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# 1 **The TFIIH components p44/p62 act as a damage sensor during** 2 **nucleotide excision repair**

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8  
9 Keywords; DNA repair, NER, fluorescence imaging, single molecule

10

## 11 **Abstract**

12 Nucleotide excision repair (NER) protects the genome following exposure to  
13 diverse types of DNA damage, including UV light and chemotherapeutics.  
14 Mutations in human NER genes lead to diseases such as xeroderma  
15 pigmentosum and Cockayne syndrome <sup>1</sup>. In eukaryotes, the major transcription  
16 factor TFIIH is the central hub of NER. The core components of TFIIH include  
17 the helicases XPB, XPD, and the five core ‘structural’ subunits <sup>2-6</sup>. Two of these  
18 core-TFIIH proteins, p44 and p62 remain relatively unstudied; although p44 is  
19 known to regulate the helicase activity of XPD during NER <sup>7-9</sup>. p62’s role is  
20 thought to be structural <sup>10</sup>; however, a recent cryo-EM structure <sup>11</sup> shows p44,  
21 p62, and XPD making contacts with each other, implying a more extensive role  
22 in DNA repair beyond the structural integrity of TFIIH. Here, we show that p44  
23 stimulates XPD’s ATPase, but upon encountering DNA damage further  
24 stimulation is only observed when p62 is in the ternary complex. More  
25 significantly, we show that the p44/p62 complex binds DNA independently of  
26 XPD and diffuses along its backbone, indicating a novel DNA-binding entity in  
27 TFIIH. These data support a role for p44/p62 in TFIIH’s mechanism of damage  
28 detection. This revises our understanding of TFIIH and prompts more extensive  
29 investigation of all of the core subunits, for an active role during both DNA  
30 repair and transcription.

## 31 **Results and Discussion**

32

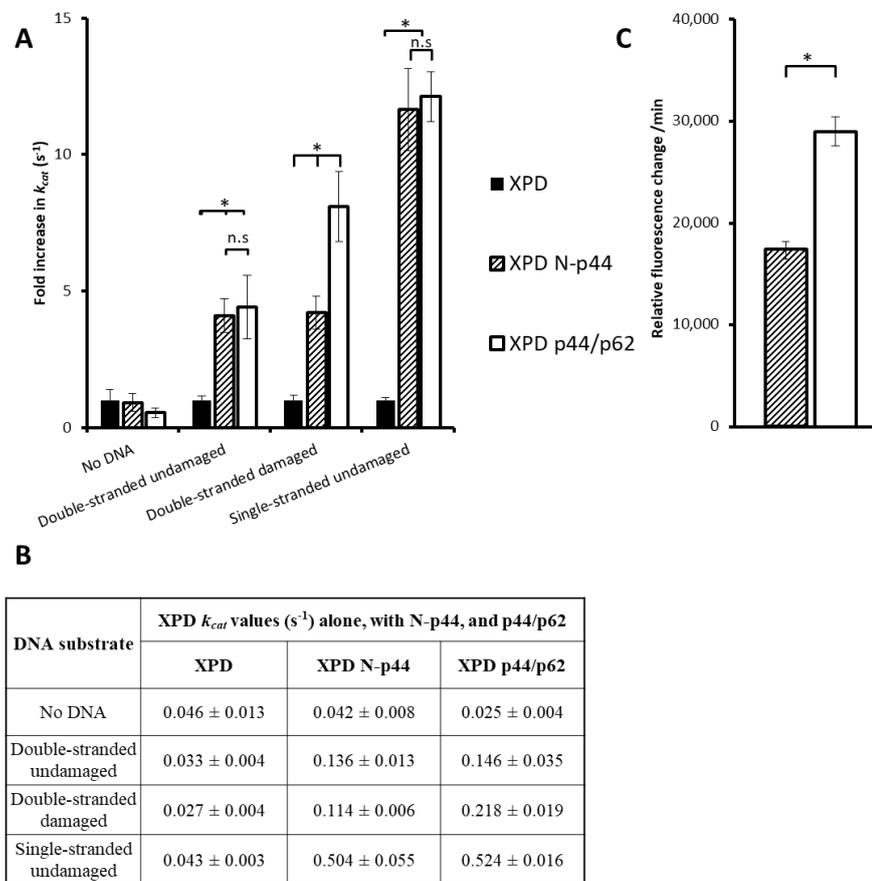
### 33 **XPD's ATPase is stimulated by p44/p62**

34 p44 contacts both p62 and XPD in TFIIH<sup>3,11,12</sup>, and mutations in XPD that  
35 disrupt p44 or p62 binding cause defects in NER and result in disease<sup>3,7,8,12</sup>. To  
36 investigate if p44/p62 was able to stimulate the ATPase of XPD, the turnover of  
37 ATP in the presence of different DNA substrates was measured using an  
38 NADH-coupled assay.

39 In the absence of p44 and p62, XPD's ATPase activity is slow even in the  
40 presence of single-stranded DNA (0.043 s<sup>-1</sup>). However, with a p44 fragment  
41 (residues 1-285 (N-p44)) containing the von Willebrand domain, XPD's  
42 ATPase was significantly stimulated in the presence of both double- and single-  
43 stranded DNA<sup>8</sup> (~0.03 s<sup>-1</sup> to 0.136 s<sup>-1</sup> and 0.504 s<sup>-1</sup> respectively p < 0.05  
44 (**Figure 1**)). No further acceleration of the ATPase was observed with full  
45 length p44 co-expressed in complex with p62 (p44/p62). However, remarkably,  
46 when damage (a fluorescein moiety shown to proxy for damage<sup>13</sup>) was  
47 introduced into a dsDNA substrate, p44/p62 accelerated XPD's ATPase two-  
48 fold more than on undamaged DNA (**Figure 1A & B**). N-p44 alone could not  
49 accelerate XPD's ATPase in the presence of damage, indicating the ternary  
50 complex (p44/p62) is responsible for this further enhancement and thus may  
51 play an important role in lesion detection. These results may explain why  
52 truncations of the yeast p62 homologue (Tfb1) sensitize the organism to UV  
53 irradiation<sup>14-16</sup>.

54 To further investigate the role of p44 and p62 in activating XPD we analyzed  
55 XPD's helicase activity on an open fork substrate. Again, p44/p62 is seen to  
56 play an active role by enhancing the helicase activity compared to N-p44 alone  
57 (**Figure 1C**). Although no damage is present in the open fork substrate, p44/p62  
58 significantly enhances XPD's ability to successfully unwind the DNA substrate

59 (two-fold more than XPD with N-p44), despite no change in ATPase activity  
 60 (**Figure 1A**).



61 **Figure 1. Steady-state ATPase and helicase activity of XPD in the presence of**  
 62 **various DNA substrates and core TFIID proteins.** A) The activity of XPD's  
 63 ATPase is stimulated by both N-p44 (dashed) and p44/p62 (white) on various  
 64 DNA substrates. Values for  $k_{cat}$  are given as a fold change from XPD alone  
 65 (black). Errors are shown as S.E.M from 3 repeats. B) Table showing  $k_{cat}$  values  
 66 ± S.E.M for XPD's ATPase. C) XPD's helicase activity is stimulated by N-p44  
 67 (dashed) and p44/p62 (white) on an open fork substrate. XPD alone displays no  
 68 helicase activity<sup>8</sup>. Errors are shown as S.E.M from 9 repeats. Statistical

69 *significance determined using a student's t-test where \* =  $p < 0.05$ , n.s = not*  
70 *statistically significant.*

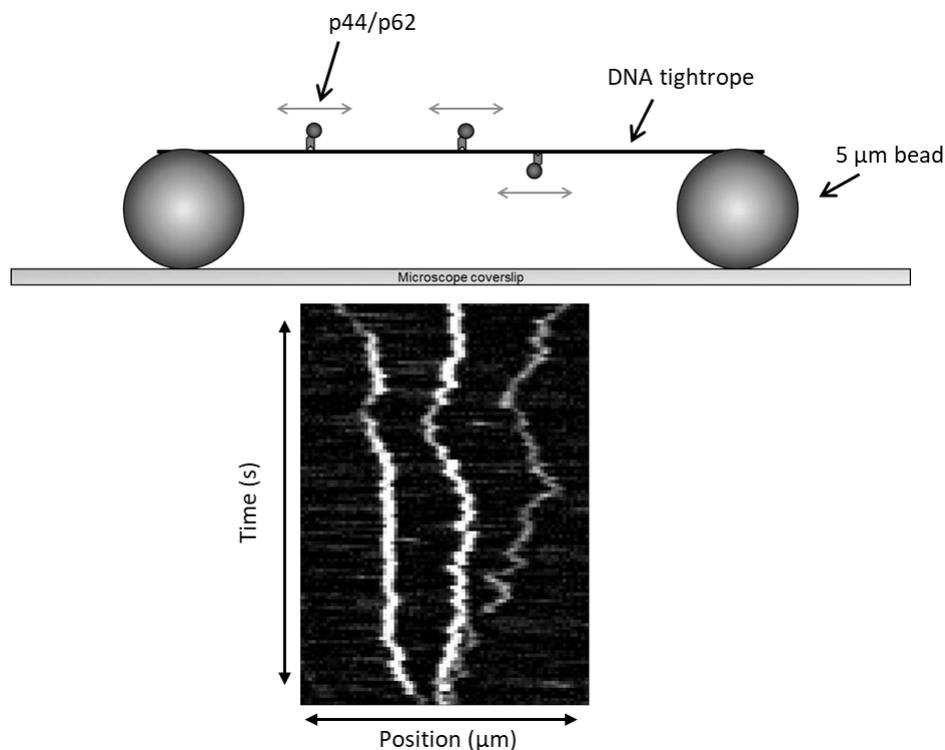
71

72

### 73 **The p44/p62 complex directly binds DNA**

74 The role of p44/p62 in the recognition of damage presents the intriguing  
75 possibility that this complex could interact with DNA independently from XPD.

76 To investigate this, we used a single molecule DNA tightrope assay <sup>17</sup> (**Figure**  
77 **2**). Conjugation of a fluorescent quantum dot (QDot) to the poly-histidine  
78 purification tag on the p44/p62 complex <sup>18</sup> was achieved using an anti-His IgG  
79 antibody. Substantial binding of p44/p62 to dsDNA was observed, and of these  
80 approximately 80% could diffuse (n = 599 total) providing the first direct  
81 evidence that these TFIIH subunits are able to bind DNA independently of  
82 XPD.



83 **Figure 2. Schematic of a tightrope and kymograph analysis.** DNA tightropes  
84 are formed between beads adhered to a coverslip. QDot labelled proteins are  
85 then observed binding to the DNA. A video can be transformed into a  
86 kymograph by plotting position through time. Diffusing molecules appear as  
87 movement in the X axis for a duration of frames (Y axis). The kymograph shown  
88 in the lower panel indicates three diffusing p44/p62 molecules.

89  
90 The p44/p62 complex displayed multiple types of behavior on DNA. Firstly, we  
91 observed complexes randomly diffusing along the DNA, unable to pass one  
92 another (**Figure 2**). Secondly while diffusing, pausing was seen, often at the  
93 same location on the tightropes. This may indicate a visit to a damage site or a  
94 specific sequence. Finally, fluorescence intensity fluctuations of the same  
95 molecule over time suggest possible oligomerization. At elevated salt  
96 concentrations (100 mM vs 10 mM KCl) fewer molecules bound to DNA, and  
97 of these, a lower percentage diffused (55%, n = 58 total). We calculated the  
98 diffusion constant using mean-squared displacement analysis<sup>17</sup> and found no  
99 significant change (p > 0.05) between salt conditions (10 mM KCl  $0.067 \mu\text{m}^2/\text{s}$   
100  $\pm 0.006$  vs 100 mM KCl  $0.042 \mu\text{m}^2/\text{s} \pm 0.010$ ), which suggests that p44/p62  
101 molecules slide along the DNA helix<sup>19</sup>. Based on the estimated size of a  
102 p44/p62 complex conjugated to a QDot, the diffusion constant appears limited  
103 by rotation-coupled diffusion around the backbone of the DNA helix<sup>20</sup>. This is  
104 consistent with the inability for complexes to pass one another on the DNA.

105  
106 In summary, we present the first mechanistic characterisation of the non-  
107 helicase TFIIH subunits p44/p62. Complexes formed by these two proteins  
108 were observed to bind and slide on dsDNA. Our bulk phase ATPase and  
109 helicase data indicate that p44/p62 is involved in damage recognition. One  
110 could speculate that p44/p62 actively enhances TFIIH activity towards scanning  
111 the opened repair bubble to position TFIIH factors for subsequent excision.

112 Nonetheless, our results clearly show that the p44/p62 complex plays an active  
113 and not just a structural role in the TFIIF complex.

114

115

## 116 **Methods**

### 117 *Purification*

118

119 The genes encoding p44 and p62 were cloned from *C. thermophilum* cDNA.  
120 p62 was cloned into the pETM-11 vector (EMBL) without a tag. p44 was  
121 cloned into the pBADM-11 vector (EMBL) containing an N-terminal hexa-  
122 Histidine tag followed by a TEV cleavage site. p62 and p44 were co-expressed  
123 in *E. coli* BL21 CodonPlus (DE3) RIL cells (Agilent) and were co-purified via  
124 immobilized metal affinity chromatography (Ni TED, Machery-Nagel),  
125 followed by size exclusion chromatography (SEC), and anion exchange  
126 chromatography (AEC). SEC was conducted with a HiLoad 16/600 Superdex  
127 200 prep grade column (GE Healthcare) in 20 mM Hepes pH 7.5, 250 mM  
128 NaCl, and 1 mM TCEP. AEC was conducted with a MonoQ 5/50 GL column  
129 (GE Healthcare). The proteins were eluted via a salt gradient ranging from 50 to  
130 1000 mM NaCl. AEC buffers were composed of 20 mM HEPES pH 7.5,  
131 50/1000 mM NaCl, and 1 mM TCEP. The p62/p44 protein complex was  
132 concentrated to approximately 20 mg/ml and flash frozen in liquid nitrogen for  
133 storage.

134 XPD and N-p44 (1-285) from *C. thermophilum* were cloned as described  
135 previously<sup>21</sup>. XPD was expressed as N-terminally His-tagged proteins in *E. coli*  
136 ArcticExpress (DE3)-RIL cells (Agilent). Cells were grown in TB medium at  
137 37°C until they reached an OD<sub>600</sub> of 0.6-0.8. Expression was started with the  
138 addition of 0.05% L-arabinose and performed at 11°C for 20 h. p44 was  
139 expressed as N-terminally His-tagged protein in *E. coli* BL21-CodonPlus  
140 (DE3)-RIL cells (Stratagene). Cells were grown as described for ctXPD and

141 expression was started by adding 0.1 mM IPTG at 14°C for 18 h. XPD and p44  
142 were purified to homogeneity by metal affinity chromatography (Ni-IDA,  
143 Macherey&Nagel) as described previously <sup>21</sup> followed by size exclusion  
144 chromatography (SEC) (20 mM HEPES pH 7.5, 200 mM NaCl) and an  
145 additional anion exchange chromatography (AEC) step in the case of XPD.  
146 AEC was performed using a MonoQ 5/50 GL column (GE Healthcare) with 20  
147 mM HEPES pH 7.5, 50 mM NaCl, and 1 mM TCEP as loading buffer and the  
148 same buffer containing 1 M NaCl was used for elution. The final buffer after  
149 AEC was 20 mM HEPES pH 7.5, 250 mM NaCl, and 1 mM TCEP. The  
150 proteins were concentrated to at least 5 mg/ml based on their calculated  
151 extinction coefficient using ProtParam (SwissProt) and then flash frozen for  
152 storage at -80°C.

153

#### 154 *ATPase assay*

155 dsDNA substrates used:

156 F26,50 contains a fluorescein moiety covalently attached to thymine (\*);

157 5`GACTACGTA CTGTTACGGCTCCATCT\*CTACCGCAATCAGGCCAGA  
158 TCTGC 3`

159 The reverse complementary sequence to F26,50;

160 5`GCAGATCTGGCCTGATTGCGGTAGCGATGGAGCCGTAACAGTACG  
161 TAGTC 3`

162 F26,50 without the fluorescein moiety;

163 5`GACTACGTA CTGTTACGGCTCCATCTCTACCGCAATCAGGCCAGAT  
164 CTGC 3`

165 The NADH-coupled ATPase assay was performed as described previously <sup>22</sup> in  
166 plate reader format. Imaging buffer containing the NADH-reaction components  
167 was supplemented with 1 mM fresh TCEP, protein (100 nM (equimolar  
168 concentrations for XPD N-p44 and XPD p44/p62)), and 50 nM of DNA  
169 substrate. The reaction was started with the addition of 1 mM ATP to each well,  
170 and the change in OD340 (NADH) was monitored every 8 seconds/well over 30

171 minutes at room temperature in a Clariostar plate reader. The rates of NADH  
172 consumption were used to calculate  $k_{cat}$ . Reactions were repeated 3 times, and  
173 S.E.M used as errors values.

174

### 175 *In vitro helicase assay*

176 Helicase activity was analyzed utilizing a fluorescence-based helicase assay.

177 We used an open fork substrate with a Cy3 label at the 3' end of the

178 translocated strand where unwinding of the DNA substrate reduces quenching  
179 of the Cy3 fluorescence.

180 5' AGCTACCATGCCTGCACGAATTAAGCAATTCGTAATCATGGTCATA

181 GC-Cy3 3' and a dabcy1 modification on the 5' end of the opposite strand

182 5' Dabcy1-

183 GCTATGACCATGATTACGAATTGCTTGGAATCCTGACGAACTGTAG

184 3'

185 Assays were carried out in 20 mM HEPES pH 7.5, 50 mM KCl, 5 mM MgCl<sub>2</sub>,

186 and 1 mM TCEP. DNA was used at a concentration of 250 nM. Helicase

187 activity was measured with equimolar concentrations of XPD, p44, and/or p62.

188 The mix of reagents, with the exception of ATP, were preincubated at 37°C and

189 the reaction was subsequently started with the addition of 5 mM ATP. Kinetics

190 were recorded with a Flourostar Optima plate reader (BMG labtech).

191 Fluorescence was detected at an excitation wavelength of 550 nm (slit width, 2

192 nm) and an emission wavelength of 570 nm (slit width, 2 nm). Initial velocities

193 were fitted with the MARS software package (BMG labtech) and represent the

194 averages of at least three different reactions and two independent protein

195 batches.

196

### 197 *Single Molecule DNA Tightrope Assay*

198 For a detailed protocol see <sup>18</sup>. p44/p62 interactions with DNA were studied in

199 imaging buffer (20 mM Tris pH 8.0, 10 mM KCl (100 mM for high salt), 5 mM

200 MgCl<sub>2</sub>, 1 mM TCEP). Videos for diffusion analysis were collected between 30  
201 seconds and 5 minutes at 10 frames per second. Video analysis was performed  
202 in ImageJ as described previously<sup>17</sup>.

203

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209 CK. The authors declare no conflict of interest.

210

## 211 **Author contributions**

212 Collected data: JTB, JK, WK. Designed experiments: JTB, JK, CK, NMK.

213 Analysed Data: JTB, JK, NMK. Wrote paper: JTB, JK, CK, NMK.

214

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216

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