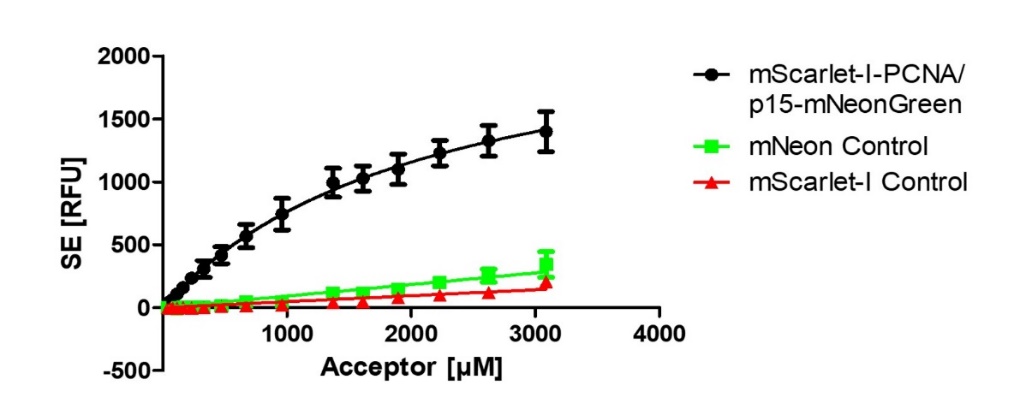
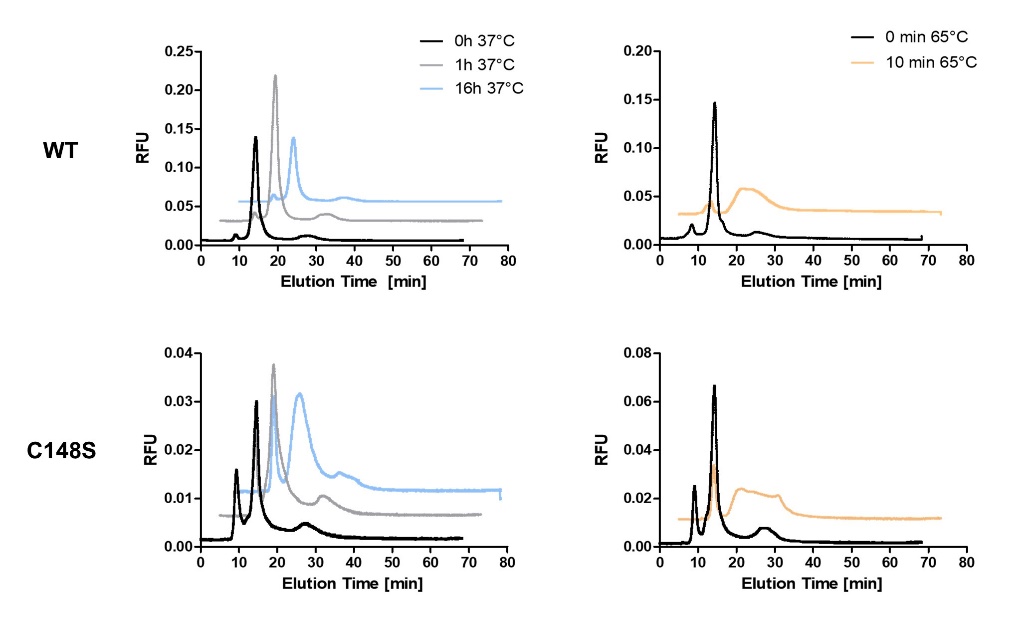
**Supplementary Materials:**

**Table S1**. List of PCNA141-155 amino acid sequences from different eukaryotic species using HomoloGene database [87]. Cysteine at position 148 was marked in red.

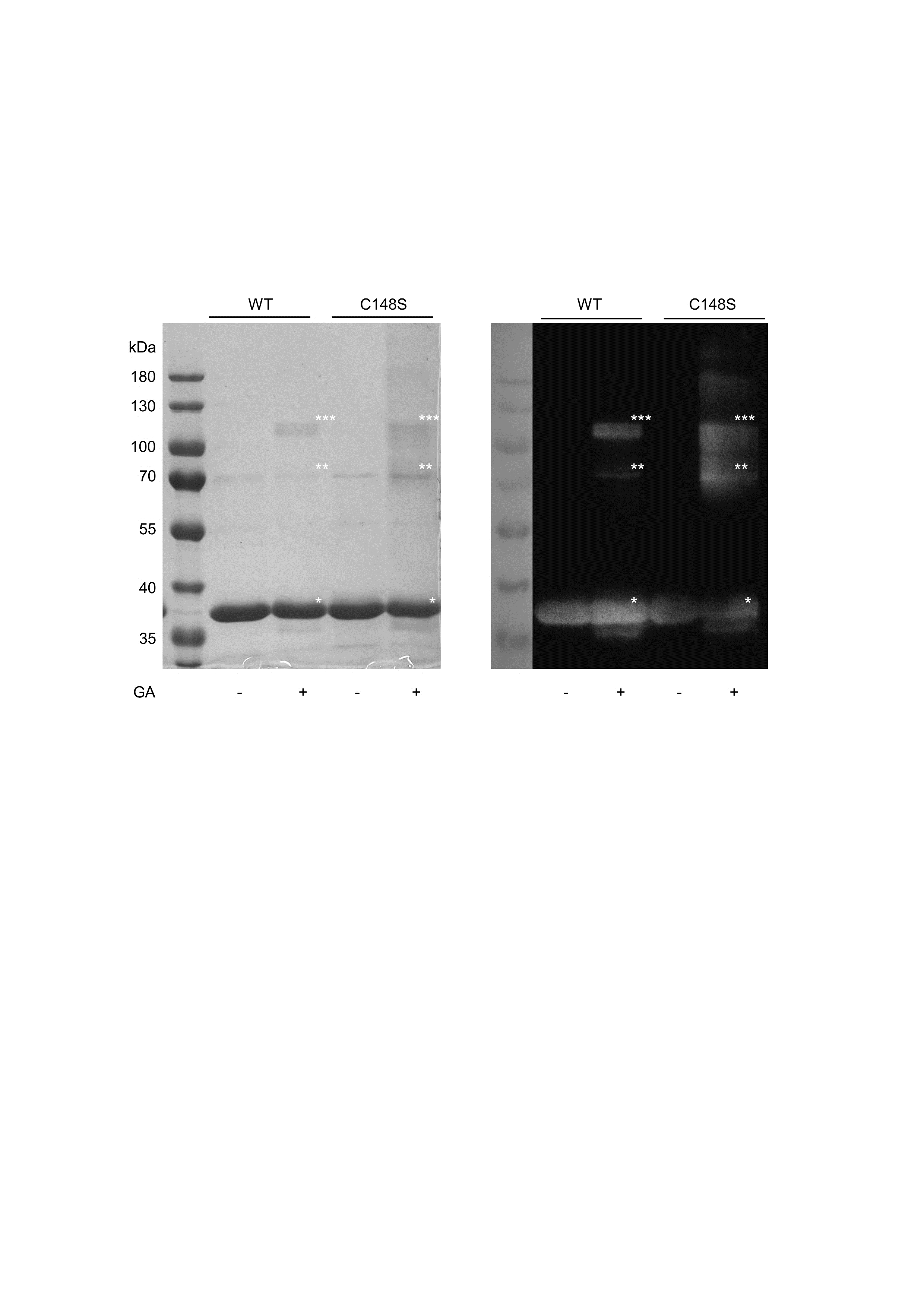
|  |  |
| --- | --- |
| **Organism** | **Sequence** |
| *Homo sapiens* | SGEFARICRDLSHIG |
| *Pan troglodytes* | SGEFARICRDLSHIG |
| *Macaca mulatta* | SGEFARICRDLSHIG |
| *Canis lupus familiaris* | SGEFARICRDLSHIG |
| *Bos taurus* | SGEFARICRDLSHIG |
| *Mus musculus* | SGEFARICRDLSHIG |
| *Rattus norvegicus* | SGEFARICRDLSHIG |
| *Gallus gallus* | SAEFARICRDLSHIG |
| *Xenopus tropicalis* | SGEFARICRDLSHIG |
| *Danio rerio* | AMEFARICRDLAQFS |
| *Drosophila melanogaster* | AMEFARICRDLSQFG |
| *Anopheles gambiae str. PEST* | AGEFQKTCKDLSTFS |
| *Caenorhabditis elegans* | SSEFSKIVRDLSQLS |
| *Saccharomyces cerevisiae* S288C | SADFAKTVRDLSQLS |
| *Kluyveromyces lactis* | SAEFAKIIRDLNQLS |
| *Eremothecium gossypii* ATCC 10895 | AAEFQRITRDLLTLS |
| *Schizosaccharomyces pombe* | SAEFRRICTDLLAMS |
| *Pyricularia oryzae* 70-15 | SSEFKRITTDLMAMS |
| *Neurospora crassa* OR74A | SGEFSRICKDLSSIG |
| *Arabidopsis thaliana* | SNEFSRICKDLSSIG |
| *Oryza sativa Japonica Group* | SGEFARICRDLSQIG |



**Figure S1**. Proof of specific binding in the FRET assay. 1 µM of p15-mNeonGreen was incubated with increasing mScarlet-I-PCNAWT concentrations (0-2.1 µM) for 1 h at 37 °C at 300 rpm. As nonbinding controls, the interaction of mNeonGreen/mScarlet-I-PCNA or mNeonGreen-p15/mScarlet-I was analysed. The sensitized emission (SE) of three experiments in triplicates was shown. For further analysis of binding, the sensitized emission of the mNeonGreen control was subtracted from the sensitized emission of the samples. RFU= Relative Fluorescence Units.



**Figure S2.** The C148S mutation decreases the thermal stability of mScarlet-I-PCNA. Proteins were incubated at 37 °C for 0 h (black), 1 h (grey) or 16 h (blue) or at 65 °C for 10 min (orange) and were afterwards injected to AF4. The induction of aggregates was evaluated by plotting the fluorescence emission at 610 nm against the elution time. The increase of thermal stress correlates with higher elution times, which can be explained by the formation of higher molecular weight complexes.

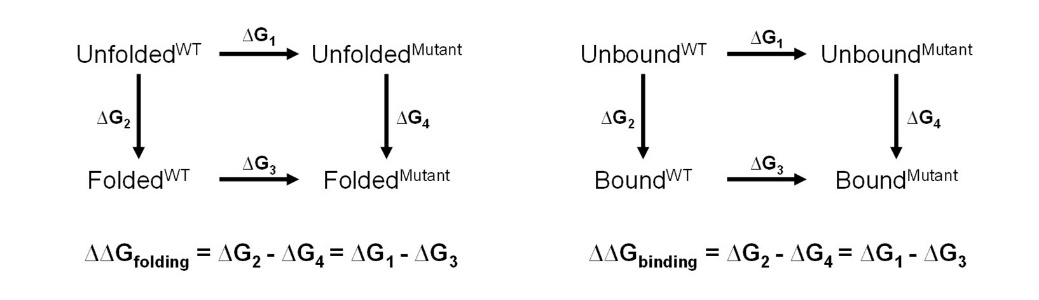


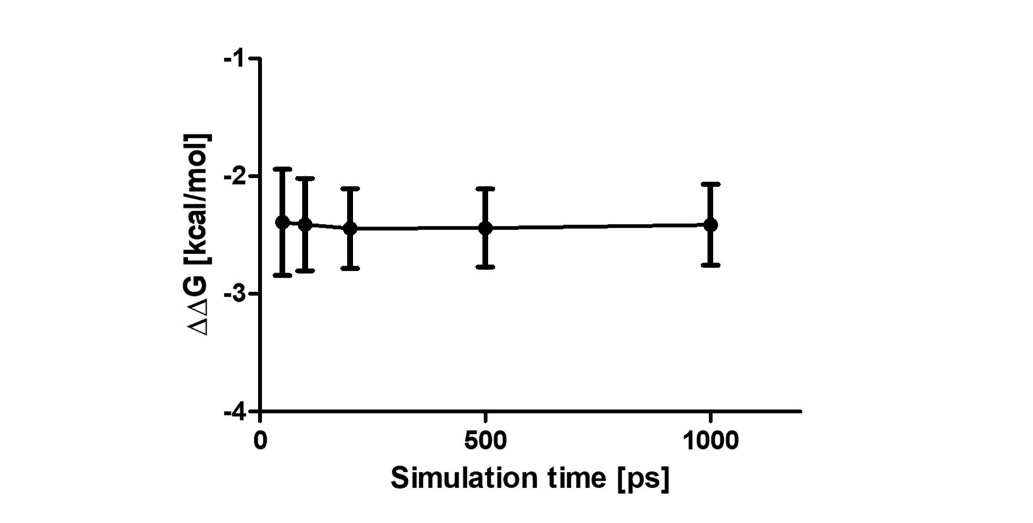
**A B**

**Figure S3**. C148S does not prevent trimerization of PCNA. PCNAWT and PCNAC148S were crosslinked using 0.004% glutaraldehyde for 10 min at RT. 5 µg protein were loaded on a 10% SDS-PAGE gel and stained by Coomassie brilliant-blue **(A)** or transferred to an PVDF membrane and immunoblotted with an antibody against the His6-epitope **(B)**. The asterisks mark the bands of the monomeric (\*, 31.2 kDa) dimeric (\*\*, 62.4 kDa) or trimeric (\*\*\*, 93.6 kDa) PCNA.

**Table S2**. List of oligonucleotides used in this work.

| **Number** | **Oligonucleotide sequence** | **Plasmid** | **Usage** |
| --- | --- | --- | --- |
| Pr2844  Pr2845 | AGATCCACTCCACCCTCTT-TCTCATCGTTGGTG; gagc-ttacaagTAAGGATCCGG-CTGCTAAC | pSR052– vector | In-Fusion: generation of p15-mNeon intracellular expression plasmid |
| Pr1878  PR1879 | GGTGAGGTGATCTATGGT-GACAAGGGCGAG; TTACT-TACAGCTCGTCCAT-GC | pSR052– insert | In-Fusion: generation of p15-mNeon intracellular expression plasmid |
| Pr427  Pr2837 | CATATGATGGTGATGGTG-GTCATGGTATATCTCCTTC; GGTGGAGGTGGATCTatg-ttcgaggcg | PSR046– vector | In-Fusion: generation of mScarlet-I-PCNA connected by the flexible linker (GGGGS). |
| Pr1963  Pr2119 | CACCATCACCATCAATGG-TGACAAGGGCGAGGC; AG-ATCCATCCACCCTGTACA-GCTCGTCCATGCC | pSR046-insert | In-Fusion: generation of mScarlet-I-PCNA connected by the flexible linker (GGGGS). |
| Pr1518  Pr2840 | Atgttcgaggcgcgcctgg; cttacagctcgtccatgc-cgc | pSR051– vector | In-Fusion: Incorporation of the hydrophilic flexible linker in mScarlet-I-PCNA sequence (GEGQGQGPGRGYAYRS) |
| Pr2838  Pr2839 | gacgagctgtacaagGGC-GAAGGCCAGGGCCAGGG-CCAGGGCCCGGGCCGCGG-CTATGCGTATCGCAGCat-gttcgaggcgcgc; gcgc-gcctcgaacatG | pSR051-Insert | In-Fusion: Incorporation of the hydrophilic flexible linker in mScarlet-I-PCNA sequence (GEGQGQGPGRGYAYRS) |
| Pr3199  Pr3200 | Tgagatctcgggatatacgtgcaaattcacc; Tttgc-acgtatatcccgagatct-cagcc | pSR080;  pSR081 | Mutagenesis: Incorporation of C148S in PCNA or mScarlet-I-PCNA sequence |

**Figure S4**. Thermodynamic cycle used for the calculation of ∆∆Gfolding (left)and ∆∆Gbinding (right). Amino acids were morphed along the non-physical path indicted by horizontal arrows to estimate the corresponding ∆G values. The ∆∆G value for a given mutation can then be obtained by the formula shown under the cycle. Vertical arrows indicate the physical folding or binding pathway. Obtaining the ∆G for these paths is considerably more computationally expensive.



**Figure S5.** Influence of the simulation time of alchemical transformations on the ∆∆Gfolding of PCNAA148C. ∆∆Gfolding was calculated from three independent equilibrium trajectories. From these transition simulations with different length were started. The mean and standard deviation of ∆∆Gfolding are plotted against the simulation time for each transition simulation.