AN IRON-SULFUR CLUSTER IS ESSENTIAL FOR THE BINDING OF BROKEN DNA BY ADDAB-TYPE HELICASE-NUCLEASES

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SUPPLEMENTARY INFORMATION

EXPERIMENTAL PROCEDURES

Mutagenesis, expression and purification of proteins-Mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene) with the pAddB-28a vector as template (1). The entire *addb* gene was sequenced to ensure the correct mutations had been introduced without additional spurious mutations (The Sequencing Service, University of Dundee,UK). Mutant *addb* genes were then cloned into pCOLADuet-1-AddA vectors generating the pCOLADuet-1-AddAB plasmids with the desired mutations (2). AddAB purification was conducted at 4^{o}C except for heparin and monoQ chromatography. Cells were lysed by sonication followed by centrifugation to remove cell debris. Polymin P was added to the soluble cell extract to a final concentration of 0.4% with stirring. The precipitated material was pelleted by centrifugation and resuspended in B-buffer (20 mM Tris-Cl pH 7.5, 0.1 mM EDTA, 0.1 mM dithiothreitol) + 500 mM NaCl followed by stirring. Following centrifugation, the insoluble material was resuspended in B-buffer + 1 M NaCl. Any remaining insoluble material was cleared by centrifugation and solid ammonium sulfate was added to a final concentration of 80% (0.54 g/ml). The precipitate was recovered by centrifugation, resuspended in B-buffer + 50 mM NaCl and dialysed extensively against B + 50 mM NaCl. The conductivity of the protein sample was adjusted to $\leq$ 7.2 mS/cm to ensure efficient binding to the heparin column. Heparin and MonoQ chromatography were performed as described previously (1). The final proteins were dialysed against B-buffer + 100 mM NaCl and the concentrations were determined using the theoretical extinction coefficient 251,900 $M^{-1}cm^{-1}$. Glycerol was added to 10% prior to storage at -80^{o}C. We report that several different AddAB preparations were found to contain different proportions of iron-free material (see Figure 5B and text for discussion). The range of proteins tested included various independent wild type AddAB preparations as well as some proteins used in previous studies that contain point mutations at catalytic residues in the ATP hydrolysis or nuclease active sites (2). For the purposes of this paper (i.e. with respect to the integrity of the iron-sulfur cluster) those proteins are all referred to as wild type.

The *addA* gene, containing the D1172A mutation described previously (2) was cloned into the pET15b vector (Novagen). The resulting pET15b-AddA^{D1172A} plasmid enables the purification of a hexa-histidine tagged AddA^{D1172A} protein. BL21 (DE3) cells transformed with pET15b-AddA^{D1172A} were grown in LB at 37^{o}C to an $A_{600\ nm}$ of 0.5 prior to induction with 1 mM IPTG. Following induction cells were incubated for 3 hours at 27^{o}C, harvested by centrifugation and resuspended in 50 mM Tris-Cl pH 7.5, 10 % sucrose. Cells were lysed by sonnication and the buffer was supplemented with 500 mM NaCl, 25 mM Imidazole, 0.1 mM DTT and 0.1 mM PMSF. Following centrifugation to remove cell debris the protein was loaded on to a 5 ml His-Trap FF column equilibrated in 20 mM Tris acetate pH 7.5, 0.1 mM EDTA and 0.1 mM DTT (B buffer) supplemented with 500 mM NaCl and 25 mM Imidazole. Bound protein was eluted with a 30 column volume (CV) gradient to B buffer containing 500 mM NaCl and 500 mM Imidazole. The eluted protein was dialysed against B buffer + 50 mM NaCl and loaded on to a 1 ml mono-Q column equilibrated in B buffer + 200 mM NaCl. AddA was eluted with a 30 CV gradient to B buffer + 600 mM NaCl. Peak fractions were subsequently buffer exchanged into B buffer + 100 mM NaCl using a 5 ml Hi-trap desalting column. Prior to the addition of glycerol to 10 % the AddA concentration was calculated using a theoretical extinction coefficient of 135,460 $M^{-1}\ cm^{-1}$ at 280 nM.

Circular dichroism spectroscopy-Measurements were conducted using a Jasco J-810 in a 1 mM cell. The traces are an average of 10 reads taken at room temperature. Proteins samples were approximately 0.16 mg/ml in buffer containing 25 mM Tris-acetate pH 7.5 and 50 mM

potassium acetate. Data was normalised for protein concentration using the equation:
Mean molar ellipticity = ellipticity / (10 x peptide bond concentration x path length)

Two-dimensional polyacrylamide gel electrophoresis-2.75 μg AddAB proteins (250 nM concentration) were run through native 6% polyacrylamide gels in TBE. The AddAB-containing lanes were excised and soaked in 1x SDS running buffer for 30 minutes. Samples were run through a second denaturing gel by laying gel slices on top of 10% SDS polyacrylamide gels with 4% stacking gels prior to electrophoresis and Coomassie-blue staining.

Streptavidin displacement assays-5′-radiolabelled 45 base substrates with biotin molecules near either the 3' or 5' termini were utilised in these experiments. The sequences were as follows (X represents a biotin-modified T residue): 5'-GXA CGT ATT CAA GAT ACC TCG TAC TCT GTA CTG ACT CGG ATC CTA and 5'-GTA CGT ATT CAA GAT ACC TCG TAC TCT GTA CTG ACT CGG ATC CXA. The biotinylated oligonucleotides were pre-bound to excess streptavidin prior to the addition of AddAB enzymes and free biotin to initiate the reactions. The reaction buffer contained 50 mM Bis-tris acetate pH 6.0, 25 mM NaCl, 5 mM magnesium acetate, 5 nM DNA (in molecules), 400 nM streptavidin, 8 μM biotin, 4 mM ATP, 1 mM dithiothreitol and 100 μg/ml BSA. Experiments were initiated with 4 nM wild type AddAB or 200 nM Fe-S mutant AddAB enzymes. Reactions were incubated at 37^{o}C for the indicated times prior to quenching with an equal volume of 5% Ficoll-400, 400 mM NaCl, 100 mM Tris Cl pH 8.8, 1% SDS and 100 mM EDTA. Products were separated on 10% native polyacrylamide gels in TBE. Gels were dried on DEAE paper, exposed to phosphor screens and visualised using a Typhoon 9400 with image quant software.

FIGURE LEGENDS

Supplementary Figure 1 SDS-PAGE analysis of purified wild type and mutant AddAB proteins. The molecular masses (kDa) of the markers are shown.

Supplementary Figure 2 Circular dichroism spectroscopy analysis of wild type and mutant AddAB enzymes.

Supplementary Figure 3 All four Fe-S mutants display identical trypsin cleavage patterns. Proteins were incubated at room temperature with 1 μg/ml trypsin for 30 seconds prior to SDS-PAGE analysis. The positions of the major products are indicated (I, II) as in Figure 5. “AddAB 1” and “AddAB 2” are independent wild-type AddAB preparations with high and low iron content respectively.

Supplementary Figure 4 Wild type and Fe-S mutant AddAB preparations run as stable heterodimers in native polyacrylamide gels. (A) Two-dimensional gels with the native and denaturing dimensions running form left to right and top to bottom respectively. AddAB is run as a marker in the denaturing gel to illustrate the positions of the AddA (141 kDa) and the AddB (134 kDa) subunits, with the AddB subunit migrating further than the AddA subunit. (B) Native polyacrylamide gel of wild type and the four Fe-S mutant AddAB complexes. The gel was stained for protein and metalloproteins with Coomassie-blue and luminol respectively. The positions of the protein bands are indicated as in Figure 5B (I, II). “AddAB 1” and “AddAB 2” are independent wild-type AddAB preparations with high and low iron content respectively.

Supplementary Figure 5 Fe-S mutant enzymes retain unidirectional ssDNA translocation activity. Graphs show time courses for the displacement of streptavidin from biotinylated oligonucleotides by wild type and Fe-S mutant AddAB enzymes. Experiments were performed with 3’- and 5’-biotinylated oligonucleotides as indicated. Standard controls were performed with no ATP and with no free biotin (to confirm that the streptavidin is not displaced by nuclease activity). The wild type and all four mutant proteins were capable of displacing streptavidin from the 5′ end of biotinylated oligonucleotides. This activity was dependent on ATP and was not observed when the streptavidin was located at the 3′ end of the oligonucleotide, demonstrating that each complex can couple ATP

hydrolysis to unidirectional translocation on single-stranded DNA. This result provides evidence that ssDNA motor activity does not require the iron-staple structure. However, this observation should be interpreted with some caution, because the rate of streptavidin displacement was severely compromised in all of the Fe-S mutant protein complexes relative to wild type (Supplementary Figure 5). Nevertheless, the residual activity of Fe-S mutants is comparable to other (wild type) DNA helicases measured using this assay (3).

Supplementary Figure 6 T-coffee multiple sequence alignment of iron-staple nuclease domains (http://tcoffee.vital-it.ch). The sequences are from representative proteins for each of the five different enzyme classes shown in Figure 1B. The Superfamily I nuclease motifs (I, II and III) are illustrated, as is the RecB-family specific motif. Conserved catalytic residues are highlighted in red and the (putative) iron-sulfur cluster cysteine ligands are highlighted in pink. The positions of the first and last amino acid shown for each sequence are indicated to the left and right of the alignment. The number of amino acids between each motif is also indicated. The uncharacterized protein NP_217718.1 is from *M. tuberculosis* and is representative of a class of enzyme containing an N-terminal SF1 helicase domain fused to C-terminal iron-staple nuclease domain.

REFERENCES

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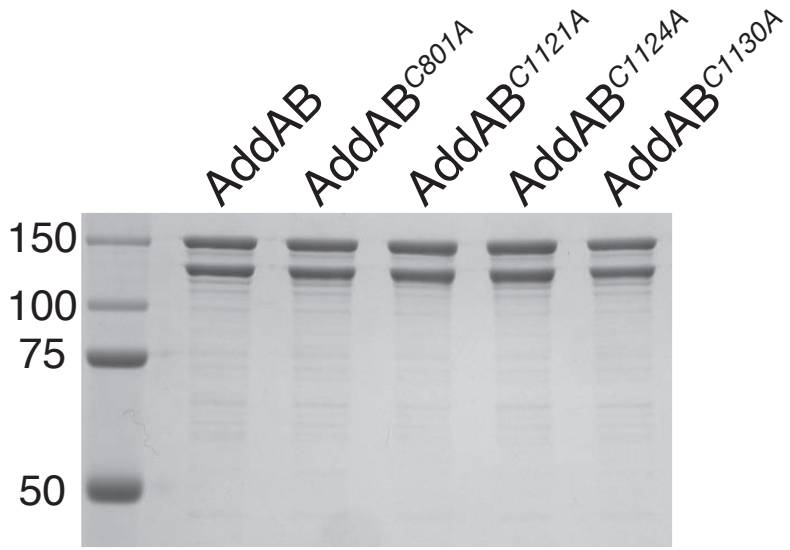
2. Yeeles, J. T., and Dillingham, M. S. (2007) *J Mol Biol* **371**(1), 66-78
3. Morris, P. D., and Raney, K. D. (1999) *Biochemistry* **38**(16), 5164-5171

Supplementary 1



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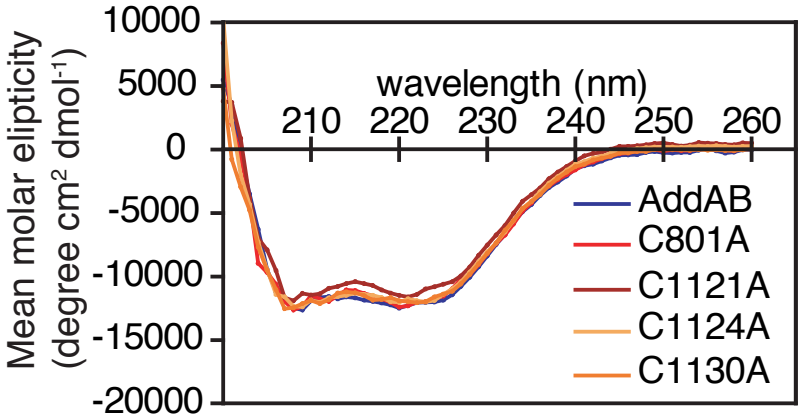

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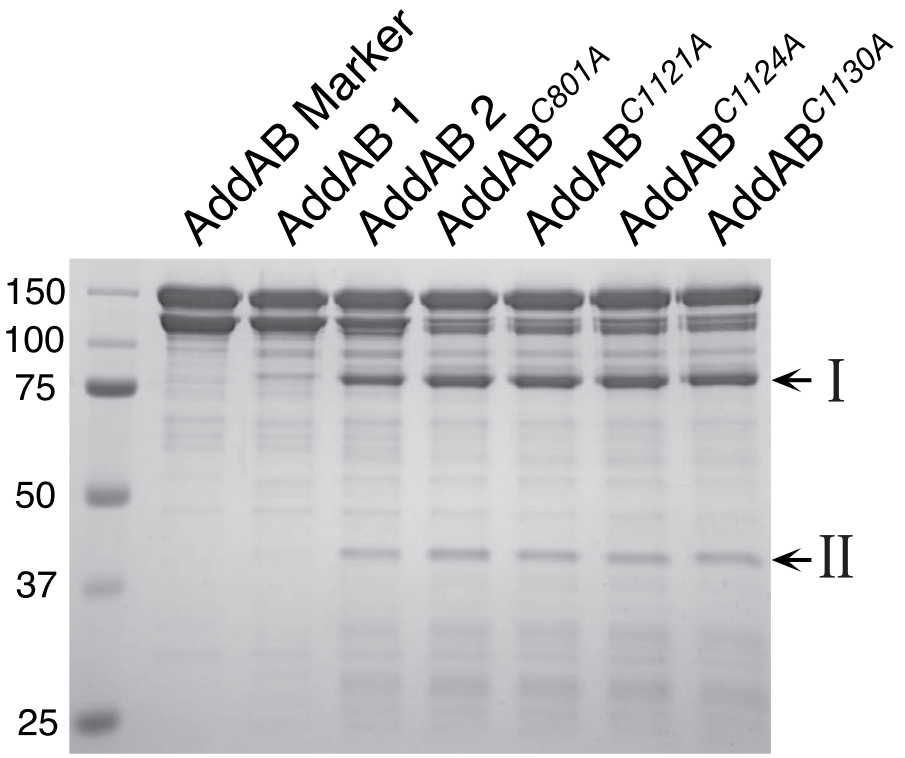

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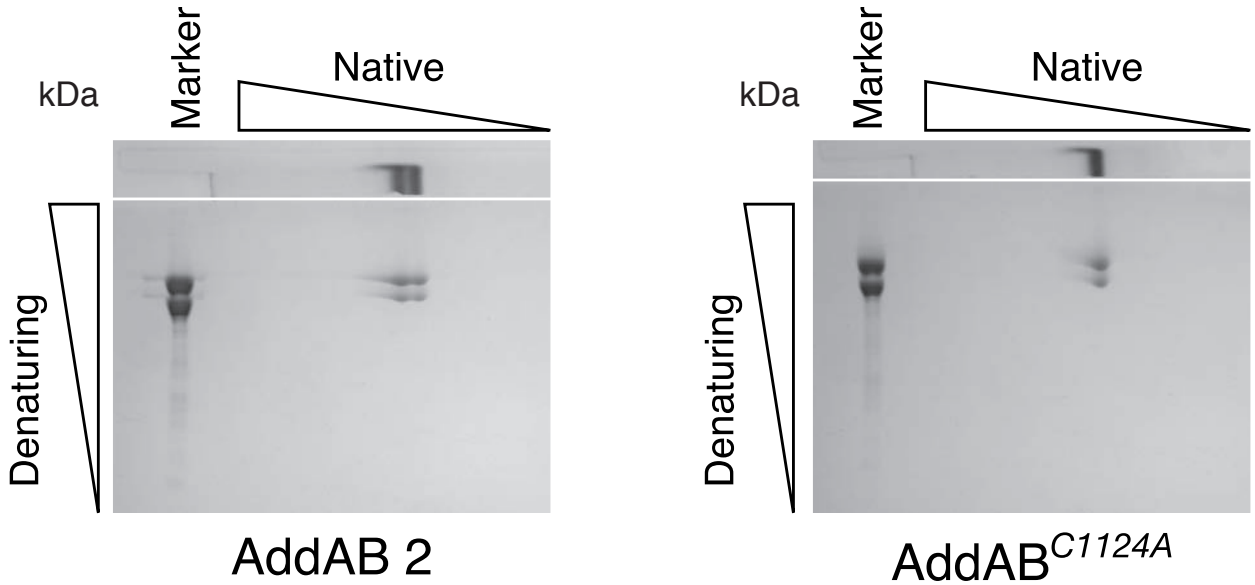

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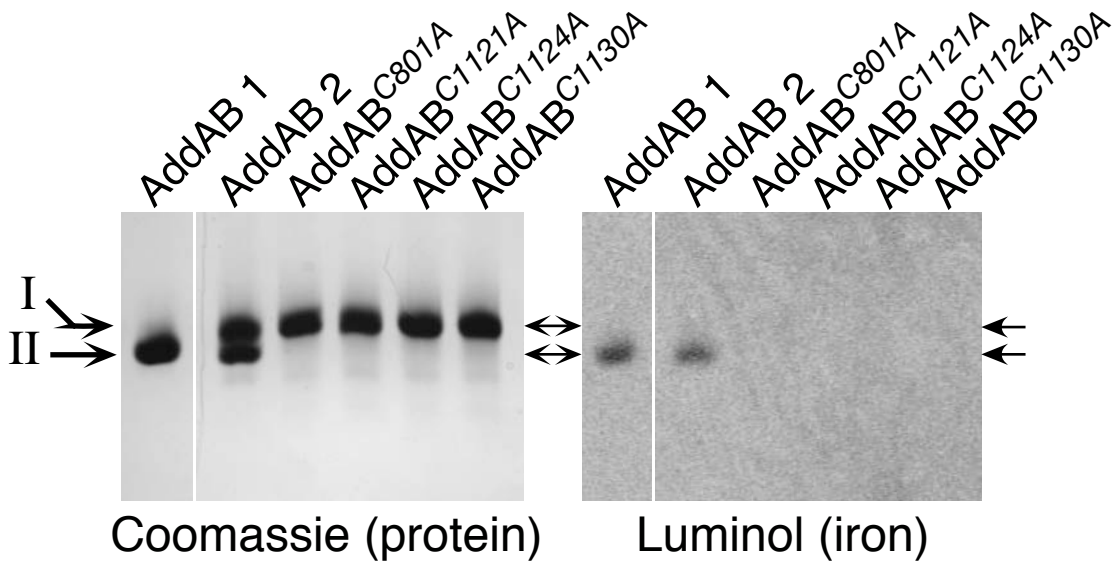

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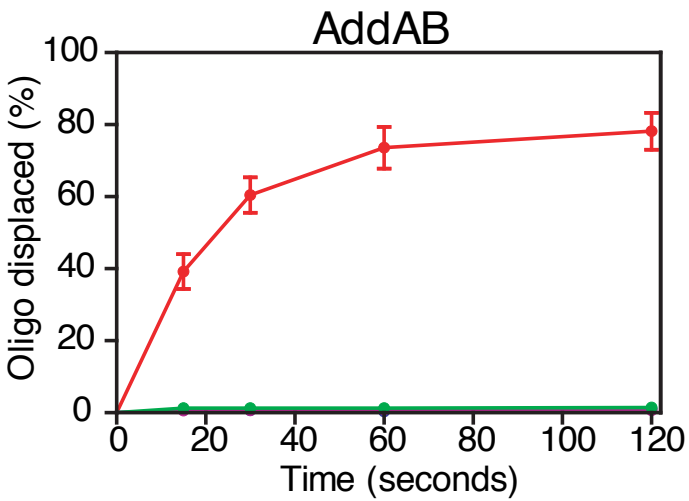

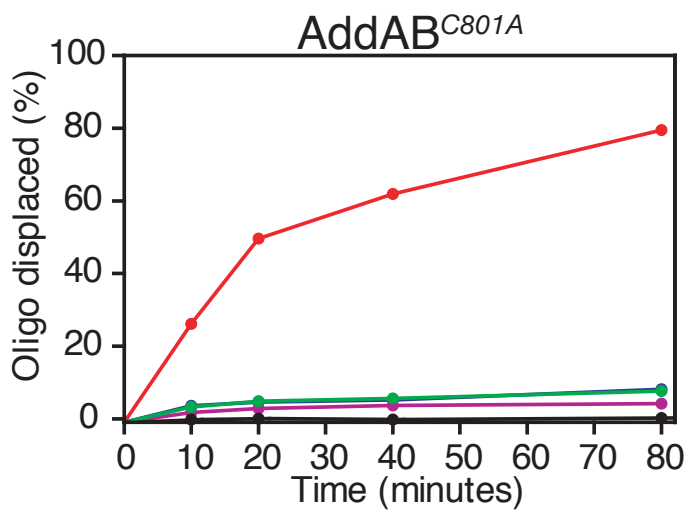

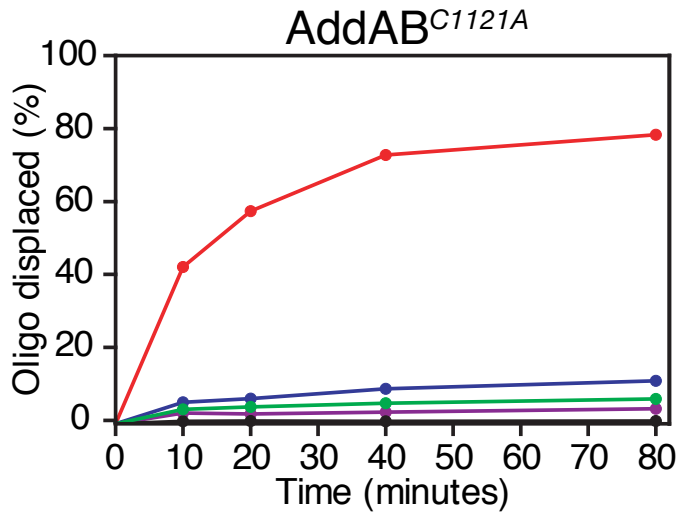

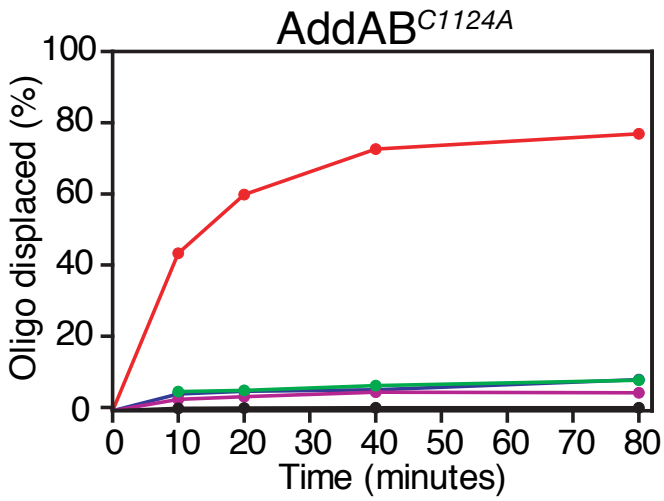

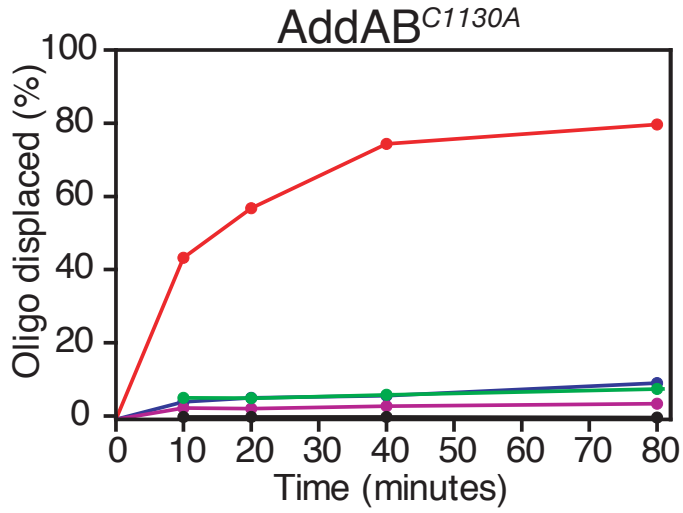

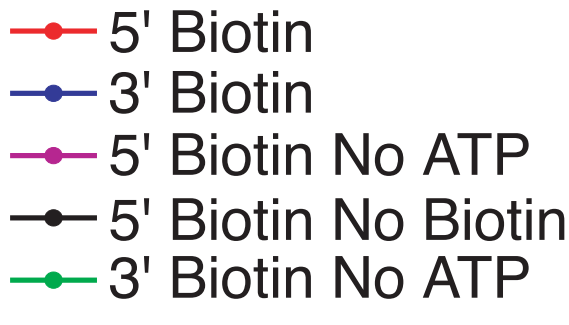

Supplementary 6