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Multiplex genome editing in *Ashbya gossypii* using CRISPR-Cpf1

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ABSTRACT

CRISPR/Cas technologies constitute essential tools for rapid genome engineering of many organisms, including fungi. The CRISPR/Cas9 system adapted for the industrial fungus *Ashbya gossypii* enables efficient genome editing for the introduction of deletions, insertions and nucleotide substitutions. However, the Cas9 system is constrained by the existence of a specific 5′-NGG-3′ PAM sequence in the target site. Here we present a new CRISPR/Cas system for *A. gossypii* that expands the molecular toolbox available for microbial engineering of this fungus. The use of Cpf1 nuclease from *Lachnospiraceae bacterium* allows a T-rich PAM sequence (5′-TTTN-3′) to be employed and facilitates implementation of a multiplexing CRISPR/Cpf1 system adapted for *A. gossypii*. The system has been validated for the introduction of large deletions with five different auxotrophic markers (*HIS3*, *ADE2*, *TRP1*, *LEU2* and *URA3*). The use of both crRNA and dDNA arrays in a multi-CRISPR/Cpf1 system is demonstrated to be an efficient strategy for multiplex gene deletion of up to four genes using a single multi-CRISPR/Cpf1 plasmid. Our results also suggest that the selection of the target sequence may affect significantly the editing efficiency of the system.

Introduction

A. gossypii is a filamentous fungus that is currently exploited industrially for the production of riboflavin [1]. It has also been proposed as a microbial cell factory for production of folic acid, nucleosides, recombinant proteins, γ-lactones and biolipids [2–6]. The development of novel molecular tools is essential for the implementation of rational system metabolic engineering approaches in *A. gossypii* and a complete toolbox for its genomic manipulation, including an adapted CRISPR/Cas9 system [7–9].

CRISPR/Cas9 systems have emerged as the foremost technique for genome engineering of many organisms, including yeasts and fungi, with applications that go further beyond the single gene modification (deletions and nucleotide substitutions) [10]. Thus, gene regulation and systems metabolic engineering approaches have been described using CRISPR/Cas9 systems in different yeasts and fungi [10,11].The CRISPR/Cas9 system for *A. gossypii* shows a high editing efficiency for introduction of gene deletions, insertions and nucleotide substitutions, which largely facilitates genomic engineering of the fungus in a markerless manner [8]. The efficiency of the system in a multinucleated syncytium such as *A. gossypii* mycelia relies on a one-vector strategy comprising (i) the expression modules for the CAS9 and the single guide RNA (sgRNA), and (ii) the donor DNA (dDNA) that can be directly used from the plasmid for homologous recombination (HR) repair of double-strand breaks (DSBs). The expression of the sgRNA is driven by regulatory sequences from the *A. gossypii SNR52* gene, which is transcribed by RNA Polymerase III.

CRISPR/Cas-mediated genome editing has largely transformed microbial engineering approaches. However, there are some limitations of these technologies regarding the editing efficiency variation between genomic sequences, off-target effects and protospacer adjacent motif (PAM) sequence restrictions [12]. The *A. gossypii* CRISPR/Cas9 system is also restricted to the presence of a 5′-NGG-3′ PAM sequence on the genomic target to generate a double-strand break. Also, multiplexing engineering for the simultaneous editing of different targets and metabolic pathways has not been described for *A. gossypii*.

Cpf1 (recently renamed as Cas12a) is a class 2/type V RNA-guided endonuclease discovered in several bacterial genomes and one archaeal genome [13,14]. Cpf1-mediated genome editing has been described in bacteria, yeasts, plants, insects and vertebrates, including human cells [15–22]. Cas9 and Cpf1 differ in evolutionary origin and also show significant structural differences (Fig. 1), resulting in different

Abbreviations: CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; dDNA, donor DNA; HR, homologous recombination; DSBs, double-strand breaks; PAM, protospacer adjacent motif; gRNA, guide RNA; sgRNA, single guide RNA; crRNA, CRISPR RNA; tracrRNA, trans-activating crRNA; SMM, synthetic complete minimal media; ORF, open reading frame; SPA, sporulation media

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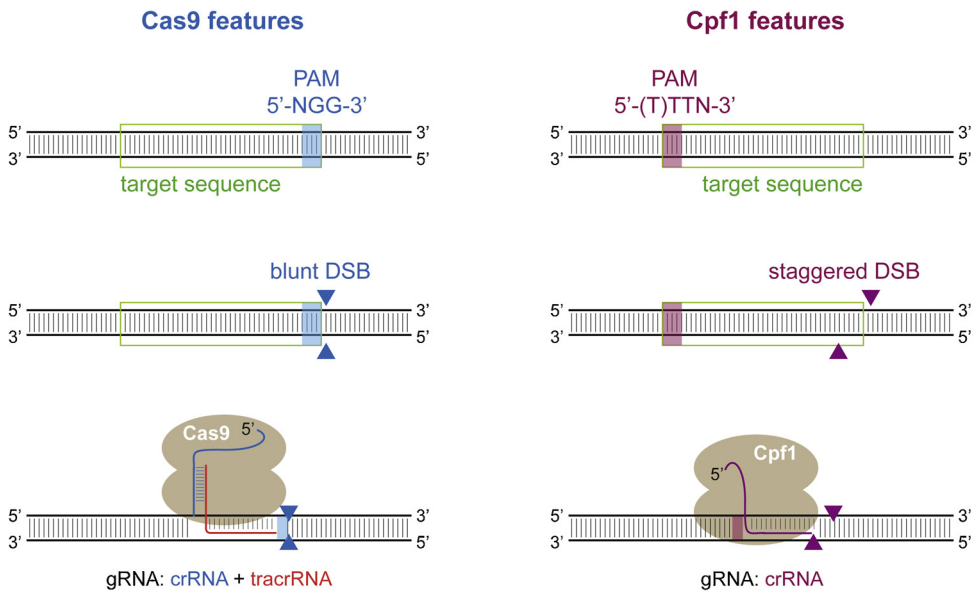

Fig. 1. Principal differences between the CRISPR/Cas9 and CRISPR/Cpf1 editing systems. Differential features of CRISPR/Cas9 (right) and CRISPR/Cpf1 (left): the location and sequence of the PAM within the target region, the type of DNA cleavage, and the structure of the gRNA.

molecular mechanisms and genome editing features [12].

a) Cpf1 recognizes T-rich PAM sequences, i.e. 5′-TTTN-3′ (AsCpf1, LbCpf1) and 5′-TTN-3′ (FnCpf1), in contrast to the G-rich PAM sequence (5′-NGG-3′) of Cas9 [14].
b) Cpf1-PAM sequences are located at the 5′ end of the target DNA sequence, instead of the 3′ end for Cas9-PAM sequences [12].
c) Cpf1 cleaves DNA after the +18/+23 position of the PAM, creating a staggered DNA overhang, whereas Cas9 cleaves DNA close to its PAM after the -3 position of the protospacer at both strands and creates blunt ends [12].
d) Cpf1 is guided by a single CRISPR RNA (crRNA) and does not require a trans-activating crRNA (tracrRNA), resulting in a shorter guide RNA (gRNA) sequence than the single guide RNA (sgRNA) used by Cas9 [12].
e) Cpf1 displays an additional RNase activity that functions in crRNA processing [23]. This might simplify multiplex genome editing, as demonstrated in [22] where a single crRNA array was used to edit up to four genes simultaneously in mammalian cells.

A G-rich PAM sequence corresponding to the Cas9 can be frequently found in the *A. gossypii* genome, which shows a GC content of 52 % [24]. However, the use of CRISPR/Cas9 can be challenging for genome editing of AT-rich regions, where the use of Cpf1 nuclease has been shown to be more effective [12].

Here we report a CRISPR/Cpf1 system adapted for *A. gossypii*. The use of a Cpf1 endonuclease allows implemention of a multiplex genome editing system that was efficient in simultaneous editing of the auxotrophic markers *HIS3*, *ADE2*, *TRP1*, *LEU2* and *URA3*.

Materials and methods

A. gossypii strains and growth conditions

The wild-type *A. gossypii* ATCC 10,895 strain was used. *A. gossypii* cultures were carried out at 28 °C in MA2 rich medium [25]. Auxotrophic mutants were analyzed in synthetic complete minimal media (SMM) complete minimal media lacking the corresponding nutritional requirement (SMM-his, SMM-ade, SMM-trp, SMM-leu, SMM-ura) [26]. *A. gossypii* transformation, sporulation and spore isolation were performed as described previously [25]. Geneticin (G418) (Gibco-BRL/Fisher Scientific, Madrid, Spain) was used where indicated at concentrations of 250 mg/L.

Assembly of the CRISPR/Cpf1 system for A. gossypii

The CRISPR/Cpf1 system was assembled in a single vector containing all the required modules for genomic editing. The *A. gossypii* CRISPR/Cas9 vector was used as a backbone that included the replication origins (yeast 2μ and bacterial ColE1) and the resistance markers (Amp^R and $G418^R$) [8]. The donor DNA and the modules for the expression of Cpf1 and crRNAs were assembled as follows. A synthetic codon-optimized open reading frame (ORF) of the Cpf1 enzyme from *Lachnospiraceae bacterium* (LbCpf1) with a SV40 nuclear localization signal was assembled with the promoter and terminator sequences of the *A. gossypii TSA1* and *ENO1* genes, respectively. The *TSA1* promoter provides strong constitutive expression, comparable to the widely used P_{GPD1} [9]. The expression of the crRNAs was driven by the promoter and terminator sequences of the *A. gossypii SNR52* gene, which is transcribed by RNA Polymerase III. Synthetic donor DNAs comprising the corresponding genome edited mutants were also assembled in the CRISPR/Cpf1 vector (Fig. 2A). The assembly of the fragments was achieved following a Golden Gate assembly method as previously described [9]. Briefly, a directional cloning strategy was used, introducing *Bsa*I sites at the ends of the fragments. The *Bsa*I sites are flanked by sequences of 4-nucleotide (nt) sticky ends. Hence, after *Bsa*I digestion, all the modules contain compatible 4-nt sticky ends that facilitate a single-step directional assembly of the CRISPR/Cpf1 vector. The sequences of all the modules are described in Supplementary Material Table S1.

CRISPR/Cpf1 genome editing in A. gossypii

Spores of indicated strains of *A. gossypii* were transformed with 5 − 15 μg of the corresponding CRISPR/Cpf1 plasmid. Heterokaryotic transformants were selected in G418-containing MA2 media. The G418

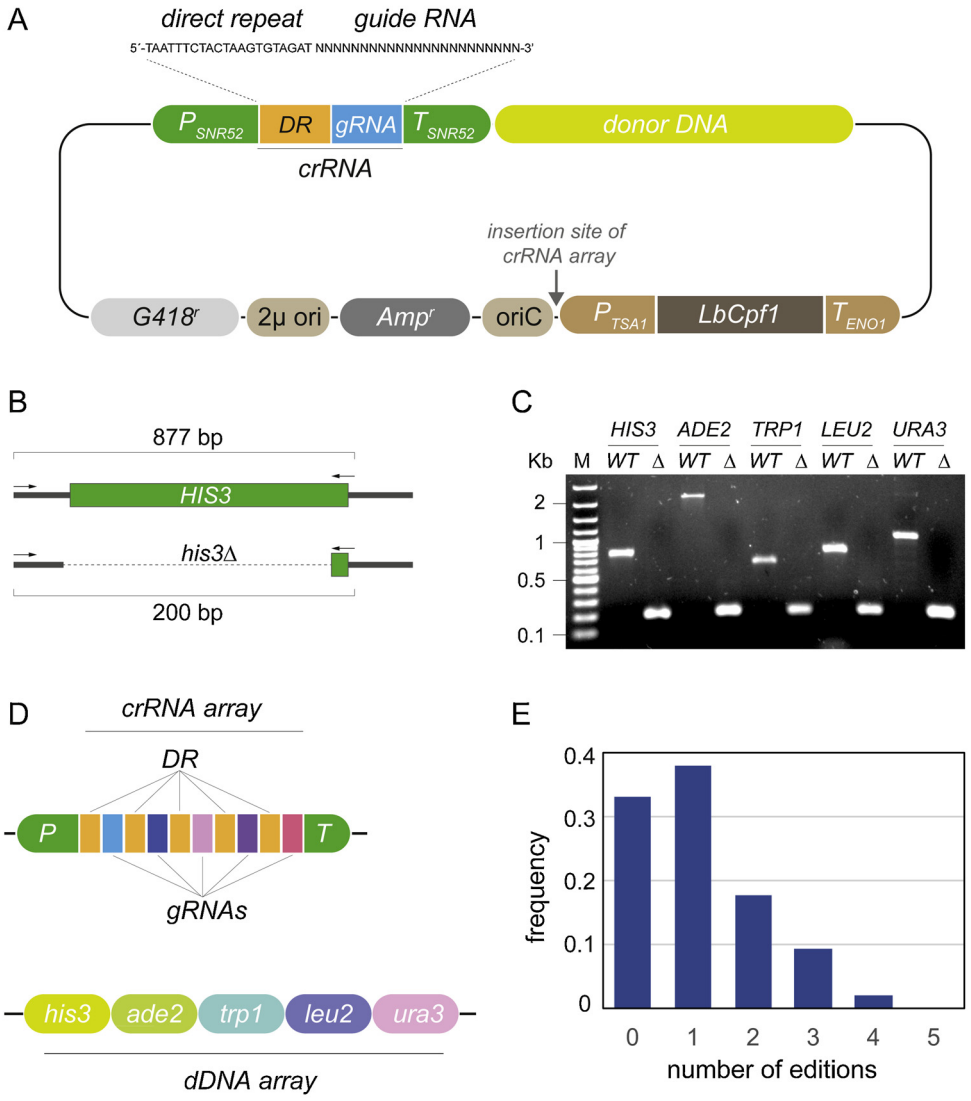

Fig. 2. The CRISPR/Cpf1 system adapted for *A. gossypii*. (A) Modular design of the CRISPR/Cpf1 vector. (B) Strategy for analytical PCR of homokaryotic clones after CRISPR/Cpf1-HIS3 editing. The PCR primers were designed to amplify different fragments from the WT *HIS3* (877 bp) and the *his3*Δ (200 bp) loci. The same strategy was used for the *ADE2*, *TRP1*, *LEU2* and *URA3* loci. (C) Analytical PCR of homokaryotic auxotrophic clones after the CRISPR/Cpf1 editing. The sizes of the PCR products correspond to wild-type (WT) and edited (Δ) colonies, respectively. *HIS3* (877 bp, 200 bp), *ADE2* (2322 bp, 200 bp), *TRP1* (798 bp, 200 bp), *LEU2* (946 bp, 200 bp) and *URA3* (1054 bp, 200 bp). (D) Organization of the crRNA array and the dDNA array in the multi-CRISPR/Cpf1 vector. Each fragment of the dDNA array is used for HR-directed repair of DSBs generated by Cpf1 in the corresponding loci. (E) Frequency of genomic editions obtained with the multi-CRISPR/Cpf1 system.

resistant colonies were isolated and grown in G418-MA2 media for 2 d to facilitate genomic editing events. The loss of the CRISPR/Cpf1 plasmids, which is essential to avoid genomic integration of the plasmid, was carried out after the sporulation of the heterokaryotic clones in sporulation media (SPA) lacking G418. Homokaryotic clones were isolated in MA2 media lacking G418. The genomic mutations leading to auxotrophic colonies were analyzed both by auxotrophic screening and analytical PCR. All genome edited mutants were further confirmed by DNA sequencing of the analytical PCR fragments.

Results and discussion

Design of a CRISPR/Cpf1 system adapted for A. gossypii

The CRISPR/Cas9 tool for *A. gossypii*, which was designed as a one-vector system, is optimized for the genomic engineering of multinucleated germlings of the fungus (Fig. 1) [8]. In order to expand the repertoire of genomic editing tools for *A. gossypii*, a CRISPR/Cpf1 system has been designed. The Cpf1 enzyme from *Lachnospiraceae bacterium*, which recognizes the PAM sequence 5′-TTTN-3′, was used and its expression was driven by the promoter and terminator sequences of the *A. gossypii* genes *TSA1* and *ENO1*, respectively. The Cpf1 module was assembled with functional sequences of the CRISPR/Cas9 vector that was used as a backbone [8] (Fig. 2A). The crRNAs comprised a 21-bp direct repeat (DR) sequence together with a target-specific 23-bp sequence that works as the guide RNA (gRNA) for the ribonucleoprotein Cpf1/crRNA (Fig. 2A). In addition, the CRISPR/Cpf1 system comprised a sequence that functions as donor DNA (Fig. 2A, dDNA), which is used for HR repair of DNA double strand breaks without the excision of the dDNA fragment from the CRISPR/Cpf1 plasmid.

Table 1
Editing efficiency of the CRISP-LbCpf1 systems.

	Editing efficiency (%)[1]			
Edition event	LbCfp1	LbCfp1-crRNA	LbCfp1-crRNA-dDNA	LbCf1-crRNA array-dDNA array
his3$^-$	–	–	68.4	19.2
ade2$^-$	–	–	77.2	29.6
trp1$^-$	–	–	19.2	10.4
leu2$^-$	–	–	62.2	24
ura3$^-$	–	–	58.8	25.6

[1] Editing efficiency was calculated by auxotrophic screening of 250 homokaryotic colonies isolated after sporulation of primary heterokaryotic transformants.

CRISPR/Cpf1-mediated single gene editing of A. gossypii

The functionality of the CRISPR/Cpf1 system was assessed for the ability to generate large gene deletions causing auxotrophic phenotypes. Thus, the screening of genome edited variants was carried out by analyisi of the corresponding nutritional requirements in selective culture media. Five different genomic targets were selected for single CRISPR/Cpf1-mediated editing events: *ADL270C* (*HIS3*), *ACR210C* (*ADE2*), *AER014W* (*TRP1*), *AAL012C* (*LEU2*) and *AEL059W* (*URA3*). The five CRISPR/Cpf1 plasmids were used to transform spores of the wild-type strain of *A. gossypii* and positive heterokaryotic clones were selected in G418-containing medium. 250 homokaryotic clones were isolated after sporulation of the primary heterokaryotic clones and the editing efficiency of each plasmid was calculated by auxotrophic screening in selective media (Table 1). The five CRISPR/Cpf1 plasmids were able to produce the corresponding editing event, confirmed by analytical PCR (Fig. 2B-C) and DNA sequencing of the amplicons (not shown). However, the editing efficiency of the CRISPR/Cpf1 plasmids differed substantially depending on the targeted sequence (Table 1). While both *HIS3* and *ADE2* editing exhibited high efficiencies (68.4 % and 77.2 %, respectively), the plasmid for CRISPR/Cpf1-*TRP1* deletion showed a 19.2 % efficiency. This variation can be explained by differences either in the recognition of the PAM sequence or in the specificity of the target DNA binding and cleavage of each crRNA, as previously described for other CRISPR/Cas9/Cpf1 systems [12]. Indeed, significant variability has already been reported in the CRISPR/Cas9 editing efficiency of the *A. gossypii ADE2* gene depending on the sequence of the sgRNA used [8]. Also, the low editing efficiency shown at the *TRP1* locus could be related to the presence of the complex chromatin structure of a neighboring centromere [24].

CRISPR/Cpf1 multiplex genome editing using a single crRNA array

Multiplex genome editing can be achieved with CRISPR/Cas9 systems, but requires more complex sgRNA modules than those of the CRISPR/Cpf1 systems, due to the intrinsic Cpf1 RNase activity that facilitates crRNA processing [22]. Hence, a crRNA array was designed for the multiplex gene deletion of the five auxotrophic markers *HIS3*, *ADE2*, *TRP1*, *LEU2* and *URA3* using a multi-CRISPR/Cpf1 vector. The crRNA array comprised specific gRNAs for each target sequence preceded by the direct repeats that enable the formation of five different crRNAs to drive the Cpf1 nuclease activity (Fig. 2D). In addition, the multi-CRISPR/Cpf1 plasmid contained an array of dDNA sequences for HR-directed repair of the double-strand breaks generated by Cpf1 in the target genes (Fig. 2D). In contrast to the singleplex CRISPR/Cpf1 plasmid, the dDNA array was assembled between the oriC and the Cpf1-expression module in the multi-CRISPR/Cpf1 plasmid (Fig. 2A).

The multi-CRISPR/Cpf1 plasmid was used to transform spores of the wild-type strain of *A. gossypii*. Primary G418-resistant heterokaryotic colonies were isolated in selective medium, thereby confirming the functionality of the plasmid. The isolation of homokaryotic clones was carried out after the sporulation of heterokaryotic transformants. The frequency of genomically edited mutants was evaluated in 250 homokaryotic clones by auxotrophic screening in selective media (Table 2). Their presence was further confirmed by analytical PCR and DNA sequencing (not shown). The results revealed a significant reduction in the editing efficiency of each genomic sequence (Table 1). As a consequence, one third of the homokaryotic clones analyzed were devoid of auxotrophies. However, the multi-CRISPR/Cpf1 demonstrated a satisfactory efficiency for the recovery of double and triple mutants (44 and 23 out of 250, respectively) (Fig. 2D). Although the system was challenged to generate up to five genomic mutations, quintuple auxotrophic mutants could not be detected among the analyzed colonies. However, while at low frequency, quadruple mutants were isolated, thus demonstrating that the multi-CRISPR/Cpf1 system can be applied as a multiplex genomic editing approach (Fig. 2E and Table 2).

A. gossypii lacks a known sexual cycle so that the construction of strains combining different gene modifications by sexual crossing is not possible. The construction of engineered strains containing multiple gene modifications relies on successive cycles of marker-mediated DNA integration followed by marker removal [27]. Although effective, this is a laborious and time-consuming procedure. Multiplex gene modification methods, as demonstrated here, enable the modification of several genes simultaneously and a rapid generation of engineered strains.

CRISPR/Cas9 technologies have been described for genome editing of many filamentous fungi using different strategies for vector construction and transformation methods [11]. In contrast, Cpf1-based systems have only been described for two Aspergilli species among filamentous fungi [28]. The Cpf1-based genomic editing of Aspergilli required the co-transformation of the Cpf1-expressing vector with an oligonucleotide (dDNA) for genomic site-directed mutagenesis [28]. The system presented is fully assembled as a one-vector method containing all the modules (Cpf1, gRNA and dDNA), which facilitates transformation events in multinucleated cells, as previously described for CRISPR/Cas9 [8]. In addition, multi-CRISPR/Cpf1 represents the first Cpf1-based system for multiplex genomic editing described to date. The use of genome-scale gRNA libraries may constitute a powerful tool for discovery and functional annotation of genetic elements that

Table 2
Recovery of multiple mutants with the multiplex CRISP-LbCpf1 system.

Double mutants	Number	Triple mutants	Number	Quadruple mutants	Number
his3-ade2	6	*his3-ade2-leu2*	3	*his3, ade2, leu2, ura3*	2
his3-trp1	1	*his3-ade2-trp1*	1	*his3, ade2, trp1, leu2*	1
his3-leu2	8	*his3-ade2-ura3*	5	*his3, ade2, trp1, ura3*	1
his3-ura3	4	*his3-trp1-leu2*	1	*ade2, trp1, leu2, ura3*	1
ade2-trp1	3	*his3-leu2-ura3*	3		
ade2-leu2	8	*ade2-trp1-leu2*	2		
ade2-ura3	3	*ade2-trp1-ura3*	1		
trp1-leu2	1	*ade2-leu2-ura3*	6		
trp1-ura3	6	*trp1-leu2-ura3*	1		
leu2-ura3	4				

modulate transcriptional activity in *A. gossypii*. Chromatin accessibility as well as target sequence composition significantly affects Cpf1 activities [29] so that the development of Cpf1 activity prediction algorithms considering these two factors substantially improves the selection of target sequences and the efficiency of multi-CRISPR/Cpf1systems. Recently, a deep learning approach, DeepCpf1, has been described to improve the accuracy of Cpf1-based genome editing, aiming to predict gRNA on-target activities and off-target effects [30].

Conclusions

A versatile CRISPR/Cpf1 method has been adapted for efficient genome editing of *A. gossypii*. It complements the CRISPR/Cas9 system, thus minimizing the limitations associated with the existence of specific PAM sequences within the target sites. In addition, a multi-CRISPR/Cpf1 has been designed and validated for multiplex gene deletion of four auxotrophic markers. The existence of two CRISPR/Cas systems adapted for microbial engineering of *A. gossypii* will contribute significantly to facilitating system metabolic engineering approaches in this industrial fungus.

Declaration of Competing Interest

The authors declare no financial or commercial conflict of interest

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