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IN VITRO DNA RESECTION ACTIVITY OF HUMAN EXONUCLEASE1 IS MODULATED BY PCNA AND 14-3-3S

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Abstract

Cellular response to DNA lesions such as double-strand break (DSB) is dictated by resection of DNA ends. DNA resection is essential for maintaining the genome; without adequate resection of the DNA ends, repair mechanisms such as HR and ATR-dependent checkpoint will not be activated. On the other hand, excessive resection produces unstable single-stranded DNA, which could cause instability of the genome. The regulatory mechanisms of DNA resection are not fully understood. This study was carried out to understand the modulatory effects of PCNA and 14-3-3 proteins on the resection activity of hExo1. Recombinant proteins were expressed from bacteria and purified to near homogeneity. The resection assays were carried out using linearized dsDNA as a substrate. Results obtained from this study show that 14-3-3 proteins bind to hExo1 and inhibit its resection activity, while PCNA interacts with hExo1 and enhances its resection activity. This suggests that the resection activity of hExo1 on dsDNA substrate is modulated through possible protein-protein interactions. The results also show that 14-3-3 proteins repress PCNA binding to hExo1. This study presents an important insight into the mechanism through which DNA resection is controlled. This coordinated control is essential in ensuring efficient and appropriate DNA end resection needed to repair DNA damage to preserve the integrity of the genome.

INTRODUCTION

Cellular genome is protected through effective detection and repair of DNA lesions. Among many types of lesions in DNA, double strand break (DSB) is the most deleterious because unrepaired DSB can cause conditions such as immunodeficiency, instability of the genome, premature aging and cancer [1,2]. Upon DSB occurrence, cells activates DNA repair mechanisms that are controlled by the resection of DNA ends [3-5]. Homologous recombination (HR) and non-homologous end joining (NHEJ) are the two major mechanisms through which DSB can be repaired. DNA end resection enhances HR but inhibit NHEJ [6,7]. This implies that the mode of response to DNA damage is directed by the resection of DSB.

Exo1 and Dna2 are the two nucleases that catalyses a long-range resection of DNA which allows DSB repair by HR [8-10]. Inadequate resection blocks the activation of HR, whereas excess resection can cause unwanted effects since single-stranded DNA (ssDNA) is unstable. Hence, it is vulnerable to degradation and this could lead to loss of genetic information due to breakage of 3'-strand as well as cell death due to persistent ssDNA-induced checkpoint signal [11,12]. To prevent over-resection of broken DNA, there must exist a mechanism to checkmate these processes. Unfortunately, no enough information available on how the resection of DNA is regulated. Exo1 belongs to the family of RAD2 nucleases that play important functions in DNA metabolism processes such as replication, recombination, repair, checkpoint activation, meiosis, class switch

recombination, maintenance of genome, regulation of telomere, and somatic hypermutation [13,14]. It is not clear how the resection activity of hExo1 is regulated.

Recently, Chen and colleagues [11] proposed a model for the regulation of hExo1 in DNA end resection. They reported that PCNA and 14-3-3 proteins interact with hExo1 and regulate its association with DNA damage. PCNA binds within the residues 751 – 846 of the C-terminal domain of hExo1 to enhance retention at sites of DNA damage and processive resection of DNA breaks while 14-3-3 proteins bind within residues 508 - 750 of the central domain of hExo1 and block binding of PCNA to hExo1, that results to limited interaction of hExo1 with DNA damage [11]. However, the understanding of the mechanism through which this regulation is achieved still remains insufficient. The aim of this research was to express, purify, and assay the *in vitro* DNA resection activity of recombinant hExo1 on dsDNA substrate and how PCNA and 14-3-3 proteins modulate its enzymatic resection activity.

MATERIALS AND METHODS

Materials

The full-length clone for the human Exo1 splice variant (*hExo1* cDNA) and human *PCNA* cDNA was a generous gift from Professor Paul Modrich. A yeast two-hybrid vector harbouring each of the 14-3-3 isoforms coding region (pHAF633 pJG 14-3-3 zeta and pRRS305 pJG 14-3-3 epsilon) were kindly provided by Professor Lene Juel Rasmussen. pTXB1 plasmid, NdeI, NotI, SphI and SapI restriction endonucleases and proteinase K were purchased from New England Biolab (NEB). *E. coli* BL21 (DE3) and pET28a+ (Novagen), SP-FF cartridge, Superose 12 10/300 GL column and HisTrap HP column were all obtained from GE Health-care. Mini-PROTEAN® TGX Stain-Free™ Precast Gels and SYBR gold were purchased from BioRad UK.

Expression of Recombinant Proteins

Human exonuclease1 was expressed in *E. coli* BL21 (DE3) using a pTXB1 plasmid following the method described previously [15]. The coding regions of PCNA, 14-3-3ζ and 14-3-3ε were individually amplified by PCR and the PCR products were individually ligated into the *NdeI/NotI* site of the pET28a (+). His-tagged PCNA, 14-3-3ζ and 14-3-3ε were expressed individually in *E. coli* BL21 (DE3) as follows: A 100 mL starter culture was transferred to a 1 L LB + 50 µg/ml kanamycin and grown to an OD_{600} = 0.6 at 37ºC with 250 rpm rotation. Protein expression was induced by the addition of IPTG to a final concentration of 0.2 mM. The culture was allowed to grow overnight at 37ºC in a shaking incubator with rotation of 250 rpm.

Purification of Recombinant Proteins

Recombinant hExo1 protein was purified by affinity chromatography using chitin resin [15] and further purified by size exclusion using Superose 12 10/300 GL column (GE Health-care). PCNA, 14-3-3ζ and 14-3-3ε proteins were all affinity purified using HisTrap HP column (GE Health care). All the purified recombinant proteins were verified by mass spectrometry using LC-ESI-tandem MS on a Q-TOF2 mass spectrometer fitted with a Nano flow ESI source (Waters Ltd).

DNA ends Resection Assays of hExo1

DNA ends resection assays were carried out with minor modifications to the method described previously [16]. A 4.8-kb pFastBac-HTA plasmid was linearized with *SphI* restriction endonuclease, which generated a 4 nucleotides long 3' overhang dsDNA. A 20 µL resection reaction containing between 2.5-30 nM hExo1 protein (as specified) and 30 nM linear dsDNA was set up on ice and transferred to 37ºC for 1 hr. Reactions were stopped by adding termination buffer (2% w/v SDS, 10 mM EDTA) in the presence of 1 µg proteinase K and allowed to stand for 1h. Reaction products were separated on 0.8% agarose gel stained with SYBR gold and visualized with Gel Doc XR+ system (BioRad UK). Other resection reactions were set up as described earlier except that either PCNA, 14-3-3ζ or 14-3-3ε were added to assess their modulatory effects on the enzymatic resection activity of hExo1.

RESULTS AND DISCUSSION

Expression and Purification of Recombinant Proteins

Recombinant proteins were expressed and purified from bacteria. The purity of recombinant proteins was analysed on a 4-20% SDS/PAGE and estimated to be about 98% (Fig. 1).

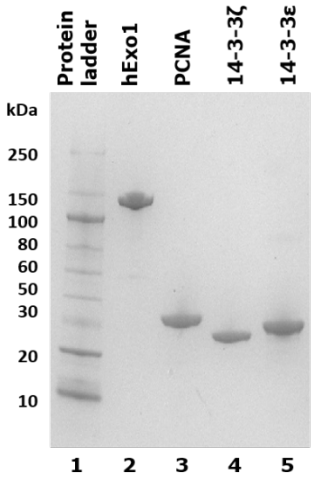

Figure 1. SDS-page analysis of the purified proteins. Protein ladder lane 1, purified recombinant hExo1 lane 2, his-tagged PCNA lane 3, 14-3-3ζ lane 4, and 14-3-3ε lane 5.

PCNA Enhances hExo1 Resection Activity on DNA

To investigate the *in vitro* resection activity of hExo1, resection assays were set up using the purified recombinant hExo1 protein and a linear dsDNA substrate. This study shows that hExo1 exhibited a 5' → 3' exonucleolytic resection activity on the linearized dsDNA substrate. Looking at lane 1 in Figure 2 where hExo1 was not added in the reaction, the substrate remained intact without being degraded compared to lanes 2, 3, 4, and 5 where hExo1 was added in the reaction in an increasing concentrations. Clearly, it can be seen that the resection products appeared more on the gel as the concentration of hExo1 increases (Fig. 2, lanes 2, 3, 4, and 5). In other sets of reactions where hExo1 and PCNA were added to the reactions in the presence of dsDNA substrate, we observed that the resection activity of the full-length hExo1 was enhanced (Fig. 2, lanes 7-10), in these reactions, dsDNA substrate was almost degraded completely even though the same concentrations of hExo1 were used as in those on lanes 2-5. To ascertain if the degradation of dsDNA substrate was exclusively carried out by hExo1, another reaction set up with the same conditions as described above except that hExo1 was not added, but only PCNA was added to the reaction and the result shows that PCNA on its own did not degrade dsDNA substrate (Fig. 2, lane 6). This confirmed that the degradation of dsDNA observed in lanes 7-10 resulted from the nuclease function of full-length hExo1. In the presence of full-length hExo1 and PCNA the substrate disappeared from the top of the gel and the resection products appeared to have migrated down to the bottom of the gel (Fig. 2, lanes 7-10), unlike in those reactions that do not contain PCNA where the remainder of the substrate can be seen on top of the gel and resection products appeared as a smear on the gel due to incomplete degradation (Fig. 2, lanes 2-5).

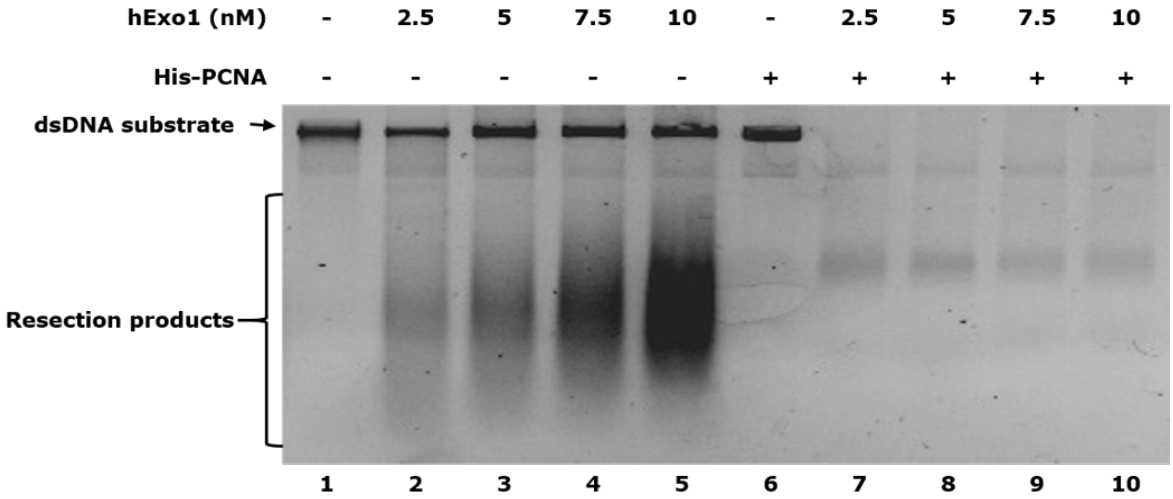

Figure 2. (A) PCNA stimulates exonuclease activity of hExo1. PCNA enhanced hExo1 resection activity on a 3' overhang dsDNA substrate. Lane 1 is substrate control; no hExo1 protein added. Lanes 2–5 are resection activity of hExo1 at varying concentrations using 30 nM dsDNA substrate, lane 6 contained 20 nM PCNA only no hExo1 protein, while lanes 7-10 are resection activity of hExo1 at varying concentrations using 30 nM dsDNA substrate in the presence of 20 nM PCNA.

14-3-3 Proteins Inhibits hExo1-mediated DNA Resection

At least six out of the seven paralogues of human 14-3-3 proteins interact with human Exo1, as previously reported [17]. It is not clear if 14-3-3 proteins directly affect the enzymatic activity of hExo1. To verify this, *in vitro* resection assays were reconstituted as described above, but here, the resection activity of hExo1 was tested in the presence of either 14-3-3ζ or 14-3-3ε. The results obtained from these assays show that both 14-3-3ζ and 14-3-3ε inhibited the exonucleolytic activity of hExo1 on linearized dsDNA substrate. Here we used a higher concentration of hExo1 (15-30 nM) to make the degradation of substrate go to completion. Interestingly, in the reactions with hExo1 alone, the substrate was degraded almost completely (Fig. 3, lanes 6-9) whereas in reactions where 14-3-3ε was added hExo1 activity was inhibited, although there was little activity as resection products appeared as smear on the gel (Fig. 3, lanes 2-5). Similarly, in the presence of 14-3-3ζ hExo1 activity was inhibited (Fig. 3, lanes 10-13). It appeared that 14-3-3ζ have stronger inhibitory effect than 14-3-3ε because more of the resection products appeared in the presence of 14-3-3ε than in the presence of 14-3-3ζ even though the same amounts of these proteins were added accordingly.

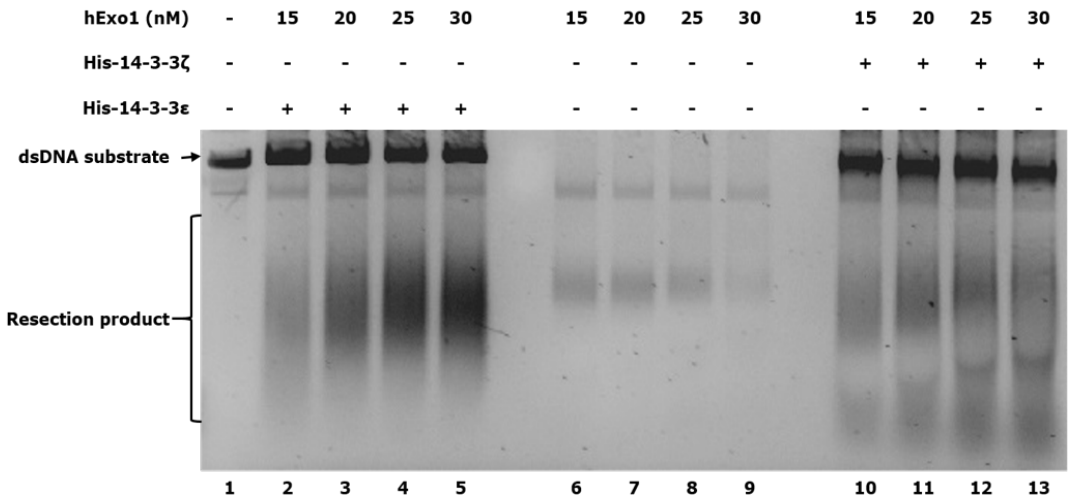

Figure 3. 14-3-3s inhibits hExo1 resection activity. The 14-3-3s inhibits hExo1 resection activities on a 3' overhang dsDNA substrate (lanes 2-5 and 10-13). Lane 1 is a substrate control; no protein added. Lanes 2-13 each contained 30 nM 3' overhang dsDNA and varying concentrations of hExo1 as indicated on the gel image. Lane 2-5, resection assay by hExo1 in the presence of 30 nM 14-3-3ε and lanes 10-13 resection assay by hExo1 in the presence of 30 nM 14-3-3ζ.

14-3-3 Proteins Repress PCNA Binding to hExo1

Since both PCNA and 14-3-3s interact with hExo1, binding one interacting partner to hExo1 may likely interfere with the binding of others. To test this assertion, resection assays were set up as described earlier. However, in some reactions, hExo1 and PCNA were added in the presence of dsDNA substrate and incubated for 5 minutes to allow hExo1/PCNA complex to form before either of 14-3-3ζ or 14-3-3ε was added to the reaction and allowed to stand for 1 hour. Another set of reactions were set up by adding hExo1 together with either 14-3-3ζ or 14-3-3ε and allowed to interact for 5 minutes then PCNA was added followed by incubation for 1 hour. The results obtained from these assays indicated that both 14-3-3ζ and 14-3-3ε repress PCNA binding/interaction with hExo1. Looking at lanes 1 and 2 in figure 4, it is clear that PCNA enhanced hExo1's resection activity while both isoforms of 14-3-3 protein inhibited the resection activity of hExo1, 14-3-3ζ exhibited stronger inhibition (lane 4) on hExo1 activity than 14-3-3ε (lane 5). In assays where hExo1 was allowed to first interact with the 14-3-3ζ before adding the 14-3-3ε or vice versa, the resection activity was inhibited at almost the same magnitude (lanes 6 and 7). Similarly, in assays where hExo1 was allowed to form a complex with PCNA before the addition of either of the 14-3-3s, the results show that resection activity of hExo1/PCNA complex was inhibited by both 14-3-3ζ and 14-3-3ε in a similar fashion to how they inhibited resection activity of hExo1 (compare lanes 8 and 9 to lanes 4 and 5). Further to this, 14-3-3ζ/14-3-3ε complex showed inhibition to both hExo1 and hExo1/PCNA complex's resection activity (lanes 10 and 11, respectively). These results revealed that both 14-3-3ζ and 14-3-3ε repress PCNA binding to hExo1.

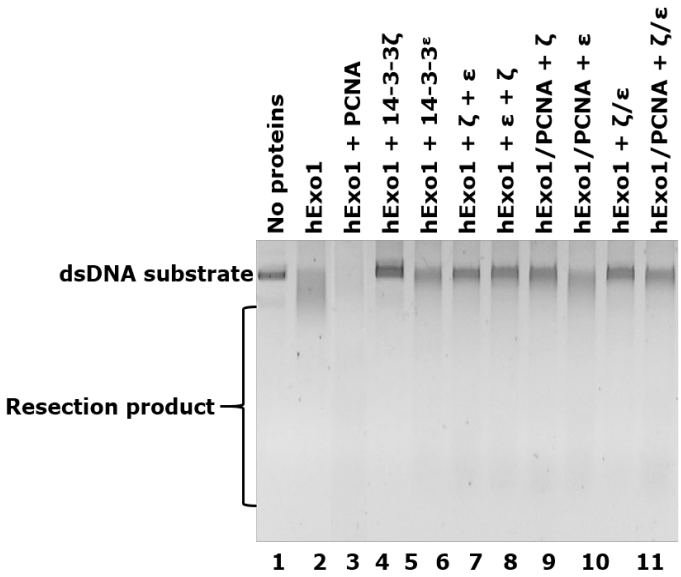

Figure 4. 14-3-3s repress PCNA binding to hExo1. Representative 1% agarose gel of three independent replicas of experiments showing 14-3-3ζ suppress PCNA binding to hExo1 in resection activities on 20 nM 3' overhang 4.8kb dsDNA substrate. Lane 1 is substrate control, no protein was added. Lane 2 contains 10 nM hExo1. Lane 3 contains 10 nM hExo1 and 10 nM PCNA. Lane 4 contains 10 nM hExo1 and 10 nM 14-3-3ζ. Lane 5 contains 10 nM hExo1 and 10 nM 14-3-3 epsilon. Lane 6 contains 10 nM hExo1, 14-3-3ζ/14-3-3ε. Lane 7 contains 10 nM hExo1, 14-3-3ε/14-3-3ζ. Lanes 8 and 9 contain hExo1/PCNA complex, 14-3-3ζ and 14-3-3ε respectively. Lane 10 contains 10 nM hExo1 and14-3-3ζ/14-3-3ε complex, while lane 11 contains hExo1/PCNA and 14-3-3ζ/14-3-3ε complex. Assays were set up on ice and reactions were activated by transferring reaction tubes to 37 ^{o}C and incubated for 60 min.

The process of DNA end resection during DSB is a multiplex mechanism through which ATR-dependent checkpoint and homology-based repair of DNA lesion are activated and initiated when DSB occurs [5,10,18,19]. This study has highlighted some essential information on the mechanism through which the enzymatic activity of hExo1 is modulated in the DNA resection process. Our results suggest that the enzymatic function of hExo1 in DNA end resection is modulated via interaction with protein partners PCNA and 14-3-3 proteins. PCNA associate with hExo1 and enhances its resection activity on dsDNA substrate but the association with 14-3-3 proteins inhibit enzymatic resection activity of hExo1 (Fig. 2 and 3). PCNA is a ring-shaped DNA sliding clamp. It is presumable that PCNA enhances hExo1 activity by sliding alongside hExo1 across the DNA substrate, thereby speeding up the resection of DNA substrate by hExo1. Our data is consistent with previous studies that reported how PCNA increases hExo1 processivity during DNA resection [11]. According to Chen and colleagues [11], when DSB occurs, PCNA bind to DSB point on the DNA and subsequently interact directly with the PIP sequence of hExo1, thereby tethering hExo1 across the DNA.

Consequently, this tethering increase hExo1 processivity. Activities of nucleases require stringent control in order to achieve appropriate DNA repair and cell survival. The positive effect of PCNA on hExo1 during DNA resection might ensure that an efficient DNA end resection is attained because resection of DNA ends is required for prompt HR and ATR checkpoint activations.

We show here that 14-3-3 proteins inhibit the *in vitro* enzymatic DNA resection activity of hExo1. Interestingly, it has been suggested that 14-3-3s do not regulate hExo1 nuclease activity at DNA damage sites. Instead, they interrupt its recruitment and retention because 14-3-3 proteins were not found localized at DNA damage sites [20]. Similarly, Andersen and colleagues reported that the interaction between 14-3-3η and 14-3-3σ with Exo1 did not modulate *in vitro* nuclease activity of recombinant Exo1 [17]. However, our results differ from these reports because we observed that 14-3-3 proteins exhibited an inhibitory effect on the enzymatic resection activity of hExo1 (Fig 3). Our study used 14-3-3ζ and 14-3-3ε, which are the most divergent among the seven paralogues of 14-3-3s. Presumably, this divergence may be why different paralogues of 14-3-3s exhibit different effects on hExo1 nuclease function. In addition to the inhibitory effect on hExo1 resection activity, 14-3-3 proteins negatively affect hExo1 association with PCNA.

The results described in this study revealed that despite the fact that hExo1 and PCNA were added in the presence of dsDNA substrate and allowed to interact and form hExo1/PCNA complex before adding 14-3-3s to the reaction, hExo1 enzymatic activity was never the less it was inhibited by the presence of 14-3-3s (Fig. 3). This effect of interfering with hExo1-PCNA association by 14-3-3s was previously reported [20]. In their study, Chen and colleagues observed that 14-3-3s limit Exo1 damage recruitment and resection by suppressing Exo1 binding to PCNA [20]. They further suggested that the decreased binding of PCNA to Exo1 may result from conformational changes in Exo1 or by modification of Exo1 or interaction with other protein factors due to the binding of 14-3-3. Our data, however, suggested that since both PCNA and 14-3-3 proteins bind to hExo1 at PIP sequence (amino acid residues 788-795) and central domain (amino acid residues 508-750) respectively [6,20], it is highly likely that binding of PCNA to hExo1 did not interfere with the binding of 14-3-3 proteins in such a way that the 14-3-3 binding domain was still available and that binding of 14-3-3s may bring a conformational change in hExo1 structure that consequently affects hExo1 nuclease activity even though it is still associated with PCNA.

While inadequate resection of DNA ends hampers activation of HR and ATR, however, excessive resection can cause negative consequences. Uncontrolled resection of dsDNA will generate excess single-stranded DNA (ssDNA) and this may drain the RPA protein pool. A further lesion can occur when an uncoated 3' ssDNA is exposed, this can lead to the instability of the genome and consequently, the genetic information will be compromised [11,21]. Recently it has been demonstrated that modulation of hExo1 activity by 14-3-3 proteins is crucial for the survival of the cell following DNA damage [20]. Considering how cancer is linked to genomic instability and replication stress, the process of DNA resection can be employed for the possible treatment of cancer.

CONCLUSION

We show that the *in vitro* enzymatic exonuclease activity of hExo1 in resection of DNA ends is a subject of positive and negative modulation by PCNA and 14-3-3 protein partners through protein-protein interactions. Interaction with PCNA enhances hExo1 function in DNA resection, whereas interaction with 14-3-3 proteins inhibits its resection activity. Also, we demonstrated that the inhibitory effects of 14-3-3 proteins on hExo1 enzymatic function in DNA resection are not affected by PCNA binding to hExo1, which suggests that both PCNA and 14-3-3s may be bound to hExo1 at the same time since their binding domains are distinct. At least, part of the process through which hExo1-mediated DNA resection is regulated has been accomplished in this study. This coordinated control is essential in ensuring efficient and appropriate DNA end resection that is needed to repair DNA damage in order to preserve the integrity of the genome.

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CONFLICT OF INTEREST

We have no conflicts of interest to declare.

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