Modular cloning of multigene vectors for the baculovirus system and yeast

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Abstract

Recombinant macromolecular complexes are often produced by the baculovirus system, using multigene expression vectors. Yet, the construction of baculovirus-compatible multigene expression vectors is complicated and time-consuming. Furthermore, while the baculovirus and yeast are popular protein expression systems, no single method for multigene vector construction is compatible with both. Here we present the modular cloning (MoClo) Baculo toolkit for constructing multigene expression vectors for the baculovirus system and, through compatibility with the MoClo Yeast toolkit, also for yeast. Vector construction by MoClo Baculo does not require PCR, primers or the sequencing of intermediate products. As a proof of principle, MoClo Baculo was used to construct baculovirus and yeast multigene vectors expressing the four- and five-subunit human Polycomb Repressive Complex 2. We show that MoClo Baculo simplifies and expedites the construction of multigene expression vectors for the baculovirus system and provides compatibility with yeast as an alternative expression system.

Introduction

An increasing number of large multi-subunit macromolecular complexes are subjected to structure determination using cryo-electron microscopy [1]. Such structural and biophysical studies often require the expression and purification of recombinant multisubunit protein complexes. When the macromolecule complex of interest is of a eukaryotic origin, its subunits are often required to be co-expressed in a eukaryotic host [2,3]. Among the most

30 commonly used eukaryotic systems for recombinant protein expression are the baculovirus
31 system [4] and yeast [5], each offering different advantages.

32 There are several widely used baculovirus expression systems. The Bac-to-Bac system is
33 one of the most commonly used systems for the expression of protein complexes with a few
34 protein subunits [6]. The Bac-to-Bac system allows the cloning of up to two genes per
35 expression vector, which is then integrated into the baculovirus DNA and packed into
36 baculovirus particles [6]. Complexes with more than two protein subunits can be expressed
37 by the Bac-to-Bac system. This is commonly done by the co-infection of insect cells using
38 multiple different baculoviruses, each carrying a different gene. Accordingly, there are many
39 examples of protein complexes that were expressed using co-infection with 3-5
40 baculoviruses (e.g. [7,8,9,10,11]). Yet, beyond that, the efficiency and reproducibility of co-
41 infections is reduced and, therefore, the expression of complexes with more than a few
42 subunits commonly requires the usage of multi-gene vectors.

43 There are several multigene vector systems for protein expression using the baculovirus
44 system. MultiBac recombines multiple genes into one vector using the Cre-lox technology
45 [12]. BiGBac enables the construction of similar multigenic vectors using a Gibson Assembly
46 [13], allowing a maximum of 25 genes in one plasmid [14]. GoldenBac utilises Golden Gate
47 assembly that simplifies vector construction, where individual genes are PCR amplified only
48 once, at the beginning of the process [15].

49 All current multigene baculovirus expression systems [6,11,12,14,15] require PCR or Gibson
50 Assembly [13], and therefore oligonucleotide synthesis. PCR-based approaches may
51 complicate the usage of GC-rich or repetitive templates for the construction of some
52 multigene vectors. Furthermore, most multigene baculovirus vectors do not have a protein
53 tag pre-incorporated in the vector. Instead, large protein tags are often added through a prior
54 cloning step while small tags are included in the cloning primers. These factors increase the
55 reliance on oligonucleotide synthesis and the subsequent sequencing of intermediate
56 products, which increase the time, cost and complexity of the project.

57 The problem of multigene vector construction has already been solved in various organisms
58 using modular cloning (MoClo) [16]. In MoClo, different ‘parts’ are functional DNA sequence
59 elements that are assembled together to form ‘devices’, which are more complex plasmids.
60 Each ‘part’ is a plasmid that includes one functional element, such as a protein-coding
61 sequence, tag, promoter, or backbone of an expression vector. Each part is generated only
62 once, usually with the aid of gene synthesis or gBlocks, and can then be used repeatedly in
63 different projects or shared between labs. Multiple plasmids, each holding a single part, are

digested together by type IIS restriction enzymes to release DNA fragments with cohesive ends that allow multiple parts to assemble together through Golden Gate assembly [17]. This process can be carried out iteratively, where plasmids are ligated, digested and ligated again to form increasingly complex plasmids. MoClo-constructed plasmids may include multiple genes and all the elements that are required for their delivery, integration into the host, selection, protein expression and protein purification. MoClo is fast, efficient, affordable, and does not require PCR, Gibson Assembly or custom primer design.

The MoClo Yeast Toolkit [18] was designed for modular cloning of multigene expression cassettes, aiming for synthetic biology applications in the yeast *S. cerevisiae*. Although the MoClo Yeast Toolkit was not designed primarily for the expression and purification of recombinant proteins, we reasoned that it would be feasible to modify it for that purpose. Based on a cloning standard inspired by the MoClo Yeast Toolkit, we set out to develop a method for cross-platform multi-gene vector building for protein expression and purification. We focused on constructing parts for modular cloning of expression vectors dedicated to the baculovirus system and yeast, given their broad applicability.

Here we present the MoClo Baculo, a multi-gene modular cloning (MoClo) method designed for the expression of proteins using the baculovirus system and in yeast. MoClo Baculo utilises Golden Gate as the only cloning method. The mutation rate is extremely low, given that MoClo does not involve the usage of PCR, Gibson Assembly, in vitro polymerases or primers. Therefore, the sequencing of intermediate product plasmids is not required, which dramatically reduces the time, cost and complexity of building multigene constructs. Modular cloning allows for flexible replacement or removal of N- or C-terminal affinity tags when developing a new purification scheme. MoClo Baculo supports the expression of up to 6 proteins from a single multigene construct, however this number can theoretically be expanded by adding adapters. The number of co-expressed subunits can further be expanded through the delivery of multiple multigene constructs. As a proof of concept, we used MoClo Baculo to build multi-gene expression vectors of the 4- and 5-subunits Polycomb repressive complex 2 (PRC2). We demonstrate the suitability of such MoClo Baculo expression vectors to express catalytically active PRC2 complexes using the baculovirus system and yeast.

97 **Results**

98 **Modification of the MoClo Yeast toolkit for better compatibility with applications of**
99 **recombinant protein expression and purification.**

100 We set out to generate a system that would utilise the same set of open reading frames
101 (ORFs) and tag constructs for building different multi-protein expression vectors.
102 Furthermore, we wished to have compatibility with protein expression using the baculovirus
103 system and yeast. We prioritised methods that do not rely on PCR, in vitro polymerase
104 reactions or primers, aiming to avoid the sequencing of intermediate products. We reasoned
105 that the MoClo Yeast Toolkit [18], originally developed for synthetic biology applications,
106 would be an ideal starting point.

107 The MoClo Baculo toolkit design is inherited from the MoClo Yeast Toolkit and therefore
108 includes the following part types that are assembled together to construct expression vectors
109 (Supplementary Fig. 1 and Supplementary Table 1): Left Connector (ConL; part 1) that
110 connects functional elements to the plasmid backbone; Promoter, designed for protein
111 expression with (part 2a) or without (part 2) N-terminal tag; an optional N-terminal tag (part
112 2b); a protein Coding Sequence (CDS; part 3); an optional C-terminal tag (part 4a); a
113 transcription Terminator sequence that is designed to be used with or without C-terminal tag
114 (part 4b or 4, respectively); a right Connector (ConR; part 5) and a backbone (part 6) that
115 can be divided into up to 4 different parts (parts 6a-d) with different functionalities (more
116 below).

117 One important difference between the MoClo Yeast Toolkit and the MoClo Baculo designs
118 is that in the latter one can use the same CDS part with or without tags(Supplementary Fig.
119 1 and Supplementary Table 1). Specifically, in the MoClo Yeast toolkit, the construction of
120 vectors for expressing untagged and N-terminally tagged proteins is done using two different
121 CDS parts (Supplementary Fig. 1). This design is undesirable for structural biologists, who
122 would benefit from the frequent removal or inclusion of N-terminal tags without having to
123 source two separate CDS parts. Therefore, to allow for removing the N-terminal tag without
124 changing the CDS part, we modified the MoCo Yeast general design of promoters and N-
125 terminal parts (Supplementary Fig. 1A).

126 To enable the modular cloning using Golden Gate assembly, each part is domesticated into
127 a chloramphenicol resistance plasmid (pYTK001) that originated from the MoClo Yeast
128 toolkit (Supplementary Fig. 1D). The resulting plasmid holds a single part and is referred to
129 as “Level 0” plasmid [18] (see methods section and Supplementary Fig. 1C,D for the flanking

130 sequences that are added to each part, to allow for its domestication and subsequent
131 usage).

132 The newly modified design of MoClo Baculo still allows the replacement of promoters
133 through modular cloning, although it is not fully compatible with the promoter design of the
134 MoClo Yeast toolkit [18]. Therefore, we generated several useful MoClo Baculo-compatible
135 *S. cerevisiae* promoters, based on promoter sequences from the MoClo Yeast toolkit
136 (Supplementary Table 1). More *S. cerevisiae* promoters can be made as needed. We also
137 designed and generated parts carrying N- and C-terminal tags that are useful for protein
138 expression and purification. Such tags for protein purification include a PreScission (3C)
139 protease cleavable maltose binding protein (MBP) tag, a dual protein A tag and a Strep-tag
140 (Supplementary Table 1). Additional tags can readily be added as individual parts, without
141 bringing the coding sequence into consideration and as long as the correct flanking
142 sequences are added to them (Supplementary Fig. 1C,D).

143 **Adding compatibility with the baculovirus system for protein expression**

144 We next sought to enable compatibility between the MoClo Yeast toolkit to the baculovirus
145 system. To this end, we modified parts of an expression vector used in the Bac-to-Bac
146 expression system (pFastBac1 [6]), aiming to enable modular cloning. For that, we first
147 aligned backbone sequences from different existing baculovirus expression systems that
148 were previously modified from the Bac-to-Bac systems. This was done to identify commonly
149 used modules (Supplementary Fig. 1B) and regions where the pFastBac1 vector can be
150 modified without negatively impacting performances. Based on this sequence analysis we
151 designed two new parts that originated from the pFastBac1 vector and are termed here BV
152 'Backbone' (part 6b-d in Supplementary Table 1 and plasmid pMB.001 in Supplementary
153 Table 2) and 'Recombination into BV DNA' (part 6a in Supplementary Table 1 and Fig. 1A,
154 top right, and plasmid pF003 in Supplementary Table 2). The BV Backbone (part 6b-d;
155 plasmid pMB.001) includes bacterial replication origin, ampicillin and gentamicin resistance
156 and the right transposition site (Tn7R), which is used for recombination into the baculovirus
157 DNA. The left transposition site (Tn7L) is included within the 'Recombination into BV DNA'
158 part (part 6a; plasmid pF003). Together, the 'Backbone' part and the 'Recombination into BV
159 DNA' part include all the functional elements required to connect to each other and to all the
160 other parts that are collectively required to express a single open reading frame, either
161 tagged or not. We envision that the usage of two parts for the backbone would facilitate
162 further backbone modifications in future works. For compatibility with the MoClo Yeast
163 cloning standard, we used codon optimization to remove BsaI and BsmBI sites from the
164 sequences that were used to construct all these parts. In the case of parts that are specific

165 for yeast, or may be used for both yeast and baculovirus expression, we also removed NotI
166 sites, as NotI is used to linearise vectors for yeast genome integration. If one wishes to use
167 the optional compatibility between MoClo Baculo to the biGBac system (more below), then
168 PmeI sites should be removed too.

169 For compatibility with the baculovirus expression system, we designed additional new parts
170 using the MoClo cloning standard: polyhedrin promoter (parts 2 and 2a) and SV40
171 terminator (parts 4 and 4b). All these parts were domesticated into Level 0 plasmids and are
172 compatible with Golden Gate assembly using the type IIS restriction enzyme BsaI. At this
173 point, the system included all the elements required to assemble expression vectors that are
174 compatible with the Bac-to-Bac system. Each of the resulting expression vectors are termed
175 "Level 1" plasmids (Fig. 1, top and Supplementary Fig. 1A). Level 1 plasmids can express a
176 single protein from an element that is termed a "Transcription Unit": a functional element
177 required to transcribe an mRNA that is coding for the expression of a desired protein.

178 To enable the assembly of multiple Transcription Units (from multiple Level 1 plasmids) into
179 a single multi-gene baculovirus expression vector (termed "Level 2" plasmid), we required a
180 new backbone that confers additional antibiotic resistance. For that, we added
181 spectinomycin resistance into the 'Recombination into BV DNA' part (part 6a). The new part
182 6a ('Recombination into BV DNA + SpecR') can now be assembled together with the
183 Backbone (part 6b-d), a Spacer (part 2-4) and Connectors (parts 1 and 5) through a Golden
184 Gate reaction using BsaI. The result is a Level 1 plasmid that is termed here 'Level 2
185 Backbone'. The Level 2 Backbone plasmid does not carry a Transcription Unit but, instead,
186 contributes the backbone during the assembly of Level 2 plasmids. Users can assemble
187 their own 'Level 2 Backbone' plasmids using parts from the MoClo Baculo toolkit, together
188 with Connector parts from the MoClo Yeast toolkit. However, for the ease of use, we
189 included in the MoClo Baculo toolkit a Level 2 Backbone plasmid (pMB.002) that includes
190 the ConLS' and ConRE' connectors. The pMB.002 plasmid allows the integration of up to 6
191 transcription units into a Level 2 plasmid (Fig. 1B, right, and Supplementary Table 2).
192 Overall, this design ensures that both single-gene (Level 1) and multi-gene (Level 2)
193 expression vectors are suitable for protein expression using the Bac-to-Bac system and,
194 therefore, confer resistance to ampicillin and gentamicin. In addition to these, multi-gene
195 (Level 2) baculovirus expression vectors also confer resistance to spectinomycin.

196 **Adding compatibility with the biGBac system**

197 We envisioned that some users may benefit from compatibility between MoClo Baculo and
198 the biGBac system [14]. This is because the biGBac system allows to connect together 5

199 multi-gene vectors using Gibson Assembly. Therefore, we decided to generate MoClo
200 Baculo parts that would include the Gibson Assembly linkers of the biGBac system. For that,
201 we modified the pMB.002 plasmid by inserting linker sequences ("A-F") from pBIG1a-e [14]
202 and PemI restriction enzyme sites (Supplementary figure 4A). We named the resulting
203 plasmids pMB.BIG1a to pMB.BIG1e. These plasmids are functionally identical to the biGBac
204 system plasmids pBIG1a to pBIG1e plasmids, respectively. The compatibility between
205 MoClo Baculo to the biGBac system may allow the optional connection of up to five Level 2
206 MoClo Baculo plasmids, each with up to six transcription units. The resulting biGBac
207 expression vectors are referred to as Level 3 plasmids here and contain up to 30
208 transcription units.

209 We believe that most users will not need the compatibility with the biGBac system, and
210 therefore it is marked here as optional. This is because compatibility with biGBac is
211 beneficial only if one wishes to increase the maximal number of transcriptional units from six
212 (Level 2 plasmids) to 30 (optional Level 3; Supplementary Fig. 4B) without adding new
213 MoClo Baculo adapters. However, the added compatibility between the MoClo Baculo to the
214 biGBac system may allow regular users of the biGBac system to construct their expression
215 vectors with the aid of Modular Cloning. This may provide biGBac users with the simplicity of
216 removing or replacing tags without PCR or gibson assembly.

217 As a proof of principle, we assembled a baculovirus vector aiming for the expression of an
218 eight subunit complex (Supplementary Fig. 5). This was done first with the MoClo Baculo
219 system, using Golden Gate assembly, to construct the three Level 2 vectors (Supplementary
220 Fig. 5B): pMB.BIG1a.PRC2 (expresses four PRC2 subunits), pMB.BIG1b.MTF2-PALI1 (for
221 the expression of two accessory subunits of PRC2: MTF2 and PALI1), and pMB.BIG1c.G9A-
222 GLP (for the expression of the H3K9me3 methyltransferase G9A and GLP). The resulted
223 three Level 2 vectors are compatible with the biGBac system, which was used to combine
224 them using Gibson Assembly into one Level 3 vector of 32 kb (pBIG2abc.PRC2.1-EHMT),
225 aiming for the co-expression of all eight subunits (Supplementary Fig. 5C). The efficiency of
226 constructing the Level 3 vector was 33% (2 out of 6 clones were correct; Supplementary Fig.
227 5A). The produced Level 3 vectors were verified using whole plasmid sequencing, with the
228 exception of sporadic mutations in the homology regions used for Gibson Assembly.

229 **Modular cloning of baculovirus expression vectors**

230 From this point, modular cloning is carried out using the same workflow that has been
231 described for the MoClo Yeast toolkit [18]. As the process is described below in brief, it is
232 highly recommended to read the description of modular cloning by Weber et al [16] and Lee

233 et al [18]. Briefly, all the Level 0 (chloramphenicol resistant) vectors that carry the parts for a
234 desired Transcription Unit are combined in a single pot Golden Gate assembly, using the
235 type IIS restriction enzyme BsaI (Fig 1a). The produced Level 1 plasmid has lost its
236 chloramphenicol resistance and now confers resistance to ampicillin in *E. coli*. The Level 1
237 baculovirus expression vectors also confer resistance to gentamicin in *E. coli*. This transition
238 between antibiotic selection markers is inherited from the modular cloning method and
239 allows selection against precursor plasmids while progressing up to the next plasmid Level
240 through iterative cloning. For the production of baculovirus expression vectors, we use
241 chloramphenicol to select Level 0 plasmids, ampicillin or gentamicin to select Level 1
242 plasmids and spectinomycin to select Level 2 plasmids. Each Level 1 plasmid can now be
243 used to express a single protein, depending on the transcription unit and the backbone.
244 Multiple Level 1 plasmids can be used to assemble one Level 2 plasmid—a multi-gene
245 expression vector—using the Golden Gate assembly region with BsmBI digestion (Fig. 1B,
246 bottom). Yet, the assembly of Level 2 vectors requires some planning, because the order of
247 the transcription units in a Level 2 vector is defined based on the Connectors (parts 1 and 5)
248 that are included in each of the Level 1 vectors that are combined to assemble a multi-gene
249 expression vector (Level 2).

250 Lee et al. [18] and Weber et al. [16] are highly recommended readings on the topic of
251 connector design, as the MoClo Baculo toolkit uses the connector parts of the MoClo Yeast
252 toolkit [18]. In brief, connectors are designed such that a right Connector (ConR, part 5) of a
253 given Transcription Unit can be connected only to a complementary left Connector (ConL,
254 part 1) of another Transcription unit. Exceptions are the ConLS' and ConRE' connectors that
255 are reserved for the backbone. For instance, ConR1 is connected to ConL1 of another
256 Transcription Unit, ConR2 is connected to ConL2 in another Transcription unit, and so forth
257 (see Supplementary Table 1 for the list of connectors). Practically, this means that the order
258 of the transcription units in a given Level 2 vector usually need to be defined at the time
259 when one designs the relevant set of Level 1 vectors that are intended to be used together in
260 a subsequent Golden Gate assembly.

261 Even when Level 2 expression vector is intended for the baculovirus system, we find it
262 convenient to use the same connector parts that were designed by Lee et al for the MoClo
263 Yeast toolkit [18]. The MoClo Yeast toolkit provides connectors for combining up to 6
264 transcription units together. If less than 6 subunits are assembled, a linker plasmid can be
265 used to connect the last transcription unit to the backbone (an example for that is described
266 in the next section). It is anticipated that more connectors can be added to increase the

267 number of transcription units in a given multi-gene expression vector, as long as the total
268 length of the construct permits Golden Gate assembly.

269 We find that the sequencing of Level 1 plasmids is not required if they are only used as
270 intermediate products for assembling a Level 2 vector. This is because mutations are
271 uncommon, since no PCR or oligonucleotides are used. Therefore, when Level 1 plasmids
272 are only used as intermediate products, we commonly validate the correct Level 1 plasmid
273 assembly by restriction digestion before proceeding to Level 2 assembly. At the end of the
274 process, the correct assembly of the Level 2 plasmid is validated by restriction digestion
275 followed by whole plasmid sequencing. The obtained Level 2 plasmids are multi-gene
276 expression vectors compatible with the bac-to-bac baculovirus expression system (Fig. 1C).

277 **Modular cloning of yeast expression vectors**

278 The construction of yeast expression vectors is carried out using the same CDS and tag
279 parts that are used for baculovirus vector building. The process of vector building for yeast
280 expression is nearly identical to the construction of baculovirus vectors, as described above.
281 An exception is that for the construction of yeast expression vectors we normally use
282 chloramphenicol to select Level 0 plasmids, ampicillin to select Level 1 plasmids and
283 kanamycin to select Level 2 plasmids, as described by Lee et al [18]. Most of the parts that
284 are used for building Level 1 and 2 plasmids for yeast expression are taken from the MoClo
285 Yeast toolkit [18] (Fig. 1A; boxes with black lines). Exceptions are the following parts that
286 were generated for the MoClo Baculo toolkit (Fig. 1A; boxes with a blue line or dashed blue
287 line): yeast promoter parts dedicated for using together with N-terminal tags according to the
288 MoClo Baculo cloning standard and the same N-terminal tag parts, CDS parts and C-
289 terminal tag parts that are used for baculovirus expression.

290 **Proof of principle: multi-gene vector construction**

291 As MoClo Baculo adapted the Modular Cloning strategy of the MoClo Yeast toolkit [18], the
292 efficiency of cloning Level 0 and Level 1 vectors is close to 100 %. As a demonstration of
293 Level 1 cloning, we constructed six different Level 1 plasmids (Fig. 2A). These included a
294 backbone plasmid for baculovirus expression (pMB.002) and five Transcription Unites for the
295 expression of each of the five subunits of the PRC2-AEBP2 complex (Fig. 2A,B). These five
296 Transcription Units designed for the expression of the PRC2 protein subunits MBP-EZH2,
297 MBP-SUZ12, MBP-EED MBP-RBBP4 and AEBP2, where MBP is a PreScission-cleavable
298 maltose binding protein tag. After the Golden Gate assembly reaction, two clones were

299 picked per each of these Level 1 plasmids. All these clones were then assayed using
300 restriction digestion, confirming a success rate of 100% (12/12; Fig 2A).

301 As with other systems of Modular Cloning [16,18], the efficiency of assembling Level 2
302 vectors may be reduced with the size and the number of parts. Here we demonstrate the
303 construction of two Level 2 vectors, for the baculovirus expression of the PRC2 core
304 complex in the presence or absence of its accessory subunit AEBP2 (Fig. 2C,D).
305 Specifically, a 19 kbp PRC2 expression vector was designed for the expression of a 4-
306 protein complex (MBP-EZH2, MBP-SUZ12, MBP-EED and MBP-RBBP4) and assembled
307 with 80 % efficiency (4/5 colonies; Fig. 2C). A 21 kbp PRC2-AEBP2 expression vector
308 designed to express five protein subunits (MBP-EZH2, MBP-SUZ12, MBP-EED, MBP-
309 RBBP4 and AEBP2) and was assembled with an efficiency of 50% (2/4 colonies; Fig. 2C).

310 Each of the Golden Gate assemblies that were carried out to generate the expression
311 vectors for the 4- and 5-subunit PRC2 complexes included a linker plasmid (L6 and L56 in
312 Fig 2B). Such a linker plasmid is required when less than 6 Transcription Units are
313 incorporated and aims to connect the last Transcription Unit to the backbone. The linker
314 plasmid is assembled in a Golden Gate reaction using BsaI, by combining an ampicillin
315 resistant *E. coli* Backbone plasmid (plasmid pYTK095 from the MoClo Yeast Toolkit), a
316 Spacer (parts 2-4, plasmid pYTK048 from the MoClo Yeast toolkit) and the appropriate
317 Connector parts (parts 1 and 5; Supplementary Table 1) that are required to connect the last
318 Transcription Unit to the Level 2 Backbone plasmid (pMB.002; Supplementary Table 2).

319 The sizes of the two Level 2 plasmids that we generated—19 kbp and 21 kbp—approach the
320 upper size of plasmids that can be efficiently assembled using Golden Gate assembly
321 (commonly reported as 20-30 kbp [16,18,19]). The expression of all subunits is driven by the
322 same polyhedrin promoter and SV40 terminator, similar to biGBac [14] and Multibac systems
323 [12]. Selected Level 2 clones were subjected to whole plasmid sequencing before
324 downstream protein expression and purification.

325 **Proof of principle: protein expression and purification**

326 As proof of principle, we used the PRC2- and PRC2-AEBP2 multigene baculovirus
327 expression vectors that were constructed above (pMB.PRC2 and pMB.PRC2.AEBP2,
328 respectively; Fig. 2D) for protein expression and purification. The obtained macromolecular
329 complexes were pure, assembled and monodispersed (Fig. 3A-D,G). We obtained about 4
330 mg and 1.5 mg of PRC2 and PRC2-AEBP2, respectively, per litre of Hi5 insect cell culture.
331 We also demonstrated PRC2 protein expression from the biGBac-compatible vector

332 pMB.BIG1a.PRC2 (Fig. 3H), which was assembled solely by Golden Gate assembly using
333 MoClo Baculo (Supplementary Figure. 5B).

334 As a proof-of-principle of protein expression using the yeast *S. cerevisiae*, we generated an
335 expression vector for PRC2 (pMY.PRC2; Supplementary Fig. 2). For vector building, we
336 used the same CDS (part 3) and Connectors (parts 1 and 5) parts that were used to build
337 the baculovirus expression vectors above. To allow for yeast expression, we replaced the
338 promoters (part 2/2a) and terminators (part 4) with *S. cerevisiae* promoters and terminators.
339 For the same reason, we replaced the parts responsible for integration into the host DNA
340 (parts 4a-d) with parts that were designed for integration into *S. cerevisiae* chromosome in
341 yeast (Fig. 1A and Supplementary Fig. 2). We also replaced the MBP tags by a dual Protein
342 A tag (Protein A) that we find more suitable in the yeast system, possibly given the lower
343 expression level of human PRC2 in S. *cerevisiae* (more below). We then integrated the
344 multi-gene PRC2 expression vector into *S. cerevisiae* chromosome through homologous
345 recombination and used the obtained strain for the expression of the human PRC2. The
346 obtained PRC2 was then purified as an assembled complex (Fig. 3E,F).

347 PRC2 is a histone methyltransferase enzyme. Therefore, we wished to determine that the
348 recombinant PRC2 protein complex that we obtained is enzymatically active *in vitro*. To this
349 end, we carried out an *in vitro* histone methyltransferase assay, to compare the enzymatic
350 activities of the recombinant 4-subunits human PRC2 complexes that we purified using three
351 different approaches: (i) Baculovirus expression, through the co-infection of four baculovirus
352 stocks that were made using standard pFastBac1 vectors, each designed to express a
353 single PRC2 subunit (Fig. 4, “pFB1 PRC2”), including the protein subunits MBP-EZH2, MBP-
354 SUZ12, MBP-RBBP4 and MBP-EED. (ii) Baculovirus expression using a single MoClo
355 Baculo-derived multi-gene vector carries Transcription Units for the same four PRC2
356 subunits with the same MBP tags (Fig. 4, “MoClo Baculo PRC2, insect cells”). (iii) A yeast
357 expression of PRC2 using a single multi-gene expression vector including four Transcription
358 Units of the same PRC2 subunits as above, with the exception that EZH2 carries a 2xProtein
359 A tag and the rest of the subunits are untagged (Fig. 4, “MoClo Baculo PRC2, yeast”). In all
360 cases, all tags were removed during the protein purification process and were not present in
361 the enzymatic assay.

362 All the PRC2 samples exhibited a substantial enzymatic activity only in the presence of an
363 allosteric effector peptide H3K27me3 (Fig. 4B, blue bars), in agreement with PRC2 being an
364 allosteric enzyme [20]. We also observed the automethylation of the catalytic subunit EZH2
365 (Supplementary Fig. 4A,B), which has to be methylated for PRC2 to become active [21,22].

366 We observed some variations in the enzymatic activity of the different recombinant human
367 PRC2 preparations. These variations in activity were rather small, within <2-fold of the
368 apparent reaction rate (Fig. 4B). These variations in activity might be attributed to the
369 different expression systems. We also cannot exclude slight variations in the quantification of
370 the protein concentration or subunit stoichiometry between samples, although SDS-PAGE
371 did not identify large differences between them (Fig 4C). Overall, we demonstrated the
372 construction of multi-gene expression vectors using MoClo Baculo and their subsequent
373 application of protein expression and purification using the baculovirus system and yeast.

374

375 **Discussion**

376 The MoClo Baculo system allows a high flexibility in building expression vectors while
377 altering tags and expression systems as needed. According to data herein (Table 1) and
378 elsewhere [23, 24], the baculovirus system provides a higher protein yield per litre than
379 yeast. On the other hand, from the point plasmids are constructed, protein expression using
380 the baculovirus system is a slow process (20-30 days under ideal conditions), while the
381 yeast expression system provides a faster progress (6-7 days; Table 1 and [24]). Another
382 advantage of the yeast expression system is the lower cost of the media: roughly 40-20% of
383 the per-litre cost compared to the baculovirus system (Table 1). We propose that one could
384 use MoClo Baculo in order to construct expression vectors for fast and cost-effective protein
385 expression trials in yeast. Subsequently, the best-performing set of ORFs and tags can be
386 assembled into baculovirus expression vectors, aiming for a high-yield protein production in
387 insect cells.

388 A benefit of MoClo Baculo, with respect to most other baculovirus systems (Table 2), is that
389 the entire vector construction process can be automated, allowing high throughput cloning.
390 Specifically, MoClo Baculo is PCR free, which reduces the likelihood of mutations and
391 eliminates the need for primer design and PCR optimization. Open reading frames can be
392 synthesised as plasmids or gene fragments (e.g. gBlocks), such that they can be directly
393 domesticated into Level 0 vectors by Golden Gate assembly. For the same reason, MoClo
394 Baculo would be beneficial also by reducing the time and training required to design and
395 construct complex expression vectors in any lab.

396 As for other MoClo cloning standards, MoClo Baculo would allow labs and institutes to
397 manufacture, store and share collections of ORFs and tags ready for their assembly into
398 multi-gene expression vectors for baculovirus and yeast. MoClo Baculo was designed as a

general cloning standard, with the end goal of expression and purification of multi-subunit complexes for applications of structural biology, biophysics and biochemistry. As such, MoClo Baculo could potentially be expanded to other protein expression systems, as done with other MoClo tools for various applications [25,26,27,28,29]. Overall, MoClo Baculo is a cost-effective, fast and robust system for constructing multi-gene expression vectors, demonstrated here using the baculovirus system and yeast.

Acknowledgements

We thank the support of the Micromon Genomics facility. This work was supported by Sylvia and Charles Viertel Senior Medical Research Fellowship (C.D.) and the National Health and Medical Research Council (NHMRC) grant numbers APP1162921, APP1184637, APP2011767 and APP2020900 (C.D.).

Competing interests

The authors declare no competing interests.

Author contributions

Z.L., M.B. and C.D. designed the project, Z.L., S.F.F. and M.B. carried out experiments while Z.L. led aspects related to the baculovirus system and M.B. led aspects related to yeast, Z.L. and M.B. analyzed data, Z.L., M.B. and C.D. wrote the manuscript and C.D. supervised the project.

Methods

Plasmid sequences

Part plasmids (Level 0) and expression vectors (Level 1 and 2) that were generated in this study are listed in Supplementary Table 2 and their annotated sequences are provided in GenBank file format in Supplementary Data 1. Plasmid sequence annotation was carried out with the aid of pLannotate [30]. Detailed information about plasmid construction is provided below. Even if one wishes to use MoClo Baculo exclusively for protein expression using the baculovirus system they would still need some plasmids from the MoClo Yeast toolkit, such as the Level 0 backbone (plasmid pYTK001), Connector part plasmids, Spacer part (plasmid pYTK048) and an *E. coli* backbone (plasmid pYTK095). The MoClo Yeast toolkit plasmids are available on AddGene (Kit #1000000061) and their sequences are provided in [18].

429 **Design of coding sequences (CDS) for MoClo Baculo**

430 All CDS sequences were designed for compatibility with the MoClo Yeast system [18]. This
431 includes the addition of overhangs that are reserved for part 3 (CDS; Supplementary Fig.
432 2C). Flanking BsmBI and BsaI sites were added to the CDS sequence, designed to generate
433 the desired overhands for Golden Gate assembly into Level 0 and Level 1 plasmids,
434 respectively (See Supplementary Fig. 1D).

435 In the MoClo Baclo system, part 3 allows for protein expression either with or without an N-
436 terminal tag (Supplementary Fig. 2A), so only one CDS part is needed per project. As with
437 the MoClo Yeast toolkit, the design of the CDS involved the introduction of synonymous
438 mutations to remove the restriction sites for NotI, BsmBI, and BsaI from within the protein
439 open reading frame (i.e., with the exception of the flanking BsmBI and BsaI sites). If one
440 wishes to use MoClo Baculo to construct biGBac plasmids, they will also have to ensure that
441 CDS and tag parts do not include PemI sites, although this would rarely be a problem as
442 PemI sites are extremely uncommon. If one wishes only to construct expression vectors for
443 the baculovirus system, not for yeast, then it is not required to remove NotI sites from CDS
444 parts. This is because NotI is only used to linearise plasmids before their homologous
445 recombination into a yeast chromosome. Finally, CDS are then domesticated into Level 0
446 plasmids, as described in the next section.

447 **Generation of new part (Level 0) plasmids**

448 This process does not need to be repeated in cases where part plasmids already exist. It
449 would typically be carried out once when a new part (e.g. a new tag) is introduced for the
450 first time. The appropriate part-specific flanking sequences (Supplementary Fig. 1C) and
451 universal flanking sequences (Supplementary Fig. 1D) were added to part sequences, either
452 through gene synthesis or PCR primers, to enable domestication into the Level 0 vector and
453 the subsequent modular cloning. DNA with the sequence of the promoter, terminator and tag
454 parts were obtained either as ampicillin-resistant plasmids from gene synthesis (GenScript),
455 gene fragments (IDT gBlocks), or PCR amplicons (Phusion® High-Fidelity DNA Polymerase,
456 NEB) that were generated from plasmids with a pFastBac1.HMBP backbone. The parts were
457 then cloned using Golden Gate Assembly (BsmBI-v2, NEB #R0739) into the Level 0
458 backbone plasmid (pYTK001) of the MoClo yeast toolkit. The Backbone part (Part 6b-c for
459 the baculovirus system; Supplementary Table 1; plasmid pMB.001) for Level 1 MoClo
460 Baculo baculovirus expression plasmids, was synthesised by Genscript to remove the BsaI
461 and BsmBI sites and insertion of GFP-dropout.

462 **Generation of level 2 backbone plasmids**

463 pMB.002 was generated by the golden gate assembly with BsaI enzyme using pMB.001 and
464 pMB.004 from the MoClo Baculo toolkit, together with pYTK048, pYTK008, pYTK073 from
465 yeast MoClo toolkit [18]. To construct the biGBac compatible backbones pMB.BIG1a to
466 pMB.BIG1e (Supplementary Fig. 4A), the same reaction was performed using modified
467 pYTK008 plasmids (ConLS'A to ConLS'E, respectively) and modified pYTK073 plasmids
468 (ConLE'B to ConLE'F, respectively), where the biGBac compatible homology regions for
469 Gibson Assembly (sequences A to F [14]; Supplementary Table 3) were inserted by BsmBI
470 linearisation and HiFi DNA assembly (NEB, #E2621L) using ssDNA templates.

471 **Golden Gate assembly using BsaI and BsmBI**

472 For Golden Gate assembly using BsaI, 25 fmol of each plasmid was added per reaction and
473 assembled with NEBridge® Ligase Master Mix (NEB #M1100) and BasI-HF-V2 (NEB
474 #R3733) according to protocol provided by the manufacturer. For Golden Gate assembly
475 using BsmBI, 25 fmol of each plasmid was added per reaction with 1 µLBsmBI-v2 (NEB
476 #R0739), 1 µL HiT4 ligase (NEB #R2622), 1.5 µL 10x T4 ligase buffer (NEB #B0202), 1 µL
477 500 mM NaCl making up a 15 µL reaction. The mixture was cycled 60 times between 16 ºC
478 and 50 ºC for 5 min each, followed by 30 min final ligation at 16 ºC and digestion at 50 ºC for
479 15 min. The reaction was held at 10 ºC and stored at -20 ºC until use.

480 XL1-Blue (Agilent) was used as the only bacterial strain for all the cloning. Transformation
481 and culturing of XL1-Blue is carried out according to the manufacturer's protocol. For the
482 propagation of pMB.001, the transformed cells were selected on an ampicillin plate. For the
483 selection of Level 1 clones, we find that it is easier to identify GFP positive colonies (i.e.
484 where Golden Gate assembly failed) on ampicillin plates.

485 **Construction of Level 3 plasmids using the biGBac system (optional)**

486 pBIG2 plasmids were linearised in PmeI (NEB #R0560) restriction digest reaction at a final
487 concentration of 10 fmol/µL. All multigene-carrying pMB.BIG1a-e plasmids were miniprep
488 (Qiagen #27106) with an extra PE buffer wash and eluted in 40 µL 60 ºC Milli-Q water twice.
489 Then, 500 fmol of each plasmids were mixed in a total of a 10 µL reaction of 1x Cutsmart
490 (NEB) and with 1 µL PmeI (NEB #R0560). If plasmid concentration was not sufficient, all
491 plasmids were mixed and dried in speedvac and re-dissolved in 9 µL 1x Cutsmart (NEB). All
492 digestions were carried out at 37 ºC for 90 minutes. Then, 1 µL of linearised backbone and 1
493 µL digested pMB.BIG1a-e mix were used to set up a 20 µL Gibson Assembly (NEB #E2611)
494 reaction. The ligation mix were used to transform chemical-competent NEB10-beta (NEB

495 #C3019) or electro-competent XL1-Blue, depending on the size of the final product.
496 Generally, for plasmid between 20-30 kbp, electroporation is beneficial but not essential. For
497 plasmid >30 kbp, electroporation is required.

498 **Expression and purification of PRC2 using the baculovirus system**

499 Baculovirus was generated using Sf9 cells according to the instructions by the manufacturer
500 (ThermoFisher). The virus titer was determined by the viability of infected SF9 cells using the
501 MTS kit (Promega #G3580).

502 Protein expression was carried out according to the method previously described by Zhang
503 et al [11]. Specifically, High Five cells were grown in Insect-XPRESS (Lonza #12-730Q)
504 media and were infected with baculovirus at a cell density of 1.8-2.0*10^6 cells/ml. 70 hours
505 post-infection, cell pellets were harvested at 1500 g and lysed in lysis buffer (50 mM Tris
506 pH8 at 25 °C, 300 mM NaCl, 15 % glycerol, 0.5 % NP-40, 1 mM TCEP, cOmplete protease
507 inhibitor (11836170001)) for 30 min at 4 °C. The lysate was clarified by centrifugation at
508 30,000 g for 30 min and the supernatant was incubated with amylose resin (NEB #E8021)
509 for 60 min with rotation. Then, the resin was washed with 5 column volume of lysis buffer,
510 10-column volumes of HSW buffer (50 mM Tris pH8 at 25 °C, 500 mM NaCl) and 10 c.v. of
511 LSW buffer (50 mM Tris pH8 at 25 °C, 150 mM NaCl). The protein was eluted from the resin
512 with LSW buffer supplemented with 10 mM maltose and 1 mM TCEP. Then the protein was
513 digested overnight with PreScission protease and loaded to a 5 ml prepacked Heparin
514 column (Cytiva #17040701) and eluted with a linear gradient over 10 c.v., starting from
515 Buffer A (150 mM NaCl, 20 mM Tris pH 8.0 at 25 °C) to 50% Buffer B (2000 mM NaCl, 20
516 mM Tris pH 8.0 at 25°C). Finally, the eluate was diluted 1:1 with 20 mM Tris pH 8.0, 1 mM
517 TCEP, concentrated using 30 kDa Amicon Ultra centrifugal filter and injected to a Superose
518 6 Increase 10/300 column (Cytiva, #29091596) in HEPES pH7.5 at 25 °C and 150 mM NaCl.
519 Fractions that contain protein of interest were supplemented with 1 mM TCEP, concentrated
520 and frozen in liquid nitrogen. The entire purification was carried out at 4-8 °C.

521 **Expression and purification of PRC2 using S. cerevisiae**

522 For the purification of the 4-subunits PRC2 from yeast, cells were picked from a single
523 colony and grown overnight in -Ura synthetic minimal media (2 g/L Yeast Synthetic Drop-out
524 Medium Supplement without uracil (Merck #1501), 67 g/L Yeast Nitrogen Base Without
525 Amino Acids (Merck #Y0626)) supplemented with 3% Glucose. The following day, cells were
526 washed twice and used to inoculate 12 L of YP-lactate (1.5% Yeast extract (Merck #Y1625),
527 3% Meat Peptone (ThermoScientific H26694.36), 2% Lactate (Chem Supply #LL008)) in 6

528 flasks. Cultures were grown at 30 ºC and 180 rpm for 31h. EZH2 expression was induced by
529 the addition of 3% galactose and the cultures were grown for 19h. Cells were harvested,
530 washed in water, pelleted (335 g), flash-frozen and stored at -80 ºC until the protein
531 purification was carried out. For protein purification, the cell pellet was thawed in an equal
532 volume of 2X lysis buffer (400 mM NaCl, 50 mM Tris pH 7.5, 0.5 mM DTT, 17 mg/mL
533 Phenylmethanesulfonyl Fluoride (Merck #P7626), 25 µg/mL Leupeptin (Merck #2884), 125
534 µg/mL Pepstatin A (Merck #516481), 33 mg/mL Benzamidine (Merck #B6506)), and lysed
535 using a DynoMill. Lysate was clarified by centrifugation at 30,000 g, followed by
536 ultracentrifugation at 45,000 rpm. The supernatant was bound overnight to 20 mL of IgG
537 resin. Beads were washed with a high salt buffer (500 mM NaCl, 20 mM Tris pH 7.5, 0.5 mM
538 DTT), followed by a low salt buffer (200 mM NaCl, 20 mM Tris pH 7.5, 0.5 mM DTT). PRC2
539 complex was eluted using an overnight cleavage by TEV protease (100 µg) and subjected to
540 heparin purification. The heparin peak was concentrated and injected into a Superose 6
541 10/300 column (Cytiva, #29091596). The protein in the peak corresponding to PRC2 was
542 concentrated and flash-frozen.

543 **SDS-PAGE**

544 To resolve all subunits using SDS-PAGE, 3-8% Tris-Acetate gel (ThermoFisher
545 #EA0375BOX) and 1X MES SDS Running buffer (ThermoFisher #NP0002) were used. 3 µg
546 or 1 µg PRC2 complexes were supplemented to a final concentration of 1X LDS sample
547 buffer (ThermoFisher #NP0007) with 1% 2-Mercaptoethanol (Sigma-Aldrich #M3148) and
548 heated at 95 °C for 5 min before loading onto a Tris-Acetate gel. The gel was run for 60 min
549 at 160 V before staining with InstantBlue Coomassie protein stain (Expedeon, #ISB1L).

550 **Nucleosome reconstitution**

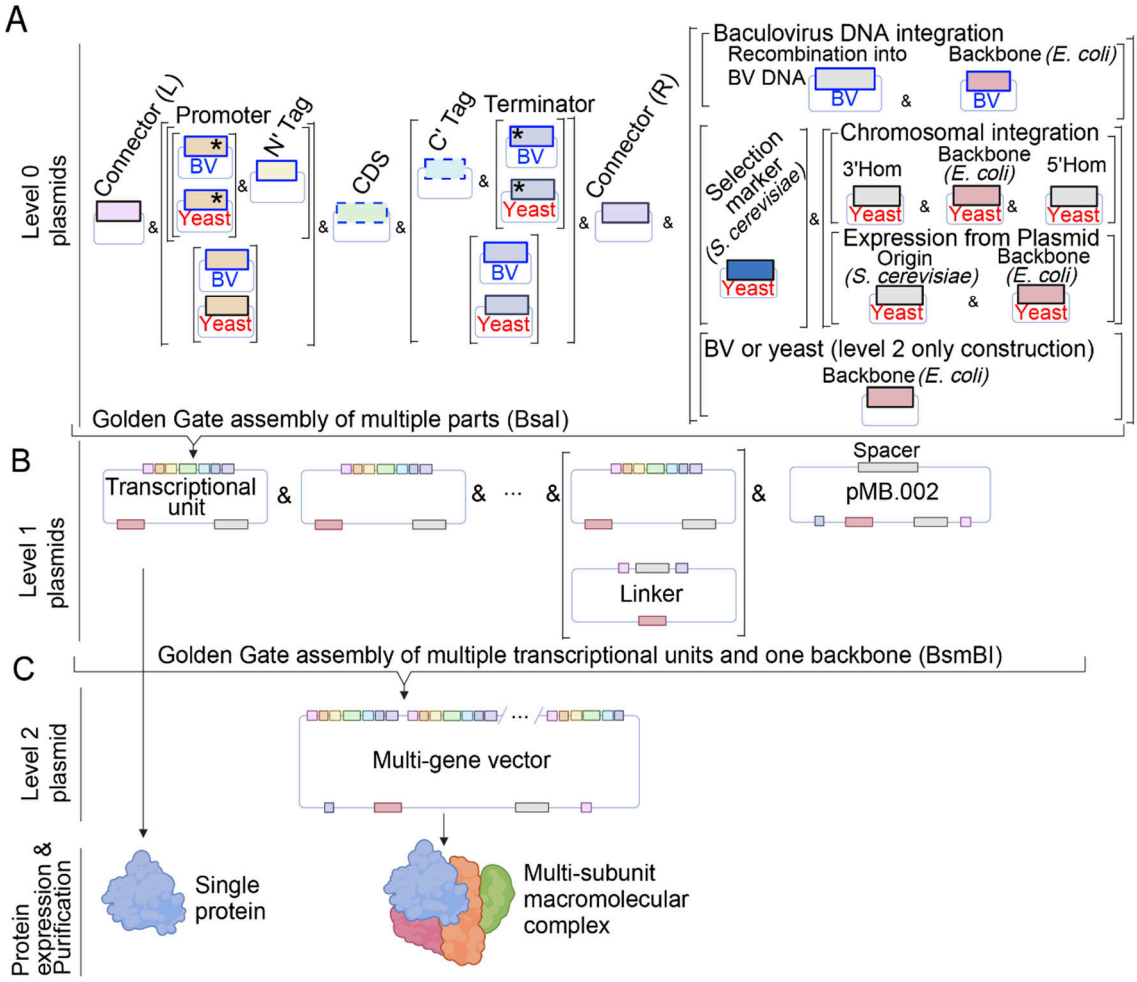
551 Histones, octamers, and polynucleosome reconstitution were carried out as previously
552 described [11]. In brief, recombinant histones were purified from inclusion bodies and
553 reconstituted into histone octamers. 182 bp DNA was amplified using Pfu DNA polymerase
554 and purified by ion exchange chromatography and isopropanol precipitation. Chromatin was
555 assembled using gradient salt dialysis at 4°C. Chromatin was reconstituted by initially
556 titrating across a range of octamer ratios (from 1:0.9 to 1:1.1 DNA:octamer molar ratio) in a
557 20 µL mixture of 3 µM DNA, 20 mM Tris pH 7.5 at 25 °C, 2 M NaCl, 1 mM EDTA and 1 mM
558 DTT. For each of these samples, gradient salt dialysis was used at 4°C in a dialysis device
559 (ThermoFisher #69572), starting from refolding buffer (20 mM Tris pH 7.5 at 25 °C, 2 M KCl,
560 1 mM EDTA and 1 mM DTT) to a medium salt buffer containing 20 mM Tris pH 7.5 at 25 °C,
561 250 mM KCl, 1 mM EDTA and 1 mM DTT over 18 h, and the final step dialysis was carried

562 out using a low salt buffer containing 20 mM Tris pH 7.5 at 25 °C, 2.5 mM KCl, 1 mM DTT.
563 The quality of mononucleosomes was assessed by 0.8 % agarose TBE and 6 %
564 polyacrylamide TBE gel electrophoresis, and the most appropriate molar ratio of
565 DNA:octamer was selected for a large-scale batch. Large-scale reconstitution was
566 conducted as above, except in a volume of 0.5 mL using dialysis tubing (Spectrum #888-
567 11527). The folded mononucleosome was concentrated with 500 µL 100 kDa MWCO
568 concentrator (Cytiva, #28932237). The concentration of the mononucleosome was
569 measured using a Qubit protein assay kit (ThermoFisher #Q3321). Mononucleosomes were
570 stored at 4 °C until use.

571 ***In vitro* HMTase activity assays using radiolabeled S-adenosyl-L-methionine**

572 For the HMTase reactions, each 10 µL reaction contained 0.6 µM PRC2 produced from
573 either yeast or insect cells, 0.6 µM chromatinised gene, and 5 µM S-[methyl-14C]-adenosyl-
574 L-methionine (PerkinElmer, #NEC363050UC). Reactions were incubated in reaction buffer
575 (50 mM Tris-HCl pH 8.0 at 30°C, 100 mM KCl, 2.5 mM MgCl2, 0.1 mM ZnCl2, 2 mM 2-
576 mercaptoethanol, 0.1 mg/mL BSA, and 5% v/v glycerol) for 30, 60, or 120 min at 30°C.
577 Reactions were stopped by adding 4X LDS loading dye (Thermo Fisher Scientific, #NP0007)
578 supplemented with 4% 2-Mercaptoethanol (Sigma-Aldrich #M3148) to a final concentration
579 of 1X LDS and heating at 95°C for 5 min. The reactions were then loaded onto 16.5 % SDS-
580 PAGE gels and ran on ice for 120 min at 160 V in 1X Tris-glycine buffer. Gels were stained
581 with InstantBlue Coomassie protein stain (Expedeon, #ISB1L) before vacuum-drying for 1 h
582 at 60°C. Dried gels were then exposed to a storage phosphor screen for six days before
583 acquiring radiograms using a Typhoon 5 Imager (GE Healthcare). All experiments were
584 performed in three independent replicates that were carried out on three separate days.
585 Densitometry was carried out using ImageLab software (Bio-Rad). Values were interpolated
586 from a standard curve using GraphPad Prism 10.2.1 and the background was subtracted,
587 then values plotted with SD displayed.

588 **Figures and Tables**



589

590 **Figure 1. Design of the MoClo Baculo system.** (A) Level 0 plasmids carrying individual
591 parts, each with a different function as indicated. Connectors: designed to connect the left
592 (L) and right (R) parts of elements from multiple Level 1 plasmids. Promoter: a promoter
593 compatible with the intended host organism. N' Tag: optional N-terminal tag. CDS: coding
594 sequence of the protein of interest. C-tag: optional C-terminal tag. Terminator: transcription
595 termination sequence. Promoter and terminator parts are marked with an asterisk if they
596 were designed to connect to an optional N-terminal or C-terminal tag, respectively. 3'Hom
597 and 5'Hom are the 3' and 5' homologous arms aiming for chromosomal integration in *S.*
598 *cerevisiae*. Origin is from an *S. cerevisiae* plasmid (e.g. 2 micron; in case the user does not
599 wish to integrate the expression cassette into a chromosome). Backbone: includes elements
600 critical for the propagation of the plasmid in *E. coli*, such as antibiotic resistance and *E. coli*
601 origin of replication. Parts specific to the baculovirus system (BV) or yeast are indicated in
602 blue and red font, respectively. Parts that were constructed for MoClo Baculo are

603 represented by boxes with a blue border. Parts that originated from the MoClo Yeast Toolkit
604 are represented by boxes of black border. Dashed blue boxes represent the ORF part, which
605 was only partially inherited from the MoClo Yeast toolkit (see Supplementary Fig. 1A), and
606 the C-terminal tag part that follows the same design as the MoClo Yeast toolkit but now
607 includes a broader selection of tags for protein purification. The ampersand (&) operator
608 represents an obligatory selection of parts that were designed to be assembled directly.
609 When parts or elements within brackets are stacked vertically, only one of them should be
610 selected. Bottom: For the assembly of Level 1 plasmids, parts are subjected to Golden Gate
611 assembly using the BsaI restriction enzyme. (B) Level 1 plasmids are transcription units,
612 each capable of expressing a single protein. An exception is where a simple backbone
613 (bottom right in (A)) is used for the assembly of the transcription unit, which leads to a
614 transcription unit that can be assembled into a functional Level 2 expression vector, although
615 the Level 1 plasmid is not designed to function as an expression vector by itself. Multiple
616 Level 1 vectors, including multiple transcription units and one backbone, are subjected to
617 Golden Gate assembly using the BsmBI enzyme to form one Level 2 vector. In some cases,
618 a 'Linker' plasmid can replace a transcription unit in order to link between another
619 transcription unit to the backbone (bottom in (B)). (C) Level 2 plasmids are multi-gene
620 expression vectors, designed to express a multi-subunit macromolecular complex. The
621 compatible expression system is determined based on the parts selected in (A). Yeast
622 expression vectors were designed to express proteins either after homologous
623 recombination into a chromosome or using a yeast plasmid. The order of the transcription
624 units is determined based on the connectors that were selected in (A).

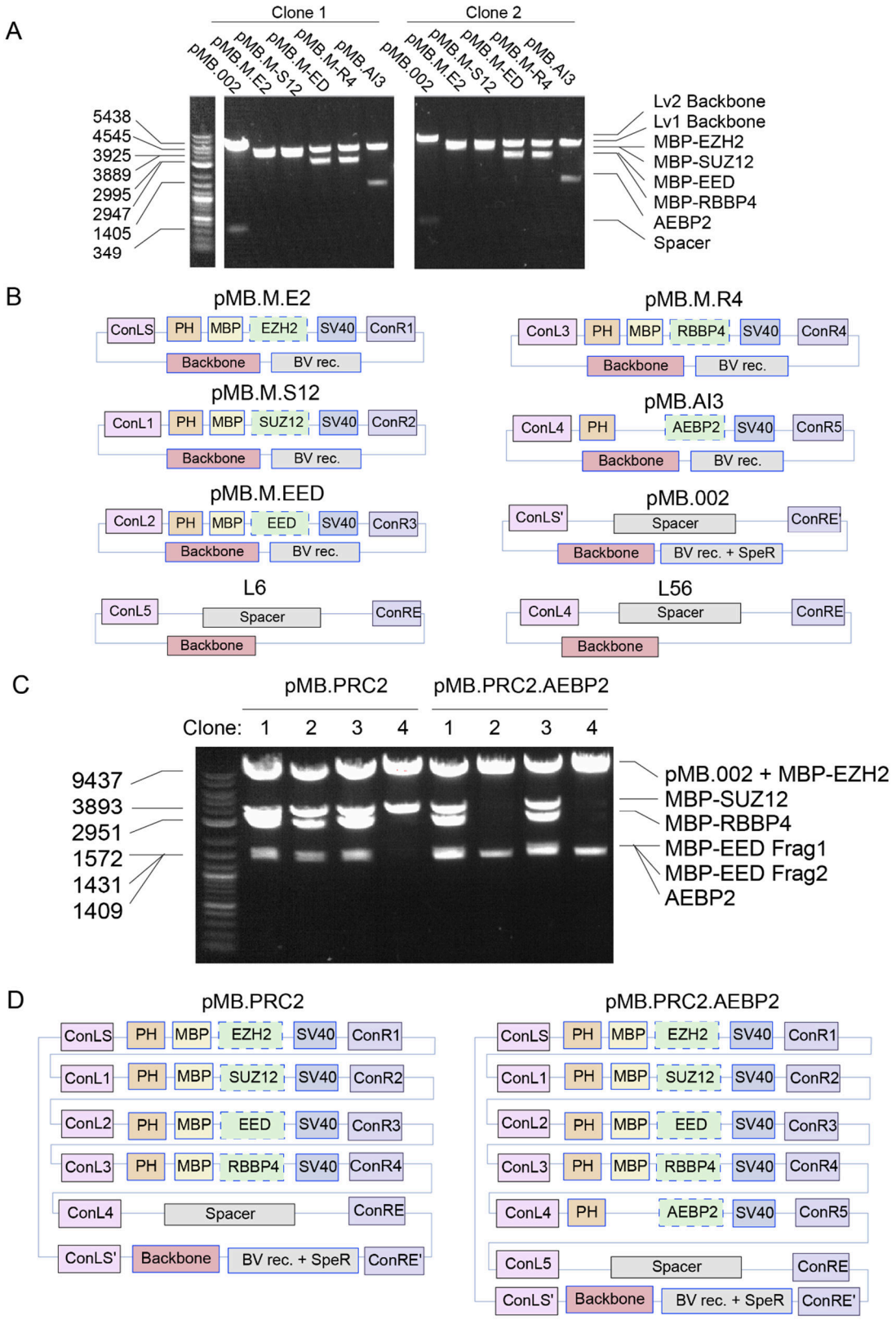


Figure 2. Proof-of-principle: construction of multigene baculovirus expression vectors for the expression of 4- and 5-subunit PRC2 complexes. (A) Golden Gate assembly of Level 1 plasmids was carried out using BsaI and two of the resulting clones were proceeded

629 for restriction digestion using BsmBI. The plasmids pMB.M.E2, pMB.M.S12, pMB.M.ED,
630 pMB.M.R4 and pMB.AI3 are Level 1 baculovirus expression vectors for the expression of
631 EZH2, SUZ12, EED, RBBP4 and AEBP2 (isoform 3). The plasmid pMB.002 is a Level 1
632 plasmid that was assembled to contribute to the backbone during subsequent assemblies of
633 Level 2 plasmids. (B) Schematic representation of the plasmids resulted from the Golden
634 Gate assembly reaction that was analysed in (A). Presented are also two 'linker plasmids',
635 L6 and L56, that are Level 1 plasmids that include a Spacer and Connector parts from the
636 MoClo Yeast toolkit [18], designed to connect the last Transcription Unit to the backbone
637 during the assembly of a Level 2 plasmid. (C) Golden Gate assembly using the Level 1
638 plasmids from (B) was carried out using BsmBI to construct Level 2 plasmids for a
639 baculovirus expression of the 4-subunit PRC2 (left) or the 5-subunit PRC2-AEBP2 (right).
640 Left: pMB.M.E2, pMB.M.S12, pMB.M.ED, pMB.M.R4, pMB.002 and L56 were assembled to
641 form an expression vector coding for PRC2. In this context, the linker L56 was designed to
642 connect the 4th transcription unit to the backbone. Right: PRC2-AEBP2 expression vector
643 was assembled from the same Level 1 plasmids, with the exception that the Level 1 plasmid
644 pMB.AI3 was added to include also the expression of AEBP2 and the L56 linker plasmid was
645 replaced by the L6 linker plasmid, to connect the 5th transcription unit to the backbone. Four
646 clones were selected from each Golden Gate reaction and were subjected to restriction
647 digestion using MfeI and SalI. (D) Schematics of the obtained Level 2 multigene expression
648 vectors, as indicated. In (B) and (D), the colour key is as in Fig. 1. PH: polyhedrin promoter;
649 SV40: SV40 polyA terminator; MBP: maltose-binding protein with PreScission cleavage site;
650 Spacer: DNA sequence that was designed to fill up a gap during vector building (originated
651 from the MoClo Yeast toolkit [18]); Backbone: vector backbone; BV rec and BV rec + SecR:
652 Tn7 recombination sites compatible with the Bac-to-Bac baculovirus expression system, in
653 the presence and absence of Spectinomycin resistance, respectively. The Level 1 plasmids
654 presented in (B) confer Ampicillin and Gentamicin resistance in *E. coli*. The Level 2 plasmids
655 presented in (D) confer Ampicillin, Gentamicin and Spectinomycin in *E. coli*. In (A) and (B),
656 fragments were analysed on 0.7% agarose TAE gel.

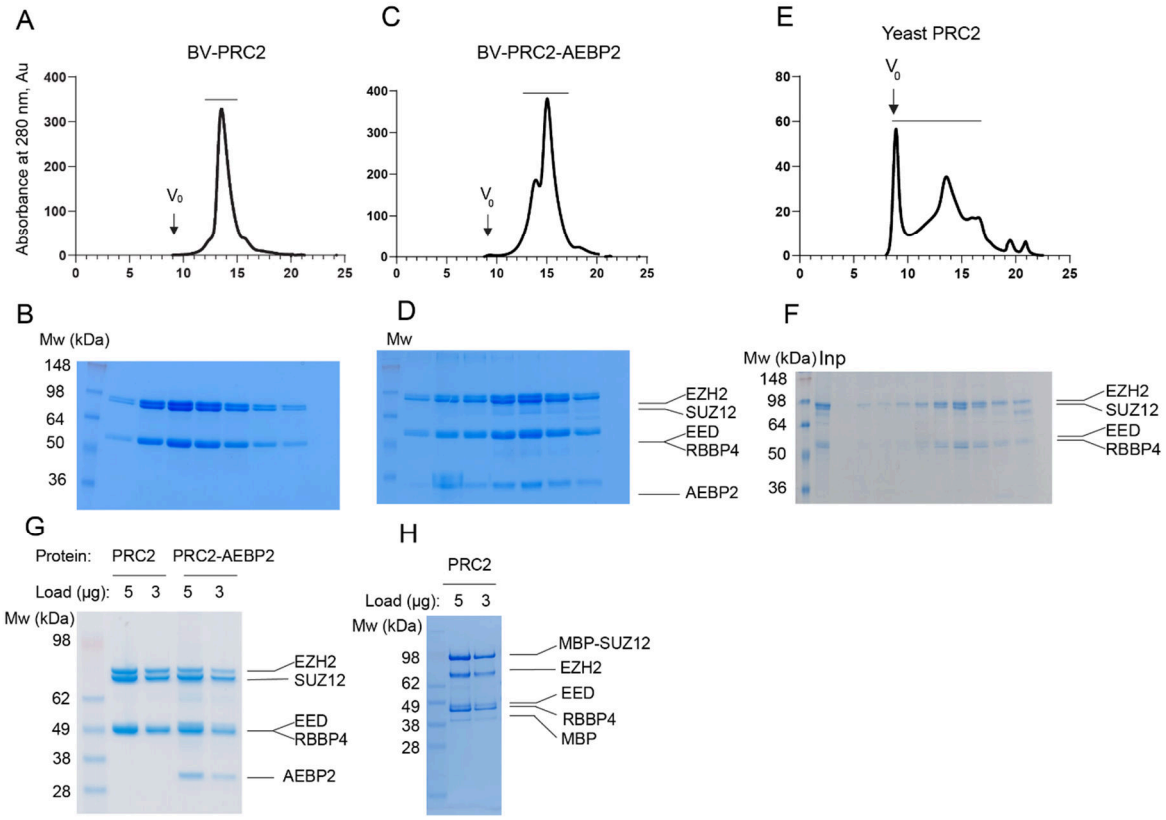


Figure 3. MoClo Baculo-expressed 4- and 5-subunit PRC2 complexes are pure and assembled. (A) PRC2 was expressed from baculovirus (BV) using the pMB.PRC2 multi-gene expression vector (Fig. 2D, left) and was fractionated over a Superose 6 Increase 10/300 column. UV absorbance at 280 nm was plotted to trace protein elution. The void volume is marked as V_0. The black horizontal line above the peak indicates fractions that were taken for SDS-PAGE analysis in (B). (B) PRC2 gel filtration fractions from (A) were analysed by SDS-PAGE (Tris-Glycine, 12% acrylamide) and stained by coomassie. (C) PRC2-AEBP2 expressed using pMB.PRC2.AEBP2 construct (Fig. 2D, right) was analysed by Superose 6 Increase 10/300 column. (D) Fractions marked by the black line in (C) were analysed by SDS-PAGE (Tris-Glycine, 12% acrylamide). (E) A PRC2 yeast expression construct (pMY.PRC2; see supplementary Fig. 2 for vector construction) was integrated into the genome of *S. cerevisiae* using homologous recombination for protein expression and then PRC2 that was purified and fractionated over a Superose 6 Increase 10/300 column. (F) Fractions that are marked by the horizontal black line in (E) were analysed on an SDS-PAGE (Tris-Glycine, 12% acrylamide). “Inp” indicates a sample before the size exclusion chromatography. (G) Purified PRC2 and PRC2-AEBP2 from (A) and (C), respectively, were analysed on SDS-PAGE (Bis-Tris, 4%-12% acrylamide) after fractions were pooled and proteins were concentrated. (H) Amylose affinity purification of PRC2 from lysate of SF9

676 cells that were infected with baculovirus carry the pMB.BIG1a.PRC2 vector (Supplementary
677 Fig. 5B), analysed on SDS-PAGE (Bis-Tris, 8%-16% acrylamide).
678

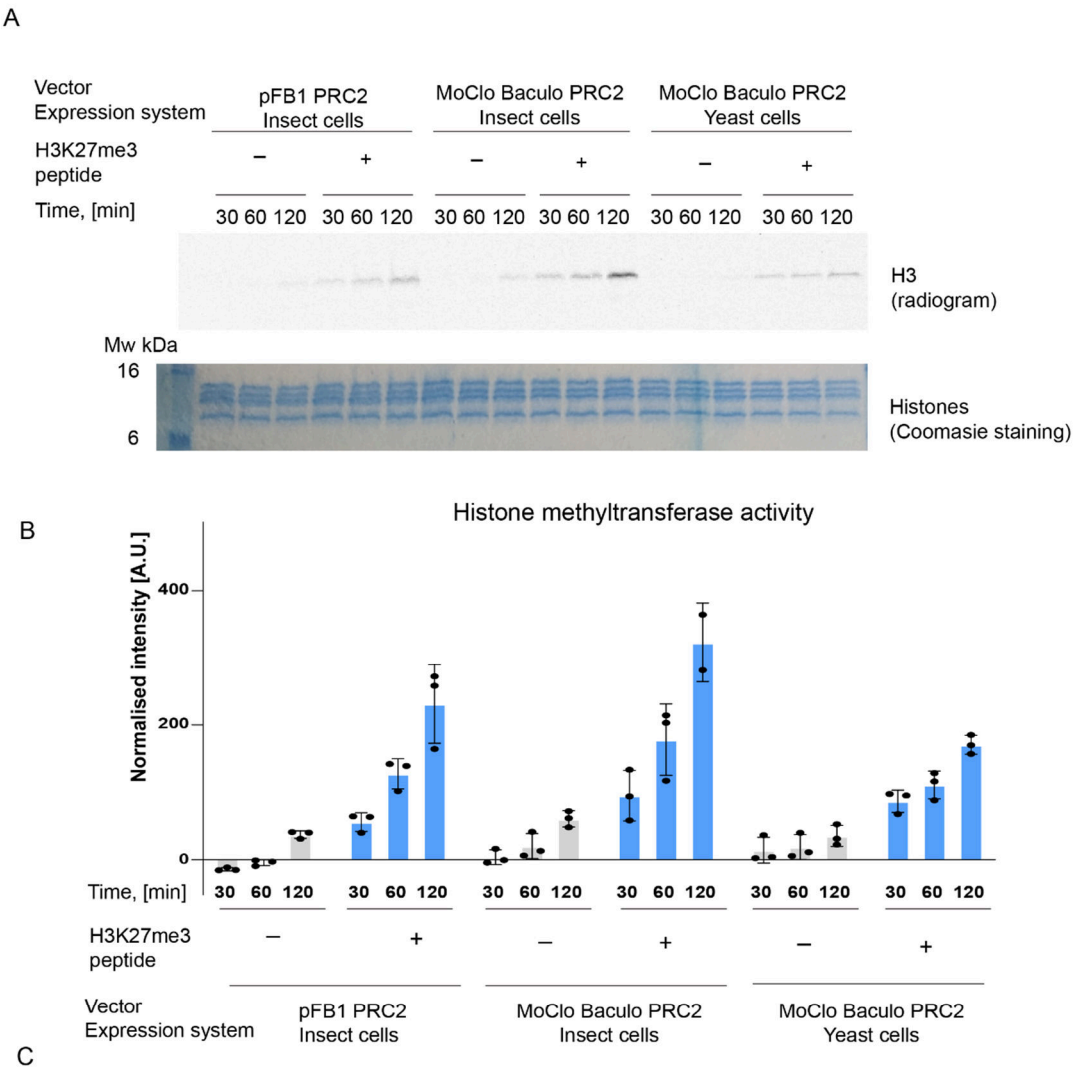


Figure 4. MoClo Baculo-expression vectors designed either for the baculovirus system or for yeast produced recombinant human PRC2 complexes that are enzymatically active. A. HMTase assay performed with 600 nM PRC2, 600 nM

683 mononucleosomes, 5 μM S-Adenosyl-L-methionine [methyl-^{14}C] and in the presence or
684 absence of 50 μM H3K27me3 peptide, as indicated. The reaction mixture was separated
685 with SDS-PAGE, stained with coomassie and exposed to a phosphor screen to produce a
686 radiogram. (B) The bar plot (bottom) represents the relative HMTase activities of PRC2 in
687 the absence (grey) or presence of H3K27me3 (blue) peptides, as indicated. Individual
688 observations are presented, mean represents the normalised densitometry and error bars
689 represent standard deviation of three independent experiments that were carried out on
690 different days. (C) Equal amounts (1 or 3 μg) of the PRC2 complexes that were used for the
691 HMTase assays were analysed on an SDS-PAGE (Tris-Acetate, 3%-8% acrylamide). In all
692 panels: “insect” and “yeast” referred to protein that was expressed using the baculovirus
693 expression system or *S. cerevisiae*, respectively. “pFB1" referred to a PRC2 complex that
694 was expressed by the co-infection of 4 baculovirus stocks, each made of one pFastBac1
695 plasmid coding for one protein. “MoClo Baculo" referred to PRC2 complexes that were
696 expressed from either baculovirus or yeast expression vectors, as indicated, constructed
697 using parts of the MoClo Baculo toolkit.
698
699

	Baculovirus	**Yeast**
Time required to build a multi-gene (Level 2) plasmid	5 days	5 days
Protein expression [a]	20-30 days	6-7 days
Yield per litre (PRC2)	3-5 mg	0.03-0.05 mg
Cost of protein expression [a]	$ 17/mg	$ 500-830/mg
Expression cost per batch [a]	$ 270 per 4 L batch	$ 300 per 12 L batch [b]
Estimated labour per batch [c]	4.6 h per 4 L batch	4.8 h per 12 L batch

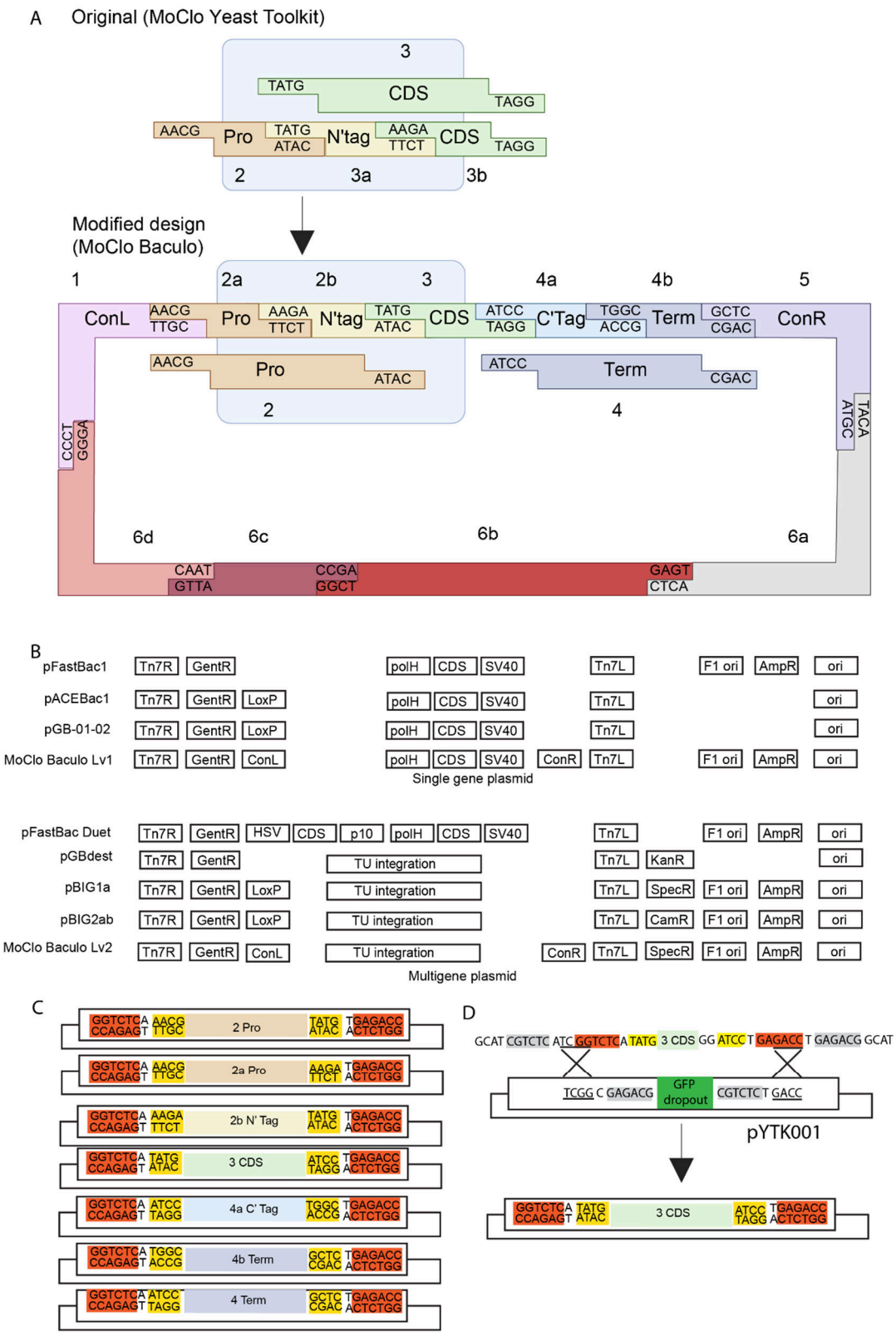
700
701 **Table 1. Comparison between the Baculovirus system to yeast in terms of protein**
702 **expression.** Values represent the best estimate based on the expression of the 4-subunit
703 PRC2 complex from multigene expression vectors generated using MoClo Baculo.
704 [a] ‘Protein expression’ and ‘Expression cost’ are defined here as the process from the stage
705 the plasmid is made until the cells that expressed the protein are pelleted and collected, but
706 before the protein purification process has started. For the baculovirus system, this process

707 includes the preparation, amplification and quantification of the baculovirus particles, insect
708 cell culture buildup, infection, incubation during the protein expression period and cell
709 harvest. For the yeast system, this process includes transformation and colony growth,
710 media prep, culture starter and inoculation, induction, growth and cell harvest. Expression
711 trial in yeast was carried out using an *S. cerevisiae* line that was generated through the
712 homologous recombination of the multi-gene expression vector into a chromosome.
713 Estimated costs are provided in US dollars.
714 [b] In this work, protein in *S. cerevisiae* was carried out using a galactose-inducible promoter
715 on one of the PRC2 subunits (EZH2). This strategy added a substantial cost because of the
716 usage of lactate (expression suppressor) and galactose (expression inducer). A usage of
717 constitutive promoter, when feasible, would avoid galactose and lactate and therefore
718 reduce the expression cost by about 50%.
719 [c] Labour was estimated in the form of cumulative working hours that were dedicated to
720 the process of protein expression, as defined in [a].

	MoClo Baculo	Multibac [12]	Bac-to-Bac [6]	BiGBac [14]	GoldenBac [15]
Expression system	BV and Yeast (*S. cerevisiae*)	BV	BV	BV	BV
Modular Cloning	Yes	No	No	Modular for the multigene level, but not for building the transcription units.	Modular for the multigene level, but not for building the transcription units.
PCR or Gibson Assembly required	N*	Y	Y	Y	Y
Maximal number of ORFs per expression vector	6 (or 30 through compatibility with biGBac)**	Theoretically unlimited	2	25	15

721 **Table 2. Comparison between MoClo Baculo to other baculovirus expression**
722 **systems.**
723 *The construction of up to Level 2 plasmids with up to 6 transcription units is done using
724 Golden Gate assembly, even if the Level 2 plasmids are compatible with the biGBac system.
725 Yet, the optional connection of multiple biGBac-compatible Level 2 plasmids with up to 6
726 transcription units each into a single Level 3 plasmids of up to 30 transcription units is done
727 using the biGBac system and required Gibson Assembly.
728 **Pre-build parts support up to 6 ORFs per MoClo Baculo vector (Level 2) and up to 30
729 ORFs if biGBac compatible parts are used to connect multiple Level 2 vectors into biGBac
730 vectors (Level 3 here). Yet, the number of ORFs per MoClo Baculo vectors (Level 2), and
731 therefore the number of ORFs in Level 3 vectors, can be expended if more MoClo Baculo
732 Connector parts are designed, as in the case of the MoClo Yeast Toolkit [18]. However, we

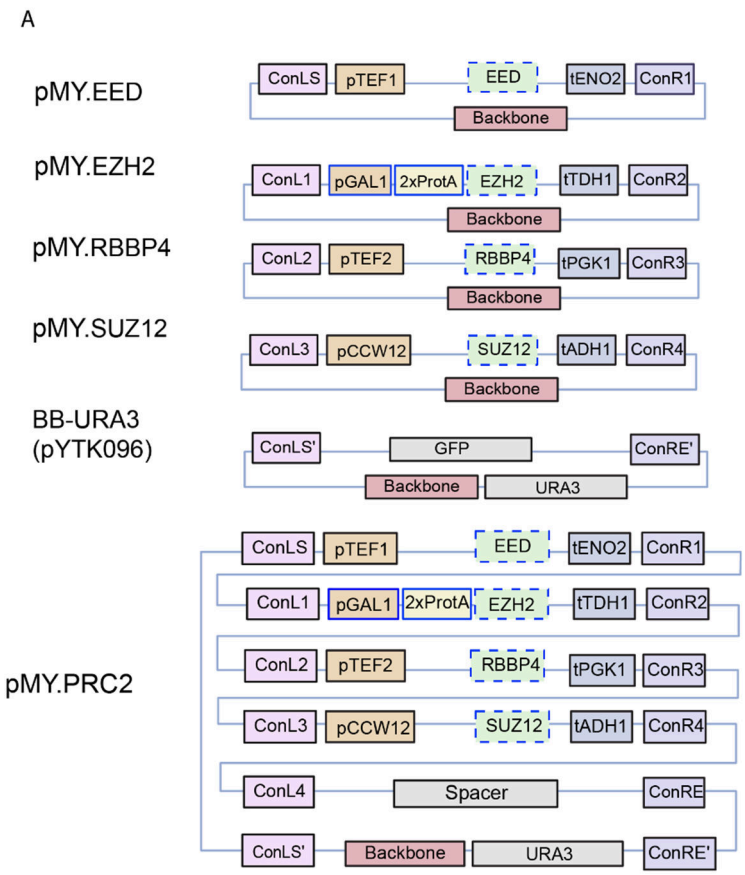
733 find that the Golden Gate assembly of Level 2 plasmids becomes very inefficient when the
734 final product size approaches 30 kbp.
735



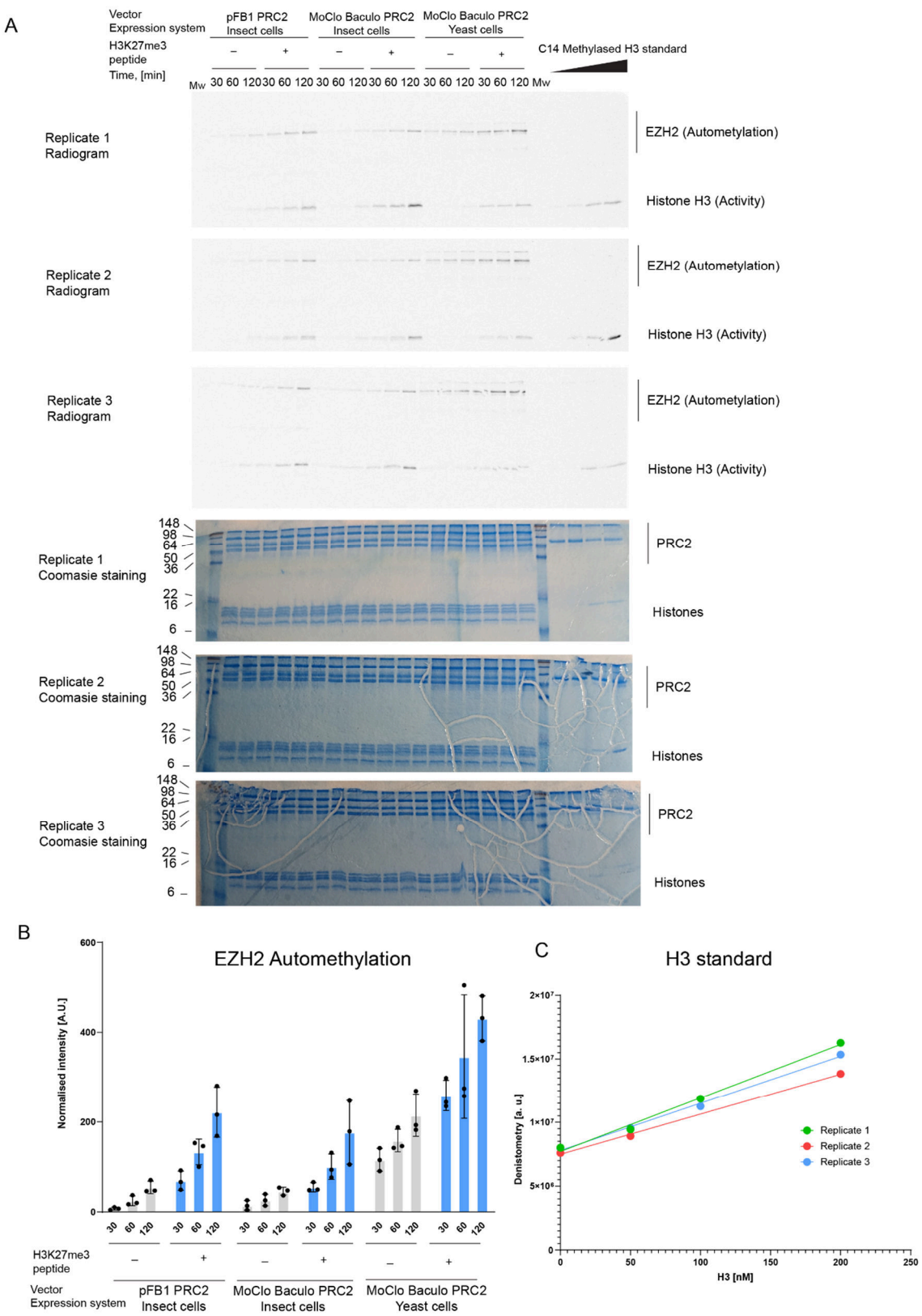
Supplementary Figure 1. Level 1 plasmid architecture of the MoClo toolkit. (A) The MoClo Baculo Level 1 plasmid design is illustrated, including the left connector (ConL), promoter (Pro), N-terminal tag (N'tag), coding sequence (CDS), C-terminal tag (C'Tag), terminator (Term) and the right connector (ConR). Changes that were made to the original

741 MoClo Yeast toolkit design [18] in order to enable the usage of the same CDS part either in
742 the presence or absence of an N-terminal tag are indicated above (highlighted in blue
743 boxes). Presented are also the four base pair overhangs that are generated in each part
744 after BsaI digestion. The parts are numbered according to the MoClo Yeast numbering
745 system, with the exception of parts that were modified (in the blue box) and the backbone
746 parts that uses the same overhangs as in the MoClo Yeast toolkit but are all collectively
747 numbered as part 6 for simplicity (specifically, the MoClo Baculo parts 6a, 6b, 6c and 6d
748 were originally termed parts 6, 7, 8a and 8b, respectively, in the Moclo Yeast toolkit). Parts
749 2-4 are related to the expressed gene, part 6 allows recombination into baculovirus DNA or
750 into the yeast genome, and parts 1 and 5 are for the integration into multi-gene vectors
751 (Level 2 plasmids). (B) A comparison between the organisation of different functional
752 modules of various baculovirus expression vectors that are compatible with the Bac-to-Bac
753 expression system for the expression of single (top) or multiple (bottom) genes and the
754 single- (Level 1; “Lv1”) and multi-gene (Level 2; “Lv2”) MoClo Baculo expression vectors.
755 Presented are the Tn7 left and right recombination sites (Tn7R and Tn7L), gentamicin,
756 kanamycin, spectinomycin, ampicillin and chloramphenicol resistance (GenR, KanR, SpecR,
757 AmpR and CamR), LoxP recombination site (LoxP), left and right connectors for Golden
758 Gate assembly (ConL and ConR), a region that hosts multiple genes or transcription units
759 (TU integration), Simian virus polyadenylation signal (SV40), Herpes Simplex Virus
760 thymidine kinase polyadenylation signal (HSV), coding sequence (CDS), baculovirus p10
761 and polyhedrin promoters (p10 and polH), f1 origin (F1 ori), and pUC origin (ori). (C)
762 Flanking sequence of promoter (Pro), N-terminal tag (N Tag), coding sequence (CDS), C-
763 terminal tag (C’Tag), terminator (Term) within the context of Level 0 vectors. BsaI restriction
764 site is highlighted in orange and the overhang in yellow. Overhang sequences are inherited
765 from the MoClo yeast toolkit, with the exception of parts 2a, 2b and 3, which were
766 redesigned. (D) In order to generate Level 0 plasmids that holds a given part (presented in
767 panel (C)), the part can be obtained as a double strand DNA (e.g. as a gene fragment or a
768 plasmid from gene synthesis) while including flanking sequences suitable for its Golden Gate
769 Assembly into the pYTK001 plasmid (originated from the MoClo Yeast Tool Kit [18]) using
770 BsmBI. As an example, presented are the complete flanking sequences that are added to a
771 CDS part (“3 CDS” representing an open reading frame sequence) that is ordered from gene
772 synthesis. BsmBI restriction sites are highlighted in grey, the overhang for assembly with
773 pYTK001 is underlined and the rest of the colours are as in panel (C). The black crosses
774 represent the site of ligation during BsmBI golden gate reaction.

775



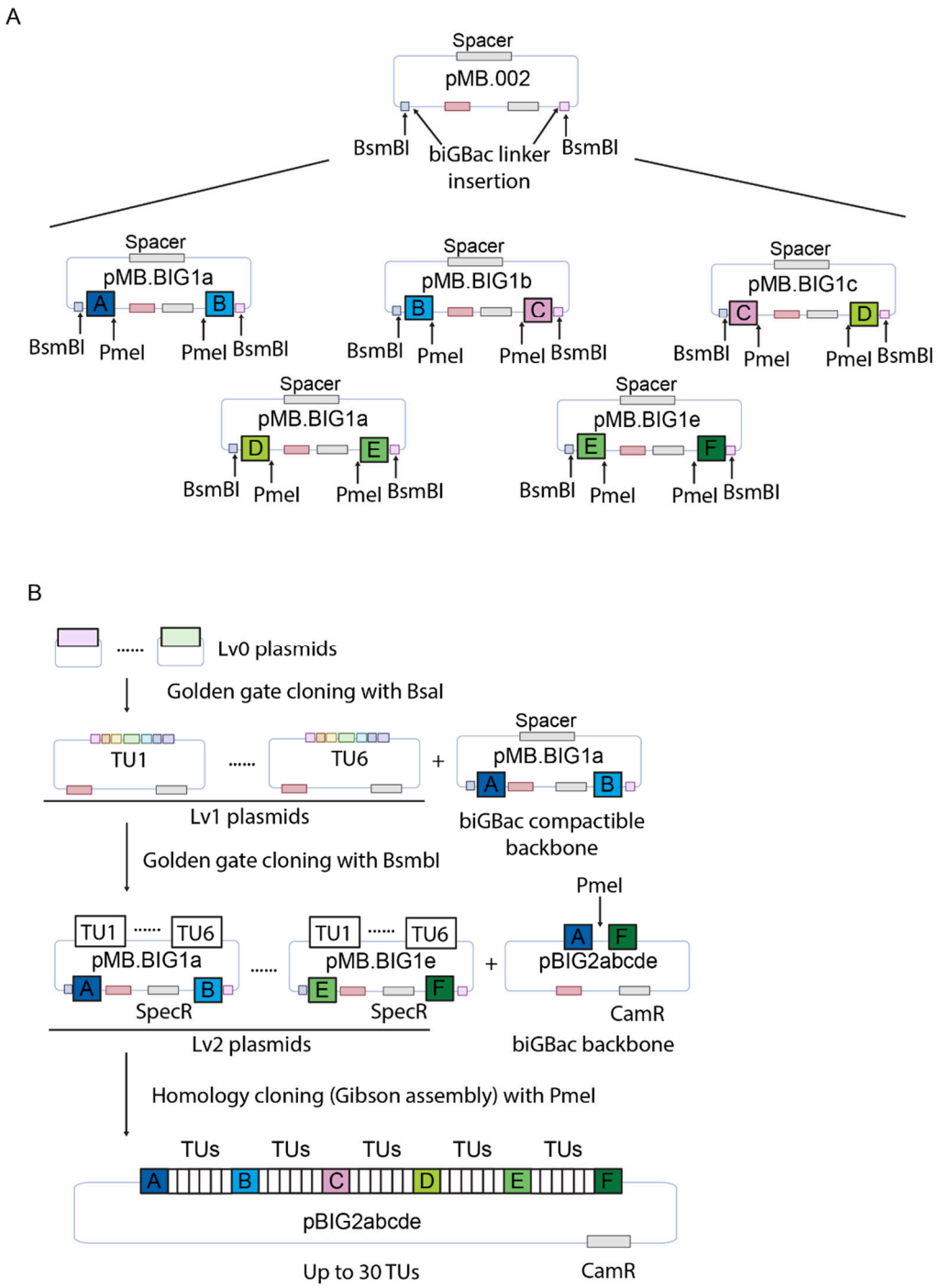
Supplementary Figure 2. A construct design of a yeast expression plasmid. Schematic of the Level 1 and Level 2 constructs that were made using parts of the MoClo Baculo and MoClo Yeast toolkits [18] for the expression of the human PRC2 in *S. cerevisiae*. Parts from the MoClo Yeast toolkit are in black boxes and parts of the MoClo Baculo (parts 2b and 2a; see in Supplementary Fig. 1A) are in blue boxes. Dashed blue boxes represent the same CDS parts that were also used to construct baculovirus expression vectors in Fig. 2. “BB-URA3” mark a backbone plasmid that originated from the MoClo Yeast toolkit [18] (pYTK096), aiming to enable genomic integration into the *URA3* locus of the *S. cerevisiae* chromosome, using selection by uracil prototrophy.



Supplementary Figure 3. Human PRC2 complexes expressed using MoClo Baculo vectors in insect cells and in yeast are enzymatically active. (A) Uncropped radiogram

790 and coomassie stained gels from three independent replicates of HMTase activity assay
791 that were carried out on three different days (the cropped gels are in Fig. 4A). The ^{14}C-
792 labelled H3 standard that is used for normalisation between different replicates is shown on
793 the right side of the gel. B. The EZH2 automethylation signal was quantified using ^{14}C-
794 labelled H3 standards and the values were plotted as a bar chart. C. H3 standard curves that
795 were used to normalise data from independent replicates of HMTase activity assays. The
796 standard curves of the different replicates are presented, with colour key as indicated.

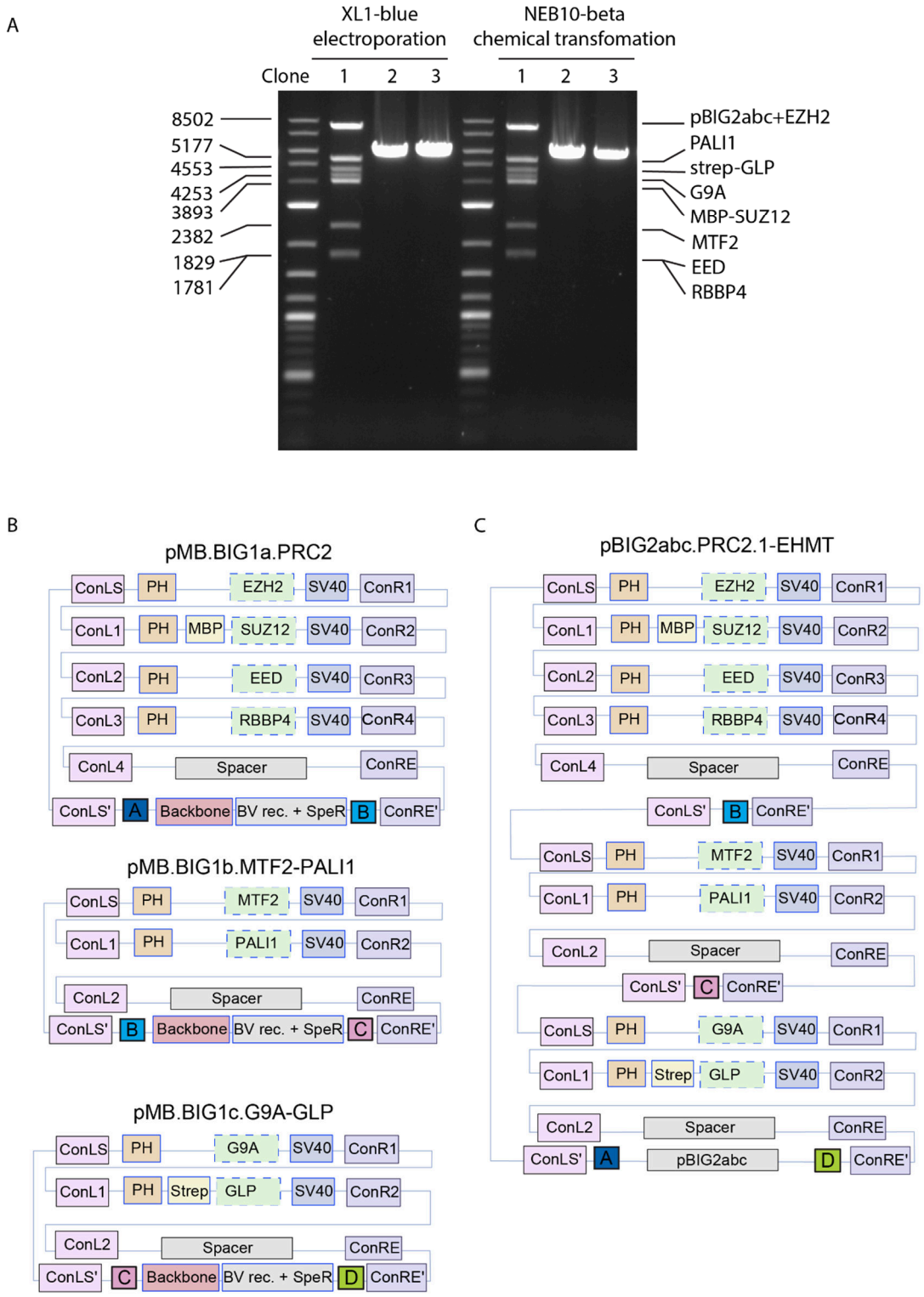
797



798

799 **Supplementary Figure 4. MoClo Baculo level 2 plasmids can be connected into larger**
800 **Level 3 plasmids through compatibility with the biGBac system.** (A) pMB.BIG1a to
801 pMB.BIG1e plasmids (bottom) were generated by inserting biGBac linker sequence (box
802 marked with A to F) and PmeI site between the pMB.002 plasmid backbone and BsmBI site.
803 (B) The workflow of constructing biGBac compatible level 2 vectors is essentially the same
804 as other MoClo Baculo vectors, except that the pMB.BIG1a to pMB.BIG1e plasmids are

805 used as Level 2 backbone instead of the pMB.002 plasmid. Colour key is as in Fig. 1 with
806 addition of biGBac linker sequences A to F in assorted colours. TU: transcriptional unit; TUs:
807 BsmBI golden gate ligated transcriptional units; SpecR: spectinomycin resistance; CamR:
808 chloramphenicol resistance.



809

810 **Supplementary Figure 5. Proof-of-principle: Construction of a Level 3 vector through**
811 **compatibility between the MoClo Baculo to the biGBac system. (A)** Gibson assembly of

812 pMB.BIG1a.PRC2, pMB.BIG1b.MTF2-PALI1 and pMB.BIG1c.G9A-GLP plasmids were
813 carried out using pBIG2abc as backbone. The final plasmid, pBIG2abc.PRC2.1-EHMT, is
814 validated by restriction digestion using MfeI and PmeI. Chemical- and electroporation
815 transformation were benchmarked here. Clone 1 are correct clones, while 2 and 3 are empty
816 pBIG2abc. (B) Schematic representation of the plasmids used for the Gibson assembly
817 reaction that was analysed in (A). (C) Schematic representation of the plasmids produced by
818 the Gibson assembly reaction was analysed in (A).

819

820 **Legends for Supplementary Tables and Data**

821 **Supplementary Table 1. Compatibility between parts for the construction of**
822 **expression vectors for baculovirus and yeast.**

823 Tabulated are relevant parts (level 0 plasmids) from the MoClo Baculo and MoClo yeast
824 toolkits, where the latter are indicated by an asterisk. Part numbers and sub-part numbers
825 are as in Supplementary Fig 1A. Expression system-specific parts, either for the baculovirus
826 (BV) system or yeast, are clustered together (see left column). All the tags, coding
827 sequences (CDS) and connector parts of the MoClo Baculo system are compatible with
828 vector building for both yeast and baculovirus expression. Promoter, terminator and
829 backbone parts are system-specific.

830 **Supplementary Table 2. Plasmids that were generated during this study.** Indicated are
831 plasmids that were constructed during this study, including new parts (Level 0 plasmids) and
832 expression vectors (Level 1 and 2 plasmids). Excluded are vectors that were constructed as
833 intermediate products of multi-gene vector buildings for the yeast expression system, as they
834 were not designed to express proteins.

835 **Supplementary Table 3. Sequences of ssDNAs that were used to generate linker**
836 **sequences for compatibility with the biGBac system.** Indicated are the sequences that
837 were added to generate the modified ConLS'A to ConLS'E and ConRE'B to ConRE'F parts
838 that were used as precursors for generating the biGBac compatible plasmids pMB.BIG1a to
839 pMB.BIG1e, respectively.

840 **Supplementary Data 1. Plasmid sequences.** Sequences of baculovirus-specific Level 0
841 plasmids that were generated during this study are included within a zipped directory as
842 GenBank files.

843 **Supplementary Data 2. Plasmid sequences.** Sequences of yeast-specific Level 0
844 plasmids that were generated during this study are included within a zipped directory as
845 GenBank files.

846 **Supplementary Data 3. Plasmid sequences.** Sequences of Level 0 plasmids that are used
847 for both yeast and the baculovirus system and that were generated during this study are
848 included within a zipped directory as GenBank files.

849 **Supplementary Data 4. Plasmid sequences.** Sequences of ORFs as Level 0 plasmids that
850 were synthesised during this study are included within a zipped directory as GenBank files.

851 **Supplementary Data 5. Plasmid sequences.** Sequences of single-gene and multi-gene
852 baculovirus expression vectors (Level 1 and Level 2) plasmids, respectively, that were
853 generated during this study are included within a zipped directory as GenBank files.

854 **Supplementary Data 6. Plasmid sequences.** The sequence of a yeast expression
855 multigene plasmid (Level 2) that was generated during this study is included within a zipped
856 directory as GenBank files.

857 **Reference**

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