Gene delivery vehicles currently in the clinic for treatment of monogenic disorders lack sufficient carrying capacity to efficiently address complex polygenic diseases. Thus, to engineer multifaceted genetic circuits for bioengineering human cells as a therapeutic option for polygenic diseases, we require new tools that are currently in their infancy. Mammalian artificial chromosomes, or synthetic chromosomes, represent a viable approach for delivery of large genetic payloads that are mitotically stable and remain independent of the host genome. Previously, we described a mammalian synthetic chromosome platform, termed the ACE system, that requires a single unidirectional integrase for the introduction of multiple genes onto the ACE platform chromosome. In this report, we provide a proof of concept that the ACE synthetic chromosome bioengineering platform is amenable to sequential delivery of off-the-shelf large genomic fragments. Specifically, large genomic clones spanning the human solute carrier family 2, facilitated glucose transporter member 1 (SLC2A1 or GLUT1, 169 kbp), and human monocarboxylate transporter 1 (SLC16A1 or MCT1, 144 kbp) genetic loci were engineered onto the ACE platform and demonstrated to express and correctly splice both gene transcripts. Thus, the ACE system provides a facile and tractable engineering platform for the development of gene-based therapeutic agents targeting polygenic diseases.

INTRODUCTION

Progress in cell bioengineering for gene-based therapies has been held back by the absence of the one indispensable tool required to address polygenicity and/or delivery of large genetic payloads (>100 kbp): a stable, non-integrating, self-replicating, and biocompatible (i.e., mammalian) intracellular synthetic chromosome that ensures controlled expression. Synthetic chromosomes provide the breakthrough in biological bandwidth required to manage such complex polygenic challenges and introduction of large genetic payloads. Synthetic chromosomes can be generated by several means, including (1) co-transfection of defined chromosomal elements (i.e., telomere elements, centromeric alpha-satellite DNA multimers, and mammalian replication origins) along with a drug-selectable marker into a permissive cell line, which then assembles the components into an artificial chromosome (e.g., mammalian artificial chromosome [MAC]); (2) dissection of individual host cell chromosomes down to minichromosomes consisting of minimal functional centromere regions or neocentromeres by a process of targeted telomere integration and excision; (3) stable maintenance of centric fragments or small accessory chromosomes modified to accept foreign genes; and (4) generation of satellite DNA-amplified chromosomes (SATACs) by targeted amplification and fragmentation of pericentromeric sequences from acrocentric chromosomes into stably maintained chromosome vectors. The overriding principle common to all of these methods is the recapitulation of functional mammalian centromeres and telomeres in a form suitable for downstream engineering. To date, engineering of synthetic chromosomes required multi-integrase systems (Cre, PhC31, and PhBT1 recombinases) to enable multigene loading.

Previously, we have described a derivation of the SATAC methodology, termed the ACE synthetic chromosome, as a means to introduce large payloads of genetic information into the cell. The ACE system can be reproducibly generated de novo in mammalian cell lines and readily purified from the host cell’s chromosomes by flow cytometry and chromosome sorting. In turn, purified mammalian ACE synthetic chromosomes can be easily introduced into a variety of mammalian cell lines by transfection and maintained for extended periods without enforced genetic selection. The ACE system consists of a synthetic chromosome (ACE synthetic chromosome), ACE targeting vectors, and the ACE integrase. The ACE synthetic chromosome contains more than 50 copies of the 245-bp bacteriophage lambda attP site-specific recombination acceptor sequence, all of which are equal in availability. The multiple copies of the acceptor site allow the ACE synthetic chromosome to be engineered to carry single or multiple copies of genes of interest using ACE targeting vectors that contain the donor attB recombination site. The ACE integrase is a derivative of bacteriophage lambda integrase (INTR) engineered to direct site-specific, unidirectional recombination in mammalian cells. In contrast to multi-recombinase systems required for other synthetic chromosome engineering platforms, the ACE...
system requires only the single ACE integrase and ACE loading vector to engineer multiple genes onto the ACE synthetic chromosome, alleviating the need for complicated synthetic chromosome designs incorporating multiple targeting vectors, each of which require unique site-specific recombination sites and recombinases. In this report, we demonstrate successful engineering of the ACE synthetic chromosome with multiple large genetic payloads. The ACE synthetic chromosome was initially engineered to contain and express a copy of the human genomic GLUT1 (SLC2A1) locus (HgGLUT1), nearly 169 kbp in size and spanning both 5' and 3' regulatory elements, designated ACEHgGLUT1. Moreover, a second large payload, the human genomic MCT1 (SLC16A1) locus (HgMCT1; approximately 144 kbp in size), was delivered onto ACEHgGLUT1 for a combined total of over 300 kbp of genomic DNA delivered to the parent ACE synthetic chromosome. We confirmed the presence of all 10 HgGLUT1 exons and all 4 HgMCT1 exons on ACEHgGLUT1/HgMCT1 as well as expression and correct splicing of the full-length HgGLUT1 and HgMCT1 transcripts. These results provide a proof-of-principle demonstration of loading multiple large genetic payloads onto the ACE synthetic chromosome with subsequent expression of engineered gene products.

RESULTS

The ACE synthetic chromosome was engineered to contain and express a genomic copy of the human GLUT1 locus in a three-step process outlined in Figure 1. Bacterial artificial chromosomes (BAC/PACs) as well as vector controls, pBACE3.6 and pCYPAC2, were purchased from the BAC/PAC Resources Center, Children’s Hospital Oakland Research Institute (CHORI; Oakland, CA). RP11-777G20 is a 169-kbp BAC clone spanning the HgGLUT1 locus on chromosome 1 and includes 39 kbp upstream and 87 kbp downstream of the HgGLUT1 transcribed region. The sequences required for INTR recombination between the ACE’s attP site and an attB site on the BAC/PAC as well as the blasticidin resistance gene were PCR-amplified as a single product from pMONO-Blasti-mcs (InvivoGen, San Diego, CA) using the primers BacRFitFor and BacRFitRev (Table S1; Figure 1, step 1). The PCR primer BacRFitFor encodes the attB sequence. The gel-purified 1.448-kbp PCR product was incorporated into the BAC/PACs using Red/ET recombination (Red/ET Recombination Kit, Gene Bridges, Heidelberg, Germany; Figure 1, step 2). Blasticidin-resistant bacterial colonies were selected and screened for the expected Red/ET recombination junctions, as shown in Figure 2A, using the primer sets BamHIFOR and attB REV for junction 1 (Figure 2B) and SV40polyAnFOR and SacBREV for junction 2 (Figure 2C). The sequences homologous to the SacB_REV and BamH1_FOR primers reside in the BAC/PAC vector, whereas the attB site and BlasticidinR resulting from step 2 onto the ACE synthetic chromosome.

Figure 1. Genomic HgGLUT1 BAC Retrofitting and ACE Integration

The conversion of a BAC clone for loading onto a synthetic chromosome requires three steps. In the first step, the desired selectable marker gene (in this case, blasticidin resistance [BlasticidinR] from pMONO-Blasti-mcs) is PCR-amplified with primers containing homology to the BAC/PAC vector backbone (black line) and the attB sequence (orange segment). Second, the BAC is retrofitted to carry the amplified PCR product containing the attB recombination site and the selectable marker gene from step 1 using Red/ET recombination. In the third step, the λINTR recombinase is used to site-specifically load the retrofitted BAC clone containing the attB site and BlasticidinR resulting from step 2 onto the ACE synthetic chromosome.
also displayed correct junctions. These retrofitted BACs/PACs were then amenable to loading onto the ACE via the attB recombination site and subsequent selection by the blasticidin drug-selectable gene.

One RP11-777G20attBSR BAC clone (Figures 2B and 2C, lane 3) and one pBACe3.6attBSR BAC (Figures 2B and 2C, lane 5) were chosen for expansion in liquid culture to produce adequate DNA for loading onto the ACE synthetic chromosome. Each BAC was co-transfected with a plasmid expressing the lINTR integrase (pCXLamIntROK7) into Y29-13D-SFS cells, a DG44 Chinese hamster ovary (CHO)-derived cell line containing the parental ACE synthetic chromosome, to site-specifically load each BAC onto an ACE synthetic chromosome (Figure 1, step 3). Four blasticidin-resistant clones from each of four independent transfections were expanded in culture to isolate genomic DNA and screened by PCR for site-specific recombination events (attP/C2attB recombination events), resulting in attR and attL junctions, as diagrammed in Figure 3A. PCR products from the attR and attL junctions (186 bp and 229 bp, respectively) were present in five of the 16 clones screened, confirming successful engineering of the ACE synthetic chromosome with RP11-777G20attBSR at a frequency of 31.25%. Junction PCR analysis of two of the five HgGLUT1 engineered clones is shown in Figures 3B and 3C, lanes 1 and 2. Both of the ACE candidates containing RP11-777G20attBSR in Figure 3 were analyzed further and also shown to contain all ten HgGLUT1 exons by PCR analysis using HgGLUT1 exon primer sets (listed in Table S1; Figure 4A, lane 3, depicts one of the two candidates for each exon). Finally, the presence of RP11-777G20attBSR on the ACE synthetic chromosome was confirmed by fluorescence in situ hybridization (FISH) colocalization of probes hybridizing to the HgGLUT1 exons and elements of the ACE synthetic chromosome (puromycin resistance gene). Figure 5 depicts the FISH analysis of the candidate in Figures 3B and 3C, lane 2. These results demonstrate that RP11-777G20attBSR correctly recombined onto the ACE synthetic chromosome; i.e., both end junctions were correct and contained the intervening sequences (all exons were present). Furthermore, RP11-777G20attBSR was only present on the ACE synthetic chromosome. We conclude that RP11-777G20attBSR was loaded onto the ACE synthetic chromosome in its entirety. This ACE synthetic chromosome containing RP11-777G20attBSR was named ACEHgGLUT1, and the resultant cell line was designated DG44-ACEHgGLUT1.

To demonstrate that multiple genomic payloads can be delivered sequentially onto an ACE synthetic chromosome, the ACEHgGLUT1 synthetic chromosome was confirmed by fluorescence in situ hybridization (FISH) colocalization of probes hybridizing to the HgGLUT1 exons and elements of the ACE synthetic chromosome (puromycin resistance gene). Figure 5 depicts the FISH analysis of the candidate in Figures 3B and 3C, lane 2. These results demonstrate that RP11-777G20attBSR correctly recombined onto the ACE synthetic chromosome; i.e., both end junctions were correct and contained the intervening sequences (all exons were present). Furthermore, RP11-777G20attBSR was only present on the ACE synthetic chromosome. We conclude that RP11-777G20attBSR was loaded onto the ACE synthetic chromosome in its entirety. This ACE synthetic chromosome containing RP11-777G20attBSR was named ACEHgGLUT1, and the resultant cell line was designated DG44-ACEHgGLUT1.

To demonstrate that multiple genomic payloads can be delivered sequentially onto an ACE synthetic chromosome, the ACEHgGLUT1
The synthetic chromosome was engineered to contain a second large payload for a combined total of over 300 kbp of genomic DNA delivered to the ACE synthetic chromosome. A BAC clone spanning the HgMCT1 locus, RP11-1151D18, was purchased from BPRC, CHORI. RP11-1151D18 is 144 kbp and contains 52 kbp upstream and 48 kbp downstream of the HgMCT1 transcribed region, respectively, in addition to the HgMCT1 coding sequences. In a manner similar to that shown in Figure 1 for HgGLUT1, the HgMCT1 BAC and the pBACe3.6 parent vector were retrofitted by Red/ET recombination to incorporate the attB site necessary for integration onto ACEHgGLUT1 and, in this case, zeocin resistance for positive selection following loading in the DG44-ACEHgGLUT1 cell background. PCR amplification of a fragment containing the attB recombination site and a GFP-zeocin resistance gene fusion transcript was performed using linearized pSELECT-GFPzeo (InvivoGen, San Diego, CA) as a template and the PCR primers HgMCT1_RflLower and HgMCT1_RflUpper (Table S1). The attB sequence was incorporated into the HgMCT1_RflUpper primer used in the PCR amplification. Following Red/ET recombination and zeocin selection in E. coli, integration of the attB recombination site and zeocin resistance gene on the BACs was confirmed using PCR amplification with primers specific to the novel junctions formed by Red/ET recombination with the attB-GFP-zeocin construct: BamHI_For and attB_Rev for junction 1 and BetaGlo_pAnFor and 3’SacB_Rev for junction 2 (Table S1; data not shown). The retrofitted BAC candidates of RP11-1151D18attBGF_Preo were also analyzed for the presence of the four HgMCT1 exons using PCR primers specific to the HgMCT1 exons (Table S1). Figure 4B depicts one of four candidates tested for the four MCT1 exons by PCR analysis and confirms their presence.
the ten HgGLUT1 exons and incorporated the four HgMCT1 exons, indicating that the loading of the 144-kbp retrofitcd HgMCT1 BAC onto ACE_HgGLUT1 was achieved for a total payload of over 300 kbp. Engineering of this double loaded ACE, ACE_HgGLUT1/HgMCT1, successfully demonstrates the ability to load multiple large DNA fragments, each greater than 100 kbp in size, onto a single ACE.

To further confirm the presence of both HgGLUT1 and HgMCT1 on the ACE synthetic chromosome of DG44-ACE_HgGLUT1/HgMCT1, FISH was used to assess the localization of the two genomic BAC sequences. Probes to HgMCT1 and HgGLUT1 were applied to metaphase spreads of the DG44-ACE_HgGLUT1/HgMCT1 cell line. The colocalization of FISH probe signals for the HgGLUT1 and HgMCT1 genes to a single chromosome, shown in Figure 7, further demonstrates that dual loading was accomplished. These results indicate that the capacity of the ACE synthetic chromosome exceeds 300 kbp, although its upper capacity limit remains to be determined.

To demonstrate correct expression from the HgGLUT1 and HgMCT1 loci on the engineered ACE, total RNA was isolated from DG44-ACE_HgGLUT1/HgMCT1 and Y29-13D-SFS, the parental ACE synthetic chromosome-containing cell line, and used to prepare cDNA. This cDNA was used as template in two PCR reactions to amplify the complete coding sequence from both HgGLUT1 and HgMCT1 using primers homologous to the 5' and 3' UTRs (HgGLUT1: GLUT1_xpt_For, GLUT1_xpt_Rev; HgMCT1: MCT1_xpt_For, MCT1_xpt_Rev; Table S1). PCR products corresponding to the correct sizes (1.667 kbp and 1.833 kbp, respectively) were obtained from DG44-ACE_HgGLUT1/HgMCT1 (Figure 8), demonstrating correct splicing of the HgGLUT1 and HgMCT1 transcripts. The PCR products amplified with PrimeStar, a high-fidelity DNA polymerase, were sequenced (HgGLUT1: hSLC2A1_Ex5_F, hSLC2A1_Ex5_R, hSLC2A1_Ex8_F, hSLC2A1_Ex8_R; HgMCT1: hSLC16A1_Ex2_For, hSLC16A1_Ex2_Rev, hSLC16A1_Ex3_For, hSLC16A1_Ex3_Rev; Table S1, see footnote) and shown to contain wild-type HgGLUT1 and HgMCT1 sequences. The HgMCT1 sequence contained the common T1470A SNP (rs1049434).12,13 These results confirm that neither gene acquired mutations during the engineering process and that both were properly spliced from ACE_HgGLUT1/HgMCT1. Taken together, these results demonstrate that both RP11-777G20 and RP11-1151D18 retained their original genomic organization throughout the engineering process.

DISCUSSION
Cell and gene therapy research directed toward monogenic disorders and select cancers have resulted in ascertainable, curative therapeutic endpoints.14–16 However, based on historical research in human genetic diseases and codified by the information gleaned from the Human Genome Project, it is apparent that multifactorial (polygenic and environmentally influenced) disorders are much more frequent than monogenic disorders.17 Although the viral and non-viral delivery vehicles utilized to treat monogenic disorders have provided a valuable engineering blueprint for gene- and cell-based therapies, these tools
systems incorporating internal ribosome entry site (IRES)\textsuperscript{19,20} elements or self-cleaving peptides\textsuperscript{21,22} have been utilized to build combinatorial arrays of multigene constructs.\textsuperscript{3,23–25} Although these systems are capable of producing a polycistronic mRNA, their utility is limited to the production of only a few proteins under the control of synthetic promoters. Furthermore, the delivery of these combinatorial arrays is reliant on current viral and non-viral delivery vehicles with their associated limitation of payload capacity and, in the case of integrating viral vectors, the liability of insertional mutagenesis.

In this report, we demonstrate for the first time that the ACE platform chromosome can be sequentially engineered with extra-large genomic fragments in a predictable manner as a first step toward the construction of complex synthetic gene circuits incorporating native regulatory elements. For this, the ACE chromosome was engineered to contain a genomic copy of HgGLUT1, approximately 169 kbp in size, including 3' and 3' regulatory regions with a loading frequency of 31.25%. Moreover, a second large payload, HgMCT1, was delivered onto ACEHgGLUT1 for a combined total of over 300 kbp of genomic DNA bioengineered onto the ACE synthetic chromosome, with a loading efficiency of 16.67%. In previous work, we demonstrated a loading efficiency of 100% for a 5-kbp ACE targeting vector. Here the loading vectors were more than 100 kbp in size; thus, the stoichiometry between the size of the DNA fragment and attB sites is significantly different. Future work will focus on determining the factors that affect loading efficiency. However, the efficiencies demonstrated here are easily managed for screening to identify clones with correct integration junctions. Any clones not meeting integration junction screening criteria were not analyzed further. We confirmed the presence of all HgGLUT1 (10) and HgMCT1 (4) exons on the ACEHgGLUT1/HgMCT1 synthetic chromosome by PCR as well as expression of the hGLUT1 and hMCT1 transcripts. These results provide a proof-of-principle demonstration of loading multiple large genetic payloads onto the ACE synthetic chromosome with subsequent expression of engineered gene products. Importantly, both large payloads retained their organization, and neither acquired mutations during the engineering process. In addition, loading of the HgMCT1 BAC clone introduced an allele of the HgMCT1 locus (A1470T) associated with endurance and blood lactate levels after intensive exercise.\textsuperscript{12,13,26}

The ACE engineering system provides a tractable engineering platform for transferring large (>100 kbp) genetic payloads with native genetic architecture onto a synthetic chromosome. Although other MAC platforms have been developed for multiple loadings onto a synthetic mammalian chromosome,\textsuperscript{2,4,6,27,28} these previously described platforms required the creation of multiple vectors with different site-specific recombination systems to achieve multiple loadings and have not reached the engineerable carrying capacity demonstrated here. For example, Honma et al.\textsuperscript{28} have demonstrated loading of genomic DNA up to 39 kbp in size. Alternatively, Hoshiya et al.\textsuperscript{27} have used homologous recombination-proficient chicken DT40 cells to engineer a human chromosome limited to the Duchenne muscular dystrophy locus (2.4 Mbp) by repeated


**Figure 5. HgGLUT1 BAC Colocalizes to the ACE**

Shown is fluorescent in situ hybridization analysis of the ACE synthetic chromosome engineered with the retrofitted HgGLUT1 BAC clone RP11-777G20\textsuperscript{osiris} in the DG44 engineering cell line (DAPI). Hybridization probes were generated to the puromycin resistance gene (PuroR; Alexa Fluor 488 signal) and HgGLUT1 BAC, RP11-777G20\textsuperscript{osiris} (HgGLUT1; Alexa Fluor 594 signal). Composite is the overlay of the three panels, indicating colocalization of the HgGLUT1 BAC clone introduced an allele of the HgGLUT1 locus (10) and HgMCT1 (4) exons on the ACEHgGLUT1/HgMCT1 synthetic chromosome by PCR as well as expression of the hGLUT1 and hMCT1 transcripts. These results provide a proof-of-principle demonstration of loading multiple large genetic payloads onto the ACE synthetic chromosome with subsequent expression of engineered gene products. Importantly, both large payloads retained their organization, and neither acquired mutations during the engineering process. In addition, loading of the HgMCT1 BAC clone introduced an allele of the HgMCT1 locus (A1470T) associated with endurance and blood lactate levels after intensive exercise.\textsuperscript{12,13,26}

are insufficient to address complex, polygenic diseases because of the limited amount of genetic payload that can be delivered as well as the need to find “safe” genomic sites of integration to maintain stability.\textsuperscript{18}

For coordinated production and expression of multiple proteins directed to the treatment of polygenic disorders, polycistronic vector

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truncation of the chromosome. In the approach presented here, the use of a single uni-directional, site-specific ACE integrase system mitigates the need for an admixture of multiple recombination and/or integration systems. Because the attP recombination acceptor site is 245 bp, the presence of a pseudosite in the human genome is less probable than recombination systems using minimal attP sites (e.g., Fc31 minimal attP sites have over 100 pseudosites in the human genome30). Furthermore, a BLAST search of the human, mouse, and CHO genomes with the 245-bp attP site returned no matches. Thus, the probability of off-target integration events is extremely unlikely. Furthermore, the ability to flow-sort-purify ACE chromosomes to high purity allows the subsequent transfer of engineered ACE chromosomes into a variety of cell types, circumventing the need for microcell transfer methodology. Finally, by engineering the ACE in the host DG44 cell background prior to flow sorting and transfer to the recipient (e.g., patient) cell background, any off-target effects and resulting toxicities are nullified. The upper limit of the capacity of the ACE platform, in both numbers of sequential loadings as well as final combined payload, remains to be tested.

MACs circumvent many of the limitations associated with plasmid- and viral-based gene expression systems and provide an alternative means to introduce large payloads of genetic information into cells without influencing the performance of the therapeutic components necessary for the development of a multi-therapeutic approach to target complex genetic disease. In addition, MACs provide a genetic focal point by which unlinked mammalian genetic loci can be brought into linkage disequilibrium. Here we demonstrate that the ACE platform chromosome provides a predictable designer therapeutic approach for efficient, tractable bioengineering of large genomic fragments onto a portable chromosome platform as a tool to address polygenic disorders.

MATERIALS AND METHODS

BAC Retrofitting with Red/ET Recombination

BACs were retrofitted to include an attB recombination site and a drug resistance gene cassette within the BAC vector backbone for site-specific loading onto the synthetic chromosome and drug selection as diagrammed in Figure 1, steps 1 and 2. RP11-1151D18 (144 kbp) was retrofitted with the blasticidin resistance gene (BSR), amplified from NotI-linearized pMONO-blasti-mcs, using primers containing BAC homology, loxP511, and attB sequences (primers BACRFitFor and BACRFitRev; Table S1). The zeocin resistance gene used to retrofit RP11-1151D18 was amplified from PacI linearized pSELECT-GFPzeo using primers containing BAC homology, loxP511, and attB sequences (primers HgMCT1_RfitUpper and HgMCT1_RfitLower;
Gene Bridges

Red/ET recombination.

This was used to transform each BAC-containing strain with the Gene Bridges electroporation method. K001 was used according to the manufacturer's protocol and then used to retro-transform the E. coli strain to express the Red/ET recombination proteins. The 1.77-kbp PCR product was gel-purifi ed (Promega, Madison, WI; Wizard SV Gel and PCR Clean-Up System) according to the manufacturer's protocol and then used to retro-transform RP11-1151D18 as outlined below using Red/ET recombination.

Table S1. A two-step PCR reaction was performed with 3 cycles at the initial annealing temperature (Ta; 60°C for pMONO-blasti-mcs and 59°C for pSELECT-GFPzeo) and 32 cycles at the final Ta (68°C for pMONO-blasti-mcs and 72°C for pSELECT-GFPzeo) using Phusion High Fidelity Polymerase (New England Biolabs). The 1.448-kbp attB-IREBSR PCR product was gel-purified (QIAGEN QIAEX II Gel Extraction Kit; Hilden, Germany) according to the manufacturer's protocol and then used to amplify RP11-777G20 as outlined below using Red/ET recombination. The 1.77-kbp attB-GFPzeo PCR product was gel-purified (Promega, Madison, WI; Wizard SV Gel and PCR Clean-Up System) according to the manufacturer's protocol and then used to retro-transform RP11-1151D18 as outlined below using Red/ET recombination.

Figure 8. HgGLUT1 and HgMCT1 Expression from the ACE Synthetic Chromosome

cDNA prepared from the DG44 cell line containing the unaltered ACE (1) or the DG44-ACEHgGLUT1/HgMCT1 cells (2) was used as template in PCR reactions using specific primer sets for HgGLUT1 or HgMCT1 that span each gene from the respective 5' UTR to the 3' UTR. Primers are listed in Table S1. HgGLUT1 and HgMCT1 products of the correct size were detected in the DG44-ACEHgGLUT1/HgMCT1 cDNA template but not in the parental Y29-13D-SFS cDNA template. These results indicate expression of both human HgGLUT1 and HgMCT1 transcripts from the ACE synthetic chromosome.

PCR Confirmation of Red/ET Recombination Junctions

Proper Red/ET recombination junctions were confirmed by colony PCR using Promega (Madison, WI) 2X GoTaq Master Mix with the following primer sets at the designated Ta for 35 cycles: RP11-777G20attBSR, 5' junction (BamHIFor + attBRev, Ta = 57°C) and 3' junction (SV40polyAnFor + 3'SacBREV, Ta = 53°C); RP11-1151D18attBFPzeo, 5' junction (BamHIFor + attBRev, Ta = 57°C) and 3' junction (BetaGlopAnFor + 3'SacBREV, Ta = 57°C). Primers are listed in Table S1. PCR was carried out in a Bio-Rad T100 PCR system.

Figure 7. Fluorescence In Situ Hybridization Analysis of DG44-ACEHgGLUT1 Cells Engineered with the Retrofitted HgMCT1 BAC RP11-1151D18attBSGFPzeo

Shown is mitotic spread of the DG44-ACEHgGLUT1/HgMCT1 cell line (DAPI). Hybridization probes were generated to the HgMCT1 (exons; Alexa Fluor 488 signal) and the HgGLUT1 BAC RP11-777G20attBSGFPzeo (HgGLUT1; Alexa Fluor 594 signal). Composite is the overlay of the three panels, indicating colocalization of HgGLUT1 with HgMCT1. The inset is a magnified view of the colocalization of the signals. Two-color hybridization and signal capture via fluorescence microscopy were performed as described previously. 2 Scale bar, 10 μm.
Thermocycler using the following conditions: step 1: 95°C for 2 min; step 2: cycles 1–35, 95°C for 30 s, Ta for 30 s, and 72°C for 25–30 s; step 3: 72°C for 2 min. Correctly retrofitted BACs were expanded in LB liquid culture supplemented with 12.5 μg/mL chloramphenicol and either 25 μg/mL blasticidin for RP11-777G20/BacNeo or 25 μg/mL zeocin for RP11-1151D18/BacNeo. BAC DNA was isolated using a Sigma-Aldrich (St. Louis, MO) PhasePrep BAC DNA Kit for use in ACE loading (Figure 1, step 3).

Cell Culture and Transfections

Cells were maintained in a 37°C incubator in the presence of 5% CO2, Y29-13D-SFS is a DG44 CHO-derived cell line containing the parental ACE synthetic chromosome provided by Calyx Bio-Ventures (https://calyxbio.com/). This cell line and cell lines derived thereof were grown in Gibco MEM Alpha medium with nucleosides and glutamine (Gibco catalog number 12571-063; Thermo Fisher Scientific, Waltham, MA) supplemented with 5% HyClone FetalClone III serum (GE Healthcare, Chicago, IL), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Corning, Corning, NY; 100× penicillin and streptomycin), subsequently referred to as “complete MEM Alpha” cell culture medium. Complete MEM Alpha culture medium was also supplemented with the appropriate antibiotics as required for ACE synthetic chromosome selection: Y29-13D-SFS, 5 μg/mL puromycin (InvivoGen, San Diego, CA); DG44-AcpHglut1, 5 μg/mL puromycin and 3 μg/mL blasticidin (InvivoGen, San Diego, CA); DG44-ACEHglut1/Hmct1, 5 μg/mL puromycin and 500 μg/mL zeocin (InvivoGen, San Diego, CA).

For targeted integration onto the ACE synthetic chromosome in the DG44 cell line, the cells were plated at a density of 500 to 1,000 cells per well of a 24-well culture dish (so that the cell density reaches 50%–80% confluency by the time of transfection) 1 day prior to transfection with Lipofectamine LTX with Plus Reagent (Invitrogen, Carlsbad, CA). On the day of transfection, the medium was aspirated from the cells and replaced with antibiotic-free Alpha MEM medium supplemented with 5% HyClone FetalClone III. For each well transfected, 0.5 μg of BAC DNA and 0.2 μg of the ACE Integrase expression vector, pCXLamIntROK,7 were complexed with 1 μl PLUS reagent in 100 μl Opti-MEM reduced serum medium (Gibco, Thermo Fisher Scientific, Waltham, MA), followed by addition of 3 μl of Lipofectamine LTX, and the complex was incubated with cells according to the manufacturer’s recommendations. Each transfected well was subsequently expanded to a 10-cm2 culture dish 24 h post-transfection. Selection medium containing 3 μg/mL blasticidin plus 5 μg/mL puromycin or 250 μg/mL zeocin plus 5 μg/mL puromycin (depending on the version of BAC used) was added the following day (i.e., 48 h post-transfection), and the medium was changed every 2–3 days thereafter. After 14 days of incubation under selection, drug-resistant colonies were harvested via a cloning ring and further expanded for analysis.

PCR for Assessment of Loading a BAC onto the ACE

To assess proper λINTR recombination junctions (attL and attR), genomic DNA was prepared from 50,000 to 200,000 cells of each candidate cell line using either the Promega (Madison, WI) Wizard SV Genomic DNA Purification System or the QIAGEN DNeasy Blood and Tissue Kit (Hilden, Germany) for use in the PCR assay. A 10 μL PCR reaction mix consisting of 1× GoTaq Green Master Mix (Promega, Madison, WI), 0.25 μM each forward and reverse primers, and 20–100 ng template was used to analyze the junctions of candidate cell lines following the targeted integration event. The primer sets and Ta used to screen the recombination junctions are listed in Table S1. PCR was carried out using the following conditions in a Bio-Rad (Hercules, CA) T100 Thermocycler: step 1: 95°C for 3 min; step 2, cycles 1–35, 95°C for 30 s, Ta°C for 30 s and 72°C for 30 s; step 3: 72°C for 2 min.

PCR was used to assess the presence of the exons, associated with the targeted integration event, in a 10 μL reaction mix consisting of 1× GoTaq Green Master Mix (Promega, Madison, WI), 0.5 μM each forward and reverse primers, and 50–150 ng template. The primer sets and Ta used for each exon are listed in Table S1. For the HgGlut1 exons, PCR was carried out using the following conditions in a Bio-Rad (Hercules, CA) T100 Thermocycler: step 1: 95°C for 2 min; step 2: cycles 1–40, 95°C for 30 s, Ta°C for 30 s, and 72°C for 20 s; step 3: 72°C for 5 min. For the Hmct1 exons, PCR was carried out using the following conditions (for Bio-Rad (Hercules, CA) T100 Thermocycler: step 1: 98°C for 5 min; step 2: cycles 1–40, 95°C for 30 s, Ta°C for 15 s, and 72°C for 30 s; step 3: 72°C for 5 min.

FISH

Conventional single-color and two-color FISH analyses and high-resolution FISH were carried out using PCR-generated probes from HgGlut1 exons 1–10, pPURattP, and Hmct1 exons 1–4 as follows.

For PCR generation of FISH probes, the following 25-μL reaction mix was used: 20 pg linearized DNA template (RP11-777G20 for HgGlut1, RP11-1151D18 for Hmct1, or pPURattP for the ACE); 1× Taq ThermoPol buffer (NEB); 200 μM each of dATP, dCTP, and dGTP; 130 μM of dTTP, 70 μM biotin-16-2'-deoxyuridine 5’-triphosphate (dUTP) or DIG-11-dUTP (Roche Biosciences); 200 nM forward and reverse primers; and 4.5 U of Taq DNA polymerase (NEB). The primer sets and Ta for amplifying FISH probes are listed in Table S1. PCR was carried out using the following conditions: step 1: 95°C for 2 min; step 2: cycles 1–35, 95°C for 30 s, Ta°C for 30 s, and 72°C for 20 s; step 3: 72°C for 10 min. The extension time was increased to 1 min to produce the 618 bp product from the pPURattP template. Following PCR production of a probe, unincorporated nucleotides were removed using a QIAquick PCR Cleanup Kit (QIAGEN) or Monarch PCR & DNA Cleanup Kit (NEB).

Actively dividing cells were treated with 10 μg/mL Karyomax for 10–18 h for metaphase arrest and then harvested by centrifugation at 200 relative centrifugal force (RCF) for 7 min in preparation for metaphase spreads. Pelleted cells were resuspended in PBS, pelleted by centrifugation at 200 RCF (7 min), resuspended in 5–10 mL of 75 mM KCl, and incubated at 37°C for 4 min. The swollen cells
were then collected by centrifugation at 100 RCF, KCl was removed, and the pellet was resuspended in 5 mL cold fixative (3:1 methanol: acetic acid). Fixed cells were collected again by centrifugation at 100 RCF, the fixative was removed, and the pellet was resuspended in fresh cold fixative and stored at –20°C. For metaphase spreads, 30 μL of cells in fixative were dropped to an angled (~45°) cold glass slide prewet with fixative. The slide was then placed in a humidified chamber for 1–3 min and air-dried for 10 min. Finally, the slides were “aged” by placing them in a 65°C slide chamber (MJ Research) overnight prior to initiating probe hybridization.

Hybridization and detection of sequence-specific DNA probes complementary to the ACE backbone and/or ΔNTR targeted genomic sequences were performed as follows. Prepared slides containing metaphase spreads were treated for 20 min with 10 μg/mL RNaseA at 37°C and then rinsed twice for 2 min each time in PBS at room temperature (RT). The slides were then dehydrated through a series of ethanol solutions (70%, 85%, and 100%) for 2 min each at RT, followed by denaturation for 2 min at 70°C in 70% formamide/2× saline sodium citrate (SSC). Finally, the slides were again denatured in a cold ethanol series (70%, 85%, and 100%) for 2 min each and air-dried prior to applying the probe.

Approximately 100 ng of each labeled probe (HgGLUT1 (digoxigenin; detected with Alexa Fluor 595 nm) plus either pPUR/RattP or HgMCT1 [biotin; detected with Alexa Fluor 488 nm]) was applied to metaphase spreads on glass slides, which were then coverslipped with DAPI was applied (Vector Laboratories, Burlingame, CA; catalog number H1200). The coverslip was applied, and the slide was air-dried (~10 min). 30 μL Vectashield Antifade Mounting Medium with DAPI was applied (Vector Laboratories, Burlingame, CA; catalog number H1200). The coverslip was applied, and the slide was stored at 4°C. Slides were viewed with a Nikon 80i fluorescence microscope, and images were acquired with Nikon Elements BR imaging software on a Nikon DS-Qi1 camera.

RT-PCR and Sequencing
Total RNA was isolated (RNasy Mini Kit, QIAGEN) from Y29-13D-SFS control cells, a DG44 CHO-derived cell line containing the ACE synthetic chromosome, and the DG44-ACEHgGLUT1HgMCT1 cell line containing the HgGLUT1 and HgMCT1 loci. First-strand cDNA was prepared according to the manufacturer’s protocol using the Applied Biosystems (Foster City, CA) High Capacity cDNA Reverse Transcription Kit with random primers or the LunaScript RT SuperMix Kit (NEB, Waltham, MA) with random hexamer and poly-dT primers following the manufacturers’ recommendations. Gene-specific PCR products were amplified from the cDNA with gene-specific primers to HgGLUT1 (1.667 kbp) spanning from the 5’ UTR through exon 10 to the 3’ UTR (GLUT1_For and GLUT1_Rev; Table S1) or to HgMCT1 (1.833 kbp) spanning from the 5’ UTR through exon 4 to the 3’ UTR (MCT1_For and MCT1_Rev; Table S1) using 1 μL of first-strand cDNA template. For confirmation of the expected PCR product size from correctly spliced transcripts, Promega GoTag DNA polymerase was used with an Ta of 55°C and the following cycling conditions: step 1: 95°C, 2 min; step 2: 35 cycles (95°C, 30 s; 55°C, 30 s, 72°C, 2 min); step 3: 72°C, 5 min. PrimeStar HS DNA Polymerase (Clontech, Mountain View, CA) was used to amplify the transcripts for sequence analysis. MCT1 amplification required 35 cycles (98°C for 10 s, 55°C for 5 s, 72°C for 2 min), whereas GLUT1 amplification required only 30 cycles (98°C for 10 s; 55°C for 5 s; 72°C for 2 min) to produce sufficient product for gel purification. The resulting PrimeStar HS Polymerase PCR products were assessed by gel electrophoresis for product presence and size, gel-purified using the NEB (Ipswich, MA) Monarch Gel Purification Kit, and sequenced (University of Texas Austin DNA Sequencing Facility, Austin, TX) using the primers indicated in Table S1 (see footnotes a and b).

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.04.006.
AUTHOR CONTRIBUTIONS
Conceptualization, A.G. and E.P.; Methodology, A.G. and E.P.; Validation, A.G., E.P., D.B., and K.P.; Formal Analysis, A.G., E.P., D.B., and K.P.; Investigation, A.G., E.P., D.B., and K.P.; Writing – Original Draft, A.G., E.P., and D.B.; Writing – Review & Editing, A.G., E.P., D.B., and K.P.; Visualization, A.G., E.P., D.B., and K.P.; Supervision, A.G. and E.P.; Project Administration, A.G. and E.P.; Funding Acquisition, A.G. and E.P.

CONFLICTS OF INTEREST
A.G. and E.P. each own over 10% interest in SynPloid Biotek, LLC.

ACKNOWLEDGMENTS
The authors would like to thank the following people for help with genomic DNA preparation and PCR screening of recombination junctions: Maria Stathopoulos, Annie Mejaes, Brittain Thompson, and Sara Pinkerton. This work was supported by NIH funding (ACes) to contain \\

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