Electronic Supplementary Material (ESI) for Molecular BioSystems

Electronic Supplementary Information

Covalently trapping MutS on DNA to study DNA mismatch recognition and signaling

Roger J. Heinze[a], Svetlana Sekerina[b], Ines Winkler[a], Christian Biertümpfel[c,d], Tatiana S. Oretskaya[b], Elena Kubareva[b], and Peter Friedhoff[a]

Table S1 Oligodeoxyribonucleotides and DNA duplexes

Abbreviation	*Sequence*[*]
G30	5'-AGCTGCCA**G**GCACCAGTGTCAGCGTCCTAT-3'
A30:	5'-AGCTGCCA**A**GCACCAGTGTCAGCGTCCTAT-3'
T29	5'-ATAGGACGCTGACACTGGTGC**T**TGGCAGC-3'
T29-SS	5'-ATAGGACGCTGACACTGGTGC**T**TGGCAGCp-$(CH_2)_3$SSR-3'
G59	5'-ACAATGCGCTCATCGTCATCCTCGTCTCAAGCTGCCA**G**GCACCAGTGTCAGCGTCCTAT-3'
B30	5'-TTGAGACGAGGATGACGATGAGCGCATTGT-3'
I (G30/T29-SS)	5'-AGCTGCCA**G**GCACCAGTGTCAGCGTCCTAT-3' 3'-RSS$(CH_2)_3$-pCGACGGT**T**CGTGGTCACAGTCGCAGGATA-5'
II (A30/T29-SS)	5'-AGCTGCCA**A**GCACCAGTGTCAGCGTCCTAT-3' 3'-RSS$(CH_2)_3$-pCGACGGT**T**CGTGGTCACAGTCGCAGGATA-5'
III (G30/T29)	5'-AGCTGCCA**G**GCACCAGTGTCAGCGTCCTAT-3' 3'-CGACGGT**T**CGTGGTCACAGTCGCAGGATA-5'
IV (A30/T29)	5'-AGCTGCCA**A**GCACCAGTGTCAGCGTCCTAT-3' 3'-CGACGGT**T**CGTGGTCACAGTCGCAGGATA-5'
V (G59/B30+T29-SS)	5'-ACAATGCGCTCATCGTCATCCTCGTCTCAA----------GCTGCCA**G**GCACCAGTGTCAGCGTCCTAT-3' 3'-TGTTACGCGAGTAGCAGTAGGAGCAGAGTT RSS$(CH_2)_3$-pCGACGGT**T**CGTGGTCACAGTCGCAGGATA-5'

*Oligonucleotides were purchased from Eurogentec (0.2 µmol-scale/HPLC purified). Oligonucleotides with a 3'-thiol modifier RSS$(CH_2)_3$- (R = $(CH_2)_3$OH) were obtained in the oxidized form with an asymmetric disulfide bond (see Fig. 1 of the main text).

Cloning and Design of single-cysteine MutS

The sites for single-cysteine modifications of *E. coli* MutS were designed by structural analysis and distance measurements between protein and DNA in the crystal structure of MutS in complex with DNA containing a G:T mismatch (PDB code 1e3m) [1]. The single-cysteine mutations A469C and N497C were chosen (Figure 1 of the main text) and prepared by site-directed mutagenesis using the QuickChange kit (Stratagene). The template was pTX412/Cys-free/C800 [2] which contains the gene for a C-terminally truncated (Δ801-853) and cysteine-free (C93A, C235S, C239A, C297S, C569S, C711V) variant of *E. coli* MutS. Oligonucleotides used as mutagenesis primers (5'-CTGAAAGTTGGCTTTAATTGTGTGCACGGCTACTACATT-3', 5'-AATGTAGTAGCCGTGCACACAATTAAAGCCAACTTTCAG-3' for A469C and 5'-CGCCAGACGCTGAAATGCGCCGAGCGCTACATC-3', 5'-GATGTAGCGCTCGGCGCATTTCAGCGTCTGGCG-3' for N497C) were purchased from the Facility for Biotechnology Resources, FDA, Bethesda, MD, USA.

Table S2 Plasmids and proteins

Plasmid	*Protein*	*Comment*	*Reference*
pTX412	MutS	MutS with N-terminal His_6 tag	[3]
pTX412/Cys-free	$MutS^{CF}$	CF = cysteine-free His-MutS C93A, C235S, C239A, C297S, C569S, C711V	[2]
pTX412/Cys-free/C800	$MutS^{CF}$(Δ801-853)	truncated His-$MutS^{CF}$ (deletion of the 53 amino acids at the carboxy terminus)	[2]
pTX412/A469C/C800	MutS(A469C/Δ801-853)	truncated His-$MutS^{CF}$ with single cysteine at pos. 469	this work
pTX412/N497C/C800	MutS(N497C/Δ801-853)	truncated His-$MutS^{CF}$ with single cysteine at pos. 497	this work
pTX412/D246C/A469C/C800	MutS(D246C/A469C/Δ801-853)	truncated His-$MutS^{CF}$ with two cysteines at pos. 246 and 469	this work

Strains: HMS174 (λDE3) (F^-, *recA1 hsdR*, $r_{K12}^- m_{K12}^+$, Rif^r, DE3) cells were used for expression of variants MutS(A469C/Δ801-853), MutS(N497C/Δ801-853) and MutS(D246C/A469C/Δ801-853). TSS competent cells were transformed with derivatives of pTX412, containing the MutS gene under control of the T7 promoter [3].

To demonstrate the feasibility of single-cysteine MutS variant (MutS(A469C/Δ801-853) or MutS(N497C/Δ801-853) for crosslinking to DNA, the protein was incubated with G:T heteroduplex I or A:T homoduplex II in the presence or absence of the indicated adenosine phosphate and analyzed by SDS-PAGE (Figure S1). DNA was first stained with ethidium bromide, which also stains to some extent proteins in the presence of SDS [4]. Thereafter, proteins were stained with Coomassie. In the absence of DNA in addition to the band of the MutS monomer (molecular mass of 91 kDa) a faint band with an apparent molecular mass of > 200 kDa was observed which corresponds to the MutS dimer since it was absent after treatment with DTT (compare lanes 2 and 7 in Figure S1). In the presence of G:T heteroduplex I, an additional band with an apparent molecular mass of 120 kDa was observed in the absence of added nucleotide (Figure S1, lane 3), with ADP (Figure S1, lane 4) or ATP (Figure S1, lane 5). This band displayed an intense staining by ethidium bromide and was not present after addition of DTT prior to electrophoresis (compare lanes 5 and 7 in Figure S1) and was assigned as the MutS-DNA crosslink. Similar results were obtained with the A:T homoduplex II, albeit the intensity of the band with apparent molecular mass of 120 kDa was weaker.

Next we addressed whether crosslinking between MutS and DNA occurs only when MutS was bound to the DNA rather than in non-specific thiol/disulfide exchange reaction. It has been shown before, that MutS is switched into a DNA binding incompetent form upon binding to ATP which can be converted into a binding proficient form upon ATP hydrolysis [5]. If ATP-hydrolysis is prevented (e.g. in the absence of Mg^{2+}) or by replacing ATP with the non-hydrolyzable analogs ATPγS or AMP-PNP no DNA binding was observed [5, 6]. Indeed, when MutS was first incubated with AMP-PNP followed by the addition of DNA no band with apparent molecular mass of 120 kDa was formed but the intensity of the band with molecular mass of > 200 kDa was increased (Figure S1, lane 6). Similar results were observed when ATP hydrolysis was prevented by the addition of EDTA (data not shown).

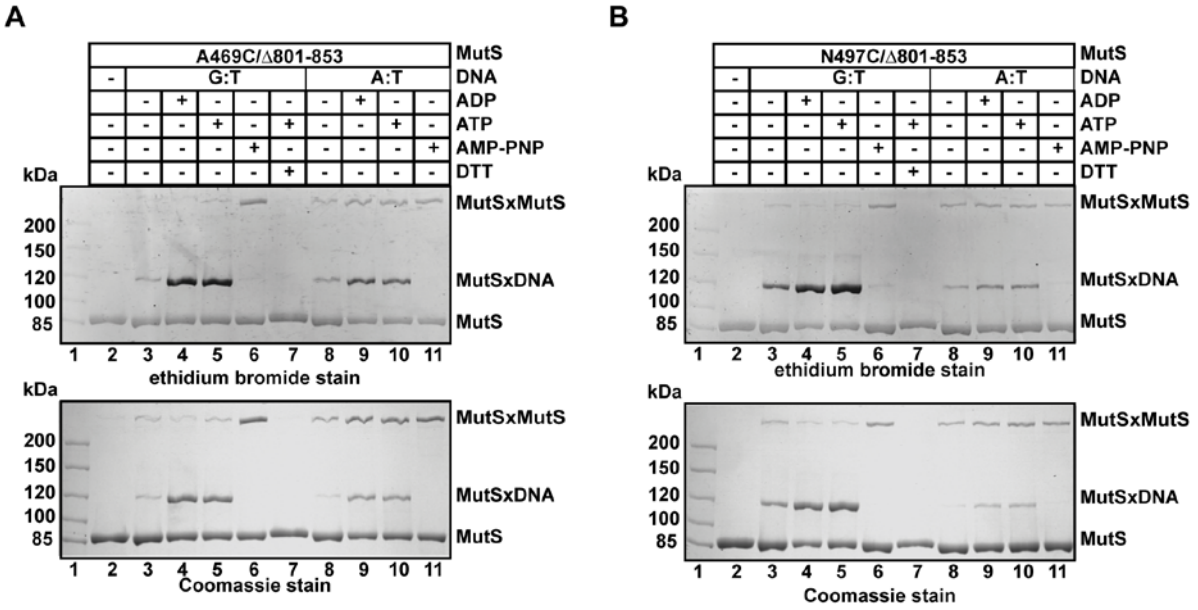

Figure S1 Crosslinking of MutS(A469C/Δ801-853) to homo- and heteroduplexes is dependent on the DNA-binding proficient form of MutS

SDS PAGE analysis of MutS(A469C/Δ801-853) (A) or MutS(N497C/Δ801-853) (B) (2 µM per monomer) incubated in the absence or presence of DNA (5 µM G:T heteroduplex (I) , lanes 3-7 or A:T homoduplex (II) , lanes 8-11) and the indicated adenosine phosphate (200 µM) for 30 min at 37 °C. DTT (10 mM) was added prior to gel electrophoresis were indicated. The gels were first stained by ethidium bromide (bottom) which stains DNA better than the protein [4] and Coomassie brilliant blue (top). In the presence of AMP-PNP no MutS-DNA crosslink was observed. Note, that the electrophoretic mobility of the MutS-MutS conjugate is much higher than expected from the molecular mass of the dimer due to the branched nature of the crosslinked complex. Low amounts of crosslinked MutS-DNA complex are also formed in the absence of any added adenosine phosphate (lane 3). In the presence of the non-hydrolysable ATP-analog AMP-PNP (or ATP and EDTA catching Mg^{2+} to prevent ATP-hydrolysis by MutS) covalently trapping of MutS on DNA is prevented due to an impaired DNA binding capacity of MutS after the proposed AMP-PNP-induced conformational changes (lane 6) [6]. MutS crosslinking to homoduplex II in a nucleotide-specific manner was also possible, although with less efficiency (lane 8-11). Finally, the addition of DTT (10 mM) results in reduction of formed disulfide-bonds and disruption of the MutS-DNA complex as well as unspecific MutS-MutS complexes that are formed in a competitive side reaction (lane 7, 12).

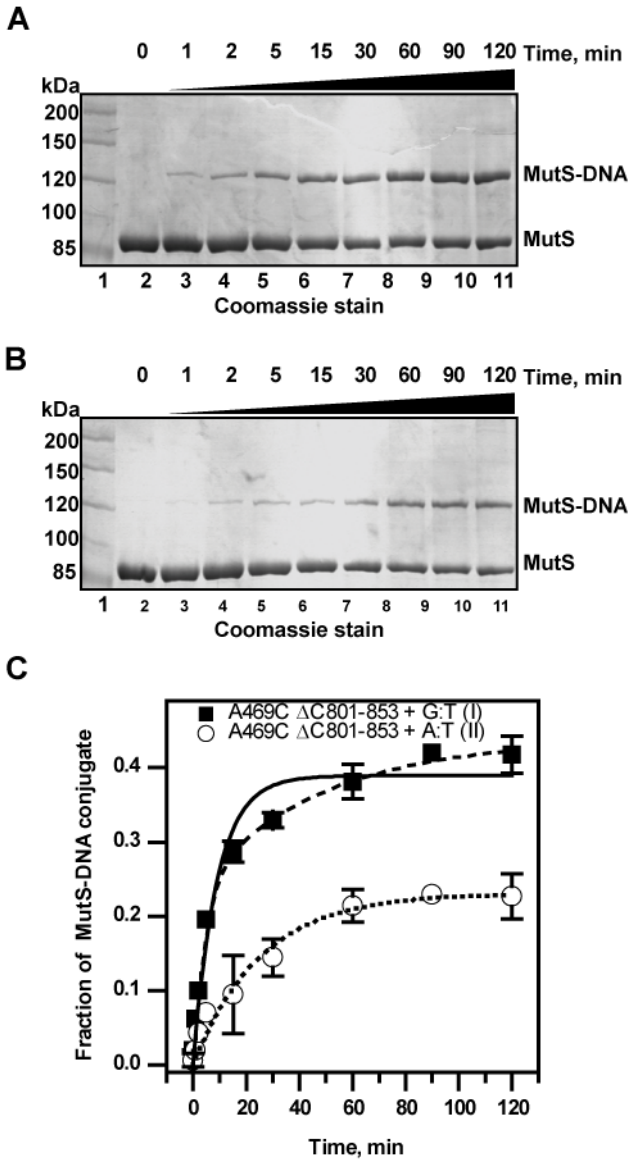

Figure S2 Crosslinking of MutS to homoduplex DNA is possible albeit slower

SDS-PAGE analysis of MutS(A469C/Δ801-853) (2 µM per monomer) incubated in the presence of 5 µM G:T heteroduplex I (A) or A:T homoduplex II (B) and 200 µM ADP at 37 °C. Crosslinking was stopped at the indicated time points by the addition of ice cooled loading dye containing SDS. (C) Quantitative analysis of the crosslinking reactions plotting the fraction of MutS-DNA conjugate to /total MutS versus time for the reaction in the presence of a G:T heteroduplex (black squares) or a A:T homoduplex (open circles). Error bars are the standard deviation from n = 2 independent crosslinking experiments. Solid or dotted lines are single exponentials fits to the data with apparent rate constants of $k_{G:T}$ = 0.11 ± 0.017 min^{-1} and $k_{A:T}$ = 0.04 ± 0.011 min^{-1}. A double exponential function (dashed line) fit to the data of the heteroduplex crosslinking much better with two apparent rate constants of $k_{G:T,fast}$ = 0.23 ± 0.04 min^{-1} and $k_{G:T,slow}$ = 0.02 ± 0.007 min^{-1}. The two rate constants observed might reflect the ability of MutS to bind to a mismatch in two different orientations one of which is only capable of forming a crosslinkg to the DNA [7].

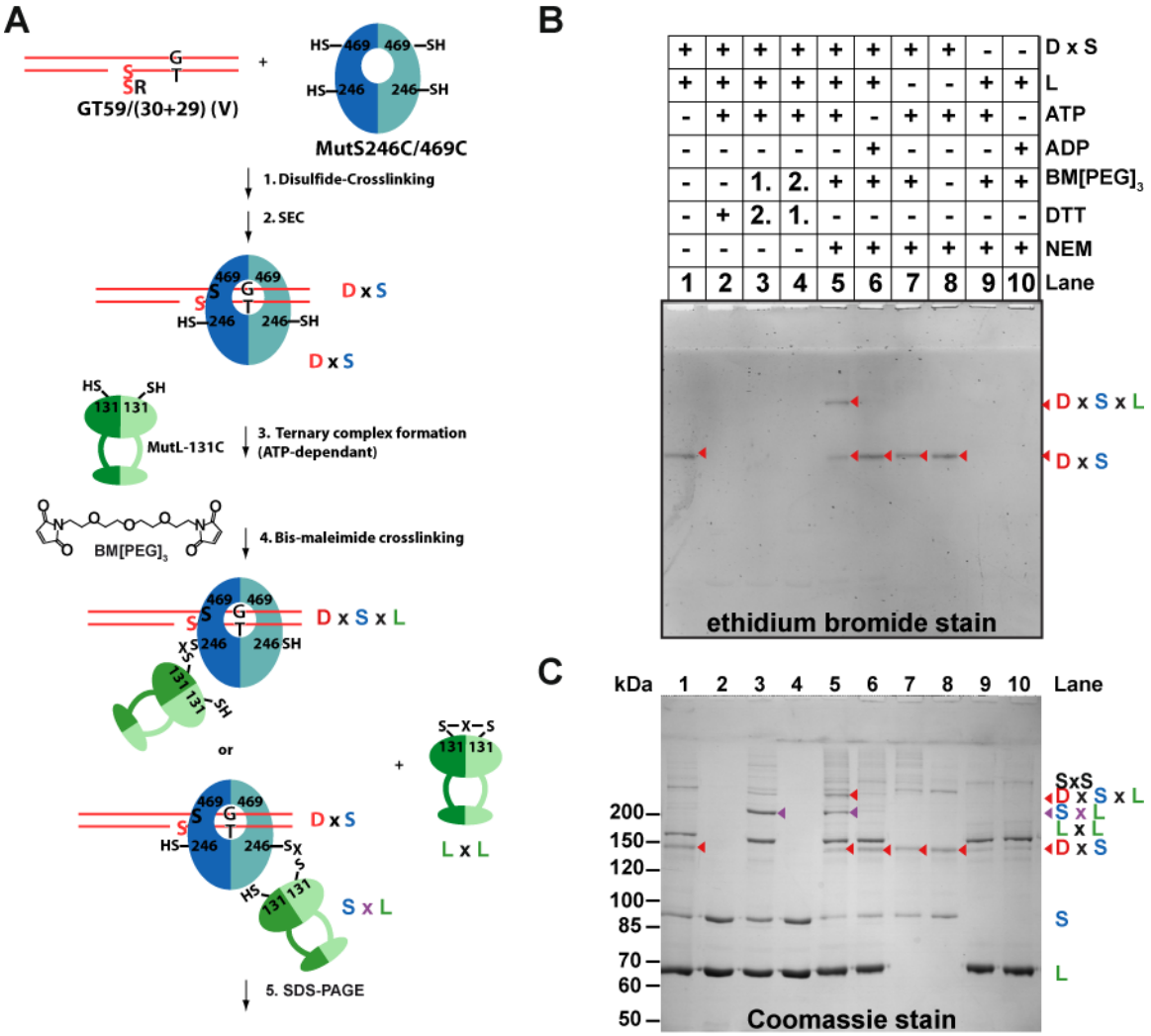

Figure S3 Trapping the ternary complex of DNA, MutS and MutL using sequential thiol/disulfide reaction exchange and bis-maleimide crosslinking

It has been shown that MutS can form a site-specific crosslink to MutL using single-cysteine variants of MutS (at position 246) and MutL (at position 131) [8] . Hence, in the present work the double-cysteine variant MutS(D246C/A469C/Δ801-853) was generated for crosslinking MutS first to DNA and then to MutL(N131C). For the MutS-MutL crosslinking reaction we used a longer DNA fragment (G:T heteroduplex V, Table S1) to account for the apparent DNA-length-dependence of MutS-MutL complex formation [8-10] . MutS(D246C/A469C/Δ801-853) was first crosslinked to duplex V via the thiol/disulfide exchange reaction, purified by size-exclusion chromatography, and then crosslinked to MutL(N131C) using the thiol-specific bifunctional crosslinker 1,11-bis(maleimido)triethylene glycol ($BM[PEG]_3$). Following SDS-PAGE analysis, we observed an additional species with lower electrophoretic mobility only in the presence of ATP, i.e., conditions that support the ternary complex formation between DNA, MutS and MutL (lanes 5, 6). These results suggest that MutS crosslinked via its clamp domain to DNA is still capable of ATP-induced conformational changes and subsequent recruitment of MutL to the site of the mismatch.

Scheme of the crosslinking reaction. (A) 59 bp heteroduplex V (Table S1) containing a 3'-thiol-modifier at the nick in the 'bottom' strand was crosslinked with the double-cysteine variant MutS(D246C/A469C/Δ801-853) (Table S2) via a thiol/disulfide exchange reaction and then the MutS-

DNA conjugate was crosslinked with MutL(N131C) using BM[PEG]$_3$. The crosslinked complex was purified by size-exclusion chromatography (SEC) using a Superdex 200TM column. To monitor ternary complex formation between DNA, MutS and MutL, the single-cysteine variant MutL(N131C) (2 µM per monomer) was incubated in the presence of 1 mM ATP or ADP with the purified MutS-DNA conjugate (400 nM) for 1 min at 37 °C prior to the addition of the homobifunctional bismaleimide crosslinker 1,11-bis(maleimido)triethylene glycol (BM[PEG]$_3$) (50 µM). After 1 min, the reaction was stopped by addition of N-ethylmaleimide (0.1 M) or dithiothreitol (DTT, 0.1 M) and the incubation mixture was loaded on 6 % SDS-PAGE followed by sequential staining by ethidium bromide (B) and Coomassie (C) to visualize DNA-containing and protein-containing bands. BM[PEG]$_3$ crosslinker was added to reaction mixture before (lane 3, 1.) or after (lane 4, 2.) DTT.

Table S3 Rate constants for ADP exchange*

MutS	*ADP exchange rate (k_{off}^{ADP}) (s^{-1})#*						
	No DNA	MutS + G:T(III)	MutSxG:T(I)	MutSxG:T(I) + TCEP	MutS + A:T(IV)	MutSxA:T(II)	Ref.
wild type	0.0072	0.076	n.a.	n.a.	n.a.	n.a.	[11]
A469C/Δ801-853	0.0069 ± 0.0002	0.046 ± 0.005	0.079 ± 0.002	0.055 ± 0.013	0.034 ± 0.001	0.034 ± 0.002	this work
D246C/A469C/ Δ801-853	n.d.	n.d.	0.051 ± 0.004	0.075 ± 0.001	n.d.	n.d.	this work

*ADP-exchange was measured by release of mant-ADP (500 nM) bound to MutS (1 μM per monomer in the absence of DNA (no DNA), in the presence of nonmodified duplexes III or IV with (+ G:T) or without a mismatch (+ A:T) or mant-ADP bound to conjugate of MutS with G:T heteroduplex I (xG:T) or with A:T homoduplex II (xA:T) in the absence or in the presence of TCEP. Mant-ADP release was monitored upon addition of an excess of unlabeled ADP competitor (1 mM) (see Fig. 5 of the main text). Unbound mant-ADP has only 40% of its fluorescence intensity compared to MutS-bound mant-ADP [11]. A single exponential function was fitted to the data to give apparent rate constants k_{off}^{ADP}. Averages and standard deviations from 2-4 independent experiments are shown. n.a. = not available; n.d. = not determined.

ESI References

1. M. H. Lamers, A. Perrakis, J. H. Enzlin, H. H. Winterwerp, N. de Wind and T. K. Sixma, *Nature*, 2000, **407**, 711-717.
2. L. Manelyte, C. Urbanke, L. Giron-Monzon and P. Friedhoff, *Nucleic Acids Res.*, 2006, **34**, 5270-5279.
3. G. Feng and M. E. Winkler, *BioTechniques*, 1995, **19**, 956-965.
4. A. Vincent and K. Scherrer, *Mol. Biol. Rep.*, 1979, **5**, 209-214.
5. S. Gradia, S. Acharya and R. Fishel, *Cell*, 1997, **91**, 995-1005.
6. M. C. Monti, S. X. Cohen, A. Fish, H. H. Winterwerp, A. Barendregt, P. Friedhoff, A. Perrakis, A. J. Heck, T. K. Sixma, R. H. van den Heuvel and J. H. Lebbink, *Nucleic Acids Res.*, 2011, **39**, 8052-8064.
7. M. Cristovao, E. Sisamakis, M. M. Hingorani, A. D. Marx, C. P. Jung, P. J. Rothwell, C. A. Seidel and P. Friedhoff, *Nucleic Acids Res.*, 2012.
8. I. Winkler, A. D. Marx, D. Lariviere, L. Manelyte, L. Giron-Monzon, R. J. Heinze, M. Cristovao, A. Reumer, U. Curth, T. K. Sixma and P. Friedhoff, *J. Biol. Chem.*, 2011, **286**, 17326-17337.
9. L. J. Blackwell, S. Wang and P. Modrich, *J. Biol. Chem.*, 2001, **276**, 33233-33240.
10. S. Acharya, P. L. Foster, P. Brooks and R. Fishel, *Mol. Cell*, 2003, **12**, 233-246.
11. J. H. Lebbink, A. Fish, A. Reumer, G. Natrajan, H. H. Winterwerp and T. K. Sixma, *J. Biol. Chem.*, 2010, **285**, 13131-13141.