Supplementary Methods

Preparation of phosphorothioate-containing *HIS4* DNA templates Phosphorothioate-containing templates in which phosphorothioate is incorporated into the non-template strand were prepared by ligating the products of two PCR reactions. The first PCR reaction using pSH515[1] as the template and upstream primer p965 (5'-biotin-TAATGCAGCTGGCACGACAGG-3') and downstream primer p515NTBio (5'-TCCGCAGCGACTGAGCATACTACTGTTCTCGAGGT-3') resulted in the production of a 356-bp fragment. The second PCR reaction with pSH515 as the template and upstream (phosphorothioate-containing) primer p515NT1 (5'-AGGCCAGTCGCTGGTGTATATaAtAgCtAtGGAACGTTCGATTCACCTCCGATGTGTGTTGTACATACATAAAAATATCATAGCACAAC-3') or various primers with the 5 phosphorothioate bases in different positions (see **Fig. 1a**) and downstream primer pNOT (5'-GGCCGCTCTAGCTGCATTAATG-3') resulted in the production of a 234-bp fragment. The AlwNI restriction site is underlined and lower case letters highlight the positions of the phosphorothioate bases. Each PCR product was purified using a Purelink PCR purification kit (Invitrogen) and digested with AlwNI (New England Biolabs) overnight at 37^0C. The digested PCR product was then purified again using a Purelink PCR purification kit (Invitrogen). AlwNI digested products were then ligated in a reaction containing 10 μg of 356-bp product and 7 μg of 234-bp product, 66 mM Tris HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 7.5% (w/v) PEG_{8000} and 2000 units of T4 DNA Ligase (NEB), overnight at room temperature. The ligation was then ethanol precipitated, washed in 80% (v/v) ethanol and the DNA pellet was dried and resuspended in 20 μl of H_20. Ligated DNA was size-separated on a 1% (w/v) agarose gel for 80 minutes and the 590-bp product was excised and gel purified using a QIAquick gel extraction kit (Qiagen). The biotinylated phosphorothioate-containing *HIS4* promoter DNA was then used in FeBABE conjugation reactions. Phosphorothioate-containing templates with phosphorothioate incorporated into the template strand were prepared using a similar strategy.

Rad25 Structure Modeling and Model Evaluation For structure modeling, the helicase domains of *S. cerevisiae* Rad25 were aligned with Archaeoglobus fulgidus XPB. For this alignment, ClustalX was first used to align Af XPB and the two helicase subdomains of human XPB, incorporating secondary structure constraints from Af XPB (2FWR)[2]. Separately, ClustalX was used to align sequences of eukaryotic XPBs including yeast Rad25. These two sequence alignments were then aligned using ClustalX. This initial alignment was used in conjunction with the program Modeller8v2[3] to generate 30 initial models. These initial models were evaluated using statistically based structure validation methods[4-6]. This analysis revealed several localized problem areas that primarily corresponded to short segments of Rad25 that weakly aligned with the Af XPB sequence. The DEVH domain of the Rad25/Af XPB alignment was next adjusted based on the NCBI alignment of the SSL2 helicase family (COG1061). This adjustment of the alignment, in conjunction with two additional rounds of modeling, resulted in several structure models which were all very similar and scored reasonably well in statistical validation tests. The final sequence alignment used for modeling is listed in **Supplementary Figure 6**. The two helicase domains shown in **Figure 5b** are the DNA damage recognition domain + N-terminal helicase subdomain (including the DEVH motif) and the C-terminal helicase subdomain. The Rad25 N-terminal domain model was based on modeling of Rad25 residues 294-714 with the full length Af XPB structure (2FWR) and truncating the model at the site of the flexible hinge region (Rad25 residue 538). The Rad25 C-terminal domain model was based on modeling Rad25 residues 548-714 with the C-terminal Af XPB structure (2FZL). Next, these two structure models were positioned in the closed orientation as described[2,7] by superimposing the models with the HCV RNA helicase structure containing single stranded RNA (1HEI).

Yeast Strains, Nuclear Extracts and Antisera The following yeast strains were used for this study: BY4705[8], wildtype (all strains are derivatives of this); SHY551 and 552 containing a triple Flag epitope at the C terminus of Rpb1 and Rpb2, respectively; SHY485 containing a triple Flag epitope at the C terminus of Rad25; SHY384 and 564 containing a triple Flag epitope at the C terminus of Tfg1 and Tfg2, respectively;

SHY772 containing a 12 Flag epitope at the C terminus of Tfg1; SHY361 and 560 containing a triple Flag epitope at the C terminus of Tfa1 and Tfa2, respectively; SHY386 containing a triple Flag epitope at the C terminus of Sua7. Yeast nuclear extracts were made as described (www.fhcrc.org/labs/hahn). Anti-Flag M2 monoclonal antibody was from Sigma, and rabbit polyclonal antisera was generated against the N-terminal 200 amino acids of *S. cerevisiae* Rpb1[9].

Supplementary References

1. Ranish, J.A., Yudkovsky, N. & Hahn, S. Intermediates in formation and activity of the RNA polymerase II preinitiation complex: holoenzyme recruitment and a postrecruitment role for the TATA box and TFIIB. *Genes Dev* **13**, 49-63 (1999).
2. Fan, L. et al. Conserved XPB Core Structure and Motifs for DNA Unwinding: Implications for Pathway Selection of Transcription or Excision Repair. *Mol Cell* **22**, 27-37 (2006).
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4. Colovos, C. & Yeates, T.O. Verification of protein structures: patterns of nonbonded atomic interactions. *Protein Sci* **2**, 1511-9 (1993).
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7. Caruthers, J.M., Johnson, E.R. & McKay, D.B. Crystal structure of yeast initiation factor 4A, a DEAD-box RNA helicase. *Proc Natl Acad Sci U S A* **97**, 13080-5 (2000).
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9. Chen, H.T. & Hahn, S. Mapping the location of TFIIB within the RNA polymerase II transcription preinitiation complex: a model for the structure of the PIC. *Cell* **119**, 169-80 (2004).

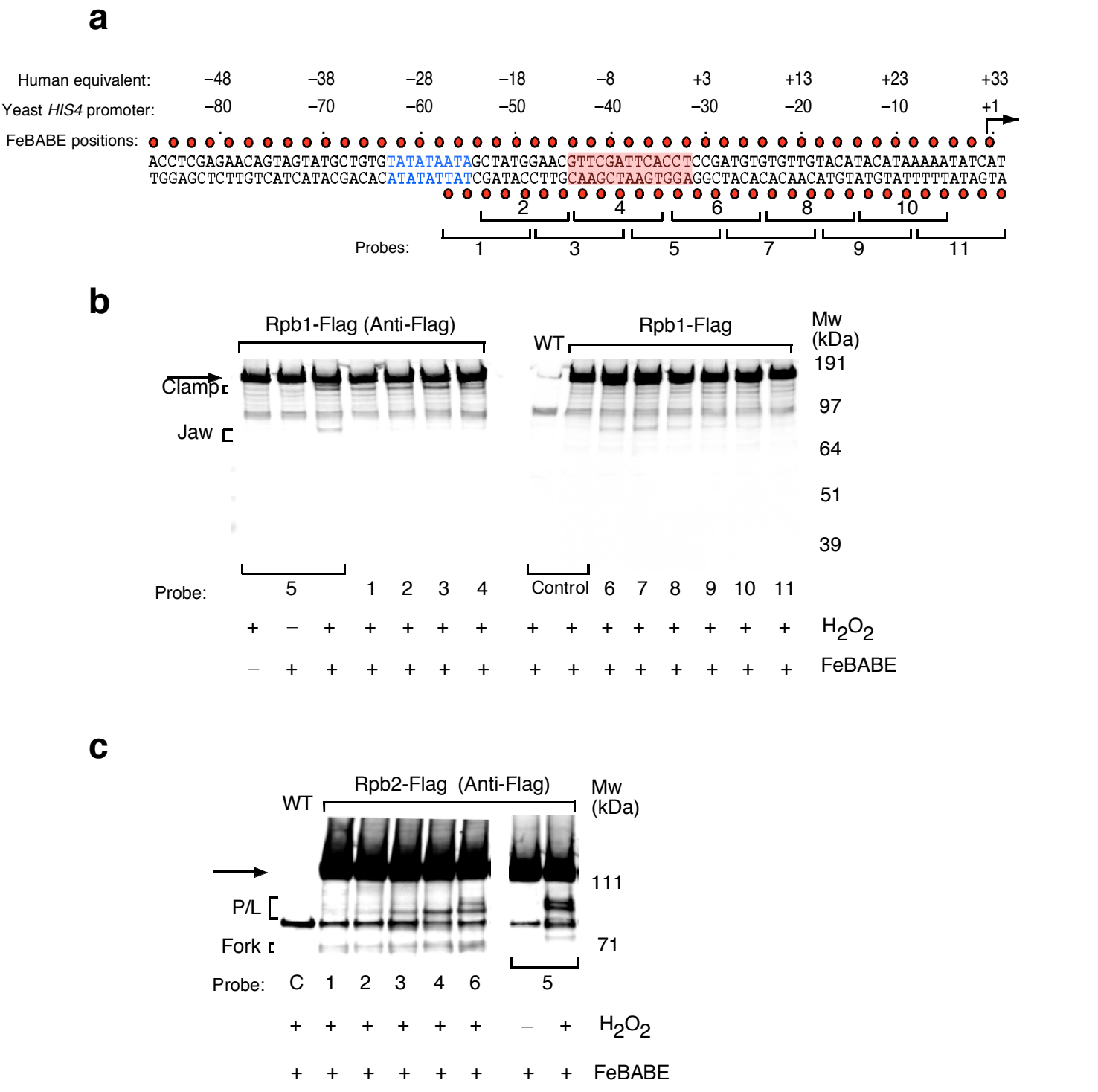
Supplementary Table 1 Miller and Hahn

PROMOTER	Rpb1 residue Int	Rpb2 residue Int	Rad25 residue Int	Tfg1 residue Int	Tfg2 residue Int	Tfa1 residue Int	Tfa2 residue Int	TFIIB residue Int
-5								
-4				331 M 421 w				
-3		180 M/S protrusion						
-2		180 M/S protrusion			292 W			
-1					292 W			core domain W
TATA				316 M				
1	262 M/S clamp core			316 M/S	292 W		224 W	
2	262 M/S clamp core			316 M/S	292 W	20 W	224 W	112 W linker 156 W core domain
3	262 M/S clamp core	180 M/S protrusion 227 M/S lobe		331 M/S	184 M	25 W	224 W	105 W linker
4	262 M/S clamp core	364 M/S lobe 523 W fork		331 M/S	184 M	25 W		99 W B-finger
5	214 M/S clamp head	195 P M/S 239 L M/S 364 L W 503 F W		421 W 528 W	196 M/S			
6	214 M/S clamp head 1187 M/S Jaw	169 P M/S 217 L M/S 532 F W	438 M/S DEAD box domain 484 M/S DEAD box domain	528 W	199 M/S			
7	214 M/S clamp head 1187 M/S Jaw		627 M/S Ct helicase domain					
8	1173 M/S Jaw		484 W DEAD box domain 511 W DEAD box domain					
9			516 W DEAD box domain					
10								
11								

Supplementary Table 1. Summary of RNA Polymerase II and General Transcription Factor cleavage sites generated by FeBABE attached to *HIS4* promoter DNA

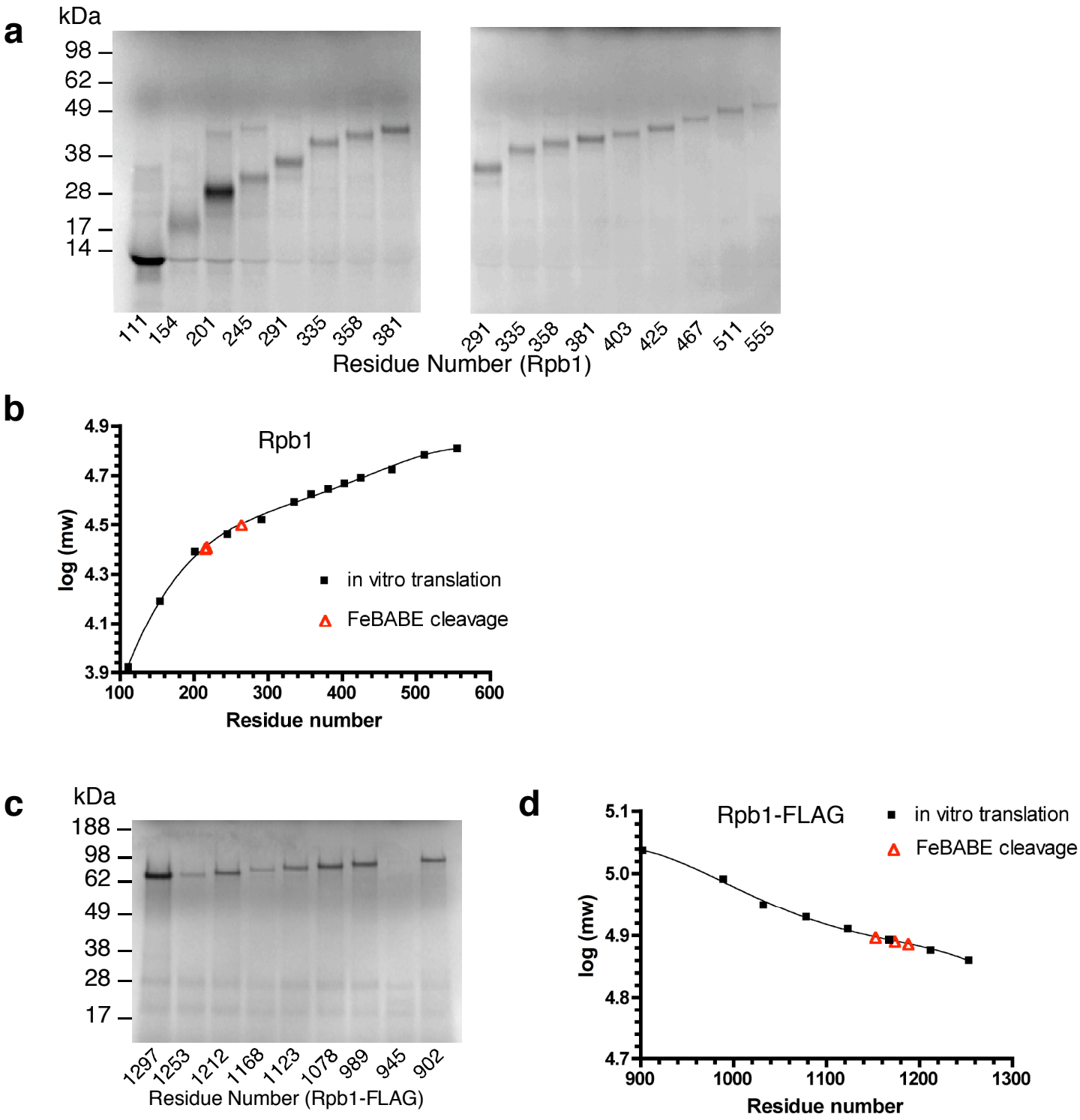
The measured residue where hydroxyl radical cleavage is observed is listed as well as the structural element, if known, where this residue is situated. Int., cleavage intensity. s, strong; m, medium; w, weak. P, protrusion; L, lobe; F, fork.

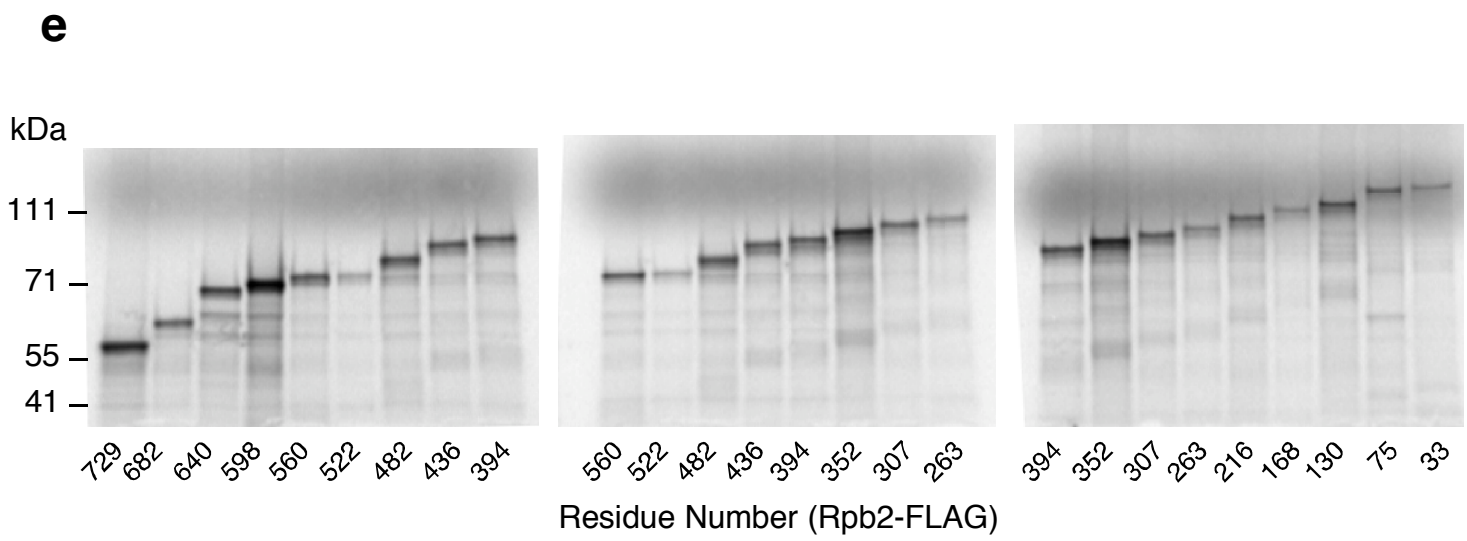
Supplementary Figure 1 Miller and Hahn

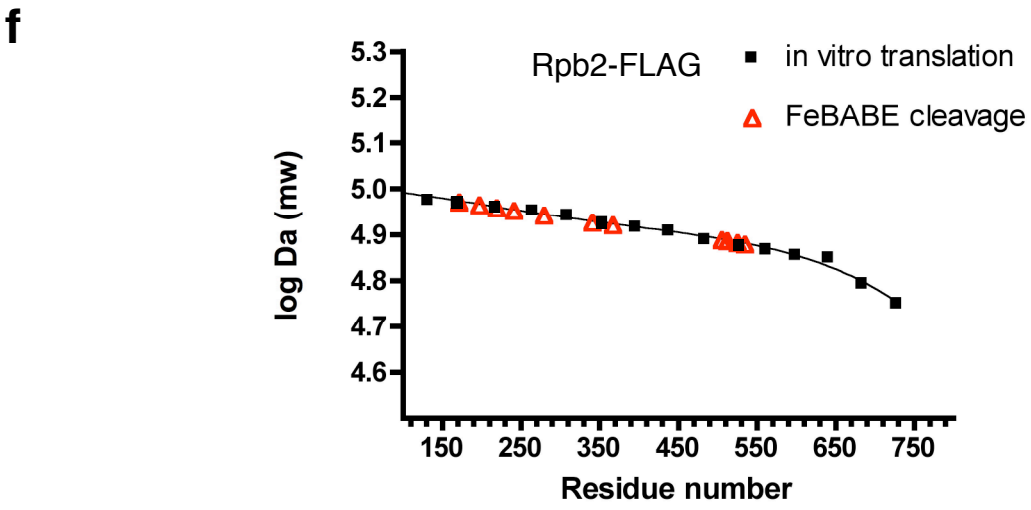


Supplementary Figure 1. Directed hydroxyl radical probing of RNA Polymerase II subunits Rpb1 and Rpb2 with FeBABE attached to the template strand (**a**) 11 FeBABE-tethered probes derived from the *HIS4* promoter where FeBABE is attached to the template strand. The color scheme is the same as in Figure 1. (**b**) and (**c**) Cleavage fragments of Rpb1-Flag and of Rpb2-Flag were visualized by Western Blotting with anti-Flag antibody as described in **Figure 2**.

Supplementary Figure 2 Miller and Hahn

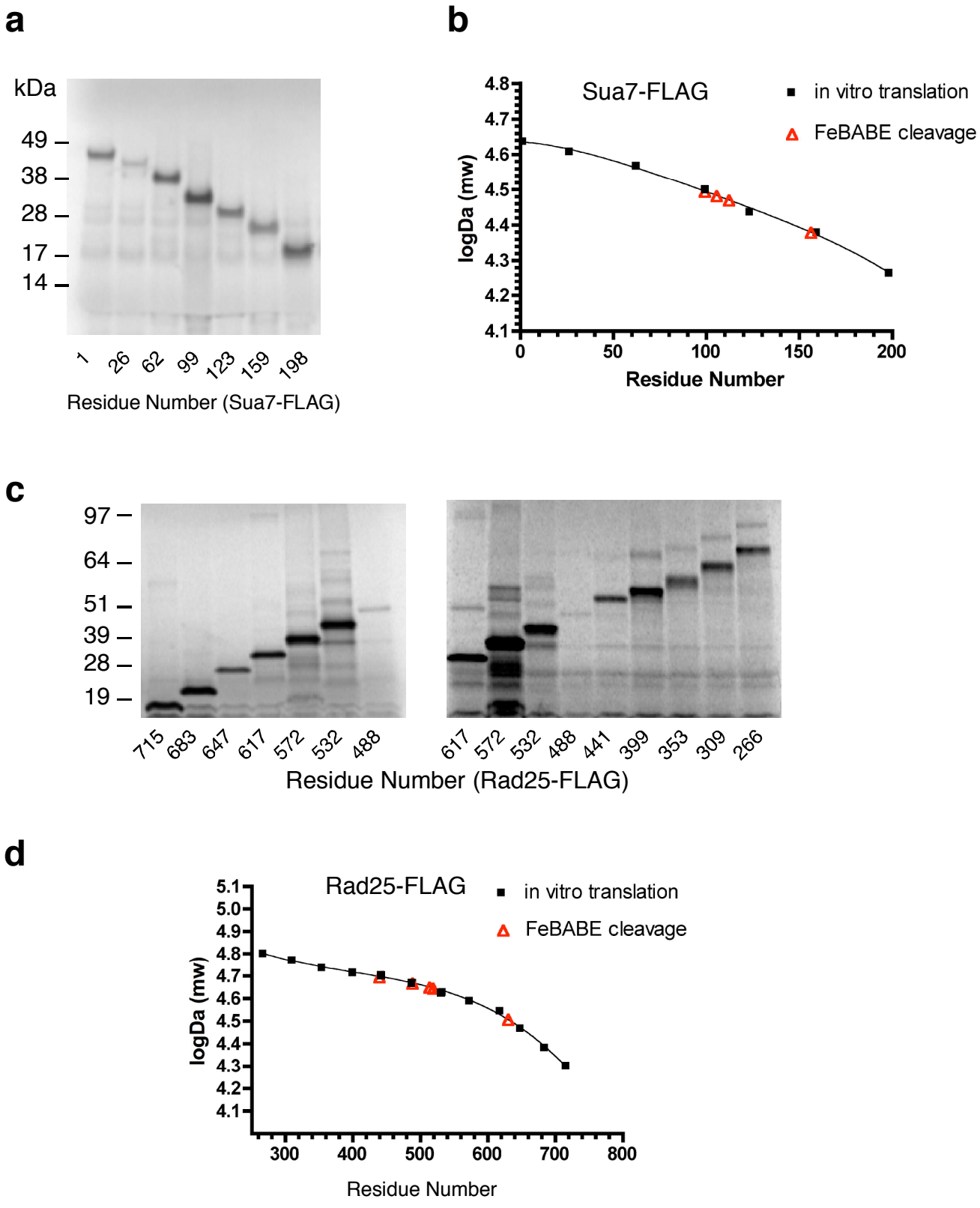




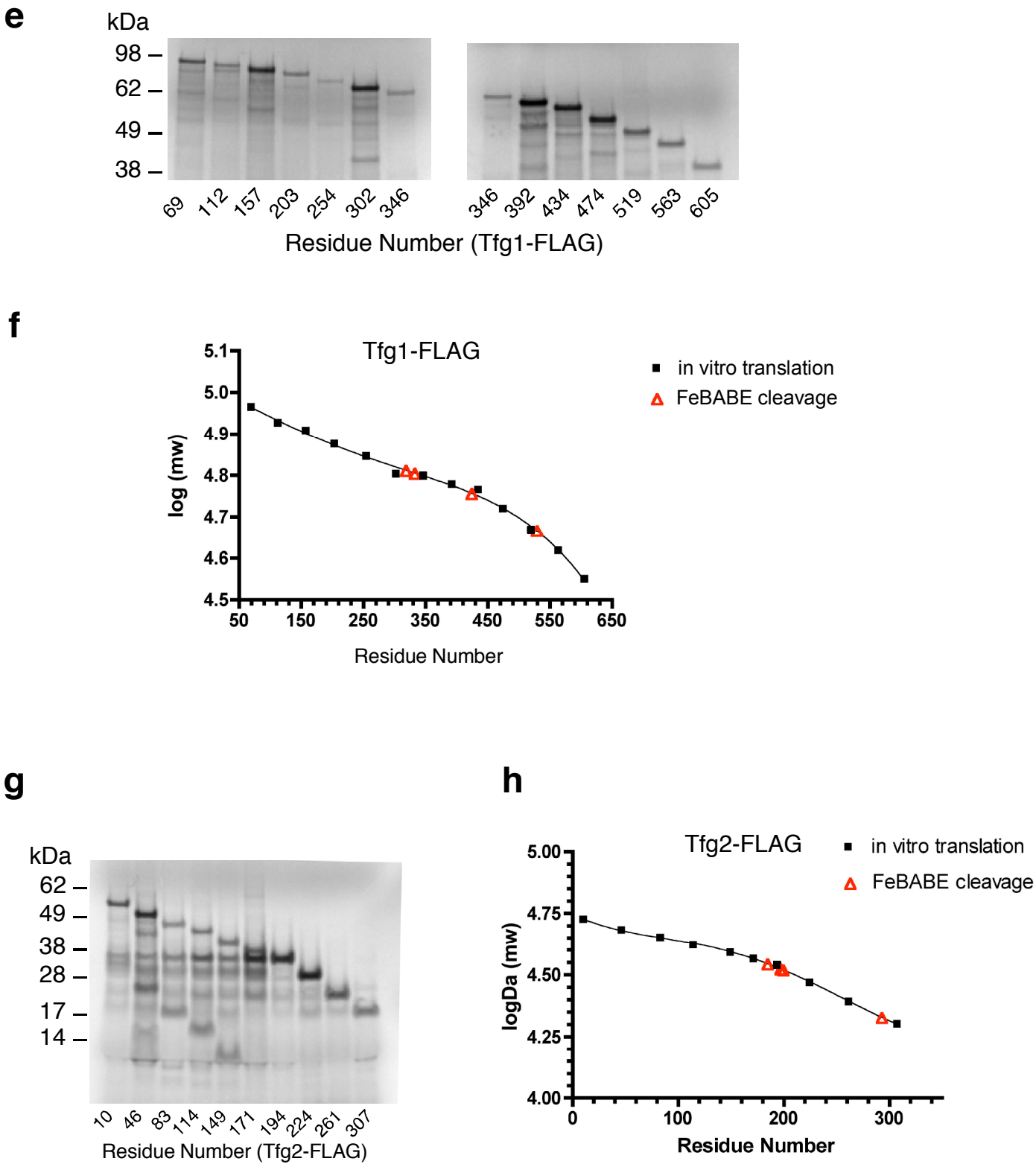


Supplementary Figure 2. In vitro translated peptide fragments of Rpb1 and Rpb2 used as molecular standards for calculating FeBABE cleavage sites (**a**, **c** and **e**) In vitro translated peptide fragments of the N-terminal region of Rpb1-Flag, the C-terminal region of Rpb1-Flag and the N-terminal region of Rpb2-Flag, respectively, analyzed by SDS-PAGE. The residue number indicates either the last (**a**) or first (**c** and **e**) residue for each individual peptide fragment. (**b**, **d** and **f**) Calibration curves for determining the amino acid numbers of FeBABE cleavage fragments. Each calibration curve is derived from a 4th order polynomial function using the sizes (log Da (mw)) of in vitro translated peptides and their corresponding residue numbers in (**a**), (**c**) and (**e**). Locations of the FeBABE cleavage fragments on the calibration curves are shown with red triangles.

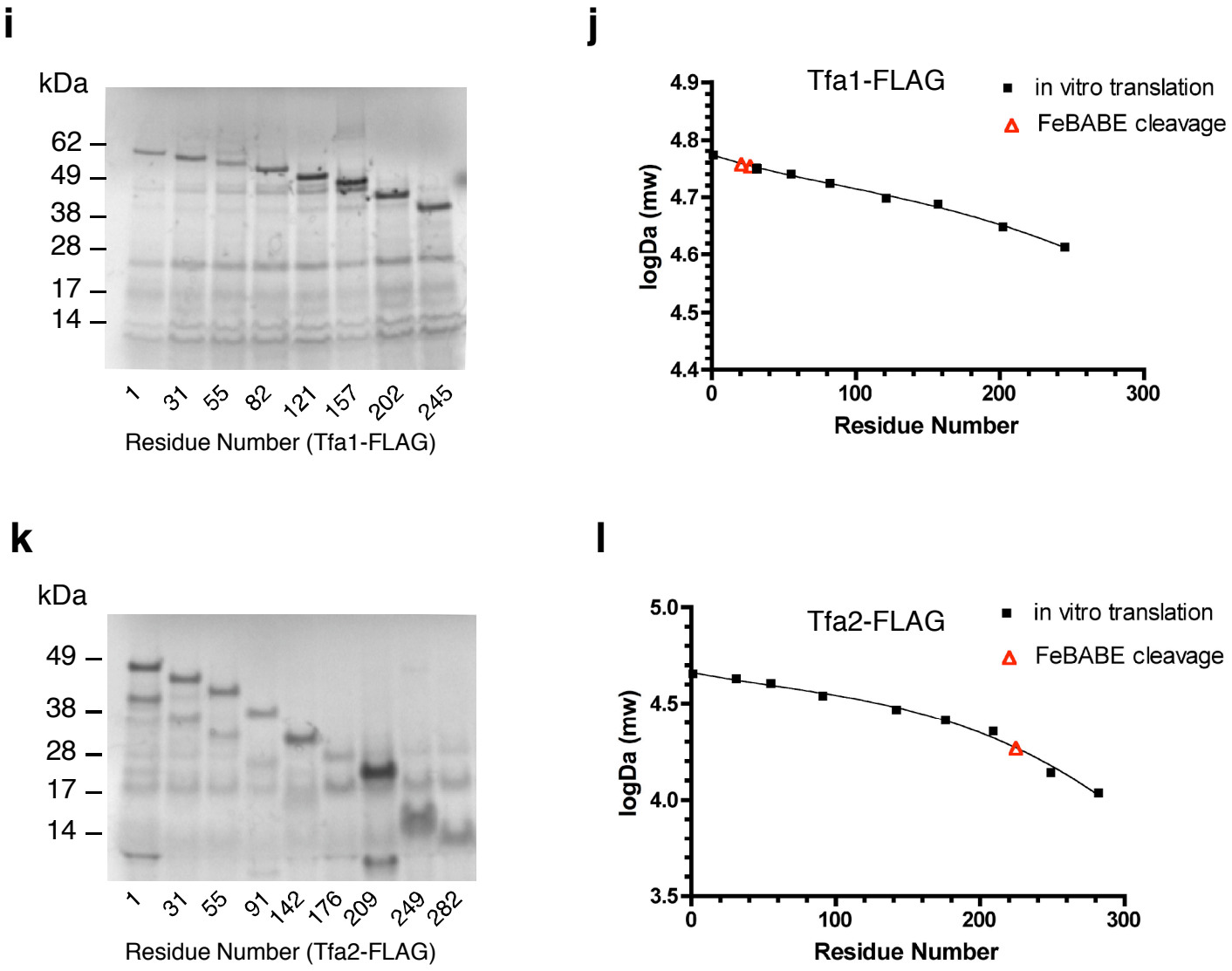
Supplementary Figure 3 Miller and Hahn



Supplementary Figure 3 contd. Miller and Hahn

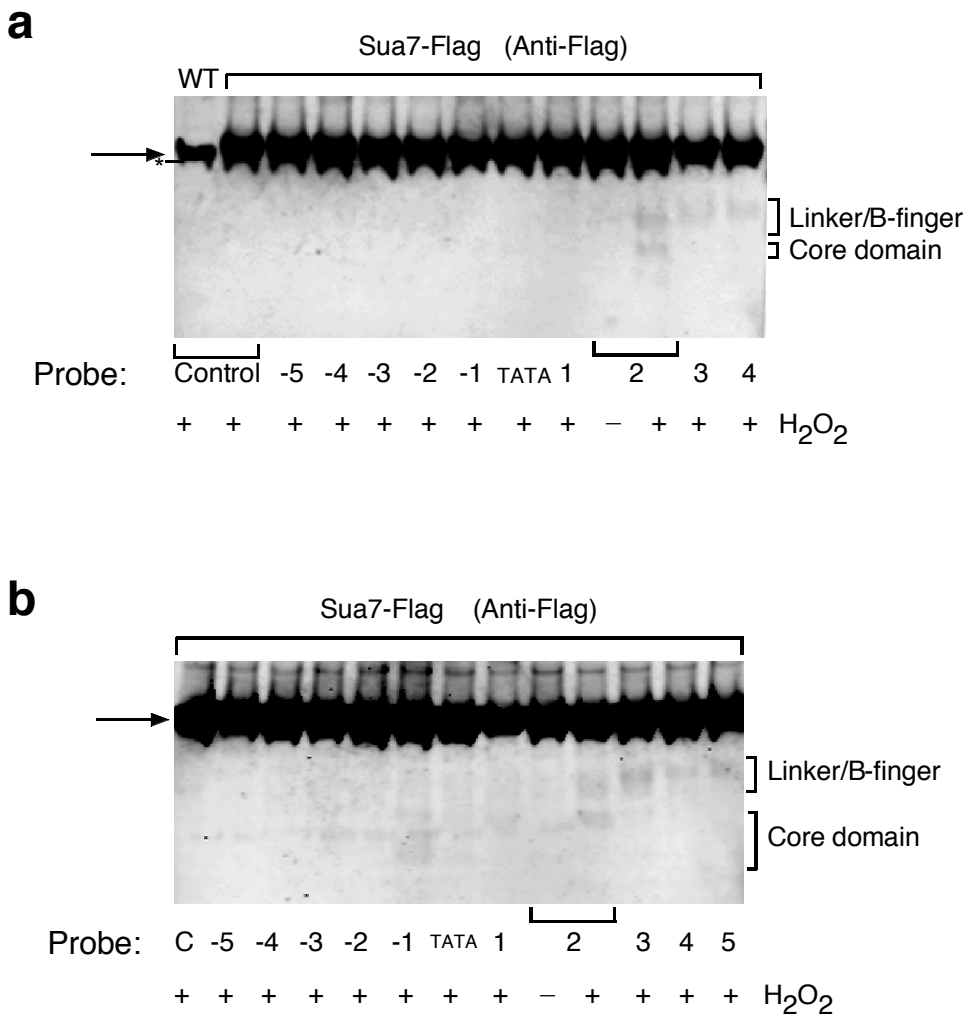


Supplementary Figure 3 contd. Miller and Hahn



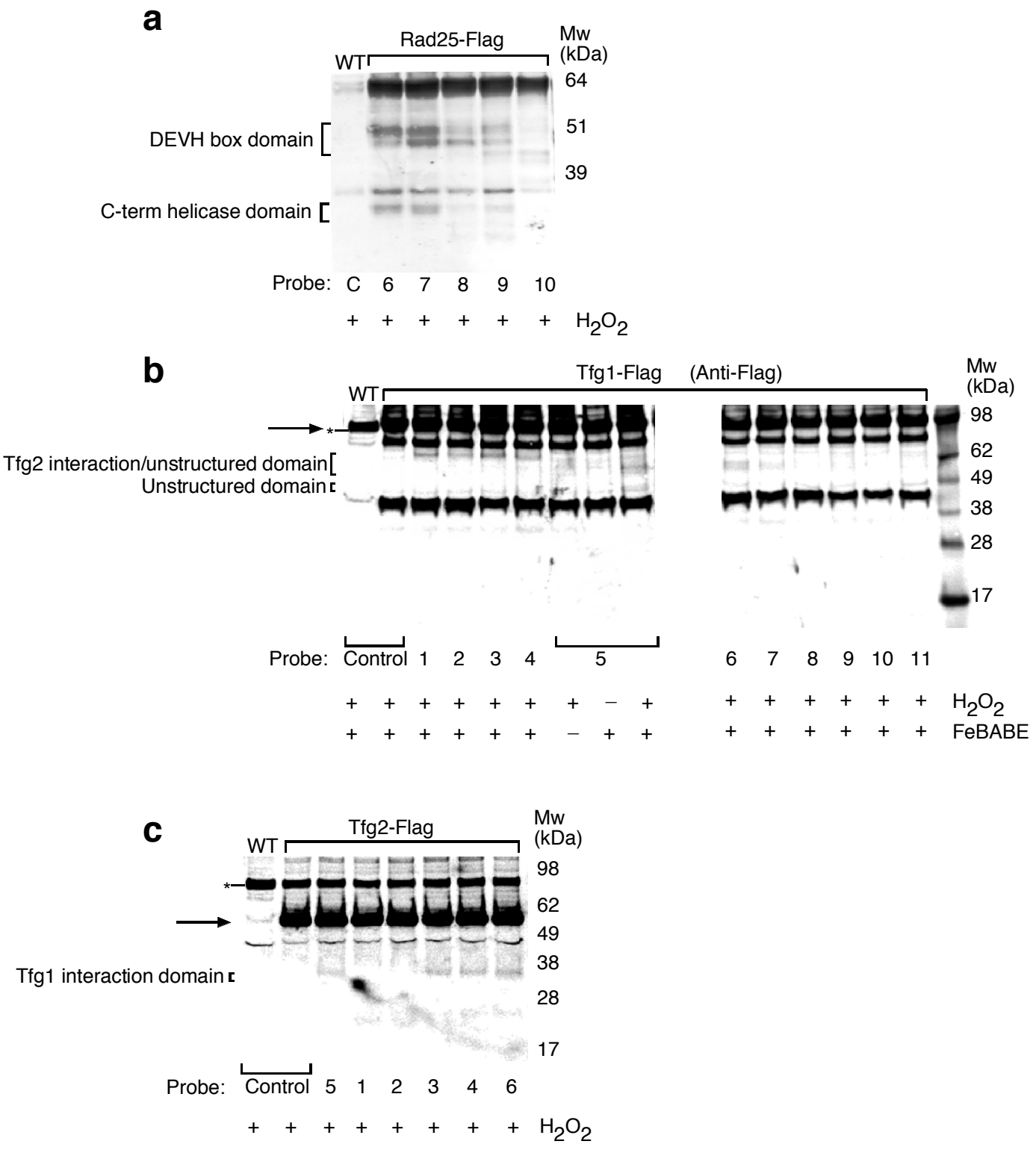
Supplementary Figure 3. In vitro translated peptide fragments of Sua7, Rad25, Tfg1, Tfg2, Tfa1 and Tfa2 used as molecular standards for calculating FeBABE cleavage sites
(**a**, **c**, **e**, **g**, **i** and **k**) In vitro translated peptide fragments of the N-terminal regions of Sua7-Flag (**a**), Tfg2-Flag (**g**) and Tfa1-Flag (**i**) and the C-terminal regions of Rad25-Flag (**c**), Tfg1-Flag (**e**) and Tfa2-Flag (**k**) analyzed by SDS-PAGE. The residue number indicates the last residue for each individual peptide fragment. (**b**, **d**, **f**, **h**, **j**, and **l**) Calibration curves for determining the amino acid numbers of FeBABE cleavage fragments, similar to those shown in **Supplementary Figure2**.

Supplementary Figure 4 Miller and Hahn



Supplementary Figure 4. Directed hydroxyl radical probing of TFIIB in the PIC
(**a**) Cleavage fragments of TFIIB were visualized by Western Blotting with anti-Flag antibody where FeBABE is either attached to the non-template strand or (**b**) template strand. Arrow points to full length TFIIB and specific cleavage fragments are indicated by the brackets and labeled as to which region in the protein is cleaved. * Indicates non-specific bands detected by antibodies.
(**c**) Schematic indicating TFIIB domains. Arrows indicate cleavage sites.

Supplementary Figure 5 Miller and Hahn



Supplementary Figure 5. Directed hydroxyl radical probing of TFIIH subunit Rad25 and TFIIF subunits Tfg1 and Tfg2 with FeBABE attached to the template strand.
(a-c), Cleavage fragments of Rad25-Flag, Tfg1-Flag and Tfg2-Flag were visualized by Western blotting with anti-Flag antibody, respectively. Same assay as described in Figure 2. * Indicates non-specific bands detected by antibodies.

```
!SS_AF_XPB                    bBBBb.....bBBBb.......--.bBBBb...bBBBb...aaaAAAaaa
AF_XPB                      MQMIAEIYYERGTIVVKGDAHVP--HAKFDSRSGTYRALAFRYRDIIEYFE
mouseXPB                    IPKDLFDFYEQMDKDEEEEEET--QTVSFEVKQEMIEELQKRCIC-LEYPL
humanXPB                    IPMDLFDFYEQMDKDEEEEEET--QTVSFEVKQEMIEELQKRCIH-LEYPL
drosXPB                     VPEDITDFYEKIDKEEEDEDEANLKTVSFEVAQEKIEVIQKRCIE-IEHPL
Scerev_rad25                VFSAVIGGDNEREEEDDDIDAVH----SFEIANESVEVVKKRCQE-IDYPV
SpombeXPB                   LFSAVVG----LQEEEDDEDAVH----LFEIKHSSVETIKKRCAE-IDYPL
                            :              .    .      *:      . :  :    :::

!SS_AF_XPB            .....bBBBb............bBBb.aaaAAAAAaaa...---bBBBb......aaaAA
AF_XPB                SNGIEFVDNAADPIPTPYFDAEISLRDYQEKALERWLVDKR---GCIVLPTGSGKTHVAM
mouseXPB              LAEYDFRNDTLNPDINIDLKPTAVLRPYQEKSLRKMFGNGRARSGVIVLPCGAGKSLVGV
humanXPB              LAEYDFRNDSVNPDINIDLKPTAVLRPYQEKSLRKMFGNGRARSGVIVLPCGAGKSLVGV
drosXPB               LAEYDFRNDTNNPDINIDLKPAAVLRPYQEKSLRKMFGNGRARSGVIVLPCGAGKSLVGV
Scerev_rad25          LEEYDFRNDHRNPDLDIDLKPSTQIRPYQEKSLSKMFGNGRARSGIIVLPCGAGKTLVGI
SpombeXPB             LEEYDFRNDNINPDLPIDLKPSTQIRPYQEKSLSKMFGNGRARSGIIVLPCGAGKTLVGI
                          :* ::  :*     :..   :* ****:* : : :   : * **** *:**: *.:

!SS_AF_XPB            AAAaaa...bBBBb..aaaAAAAaaa.....---...bBBb..bBBb.-..----bBBBB
AF_XPB                AAINELSTPTLIVVPTLALAEQWKERLGIFG---EEYVGEFSGRIKELKP----LTVSTY
mouseXPB              TAACTVRKRCLVLGNSAVSVEQWKAQFKMWSTIDDSQICRFTSDAKD-KPIGCSVAISTY
humanXPB              TAACTVRKRCLVLGNSAVSVEQWKAQFKMWSTIDDSQICRFTSDAKD-KPIGCSVAISTY
drosXPB               TACCTVRKRALVLCNSGVSVEQWKQQFKMWSTADDSMICRFTSEAKD-KPMGCGILVTTY
Scerev_rad25          TAACTIKKSVIVLCTSSVSVMQWRQQFLQWCTLQPENCAVFTSDNKEMFQTESGLVVSTY
SpombeXPB             TAACTIKKSVIVLCTSSVSVMQWRQQFLQWSNIKPDHIAVFTADHKERFHSEAGVVVSTY
                      :*   : .  :::  : . . **: ::  :     .    *:.  *:

!SS_AF_XPB            b------aaaAAAaaa-----....bBBBb...........aaaaaa....bBBBBb...
AF_XPB                D------SAYVNAEKL-----GNRFMLLIFDEVHHLPAESYVQIAQMSIAPFRLGLTATF
mouseXPB              SMLGHTTKRSWEAERVMEWLKTQEWGLMILDEVHTIPARMFRRVLTIVQAHCKLGLTATL
humanXPB              SMLGHTTKRSWEAERVMEWLKTQEWGLMILDEVHTIPAKMFRRVLTIVQAHCKLGLTATL
drosXPB               SMITHTQKRSWEAEQTMRWLQEQEWGIMVLDEVHTIPAKMFRRVLTIVQSHCKLGLTATL
Scerev_rad25          SMVANTRNRSHDSQKVMDFLTGREWGFIILDEVHVVPAAMFRRVVSTIAAHAKLGLTATL
SpombeXPB             SMVANTRNRSYDSQKMMDFLTGREWGFILLDEVHVVPAAMFRRVVTTIAAHTKLGLTATL
                             : ::         ..: ::::**** :**  : ::     :  :******:

!SS_AF_XPB            .......aaaAaaa...bBBBb......-.....bBBBBBBb..................
AF_XPB                EREDGRHEILKEVVGGKVFELFPDSLAG-KHLAKYTIKRIFVPLAEDERVEYEKREKVYK
mouseXPB              VREDDKIVDLNFLIGPKLYEANWMELQNNGYIAKVQCAEVWCPMSPEFYREYVAIKTKKR
humanXPB              VREDDKIVDLNFLIGPKLYEANWMELQNNGYIAKVQCAEVWCPMSPEFYREYVAIKTKKR
drosXPB               LREDDKIADLNFLIGPKLYEANWLELQKKGYIARVQCAEVWCPMSPEFYREYLTTKTSKK
Scerev_rad25          VREDDKIGDLNFLIGPKLYEANWMELSQKGHIANVQCAEVWCPMTAEFYQEYLRETARKR
SpombeXPB             VREDDKIDDLNFLIGPKMYEANWMDLAQKGHIAKVQCAEVWCAMTTEFYNEYLRENSRKR
                       ***.:   *: ::* *::*    .*  : ::*.    .:: .:: :   **       :

!SS_AF_XPB            ..............................aaaAAAAAAAAAAAAAAAAAAAaaa.
AF_XPB                QFLRARGITLRRAEDFNKIVMASGYDERAYEALRAWEEARRIAFNSKNKIRKLREILERH
mouseXPB              ILLYTMN-------------------------------------PNKFRACQFLIKFHER
humanXPB              ILLYTMN-------------------------------------PNKFRACQFLIKFHER
drosXPB               MLLYVMN-------------------------------------PSKFRSCQFLIKYHEQ
Scerev_rad25          MLLYIMN-------------------------------------PTKFQACQFLIQYHER
SpombeXPB             MLLYIMN-------------------------------------PKKFQACQFLIDYHEK
                       :*   .                                     .* :  ::    ..:

!SS_AF_XPB            ...bBBBb.....aaaAAaaa...bBBBb.......aaaAaaa...-.bBBBBBBb....
AF_XPB                RKDKIIIFTRHNELVYRISKVFLIPAITHRTSREEREEILEGFRTG-RFRAIVSSQVLDE
mouseXPB              RNDKIIVFADNVFALKEYAIRLNKPYIYGPTSQGERMQILQNFKHNPKINTIFISKVGDT
humanXPB              RNDKIIVFADNVFALKEYAIRLNKPYIYGPTSQGERMQILQNFKHNPKINTIFISKVGDT
drosXPB               RGDKTIVFSDNVFALKHYAIKMNKPFIYGPTSQNERIQILQNFKFNSKVNTIFVSKVADT
Scerev_rad25          RGDKIIVFSDNVYALQEYALKMGKPFIYGSTPQQERMNILQNFQYNDQINTIFLSKVGDT
SpombeXPB             RGDKIIVFSDNVYALRAYAIKLGKYFIYGGTPQQERMRILENFQYNELVNTIFLSKVGDT
                      * ** *:*: :   :   :  :    *   *.: ** .**:.*: .  ..:*. *:* *

!SS_AF_XPB            .........bBBBb...-..aaaAAAAaaa......----...bBBBBBb.....
AF_XPB                GIDVPDANVGVIMSGSG-SAREYIQRLGRILRPSKG----KKEAVLYELISRGTG
mouseXPB              SFDLPEANVLIQISSHGGSRRQEAQRLGRVLRAKKGMVAEEYNAFFYSLVSQDTQ
humanXPB              SFDLPEANVLIQISSHGGSRRQEAQRLGRVLRAKKGMVAEEYNAFFYSLVSQDTQ
drosXPB               SFDLPEANVLIQISSHGGSRRQEAQRLGRILRAKKGAIAEEYNAFFYTLVSQDTM
Scerev_rad25          SIDLPEATCLIQISSHYGSRRQEAQRLGRILRAKR-RNDEGFNAFFYSLVSKDTQ
SpombeXPB             SIDLPEATCLIQISSHYGSRRQEAQRLGRILRAKR-RNDEGFNAFFYSLVSKDTQ
                      .:*:*:*.  : :*.   * *:  *****:**..:       :*.:* *:*:.*
```

Supplementary Figure 6. Rad25/XPB alignment used in structure modeling of the Rad25 helicase domains

Rad25/XPB sequence alignment used in structure modeling of the Rad25 helicase domains. Shown is the alignment of the Rad25/XPB helicase subdomains with Archaeoglobus fulgidus XPB (Af_XPB). Shown at the top is the secondary structure of the Af XPB structure (2FWR). The alignment was derived as described in **Supplementary Methods**.

Supplementary Methods

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SHY772 containing a 12 Flag epitope at the C terminus of Tfg1; SHY361 and 560 containing a triple Flag epitope at the C terminus of Tfa1 and Tfa2, respectively; SHY386 containing a triple Flag epitope at the C terminus of Sua7. Yeast nuclear extracts were made as described (www.fhcrc.org/labs/hahn). Anti-Flag M2 monoclonal antibody was from Sigma, and rabbit polyclonal antisera was generated against the N-terminal 200 amino acids of *S. cerevisiae* Rpb1[9].

Supplementary References

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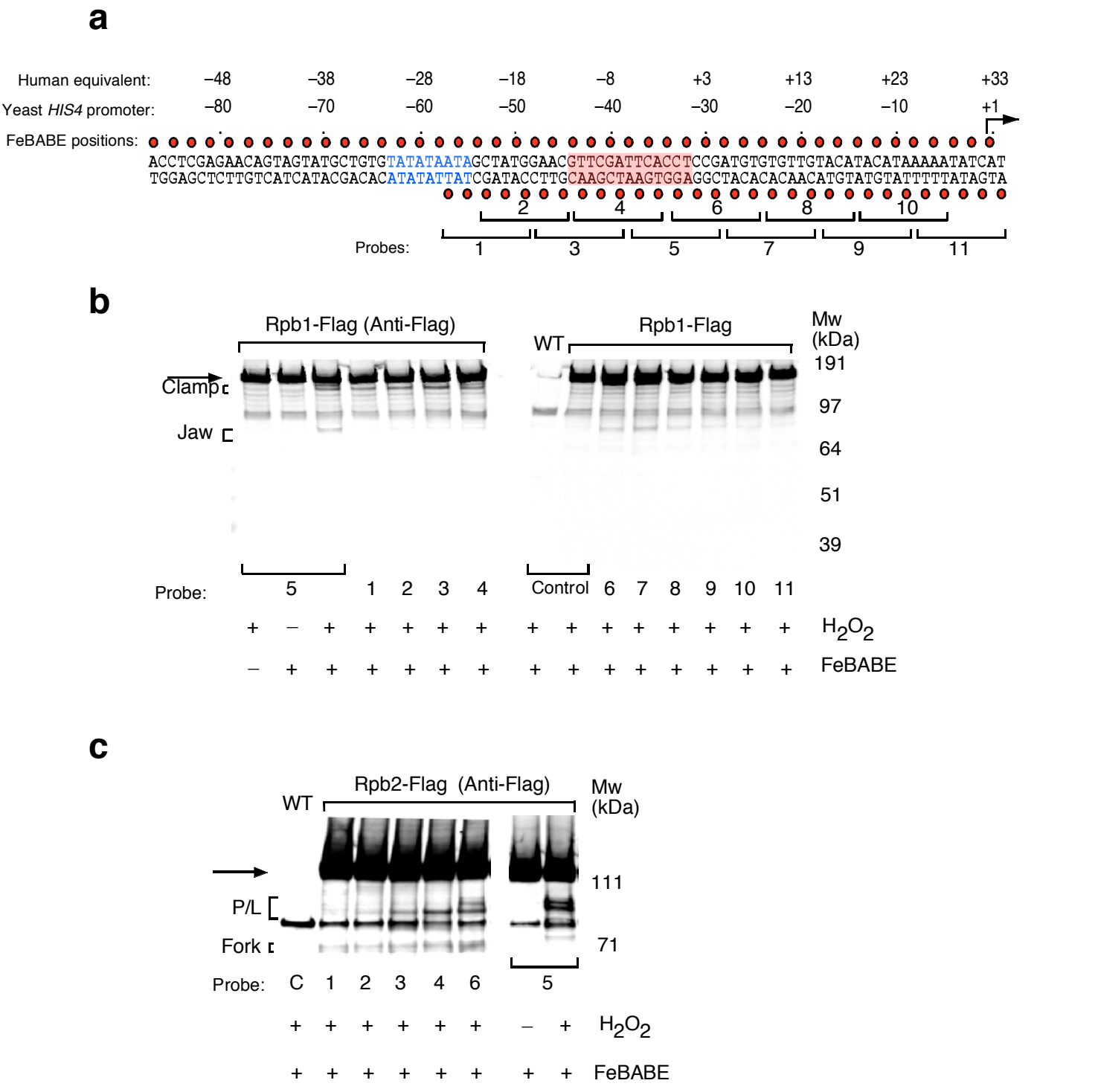
Supplementary Table 1 Miller and Hahn

PROMOTER	Rpb1 residue Int	Rpb2 residue Int	Rad25 residue Int	Tfg1 residue Int	Tfg2 residue Int	Tfa1 residue Int	Tfa2 residue Int	TFIIB residue Int
-5								
-4				331 M 421 w				
-3		180 M/S protrusion						
-2		180 M/S protrusion			292 W			
-1					292 W			core domain W
TATA				316 M				
1	262 M/S clamp core			316 M/S	292 W		224 W	
2	262 M/S clamp core			316 M/S	292 W	20 W	224 W	112 W linker 156 W core domain
3	262 M/S clamp core	180 M/S protrusion 227 M/S lobe		331 M/S	184 M	25 W	224 W	105 W linker
4	262 M/S clamp core	364 M/S lobe 523 W fork		331 M/S	184 M	25 W		99 W B-finger
5	214 M/S clamp head	195 P M/S 239 L M/S 364 L W 503 F W		421 W 528 W	196 M/S			
6	214 M/S clamp head 1187 M/S Jaw	169 P M/S 217 L M/S 532 F W	438 M/S DEAD box domain 484 M/S DEAD box domain	528 W	199 M/S			
7	214 M/S clamp head 1187 M/S Jaw		627 M/S Ct helicase domain					
8	1173 M/S Jaw		484 W DEAD box domain 511 W DEAD box domain					
9			516 W DEAD box domain					
10								
11								

Supplementary Table 1. Summary of RNA Polymerase II and General Transcription Factor cleavage sites generated by FeBABE attached to *HIS4* promoter DNA

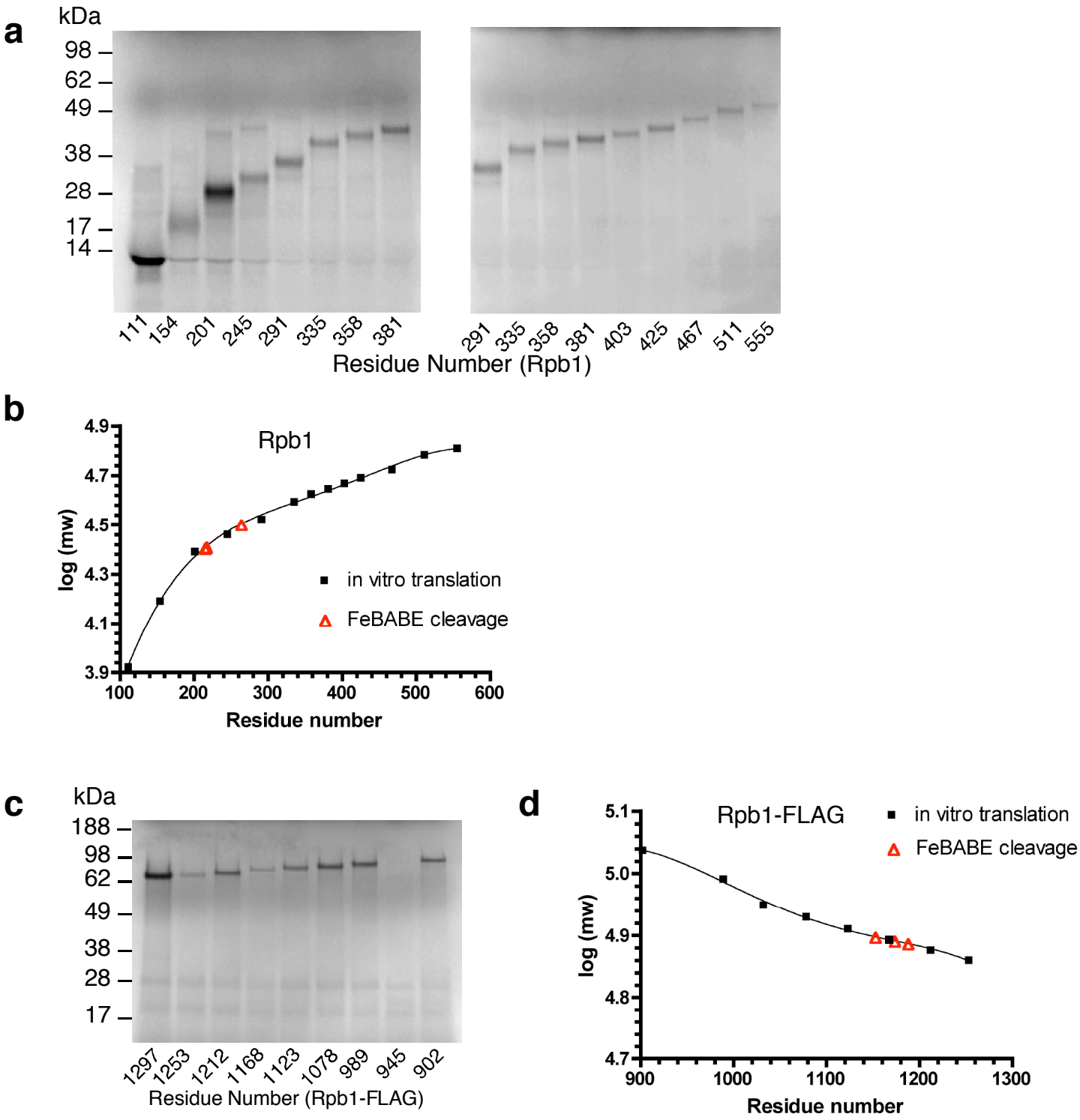
The measured residue where hydroxyl radical cleavage is observed is listed as well as the structural element, if known, where this residue is situated. Int., cleavage intensity. s, strong; m, medium; w, weak. P, protrusion; L, lobe; F, fork.

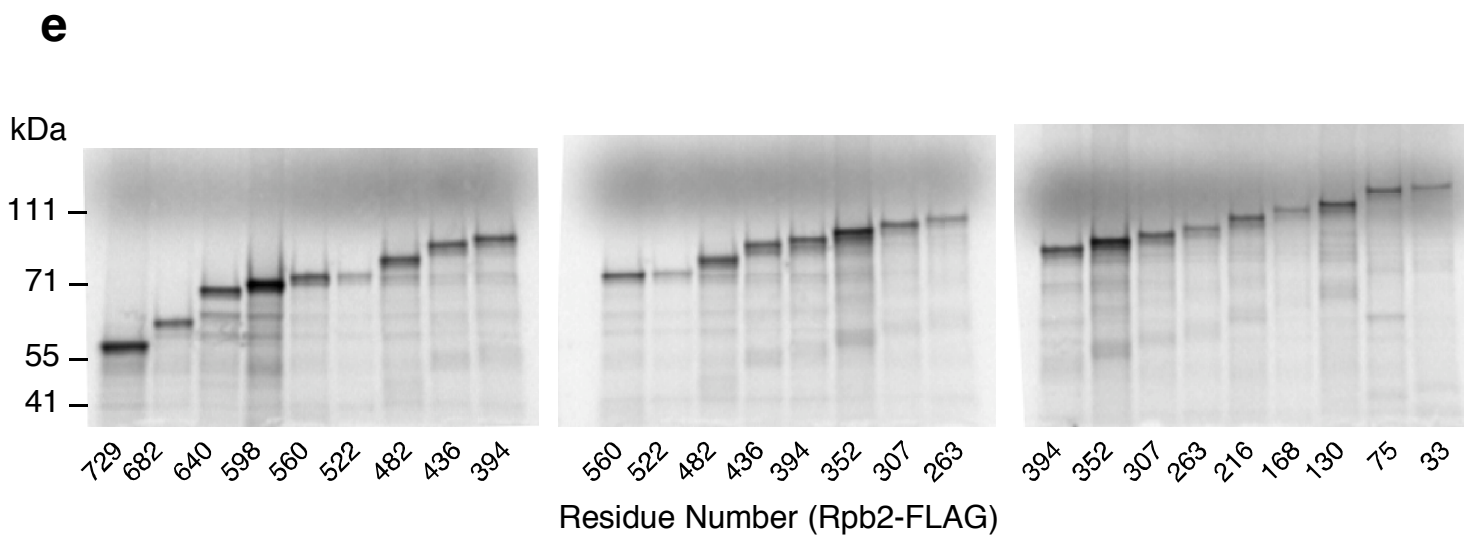
Supplementary Figure 1 Miller and Hahn

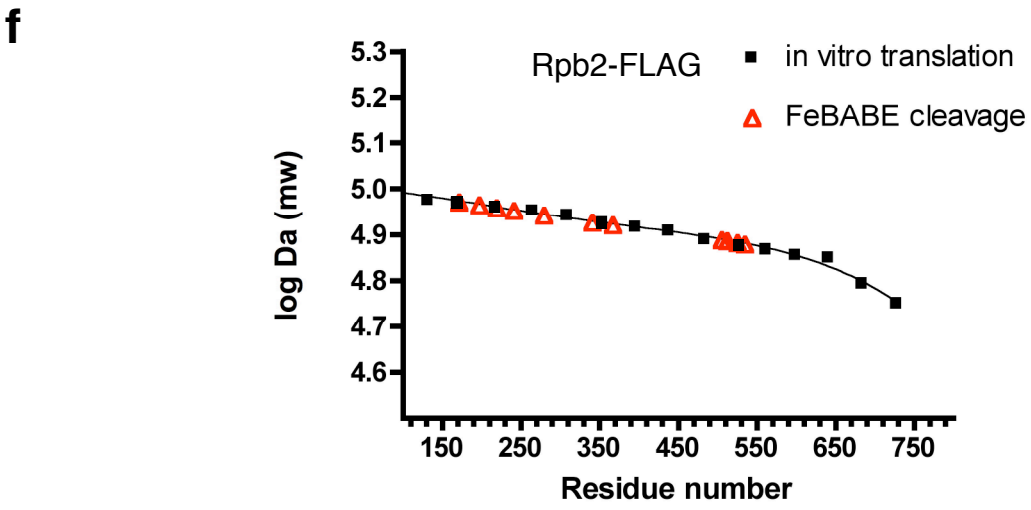


Supplementary Figure 1. Directed hydroxyl radical probing of RNA Polymerase II subunits Rpb1 and Rpb2 with FeBABE attached to the template strand (**a**) 11 FeBABE-tethered probes derived from the *HIS4* promoter where FeBABE is attached to the template strand. The color scheme is the same as in Figure 1. (**b**) and (**c**) Cleavage fragments of Rpb1-Flag and of Rpb2-Flag were visualized by Western Blotting with anti-Flag antibody as described in **Figure 2**.

Supplementary Figure 2 Miller and Hahn

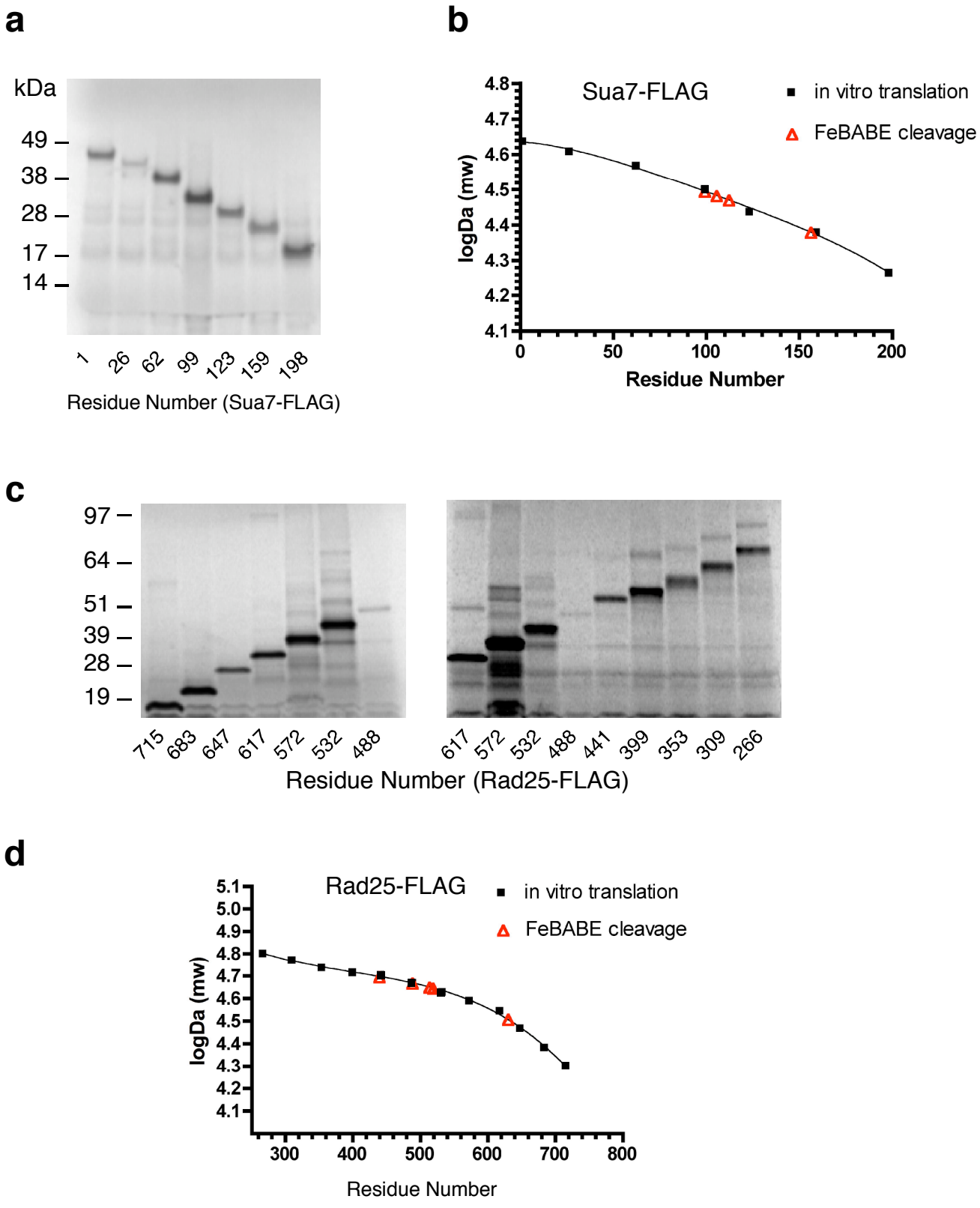




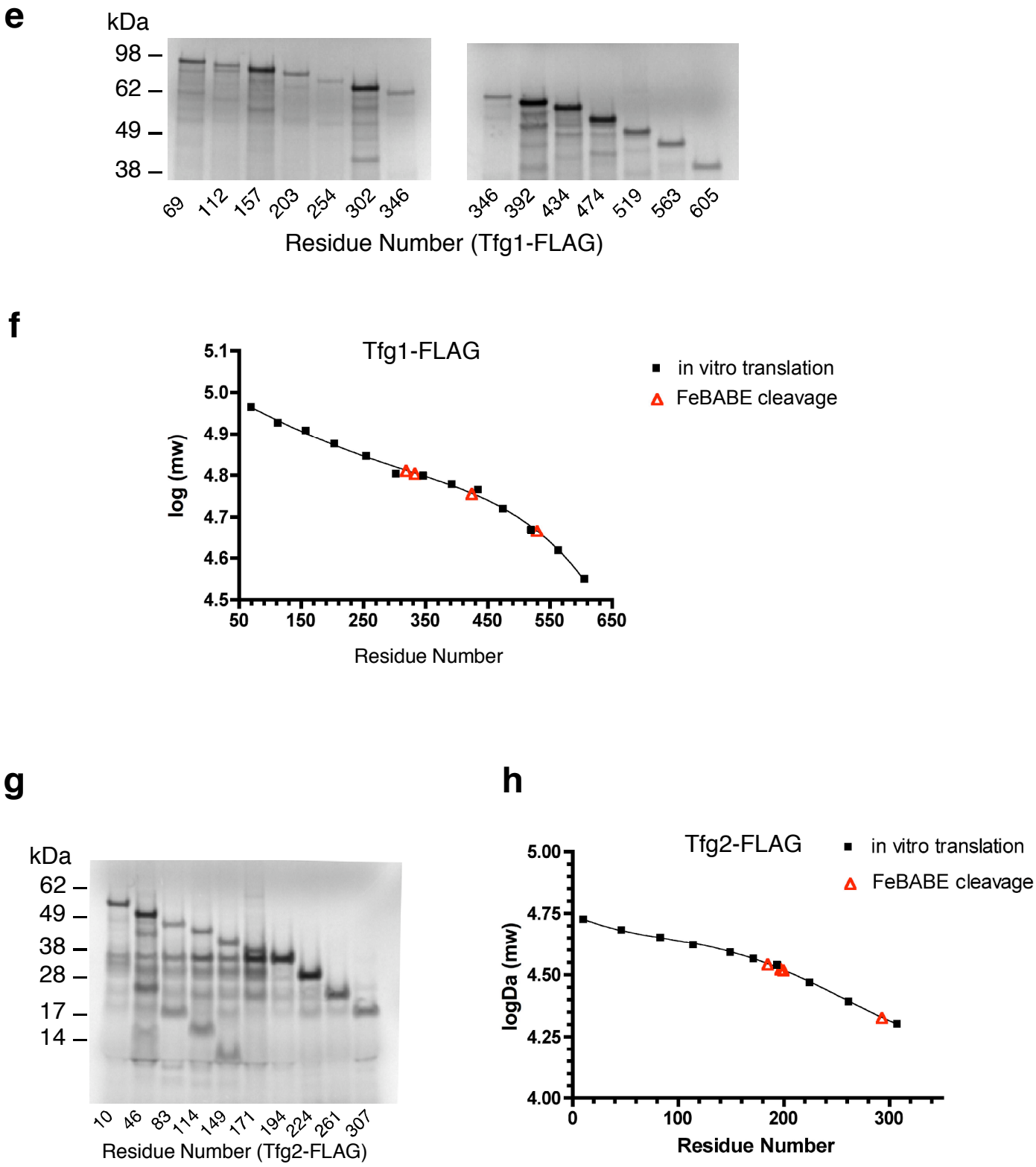


Supplementary Figure 2. In vitro translated peptide fragments of Rpb1 and Rpb2 used as molecular standards for calculating FeBABE cleavage sites (**a**, **c** and **e**) In vitro translated peptide fragments of the N-terminal region of Rpb1-Flag, the C-terminal region of Rpb1-Flag and the N-terminal region of Rpb2-Flag, respectively, analyzed by SDS-PAGE. The residue number indicates either the last (**a**) or first (**c** and **e**) residue for each individual peptide fragment. (**b**, **d** and **f**) Calibration curves for determining the amino acid numbers of FeBABE cleavage fragments. Each calibration curve is derived from a 4th order polynomial function using the sizes (log Da (mw)) of in vitro translated peptides and their corresponding residue numbers in (**a**), (**c**) and (**e**). Locations of the FeBABE cleavage fragments on the calibration curves are shown with red triangles.

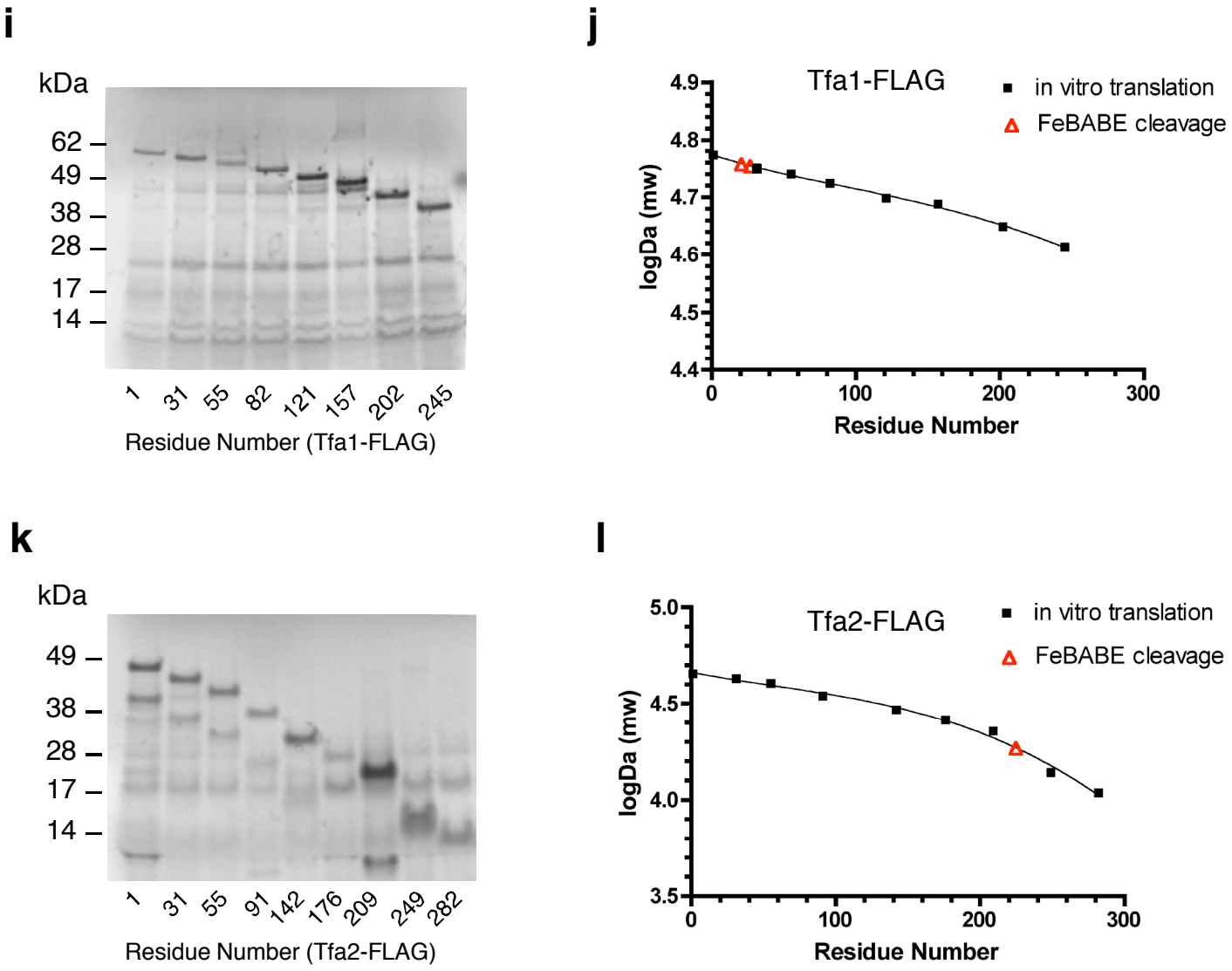
Supplementary Figure 3 Miller and Hahn



Supplementary Figure 3 contd. Miller and Hahn

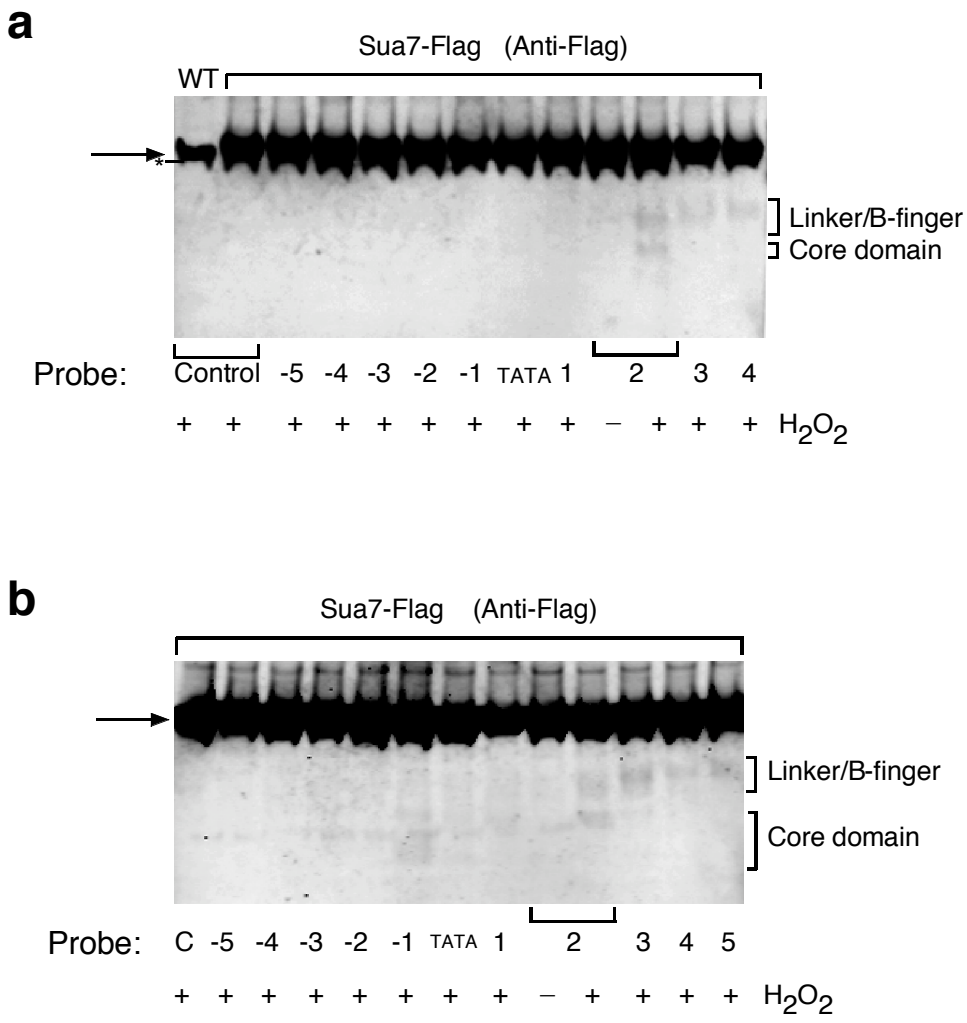


Supplementary Figure 3 contd. Miller and Hahn



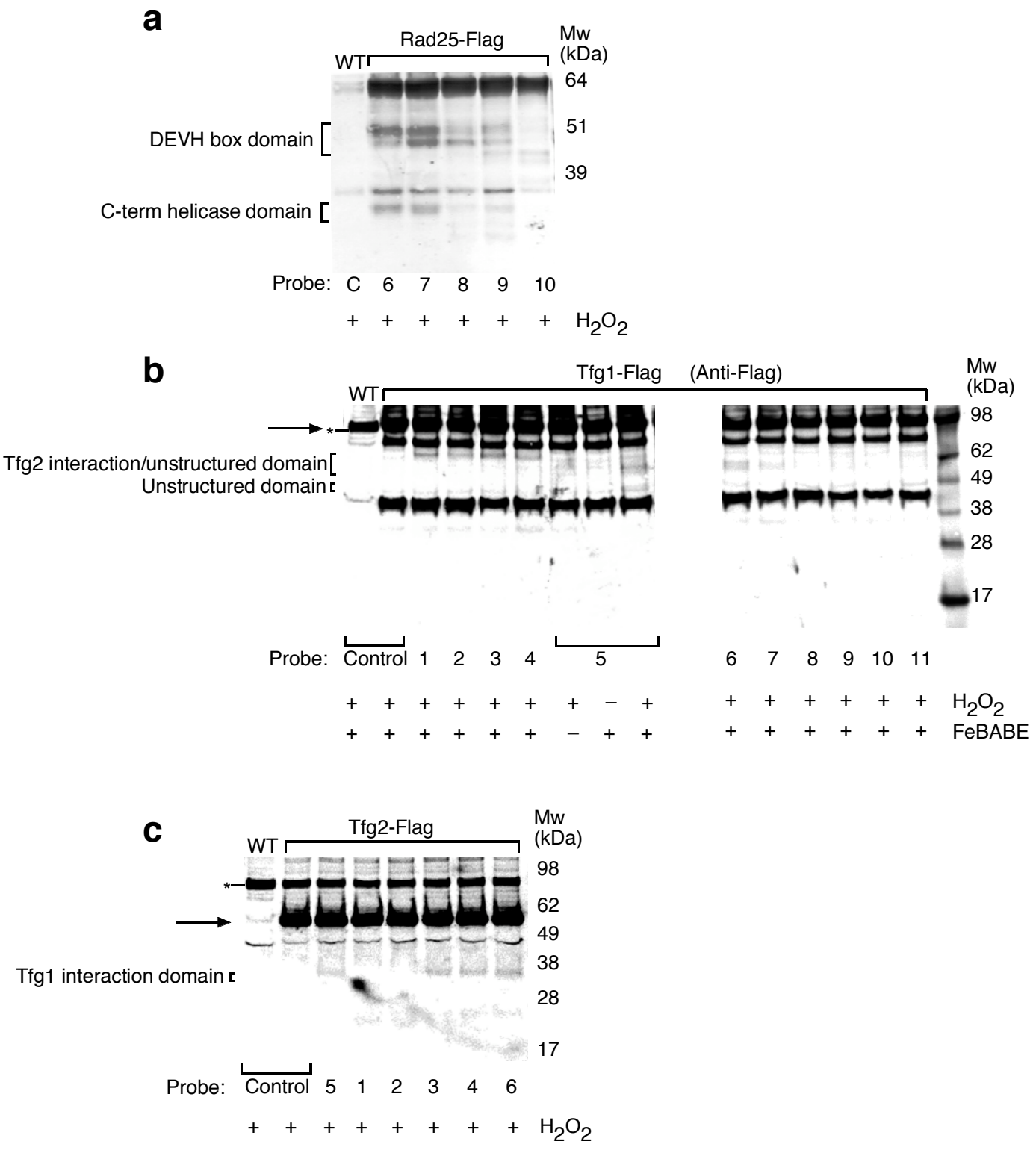
Supplementary Figure 3. In vitro translated peptide fragments of Sua7, Rad25, Tfg1, Tfg2, Tfa1 and Tfa2 used as molecular standards for calculating FeBABE cleavage sites
(**a**, **c**, **e**, **g**, **i** and **k**) In vitro translated peptide fragments of the N-terminal regions of Sua7-Flag (**a**), Tfg2-Flag (**g**) and Tfa1-Flag (**i**) and the C-terminal regions of Rad25-Flag (**c**), Tfg1-Flag (**e**) and Tfa2-Flag (**k**) analyzed by SDS-PAGE. The residue number indicates the last residue for each individual peptide fragment. (**b**, **d**, **f**, **h**, **j**, and **l**) Calibration curves for determining the amino acid numbers of FeBABE cleavage fragments, similar to those shown in **Supplementary Figure2**.

Supplementary Figure 4 Miller and Hahn



Supplementary Figure 4. Directed hydroxyl radical probing of TFIIB in the PIC
(**a**) Cleavage fragments of TFIIB were visualized by Western Blotting with anti-Flag antibody where FeBABE is either attached to the non-template strand or (**b**) template strand. Arrow points to full length TFIIB and specific cleavage fragments are indicated by the brackets and labeled as to which region in the protein is cleaved. * Indicates non-specific bands detected by antibodies.
(**c**) Schematic indicating TFIIB domains. Arrows indicate cleavage sites.

Supplementary Figure 5 Miller and Hahn



Supplementary Figure 5. Directed hydroxyl radical probing of TFIIH subunit Rad25 and TFIIF subunits Tfg1 and Tfg2 with FeBABE attached to the template strand.
(a-c), Cleavage fragments of Rad25-Flag, Tfg1-Flag and Tfg2-Flag were visualized by Western blotting with anti-Flag antibody, respectively. Same assay as described in Figure 2. * Indicates non-specific bands detected by antibodies.

```
!SS_AF_XPB                    bBBBb.....bBBBb.......--.bBBBb...bBBBb...aaaAAAaaa
AF_XPB                      MQMIAEIYYERGTIVVKGDAHVP--HAKFDSRSGTYRALAFRYRDIIEYFE
mouseXPB                    IPKDLFDFYEQMDKDEEEEEET--QTVSFEVKQEMIEELQKRCIC-LEYPL
humanXPB                    IPMDLFDFYEQMDKDEEEEEET--QTVSFEVKQEMIEELQKRCIH-LEYPL
drosXPB                     VPEDITDFYEKIDKEEEDEDEANLKTVSFEVAQEKIEVIQKRCIE-IEHPL
Scerev_rad25                VFSAVIGGDNEREEEDDDIDAVH----SFEIANESVEVVKKRCQE-IDYPV
SpombeXPB                   LFSAVVG----LQEEEDDEDAVH----LFEIKHSSVETIKKRCAE-IDYPL
                            :              .    .      *:      . :  :    :::

!SS_AF_XPB            .....bBBBb............bBBb.aaaAAAAAaaa...---bBBBb......aaaAA
AF_XPB                SNGIEFVDNAADPIPTPYFDAEISLRDYQEKALERWLVDKR---GCIVLPTGSGKTHVAM
mouseXPB              LAEYDFRNDTLNPDINIDLKPTAVLRPYQEKSLRKMFGNGRARSGVIVLPCGAGKSLVGV
humanXPB              LAEYDFRNDSVNPDINIDLKPTAVLRPYQEKSLRKMFGNGRARSGVIVLPCGAGKSLVGV
drosXPB               LAEYDFRNDTNNPDINIDLKPAAVLRPYQEKSLRKMFGNGRARSGVIVLPCGAGKSLVGV
Scerev_rad25          LEEYDFRNDHRNPDLDIDLKPSTQIRPYQEKSLSKMFGNGRARSGIIVLPCGAGKTLVGI
SpombeXPB             LEEYDFRNDNINPDLPIDLKPSTQIRPYQEKSLSKMFGNGRARSGIIVLPCGAGKTLVGI
                          :* ::  :*     :..   :* ****:* : : :   : * **** *:**: *.:

!SS_AF_XPB            AAAaaa...bBBBb..aaaAAAAaaa.....---...bBBb..bBBb.-..----bBBBB
AF_XPB                AAINELSTPTLIVVPTLALAEQWKERLGIFG---EEYVGEFSGRIKELKP----LTVSTY
mouseXPB              TAACTVRKRCLVLGNSAVSVEQWKAQFKMWSTIDDSQICRFTSDAKD-KPIGCSVAISTY
humanXPB              TAACTVRKRCLVLGNSAVSVEQWKAQFKMWSTIDDSQICRFTSDAKD-KPIGCSVAISTY
drosXPB               TACCTVRKRALVLCNSGVSVEQWKQQFKMWSTADDSMICRFTSEAKD-KPMGCGILVTTY
Scerev_rad25          TAACTIKKSVIVLCTSSVSVMQWRQQFLQWCTLQPENCAVFTSDNKEMFQTESGLVVSTY
SpombeXPB             TAACTIKKSVIVLCTSSVSVMQWRQQFLQWSNIKPDHIAVFTADHKERFHSEAGVVVSTY
                      :*   : .  :::  : . . **: ::  :     .    *:.  *:

!SS_AF_XPB            b------aaaAAAaaa-----....bBBBb...........aaaaaa....bBBBBb...
AF_XPB                D------SAYVNAEKL-----GNRFMLLIFDEVHHLPAESYVQIAQMSIAPFRLGLTATF
mouseXPB              SMLGHTTKRSWEAERVMEWLKTQEWGLMILDEVHTIPARMFRRVLTIVQAHCKLGLTATL
humanXPB              SMLGHTTKRSWEAERVMEWLKTQEWGLMILDEVHTIPAKMFRRVLTIVQAHCKLGLTATL
drosXPB               SMITHTQKRSWEAEQTMRWLQEQEWGIMVLDEVHTIPAKMFRRVLTIVQSHCKLGLTATL
Scerev_rad25          SMVANTRNRSHDSQKVMDFLTGREWGFIILDEVHVVPAAMFRRVVSTIAAHAKLGLTATL
SpombeXPB             SMVANTRNRSYDSQKMMDFLTGREWGFILLDEVHVVPAAMFRRVVTTIAAHTKLGLTATL
                             : ::         ..: ::::**** :**  : ::     :  :******:

!SS_AF_XPB            .......aaaAaaa...bBBBb......-.....bBBBBBBb..................
AF_XPB                EREDGRHEILKEVVGGKVFELFPDSLAG-KHLAKYTIKRIFVPLAEDERVEYEKREKVYK
mouseXPB              VREDDKIVDLNFLIGPKLYEANWMELQNNGYIAKVQCAEVWCPMSPEFYREYVAIKTKKR
humanXPB              VREDDKIVDLNFLIGPKLYEANWMELQNNGYIAKVQCAEVWCPMSPEFYREYVAIKTKKR
drosXPB               LREDDKIADLNFLIGPKLYEANWLELQKKGYIARVQCAEVWCPMSPEFYREYLTTKTSKK
Scerev_rad25          VREDDKIGDLNFLIGPKLYEANWMELSQKGHIANVQCAEVWCPMTAEFYQEYLRETARKR
SpombeXPB             VREDDKIDDLNFLIGPKMYEANWMDLAQKGHIAKVQCAEVWCAMTTEFYNEYLRENSRKR
                       ***.:   *: ::* *::*    .*  : ::*.    .:: .:: :   **       :

!SS_AF_XPB            ..............................aaaAAAAAAAAAAAAAAAAAAAaaa.
AF_XPB                QFLRARGITLRRAEDFNKIVMASGYDERAYEALRAWEEARRIAFNSKNKIRKLREILERH
mouseXPB              ILLYTMN-------------------------------------PNKFRACQFLIKFHER
humanXPB              ILLYTMN-------------------------------------PNKFRACQFLIKFHER
drosXPB               MLLYVMN-------------------------------------PSKFRSCQFLIKYHEQ
Scerev_rad25          MLLYIMN-------------------------------------PTKFQACQFLIQYHER
SpombeXPB             MLLYIMN-------------------------------------PKKFQACQFLIDYHEK
                       :*   .                                     .* :  ::    ..:

!SS_AF_XPB            ...bBBBb.....aaaAAaaa...bBBBb.......aaaAaaa...-.bBBBBBBb....
AF_XPB                RKDKIIIFTRHNELVYRISKVFLIPAITHRTSREEREEILEGFRTG-RFRAIVSSQVLDE
mouseXPB              RNDKIIVFADNVFALKEYAIRLNKPYIYGPTSQGERMQILQNFKHNPKINTIFISKVGDT
humanXPB              RNDKIIVFADNVFALKEYAIRLNKPYIYGPTSQGERMQILQNFKHNPKINTIFISKVGDT
drosXPB               RGDKTIVFSDNVFALKHYAIKMNKPFIYGPTSQNERIQILQNFKFNSKVNTIFVSKVADT
Scerev_rad25          RGDKIIVFSDNVYALQEYALKMGKPFIYGSTPQQERMNILQNFQYNDQINTIFLSKVGDT
SpombeXPB             RGDKIIVFSDNVYALRAYAIKLGKYFIYGGTPQQERMRILENFQYNELVNTIFLSKVGDT
                      * ** *:*: :   :   :  :    *   *.: ** .**:.*: .  ..:*. *:* *

!SS_AF_XPB            .........bBBBb...-..aaaAAAAaaa......----...bBBBBBb.....
AF_XPB                GIDVPDANVGVIMSGSG-SAREYIQRLGRILRPSKG----KKEAVLYELISRGTG
mouseXPB              SFDLPEANVLIQISSHGGSRRQEAQRLGRVLRAKKGMVAEEYNAFFYSLVSQDTQ
humanXPB              SFDLPEANVLIQISSHGGSRRQEAQRLGRVLRAKKGMVAEEYNAFFYSLVSQDTQ
drosXPB               SFDLPEANVLIQISSHGGSRRQEAQRLGRILRAKKGAIAEEYNAFFYTLVSQDTM
Scerev_rad25          SIDLPEATCLIQISSHYGSRRQEAQRLGRILRAKR-RNDEGFNAFFYSLVSKDTQ
SpombeXPB             SIDLPEATCLIQISSHYGSRRQEAQRLGRILRAKR-RNDEGFNAFFYSLVSKDTQ
                      .:*:*:*.  : :*.   * *:  *****:**..:       :*.:* *:*:.*
```

Supplementary Figure 6. Rad25/XPB alignment used in structure modeling of the Rad25 helicase domains

Rad25/XPB sequence alignment used in structure modeling of the Rad25 helicase domains. Shown is the alignment of the Rad25/XPB helicase subdomains with Archaeoglobus fulgidus XPB (Af_XPB). Shown at the top is the secondary structure of the Af XPB structure (2FWR). The alignment was derived as described in **Supplementary Methods**.