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Research Article

biGMamAct: efficient CRISPR/Cas9-mediated docking of large functional DNA cargoes at the *ACTB* locus

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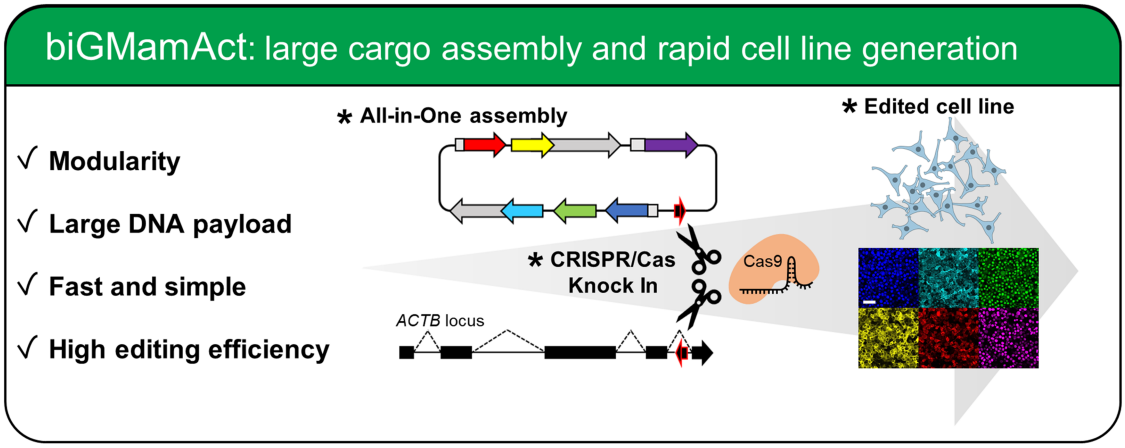
Abstract

Recent advances in molecular and cell biology and imaging have unprecedentedly enabled multiscale structure-functional studies of entire metabolic pathways from atomic to micrometer resolution and the visualization of macromolecular complexes *in situ*, especially if these molecules are expressed with appropriately engineered and easily detectable tags. However, genome editing in eukaryotic cells is challenging when generating stable cell lines loaded with large DNA cargoes. To address this limitation, here, we have conceived biGMamAct, a system that allows the straightforward assembly of a multitude of genetic modules and their subsequent integration in the genome at the *ACTB* locus with high efficacy, through standardized cloning steps. Our system comprises a set of modular plasmids for mammalian expression, which can be efficiently docked into the genome in tandem with a validated Cas9/sgRNA pair through homologous-independent targeted insertion. As a proof of concept, we have generated a stable cell line loaded with an 18.3-kilobase-long DNA cargo to express six fluorescently tagged proteins and simultaneously visualize five different subcellular compartments. Our protocol leads from the *in silico* design to the genetic and functional characterization of single clones within 6 weeks and can be implemented by any researcher with familiarity with molecular biology and access to mammalian cell culturing infrastructure.

Keywords: genetic engineering; *in situ* structural biology; correlative microscopy; CRISPR/Cas9; modular cloning system; multigene expression

Graphical Abstract



Introduction

Integration of structural and cellular biology has recently enabled the characterization of large and complex systems at different resolution scales, *in situ* [1, 2]. Studying biological macromolecules within their cellular environment enables the visualization of vital biological processes in physiological conditions and consequently

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improves our understanding of fundamental biological mechanisms. However, *in situ* molecular characterization faces specific challenges and requires new technological development.

An important challenge when studying multicomponent macromolecular systems is the number of genetic modules that need to be engineered, assembled, and expressed in the cell. For generating stable cell lines, assembling genetic cassettes in a way compatible with genomic integration is a limiting factor. Available cloning systems designed for easy and rapid assembly of multiple DNA elements are meant to be used in insect cells and can rarely be deployed in mammalian cells [3, 4]. Moreover, despite their robustness, specific skills in molecular biology, such as site-specific recombination or multisite gateway cloning, are required to establish such tools [3, 5–7], which limits their application.

Precise gene editing has become possible thanks to techniques such as CRISPR/Cas9 [8], TALEN [9, 10], and Cas-associated systems [Prime Editing [11] or CRISPR-associated transposases (CASTs) [12, 13]], which enable the modification of endogenous genes and the insertion of foreign DNA. These tools facilitate knock-outs, knock-ins, targeted mutation, and tagging and even the correction of genetic diseases [14, 15]. The generation of edited pools and clonal subpopulations allows researchers to work with homogenous cell samples, unlocking the study of subtle biological process, even at the single-cell level. However, techniques relying on these nucleases suffer from pitfalls and limitation in the experimental design. Indeed, TALENs require complicated protein design [16, 17] and validation. CRISPR/Cas9 is limited by sgRNA selection and validation [18, 19] or template design inherent to the choice of the repair mechanism exploitable in given cell types (dividing vs nondividing).

Furthermore, when working in living systems, inherent variability is increased by some other time-dependent factors, such as silencing [20].

To address these limitations and maximize the potential of currently available genetic engineering technology, here, we have designed a method for multicomponent assembly and tunable expression in mammalian cells, with highly efficient and precise CRISPR/Cas9-mediated insertion into the genome. Our method enables the rapid and straightforward generation of stable cell lines carrying large DNA payloads (Fig. 1). We expect this approach to facilitate metabolic engineering in mammalian cells and the study of complex biological processes at a multiscale cellular level, especially those requiring the simultaneous visualization of multiple macromolecules [21].

Materials and methods

Plasmid engineering

Plasmids for biGMamAct were generated by shortening biGBac plasmids [22]. Through polymerase chain reaction (PCR) and subsequent backbone self-ligation, the ampicillin resistance cassette and flanking noncoding sequences were removed, yielding psLIB, psBIG1 (a to e), and psBIG2 (ab to abcde). Furthermore, the polH promoter was exchanged for CMV, EF1α or, Tet responsive element (TRE) promoter for use in mammalian cells. The recipient plasmid psBIG1a *ACTB*T2ATetP2AP-mAGH was assembled by introducing the last intron of *ACTB* with selected sgRNA sequence in reverse orientation and the last exon of *ACTB* fused through a T2A peptide to the tetracycline-controlled transactivator rTetR and to a P2A-peptide puromycin resistance marker. Moreover, we have introduced a cassette expressing mAzamiGreen (mAG) T2A-hygromycin in opposite orientation. psBIG1a *ACTB*T2ATetP2AP-mAGH includes the biGBac α and ω sequences [22] flanking a Swa1 restriction site, enabling the simultaneous insertion of up to five PCR-amplified cassettes from psLIB via Gibson cloning using the biGBac primers. This design leverages spCas9 for linearization of the exogenous DNA to be knocked-in (pDONOR) and ensures the presence of two selection markers (puromycin and hygromycin) surrounding the DNA cargo of interest. The psBIG1a *ACTB*T2ATetP2AP-mAGH also carries biGBac A and B sequences [22], and therefore cassettes cloned into this plasmid can be further combined with other cassettes inserted into psBIG1 plasmids (b to e) via another step of standardized Gibson cloning into psBIG2 plasmids (ab to abcde). *ACTB* sgRNA cloning is achieved by annealing two ssDNA primers (*ACTB*_For 5′ CACCGACAGCTCCCCACACACCAC 3′ and *ACTB*_Rev 5′ AAACGTGGTGTGTGGGGAGCTGTC 3′) and subsequent ligation in a Bbs1-digested pX458 (Addgene #48138).

Gibson assembly of DNA elements

Genes of interest (GOIs), i.e. mito-mCherry, eYFP-Tubulin, H2B-IRFP720, GTS-mTagBFP, and mTFP1-Actin were, respectively, amplified by PCR from Addgene plasmids #206256, #206254, #206253, #206259, and #206255. They were subsequently cloned by Gibson cloning using NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs, cat. no. E2621S) into psLIB TRE digested with StuI (New England Biolabs, cat. no. R0187S) following manufacturer's recommendations. Briefly, we mixed on ice 0.05 pmol backbone with 0.1 pmol insert and 5 µl of NEBuilder® HiFi DNA Assembly Master Mix in a 10 µl-reaction mixture. We incubated the reaction for 15 min at 50°C in a thermocycler. We amplified individual cassettes contained inside psLIB shuttle plasmids using biGBac standard primers and assembled through Gibson cloning into the Swa1-digested psBIG1a *ACTB*T2ATetP2AP-mAGH. We mixed 0.06 pmol of each amplified cassette and backbone with 10 µl of NEBuilder® HiFi DNA Assembly Master Mix in a 20 µl–reaction mixture and then incubated the reaction mixture for 60 min at 50°C in a thermocycler.

To convert 0.06 picomoles into microliters of plasmids of interest, we used the following formula:

$$Vol\,(\mu L) = \frac{0.06\,pmoles \times 643 \times DNA\,fragment\,length\,(bp)}{Concentration\,(ng/\mu L) \times 1000}$$

We then transformed 10 µl of the reaction mixture into homemade Top10 chemically competent *Escherichia coli* cells. After 1-h recovery at 30°C, we plated the cells onto Luria-Bertani/Agar plates with gentamycin and incubated them overnight at 30°C. The following day, we picked individual colonies and grew them at 30°C overnight for DNA isolation. We screened for correct DNA assembly through unambiguous restriction digest (here SwaI was used for excision of each individual cassette), and positive clones were sent for validation by sequencing (Eurofins).

Cell culture

We maintained HEK293T cells (ATCC CRL 3216) in Dulbecco's Modified Eagle medium (DMEM) high glucose + 10% Fetal bovine serum (FBS) + pen/strep (complete medium) in a 37°C, 5% CO_2 incubator and passaged them every 3–4 days by trypsinization when reaching 90% confluency.

Transfection

We transfected HEK293T cells in one well of a 6-well plate using 800 ng pDONOR 6colors and 250 ng pX458 *ACTB*. We diluted the DNA into 50 µl of DMEM (without FBS and pen/strep) prior addition of 50 µl of DMEM (without FBS and pen/strep) containing 3 µl of LipoD293 (SygnaGen, cat. no: SL100668) transfection reagent.

Table 1. biGMamAct plasmid library

Plasmids	Description
psLIB CMV	biGMamAct shuttle
psLIB EF1α	biGMamAct shuttle
psLIB TRE	biGMamAct shuttle
psLIB U6	biGMamAct shuttle
psBIG1a	biGMamAct recipient
psBIG1b	biGMamAct recipient
psBIG1c	biGMamAct recipient
psBIG1d	biGMamAct recipient
psBIG1e	biGMamAct recipient
psBIG2ab	biGMamAct recipient
psBIG2abc	biGMamAct recipient
psBIG2abcd	biGMamAct recipient
psBIG2abcde	biGMamAct recipient
psLIB TRE mito-mCherry	Assembled shuttle
psLIB TRE EYFP-tubulin	Assembled shuttle
psLIB TRE H2B iRFP720	Assembled shuttle
psLIB TRE GTS-mTagBFP	Assembled shuttle
psLIB TRE mTFP1-actin	Assembled shuttle
psBIG1a ACTBT2AmcherryP2AP-TagBFPH	biGMamAct recipient for *ACTB* docking (not used here)
psBIG1a ACTBT2ATetP2AP-H	biGMamAct recipient for *ACTB* docking (not used here)
psBIG1a ACTBT2ATetP2AP-mAGH	biGMamAct recipient for *ACTB* docking
pDONOR 6colors	Assembled DONOR template for *ACTB* docking
pX458 *ACTB*	spCas9 and sgRNA *ACTB* encoding plasmid

We incubated the mixture for 15 min at room temperature before being added onto 1.5 million HEK293T cells, in 1.5 ml of complete medium before attachment of the cells. We kept the plate at 37°C, supplied with 5% CO_2 for 24 h, and then replaced the medium by 2 ml of complete medium.

Stable cell line establishment, clonal isolation, and genotyping

We dissociated cells 72 h post-transfection and pelleted and seeded all cells into a 10-cm dish with 12 ml of complete medium containing 0.5 μg/ml puromycin and 100 μg/ml hygromycin. Every 2–3 days, we monitored cell selection and exchanged the medium while maintaining 0.5 μg/ml puromycin and 100 μg/ml hygromycin. After 15 days of selection, HEK293T cells started recovering.

We dissociated the cells at ∼30% confluency, maintained the pool and additionally seeded two 10-cm dishes at clonal density (500 cells/dish). After 10 days, we manually picked individual clones, visible by eye, and further expanded them for genotyping. We performed genomic DNA extraction on clones grown in 12-well plates using the Monarch® Genomic DNA Purification Kit (New England Biolabs, cat. no. T3010S) and following the manufacturer's recommendation. We achieved genotyping of 5′-, 3′-, and wild type (WT) junctions by running PCRs on extracted gDNA of each individual clone. We amplified the loci of interest using KAPA2G Fast HotStart ReadyMix (Roche, cat no. 2GFHSGKB) following the manufacturer's recommendations. Briefly, we added 50–100 ng of gDNA to 12.5 μl of the 2× enzyme mix, 1.25 μl of forward and revers primers (10 μM), and 25 μl of dH_2O.

After a 3-min denaturation step, we performed 25 cycles of denaturation (98°C, 15 s), annealing (60°C, 10 s) and elongation (72°C, 10 s), and then a final elongation at 72°C for 1 min. For genotyping the 5′-, 3′-, and WT junctions, we used the following primer pairs: For1/Rev1, For2/Rev2, and For1/Rev2, respectively (Table 2). We analyzed the final PCR products on 1% agarose gels.

Confocal microscopy

We cultured positive clones in complete medium containing 0.5 μg/ml puromycin and 100 μg/ml hygromycin onto collagen-coated glass coverslips (Ø 20 mm). Once reaching ∼50% confluency, we replaced the medium with complete medium containing 0.5 μg/ml puromycin, 100 μg/ml hygromycin, and 1 μg/ml doxycycline. After 24 h, we removed the medium and washed the cells with phosphate-buffered saline (PBS) prior to fixation. Fixation was achieved using paraformaldehyde (PFA) (4% in PBS) for 15 min at 37°C. After PFA removal and two PBS washes, we mounted coverslips onto glass slides using ProLong™ Diamond Antifade Mountant with 4′,6-diamidino-2-phenylindole (DAPI) (ThermoFisher, cat. no. P36966). We stored mounted glass slides in the dark at 4°C for 24 h. We imaged cells using a Leica TCS SP5 AOBS confocal microscope equipped with 405-, 458-, 478-, 488-, 496-, 514-, 561-, 594-, and 633-nm lasers, and we monitored characteristic fluorescence and localization corresponding to every module inserted into the pDONOR 6colors, i.e. mTagBFP at the Golgi apparatus, mTFP-actin, mAG at the nucleus, eYFP tubulin, mCherry at the mitochondria, and iRFP720-Histone2B.

Results

Generation of a novel multicassette vector for mammalian expression of large functional DNA cargoes

Working with several isolated vectors may lead to uncontrolled variability when considering relative transfection efficiency of individual plasmids and stoichiometric recombinant expression of encoded proteins inside cells. Indeed, using several plasmids often leads to biased results due to variable DNA uptake by the cells [23]. To avoid these biases, it is strongly preferrable to generate one large single DNA vector encompassing all GOIs. Based on these considerations, we have leveraged our experience with the MultiBac [24] and biGBac [22] baculovirus expression vector systems (BEVSs), which can successfully harbor multicassettes for expression of large multiprotein complexes in insect cells for structure determination [25, 26]. Here, we developed the novel multicassette vector biGMamAct, for mammalian expression of large functional DNA cargoes. Because biGBac uses standardized, bioinformatically validated DNA sequences for ligation independent cloning (LIC) [27, 28] and minimizes the carryover of noncoding sequences (plasmid backbone) during sequential assemblies, we decided to adapt it for deployment in mammalian cells. The biGMamAct system encompasses a set of plasmids derived from the biGBac cloning system [22], which we have considerably reduced in size. From the existing pLIB, pBIG1 (a to e), and pBIG2 (ab to abcde) plasmids from biGBac [22], we created two smaller versions, named psLIB (32% smaller) and psBIG 1–2 series (28% smaller), allowing easier manipulation and/or increasing loading capacity. To shorten these vectors, we first removed their ampicillin resistance cassette, as well as flanking noncoding sequences. Additionally, we replaced the polH promoter for insect cells with various promoters (CMV, EF1α, or TRE) to match user needs (Table 1). The inclusion of TRE as a promoter enables the use of cytotoxic proteins and limits silencing that may occur when establishing a stable cell line. The psLIB backbone still allows single cassette amplification by PCR using the standardized, bioinformatically designed set

Table 2. biGMamAct cloning and genotyping primers

Primers	Use	Sequence (5′–3′)
Stu1_For	Cloning in psLIB TRE	GTCCGAAGCGCGCGGAATTCAAAGGNNNNNNNNNNNNNNNNNNN
Stu1_Rev	Cloning in psLIB TRE	CGACTAGTGAGCTCGTCGACGTAGGNNNNNNNNNNNNNNNNNNN
sgRNA ACTB_For	sgRNA cloning	CACCGACAGCTCCCCACACACCAC
sgRNA ACTB_Rev	sgRNA cloning	AAACGTGGTGTGTGGGGAGCTGTC
CasI_for	biGBac cloning	AACGCTCTATGGTCTAAAGATTTAAATCGACCTACTCCGGAATATTAATAGATC
CasI_rev	biGBac cloning	AAACGTGCAATAGTATCCAGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCG
CasII_for	biGBac cloning	AAACTGGATACTATTGCACGTTTAAATCGACCTACTCCGGAATATTAATAGATC
CasII_rev	biGBac cloning	AAACATCAGGCATCATTAGGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCG
CasIII_for	biGBac cloning	AAACCTAATGATGCCTGATGTTTAAATCGACCTACTCCGGAATATTAATAGATC
CasIII_rev	biGBac cloning	AAACTAAGCTATGTGAACCGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCG
CasIV_for	biGBac cloning	AAACGGTTCACATAGCTTAGTTTAAATCGACCTACTCCGGAATATTAATAGATC
CasIV_rev	biGBac cloning	AAACCAAGTCAATGTCAGTGTTTATTTAAATGGTTATGATAGTTATTGCTCAGCG
CasV_for	biGBac cloning	AAACACTGACATTGACTTGGTTTAAATCGACCTACTCCGGAATATTAATAGATC
CasV_rev	biGBac cloning	AACCCCGATTGAGATATAGATTTATTTAAATGGTTATGATAGTTATTGCTCAGCG
For_1	Genotyping	ATGAAGATCAAGGTGGGTGTCTTT
For_2	Genotyping	CTGCGAACGGACGTGAAGAATG
Rev_1	Genotyping	CCTGTCCAGCATCTCGATTGGC
Rev_2	Genotyping	AGGTTTTGTCAAGAAAGGGTGTAAC

of biGBac primers. We have shown that up to five PCR-amplified cassettes from psLIB can be inserted simultaneously by Gibson cloning [28] (LIC) into psBIG1a plasmids (Fig. 2a). The psBIG1a *ACTB*T2ATetP2AP-mAGH for stable and precise CRISPR/cas9 integration at the *ACTB* locus is also compatible, as it still carries the biGBac α and ω sequences [22]. The former vector also contains biGBac A and B sequences, so that cassettes cloned into this locus can further be combined with other cassettes inserted into psBIG1 plasmids (b to e) through a second step of standardized Gibson cloning into psBIG2 plasmids (ab to abcde). Similar to biGBac, our system can accommodate up to 25 independent cassettes, assembled in only two cloning steps, as exemplified in the original study [22]. Our method also allows for the use of self-cleaving peptides to reduce the number of individual cassettes, limiting repeated sequences (e.g. promoters) that could lead to unwanted recombination events during cloning (Fig. 2b). Importantly, as part of our biGMamAct cloning system, the newly created psLIB_CMV and psLIB_EF1α can be used and assembled with the smaller psBIG1 and psBIG2 vectors for transient expression of dozens of independent cassettes. BiGMamAct plasmids psBIG1 and psBIG2 offer the possibility to insert very large constructs into baculovirions for high transduction efficiency in a variety of cell types [4, 29–31].

Using the novel methodology presented here, it is now possible to generate stable cell lines with precisely integrated functional DNA cargoes of several tens of kilobases, expressing tens of macromolecular subunits. Here, we benchmarked the protocol by inserting an 18.3-kb-long DNA cargo expressing six fluorescent proteins to simultaneously visualize five different cellular compartments.

Delivery and integration of biGMamAct vectors

To tackle delivery challenges inherent to the increasing size of DNA payloads for genomic integration into specific cell lines through engineered baculovirus [29], we validated a highly efficient method for knocking in foreign DNA at the *ACTB* locus [4] via homology-independent targeted insertion (HITI) [32] following Cas9-induced double-strand breaks. We combined these two methodologies to design a robust tool for easy and quick assembly of multiple genetic modules into a large construct, which can be efficiently and precisely integrated into both dividing and nondividing cells.

When a double-strand break occurs in the genome, an exogenous piece of DNA can be introduced through two independent mechanisms: homology-directed repair (HDR) or nonhomologous end joining (NHEJ), each relying on different sets of proteins [33, 34]. Briefly, NHEJ happens throughout the cell cycle and relies on the Ku protein to recruit protein complexes, containing nucleases or polymerases, for DNA resealing by DNA ligase IV complex [35, 36]. To make our method as universal and efficient as possible, we not only chose to rely on NHEJ-repair mechanisms but also incorporate HITI [32]. Indeed, HITI has been reported as the most efficient strategy for KIs [32, 37]. In contrast to low efficiency HDR, HITI occurs at every stage of the cell cycle, making this method applicable to both dividing and nondividing cells. This approach eliminates the need for long homology arms to be cloned into donor plasmids, increasing the adaptability of our method for targeting other genomic loci (only sgRNA sequence exchange required), as well as for enhancing the cargo capacity and transfection efficiency of pDONOR. By introducing into the pDONOR plasmid the same targeted sequence (sgRNA + PAM) as the one used by spCas9 in the genome, but in reverse orientation, we exploit spCas9 to linearize the insertion cassette, ensuring its correct integration and orientation (Fig. 2a). Indeed, pDONOR insertion in reverse orientation results in restoration of the sgRNA + PAM sequence, leading to spCas9 cleavage and excision of the wrongly inserted pDONOR (Fig. 2a). Because HITI is not a scar-free integration mechanism, we selected the best sgRNA within the last intron of *ACTB* by combining E-CRISP and IDT's gRNA design checker [38]. Using the NHEJ-dependent mechanism, HITI, subsequent to spCas9 cuts into both the genome and pDONOR, underlines the power of the method described here, as we demonstrate precise integration of functional DNA of unprecedented size, with 100% of the screened clones being homozygotes (Fig. 3). We note that it is unlikely for cells to be heterozygous with large deletions or partial insertions. Primers used for genotyping are located far from the editing site, so we would have observed WT bands of various sizes. Additionally, we did not observe any cells without fluorescence from any integrated module, which would indicate partial insertions. Furthermore, the method ensures that the entire linearized pDONOR is integrated, preventing "backbone-only" integrations that could lead to false

positive in genotyping. Other methods involving RNPs or Cas9 protein delivery have been reported to achieve KIs with lower efficiency (89%) after selection, without homozygosity confirmation [39].

The psBIG1a *ACTB*T2ATetP2AP-mAGH contains the same sgRNA sequence as the *ACTB* locus targeted (Fig. 2a). This feature enables the use of Cas9 for linearizing of the exogenous DNA to be knocked-in (pDONOR). The psBIG1a *ACTB*T2ATetP2AP-mAGH sequence also includes two selection markers (puromycin and hygromycin) surrounding the sgRNA sequence. This arrangement ensures that once the DNA cargo of interest is cut, it is flanked by two selection markers. Importantly, the puromycin gene lacks an ATG start codon, meaning that resistance can only be acquired when integrated at the *ACTB* locus after correct mRNA editing. This strategy minimizes the likelihood that insertion occurs at other genomic location or that pDONOR persists as an episome after selection. Importantly, our plasmids lack SV40 and F1 origins of replication, which could otherwise lead to plasmid persistence as episomes. The use of two selection markers is needed, as we previously observed silencing at the 3′ end of large donors when applying selection pressure only at 5′ [4]. The *ACTB* sgRNA sequence is inserted in reverse orientation when compared to its genomic location, resulting in the locking of the DNA cargo once correctly inserted at the *ACTB* locus. This design ensures complete integration of the DNA cargo at the correct genomic location. Using all-in-one pDONOR encoding plasmid eliminates the drawbacks of other methods, such as viral delivery, which sometimes leads to excess backbone genomic integration [4]. Applying selection pressures at both 5′ and 3′-ends of the DNA cargo further minimizes the chance of silencing by keeping the genomic region active. If cell sorters are available, fluorescent proteins can easily be inserted or removed as fusion constructs with the selection markers genes. The use of double selection markers, which can be combined with the use of two fluorescent markers (e.g. mCherry and mTagBFP-NLS or mAG-NLS), enables efficient and reliable selection during stable cell line generation and clonal characterization. By relying on both CRISPR/Cas9 and HITI for cutting and insertion, we can precisely integrate up to 18.3-kb functional DNA payload with 100% (24/24) of the screened clones being homozygous (Fig. 3).

Integration of multicassette cargo DNA at the ACTB locus

In our method,12 we selected the *ACTB* locus as the landing pad for our large DNA cargo based on the following rationale. Actin β is an essential gene, highly expressed throughout the cell cycle. Moreover, it has been shown before that ACTB can accommodate large DNA cargo by CAST [13]. However, functionality of the foreign DNA was not tested in that study and the strategy was fundamentally different, leaning on 20 sequential insertions of an integration site followed by KI of transgene via Bxb1 integrase. Furthermore, the *ACTB* locus has been reported as an excellent candidate for docking exogenous DNA sequences for stable cell line generation. Indeed, it is reported to be less—or equally—prone to silencing than other well-established docking sites [40, 41]. To further reduce the risk of silencing over time, we integrated into our portfolio of shuttle plasmids some harboring Tet-inducible promoters. Selecting *ACTB* as the targeted genomic locus also makes this technology applicable to already established cell lines relying on AAVS1 [42], hRosa26 [43], CCR5, and others [44]. Certainly, our method can be extended to other genomic loci, either simultaneously or sequentially, by using additional selection markers and sgRNAs (e.g. housekeeping loci such as GAPDH or safe harbor sites like AAVS1 [44, 45]). Our data highlight the high efficacy of the method design, which relies on a robust Cas9/sgRNA pair targeting the *ACTB* locus and employing HITI for genomic integration. After antibiotic treatment, PCR analysis showed a barely detectable band for nonedited *ACTB* allele (Fig. 3), suggesting a very low abundance of unedited alleles, even in the multiploid HEK293 cell line. We note that our method is not dependent on high transfection efficiency. Thus, while HEK293 is particularly easy to transfect, our method should be applicable to other cell lines too. Also, as HEK293 can display multiploidy, it can be challenging to edit them as homozygous. In this context, the isolation of 100% homozygous clones ($n = 24$) demonstrates the strength of the chosen sgRNA paired with spCas9 when following the procedure described herein. For comparison, targeted integration at the widely used AAVS1 safe harbor locus using an established sgRNA/spCas9 pair resulted in 60–89% positive clones, including both heterozygotes and homozygotes [39]. Furthermore, confocal microscopy of all cells from the selected pool and clones confirmed the integration of the six modules for fluorescent labeling of cellular compartments. This indicates the complete and unaltered insertion of the pDONOR 6colors sequence, underscoring HITI as a reliable cut-and-paste mechanism for precise DNA cargo integration.

Selection of cells that reliably express all integrated DNA modules

Our method targets the last *ACTB* intron and thus uses the final exon of *ACTB* to fuse an in-frame selection marker and the gene of interest. This strategy abolishes off-target insertions that could be observed depending on sgRNA sequences or when using the highly processive spCas9. The introduction of a synthetic 3′-terminal exon fused with a self-cleaving peptide followed by a puromycin marker eliminates unpredictable indels and random integration, thereby maximizing correctly edited alleles. The number of homozygous clones identified among the tested ones (24/24) confirms the robustness of the method, even in polynuclear cell lines like HEK293. Additionally, the absence of a wild-type band in all tested clones demonstrates that the method induces very low, if any, random integrations and yields highly precise DNA cargo insertion with high efficacy.

Thanks to the two selection markers flanking the *ACTB* sgRNA + PAM sequence in pDONOR, cleavage by the spCas9 nuclease produces a 5′-end fusion of the last *ACTB* exon with puromycin resistant marker and a 3′-end consisting of a hygromycin selection cassette. Applying both selections ensures the precise and complete integration of the entire pDONOR cargo. Applying selection pressure flanking the integrated DNA sequence maintains transcriptional processivity of the edited region [4], thereby reducing the risk of silencing over time. After generating the cell line with the pDONOR 6colors integration, we induced expression of the inserted cassettes in several clones and in the pool. Confocal microscopy confirmed the expression of all modules and correct fluorescent tagging of all targeted cellular compartments. Even 1-month post-transfection, every imaged cell displayed comparable expression levels for all six fluorescent proteins, which were all correctly localized to their respective cellular compartments (Fig. 4). This underlines the utility of dual flanking selection markers and TRE promoters for mitigating silencing issues when generating stable cell lines, particularly when working with large DNA payloads or toxic proteins.

Expression of an 18.3-kb DNA cargo from the ACTB locus

We demonstrated that six coloring modules targeting different subcellular compartments, with tunable expression, can be assembled in a single step, despite their highly repetitive sequences (e.g. 6 GFP-derived fluorescent proteins and 40 TRE repeats). After assembling pDONOR 6colors, we highlighted the strength of biGMamAct through CRISPR/Cas-mediated insertion at the *ACTB* locus, achieving high efficiency and precision. Using the sgRNA/spCas9 and HITI tandem for stable integration of >18-kb DNA cargo at the *ACTB* locus, we achieved very high efficiency (100% homozygotes, Fig. 3). The biGMamAct system, with its portfolio of shuttle plasmids, enabled precise KI of an 18.3-kb functional DNA payload for the first time. Docking such a large and functional multicomponent DNA payload remains a significant challenge in metabolic engineering of mammalian cells. We previously showed successful insertions of large cargoes into human genomes using baculovirus, but in those cases, the viral genome was often partly integrating along with the gene of interest, homozygosity post-selection was limited to 60%, and we had not tested for DNA functionality [4]. Other groups reported the successful integration of large DNA cargoes at *ACTB* using CAST [13], but also in those cases, the DNA functionality was not tested. Here, we evaluated the capacity of the biGMamAct method to achieve precise DNA docking of a very large DNA payload at the *ACTB* locus while retaining its protein-coding functions. We designed and cloned into psLIB TRE six individual cassettes for fluorescent tagging of five different cellular substructures. All cassettes were assembled into psBIG1a ActBT2ATetP2AP-mAGH via Gibson cloning, resulting in the all-in-one vector pDONOR 6colors of 18.3 kb. Cotransfection with spCas9 and ACTB_sgRNA containing plasmid (pX458 *ACTB*) followed by 10 days of double selection successfully generated the desired polyclonal cell line. Clones were isolated, expanded, and genotyped, with all confirmed as homozygotes (24/24). Doxycycline induction in edited cells triggered the expression of the six independent genetic modules spanning over 18.3 kb, fluorescently labeling distinct cellular compartments. Across all the visible light spectra, homogenous expression and correct subcellular localization were observed for GTS-mTagBFP at the Golgi, mTFP1-Actin at adhesion junctions, mAG-NLS and H2B-iRFP (histones) in the nucleus, YFP-Tubulin at the cytoskeleton (microtubules), and mito-mCherry at mitochondria (Fig. 4). Notably, we were able to observe, within the cell population, the different appearance of tagged cellular compartments varying with cell cycle stages. For example, during metaphase, the Golgi apparatus is fragmented and no more visible, actin filaments are mostly visible at the cell periphery, GFP-NLS is evenly distributed within the cell, microtubules form spindle spanning from centrosomes to chromosomes, mitochondria are highly fragmented, and H2B colocalizes with the condensed chromosomes gathered at the metaphase plate (Fig. 4). These results demonstrate that biGMamAct enables the assembly and subsequent precise and efficient integration of large, complex DNA cargo at the *ACTB* locus while retaining functionality. This paves the way for future advanced engineering in human genomes involving large synthetic gene regulatory networks or matching emerging needs for *in situ* structural biology.

Discussion

Here, we report a new method that enables the efficient integration of large multicassette DNA cargoes into the *ACTB* locus of mammalian cells.

The method presented here offers the possibility to (i) easily assemble, in one standardized step through LIC, up to six genetic modules, which can then be combined with the use of self-cleaving peptides [46] and (ii) reliably and stably integrate these modules into the genome at the *ACTB* housekeeping locus using a CRISPR/Cas9-based approach [4]. Importantly, our assembly system preserves the highly modular properties of the previously reported biGBac system. Thus, up to 25 modules can be assembled in only two standardized LIC steps to create a single all-in-one carrier vector [22].

To overcome the challenges of working with multiple plasmids—often leading to biased results due to variable DNA uptake by the cells [23]—we generated a single large DNA vector encompassing all GOIs. Since this approach can involve tedious cloning methodologies, we adapted the reliable and standardized biGBac cloning system for mammalian cells, thereby generating the new biGMamAct system. Using this approach, any scientist can assemble up to six genetic modules in one single step within a week, leading to the generation of all-in-one coding plasmids for subsequent integration at the *ACTB* locus (Figs. 1 and 2a). To achieve this result, the assembled plasmid (pDONOR) is cotransfected with standard pX458 *ACTB*, which encodes for spCas9 and sgRNA targeting the last intron of the human *ACTB* locus. This transfection-based approach is less time-consuming than RNP-based methods that involve extra experimental steps in producer cells [39, 47]. Thanks to our design, spCas9 not only

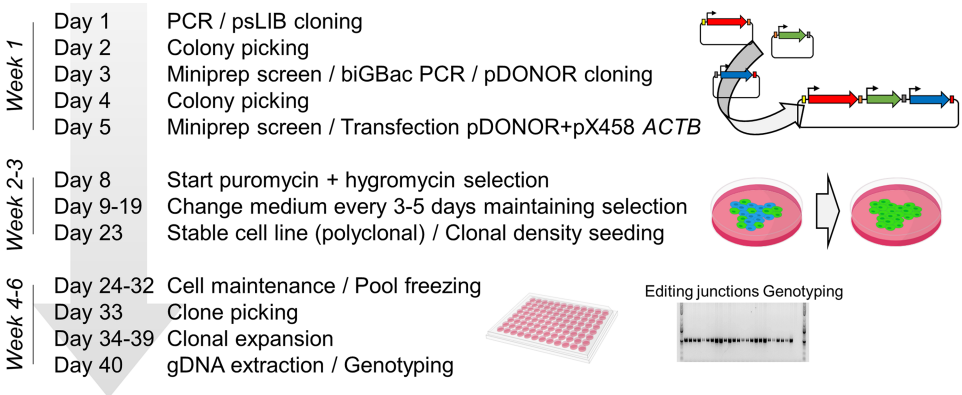

Figure 1. Workflow of the biGMamAct method. The first week of cloning is followed by 2 weeks of selection to establish a polyclonal stable cell line. Two more weeks are required to expend, isolate and genotype clonal populations. The figure was partially produced using BioIcons.

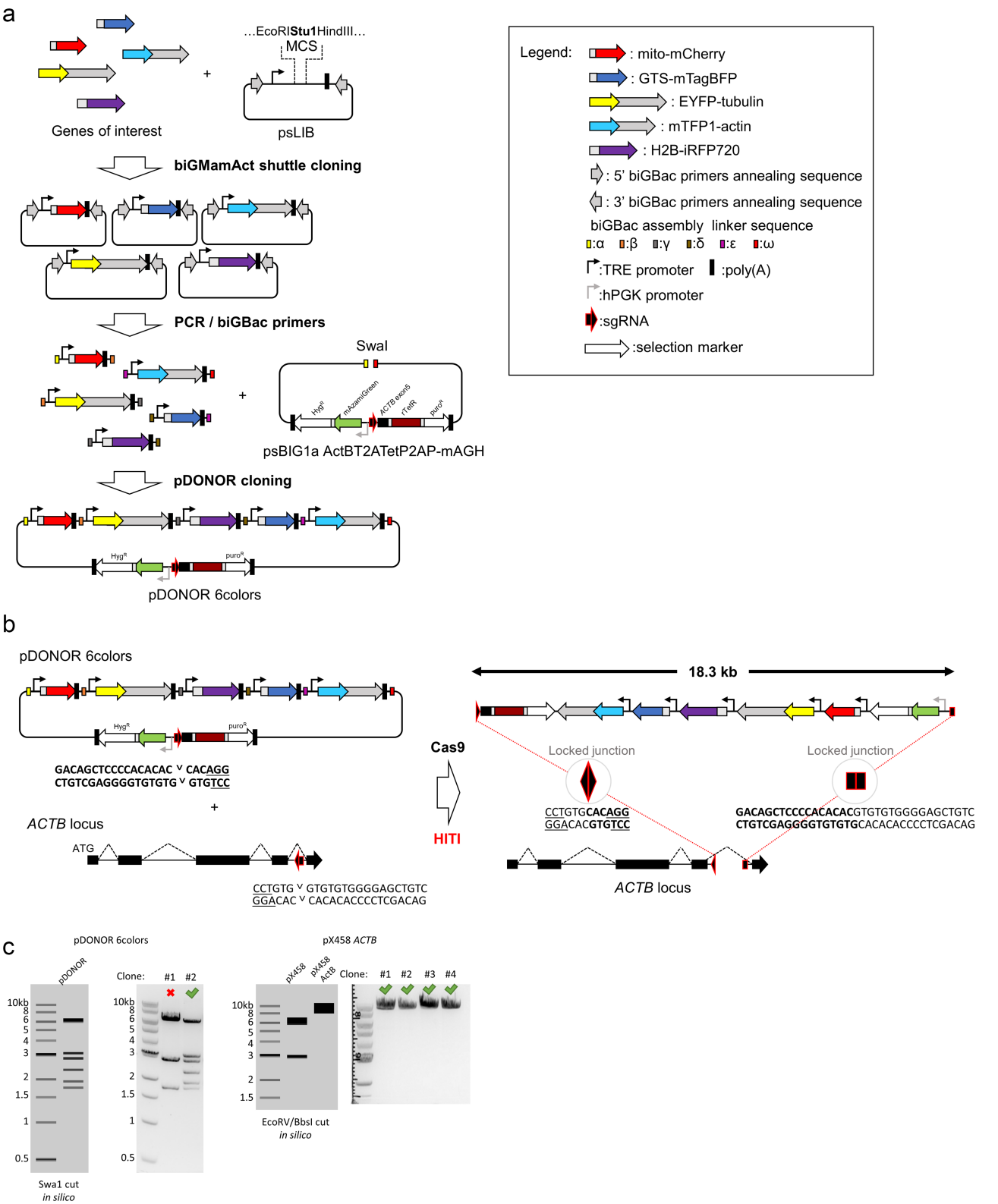

Figure 2. (a) biGMamAct assembly. GOIs inserted into the psLIB shuttle plasmids of the biGMamAct expression system can be PCR amplified using the standard bioinformatically designed primers of the biGBac system for further assembly into the recipient psBIG1a *ACTB*T2ATetP2AP-mAGH leading to all-in-one pDONOR generation. (b) KI design. Cotransfection of the pDONOR 6colors plasmid with a Cas9-containing one targeting *ACTB* locus will lead to pDONOR stable and precise integration via HITI at the *ACTB* locus with high efficiency. Indeed, *ACTB* sgRNA targeted sequence is present in reverse orientation in pDONOR for linearization by Cas9 endonuclease and orientation-controlled integration in the genome. sgRNA target sequence and resulting junctions are depicted (PAM is underlined, and DNA sequence from pDONOR in bold). (C) Cloning validation. Agarose gels showing recombining and positive pDONOR 6colors clones (left) and correct sgRNA *ACTB* cloning in pX458 (right).

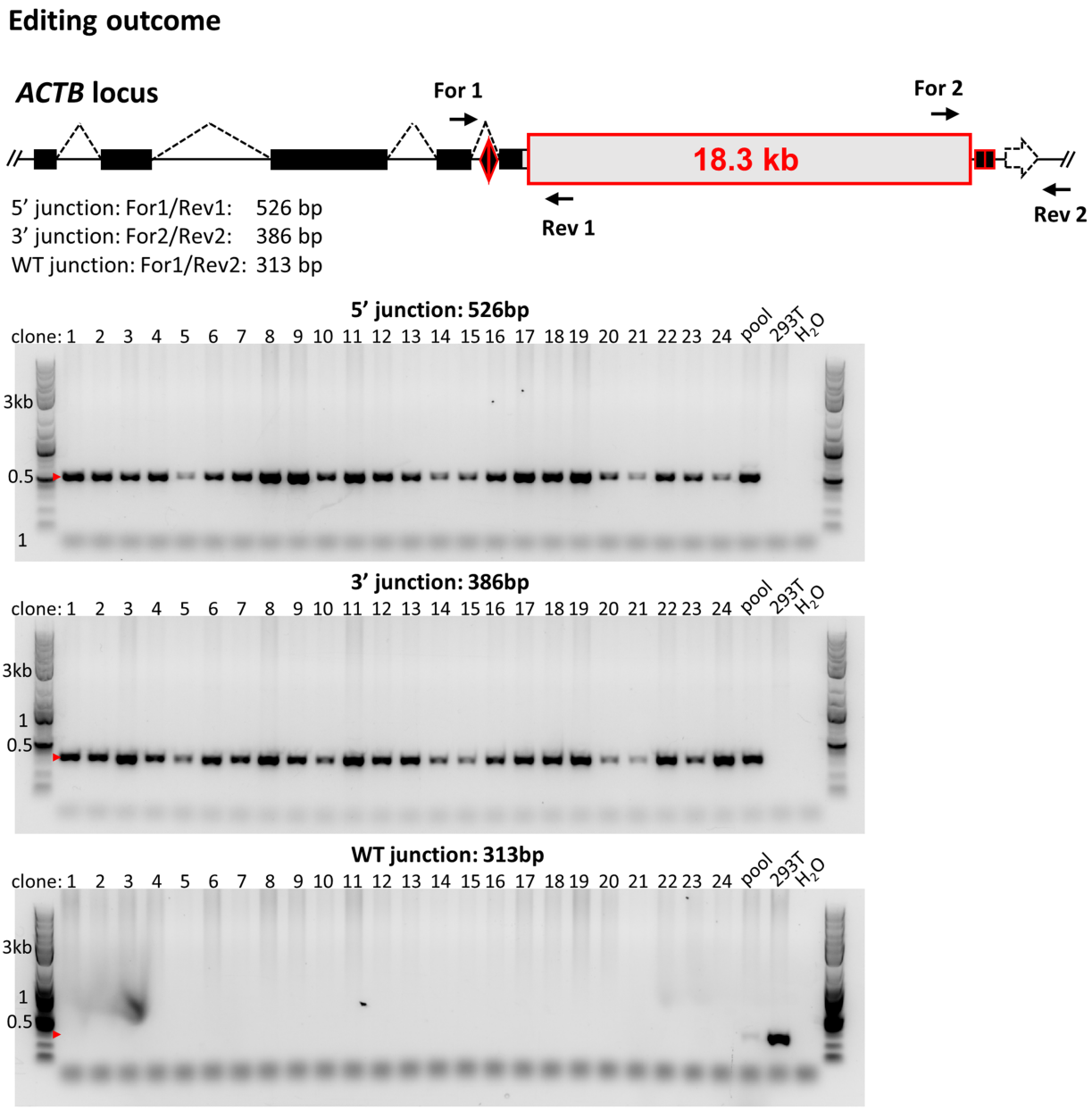

Figure 3. Genotyping of clone highlight biGMamAct high efficiency KI at *ACTB* loci. Schematic representation of edited human *ACTB* locus. Triangles and boxes represent the locked 5′ and 3′ junction resulting from HITI docking. Primers used for genotyping are depicted as arrows surrounding these junctions. Correct DNA cargo integration leads to visualization of bands at 526 and 386 bp for the 5′ and 3′ junctions, respectively. Heterozygotes still show a band at 313 bp outlining the persistence of the WT junction (only in pool and HEK293T WT). Data represent screening of 24 clones from one selected pool.

induces a double-strand break in the genome but also linearizes the pDONOR, allowing its stable and precise integration at the *ACTB* locus (Fig. 2a). After 2 weeks of double selection applied to the transfected cells, the desired stable cell line is established, and two additional weeks are required for selection and genomic validation of clonal subpopulations (Fig. 1).

We validated the method by generating stable cell lines harboring inducible, tunable reporters that enable simultaneous tagging of five different subcellular compartments: mitochondria, the Golgi, the cytoskeleton (actin), microtubules (tubulin), and the nucleus. We envisage that the method presented herein can be used to generate cell lines as efficient cell factory, loaded with the components of entire metabolic pathways [48], which can be turned on and off through the use of tunable promoters (Table 1). BiGMamAct will undoubtedly integrate quickly into the synthetic biology toolbox for engineering mammalian cell systems, addressing critical needs in this emerging field [49–51]. This method will also open new avenues for *in situ* structural biology studies, providing the opportunity to integrate tools for the localization of macromolecular complexes into cells [52, 53].

The method exploits the use of biGBac standardized and bioinformatically validated oligos for cloning, alongside a well-established genome engineering design for the quick and efficient generation of stable cell lines harboring large functional DNA payloads. Indeed, in our hands, biGBac is the cloning system that

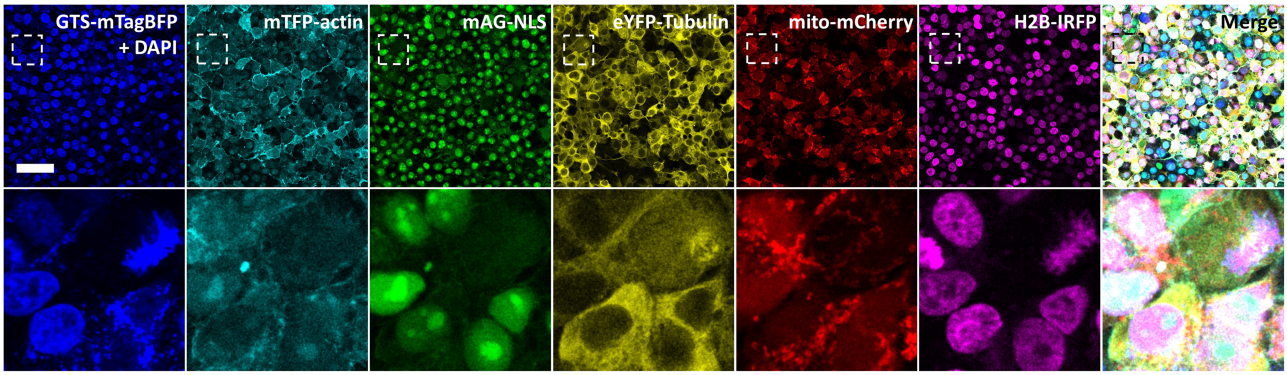

Figure 4. Confocal imaging of HEK293T edited clone. Pictures evidencing in all cells the stable integration of a large and functional DNA payload through homogeneous tunable expression and correct subcellular localization of GTS-mTagBFP (Golgi), mTFP1-Actin (cytoskeleton/adhesion), mAG-NLS (nucleus), YFP-Tubulin (microtubules), mito-mCherry (mitochondria), and H2B-iRFP (histones) on top of DAPI staining. Lower panel is a zoom into a larger field of view from above (dashed box) and showing different appearance of tagged cellular compartments during the cell cycle. For example, during metaphase, the Golgi apparatus is fragmented and no more visible, actin filaments are mostly visible at the cell periphery, GFP-NLS is evenly distributed within the cell, microtubules form spindle spanning from centrosomes to chromosomes, and mitochondria are highly fragmented and H2B colocalizes with the condensed chromosomes gathered at the metaphase plate. Scale bar: 50 μm. Images were obtained imaging one clone from the selected pool. Similar data were obtained for two other clones, $n = 3$.

allows the easiest assembly of a large number of genetic modules. Moreover, genomic integration via HITI following a Cas9-induced double-strand break is the most efficient reported approach for knocking in foreign DNA into the genome of any cell types [4, 54].

Using a single all-in-one plasmid eliminates uncontrolled stoichiometry of cellular uptake of plasmids, which can lead to artifactual results or experimental failure. Some cloning systems designed for easy and rapid assembly of multiple DNA elements are available. However, most of them are part of BEVSs and solely intended for multiprotein complex expression in insect cells [22, 24, 55]. Very few can be deployed in mammalian cells [3, 4], and despite their robustness and efficacy, they remain isolated in use due to the extensive molecular biology expertise required. Multigene assembly using MultiMAM is achieved via Cre/Lox recombination, while MuliMATE requires another recombinatorial cloning, multisite gateway. In contrast, our biGMamAct technology introduces an optimized version of the biGBac cloning system suitable for expression in mammalian cells and compatible with CRISPR/Cas-mediated insertion at the *ACTB* locus.

By relying on CRISPR/Cas9 for precise integration instead of random integration, donor sequences are integrated as a whole. When considering large DNA payloads for genomic introduction, random integration often limits insertion to sequences surrounding the selection markers. CRISPR/Cas9 mediated docking of the all-in-one pDONOR also reduces the number of selection markers required when working with several plasmids. BiGMamAct relies on standardized oligos for cloning and a validated spCas9/sgRNA system targeting *ACTB* to ensure high efficiency and time saving [4, 30]. Indeed, other approaches targeting different loci would require sgRNA design and validation. In contrast, methods involving nucleases such as TALEN would necessitate laborious cloning and validation before becoming applicable. The *bona fide* spCas9/sgRNA pair used in this study resulted in higher homozygosity of the tested clones compared to other validated pairs targeting the widely used AAVS1 locus [39, 56].

Other available methods, such as PrimeEditing [57], can be used for precise integration of smaller DNA fragments but are not suitable for large DNA cargo. Similarly, PiggyBac transposon-based methods [58] or lentiviral approaches can achieve stable integration into genomes but lack precision regarding their targeted sequences and cannot handle the DNA payloads addressed herein. Recent development using CASTs has demonstrated the capability to integrate large DNA payload in mammalian cells [13]. However, this approach requires a more complex implementation than biGMamAct, involving multiple sgRNAs to initially add the attB integration site, which is subsequently used for docking via the Bxb1 integrase to insert DNA cargo for functional assessment. Most current methods for precise genome editing rely on HDR following a CRISPR/Cas-induced double-strand break. However, it must be noted that repair through NHEJ often results in small insertions or deletions (indels) at the cut site. While genome editing for therapeutic purposes should avoid reliance on such repair pathways, NHEJ occurs in both dividing and nondividing cells, unlike HDR. Notably, methods based on NHEJ, such as HITI, have demonstrated one to two orders of magnitude higher efficiency than HDR-based methods and applicable to any cell type.

Finally, silencing over time is another critical challenge faced by biologists when generating stable cell lines. To address this issue, we combined targeting of the *ACTB* locus—a gene constantly expressed throughout the cell cycle—with inducible and tunable promoters. This approach not only mitigates silencing but also enables the use of toxic proteins.

In summary, compared to existing genetic engineering technologies, our method uniquely addresses three significant limitations: the difficulty of generating large DNA cargo, the precision of integration, and the constitutive expression of GOIs.

However, certain limitations of this method should be acknowledged. First, NHEJ introduces small indels, which in this case are confined to the last intron of the *ACTB* gene. Additionally, this strategy does not replace the last *ACTB* exon by a fusion one but instead shifts it to the 3′UTR, representing another marker induced by our editing method. Although the *ACTB* housekeeping gene has been used successfully here and by others for docking transgenes, cell viability and growth under nonstandard conditions should be assessed. Second, establishing a stable cell line following the method presented here requires two selection markers and takes 15 days, which may be lengthy for primary cells. To overcome this, we recommend replacing selection markers with fluorescent ones and combining them with

fluorescence-activated cell sorting. Third, the procedure relies on lipofection of the pDONOR and Cas9 + sgRNA plasmids. KI of very large fragment remains particularly challenging, if not impossible, when using viral delivery systems, RNPs, or Cas9 protein delivery systems, which are all constrained by their inherent packaging constrains [39, 59]. For example, adeno-associated virus and lentivirus (LVs) have maximum cargo capacities of, respectively ∼5 and ∼9 kb. This might not be ideal for cell types for which nucleofection should be favored. However, the biGMamAct system is fully compatible with other transfection methods and can be deployed in cells with low transfection efficiency since HITI achieves the highest precise integration efficiency. Fourth, a limitation inherent to the high modularity of the biGBac system [22] is the potential of recombination during the initial cloning steps when assembling dozens of genetic modules. To mitigate plasmid recombination due to repetitive elements, we recommend leveraging self-cleaving peptides during construct design and provide practical tips to address this issue. In this study, the all-in-one vector assembled contains 40 repeats of the Tet operator sequence, suggesting that recombination-related issues are minimal, if at all relevant.

In summary, using biGMamAct, we successfully demonstrated the assembly of six coloring modules targeting different subcellular compartments with tunable expression in a single step, despite their highly repetitive sequences. Following the assembly of pDONOR-6colors, we showcased the strength of biGMamAct through CRISPR/Cas-mediated insertion at the *ACTB* locus with high efficiency and precision. The combination of the *bona fide* sgRNA/spCas9 and HITI tandem enabled the stable integration of >18-kb functional DNA cargo at *ACTB* locus with unprecedented efficiency (100% homozygotes). This level of efficiency has not been achieved using high-end viral delivery vehicle [4], RNPs, or the well-established AAVS1 safe harbor locus [39]. The biGMamAct system, with its portfolio of shuttle plasmids, enabled the precise KI of an 18.3-kb functional DNA payload for the first time. Furthermore, the use of doxycycline-inducible promoters likely alleviated silencing barriers, which are particularly relevant for large DNA integrations. We anticipate that biGMamAct will be a promising tool for characterizing unelucidated biological processes, especially through *in situ* structural investigations [2, 53], and will unlock previously insurmountable challenges in synthetic biology for metabolic engineering in mammalian cells [21, 50, 51], similar to recent advances in prokaryotes [60] or plants [61].

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Author contributions

Martin Pelosse (Conception, Design and performing of all experiments, Writing—first and final draft of the manuscript, Approval of final draft of the manuscript) and Marco Marcia (Conception, Supervision of research, Writing—final draft of the manuscript, Approval of final draft of the manuscript).

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Data availability

All vectors used in this protocol will be available from Addgene (deposition 85144) and from the corresponding authors on request.

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