircle DNA cargo, we decided to investigate the use of these variant recombination sites to allow specific integration of a defined part of a plasmid. We decided to test mNeonGreen integration at the ACTR10 site by using the GA attP, which has been reported to have greater efficiency than the WT GT attP sequence (Jusiak, B. et al., 2019), and explored the specificity of matched and unmatched attB/attP dinucleotide interactions. We found that both GA and GT attP variant sites efficiently integrated cargo only when paired with the corresponding attB/attP pair, with minimal integration across mismatched combinations, with 10 fold less integration of GA donor into GT site, indicating low levels of crosstalk between these variants (Fig S5).

Based on this observation, we designed a directional tagging strategy by adding two variant attP sites (attP-GA and attP-GT) onto the 200 nt ssDNA used as an HDR template still preserving ~50 bp homology arms on each side. This is combined with a plasmid containing the cargo flanked by the two variant sites as a donor (Fig S6). We compared the original ONE STEP technology with the directional tagging strategy and achieved similar tagging efficiency (Fig S7) with the advantage that now we can generate a more scalable, pure and reproducible plasmid library of donor cargos.

Versatile tagging at multiple loci in different cell types and with large cargo sizes

Since our technology does not require the assembly of long ssDNA HDR templates but instead uses a short ssODN (200 nt) with approximately 50 bp of homology arms surrounding the recombination cassettes, integration of the cargo can be easily scaled across different loci. We tested and confirmed integration of mNeonGreen at three other genomic locations (LMNA, FBL and MAP4). To assess correct gene tagging, we used fluorescence imaging to compare the subcellular localisation of mNeonGreen with the reported location of the tagged protein. For all four targeted loci, mNeonGreen localised as expected, indicating successful tagging (Fig1e).

We also expanded ONE STEP tagging to additional cell types beyond hIPSC lines (A1ATD and Kolf2.1s) and tested it in the K562 lymphoblast line and the HAP1 near-haploid human myeloid leukaemia cell line. We observed varying efficiencies in the integration of a 0.8 kb fluorescent tag at the MAP4 locus, with integration rates ranging from 1.4% in HAP1 cells to nearly 17% in K562 cells (Fig. 1f). Despite the lower integration efficiency in some cell lines, we mitigated this issue by sorting and expanding the cells, thereby enriching for a pure population.

Another advantage of the recombinase tagging methodology is that integration efficiency is less dependent on length than with HDR. We therefore tried integration of larger cargo plasmids, and we were also able to achieve precise integration of cargos as large as ~14kb into the ROSA26-BFP locus

in Kolf2.1s (Fig S8/Fig 1g), with ~14.5% complete integration of the full cargo confirmed by FACS/genotyping.

Multiplexed tagging and simultaneous CRISPR editing

Multiplexed gene integration is a valuable technique for labelling different proteins, enabling the visualisation of their intracellular localization and interactions within the same cell. We employed directional tagging to simultaneously tag ACTR10 with mNeonGreen and MAP4 with mCherry in the same cell (Fig. 2a). Using FACS, we observed successful simultaneous tagging of MAP4-mCherry and ACTR10-mNeonGreen (Fig S9). Interestingly, there was a greater percentage of double tagging events (2,41% mChr-mNg+) than you would expect from the combination of the efficiency at each individual site (1,81% mCh+, 8.92% mNg+), suggesting that successful tagging at one site might enrich for a second tagging event. The double-positive cells were sorted, and the tagged genes were confirmed to be visible in their respective cellular compartments, consistent with the known subcellular localizations of ACTR10 and MAP4 protein products by imaging (Fig S9).
Another valuable application of the ONE STEP tagging technology is the simultaneous tagging of a primary gene along with a knockout (KO) or homology-directed repair (HDR) editing event at a second gene). We delivered reagents designed to tag MAP4 with mCherry into the BFP reporter line. The presence of the mCherry signal, combined with the BFP-GFP reporter assay, enabled us to monitor KO and HDR rates by detecting the loss of BFP or the gain of GFP signals, respectively. We observed that 70% of the cells successfully tagged with mCherry also exhibited either a BFP KO (Fig2c) or GFP HDR event (Fig2d).

ONE STEP tagging can be used to integrate transgenes in primary T cells

We also expanded the ONE STEP tagging system to edit more clinically relevant cells, such as primary human T cells, at therapeutically relevant loci, such as the TCR. To achieve best efficiency of tagging, we changed nucleofection conditions. Higher concentrations of Cas9, gRNA and ssODN HDR template were used. The P3 buffer (Lonza) was replaced by an optimised buffer (B1mix) shown to improve HDR efficiency notably when integrating large DNA fragments (An et al. 2023). The evo Bxb1 recombinase was used instead of the WT version (Pandey et al. 2024). We successfully integrated a cargo of 4.4kb (expressing EGFP) at the TCR locus (Fig 2d), using both a sense and an antisense ssODN as HDR templates containing the attP site, targeting the TCR locus (Fig S10). We observed a tagging efficiency of ~6% using the antisense ssODN. suggesting the potential to adapt this technology for the introduction of chimeric antigen receptor (CAR) transgenes at the TCR locus in primary T cells. This advancement could enhance current strategies for cancer immunotherapy through adoptive transfer. Additionally, the ability to simultaneously tag the TCR locus while performing B2M and PD1 knockouts opens the possibility of generating off-the-shelf CAR-T cells.

Conclusion

Our study demonstrates the successful development and application of the ONE STEP tagging system, a streamlined and efficient method for site-specific integration and tagging of genes. This system leverages the precision of CRISPR-Cas9-mediated editing and the unique capabilities of Bxb1 serine integrase, enabling the integration of DNA cargos in a single step. The use of completely off-the-shelf reagents, from commercial suppliers and a common set of cargo plasmids which we made available through Addgene, highlights the accessibility and practicality of this approach for a wide range of research and clinical applications.

ONE STEP tagging has proven effective in different cell types from hiPSCs to cancer cells and primary human T cells. This system allows for large DNA insertions and the tagging of multiple genomic sites simultaneously. We demonstrated the integration of a 0.8 kb fluorescent tag at the MAP4 locus with varying efficiencies across different cell lines, achieving up to 17% in K562 cells. Furthermore, the

system facilitated the precise insertion of larger cargos, up to ~14 kb, at specific loci in hiPSCs, underscoring its versatility and efficiency.

The ability to combine tagging with knockout (KO) and homology-directed repair (HDR) events further enhances the utility of the ONE STEP system. For instance, we achieved simultaneous tagging of a primary gene and a KO or HDR event at a secondary gene, with high rates of co-occurrence. This dual functionality is particularly valuable for complex genetic modifications and studies of gene interactions.

Our results also demonstrate the applicability of ONE STEP tagging in clinically relevant cells. We successfully integrated a cargo of 4.4kb (expressing EGFP) at the TCR locus in primary human T cells, paving the way for potential cell therapeutics adaptations, for example introduction of chimeric antigen receptor (CAR) transgenes at the TCR locus. Additionally, the use of Cas9 nuclease offers simultaneous tagging of the TCR locus and performing B2M and PD-1 knockouts that opens the possibility of generating off-the-shelf CAR-T cells, which are able to resist alloresponsive T cell attacks and with reduced T cell exhaustion (Eyquem et al. 2017), offering new avenues for cancer treatment.

An important comparison between recently published PE-based systems and our HDR-based recombinase-mediated system is the limitation of insert size when integrating recombination sites. ssODNs, which can now be routinely synthesised up to 200 bp (with a cargo capacity of ~100-120 bp), can insert dual recombination sites, whereas PE is limited to a single recombination site. This poses a drawback for recombination donor production in PE-based methods. In the dual cassette version of our system, recombination donors are readily produced and do not need modification prior to transfection, maintaining insert specificity. This quality makes them truly off-the-shelf integration vectors, as only the ssODN template and the sgRNA need to be designed.

In conclusion, the ONE STEP tagging system represents a significant advancement in genetic engineering, providing a fast, efficient, and versatile method for precise site-specific gene integration. Its ability to facilitate large insertions, multiple site tagging, and combination with other genetic modifications makes it a powerful tool for both research and clinical applications. The system's success in hiPSCs and primary T cells underscores its potential for advancing gene therapy and regenerative medicine.

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Conflicts of interest

AB is a founder of and consultant for Ensocell therapeutics.

Figure 1

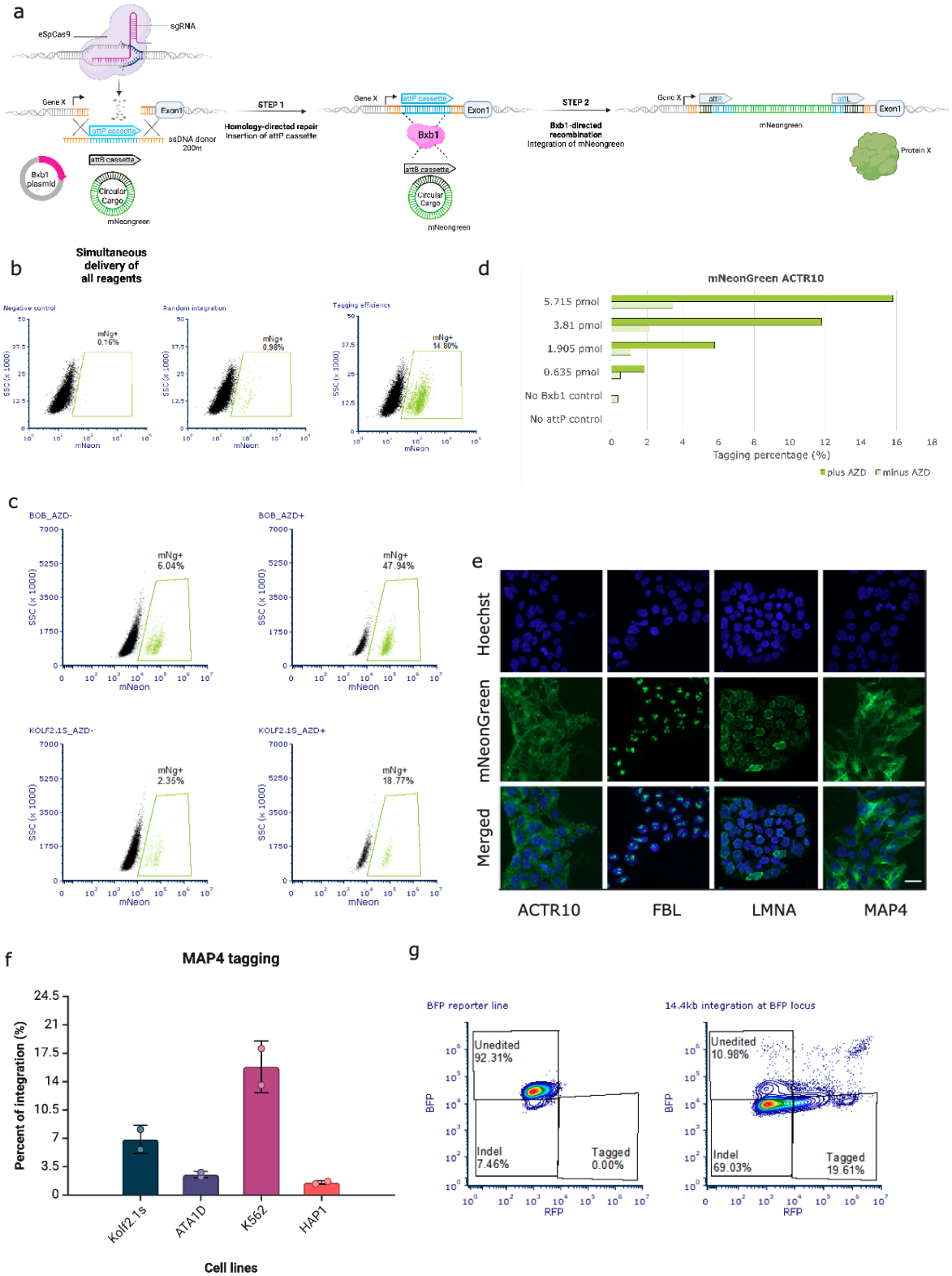

Figure 1 - Principle and optimisation of ONE-STEP tagging in different genomic locations, cell lines and cargo types. a, Schematic of ONE-STEP tagging technology. The system involves insertion of landing site via Crispr-Cas9, followed by landing site recognition and integration of cargo by Bxb1 integrase. All reagents are delivered simultaneously. **b,** ONE-STEP tagging efficiency at the 5' end of the ACTR10 locus using mNeonGreen as cargo donor. 'Negative control' omits Bxb1 and circular donor; 'Random integration control' omits the ssDNA donor indicating unspecific donor integration of

<1% and 'Tagging efficiency' includes delivery of all components, resulting in on-target tagging efficiency of 11%. **c,** AZD-7648 is a potent DNA-PK inhibitor that improves gene tagging in iPSCs. mNeonGreen integration efficiency at the 5'end of ACTR10 increases to 47.31% (A1ATD) and 19.36% (Kolf2.1s) from 5.98% and 2.30% respectively using 0.5uM of DNA-PK inhibitor AZD-7648. **d,**Testing of cargo donor concentration to achieve optimal tagging efficiency. Using higher concentrations of mNeonGreen dsDNA cargo donor significantly enhanced tagging efficiency at the ACTR10 locus, even in the absence of AZD-7648, thus demonstrating that an excess of cargo donor alone is adequate to improve results. **e,** Endogenous protein tagging with mNeonGreen via ONE STEP tagging at four loci (*ACTR10*, *FBL*, *LMNA* and *MAP4*). Fluorescence images of representative cells are shown. Cells have nuclear Hoechst staining. Endogenous expression of mNeonGreen localise according to known, literature based, location of the four tagged genes. Scale bar 25µm. **f,** ONE-step Tagging across multiple cell types. Percentage of integration of a 0.8kb tag at the MAP4 locus across different human cell lines (Kolf2.1s, A1ATD, K562, HAP1). **g,** FACS plot showing the integration of a 14.4 kb cargo into the BFP locus of the Kolf21s BFP reporter line. Cells that undergo integration are characterised by the loss of BFP signal and the gain of RFP signal, indicating successful integration. The tagging efficiency is approximately 14.5%.

Figure 2

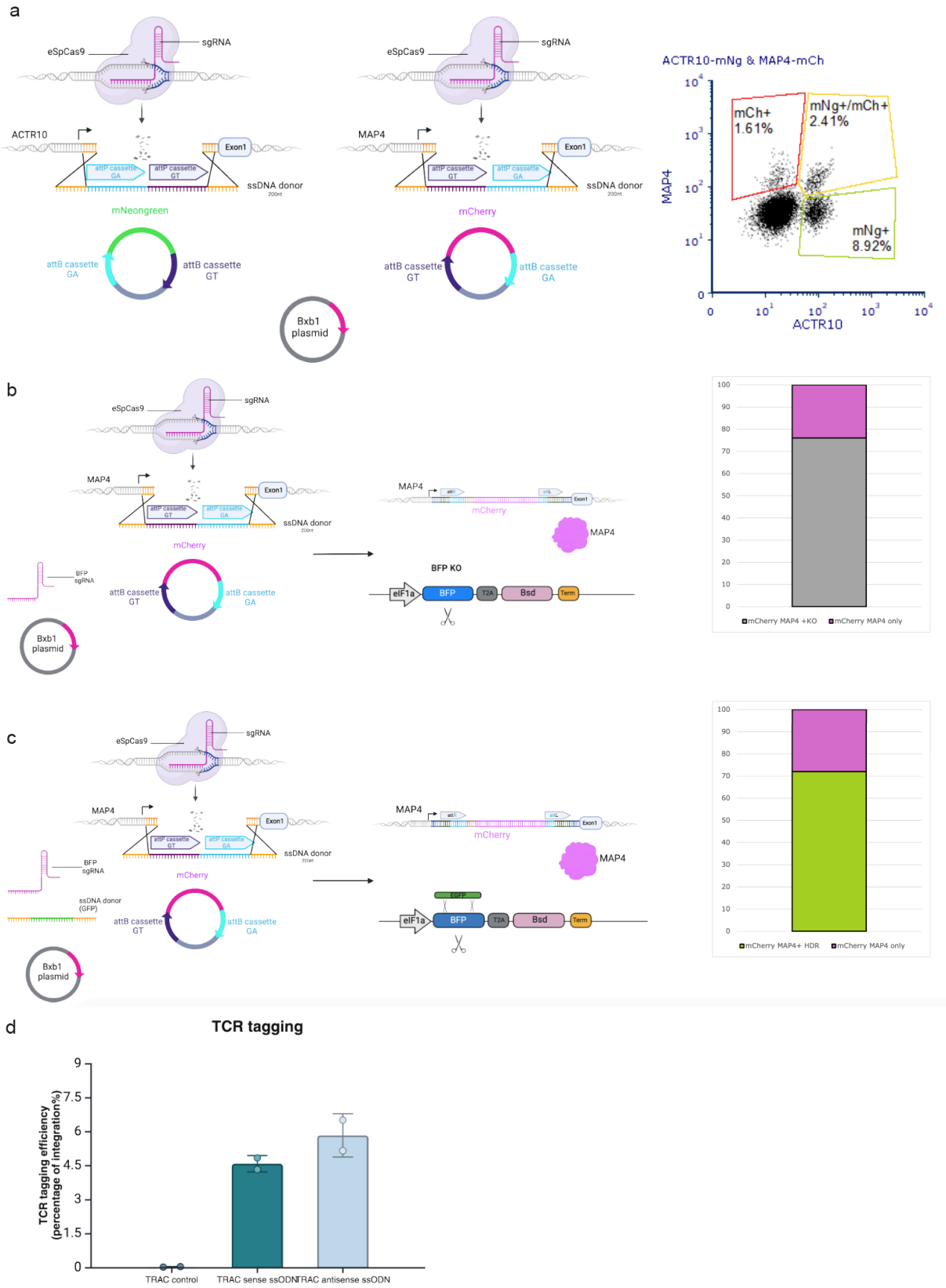

Figure 2 - ONE-step tagging can be used for double tagging, simultaneous tagging and endogenous gene editing and in primary T cells. a, ONE-step Directional Tagging allows for

simultaneous tagging of multiple loci with different cargo vectors. Schematic of experimental design on the left. FACS analysis showing efficiency of multiplexed tagging insertion of combinations of fluorophores (mNeonGreen and mCherry) at two different loci (ACTR10 and MAP4), on the right. **b,** Simultaneous tagging of a primary gene (MAP4) along with a knockout (KO) event at a secondary gene (BFP). Schematic of experimental design on the left. Reagents designed to tag MAP4 with mCherry were delivered simultaneously with a BFP sgRNA into a BFP reporter line. The presence of the mCherry signal, combined with the BFP-GFP reporter assay, enabled us to monitor KO rates by detecting the loss of BFP. Roughly 76% of the cells successfully tagged with mCherry also exhibited BFP KO. **c,** Simultaneous tagging of a primary gene (MAP4) along with a HDR event at a secondary gene (BFP to GFP). Schematic of experimental design on the left. Reagents designed to tag MAP4 with mCherry were delivered simultaneously with a HDR template employed to introduce the 2-nucleotide mutation in the BFP reporter gene, resulting in subsequent conversion to GFP into a BFP reporter line. The presence of the mCherry signal, combined with the BFP-GFP reporter assay, enabled us to monitor HDR rates by detecting the gain of GFP. Roughly 72% of the cells successfully tagged with mCherry also exhibited GFP HDR events. **d,** ONE-STEP tagging integration efficiency of a 4.4kb insert into the TCR locus (EGFP expression) in primary human T cells, using sense and antisense ssODNs.

Materials and Methods

ssODN design

ssODNs that contain a single recombination site use an attP variant sequence that is 58bp long. ssODNs that contain two recombination sites use core attP variant sequences that are 48bp long (derived from the 58bp variant). When convenient, the length of one of the two 48bp long sites in the dual recombination cassette is extended by including an additional 5bp from the 58bp variant as described below.

In this study, the recombination sites used in each part of the doublet pair of attP-attB sites contain different central dinucleotide mutants (heterotypic sites) in order to ensure the efficient integration of only the insert portion and not the backbone of the donor vector. This theoretically improves recombination efficiency by 50%. Table S1 contains a breakdown of the recombination sites used throughout this work - attP, attB, resulting attR and attL sites and the lengths of DNA spacers used to keep insertions in-frame in the case of gene tagging.

Due to the order of insertion of recombination sites in the genome - attP(s) being inserted in the genome first, and subsequently being recombined with a donor molecule containing attB(s) - the resulting recombinant sites and cassette are of the following structure: attR-insert-attL (this is the case for both the single and double pair of attP-attB site systems). In the case of 5' end tagging (where an N-terminal protein fusion is desired), either the single or dual attP cassettes are designed to replace the start codon of the targeted CDS. In the case of 3' end tagging (where a C-terminal protein fusion is desired), either the single or dual attP cassettes are designed to replace the stop codon of the targeted CDS.The single attP-attB pair results in attR and attL sites that are 52bp long post-recombination. The double attP-attB pair results in attR and attL sites which are 47bp long post-recombination. This difference necessitates a slightly different approach in the design of either ssODN oligos, dsDNA donor cargos or both when keeping inserts in-frame with a native gene is of concern. In this study, we address this difference by implementing the necessary changes in the ssODN design and keep the dsDNA donor plasmids similar. For information on how single pair N-terminal tagging and C-terminal constructs are kept in-frame see the "dsDNA donor design" section. To keep double pair N-terminal tagging constructs in-frame, a DNA spacer is included after the second attP of the ssODN in order to influence the length of the resulting attL. The preferred sequence used in this study is the final 5bp of the 58bp-long attP - effectively the ssODN contains a single 48bp attP followed by another 53bp attP. However, an alternative spacer can be used as long as it fulfils the requirements outlined in the "dsDNA donor design" section. To keep double pair C-terminal tagging constructs in-frame, a DNA spacer is included before the first attP of the ssODN in order to influence the length of the resulting attR. The sequence used in this study is the first 5bp of the 58bp attP variant. This effectively results in a ssODN that contains a single attP of 53bp, followed by another 48bp attP. However, an alternative spacer can be used as long as it fulfils the requirements outlined in the "dsDNA donor design" section.

sgRNA sequences for CRISPR cuts are designed to cut as close to the insertion site as possible, whilst minimising off-target effects, and ideally with the sgRNA sequence spanning the insertion site to block recutting after correct integration. If this is not possible, synonymous or non-coding mutations should be introduced in the PAM site within the homology arms of the ssODN. sgRNAs are synthesised as chemically modified RNAs (Synthego) to minimise toxicity and maximise editing efficiency.

All sgRNA sequences are listed in table S2, and ssODN sequences used for single attP, dual attP and BFP targeting are listed in tables S3, S4 and S5.

dsDNA donor design

Recombination donor constructs used in this study are circular dsDNA molecules and contain attB sites which are 46bp long (plasmid maps used in this study in Table S6). In the case of the single attP-attB pair system, a circular plasmid is cut and self-ligated to create a circular donor (containing a single attB site) - the entire length of the circularised cargo is integrated into the genome (the circularised cargo

does not contain any bacterial vector backbone sequence). The parent attB cargo vector also contains single attB site and inward facing Type IIS (BbsI) restriction sites that flank the attB-insert sequence.
The double attP-attB pair system contains two attB sites flanking the insert. It can be used without *in vitro* circularisation as upon completion of the recombination the insert cassette is inserted and the bacterial backbone sequence is excised.
N-terminal tagging constructs are designed to be in-frame with the gene-of-interest post-recombination. The length of the attR and attL sites that result from recombination is 52bp in the case of the single attP-attB pair system and 47bp in the case of the dual attP-attB system.
To keep N-terminal tagging constructs in-frame a DNA spacer is included in the 3' end of the dsDNA donor construct. Importantly, the spacer shouldn't contain stop or start codons and its length should complement the attL to a length divisible by 3. A quick way to check that this is possible is to perform a modulo operation with a divisor of 3 on the sequence length - it should return a remainder of 2. This is because 52 (the length of attR and attL resulting from recombination of the single attP-attB system) has a remainder of 1 when divided by 3, their sum is 54, which is divisible by 3 and hence would be in-frame in the absence of stop codons.
To keep C-terminal tagging constructs in-frame a DNA spacer is included in the 3' end of the dsDNA donor construct. As in the case of N-terminal tagging, the spacer's length is designed to return a remainder of 2 when divided by 3 and not to avoid stop codons.

dsDNA donor circularisation

The dsDNA donors used in the single attP-attB pair system were generated from producer plasmids. The producer plasmids were purified from bacterial cultures. The plasmids were then digested with BbsI and the fragment corresponding to the insert was gel purified. The purified insert fragment was self-ligated using T4 DNA Ligase. Low molar concentrations of DNA substrate and high concentrations of ligase improve the yield of the self-ligated circularised monomer. High DNA concentrations increase the accumulation of intermolecular ligation and the formation of linear and circular dimers, trimers and higher multimers. The ligation reaction was then purified using silica columns. In some experiments, the ligation reactions were treated with T5 exonuclease to remove carryover linear monomers and ligated linear multimers as well as open circles (nicked DNA) and ssDNA.

Endotoxin removal from DNA preparations

DNA extractions were purified using the TXS method(Ma et al. 2012) prior to nucleofections. Briefly, a 0.25 volume of TXS solution was added to 1 volume DNA solution and mixed thoroughly by inverting. The solution was then incubated at room temperature for 5-10 minutes or for as long as overnight at 4°C.
A 0.25 volume of 5M NaCl was then added and mixed thoroughly by inverting. The sample was then centrifuged for at least 10 minutes at maximum speed (>15,000 x g) at 4°C. The clear upper layer, which contains the purified plasmid, was aspirated into a clean tube (care was taken not to take up the red tinted solution at the bottom of the tube). The clear supernatant was then precipitated using 100% isopropanol, washed with 70% v/v ethanol and dried. The DNA pellet was then resuspended with nuclease-free water or TE buffer (pH 8).

Mammalian cell culture

Kolf2.1s is an edited induced pluripotent stem cell (iPSC) line, corrected for a 19bp deletion in one copy of ARID2, which has undergone extensive characterisation and is commonly used for editing. Kolf2.1s are derived by the Human Induced Pluripotent Stem Cell Initiative (HipSci) consortium (ref). Kolf2.1s BFP/GFP reporter line contains a BFP reporter inserted in the ROSA26 locus under a EF1a promoter (Bassett's lab).
iPSC lines were cultured under feeder free condition in Stemflex medium (combo kit, Gibco ™ A3349401) on Vitronectin substrate. Vitronectin is used at 1:100 dilution of a 1mg/ml stock solution in

PBS, using 1ml per 6w well for coating. Incubate at RT for 1 hour before aspirating and replacing with culture media immediately. After initial thaw, cells were clump-passaged 1:10 every 4-5 days and cultured in a humidified incubator at 37°C and 5% CO_2.
K562 and HAP1 were cultured respectively in RPMI and DMEM/F12 medium both supplemented with 10% (vol/vol) fetal bovine serum (FBS).

Nucleofection

Nucleofection of Cas9 RNP, ssODN containing the attP site, Bxb1 plasmid and cargo donor containing the attB site in iPSCs (200000 cells) were carried out in P3 Primary Cell buffer (program CA137) in 16-well cuvettes (Lonza) using an Amaxa 4D-Nucleofector (Lonza). Synthetic sgRNA (Synthego) and end-blocked ssODN (ultramer; IDT) were diluted in IDT duplex buffer to a concentration of 200mM and 100mM, respectively. eSpCas9 protein was in house produced and diluted in PBS to a final concentration of 4mg/ml, but equivalent results can be obtained with commercial Cas9 proteins (Cas9 HiFi, IDT).

Small molecule titration

All small molecule inhibitors tested are commercially available. AZD-7648 (HY-111783, MedChem Express), M3814 (HY-101570, MedChem Express) and NU7441 (S2638, Selleckchem) were dissolved in DMSO (Thermofischer Scientific) at a concentration of 5mM. IDT HDR Enhancer v2 (10007910, IDT) was purchased as a 0.69 mM concentrated solution in DMSO. IDT HDR Enhancer v1 is no longer commercially available.
Optimal concentrations of small molecule inhibitors were determined using the BFP-GFP reporter assay. Nucleofection of sgRNA (Synthego), Cas9 protein (produced in-house) and ssODN (Ultramer DNA oligo, IDT) into iPSC BFP reporter line were carried out in 100uL cuvettes (Lonza) using an Amaxa 4D-Nucleofector (Lonza), P3 Primary Cell buffer and program CA137. Final amounts per nucleofection: $1x10^6$ cells in 100 µL P3 solution, 20 µg Cas9 protein, 20 µg sgRNA and 500pmol ssODN. Post-nucleofection, cells were maintained in small molecule inhibitor supplemented culture media at various concentrations (0, 0.5, 1, 2, 4, 10, 20, 30 and 50uM) for 24 hours.
3 days post-nucleofection, cells were analysed for presence or absence of GFP and BFP respectively by FACS (CytoFLEX, Beckman Coulter).

Genomic DNA extraction and characterisation by PCR

DNA was extracted from nucleofected cells using the DNAeasy Bood and Tissue kit from Qiagen (Cat. No. / ID: 69504) according to manufacturer's instructions. After purification, genomic DNA was eluted in 50µl of water. To confirm correct integration, target regions were PCR amplified (primers in table S7) and analysed by gel electrophoresis. 10ng of gDNA was used to set up 50µl PCR reactions using KAPA HiFi HotStart ReadyMix (2x) (KAPA Biosystems, KK2601). The thermal cycling profile of the PCR was: 95°C 3 min; 35x (98°C 20 s, 65°C 15s, 72°C 15s); 72°C 1 min.

Genome-editing characterisation by ICE analysis

Quantification of ssODN integration
A few days post-electroporation, edited cells were pelleted by centrifugation and the DNA isolated using PureLink™ Genomic DNA Mini Kit according to manufacturer's instructions.
Amplicons were generated using indicated PCR primers (Table S7), designed to amplify an approximately 1000 bp region of genomic DNA surrounding the target site. 10ng of gDNA was used to set up 50µl PCR reactions using KAPA HiFi HotStart ReadyMix (2x) (KAPA Biosystems, KK2601). The thermal cycling profile of the PCR was: 95°C 3 min; 35x (98°C 20 s, 65°C 15s, 72°C 15s); 72°C 1 min.
Resulting PCR product was purified using Monarch® PCR & DNA Cleanup Kit (5 µg) according to manufacturer's instructions and shipped to Genewiz (Leipzig, Germany) for Sanger sequencing. Trace sequencing files were uploaded to ICE v2 (https://ice.synthego.com/, Synthego) to quantify efficiency of ssODN integration.

Imaging

Cells for imaging were plated on PhenoPlate 96-well black walled microplates (Revvity, cat. #6005182). Once confluent cells were washed with 100µl PBS per well and fixed at room temperature for 10 minutes with 4% PFA supplemented with Hoechst at 1 µg/ml for nuclei staining (40µl/well). Post-fixing, cells were washed 3 times then stored in 100µl PBS. Cells were imaged on an Opera Phenix (Perkin Elmer) using a 40x/1.1 NA water lens, or as specified in the figure legends. Hoechst and mNeonGreen were excited at 375nm and 488nm, respectively. Hoechst fluorescence was collected at 435-480nm with an exposure time of 60ms, and mNeonGreen fluorescence was collected at 500-525nm with an exposure time of 100ms.

Primary T cell isolation and stimulation

Human biological samples were sourced ethically, and their research use was in accord with the terms of informed consent under an institutional review board/ethics committee-approved protocol (15/NW/0282). Peripheral blood mononuclear cells (PBMCs) were isolated from fresh leukapheresis products from human healthy donors (Leukopaks, BioIVT) using a Ficoll-Paque PLUS (GE Healthcare, cat. #GE17-1440-03) density gradient centrifugation. Cells were cryopreserved in freezing media (RPMI 1640 (Gibco, cat. #52400025), 10% DMSO, 50% Fetal Bovine Serum (FBS, SIGMA-ALDRICH, cat. #F9665)) and stored in liquid nitrogen. PBMCs were thawed a day before T cell isolation, resuspended in complete RPMI media at 20e6 cells/mL (RPMI 1640, 10% FBS, 100 U/mL Penicillin-Streptomycin (Gibco, cat. #15140122), 2 mM L-Glutamine (Merck, cat. #G7513)) and incubated at 37°C 5% CO2 overnight. PBMCs were collected and washed twice with DPBS without calcium or magnesium (Gibco, cat. #14190144). Total CD4+ T cells were isolated by immunomagnetic negative selection using the EasySep™ Human CD4+ T Cell Isolation Kit (STEMCELL Technologies, cat. #17952) according to the manufacturer's instructions. After isolation, cells were cultured in media consisting of StemPro™-34 SFM (Gibco, cat. #10639011), 10% FBS, 100 U/mL Penicillin-Streptomycin, 2 mM L-Glutamine, recombinant Human IL-2 at 40U/mL 10 ng/mL (PeproTech, cat. #200-02BiolegendR&D systems) (named as complete StemPro) at 1e6 cells/mL. Cells were then stimulated with 12 µL/mL ImmunoCult™(TM) Human CD3/CD28 T Cell Activator (STEMCELL Technologies, cat. #10971).

Nucleofection of primary T cells

Lyophilized sgRNAs (Synthego) and ssODNs (IDT) were resuspended in water to a stock concentration of 100µM and stored at -20°C until use. RNPs were produced by mixing sgRNAs (180 pmol) and Cas9 (Alt-R™ S.p. Cas9 Nuclease V3, IDT, 61 pmol) at a 3:1 sgRNA:Cas9 molar ratio. The following ONE STEP components were added to the RNP complexes: ssODN GA GT donor template (100 pmol), Bxb1 plasmid (500 ng), and GA mNeonGreen GT plasmid (1.5µg). Primary T cells were spun down for 5min at 400g and washed twice with DPBS without calcium or magnesium, before being resuspended in B1mix buffer (buffer B1 (5mM KCl, 15mM $MgCl_2$, 120mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 10mM sodium succinate and 25mM mannitol) were combined with mix solution (0.5mM sodium pyruvate, 0.8mM $Ca(NO_3)_2$, 0.26mM i-inositol (myo-inositol), 4mM GlutaMAX (L-alanyl-L-glutamine dipeptide in 0.85% NaCl), 20mM d-glucose) in a v/v ratio of 72.3 to 27.7) at 7e5 cells per 20µL and added to the ONE STEP pre-mix. The cells in the buffer were then transferred to a 16-well Nucleocuvette™ Strip (Lonza, 4D-Nucleofector™ X Kit) for nucleofection using the pulse code EH-115. Immediately after nucleofection, 80µL of pre-warmed complete StemPro media was added to each well and incubated at 37°C with 5% CO2 for 15 min. The cells were then transferred to a 96-well round-bottom plate containing 145µL of media with 0.5µM AZD-7648 and incubated at 37°C with 5% CO2 for 24 hours. AZD-7648 was then washed out by removing supernatants without disturbing the pellets and resuspending the cells in 250µL of pre-warmed media.