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Baculovirus expression system for heterologous multiprotein complexes

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The discovery of large multiprotein complexes in cells has increased the demand for improved heterologous protein production techniques to study their molecular structure and function. Here we describe MultiBac, a simple and versatile system for generating recombinant baculovirus DNA to express protein complexes comprising many subunits. Our method uses transfer vectors containing a multiplication module that can be nested to facilitate assembly of polycistronic expression cassettes, thereby minimizing requirements for unique restriction sites. The transfer vectors access a modified baculovirus DNA through Cre-loxP site-specific recombination or Tn7 transposition. This baculovirus has improved protein expression characteristics because specific viral genes have been eliminated. Gene insertion reactions are carried out in *Escherichia coli* either sequentially or concurrently in a rapid, one-step procedure. Our system is useful for both recombinant multiprotein production and multigene transfer applications.

The identification of novel multiprotein cellular complexes has accelerated considerably as a consequence of genome-wide analysis of protein-protein interactions[1,2], powerful multiple-affinity protein purification methods[3,4] and ultrasensitive mass spectrometry[5,6]. Based on extensive two-hybrid searches, the average number of interaction partners for any given protein is estimated in baker's yeast to be five to eight[7–9]. The concept of the cell as a collection of multicomponent protein machines, one for essentially every major process, has thereby emerged[10]. This poses formidable challenges for protein production technologies aimed at molecular level structure and function studies of eukaryotic multiprotein complexes with intracellular quantities refractory to large-scale extraction from source.

Components of multiprotein complexes often display low solubility and activity in isolation, necessitating recombinant technologies that permit simultaneous expression of several genes. Consequently, considerable effort has been invested in generating polycistronic vectors carrying multiple expression cassettes. Recently, a polycistronic vector has been used for expression of a transcription factor complex composed of four subunits in *E. coli*[11]. Moreover, polycistronic vectors have been recognized as useful for therapeutic gene delivery[12,13]. DNA constructs used in these studies were generated by conventional methods using endonucleases and ligase. Protein complexes in eukaryotes, however, often contain ten or more subunits with individual polypeptides ranging in size up to several hundred kDa, severely restricting the applicability of conventional cloning strategies and largely ruling out *E. coli* as an expression host.

Recombinant baculoviruses are particularly attractive for high-level production of large eukaryotic protein assemblies[14]. Because of the flexibility of the *Autographa californica* nuclear polyhedrosis virus (AcNPV) envelope, large DNA insertions can be accommodated in its 130 kb dsDNA genome. Genes driven by AcNPV late promoters are typically abundantly expressed, as well as authentically processed and targeted in insect cells, including those encoding architecturally complex particles such as capsid structures[15]. Expression of several genes in one cell can be achieved by coinfection with several viruses, each carrying a single foreign gene. However, the logistical demand of maintaining many viruses at known titers and defined relative expression levels renders reproducible large-scale multiprotein complex production nearly impossible. In contrast, infection with one baculovirus containing all heterologous genes of choice greatly simplifies virus handling, leads to reproducible expression and has been shown to result in up to a 30-fold higher protein yield as compared to multiple single-gene viruses[15,16].

Traditionally, recombinant baculovirus generation is carried out in two steps. Foreign genes are first cloned into a small transfer vector propagated in *E. coli* and then inserted into the baculovirus genome by homologous recombination in insect cells yielding 30–80% recombinant progeny[14]. This procedure was simplified considerably by the introduction of a shuttle bacmid (bMON14272) propagated in *E. coli* containing the Tn7 attachment site from a transfer vector (Bac-to-Bac; Invitrogen) for transposition of foreign genes[17,18]. A bicistronic transfer vector, pFastBacDUAL, which contains the polyhedrin (polh) and p10 late viral promoters in two separate cassettes with restriction sites for sequentially subcloning two foreign genes for coexpression, was also introduced[18].

For production of eukaryotic multiprotein complexes containing large subunits, we recognized the need for transfer vectors permitting assembly of genes in a nonsequential, modular manner in order to alleviate the requirement for compatible restriction sites occurring in a collection of long coding sequences. Therefore, we constructed transfer vectors pUCDM and pFBDM featuring a multiplication module between two expression cassettes driven by polh and p10 late viral promoters (**Fig. 1a**). The logic of adding genes proceeds in two steps. First, the bicistronic expression cassette containing genes of interest can

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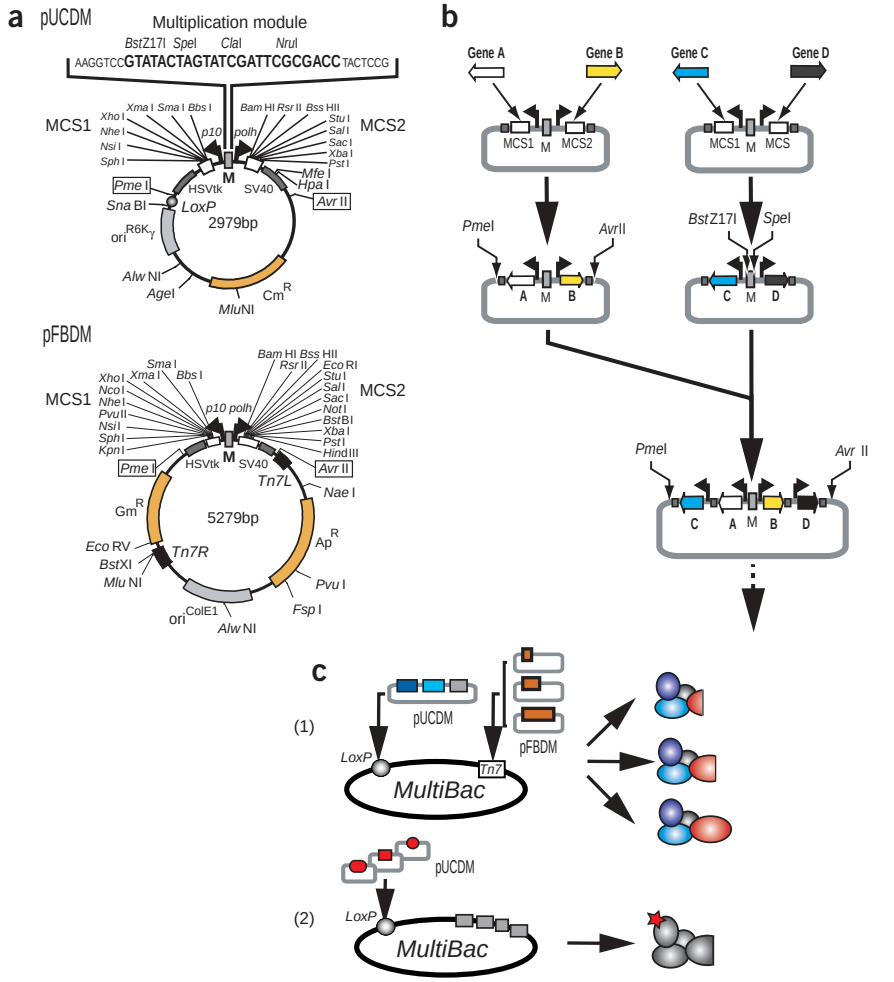

Figure 1 Assembly of polycistronic vectors with the MultiBac system. (**a**) Vectors pUCDM and pFBDM contain expression cassettes comprising a central multiplication module (M), promoters (polh, p10), multiple cloning sites (MCS1, MCS2), and terminators (SV40, HSVtk) flanked by unique *Pme*I and *Avr*II (boxed) endonuclease sites. pUCDM also contains the inverted repeat for Cre-loxP site-specific recombination (*LoxP*) and a resistance marker for chloramphenicol. pFBDM contains transposon elements (*Tn7L*, *Tn7R*) and resistance markers for ampicillin and gentamycin. Genes of interest are cloned into MCS1 or MCS2. (**b**) Derivatives of pUCDM or pFBDM containing multigene expression cassettes are assembled by excising the expression cassette containing two genes (Gene A, Gene B) using *Pme*I and *Avr*II digestion, and then inserting the fragment generated into another cassette containing more genes (Gene C, Gene D) via the *Bst*Z17I/*Spe*I or the *Nru*I/*Spe*I sites (both pairs *Pme*I/*Avr*II compatible) present in the multiplication module (M). The process is iterative. (**c**) MultiBac is adaptable to combinatorial applications of protein production. Example (1) shows multiple genes encoding for protein subunits cloned in pUCDM using the multiplication module and inserted into MultiBac bacmid by Cre-loxP site-specific recombination. In the same reaction, genes cloned singly in pFBDM encoding for a series of truncation variants of an additional protein are inserted into the bacmid via Tn7 transposition. Example (2) shows different enzymes cloned singly in pUCDM for insertion at *LoxP* with the purpose of modifying the proteins already expressed from *Tn7*.

be excised by digestion with unique endonucleases outside the gene terminators (**Fig. 1b**). Second, this cassette is inserted via compatible sites into the multiplication module of a pUCDM or pFBDM derivative containing further genes of choice. The restriction sites used for integration are eliminated in this process, and multiplication can be repeated iteratively using the new multiplication module in the inserted cassette. This modular strategy for combinatorial assembly is carried to the level of the baculovirus as well by creation of bacmid MultiBac with independent integration sites for the pUCDM and pFBDM multiplied cassettes (**Fig. 1c**). The Tn7 transposition site (*Tn7*) is used for pFBDM transfers, and we have incorporated a Cre-loxP site-specific recombination site (*LoxP*) for pUCDM transfers. We chose the Cre-loxP system for the second integration site as it is well-established for the effective construction of recombinant DNA without using endonucleases[19]. pUCDM contains a replication origin derived from R6Kγ, making its propagation dependent on a host expressing the *pir* gene[20]. pUCDM also carries the chloramphenicol resistance marker. Therefore, propagation of a bacmid in *pir*-negative cells in the presence of chloramphenicol is entirely dependent on successful Cre-loxP-mediated integration of pUCDM. The pUCDM and pFBDM cassette transfers into bacmid MultiBac can be done concurrently or in separate steps, facilitating different applications (**Fig. 1c**).

We validated our system by coexpressing multiple genes encoding the chromatin remodeling complexes, yeast Isw2 and Isw1b, and a transcription factor subcomplex of human TFIID. These complexes comprise two, three and five proteins, respectively. Viruses for each complex were prepared with all genes for proteins of a complex integrated in *Tn7*, and with or without a polh-driven enhanced yellow fluorescent protein (EYFP) gene in *LoxP* (**Fig. 2a**). For comparison, viruses were also made with only EYFP in *LoxP*, with the two subunits of Isw2 integrated separately in *Tn7* and *LoxP*, and with the TATA box–binding protein associated factor (TAF) protein genes of the TFIID subcomplex split three and two between *Tn7* and *LoxP*. Both viruses for Isw2 expression, with recombinant genes at the same site or apart, resulted in similar yields of purified, active complex (**Fig. 2a**), demonstrating that the gene arrangement in the bacmid does not dramatically affect relative production of subunits or their assembly. The EYFP gene was used to monitor the effect of expression from multiple promoters on protein yield. The level of EYFP was virtually constant at 6–8 mg/l over all the viruses containing it. Protein production for the complexes remained constant irrespective of EYFP expression (**Fig. 2a**). These results indicate that expression is not yet saturating, and suggest that still larger complexes can be expressed without reduced production. In cases where the yield of a particular protein is limiting, multiple gene copies can be introduced. We find a linear increase in fluorescent protein expression with gene copy number (**Supplementary Note** online).

During production of Isw2 using the Bac-to-Bac system, we observed by Edman analysis viral-dependent proteolytic breakdown

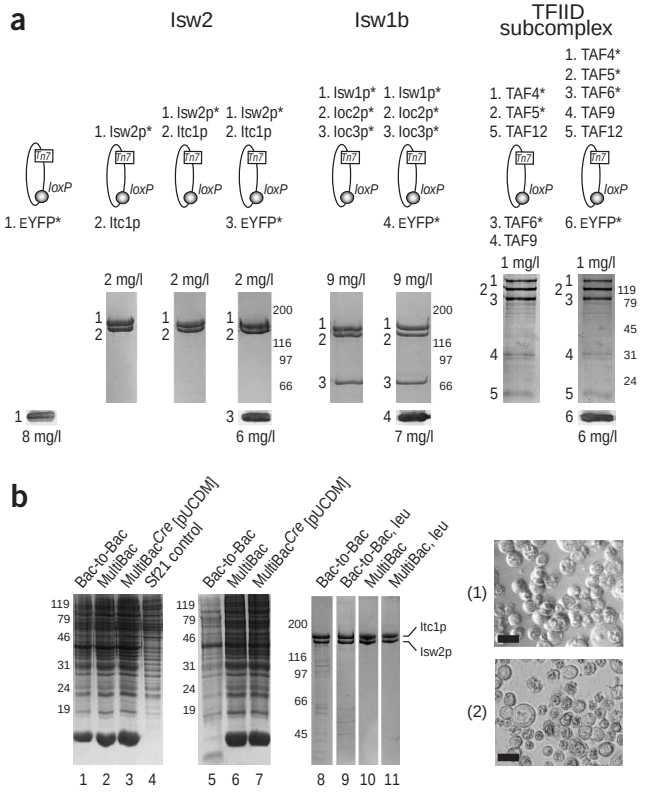

Figure 2 Expression of multiprotein complexes. (**a**) Recombinant protein production using bacmid MultiBac is shown for three different multiprotein complexes: yeast Isw2 (2 subunits, 275 kDa), yeast Isw1b (3 subunits, 300 kDa), and a subcomplex of human TFIID (5 subunits, 331 kDa). Additionally, EYFP was expressed alone and in conjunction with each complex. The genes integrated in *LoxP* and *Tn7* are shown above the Coomassie-stained, SDS gel sections with bands indicating the protein components after expression and purification of the individual complexes. Genes were expressed from polh (*) or p10 promoters. The gel band numbering corresponds to the genes listed above. Approximate yields for each purified complex are indicated above the gel sections as estimated by Bio-Rad protein assay of the purified complexes. EYFP expression is shown by western blot, and approximate yields indicated below are estimated from absorbance measurements (A_{513}) of cell lysates. Molecular weights in kDa shown to the right of gel sections are based on marker lanes not shown. (**b**) Stabilities of proteins are compared for Sf21 cells transfected with bacmids Bac-to-Bac, MultiBac, and MultiBac with pUCDM inserted (lanes 1–3). Viral-dependent protein expression is evident in all infected cells compared to uninfected cells (lane 4) at 48 h after infection as visualized by Coomassie-stained, SDS gels of whole cell extracts. The same extracts (lanes 5–7) incubated for 96 h at 37 °C show the positive effect of the *chiA* and *v-cath* deletions in bacmid MultiBac as compared to the presence of these genes in Bac-to-Bac. Yeast Isw2 complex was purified at 4 °C using Bac-to-Bac or MultiBac, respectively, revealing substantial degradation of the complex in the case of Bac-to-Bac (lane 8) in comparison to that for MultiBac (lane10). Addition of the protease inhibitor leupeptin (leu) to 50 μM reduced protein degradation in the Bac-to-Bac sample (lane 9), but not to the level achieved with MultiBac in the presence or absence of leupeptin (lanes 10 and 11). Micrographs (1) and (2) show cells 72 h after infection with bacmids MultiBac and Bac-to-Bac , respectively. MultiBac-infected cells are uniformly round and appear intact in contrast to the cell lysis prevalent for the Bac-to-Bac–infected cells. Scale bars, 20 μm.

consistent with the action of a cysteine protease. The baculovirus *v-cath* gene encodes such a protease directly involved in liquefaction of the insect cell host[21], and its deletion has been shown to improve protein production from a *Bombyx mori* polyhedrosis virus[22]. V-CATH is activated upon cell death by a process dependent on *chiA*, a juxtaposed gene encoding for a chitinase[23]. We disrupted both genes to eliminate V-CATH activity in the procedure adding the Cre-loxP integration site to create bacmid MultiBac (**Supplementary Fig. 1a** online). Integration of the expression cassettes contained in pUCDM and pFBDM into the bacmid is performed by providing electro-competent DH10MultiBac^{Cre} cells containing the bacmid with Cre recombinase, via transient expression from pBADZ-His$_6$Cre under arabinose control (**Supplementary Fig. 1b** online), and supplying Tn7 transposition functions by including the helper plasmid pMON7124[18]. Infection of Sf21 cells with bacmid MultiBac with or without a copy of pUCDM integrated yielded viable virus with handling characteristics identical to bMON14272 from the Bac-toBac system and no noticeable deficits due to the modifications (**Fig. 2b**). On the contrary, cells remained largely intact 72 h after infection, and showed a dramatic improvement in recombinant protein quality. Proteolytic breakdown was greatly reduced without the addition of protease inhibitors.

The three complexes expressed were purified by affinity chromatography based on histidine tags for Isw2[24] and Isw1b (D.J.F. & T.J.R., unpublished data), and histidine and calmodulin-binding peptide tags for the TFIID subcomplex (I.B. & T.J.R., unpublished data). The purified Isw2 and Isw1b complexes are highly active in ATP hydrolysis and chromatin remodeling. The TFIID subcomplex was found to be a dimer of approximately 700 kDa by gel permeation chromatographic analysis. The presence of all respective subunits in each purified complex suggests that all components were expressed and assembled in each infected cell. Each of these complexes is equimolar in subunit stoichiometry.

To probe questions concerning coexpression in single cells and heterologous promoters, we assayed protein production qualitatively by expressing fluorescent proteins. We inserted two enhanced cyan fluorescent protein (ECFP) genes and one EYFP gene (EYFP is 5 times brighter than ECFP[25]) into *Tn7* and *LoxP*, respectively, and monitored their fluorescence in individual whole cells. Single cells either displayed expression from both ECFP and EYFP, or none at all (**Fig. 3a**). Next, we inserted a fourth gene encoding the red fluorescent protein, dsRED, driven by a cytomegalovirus (CMV) promoter into the multiplication module between ECFP cassettes (**Fig. 3b**). Lysate from Sf21 insect cells infected with this virus shows the characteristic fluorescence of ECFP and EYFP proteins (**Fig. 3c**). Mammalian COS cells infected with this virus amplified in insect cells displays strong expression of dsRED protein.

Modern molecular biology depends often on modifying heterologous gene products, for example, to define protein functional domains and to reduce heterogeneity by eliminating low complexity regions. Additionally, post-translational modification of the proteins studied by specific enzymes may be required to gain activity. The logic of the modular and flexible expression system presented here, both for the generation of transfer vectors and bacmid, allows an approach whereby such adjustments can be realized with comparative ease. We anticipate the construction of multigene transfer vector libraries facilitated by using robotics for cloning and high-throughput sequencing. Recombinant baculovirus vectors have also recently emerged as a powerful tool for mammalian cell gene delivery[26]. The demand has arisen for polycistronic viral vectors for combined gene therapy as an alternative to single gene therapy[12]. The requirement for accessory genes in gene therapy, for instance, to block inactivation

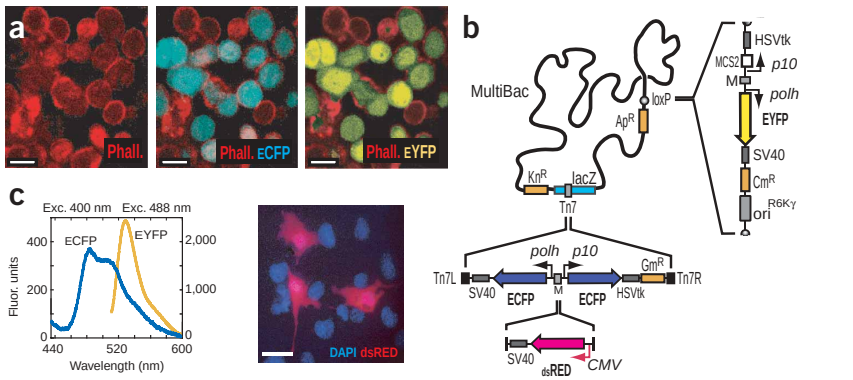

Figure 3 Fluorescent protein monitors of gene expression. (**a**) Genes encoding EYFP and ECFP were inserted into bacmid MultiBac via pUCDM at *LoxP* and pFBDM at *Tn7*, respectively. The micrographs were taken of the same field of Sf21 cells infected with the composite bacmid. Staining with Alexa Fluor 633 phalloidin indicates cell locations (left). The specific fluorescence signals for ECFP (center) and EYFP (right) are detected concurrently from infected cells. Scale bars, 20 μm. (**b**) The MultiBac bacmid used in **a** is shown schematically with the addition of a gene expressed from a mammalian promoter. The MultiBac bacmid was created by substituting the Cre-loxP site-specific recombination sequence for the *v-cath* and *chiA* genes. The gene for EYFP was inserted by pUCDM transfer. The dsRED gene and a CMV promoter were inserted into the multiplication module (M) separating the two ECFP genes and associated promoters, and combined with the bacmid at *Tn7*. (**c**) The MultiBac derivative described in **b** expresses ECFP and EYFP in insect cells and dsRED in mammalian cells. The fluorescence spectra (left) measured on lysates from infected Sf21 cells are characteristic of ECFP and EYFP. The micrograph (right) shows that mammalian COS cells infected with the same virus express dsRED. Nuclei were stained blue with DAPI.

by the complement system[27], has also been recognized. By including promoters active in mammalian cells in pFBDM and pUCDM, as demonstrated here with a CMV promoter, the MultiBac system is applicable to multigene delivery in mammalian cells.

METHODS

Vector construction. Transfer vector pFBDM was derived from pFastBacDUAL (Invitrogen). The sequence 5′-TACTAGTATCGATTCGCGACC-3′ was inserted into the *Bst*Z17I site between the polh and p10 promoters generating the multiplication module containing, in order, *Bst*Z17I, *Spe*I, *Cla*I and *Nru*I cleavage sites (**Fig. 1a**). A site for the rare-cutter *Pme*I was added by replacing the *Sna*BI recognition sequence with the oligonucleotide 5′-AGCTTTGTT-TAAACAAAGCT-3′. Vector pUCDM was generated by combining the 998 bp *Eco*RV/*Alw*NI fragment of pUNI10 univector (gift from S. Elledge) containing the R6Kγ origin with the 1,258 bp *Alw*NI fragment from pLysS (Novagen) after treatment with mung bean nuclease (New England Biolabs). This pLysS fragment contains the chloramphenicol resistance marker. The resulting construct was digested with *Sma*I and *Xba*I, and ligated to the 1,016 bp *Pme*I/*Avr*II fragment of pFBDM. Unique restriction sites for *Avr*II and *Pme*I restriction endonucleases were reintroduced by site-directed mutagenesis using the QuickChange protocol (Stratagene). The vectors pUCDM and pFBDM contain identical expression cassettes including the multiplication module (**Fig. 1a**).

Plasmid pKIloxP contains the linear fragment used to replace the *v-cath* and *chiA* genes in bMON14272 by ET recombination[28] and was generated from pFastBacDUAL by recircularization of the 3,385 bp *Stu*I/*Fsp*I fragment thus eliminating these sites and disrupting the ampicillin resistance gene. This pKI vector has a gentamycin marker and the original pFastBacDUAL p10 MCS containing *Xho*I, *Nco*I and *Kpn*I restriction sites. pKI was modified to yield pKIloxP (**Supplementary Fig. 1a** online) as follows. The 1,008 bp *Bsp*HI fragment of pFastBacDUAL containing the ampicillin resistance marker was introduced into the *Nco*I site. Next, homology region HomA from the baculovirus *chiA* gene was amplified from bMON14272 with primers 5′-GCGCGCCTCGAGGCCTCCCACGTGCCCGACCCCGGCCCG-3′ and 5′-GCGCGCCTCGAGGGAGGAGCTGCGCGCAATGC-3′, and digested with *Xho*I. The resulting 408-bp fragment was inserted into the *Xho*I site of pKI. Homology region HomB from the *v-cath* gene was amplified with primers 5′-GCGCGCGGTACCGCGTTCGAAGCCATCATTA-3′ and 5′-GCGCGCGGTAC-CAGGCCTGAAAAATCCGTCCTCTCC-3′, digested with *Kpn*I and the resulting 372-bp fragment inserted into the pKI *Kpn*I site. Finally, a Cre-loxP sequence was introduced into the *Nhe*I site by the oligonucleotide 5′-CTAGAATAACTTCGTATAGCATACATTATACGAAGTTAGTTTAAACT-3′. The gene encoding for Cre recombinase and a six-histidine tag directly adjacent to the start codon was generated from synthetic oligonucleotides (Microsynth) and ligated to *Nco*I/*Kpn*I digested pBAD22 vector[29]. The ampicillin resistance marker was inactivated by *Fsp*I/*Sca*I digestion. A zeocin resistance gene under control of a synthetic prokaryotic promoter (EM7) was excised from pPICZA (Invitrogen) by *Pvu*II/*Eco*RV digestion and ligated to the 5,388-bp *Fsp*I/*Sca*I fragment yielding pBADZ-His$_6$Cre (**Supplementary Fig. 1b** online). Transfer vector pUCDM-EYFP was generated by subcloning the 909 bp *Eco*47III/*Mfe*I fragment of pEYFPC1 (Clontech) into pUCDM at the *Stu*I and *Mfe*I sites. The 743 bp *Age*I/*Xho*I fragment of pECFPC1 (Clontech) was subcloned into pFBDM at the *Xma*I and *Xho*I sites, then excised by *Bbs*I/*Xho*I digestion and placed into the *Bam*HI/*Sal*I sites to yield pFBDM-ECFP. The gene encoding dsRED was PCR amplified from pdsRED-N1 (Clontech) together with the CMV promoter and blunt ligated into *Nru*I/*Stu*I digested-pFBDM replacing the polh promoter and MCS2.

Constructs for expression of Isw2 chromatin remodeling complex were made by inserting the gene encoding for Isw2p with a C-terminal, six-histidine tag into the *Nco*I and *Pvu*II sites of MCS2 creating pFBDM-Isw2p, and by placing the gene encoding for Itc1p into the *Stu*I site of pUCDM to create pUCDM-Itc1p. The dual transfer vector pFBDM-ISW2 was generated from pFBDM-Isw2p by insertion of the Itc1p gene into the *Stu*I site of MCS1. Cloning of constructs encoding for yeast Isw1b and the human TFIID subcomplex will be described elsewhere. All vectors were propagated in TOP10 cells (Invitrogen) with the exception of pUCDM and its derivatives, which were propagated in bacterial hosts BW23473 or BW23474 (gift from S. Elledge) expressing the *pir* gene.

Generation of the MultiBac bacmid. The vector pBAD-ETγ carrying truncated recE under the arabinose-inducible PBAD promoter and recT under the EM7 promoter (gift from A.F. Stewart) was modified by placing the zeocin resistance gene from pPICZA into the *Fsp*I and *Sca*I sites as described for pBADZ-His$_6$Cre yielding pBADZ-ETγ. This vector was transformed into DH10BAC (Invitrogen) cells harboring the bacmid bMON14272 and the helper plasmid pMON7124[17,18] yielding colonies resistant to kanamycin, tetracyclin and zeocin. A single colony was used to inoculate 500 ml 2× TY (1.6% bacto tryptone, 1% yeast extract, 0.5% NaCl) in the presence of these three antibiotics at 20 °C. At OD_{600} = 0.25, l-arabinose (Fluka BioChemicals) was added to 0.1% and the culture incubated until OD_{600} = 0.5. Electro-competent DH10BacET cells were then generated following standard procedures and stored at −80 °C. JM110 cells (Stratagene) lacking *dam* and *dcm* activity were transformed by the vector pKIloxP (**Supplementary Fig. 1a** online). Unmethylated plasmid DNA was digested with *Stu*I to yield a 1,827-bp fragment containing the ampicillin resistance marker and a Cre-loxP sequence flanked by *chiA* and *v-cath* homology regions. A minimum of 0.3 μg linear DNA was then electroporated into DH10BacET cells. Transformed cells were grown for 4 h at 37 °C and plated on agar plates containing kanamycin, tetracycline and ampicillin. Bacmid DNA from two single triple-resistant colonies was analyzed by PCR amplification using primers 5′-AGGTACTAAATATGGCG-3′ and

5′-CTGAGCGACATCACT-3′, which anneal outside the homology regions and by sequencing the PCR fragment to confirm correct integration. Frozen electro-competent DH10MultiBac cells containing the modified bacmid having the *v-cath* and *chiA* genes replaced by an ampicillin marker and *LoxP* were prepared as above with ampicillin replacing zeocin in the culture.

Composite MultiBac bacmids. For expression of Cre recombinase, DH10MultiBac was transformed by pBADZ-His$_6$Cre through electroporation yielding clones resistant to kanamycin, tetracyclin and zeocin. A single colony was used to inoculate 500 ml 2× TY in the presence of these antibiotics at 20 °C. L-arabinose was added to 0.1% at OD_{600} = 0.25. At OD_{600} = 0.5, frozen electro-competent DH10MultiBac^{Cre} cells containing overexpressed Cre recombinase (**Supplementary Fig. 1b** online) were prepared. Plasmid pUCDM or derivatives were electroporated into DH10MultiBac^{Cre} cells and plated on agar containing kanamycin, tetracyclin and chloramphenicol. Triple-resistant clones were assayed on plates containing IPTG and Bluogal for integrity of the *lacZα* gene with *Tn7*, yielding blue colonies in all cases tested. Clones were analyzed for the correct Cre-loxP site-specific recombination event by PCR amplification and sequencing as described above. Loss of pBADZ-His$_6$Cre was confirmed by replating in the presence of zeocin. Electro-competent cells were prepared from the individual clones in the presence of kanamycin, tetracyclin and chloramphenicol. Transposition of Tn7 elements from pFBDM derivatives into *Tn7*, identification of recombinants by blue/white screening, and transfection of purified composite bacmid was carried out as recommended[18]. For simultaneous transformation of DH10MultiBac^{Cre} with pUCDM and pFBDM derivatives, at least 1 μg of each was used. Yeast Isw2 was expressed and purified as described[24]. Expression and purification of yeast Isw1b and the human TFIID subcomplex will be described elsewhere.

A function of the F-replicon on the bacmid is to limit the copy number to one or two, reducing the potential for undesired recombination[30]. Introduction of pFBDM derivatives into MultiBac disrupts the *lacZα* gene allowing unambiguous identification of cells containing only composite bacmid. However, in the case of Cre-catalyzed fusion of pUCDM derivatives, coexistence of a copy of both the composite and parent bacmids cannot be ruled out based on chloramphenicol resistance. Virus from initial transfections with MultiBac containing the EYFP gene inserted by Cre catalysis was therefore clonally separated by plaque purification. As 29 of 32 (91%) plaque-purified specimens expressed EYFP, Cre-loxP site-specific recombination occurred with nearly saturating efficacy. Notwithstanding the presence of several copies of viral promoters (p10, polh) and terminators (SV40, HSVtk), MultiBac derivatives could be serially passaged at least five times in Sf21 cells at a multiplicity of infection (MOI) $\leq$ 1.

For fluorescence assays of EYFP and ECFP, 0.9×10^6 Sf21 cells were plated on 35 mm tissue culture plates (Corning) and infected with 100 μl virus stock ($\geq 10^9$ plaque-forming unit (pfu)/ml). Cells were fixed 48 h after infection for 30 min with PBS containing 2% paraformaldehyde. As blocking solution, PBS supplemented with 0.5% saponin (Sigma) and 10% fetal calf serum was applied for 1 h at 20 °C. Cells were stained with Alexa Fluor 633 phalloidin (Molecular Probes) diluted 1:200 in blocking solution. After washing with PBS, glycerol/PBS solution AF1 (CITIFluor) was applied and the wells sealed. Fluorescence analysis of Alexa Fluor 633 phalloidin, ECFP and EYFP was performed with a Leica TCS SP confocal microscope. For mammalian cell infection, COS cells were plated at a density of 30% and infected 18 h later at a MOI of 50 with baculovirus amplified in Sf21 insect cells to 4×10^8 pfu/ml. The virus-containing supernatant was replaced with fresh medium after 4 h. Cells were fixed after 40 h with 2% PFA/PBS. Expression of EYFP was confirmed by western blotting using anti–green fluorescent protein antibody (Roche). Expression of EYFP and ECFP was quantified by absorbance spectrometry (**Supplementary Fig. 1c** online).

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Biotechnology* website for details).

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