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In-depth Characterization of the Homodimerization Domain of the Transcription Factor THAP1 and Dystonia-Causing Mutations Therein

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Abstract Mutations in the THAP1 gene encoding the transcription factor THAP1 have been shown to cause DYT6 dystonia. THAP1 contains a highly conserved THAP zinc finger at its N-terminal region which allows specific binding to its target sequences as well as a coiled-coil domain (amino acids 139–190) towards its C-terminus postulated as a protein-protein-binding motif. While several DYT6-causing mutations within the THAP domain were shown to decrease THAP1 activity in transcriptional regulation and DNA-binding, the role of mutations within the coiled-coil domain is rather unknown. Therefore, assigning a function to this domain may enable functional testing of mutations in this region. Notably, THAP1 and other THAP proteins form homodimers; however, the responsible domain has not been elucidated in detail. We show that the region of amino acids 139–185 is involved in formation of THAP1 homodimers by using yeast-two-hybrid, GST pull-down, and cross-linking assays. Surprisingly, all nine reported DYT6-causing missense mutations within this region had no effect on dimerization of THAP1 in GST pull-down and formaldehyde cross-linking assays. In conclusion, we demonstrated that a region of 47 amino acids is involved in THAP1 homodimerization but mutations in this region seem not to impair this mechanism.

Keywords Homodimerization · Transcription · Dystonia · Mutation

Abbreviations

THAP1	Thanatos-associated [THAP] domain-containing apoptosis-associated protein 1
DTT	Dithiothreitol
DYT6	Dystonia 6
TOR1A	Torsin1A
NLS	Nuclear localization signal
Wt	Wildtype
Aa	Amino acids
TE	Tris/EDTA

Introduction

Several mutations in the transcription factor THAP1 (Thanatos-associated [THAP] domain-containing apoptosis-associated protein 1) have been reported in patients with DYT6 (dystonia 6) (Fuchs et al. 2009; LeDoux et al. 2012; Lohmann et al. 2012). This form of dystonia, also known as DYT-THAP1 (Marras et al. 2016), is an autosomal dominant movement disorder characterized by sustained or intermittent muscle contractions causing abnormal postures, often with childhood onset in craniofacial muscles and generalization involving the arms and trunk (Albanese et al. 2013). Laryngeal dystonia that causes speech difficulties is frequent (Bressman et al. 2009). THAP1 consists of 213 amino acids including a characteristic atypical THAP zinc finger domain in the N-terminus (amino acids 1–81), a central proline-rich region (amino acids 96–108), and a coiled-coil domain (amino acids 139–190) with a bipartite NLS (amino acids 147–162) in

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the C-terminal part of the protein. Most of the more than 80 reported mutations are missense mutations located within the THAP domain or truncating mutations that may affect the transcriptional activity of THAP1 (LeDoux et al. 2012; Lohmann et al. 2012; Osmanovic et al. 2011). On the other hand, functional consequences of missense mutations affecting more C-terminal regions of the protein are largely unknown which can partly be explained by lack of a read-out. Therefore, only computational prediction programs can be applied to predict pathogenicity of variants within this region. However, these programs have clear limitations in terms of accuracy (Thusberg et al. 2011).

It can be hypothesized that the coiled-coil domain of THAP1 is involved in homodimerization that was shown for THAP proteins (Dejosez et al. 2008; Lanati et al. 2010). In a previous study, it was suggested that the homodimerization of THAP1 is mediated by only 13 amino acids (amino acids 154–166, Sengel et al. 2011). To further characterize the THAP1 homodimerization domain, we performed yeast-two-hybrid and GST pull-down assays to narrow down the minimal region responsible for THAP1 homodimerization. Further, we investigated the functional consequence of nine reported DYT6-causing missense mutations within the homodimerization region of THAP1 by GST pull-down and formaldehyde cross-linking assays.

Materials and Methods

Plasmid Constructs

The full-length open reading frame of THAP1 was amplified using a marathon ready human fetal brain cDNA library (Clontech; Mountain View, CA, USA) and inserted into the pGEX-4 T2 (GE-Lifesciences; Fairfield, CT, USA), pGBKT7, pGADT7 (Clontech; Mountain View, CA, USA), and pFLAG-N3 (3xFLAG, Invitrogen) expression plasmids. The truncated THAP1 constructs were amplified with the appropriate primers and inserted into the pGBKT7 expression plasmid. Mutated THAP1 constructs were generated using the QuikChange® Site-directed Mutagenesis Kit (Stratagene) and the full-length THAP1-pGBKT7 expression plasmid as template. These mutated THAP1 constructs were also subcloned into pFLAG-N3 (3xFLAG). All constructs were verified by direct sequencing (PE Applied Biosystems).

Yeast-Two-Hybrid Assay

Yeast cells (AH109) were transformed using lithium-acetate transformation. Briefly, yeast cells were grown in YPD full medium. Cells were harvested by centrifugation and after several washing steps resuspended in 250–300 μl TE buffer/lithium acetate reagent. Each transformation reaction contained 50 μl yeast cells, 300 μl polyethylenglycol reagent (100 mM TE, 100 mM lithium acetate, 50% polyethylenglycol 4000), 5 μl salmon sperm DNA (Sigma), and 1 μl of each construct of interest in pGADT7 and pGBKT7. The samples were carefully mixed and incubated at 30 °C for 30 min followed by a heat shock (42 °C for 15 min). Next, cells were put on ice for 1 min and plated on -2SD agar (-leucine, -tryptophane). After 3–4 days of incubation at 30 °C, yeast colonies were picked and plated on -4SD agar (-leucine, -tryptophane, -adenine, and -histidine). If an interaction between the analyzed proteins occurred, yeast growth on -4SD agar was observed after 4–6 days incubation at 30 °C.

GST Pull-down Assay

GST-THAP1 fusion protein as well as GST protein as control were overexpressed in *Escherichia coli* BL21 and purified by affinity chromatography with glutathione sepharose 4B (GE-Lifesciences; Fairfield, CT, USA) following the manufacturer's instructions. The amount of protein was determined with the colorimetric Bradford assay and specificity of the proteins was confirmed with SDS-PAGE and Western blot analysis using an anti-GST antibody (Santa Cruz, Biotechnology; Santa Cruz, CA, USA). Next, 5 μg of purified protein was loaded onto 25 μl of glutathione sepharose 4B beads in each GST pull-down assay and incubated overnight at 4 °C. In vitro transcribed/translated and [^{35}S]-labeled THAP1 proteins were generated in the presence of [^{35}S]-labeled methionine (Hartmann Analytic GmbH; Braunschweig, Germany) using the TNT-coupled reticulocyte lysate system (Promega; Madison, WI, USA). Wildtype and mutated full-length and truncated THAP1 constructs in pGBKT7 plasmids served as templates. In each GST pull-down assay, 5 μl of in vitro transcribed/translated THAP1 protein was added to the immobilized GST-THAP1 or GST protein as control, respectively, and incubated overnight at 4 °C. After washing, 1 μl of each in vitro transcribed/translated protein was diluted in 30 μl and 10 μl Laemmli buffer, respectively, separated by 10–16% SDS-PAGE and visualized with Coomassie Brilliant Blue staining. The dried gel was autoradiographed (10–48 h) and the [^{35}S]-labeled proteins were analyzed by phosphorimaging (CR 35 Bio; Dürr Medical, Bietigheim-Bissingen, Germany) and visualized with the AIDA Image Analysis v.4.27 (Raytest, Straubenhardt, Germany) software.

Formaldehyde Crosslinking

HEK293 cells were transfected with 3xFLAG-tagged wildtype THAP1 or mutated THAP1. After 24 h, cells were pelleted and resuspended in 0.5% formaldehyde/PBS. Next, cells were incubated for 7 min at room temperature (RT) on a rotator and sedimented at 1800×*g* at RT for 3 min. The cell pellet was resuspended in 0.5 ml of 1.25 M glycine/PBS to quench the cross-linking reaction. Then, cells were centrifuged at 4000×*g* at RT for 5 min and washed once with 1.25 M glycine/PBS. Pellets were lysed in an adequate volume of RIPA buffer and protein concentration was determined using the Dc Protein Assay (BioRad). Samples were prepared for SDS-PAGE (without boiling of the samples) and subsequent Western blot analysis. For SDS-PAGE, NuPAGE 4–12% Bis-Tris gels (Life technologies) were used. After electrophoresis, proteins were transferred to the nitrocellulose membrane (Protran) and probed with primary antibodies raised against FLAG (Sigma-Aldrich) and Neomycin phosphotransferase II (NPTII, Millipore) as control for transfection efficiency.

Results

Evidence for THAP1 Homodimerization in Yeast

In a first step, THAP1 homodimerization was analyzed in yeast cells (AH109) using the Matchmaker™ GAL4 two-hybrid system 3 (BD Bioscience) to confirm homodimerization (Lanati et al. 2010). Cells were transformed with full-length THAP1 in pGADT7 and pGBKT7 expression plasmids and growth on -4SD agar was observed after 4–6 days providing evidence for a THAP1-THAP1 interaction (Fig. 1). Next, we narrowed down the interacting domain by co-transformation of yeast cells using wildtype THAP1 (in pGADT7) and different truncated THAP1 constructs (in pGBKT7) (Fig. 1a). Growth on -4SD agar was only observed after co-transformation of yeast with wildtype THAP1 and fragment C which represented the C-terminal region of THAP1 (Fig. 1b).

Identification of the Minimal Region for THAP1 Homodimerization in GST Pull-down Assays

To confirm the results of the yeast-two-hybrid assay and to further narrow down the minimal region responsible for the THAP1 homodimerization, we performed GST pull-down assays. Homodimerization of THAP1 was verified by pull-down of full-length THAP1 protein. In addition, five of the 12 THAP1 protein fragments (fragments C, D, D3, D6, D7) were able to interact with full-length THAP1 whereas the remaining seven fragments (fragments A, B, D1, D2, D4, D2.1, D5) did not bind to full-length THAP1 (Fig. 2). Notably, fragment D4 represented the previously reported interaction domain (Sengel et al. 2011). The shortest fragment still able to form THAP1-THAP1 homodimers was represented by a stretch of 47 amino acids (amino acids 139–185;

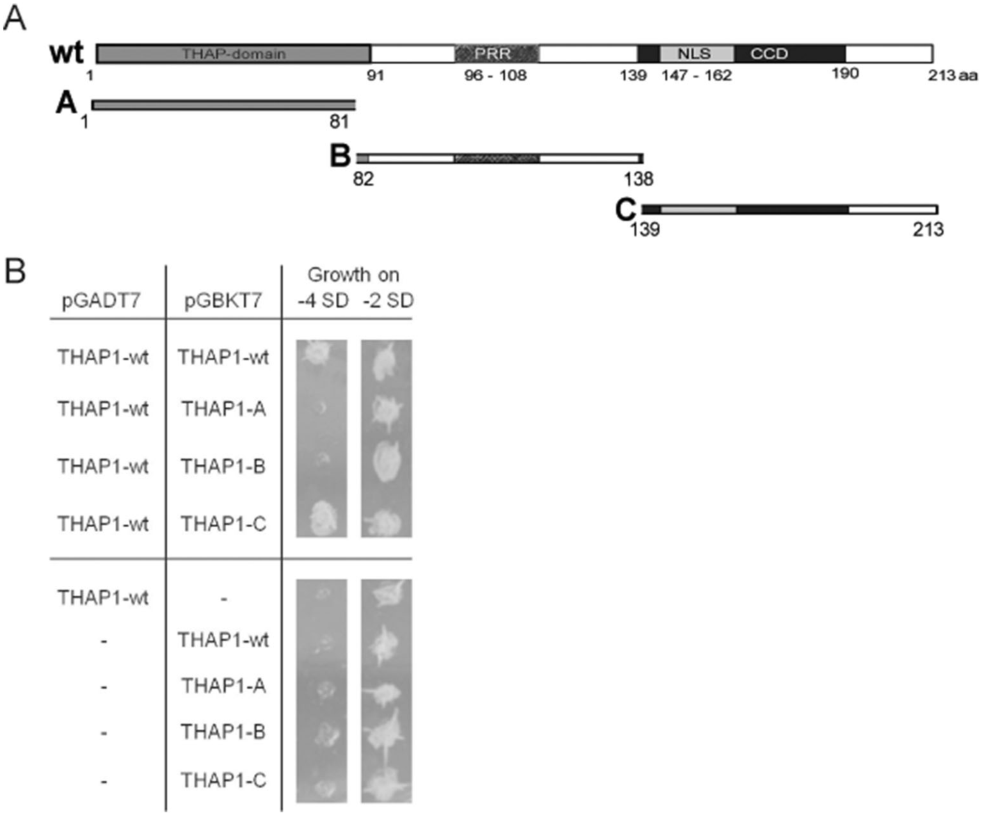

Fig. 1 Homodimerization of THAP1 using yeast-two-hybrid assays. **a** Schematic representation of the THAP1 protein and the analyzed fragments *A*, *B*, and *C*. *wt* wildtype, *PRR* proline-rich-region, *NLS* nuclear localization signal, *CCD* coiled-coil domain, *aa* amino acids. **b** Yeast (AH109) was co-transformed with pGADT7 and pGBKT7 constructs as indicated (*left* and *middle columns*). The growth on -4SD and -2SD agar (as control) is shown in the *right column*. Only yeast transformed with wildtype THAP1-wildtype THAP1 or wildtype THAP1-fragment C were able to grow on -4SD agar. The *lower panel* indicates the control transformation with an empty vector (−)

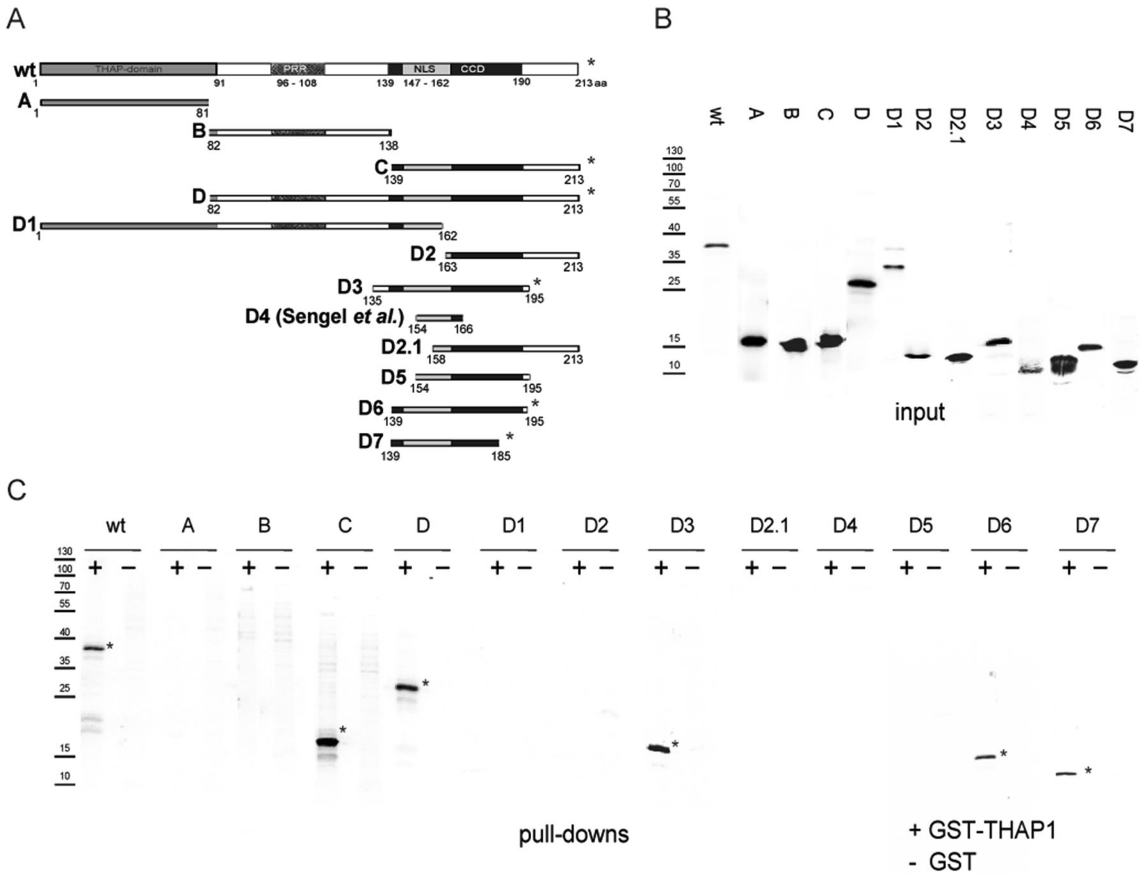

Fig. 2 Homodimerization domain of THAP1 investigated by GST pull-down assays. **a** Schematic representation of the THAP1 protein with the N-terminal THAP-domain, the central proline-rich region (PRR), and the C-terminal coiled-coil domain (CCD) including the bipartite nuclear localization signal (NLS). The GST pull-down assays were performed with full-length THAP1 (wt) protein and 12 truncated fragments (*A*—D7). The *stars* highlight the protein constructs that interact with full-length THAP1 protein. **b** The quality and quantity of the [^{35}S]-radiolabelled, in vitro transcribed/translated THAP1 proteins (input) was tested via SDS-PAGE and autoradiography. **c** Protein-protein interaction between GST-tagged full-length THAP1 and the radiolabelled in vitro transcribed/translated THAP1 proteins. The proteins/fragments that interact with full-length THAP1 are marked with a star representing the same fragments like in (*A*). As negative control, empty GST protein was loaded to the glutathione sepharose beads

fragment D7). Interestingly, this region comprises almost the entire coiled-coil domain of THAP1 (amino acids 139–190). Smaller fragments representing truncated parts of this region were unable to bind to full-length THAP1 (Fig. 2c).

Investigation of DYT6-Causing Missense Mutations by GST Pull-down Assays and Formaldehyde Crosslinking

Next, we tested whether nine reported DYT6-causing missense mutations within the 47-amino acid spanning homodimerization region (p.M143 V (Söhn et al. 2010), p.I149T (Xiao et al. 2010), p.H150P (Cheng et al. 2010), p.A166T (Xiao et al. 2010), p.R169Q (Houlden et al. 2010), p.C170R (Bonetti et al. 2009), p.E174G (Song et al. 2011), p.L177P (Newman et al. 2013), p.L180S (Cheng et al. 2012); Fig. 3a) were able to form THAP1-THAP1 dimers. GST pull-downs revealed that none of the mutations in the coiled-coil domain of THAP1 did significantly change the ability of THAP1-THAP1 binding (Fig. 3b). Further, formaldehyde crosslinking showed that overexpressed THAP1 wildtype and all nine mutated THAP1 proteins were able to form THAP1-THAP1 dimers in HEK cells (Fig. 3c).

Discussion

While mutations within the DNA-binding (THAP) domain of THAP1 were shown to decrease the DNA-binding ability of THAP1, no functional consequences have yet been demonstrated for missense mutations within the C-terminal part of THAP1. Certainly, the C-terminal coiled-coil domain of THAP1 is a potent protein-binding motif and 13 amino acids within this motif have been shown to be involved in THAP1 homodimerization (Sengel et al. 2011). Of note, the

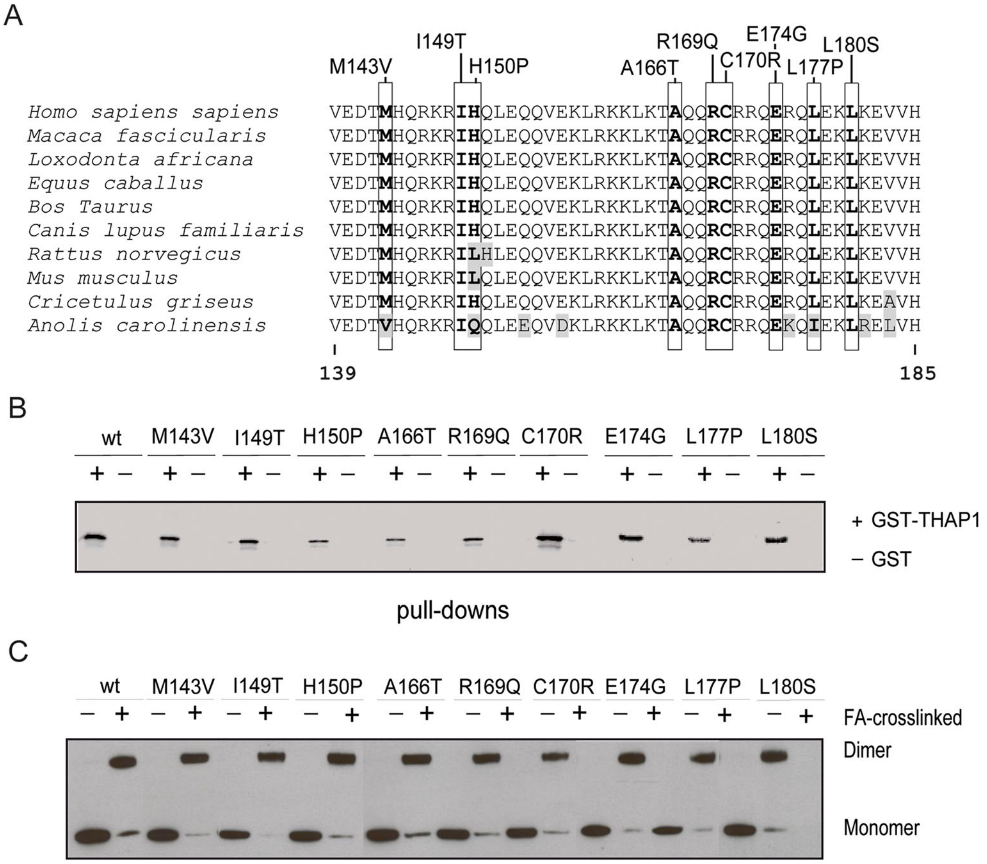

Fig. 3 Impact of nine reported mutations within the homodimerization domain of THAP1. **a** The analyzed missense mutations within the minimal homodimerization region (fragment D7) are boxed and their conservation across species is indicated. **b** Protein-protein interactions between wildtype (wt) and different mutant THAP1 proteins were analyzed by GST pull-downs showing that none of the missense mutations significantly affects the ability of THAP1 to interact with itself. As negative control, empty GST protein was loaded to the glutathione sepharose beads. **c** The Western blot shows the dimerization of wildtype (wt) THAP1 and mutated THAP1 by the formaldehyde cross-linking assay. Without formaldehyde treatment, only the monomers are visible. None of the THAP1 mutations led to a reduced ability for homodimerization of THAP1. THAP1 was targeted with an antibody against FLAG

authors of that study (Sengel et al. 2011) tested only two different truncated fragments of THAP1 including a fragment with a frameshift mutation starting at amino acid 154 and a truncated form of THAP1 with only 166 amino acid. While the latter was still able to form dimers, the shorter form failed to interact with THAP1. Based on these data, the authors concluded that only the 13 amino acids distinguishing these two truncations are necessary for dimer formation. However, they did not provide confirmatory data for dimerization of this short fragment. Therefore, we aimed for a more systematic and in-depth analysis of the homodimerization domain of THAP1.

Using yeast-two-hybrid and GST pull-down assays, we confirmed homodimerization of THAP1 and extended the homodimerization domain to a region of 47 amino acids (amino acids 139–185) representing almost the entire coiled-coil domain of THAP1. Notably, nine missense mutations within this homodimerization domain have been reported in DYT6 patients during the past years. Surprisingly, none of these mutations within the coiled-coil domain significantly altered THAP1 dimerization neither in GST pull-down nor in formaldehyde cross-linking assays. It is conceivable that lack of an effect of the mutations is related to the large amount of ectopically expressed THAP1 protein in our assays, e.g., there is an overload of THAP1 protein that dimerizes despite single missense variants. Alternatively, the THAP1 mutations may indeed not hamper the THAP1-THAP1 interaction but may influence the interaction with other, as yet unidentified, THAP1-binding proteins within the coiled-coil domain. Notably, two (p.I149T, p.A166T (Xiao et al. 2010)) of the nine missense mutation tested in our study did also not show alterations on homodimerization in a previous study (Sengel et al. 2011).

Taken together, we further characterized the THAP1 homodimerization using three independent methods and demonstrate that 47 are essential for the THAP1-THAP1 interaction. Thus, we assign a physiological function to the coiled-coil domain of THAP1 which represents a highly potent protein-binding motif (Burkhard et al. 2001).

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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