

Conserved C-terminal segment of human single-stranded DNA-binding proteins facilitates protein condensation

OTDK dissertation

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Summary

Recent studies highlighted the central importance of liquid-liquid phase separation (LLPS)-based protein condensation in cellular physiology, as it has been proven to be a key mechanism of internal compartmentalization. LLPS condensates are membraneless, dynamic, and liquid-like and are found in all organisms. Our group has shown that single-stranded DNA (ssDNA)-binding (SSB) proteins undergo LLPS in both bacteria and humans. Their presence is indispensable in DNA metabolism, for instance in replication, recombination and DNA-repair processes. The prototypical, tetrameric SSB from *E. coli* harbours three structural elements: the OB (oligonucleotide binding) domain, which binds to single-stranded DNA; the IDL (intrinsically disordered linker), that modulates cooperative DNA-binding by SSB tetramers and drives LLPS, and the CTP (C-terminal peptide), which is the last 9 amino acids on the protein's C-terminus. The highly conserved CTP is responsible for protein-protein interactions and recruiting proteins to single-stranded DNA. CTP-OB interactions between tetramers also facilitate LLPS. Human SSB (hSSB1/2) proteins, homologs of the *E. coli* SSB, play essential roles in the maintenance of the genome, specifically in homologous recombination and base excision repair of oxidative DNA damages. Interestingly, the last 13 amino acids at their C-terminus are conserved among vertebrates, but the function of the CTP in hSSB function and LLPS propensity is unknown. Therefore, I created CTP-truncated variants of hSSB1 (hSSB1-dC) and hSSB2 (hSSB2-dC) by molecular cloning. I expressed and purified the proteins. I examined their LLPS propensities by epifluorescence and DIC microscopy and assessed the change in their ability of condensate formation. I also examined proteins in the presence of molecular crowders, as the crowder effect is a known driving force of LLPS. I found that hSSB2-dC is poorly soluble and easily aggregates, while hSSB1-dC remains soluble but is only capable of LLPS in the presence of molecular crowders. I also showed via fluorescence anisotropy titrations that the binding affinity of hSSB1 to ssDNA is significantly reduced when the CTP is truncated, indicating that it is also involved in DNA binding. My results highlight that the CTP of human SSBs has essential roles in LLPS regulation, protein stability and ssDNA binding, with characteristic differences from the bacterial EcSSB.

List of abbreviations

- CTP: C-terminal peptide
- EcSSB: *Escherichia coli* SSB
- hSSB1: human SSB1
- hSSB1-dC: human SSB1 lacking the C-terminal peptide
- hSSB2: human SSB2
- hSSB2-dC: human SSB2 lacking the C-terminal peptide
- IDL: intrinsically disordered linker
- IDR: intrinsically disordered region
- LLPS: liquid-liquid phase separation
- OB domain: oligonucleotide-binding domain
- PEG: polyethylene-glycol
- SSB: single-stranded DNA-binding protein
- ssDNA: single-stranded DNA
- WT: wild type

Introduction

Eukaryotic cells were thought to rely primarily on phospholipid membranes for internal compartmentalization and for the spatial or temporal separation of their biochemical processes. Membrane-bound organelles are essential building blocks of a eukaryotic cell and are indispensable for cell development and turn-over, maintaining functional diversity and long-term regulation of metabolism¹. Conventionally, all intermembrane space was considered to be organized by diffusion, membrane transport and stoichiometric, specific interactions². However, in previous years, a different principle in the compartmentalization of intracellular space has gained significant attention. More and more proteins are being identified to form condensates by a phenomenon previously unknown in the aspect of biomolecular systems. These condensates are membrandelles, their behaviour is dynamic and liquid-like³. They usually form or disassemble rapidly upon changes in solution conditions, and they are able to fuse with one another⁴. This is due to the process of liquid-liquid phase separation (LLPS), which is a fundamental and long-known phenomenon in physics and material sciences, but its relevance was only recently proposed and proven in cell biology⁵.

Biomolecular condensates as organizers of intracellular space

Protein condensates have several distinct features. Their formation is driven by multivalent, non-stereospecific and non-stoichiometric, relatively weak interactions³. These interactions allow the protein to form a separate liquid phase under certain solution conditions⁵. Protein droplets behave similarly to oil droplets in water. They are able to fuse and move around freely⁵. Due to multivalent interactions, molecules are dynamically rearranged within the condensates⁵. In recent years, several biomolecular condensates have been identified in cells, such as postsynaptic densities, signalling clusters, stress granules and several ribonucleoprotein granules (**Figure 1**)³. Their formation is mostly driven by proteins that are capable to undergo LLPS⁵. Several of these membraneless organelles are currently among the highly researched topics of cell biology^{4,6}, and they have been associated with neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS)⁷⁻⁹, frontotemporal dementia^{7,9}, Parkinson's disease^{7,10,11}, Alzheimer's disease^{7,12,13}, and Huntington's disease^{7,14}. Furthermore, LLPS condensates are evidenced to contribute to the survival of cancer cells in different types of cancer^{7,15} – e. g. prostate cancer¹⁶, skin cutaneous melanoma¹⁷, and leukaemia¹⁸ - and were also shown to be present during viral infections⁷ and DNA repair¹⁹. Protein condensates can selectively recruit and enrich interaction partners and can create the optimal conditions for the occurring

biochemical reaction³. Many proteins that are already known to undergo LLPS harbour intrinsically disordered regions (IDR). IDRs have low sequence complexity and usually contain aromatic or charged linear motifs that are able to form dynamic, multivalent and non-stoichiometric intermolecular interactions, which are the requirements of LLPS³.

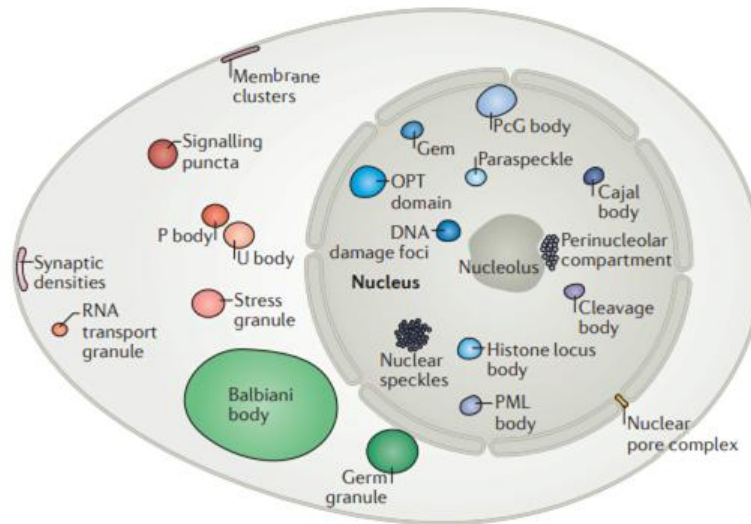


Figure 1 – Biomolecular condensates in eukaryotic cells. Not all condensates appear in every cell, some are cell-type specific. Figure was taken from S. F. Banani et al.³.

Single-stranded DNA-binding proteins undergo LLPS

Single-stranded DNA-binding (SSB) proteins are generally found in all organisms of the biosphere. Their purpose is to stabilize single-stranded DNA sections that form during the maintenance of the genome; they bind to the DNA and protect it from nucleolytic degradation or chemical alterations that can result in, loss of genetic information, genomic instability and mutation²⁰. Furthermore, they interact with many proteins that are involved in DNA metabolism and are able to recruit them to the site of action²⁰. SSBs are therefore considered as central players or organizers of genome maintenance²⁰. The vast majority of known SSBs contain or are predicted to contain IDRs²¹.

The SSB protein of the bacterium *Escherichia coli* is the most well-known and researched. It forms homotetramers, and the monomer (19 kDa, 178 amino acid) contains three important structural motifs (**Figure 2A**)^{21–23}. The largest is the N-terminal OB (oligonucleotide binding, 1-111 amino acid positions) domain that accounts for the third of the total molecular weight and is responsible for the binding of ssDNA²². It is followed by a relatively long IDR (~112-169 amino acid positions)^{21,23,24}. The last 9 amino acids on the protein's C-terminus are highly conserved among bacteria compared to the rest of the IDR^{20,22,25}. It mainly consists of

negatively charged amino acids, so it is usually referred to as the acidic tip, or the C-terminal peptide (CTP)²². Since the IDR connects the OB domain to the CTP, the intrinsically disordered linker (IDL) term is also used. The CTP is the conserved binding site for more than a dozen interaction partners and is responsible for their recruitment to the DNA^{20,25}. Besides, it interacts with the OB domain in the same region where the OB-ssDNA interaction takes place²¹.

Our group reported that EcSSB forms liquid-like droplets by LLPS in physiological conditions (**Figure 2B**)²². The presence of IDL is indispensable for LLPS propensity, and the intertetrameric OB-CTP interactions facilitate LLPS and the entanglement of IDLs²². It was also evidenced that ssDNA inhibits the formation of condensates (**Figure 2B**)²². This can be explained based on available structural information about EcSSB. ssDNA binding by the OB domain inhibits OB-CTP interactions needed for LLPS²². Furthermore, it was shown that EcSSB condensates selectively enrich interaction partners²². In cells, when the exposed ssDNA is relatively low under normal conditions^{22,26}, EcSSB is stored in LLPS condensates, and they were shown to associate to the inner membrane^{22,27}. Upon genomic stress, when unbound ssDNA appears the droplets disassemble, and EcSSB, together with the content of the condensates localize to the DNA²².

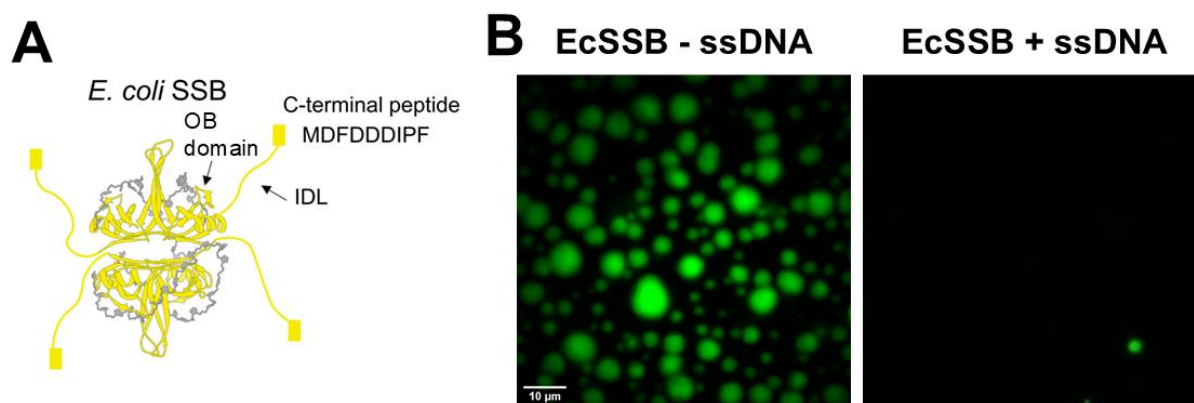


Figure 2 – EcSSB undergoes LLPS that is inhibited by ssDNA. (A) The structure of an EcSSB tetramer bound to ssDNA (gray). Figure is taken from G. M. Harami et al.²². (B) EcSSB forms liquid-like droplets that are inhibited by ssDNA. Fluorescence microscopy pictures of fluorescently labelled, recombinant EcSSB in vitro.

The eukaryotic orthologs of EcSSB have been unknown for quite some time. The RPA protein, which is considered to be the main single-stranded DNA-binding protein in eukaryotic cells, is only functionally similar to the bacterial SSB and it is not an EcSSB ortholog²⁸. It has a different structure and exhibits different DNA binding mechanisms²⁹. Recently, human SSB (hSSB) proteins, hSSB1 and hSSB2 were identified as bacterial SSB orthologs (**Figure 3 A-B**)³⁰. They show structural similarities to bacterial SSBs, and the OB and ID regions can both be found in

them^{29,30}. They are essential in DNA repair mechanisms during homologous recombination³⁰ and during oxidized DNA damage responses³¹. Upon oxidative DNA damage, hSSB1 recruits human 8-oxoguanine glycosylase (hOGG1) to the site of damage, which accounts for the base excision repair of 8-oxoguanine³¹. It is also present during the repair of double-stranded DNA breaks by homologous recombination, where it is indispensable for the localization and function of Exo1³² or the MRN nuclease complex^{31,33}. Both hSSB1 and hSSB2 was shown in our lab recently to undergo phase separation, however – unlike EcSSB – droplet formation is highly redox-dependent and occurs only in the presence of ssDNA^{34,35}. Reducing conditions inhibit hSSB1 to undergo LLPS, while oxidative conditions initiate nucleoprotein condensation (**Figure 3C**)³⁴. It was also evidenced that the redox-sensitive LLPS is regulated by covalent oligomerization via cysteine oxidation (**Figure 3A**)³⁴. hSSB2 is not as sensitive to redox changes as hSSB1 is³⁵. It can phase separate on a wider range of redox conditions, but the nucleoprotein condensates are less dynamic and more gel-like under oxidative conditions³⁵ (**Figure 3D**). In both proteins it was found, that deleting the IDR results in the loss of LLPS propensity (**Figure 3C-D**)^{34,35}. Similarly to EcSSB condensates, hSSB proteins also selectively enrich interaction partners in the droplets^{29,34,35}.

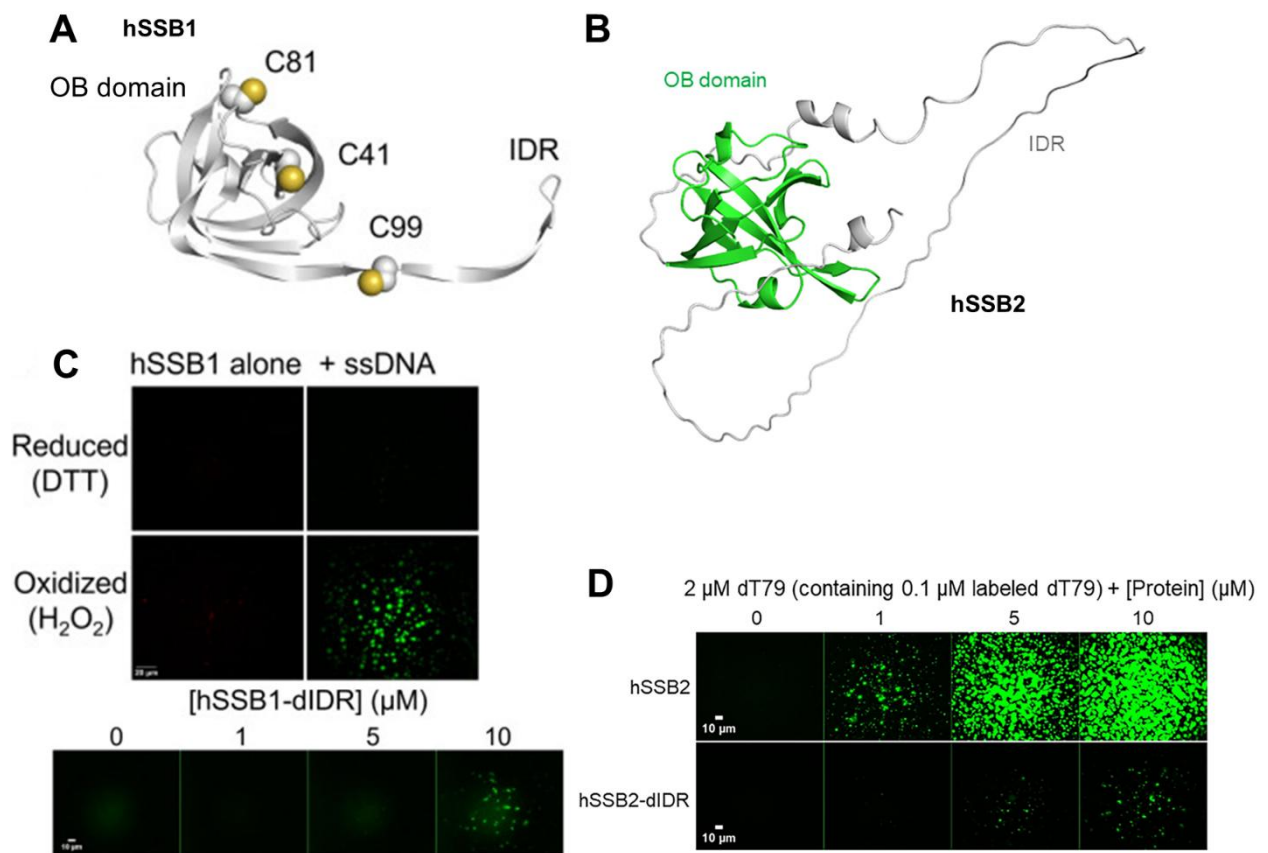


Figure 3 – hSSB1 and hSSB2 are structurally similar to EcSSB and form nucleoprotein condensates regulated by redox conditions. (A-B) Ribbon diagrams of hSSB1 and hSSB2 structures. The cysteines regulating redox sensitive condensation are highlighted in hSSB1. A crystal structure of a truncated protein is shown in case of hSSB1, while hSSB2 structure was predicted by AlphaFold. (C-D) hSSB1 and hSSB2 form liquid-like droplets but are incapable of LLPS when the IDR is removed. All figures are taken from G. M. Harami et al.³⁴ and Z. J. Kovács et al.³⁵.

Oxidative conditions are harmful to cells; therefore, it induces a stress response³⁶. The protection of the genome is a vital task, because the genetic material could be a victim of oxidative damage induced mutations^{29,30}. Our previous results indicate that hSSB1 upon oxidative stress response, may form LLPS nucleoprotein condensates together with several interaction partners at the site of damage³⁴. They bind exposed ssDNA and enhance DNA repair mechanisms. This is supported by the observation, that hSSB1 is involved in oxidized DNA damage responses^{30,31,37}. Also, hSSB1's role in DNA metabolism is further emphasized by the fact that it is ubiquitously expressed in all tissues³⁸. The role of hSSB2 is less established, but it has been demonstrated to be involved in DNA repair mechanisms upon UV or gamma irradiation^{35,39}. Unlike hSSB1, it is only expressed in specific tissues, such as the reproductive system (both male and female), the lymphoid tissues and the spleen, and the gastrointestinal tract^{35,40}.

Interestingly, the C-terminus of vertebrate SSB1/2 orthologs, compared to the rest of the IDR, are also conserved (**Figure 4A-B**) and hSSB1 and hSSB2 harbours similar CTP sequences (**Figure 4C**). However, compared to the acidic EcSSB CTP (**Figure 1**), the CTP of hSSB1 and hSSB2 contains basic amino acids. The negative EcSSB CTP binds to the positive ssDNA binding site on the OB domain²². This is not possible for a positively charged CTP. Thus, the precise role of hSSB CTP in protein function is not known, but the level of conservation indicates an important regulator role for this region.

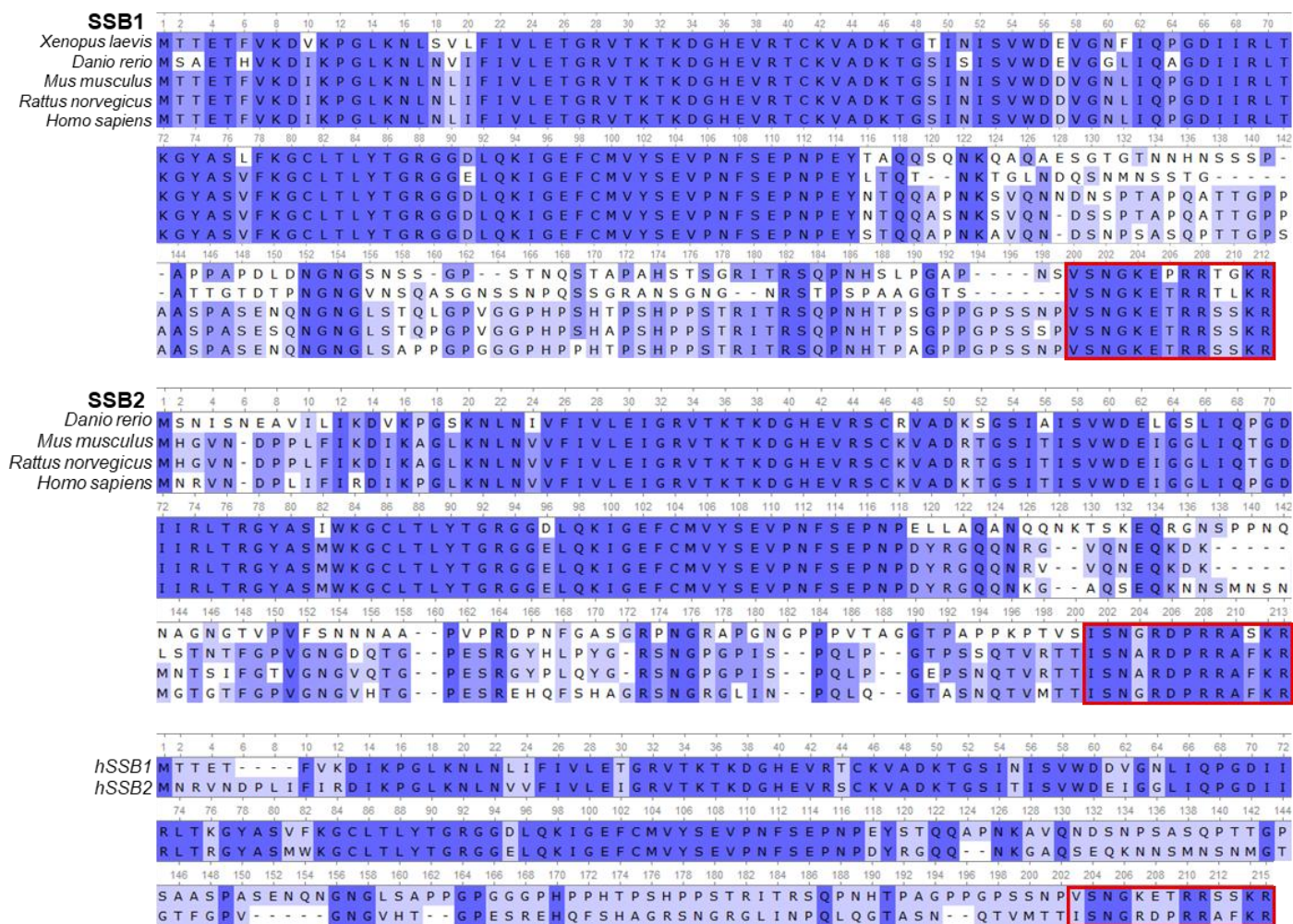


Figure 4 – Sequence alignments of vertebrate SSB1 and SSB2 orthologs. The SSB1 (A) and SSB2 (B) proteins of indicated species were aligned with the human orthologs. (C) The sequences of hSSB1 and 2 were aligned with each other as well. Blue colour indicates conservation. CTP is highlighted in red. The OB domain and the CTP are highly conserved, while most of the IDR is only partially. Sequence alignment was carried out by me and aligned with ClustalW in Unipro UGENE software.

Objective

During my research my objective was to uncover the role of the basic CTP sequence of hSSB1 (VSNGKETRRSSKR) and hSSB2 (ISNGRDPRRAFKR) in LLPS regulation and DNA-binding by creating CTP-truncated protein variants. Based on amino acid sequence alignments (**Figure 4**) I truncated the last 13 amino acids from hSSB1 and hSSB2.

Aims

- Creating hSSB1 and hSSB2 variants that do not contain the CTP (hSSB1-dC and hSSB2-dC) by molecular cloning, with subsequent protein expression and purification
- Examine their ability to undergo LLPS compared to their wild type variants using epifluorescence and DIC microscopy
- Measure their ssDNA-binding affinity by fluorescence anisotropy

Materials and Methods

Determining the sequence of the CTP hSSB-dC variants

I started by defining the CTP for both hSSB1 and hSSB2. I performed amino acid sequence alignments with ClustalW in the Unipro UGENE software v33.0, comparing the SSB proteins of various vertebrates to each other as seen in **Figure 4**. I used the SSB sequences of *Danio rerio*, *Mus musculus*, *Rattus norvegicus*, and *Homo sapiens*, as well as the SSB1 sequence of *Xenopus laevis*. I also performed sequence alignments between hSSB1 and hSSB2 with the same method. The alignments showed a distinct conserved CTP region of 13 amino acids in both proteins. VSNGKETRRSSKR in hSSB1 and ISNGRDPRRAFKR in hSSB2.

Molecular cloning

First, I amplified my inserts encoding hSSB1-dC and hSSB2-dC with a C-terminal Histidine-tag from the WT sequence encoded in pET29a plasmids by PCR. The used primers are shown in **Figure 5A**. KOD polymerase was used with the reported primers. After 3 minutes of initial denaturation at 95 °C, thermal cycling was performed as follows: 95 °C for 30 seconds, 55 °C for 30 seconds, 70 °C for 25 seconds in 30 cycles. Final elongation was achieved by 7 minutes at 70 °C. All PCR reactions were carried out on a BLUE-RAY Turbocycler PCR device. The PCR products were purified with a QUIAGEN QIAquick PCR Purification Kit. pET29a vectors were used for both hSSB1-dC and hSSB2-dC, which contains the antibiotic resistance gene for kanamycin. Both the inserts and vector were digested with restriction enzymes NdeI and XhoI. The digested vector was purified with a QUIAGEN QIAquick Gel Extraction Kit, and the digested inserts were purified with a QUIAGEN QIAquick PCR Purification Kit, with subsequent ligation by T4 ligase. Ligation reactions were transformed into DH5α *E. coli* cells. I performed a colony screen PCR with the DreamTaq polymerase with vector specific forward primer (annealing to T7 promoter) and insert specific reverse primer (**Figure 5A**). After 3 minutes of initial denaturation at 95 °C, thermal cycling was performed as follows: 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 1 minute in 30 cycles. Final elongation was achieved by 7 minutes at 72 °C. The products were analysed by agarose gel electrophoresis. The colonies where the ligation was successful were inoculated into 5 ml LB (Luria-Bertani media) containing 30 µg/ml kanamycin and were left overnight in a shaking incubator at 37 °C. On the next day, the plasmids were purified with a QIAprep Spin miniprep kit and were sent for sequencing.

A

pET29a-hSSB1-dC forward	5'	gaaggagatatatcatgacgacggagacctttgtgaaggatc	3'
pET29a-hSSB1-dC reverse	5'	ctcgagtcaatggatggtgatggtaggggtgctggaagggccagg	3'
pET29a-hSSB2-dC forward	5'	ctttaagaaggagatatatgaatagggtcaacgacccac	3'
pET29a-hSSB2-dC reverse	5'	ctcgagtcaatggatggtgatgggtgtgtggatcactgtttgattac	3'
Nde1 restriction site, START codon, Xho1 restriction site, STOP codon, Histidine-tag			



Figure 5 – The primers used for KOD PCR and the domain structures of the used protein variants. (A) These primers were designed to amplify hSSB1-dC and hSSB2-dC with a C-terminal Histidine-tag used for subsequent protein purification steps. Restriction sites, START and STOP codons and the His-tag were colour-coded as seen in the table above. (B) The figure illustrates the domain composition of each protein, with the position of the amino acids at the start and end of the domains. The Histidine-tag is referred to as His6.

Protein expression and purification

The plasmids encoding hSSB1-dC and hSSB2-dC (**Figure 5B**) were transformed into E. coli Rosetta 2 cells. Starter cultures were grown overnight in 6 ml LB supplemented with 30 µg/ml kanamycin and 30 µg/ml chloramphenicol on 37°C. Then 6 x 1 ml starter culture was

transferred into 6 x 1 l LB supplemented with 30 µg/ml kanamycin and 30 µg/ml chloramphenicol. Cells were grown on 37 °C with continuous shaking until OD₆₀₀ = 0,6. At this point, 1 mM IPTG was added to each culture to induce protein expression. The temperature was lowered to 18 °C, and the cells were left in the shaking incubator overnight. Next, the cells were harvested by centrifugation at 4000 rpm and resuspended in buffer A (25 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 0,1% TWEEN 20) supplemented with Protease Inhibitor Tablets (Pierce). Cells were lysated by sonication and cell debris were collected by centrifugation on 18000 rpm for 30 minutes. I loaded the supernatant onto a 5 ml Ni-NTA column (1 ml/min), which I equilibrated with buffer A. From here, the preparation of the two proteins differs in some steps, as different buffer solutions were used.

For hSSB1-dC, the column was washed with buffer B1 (25 mM Tris-HCl pH 8.0, 50 mM NaCl, 10% glycerol, 0,1% TWEEN 20, 50 mM imidazole) at 3 ml/min. Then, it was eluted by a gradient elution, 1 ml/min, increasing 2% per minute, going from buffer B1 to buffer B2 (25 mM Tris-HCl pH 8.0, 50 mM NaCl, 10% glycerol, 0,1% TWEEN 20, 250 mM imidazole). The elution peak was collected and loaded onto a 5 ml Hi-Trap heparin column, which was equilibrated with buffer HP1 (25mM Tris-HCl pH 8.0, 50 mM NaCl, 10% glycerol). The utilization of the heparin column is common in the preparation of DNA-binding proteins, as its structure is highly similar to the backbone of DNA. By this method, DNA-binding proteins can be captured easily, as they bind to the polysaccharide chain of heparin. I eluted the protein with a gradient elution, from buffer HP1 to HP2 (25 mM Tris-HCl pH 8.0, 1 M NaCl, 10% glycerol) at 1 ml/min, increasing 2% per minute.

For hSSB2-dC, the column was washed with a modified buffer B1 (500 mM NaCl instead of 50 mM). For purification I used an alternative buffer B2 (25 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 0,1% TWEEN 20, 1 M imidazole). I performed gradient elution going from buffer B1 to B2 at 1ml/min increasing 2% per minute and pooled the fractions containing the protein. In case of hSSB2-dC, no heparin chromatography was performed as hSSB2 irreversibly precipitates on heparin column³⁵.

The purified samples were dialyzed against storage buffer (for hSSB1-dC: 25mM Tris-HCl pH 8.0, 50 mM KCl, 10% glycerol, 100 mM MgCl₂, 1mM DTT; for hSSB2-dC, I replaced Tris-HCl with 25 mM HEPES pH 7.0) overnight at room temperature. Then I concentrated the proteins by centrifugation on an Amicon Ultra 10kDa column. Proteins were frozen and stored in liquid N₂.

An AKTA-START system was used for chromatography. Purification protocol was based on G. M. Harami et al.³⁴ and Z. J. Kovács et al³⁵. Protein concentration was measured by the Bradford-

method. SDS-PAGE was used to examine the purity of samples. Purified hSSB1 and hSSB2 WT (**Figure 5B**) were already available to use in the laboratory.

Epifluorescence microscopy

Examining LLPS in an epifluorescence microscope is a commonly used technique^{22,34,35}. For my measurements I used a Nikon Eclipse Ti-E TIRF microscope in epifluorescence mode with an apo TIRF 100x oil immersion objective (numerical aperture (NA) = 1.49). For excitation, I used a 543-nm laser (25-LGP-193–230, Melles Griot), fluorescence was deflected to a ZT405/488/561/640rpc BS dichroic mirror, and it was recorded by a Zyla sCMOS (ANDOR) camera. I captured the images with NIS-Elements AR (Advanced Research) imaging software. 20 µl of sample was loaded into wells of µ-Slides at 25°C. 100 nM Cy3-dT₇₉ (79-mer deoxythymidine homopolymer labelled with Cy3 fluorophore) was used for the detection of nucleoprotein droplets. Experiment contained 2 µM dT₇₉ (unlabelled) and 100 nM Cy3-dT₇₉, unless otherwise indicated. Experiments without molecular crowder contained 25 mM Tris-HCl pH 7.4, 10 mM MgCl₂ and 50 mM KCl.

In experiments with molecular crowders present, 25 mM Tris-HCl pH 7.4 was applied with 5% PEG₂₀₀₀₀ and no additional salts. The oxidizing agent was 1 mM H₂O₂, and the reducing agent was 1 mM DTT.

DIC microscopy

DIC microscopy is suitable for the visualization of condensates in the absence of fluorescently labelled reporters. DIC microscopy was carried out with the help of Dr. Tibor Kovács from ELTE TTK, Department of Genetics. Reaction conditions were the same as described above. A Zeiss Axioimager M2 microscope was used with a Plan-APOCHROMAT 20x 0,8 NA objective, equipped with ApoTome2. The software used for capturing images was Zeiss Zen Blue lite.

Image processing

Images were processed with the software ImageJ. A representative image was selected from each experiment. Image stack was generated from raw images, so they can be edited identically. A 600x600 px square was cut from the centre of epifluorescence images as the illumination of the laser was the most even in this area. A 500x500 px square was cut from DIC images. The stack was converted into a montage. The background has been evenly subtracted from all epifluorescence images with the rolling ball method.

Fluorescence anisotropy measurements

I utilized fluorescence anisotropy titration to measure the ssDNA-binding affinity of the proteins. The reaction was carried out in FP buffer (25 mM Tris-HCl pH 7.4, 50 mM KCl, 1 mM DTT) with 10 nM ssDNA (ss36: ATTTTTCGGATGGCTTAGAGCTTAATTGCGCAACG-fluorescein) as ligand. 12 μ l samples were loaded in a 384-well low-volume nontransparent microplate (Greiner) at 25 °C and measured on a BioTek Synergy H4 Hybrid Multi-Mode Microplate Reader, using the software BioTek Gen5. Fluorescence anisotropy was measured, which was later converted into anisotropy values. For the evaluation of the results, I used Origin software. Datapoints were fitted with the Hill equation.

Results

CTP truncation results in the loss of LLPS propensity for hSSB1

To examine the effect of the CTP truncation on LLPS propensity, I examined hSSB1-dC by epifluorescence microscopy using 5 μ M of protein in the presence of fluorescently labelled ssDNA (Figure 6A).

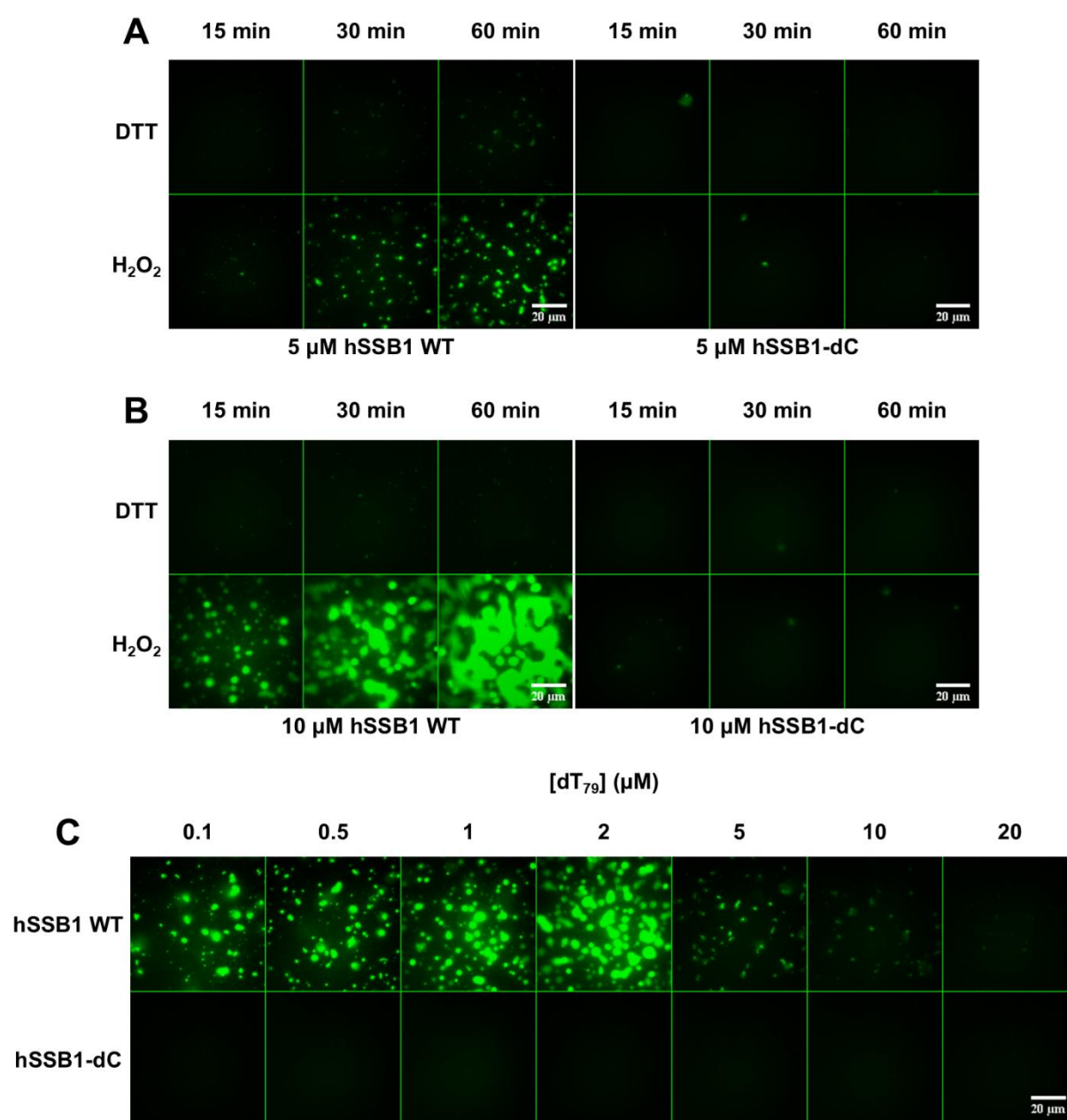


Figure 6 – hSSB1-dC does not undergo phase separation. (A) hSSB1 WT can only undergo LLPS in an oxidative environment, while hSSB1-dC is unable to form droplets in either condition, independently of protein concentration. Timelapse epifluorescence microscopy images of 5 μ M (A) or 10 μ M (B) protein condensates in the presence of 2 μ M dT₇₉ containing

0.1 μ M Cy3-dT₇₉. (C) hSSB1-dC is unable to undergo LLPS independently of ssDNA concentration. 5 μ M proteins are shown in the presence of indicated concentrations of dT₇₉ containing 0.1 μ M Cy3-dT₇₉ and 1 mM H₂O₂.

I captured timelapse images after 15, 30 and 60 minutes of incubation time. While the wild protein showed progressive droplet formation under oxidative conditions, no droplet formation was observed for hSSB1-dC. Measuring droplet formation on higher protein concentrations (10 μ M) gave similar results (**Figure 6B**). These data indicate that CTP truncation significantly reduces the LLPS propensity of the protein.

Since the deletion of CTP may also influence the ssDNA-binding stoichiometry, I examined droplet formation of hSSB1-dC under a series of different ssDNA concentrations and observed no LLPS condensates (**Figure 6C**).

hSSB1-dC's LLPS propensity is restored in the presence of molecular crowder

The intracellular crowding effect brought about by the protein-rich cytoplasmic or nucleolar space is usually considered a driving force of LLPS⁴¹. This phenomenon alters the properties of macromolecules in a highly concentrated solution, for instance it can influence protein folding, solubility and the affinity of interactions⁴². Crowder molecules could be proteins (e.g, BSA), nucleic acids or polysaccharides⁴² (e.g. dextran), that are present in the intracellular space or synthetic polymers (polyethylene glycol) that mimic the crowding effect of biomolecules. In a solution these molecules reduce available solvent, which mostly affects larger molecules. It also affects the association rate of different proteins^{42,43}, along with protein-DNA interactions^{42,44}. Therefore, I tested whether hSSB1-dC is able to undergo LLPS in the presence of 5% PEG₂₀₀₀₀, which is generally used as a crowding agent²². This experiment was carried out to test whether the structure of hSSB1-dC is suitable enough for LLPS transition in an assistive environment. I captured similar timelapse images as before and found that hSSB1-dC does undergo LLPS in the presence of molecular crowders (**Figure 7**). It is worth noting that the presence of PEG also influenced the redox dependence of LLPS, as the WT protein was able to undergo LLPS even in reducing conditions, although the effect of oxidation is still observable. This suggests, that molecular crowders may also regulate the redox sensitivity of hSSB1 condensates. Nevertheless, no redox-dependence is seen in case of hSSB1-dC. Taken altogether, we can conclude that the presence of a crowding effect restores the LLPS propensity of hSSB1-dC.

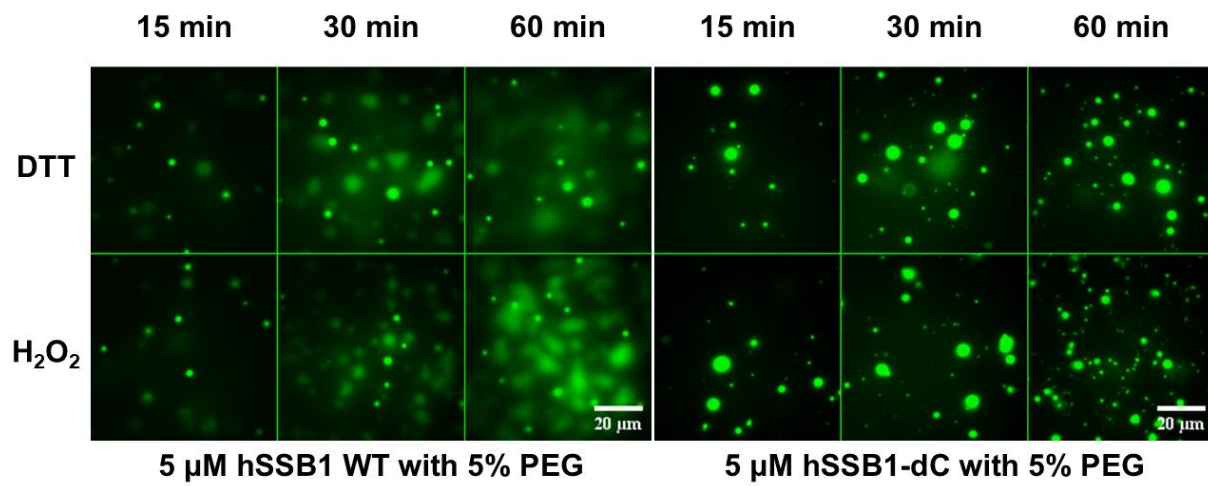


Figure 7 – *hSSB1-dC* is able to form droplets in the presence of *PEG*₂₀₀₀₀. Epifluorescence microscopy images of protein condensates in the presence of 2 μM dT79 containing 0.1 μM Cy3-dT79.

hSSB2-dC aggregates

In the case of hSSB2-dC, I examined LLPS under several protein concentrations with epifluorescence microscopy. Compared to hSSB2 WT that showed droplet like condensates under a wide range of redox conditions, hSSB2-dC was poorly soluble and formed amorphous, solid-like aggregates of different sizes in all conditions (**Figure 8A-B**). There was no visual evidence of spherical droplets, that is typical for LLPS condensates.

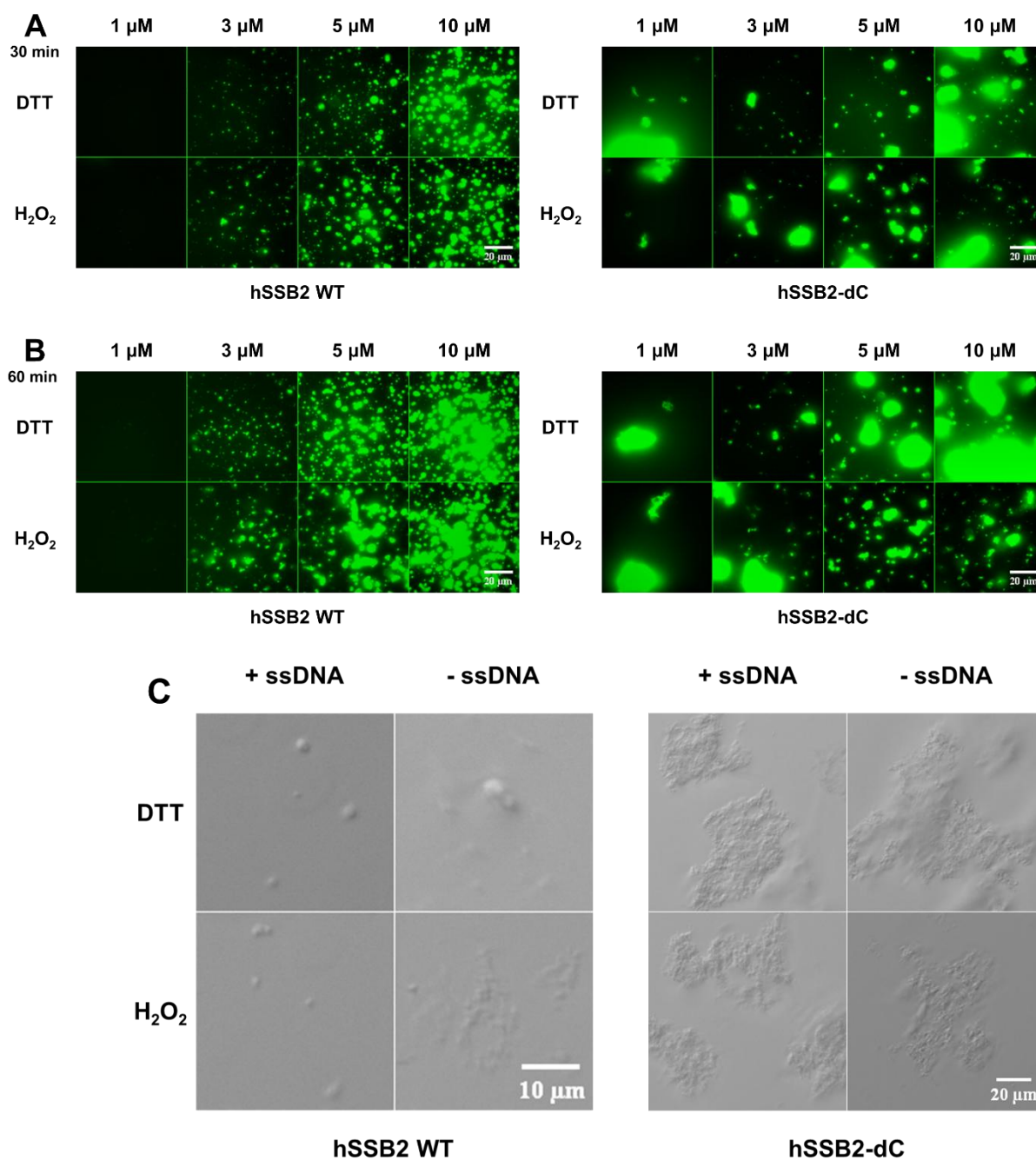


Figure 8 – hSSB2-dC aggregates in all examined conditions. Epifluorescence microscopy images of protein condensates in the presence of 2 μM dT79 containing 0.1 μM Cy3-dT79 after

(A) 30 and (B) 60 minutes incubation. (C) DIC microscopy images of indicated proteins in the presence or absence of ssDNA after a 60-minute incubation in the same experimental conditions as in panel A-B.

I performed experiments using DIC microscopy, in order to examine hSSB2-dC in the absence of ssDNA (**Figure 8C**). I detected hSSB2-dC aggregates even when there was no ssDNA present, indicating that the solubility and stability of hSSB2 is significantly decreased upon CTP truncation and aggregation is not induced by ssDNA. Interestingly, the WT protein also formed small amorphous structures on its own, but those completely disappeared upon the addition of ssDNA and spherical droplets were observed.

Molecular crowders do not restore hSSB2-dC's LLPS propensity

The aforementioned molecular crowding effect was tested in the case of hSSB2 as well. The images show, that molecular crowders do not restore the protein's ability to undergo LLPS (**Figure 9**). The appearance of the observed structures are still less spherical. Fusion events, as seen in the case of the WT protein, were not observed. Taken altogether, we can conclude that CTP truncation greatly destabilizes protein structure, that results in decreased solubility and the complete loss of LLPS propensity.

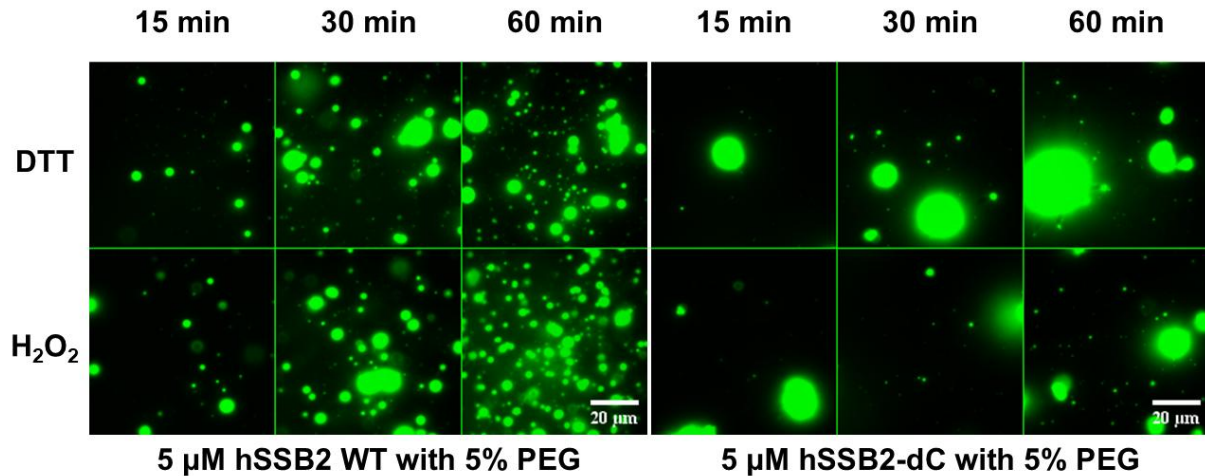


Figure 9 – hSSB2-dC's LLPS propensity is not restored by 5% PEG₂₀₀₀₀. Epifluorescence microscopy images of protein condensates in the presence of 2 μ M dT79 containing 0.1 μ M Cy3-dT79.

CTP of hSSB1 is required for strong ssDNA-binding

To investigate whether CTP truncation affects the DNA-binding of hSSB1, I utilized fluorescent anisotropy measurements with a fluorescently labelled ssDNA as substrate. I compared the DNA-binding affinities of the WT and dC variants and concluded that CTP truncation resulted in significantly weaker DNA-binding (**Figure 10**). This finding indicates that the CTP does not only contribute to the protein condensation, but it is involved in DNA-binding as well.

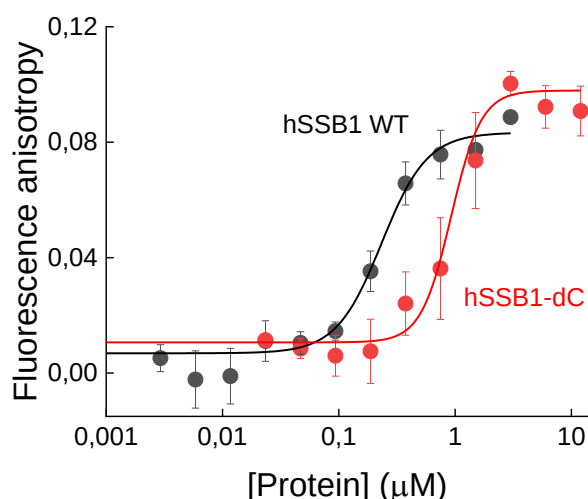


Figure 10 – The ssDNA-binding of hSSB1-dC is reduced compared to the WT. Means and SEM are reported. Data were fitted with the Hill-equation. WT: $K_d = 0,23 \pm 0,04 \mu M$, $n = 2,23 \pm 0,73$; dC: $K_d = 0,92 \pm 0,19 \mu M$, $n = 3,35 \pm 1,62$.

Discussion

The OB domain and the IDR are well known structural elements of both hSSB proteins. The comparison of vertebrate SSBs reveals that the C-terminus of the proteins, mostly comprised of basic amino acids is conserved (**Figure 4**). The acidic CTP of EcSSB is also highly conserved among bacterial SSBs, and it is responsible for interacting with the DNA-binding site on the OB-domain and with several DNA-maintenance proteins^{20,22,25}. OB-CTP interactions are important drivers of LLPS, as it facilitates the scaffolding and the entanglement of the IDRs²². ssDNA inhibits EcSSB condensation by competing with CTP-OB interactions, abolishing the scaffolding effect²².

In contrast, the presence of ssDNA is needed for hSSB LLPS, where the binding of hSSB molecules to the same ssDNA strand increases IDR-IDR interactions and therefore LLPS³⁴. In case of hSSB proteins, the scaffolding effect is provided by DNA-binding. The hSSB CTP cannot bind to the positively charged DNA-binding site on the OB-domain since the CTP is also positively charged, but it could influence DNA-binding in order to increase scaffolding and LLPS propensity.

Based on my results we can conclude that the hSSB CTP is an important regulator of protein function. CTP truncation results in significant changes in the solubility and LLPS propensity of the proteins. hSSB1-dC and hSSB2-dC are unable to undergo LLPS in the presence of ssDNA. Moreover, hSSB2-dC forms aggregates, further indicating that the CTP is an important factor in protein stability. Molecular crowders are able to restore the LLPS-propensity of hSSB1-dC, but not hSSB2-dC. I also found that CTP is involved in DNA-binding. Truncation of CTP decreased ssDNA-binding affinity to a similar extent when the whole IDR was deleted³⁴, indicating that the CTP is the region that is involved in ssDNA binding and not the whole of the IDR. Based on my results we concluded that CTP enhances the scaffolding effect of DNA-binding, and therefore the proteins' LLPS propensity.

Furthermore, it is still of question whether hSSB CTP is also involved in protein-protein interactions as well, similarly to EcSSB CTP. In the future, I plan to investigate whether CTP can bind to known interaction partners of hSSB.

hSSB1 is a key player in DNA-repair mechanisms and is expressed in all tissues³⁸. Its expression is highly upregulated in cancerous tissues, suggesting that its function in DNA-repair and its LLPS propensity is advantageous for cancerous cells^{6,45}. Learning to influence LLPS in cells is a highly researched topic currently⁵ and our results about hSSB1 LLPS may

contribute to clinical research efforts increasing the effectiveness of treatments used against cancerous tumours in chemotherapy.

Author's Contribution

- I created the constructs of the hSSB1-dC and hSSB2-dC variants by molecular cloning, then I expressed and purified the proteins.
- I used epifluorescence and DIC microscopy to examine the proteins' LLPS propensity.
- I performed fluorescence anisotropy measurements.

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