

Establishing knockout cell lines to examine the cellular function of human single-stranded DNA binding proteins

OTDK work

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Abbreviations

8-oxoG	-	8-oxo-7,8-dihydroguanine
ATM	-	ataxia telangiectasia mutated
BER	-	base excision repair
CRISPR	-	clustered regularly interspaced short palindromic repeats
DSB	-	double-strand break
HDR	-	homology-directed repair
hOGG1	-	oxo-guanine glycosylase 1
HR	-	homologous recombination
IDR	-	intrinsically disordered region
KO	-	knockout
LLPS	-	liquid-liquid phase separation
mtDNA	-	mitochondrial DNA
NER	-	nucleotide excision repair
NHEJ	-	non-homologous end joining
OB	-	oligosaccharide/oligonucleotide-binding
PAM	-	protospacer adjacent motif
ROS	-	reactive oxygen species
RPA	-	replication protein A
sdDNA	-	double-stranded DNA
SG	-	stress granule
SSB	-	single-stranded DNA-binding protein
ssDNA	-	single-stranded DNA

Introduction

DNA requires constant protection from endogenous and exogenous threats to preserve its integrity and ensure proper cellular function. Failure to prevent or repair DNA damage leads to mutations that can disrupt cellular functions and contribute to tumorigenesis. DNA unwinds from double-stranded to single-stranded form (ssDNA) during replication and transcription, making it more vulnerable to damage (Marceau et al., 2012). ssDNA is particularly susceptible to nucleolytic damage and prone to form secondary structures that may stall replication or transcription (Marceau et al., 2012). A key protective mechanism involves specific single-stranded DNA-binding proteins (SSBs) that form a coat around ssDNA (Olson & Wuttke, 2024). These proteins protect ssDNA from secondary structure formation and decrease the DNA exposure to enzymatic activity while prevent premature re-annealing, ensuring sufficient time for accurate duplication of the template strand (Xu et al., 2023).

SSBs are essential for protecting ssDNA during replication, transcription, and particularly DNA repair, which is critical for genomic stability. The cell cycle is tightly regulated by checkpoints at the G1, G2/M, and metaphase stages, ensuring that DNA damage, including DSBs, is detected and repaired before progression. Depending on the damage type and cell cycle phase, repair pathways including HDR, NHEJ, and BER are activated, requiring ssDNA exposure where the involvement of SSBs is essential (Ceccaldi et al., 2016). Thus, SSBs are crucial not only for replication and transcription but also for maintaining genomic stability.

SSBs are present in all living organisms, emphasizing their importance. A key structural feature shared by all SSBs is the oligosaccharide/oligonucleotide-binding (OB) fold domain which is conserved across all kingdoms of life (Marceau et al., 2012) (Xu et al., 2023). The OB-fold domain consists of five anti-parallel β -strands forming a β -barrel structure and an α -helix, responsible for ssDNA or ssRNA binding specificity (Murzin, 1993) (Ren et al., 2014) (Lawson et al., 2019). Aside from DNA binding property, the OB domain facilitates critical interactions including self-oligomerization into dimers and tetramers, as well as complex formation with other proteins (Xu et al., 2023). SSB's activity is tightly regulated by post-translational modifications (like acetylation, SUMOylation, and ubiquitylation), which influence their ssDNA binding capacity, interactions with other proteins, and their lifespan (Olson & Wuttke, 2024).

The most well-known SSB in humans is the heterotrimeric replication protein A (RPA), a complex composed of RPA70, RPA32, and RPA14. It is mostly studied for its direct and indirect involvement in replication and in several DNA repair pathways and recruiting repair proteins to damage sites (Wu et al., 2016). In addition to RPAs, which contain multiple OB-fold domains, there is a group of SSBs that has a simpler structure containing only one OB-fold (Croft et al., 2019). This group includes the mitochondrial SSB (mtSSB/SSBP1), which participates exclusively in the mitochondrial DNA repair. There are also nuclear counterparts of mtSSB with overlapping functions with RPAs in humans, namely hSSB1 (NABP2) and hSSB2 (NABP1). They contain an additional functionally important element, an intrinsically disordered region (IDR) at their C-terminal (Xu et al., 2023). This thesis focuses on human single OB-fold-containing SSBs, and the following section briefly overviews the current knowledge regarding their cellular and molecular functions.

The hSSB1/2 has a diverse role in DNA damage repair

hSSB1 was first described as functioning in homologous recombination (HR), where it is recruited to double-strand break (DSB) sites and facilitate the activation of ATM- dependent checkpoint pathway to initiate DNA repair (Croft et al., 2019) (Richard et al., 2008). The checkpoints are regulated by the ATM and ATR protein kinases, which serve as key controllers of the primary pathways involved in DNA repair (Wu et al., 2016). hSSB1 regulates cell cycle checkpoints via the ATM and p53 pathways by stabilizing p21 and p53 and protecting them from degradation (Wu et al., 2016). hSSB1 can bind to NBS1, the component of the MRN complex along with Mre11 and Rad50 in response to DNA damage and recruits the complex via this direct interaction to the DSB site (Richard et al., 2011), supporting the DNA repair and strand rejoining (Richard et al., 2008). This interaction is promoted through modification on hSSB: SUMOylation of lysin 79/94 and phosphorylation of threonine 117 by ATM kinase. The SUMOylation of hSSB1 enhancing the NBS1 and subsequently the ATM recruitment to the damage site while the phosphorylation augments the hSSB stability during the process (Richard et al., 2008) (Zhou et al., 2020). hSSB1 is also directly phosphorylated by DNA-PK at serine residue 134 following the disruption of replication forks and promotes cellular survival (Ashton et al., 2017).

The hSSB1 is capable of self-oligomerization into dimers and tetramers following oxidative stress, driven by the formation of disulfide bonds between cysteine residues in its structure. This disulfide bond formation alters hSSB1's conformation, enhancing its affinity for ssDNA and facilitating its interactions. This process is crucial for the repair of 8-oxo-7,8-

dihydroguanine (8-oxoG) lesions through interactions with oxo-guanine glycosylase 1 (hOGG1), an enzyme responsible for recognizing and excising oxidized guanine bases during BER (Vidhyasagar et al., 2016) (Touma et al., 2017) (Paquet et al., 2015). The 8-oxoG is one of the most common damage lesions originating from the formation of reactive oxygen species (ROS). These lesions affect the progression of the replication fork, reducing replication fidelity, or even stalling the polymerase, and subsequent DSB if it is not properly managed (Saxena & Zou, 2022).

Both hSSB1 and hSSB2 form a heterotrimeric complex with INTS3 and C9orf80, known as the SOSS complex, which is involved in DNA maintenance. The hSSB1 and 2 have competitive binding to INTS3, regulating their abundance, as in complex they have increased stability by the preventing proteasomic degradation (Zhang et al., 2023). The complex prevents stalling of DNA polymerase, resolves DNA G-quadruplex structures in ssDNA, and protects G-overhangs at telomere ends (Li et al., 2009) (Gu et al., 2013). The SOSS complex plays a role in double-strand break (DSB) repair by forming phase-separated compartments with RNAPII at damage sites, enabling the recruitment of repair machinery and transcriptional regulation (Long et al., 2023).

In homologous recombination, the SOSS complex facilitates RAD51 recruitment and nucleoprotein filament formation on ssDNA. It also interacts with the MRN complex to promote DSB end resection and ssDNA production (Pálinkás et al., 2024). The complex stabilizes stalled replication forks to prevent nucleolytic degradation and supports replication restart (Harami et al., 2024) (Kovács et al., 2024). Post-translational modifications, including phosphorylation, SUMOylation, and ubiquitylation, regulate the SOSS complex's activity by influencing DNA binding and protein-protein interactions (Banani et al., 2017). Disruption of the SOSS complex has been implicated in genomic instability and telomere-related dysfunction (Alberti & Hyman, 2021).

The hSSB1 known to interact with BLM (Bloom's syndrome helicase) from the RecQ family of DNA helicases, which is also function in genome maintenance by protecting stalled replication forks. hSSB1 and BLM helicase form a complex in cells and the interaction is altered in response to ionising radiation (IR). BLM and hSSB1 also co-localised at nuclear foci following IR-induced double strand breaks and stalled replication forks. hSSB1 depleted cells contain less BLM protein and that this deficiency is due to proteasome mediated degradation of BLM (Croft et al., 2017).

hSSB1 new regulation ability emerging from its cellular localization

Our recent studies have shown that hSSB1/2 can undergo liquid-liquid phase separation (LLPS) under certain conditions (Pálinkás et al., 2024) (Harami et al., 2024) (Kovács et al., 2024). During LLPS, the biomolecules condense into distinct phases, separated by phase boundary, which can be characterized by surface tension. The process is driven by weak, multivalent interactions, and can lead to the organisation of membrane-less compartments within the cells (Banani et al., 2017). The proteins involved in LLPS may have a kind of folded interaction domains or IDRs, which enable dynamic interactions and contribute to the liquid-like behaviour of these condensates (Alberti & Hyman, 2021).

LLPS-based compartmentalization provides a highly dynamic organization and regulation of certain organelles, such as processing bodies (p-bodies), stress granules (SGs), and the nucleolus. SGs form in response to cellular stress, which leads to suspended translation, as various RNA-binding proteins, like G3BP1 and G3BP2, bind untranslated mRNA to organize granules (Protter & Parker, 2016). It contains ribosomal subunits, translation initiation factors, and additional proteins, while the exact composition of SGs depends on the type of stressor, the cell type, and various other factors (Baymiller & Moon, 2023). SGs play roles also in various signaling pathways, ultimately promoting cell survival during acute stress (Protter & Parker, 2016).

In a large-scale interactome study, a direct interaction between hSSB1 and G3BP1, the main organizer of SGs, was demonstrated (Havugimana et al., 2022). Our research group recently showed that hSSB1 is present in SGs under oxidative stress (Harami et al., 2024). From this perspective, the LLPS propensity of hSSB1 may be essential, but this remains to be explored.

Now, our research focuses on the roles of hSSBs and their effects in SGs, including interactions with partner proteins. Recently, we identified a link between hSSB1 and SG formation (Harami et al., 2024), likely through its interaction with G3BP1, a key SG protein (Yang et al., 2020). This suggests a potential regulatory role for hSSBs within SGs through these interactions.

hSSB2 (NABP1/OBFC2A) functional insights

The hSSB2 and hSSB1 primarily differ in their carboxyl-terminal tails, which determine the specific proteins they interact with (Lawson et al., 2020). The hSSB2 has been shown to play a distinct role in the repair of UVB damage causing cyclobutene pyrimidine dimers in the DNA, by requiring nucleotide excision repair (NER) pathway-associated proteins like RPA32 to the damage site, implying its role in separate processes from hSSB1 (Boucher et al., 2021).

The gene OBFC2A (NABP1) which encodes the hSSB2, also produces a non-coding transcript called lncRNA OBFC2A, which plays a regulatory role in cellular stress responses and genomic stability. The expression of OBFC2A is regulated by miRNA response elements (MREs), including four putative MREs for miR17, miR21, miR23b, and miR27b. Ectopic overexpression of miR17 and miR21 has been shown to reduce endogenous OBFC2A (hSSB2) mRNA levels. Notably, downregulation of OBFC2A expression through miR17- and miR21-dependent mechanisms, promoting cell proliferation and genomic instability (Vernin et al., 2014). Oxidative stress has also been shown to regulate lncRNA OBFC2A, further linking its function to cellular stress responses.

Contrasting the essentiality of hSSB1 and 2 in repair and development

Cells depleted of hSSB1 exhibit increased radiosensitivity, defective checkpoint activation, and enhanced genomic instability (Wu et al., 2016). *In vivo* studies showed that depleting hSSB1 in mice leads to perinatal lethality (Feldhahn et al., 2012), while the double knockout of hSSB1 and hSSB2 is lethal in the early embryonic phase. The conditional KO in adult rodents also lead to death by depletion of hematopoietic progenitor cells, which are known for their extremely high proliferative capacity and consequently a high vulnerability to DNA damage (Shi et al., 2017). In accordance, hSSB1-deficient mice showed increased susceptibility to cancerous leukocyte development (Wu et al., 2016).

The hSSB2 is a paralog of hSSB1 and has mostly redundant functions with it. While hSSB1 is expressed in high abundance in all tissues and seems to be essential for survival, hSSB2 is present only in certain cell types and dispensable in tissue development and DNA repair (Feldhahn et al., 2012) (Boucher et al., 2015).

Clinical significance of hSSB1/2 in cancer progression

As mentioned above, the appropriate expression levels of hSSBs are essential for cellular survival; however, their overexpression has been linked to enhanced tumor cell survival. hSSB1 overexpression is correlated with a poor prognosis in non-small cell lung cancer (NSCLC) at both the mRNA and protein levels. Studies have shown that depletion of hSSB1 inhibits cell proliferation in lung cancer cell lines, indicating its critical role in cancer progression (Boucher et al., 2022). Recent research suggests that targeting epigenetically upregulated proteins, like hSSB1, using therapeutic oligonucleotides such as DKLS02 represents a novel approach to treating lung cancer (O'Byrne et al., 2023).

Similarly, the mRNA and protein expression of NABP2 (hSSB2) have been found to be significantly upregulated in hepatocellular carcinoma (HCC), correlating with a poor prognosis. Additionally, NABP2 is closely associated with immune cell infiltration and immune checkpoint regulation in HCC, highlighting its potential as a biomarker and therapeutic target in cancer treatment (Li et al., 2023).

OBFC2A gene has been implicated in variant acute promyelocytic leukemia (APL) cases through its rearrangement with RARA, resulting fusion proteins with retained OB fold (Won et al., 2013). Additionally, lncRNA OBFC2A has been found to mediate apoptosis and autophagy induced by the benzene metabolite 1,4-Benzoquinone (1,4-BQ) through interaction with LAMP2, a glycoprotein essential for lysosomal stability. The research suggests that lncRNA OBFC2A could serve as a biomarker for hematotoxicity caused by benzene exposure (Wang et al., 2023). Emerging evidence positions OBFC2A as a potential prognostic biomarker for triple-negative breast cancer (TNBC) as well (Wu et al., 2021).

The mtSSB is the exclusive for mitochondrial genome maintenance

mtSSB is the only known single-stranded DNA-binding protein involved in mitochondrial DNA (mtDNA) maintenance (Maier et al., 2001). The mtDNA, approximately 16 kb in length and present in high copy numbers in the mitochondrial matrix, is essential for energy production via oxidative phosphorylation (Park et al., 2023). mtSSB is chromosomally encoded and translocated to the mitochondria (Stojković et al., 2016).

Together with Twinkle DNA helicase and DNA polymerase gamma (Pol γ), the mtSSB is a core component of the mitochondrial replisome, a complex essential for mtDNA replication and transcription. The main roles of mtSSB are stabilizing the single-stranded mtDNA and

preventing the collapse of displacement loops (D-loops) while limiting nonspecific RNA primers from being synthesized at incorrect sites (Herbers et al., 2019). D-loops are key intermediate structures during mtDNA replication and transcription, formed when a short segment of mtDNA is displaced to form a single-stranded loop. The formation and maintenance of D-loops are critical for initiating mtDNA replication (Herbers et al., 2019). By interacting with mitochondrial RNA polymerase (POLRMT), mtSSB ensures the synthesis of primers only at designated origins of replication. This precise regulation is essential for the recruitment of Pol γ and the coordinated initiation of replication (Roy et al., 2022). The mtSSB also plays a significant role in the regulation of the mtDNA copy number, ensuring proper replication and inheritance of mitochondrial genomes (Ruhanen et al., 2010).

Absence of mtSSB leads to replication initiation failure and unregulated RNA synthesis, as it restricts primer formation to specific replication origins, maintaining mitochondrial genome stability and fidelity (Jiang et al., 2021). In fruit flies, mtSSB depletion slows DNA synthesis by Pol γ , reducing mtDNA levels (Korhonen et al., 2004), while mtSSB mutant *Drosophila* exhibit impaired respiration, failing to survive metamorphosis (Maier et al., 2001). In mice, mtSSB knockout causes embryonic lethality, and conditional knockout in muscle tissues drastically reduces lifespan due to maldevelopment (Jiang et al., 2021). In humans, SSBP1 mutations lead to severe phenotypes like hearing loss, optic atrophy, retinal degeneration, and kidney issues, with mtSSB malfunction causing mtDNA copy number depletion leading to developmental problems (Gustafson et al., 2019).

Examination of SSBs to get insight into cellular regulation

The single OB-fold SSBs (hSSB1/2 and mtSSB) are crucial for DNA replication, repair, and proper development. hSSB1/2 play significant roles in genome stability, DNA repair, stress response, and phase separation, with hSSB1 essential for survival. mtSSB is critical for mitochondrial genome maintenance, with links to various diseases underscoring its importance in medical research. These proteins are thus central to studies on genome maintenance, stress response, development, and cancer biology.

Our research center of attention revolving around the hSSBs' roles and their effects in SGs, including interactions with partner proteins. Recently, we identified a link between hSSB1 and SG formation (Harami et al., 2024), experimentally confirming its interaction with G3BP1, a key SG protein (Yang et al., 2020). This suggests a potential regulatory role for hSSBs within SGs through these interactions.

Aims

The focus of the present research is to investigate the functions of hSSB1, hSSB2, and mtSSB in stress response at cellular and molecular levels. Generally, to understand the function of a protein-coding gene the first step is examining what phenotypic changes are caused in their absence. For this purpose, we decided to establish and characterise knockout (KO) cell lines for each protein.

Applying CRISPR-Cas9 gene editing technique, we approached to introduce non-sense mutations in both alleles of hSSB1, hSSB2, and mtSSB, respectively, in a diploid human retinal pigment epithelium cell line (RPA1). For further investigation, of the function of hSSB1 in the regulation of stress granule formation, we also targeted for deletion its stress granule-associated interacting partner, the protein G3BP1, which was to serve as a reference for stress granule formation.

I designed a series of guide RNAs for Cas9 to specifically target the genes and cloned them into a plasmid encoding both Cas9 and an eGFP reporter protein. Our goal was to establish single-cell clone-derived cell lines, each containing a single nonsense mutation in the target genes. To achieve this, we sorted eGFP-positive cells using fluorescence-activated cell sorting (FACS). To verify successful cleavage events in the targeted genomic regions, we conducted a screening PCR followed by an endonuclease assay, as described below. Initially, these assays were performed on the eGFP-positive bulk population to assess the efficiency of the guide RNAs and Cas9 in cleaving the targeted DNA sequences. For constructs that showed effective cleavage, we repeated the assays on all single-cell clones generated in parallel. For precise genetic analysis, samples from the most promising clones were sent for sequencing. Finally, we aimed to validate the presence of the nonsense mutations at the protein level using Western blotting and immunocytochemistry techniques.

The resulting, well-characterized mutant cell lines are suitable for functional assays, to further examinations of the role of SSBs in genomic stability and stress response mechanisms. Since there are no reliable knockout cell lines for hSSB1/2 and mtSSB available so far, the present project provides a robust tool for future research in this field.

Methodological background

CRISPR-Cas9 technique

Deletion of a gene is a general approach for investigating its phenotype. CRISPR-Cas9 offers a powerful tool for knocking out a gene, using an RNA-guided nuclease, Cas9, to cleave DNA at a site defined by complementarity between the single guide RNA (sgRNA) and the target region (Ran et al., 2013) (Ryan et al., 2023). The presence of a protospacer adjacent motif (PAM) at the targeted genomic region is also an essential condition for Cas9 to bind to DNA. The sgRNA sequence is designed to begin 20 nucleotides upstream of the PAM, as the Cas9 cleaves approximately 3 nucleotides upstream of the PAM.

This project utilized a pre-designed plasmid encoding Cas9, a reporter eGFP, and the tracrRNA recognition sequence. We designed and cloned specific crRNA sequences into the plasmid to form functional sgRNAs with the tracrRNA, enabling a single-plasmid-based expression system with all components to knock out the targeted gene.

Cas9 cleavage at the target site causes DSB that is repaired by either NHEJ or HR, depending on the cell cycle state (Haapaniemi et al., 2018). Genome editing precision is higher via HR and more efficient in many tumor cell lines; however, non-tumor cells are more likely to use the error-prone NHEJ pathway, which has a higher mutation rate (Haapaniemi et al., 2018). The error-prone NHEJ repair is useful for creating random mutations in the target region, allowing gene knockouts by disrupting protein-coding regions and producing loss-of-function cells (Haapaniemi et al., 2018).

T7 endonuclease assay for validation

Repairing DSBs caused by CRISPR-Cas9 can lead to varied genomic events across cells, making it challenging to identify specific mutations in each clone. Sequencing the target region is thorough but costly and time-intensive for every single-cell clone. To expedite validation of CRISPR-Cas9 edits, we used the T7 endonuclease assay, which detects and cleaves distorted sites in heteroduplex DNA (Sentmanat et al., 2018). In the case of diploid cells, heteroduplexes may form between the amplicon of mutated and wild-type alleles or between differing mutations during PCR, creating distortions that T7 endonuclease can recognize and cleave.

After T7 enzyme treatment, the appearance of DNA fragments shorter than the original PCR product indicates a mutation introduced during DSB repair (Sentmanat et al., 2018). This method provides a rapid way to validate CRISPR-Cas9 editing efficiency in cells. However, further validation is needed, as the assay only detects DNA distortions without detailing the exact mutations; thus, T7-positive samples are sequenced to identify precise genomic changes.

Methods and materials

sgRNA and primer design

The sgRNA sequences are designed by using the Brunello library for G3BP1, Crispor, and Chop-chop sgRNA prediction tools for NABP1, NABP2, and SSBP1. The target sites were chosen based on the efficiency scores and localization of the exons. The experimentally validated sgRNA sequences are shown in *Table 1*. The primers were mainly designed with the help of NCBI primer blast. Due to the highly repetitive DNA sequence of the SSBP1 gene, the primers were adjusted manually. The primers were designed 1:3 from the PAM region, aiming for ~1000bp PCR product. The used primers are in *Table 2*. The sequences were checked with the UCSC Genome Browser BLAT tool to avoid the ones with off-target properties. The primer and sgRNA (converted to cDNA) forward and reverse oligos were ordered from IDT, containing the Bbs1 restriction enzyme cleavage site (CACCG for forward and AAAC for reverse oligos). The exact cleavage design is explained in the Recombinant plasmid construction chapter.

Table 1: Experimentally validated sgRNA sequences (successful targets highlighted with green)

Target name	Location (human hg38 ref.)	sgRNA (presented as cDNA sequence)	PAM sequence
G3BP1 Exon 4	chr5: 151795507-151795526	AGAAAGACAGCAAACACCTG	AGG
G3BP1 Exon 8	chr5: 151790965-151790984*	TACCACACCATCATTTAGCG	TGG
NABP1 Exon 3/1	chr2: 191679106-191679125	GATATTATTCGGTTGACCAG	AGG
NABP1 Exon 3/2	chr2: 192546682-192546743	GTCTGACACTTTATACTGGA	AGG
NABP1 Exon 4/1	chr2: 191679053-191679072	GCAGCATCACTATTTCCGTG	TGG
NABP1 Exon 4/2	chr2: 192548455-192548530	GAACCCAACCCAGATTATCG	AGG
NABP2 Exon 2	chr12: 56224844 -56224863	AGGCTCAGGCAGCATGACGA	CGG
NABP2 Exon 3/1	chr12: 56225437-56225456	CAGCATCAATATCTCTGTCT	GGG
NABP2 Exon 3/2	chr12: 56225489 -56225508	GACATTATCCGGCTCACCAA	AGG
NABP2 Exon 4	chr12: 56225401-56225420	TGAGGTTCCGGACCTGCAAAG	TGG
SSBP1 Exon 3/1	chr7: 141441969-141442029	GAACCCAACCCAGATTATCG	GGG
SSBP1 Exon 3/2	chr7: 141743563-141743582	CTGAATCGTGTGCACTTACT	TGG
SSBP1 Exon 3/3	chr7: 141743571-141743590	TGTGCACTTACTTGGGCGAG	TGG
SSBP1 Exon 4	chr7: 141743957-141743976	ATTGATATGCCACGTCTCTG	AGG

Table 2: The primers used for the PCR amplification (grey indicates primers with aspecific targets)

Primer	Forward	Reverse
G3BP1 Exon 4	GTGGTAGAGCCTGCATTAGATAACTGCC	GGTCTCTGTCCACTTCATATTCCTC
G3BP1 Exon 8	GGGGGATTGGATTCAAATGGAAA	GGGCAAGTGGTGACAGCTGC
NABP1 Exon 3/1	CCTGCCCAAGGTCGCCCCTC	CACTGCACTCCAGCTTGGCG
NABP1 Exon 3/2	TGTTCGGAGTAAACATTGCCAG	CTTCCACTAGGGCTGGTCTC
NABP1 Exon 4/1	CCTGCCCAAGGTCGCCCCTC	CACTGCACTCCAGCTTGGCG
NABP1 Exon 4/2	GCACTTGGATAAACTGGTGGC	CGTTTCCCAAACCTTTCAGAGC
NABP2 Exon 2	CTCTGATCCCGATTGGCTGG	CTGAGTGGAAGAGGCCCG
NABP2 Exon 3/1	GACGACGGAGACCTTTGTGAA	CACCCTATGCTGCCTCCTTG
NABP2 Exon 3/2	CCAGTGGGGTGCCGAGGCTC	ACACCCTATGCTGCCTCCTTGC
NABP2 Exon 4	CCAGTGGGGTGCCGAGGCTC	ACACCCTATGCTGCCTCCTTGC
SSBP1 Exon 3/1	ACTCTCCTTTGGTCTAATGATGGA	CCCGGGAAGACTCAGATAACC
SSBP1 Exon 3/2	ATTCGTGTGCCAGTATGATTGAG	CTTCCCTCTTCAACTACCCCT
SSBP1 Exon 3/3	ATTCGTGTGCCAGTATGATTGAG	CTTCCCTCTTCAACTACCCCT
SSBP1 Exon 4	AGGGGCACAACTATTCAGTC	CACCCTGTTTGCCCTAAACTG

Recombinant plasmid construction

The Cas9pX458-eGFP vector plasmid (provided by Dávid Szüts) was utilized for the insertion of the sgRNA coding oligos (Figure 1). The plasmid contains an ampicillin resistance gene, the coding sequences for Cas9 and eGFP, both driven by a viral CMV promoter, and a U6 promoter, driving the expression of the sgRNA coding sequence. It also has two closely positioned BbsI restriction sites, which, when digested, create sticky ends with mirrored symmetry, preventing spontaneous closing of the plasmid. The digestion removes a 23 bp fragment, which is roughly the same length as the designed sgRNA, allowing the sgRNA to be inserted in place of the excised fragment.

The sgRNA oligos were first diluted to 100µM concentrations. The forward and reverse sgRNAs were annealed and phosphorylated according to the given protocol, and the final inserts were diluted to 10,000×. The vectors were digested, using a mixture containing nuclease-free (MilliQ water, BbsI 10 × (NEB), and 10× CutSmart buffer (NEB), incubating at 37°C for 6 hours. Additional 10× CutSmart buffer was added and treated with CIP (NEB) for 30 min at

37°C to inhibit self-ligation. The enzymes inactivated at 65°C for 20 min. The digested vectors were run on agarose gel and cut out from the gel to isolate from the obliterated fragments. The plasmid purification was done with a gel purification kit (Qiaquick® Gel Extraction Kit).

The purified, digested plasmids were ligated with the sgRNA inserts. The ligation mixture contained the plasmid, the diluted sgRNA sample, T4 ligase buffer, T4 ligase, and MilliQ water. For each sgRNA sample, a separate ligation mixture was created, and a control was also prepared without a sgRNA sample. The ligation mixtures were incubated at overnight 16°C.

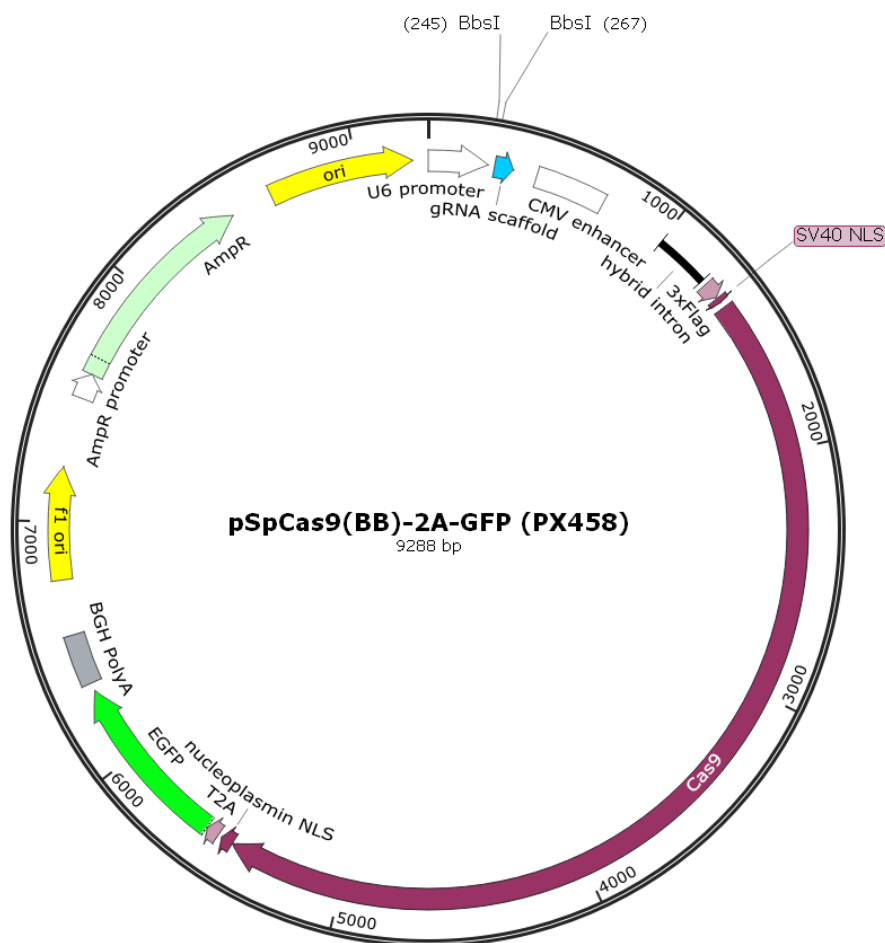


Figure 1: Map of the Cas9pX458-GFP plasmid (Ran et al., 2013).

Plasmid transformation into competent cells

The recombinant DNA plasmids were transfected into *E. coli* DH5- α competent cells. The cells constantly remained cold (on ice) when the plasmids were added to them. After 15 minutes, the cells heat shocked for 50 sec at 42°C, then put back on the ice for 5 minutes. The cells spread on agar plates containing ampicillin for the selection of plasmid-containing/resistant ones and incubated at 37°C overnight.

Inoculation of the colonies and purification for sequencing

After the colonies appeared, 1-1 individual colony from each agar plate was inoculated to tubes filled with 3ml Luria-Bertani (LB) medium containing ampicillin in 1000x dilution. They incubated for ~20h before purification at 37°C. The purification was done by a miniprep protocol using the QIAprep® Spin Miniprep Kit. The DNA eluted with MilliQ water. The final products were sent to sequencing alongside the primers specific to the part of the vector and insert. The sequencing results showed successful insertion of sgRNA sequence into the plasmid.

Sorting and isolation (single-cell cloning) of the cells

After 24 hours post-transfection, the eGFP-positive cells were sorted by FACS with the help of György Várady. Each positive cell was placed individually in a separate well. For this, 96-well plates were prepared, containing prewarmed media. The cells were incubated until they became confluence. After passaging them, part of the cells was used up for knockout validation.

T7 assay for bulk cells and single-cell colonies

The cells were centrifugated for 5 minutes at 5000 rpm, the supernatant was discarded, and then the cells were lysed by adding dilution and release buffer. The forward and reverse primers for each target were previously solved in MilliQ water and diluted to 100 μ M concentrations.

The PCR mixture was prepared to amplify the target sequence from the genome. In PCR tubes, 2x Tissue Master Mix (Thermo Fisher), nuclease-free water, the corresponding forward and reverse primers, and the relevant lysate were added according to the protocol. A PCR mix with wild-type lysate was also used as a control for each primer pair. The sequences were amplified using gradient PCR, optimizing each tube for its primer pair annealing temperature. After amplification, a portion of the PCR products was run on a 1.5% agarose-TMB gel to assess product quality.

According to the protocol, the T7E1 digestion mixture was prepared by combining Neb2 buffer (NEB), T7E1 enzyme (NEB), and MQ water with each PCR product. The mixtures were incubated for 5 minutes at room temperature, followed by 30 minutes at 30°C. After incubation, the mixtures were cooled on ice and directly loaded onto a 1.5% agarose-TMB gel. On the gel, a single band for the PCR product means no mutation occurred, so the knockout was unsuccessful. If three bands appear, it indicates a successful knockout, as the T7 endonuclease cuts the mutated sequence into two shorter fragments, producing three visible bands.

Western blot for protein level validation

The lysed cells were added to Laemmli buffer (Bio-Rad) and distilled water mixture, run in SDS-PAGE (Mini-Protean Precast Gels 4-20%) for an hour at 100V, and were blotted for an hour at 100V. The samples were checked with Ponceau dye, washed with TBST buffer for 3×10 minutes, and blocked with 5% BSA in TBST-Na-azide for an hour. The samples were then treated with the primer antibodies (α -G3BP1 (Abcam; ab56574), α -hSSB1 (Sigma-Aldrich; HPA044615), α -hSSB2 (Sigma-Aldrich; HPA054978)) and incubated overnight at 4°C. For control, α -GAPDH (Sigma-Aldrich; G9545) was used. The samples were washed for 3×10 minutes with TBST buffer, incubated with a secondary antibody (α -rabbit/mouse IgG, conjugated with HRP $\frac{1}{2}$) for an hour, and then washed again for 3×10 minutes with TBST buffer. The results were retrieved with GelDoc using Immobilon Crescendo Western Blot HRP substrate.

Cell culturing

Retinal pigment epithelium (RPE1) cells were cultured in DMEM/F-12 medium, completed by 10% FBS, 1% penicillin-streptomycin, and 0.01% hygromycin. For passage, we applied TrypLE Express enzyme. Following enzymatic detachment cells were pelleted by centrifugation at 1100 rpm for 5 minutes. For long-term storage cells were frozen in 10% DMSO-containing culture medium. They were initially frozen at -80°C, but after 24h transferred to liquid nitrogen.

Immunocytochemistry

After the growth medium was removed and rinsed with PBS, the cell was fixed with 4% paraformaldehyde (dissolved in DPBS) for 15 minutes and washed with DPBS 3 times for 10 minutes. The cells were then permeabilized with 0.1% Triton X-100 in DPBS for 10 minutes at RT, then washed 3 times with PBS. The samples were then blocked with PBS-5% BSA for 1

hour to reduce unspecific binding. The primary antibodies (same used for western blot) diluted to optimal concentrations (hSSB1 1:750, G3BP1 1:500) and incubated with the cells overnight at 10°C. The cells then washed with DPBS (3 times for 5 minutes). The secondary antibodies (α -mouse/rabbit IgG, conjugated with Alexa488 and Alexa568 fluorophores respectively) were diluted in 2.5% BSA-DPBS and incubated with the cells for 2 hours, protected from light. The nuclei were stained by Hoechst, then samples were washed with DPBS 3 times. For examination Zeiss LSM 710 confocal microscope was used.

Overview of experiment workflow

The experimental workflow is summarized in *Figure 2*. After selecting and inserting the designed sgRNA coding sequence into the plasmid, RPE1 cells were transfected via nucleoporation and sorted based on eGFP signal. For the selected single-cell clone populations, a T7 assay was conducted to identify potential successful knockouts. T7-positive cells were then sequenced and further validated through Western blotting and fluorescence imaging.

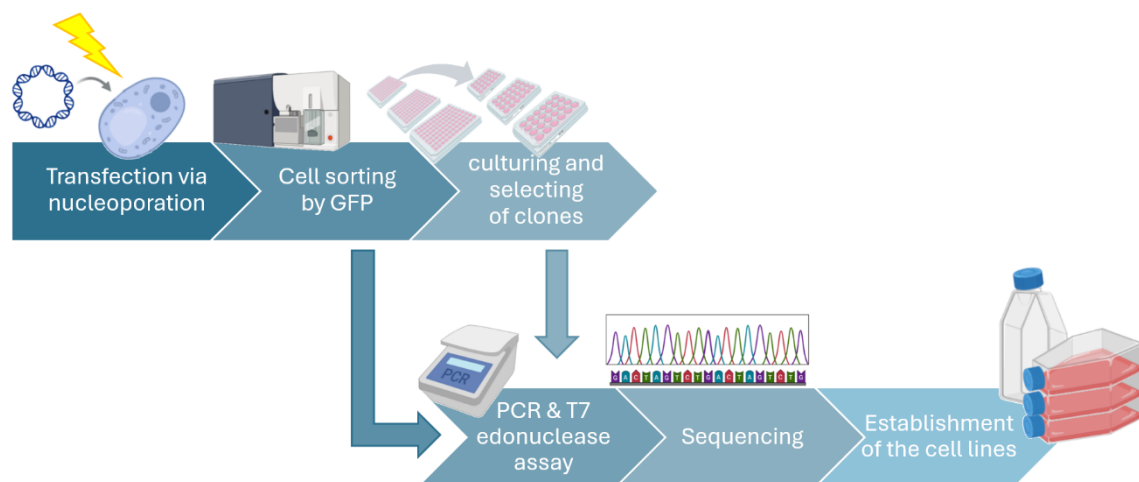


Figure 2: Applied workflow for KO cell line generation.

Results

Confirming mutation event: T7 endonuclease assay for mixed/bulk cell population

A day after nucleoporation, eGFP-positive cell bulks (not sorted into single wells) were lysed for a T7 assay. Each bulk represented a transfection with a specific sgRNA. The goal was to assess sgRNA performance and confirm Cas9 cleavage by detecting mutations near the target site. The ~1000 bp PCR products were cleaved by T7 nuclease at mismatches, producing three bands on the gel: the first band represents the original sequence, while the two additional bands indicate successful cleavage, suggesting a mutation. The bands appear at distinct sizes, as the primers amplify a mutation-containing sequence approximately 1/3 from one end, clearly showing cleavage.

There was successful mutation of G3BP1 exon 4, but not in G3BP1 exon 8, NABP2 (exon3/1, exon4/1), and NABP1 (exon3/1, exon4/2) in the first session (*Figure 3/A*). The G3BP1 exon 4 showed 2 additional bands representing the shorter sequences cut by the enzyme, but not in the control. For G3BP1 exon 8, both transfected and control cells showed mutation presence, likely due to a mismatch during PCR amplification. In hSSB1 and hSSB2 samples, all lines, including controls, showed an unspecific band, possibly from primer binding to an off-target genomic region.

The T7 was positive for the NABP2 exon 2, but not for NABP2 exon 3/2 and NABP1 exon 3/2 in the second session (*Figure 3/B*). The NABP2 exon3/2 was transfected again because the negative result originated from the application of an empty vector. In the third session, each transfection was successful, both NABP1 exon 3/2 and NABP1 exon 4/1 were T7 positive (*Figure 3/C*).

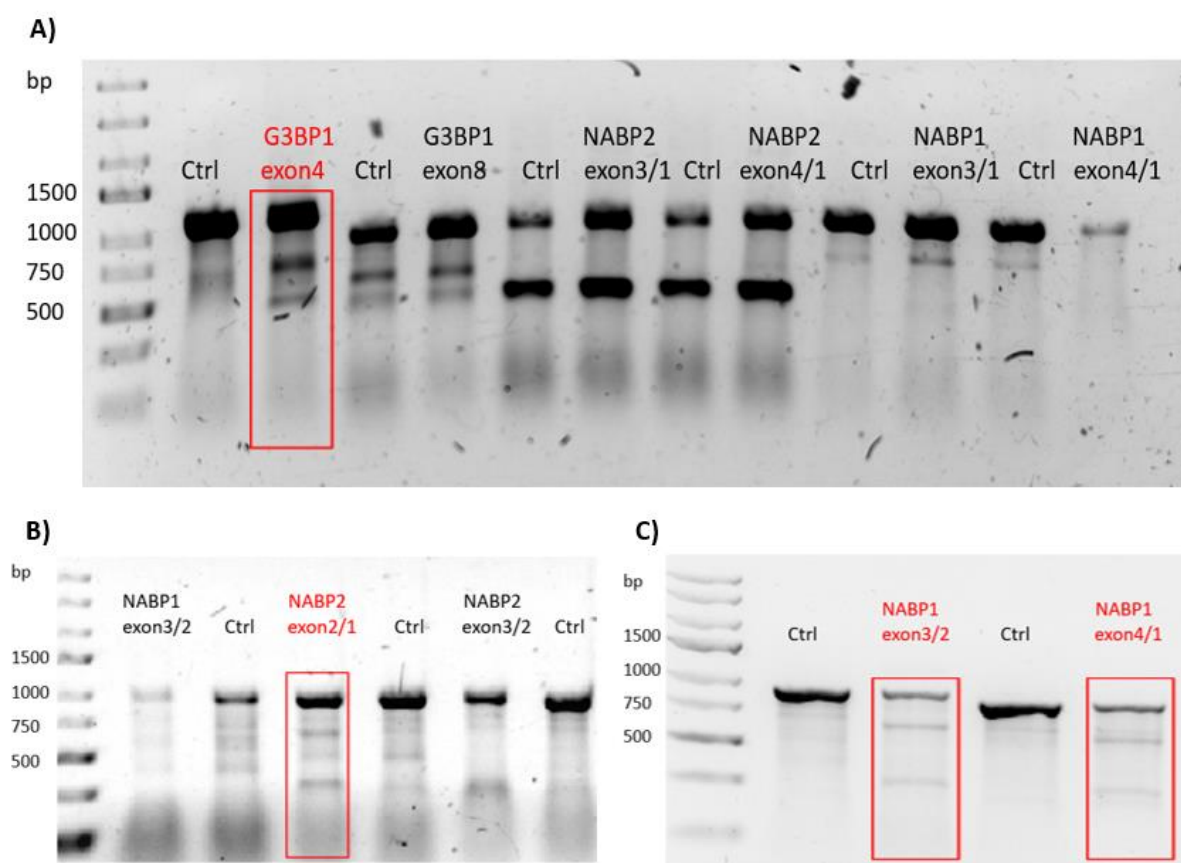


Figure 3: The T7 assay results for the bulk cells. G3BP1 exon 4, NABP2 exon 2, NABP1 exon 3/2, and NABP1 exon 4/1 were T7 positive (highlighted in red).

Confirming mutation event: T7 endonuclease assay for single-cell colonies

The sorted single-cell colonies, previously identified as T7-positive bulk populations, were tested individually to find mutated cell lines with the same genomic alterations. From the G3BP1 exon 4-mutated population, 24 cell lines were tested, with 8 positives in the T7 assay (Figure 4). In the NABP2 exon 2 population, 24 lines were tested, yielding 7 positives (Figure 5). For NABP1 exon 3/2, 13 lines were tested, with 6 showing T7 positivity (Figure 6/A). Lastly, in the NABP1 exon 4/1 group, 24 colonies were tested, and 7 were T7 positive (Figure 6/B).

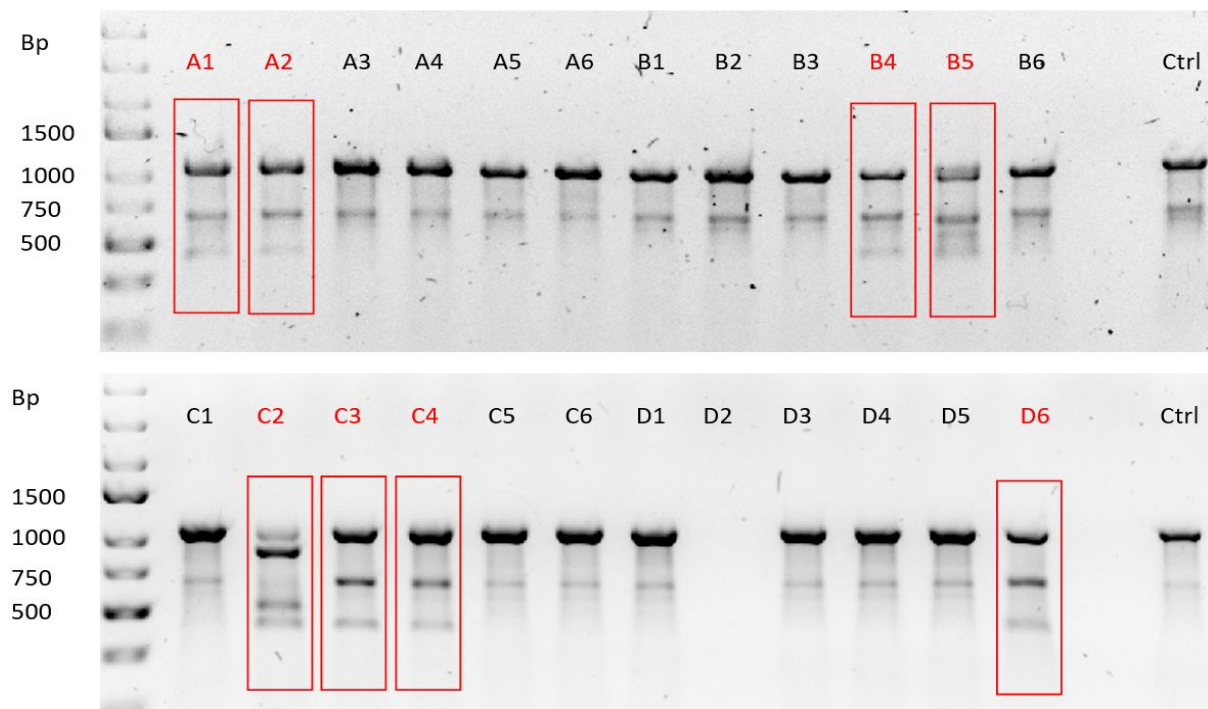


Figure 4: T7 assay results for single cell clones of G3BP1 knockout experiment. The G3BP1 exon 4 mutated cell lines showing positive results are highlighted in red.

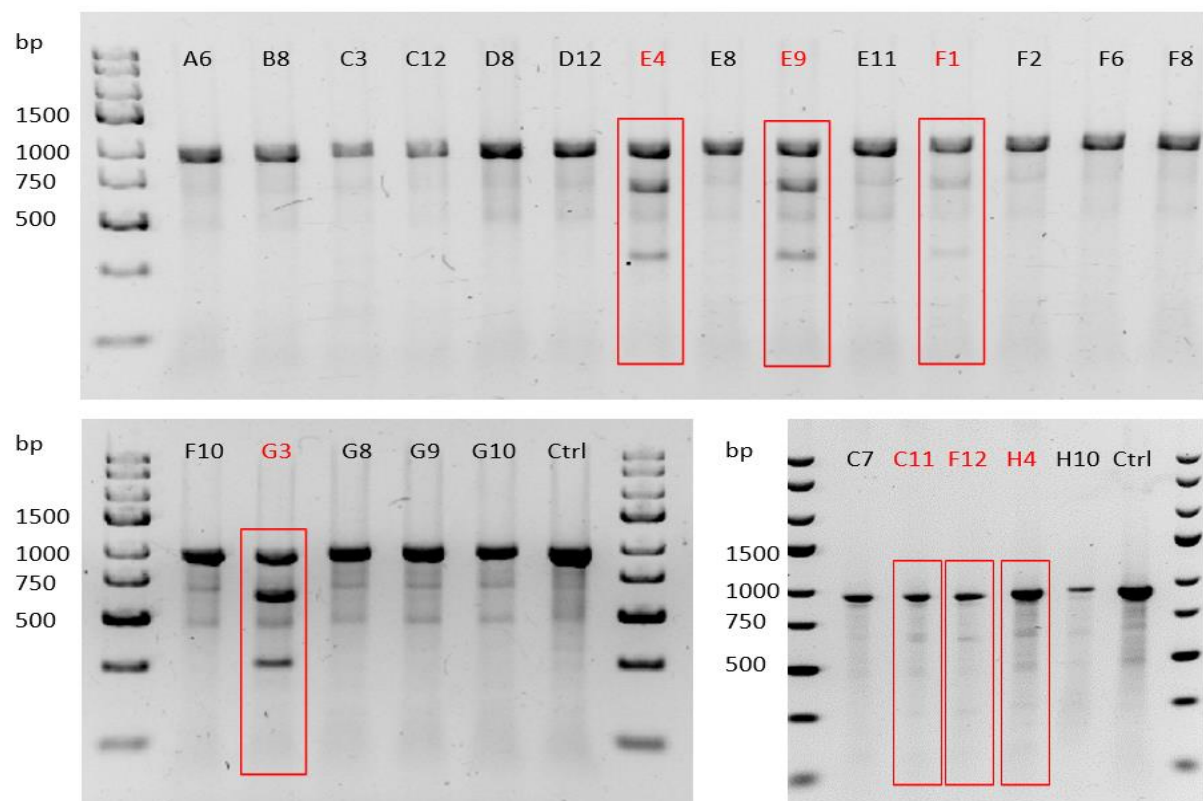


Figure 5: T7 assay results for single cell clones of NABP2 knockout experiment. The NABP2 exon 2 mutated cell lines showing positive results are highlighted in red.

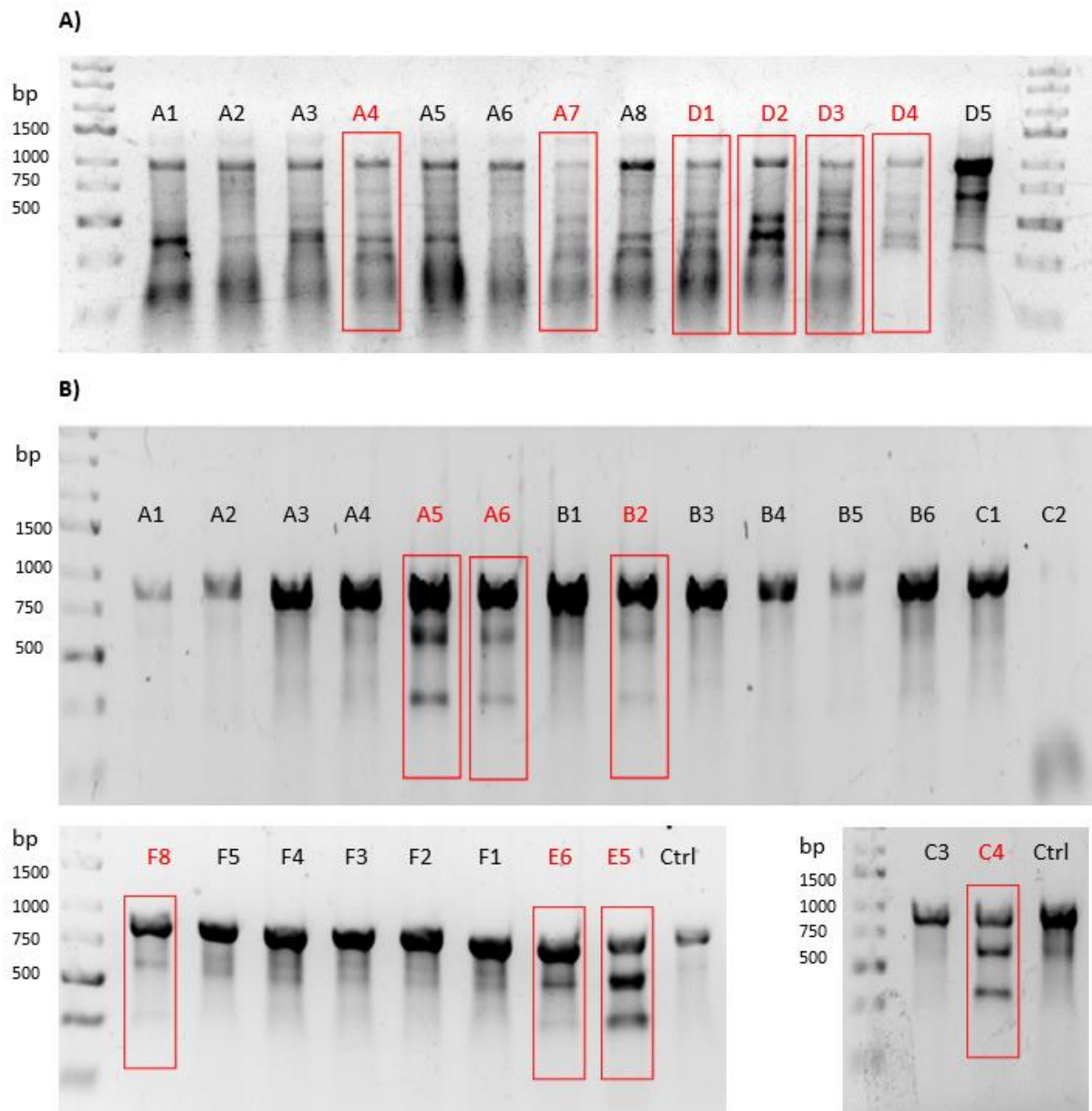


Figure 6: T7 assay results for single cell clones of NABP1 knockout experiment. The NABP1 exon 3 (A) and exon 4 (B) mutated cell lines showing positive results are highlighted in red.

Examining genomic events: sequencing results in the T7 positive clones

The PCR products of T7-positive clones were sent for Sanger sequencing with their reverse PCR primers. It was essential for identifying clones with heterozygous mutations, indicating different genomic events in each allele. Cas9 typically cleaves DNA 3–6 nucleotides downstream of the PAM sequence. For further analysis, clones with out-of-frame shift mutations were selected, except for NABP2/hSSB1 E9 cells, which contained an in-frame mutation in one allele (*Figure 7*).

A)

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Ref seq T G A T C A T A G A A A G T T C C A G A A T C A T C A G G T A C C A C C T C A G G T - G T T T G C T G T C T T T C T T
Alt1 A1 T G A T C A T A G A A A G T T C C A G A A T C A T C A G G T A C C A C C T C A G G T T G G T T G C T G T C T T T C T T
Alt2 A1 T G A T C A T A G A A A G T T C C A G A A T C A T C A G G T A C C A C C T C G T G T - - - T T G C T G T C T T T C T T

Ref seq T G A T C A T A G A A A G T T C C A G A A T C A T C A G G T A C C A C C T T T T C G A C A G G A T C T T A - - - - -
Alt1 C2 T G A T C A T A G A A A G T T C C A G A A T C A T C A G G T G C C A C C - - - - -
Alt2 C2 T G A T C A T A G A A A G T T C C A G A A T C A T C A G G T A C C A C C T C A G G T G T T T G C T G T C T T T C T T

Ref seq T G A T C A T A G A A A G T T C C A G A A T C A T C A G G T A C C A C C T C A G G T - G T T T G C T G T C T T T C T T
Alt1 C3 T G A T C A T A G A A A G T T C C A G A A T C A T C A G G T A C C A C C T C A G G T T G T T T G C T G T C T T T C T T
Alt2 C3 T G A T C A T A G A A A G T T C C A G A A T C A T C A G G T A C C A C C T C G T - - - G T T T G C T G T C T T T C T T

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B)

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Ref seq T C A C A A A G G T C T C C G T C G T - - C A T G C T G C C T G A G C C T C G G C A C C C C A C T G G A T G G G G A A T A G A
Alt1 C11 T C A C A A A G G T C T C C G T C G T A - C A T G C T G C C T G A G C C T C G G C A C C C C A C T G G A T G G G G A A T A G A
Alt2 C11 T C A C A A A G G T C T C C G T C G T T T T C A G G C T G C C T G A G C C T C G G C A C C C C A C T G G A T G G G G A A T A G A

Ref seq T C A C A A A G G T C T C C G T C G T - C A T G C T G C C T G A G C C T C G G C A C C C C A C T G G A T G G G G A A T A G A
Alt1 E9 T C A C A A A G G T C T C C G T C G T C A T G C T G C C T G A G C C T C G G C A C C C C A C T G G A T G G A G A A T A G A
Alt2 E9 T C A C A A A G G T C - - - - - A T G C T G C C T G A G C C T C G G C A C C C C A C T G G A T G G G G A A T A G A

Ref seq T C A C A A A G G T C T C C G A C G G G T T A G G C A C T G G - - - - - G C T T A G A C A C C C C G A T G G A T G G G G A A T A G G G
Alt1 G3 T C A C A A A G G T C T C C C A G T C C A G G - - - - - G C T T A G A C A C C C C G A T G G A T G G G G A A T A G G G
Alt2 G3 T C A C A A A G G T C T C C G T C G T C A T G C T G C C T G A G C C T C G G C A C C C C A C T G G A T G G G G A A T A G A G G

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C)

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Ref seq T A A A A G T A T A C T A C A C A A T T A C C C C T T T G T T C T G C T G T C C T C G A T A A T C T G G G T
Alt1 E5 T A A A A G T A T A C T A C A C A A T T A C C C C T T T G T T C T G C T G T C C T T C G A T A C A A T C T G
Alt2 E5 T A A A A G T A T A C T A C A C A A T T A C C C C T T T G T T C T G C T G T T C A T C T A T T C A T A C A C

Ref seq T A A A A G T A T A C T A C A C A A T T A C C C C T T T G T T C T G C T G T C C T C G A T A A T C T G
Alt1 C4 T A A A A A G T A T A C C T A C A C A A T T A C C C C T T T T G T T C T G C T G T C C T C G A T A A T C C C
Alt2 C4 T A A A A A G T A T A C C T A C A C A A T T A C C C C T T T T G T T C T G C T G T C C T C G A T T T A T A T

Ref seq T A A A A G T A T A C T A C A C A A T T A C C C C T T T G T T C T G C T G T C C T C G A - T A A T C T G G G T
Alt1 A5 T A A A A G T A T A C T A C A C A A T T A C C C C T T T T G T T C T G C T G T C C T C G - - T A A T C T G G G T
Alt2 A5 T A A A A G T A T A C T A C A C A A T T A C C C C T T T G T T C T G C T G T C C T C G A T T A A T C T G G G T

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Figure 7: Sanger sequencing results for single-cell clones. Three representative cases of heterozygous mutations are shown for G3BP1 KO cells (A), NABP2/hSSB1 KO cells (B), and NABP1/hSSB2 KO cells (C). The PAM sequence used for CRISPR-Cas9 editing is highlighted in red.

Protein level validation: results of western blot and immunofluorescent visualization

Western blot analysis confirmed the absence of targeted proteins, using GAPDH as a positive control (*Figure 8*). In the three selected Δ G3BP1 cell lines, G3BP1 protein was not detected. Among the three Δ hSSB1 cell lines, two lacked hSSB1 expression, while E9 cells retained it (not shown), indicating an in-frame deletion that likely removed the first amino acid from the N-terminal region, which is reported not to impair its function. Western blot analysis did not detect hSSB2 in either wild-type or KO samples, leaving its validation unresolved.

Fluorescence imaging further confirmed the absence of hSSB1 and G3BP1 proteins in the Δ hSSB1 and Δ G3BP1 KO cell lines, verifying effective protein elimination *in vitro* (*Figure 9*).

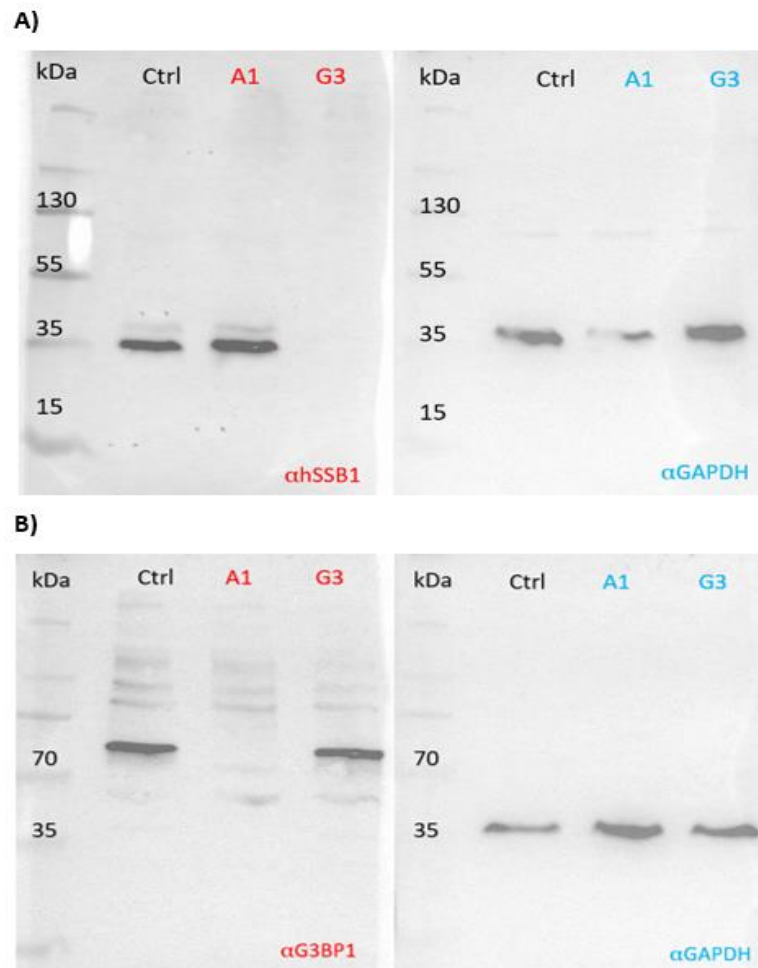


Figure 8: Western blot analysis of Δ hSSB1 G3 and Δ G3BP1 A1 clones. (The other cell lines are not included for better comprehensibility).

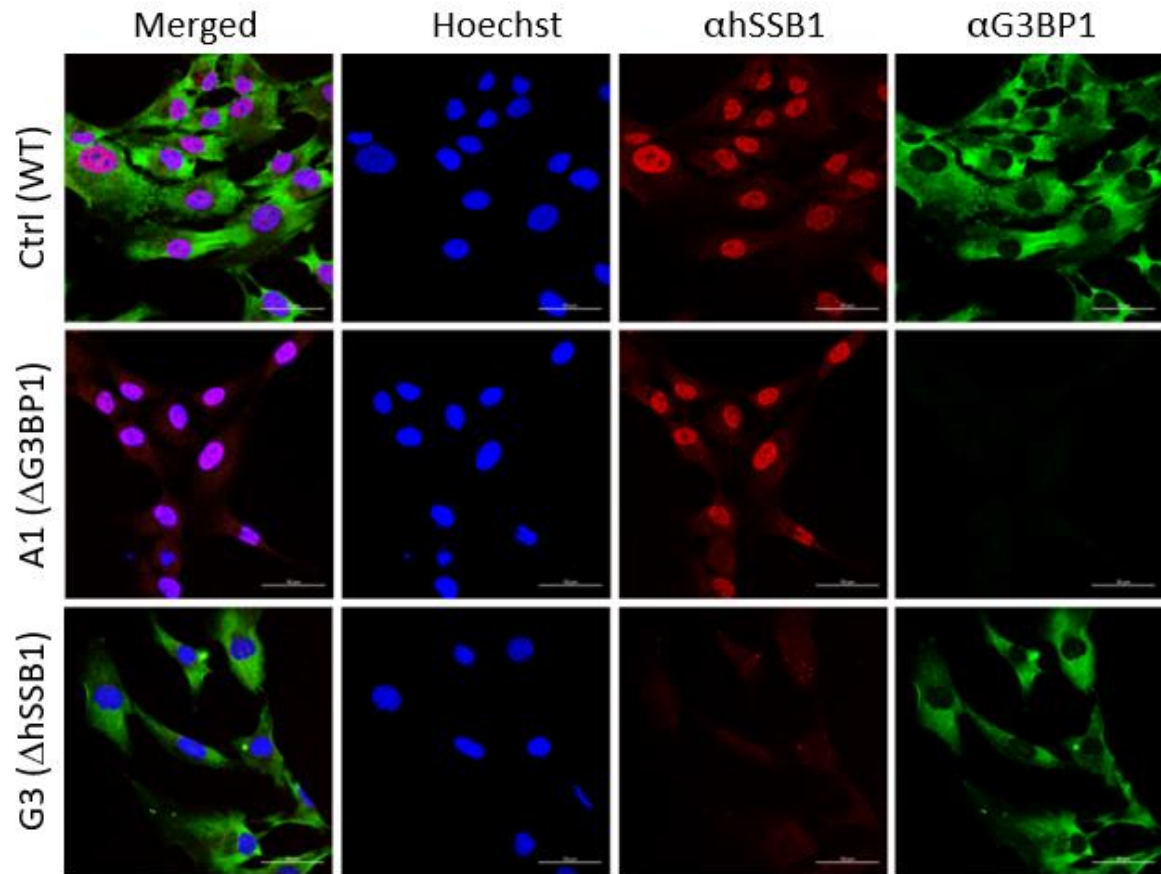


Figure 9: Immunofluorescence labelling of hSSB1 and G3BP1 on wild-type RPE1, the G3 Δ hSSB1, and the A1 Δ G3BP1 clones (scale bar: 20 μ m).

Discussion

Generation of NABP2/hSSB1, NABP1/hSSB2, and G3BP1 knockout cell lines was successfully achieved using CRISPR-Cas9 gene editing. To ensure the robustness of our results, we maintained multiple parallel cell lines with distinct mutation events. This approach reduces the risk of bias, ensuring that the cell line phenotype is not affected by other mutations outside the targeted region, making the results more reliable. Depletion of SSBP1/mtSSB is still in progress due to the gene's repetitive regions, which complicate the sgRNA and screen PCR primer design.

The efficiency of CRISPR-Cas9 editing heavily relies on the proper design of the sgRNA sequence it got special attention. This involved selecting sgRNAs that are less prone to secondary structure formation and circularization while maintaining an appropriate GC content. As the Cas9 exhibits a bias toward specific PAM sequences it is also considered for the selection (Ran et al., 2013). To avoid off-target effects that could result in unintended mutations, several bioinformatic prediction tools were employed for sgRNA design, supplemented by manual corrections when it was necessary. This careful design process was essential for optimizing the efficiency and specificity of CRISPR-Cas9 editing.

The T7 endonuclease assay proved effective in the initial screening of bulk cell populations for cleavage events, and later for the analysis of subsequent single-cell clone populations confirming the presence of specific mutations in these cells. Although we further validated the T7 positive cell lines, the off-target amplification caused by improper primer sets occasionally made it difficult to interpret the gel results, as additional non-specific bands could obscure the relevant ones. The observed mutations, primarily deletions near the Cas9 cleavage sites, were confirmed through Sanger sequencing, which revealed a range of insertion-deletion (indel) events, many resulting in frameshift mutations. The distance between the cleavage site and PAM aligned with previously reported findings.

Western blot analysis further validated the gene disruptions at the protein level, particularly for clones harbouring frameshift mutations, with most KO cell lines showing a complete absence of the targeted proteins. This type of mutation is desirable because it introduces an early stop codon in the mRNA, triggering nonsense-mediated mRNA decay. This mechanism degrades the mRNA before translation, preventing the production of any protein product. Interestingly, one cell line (E9) containing a non-frameshift deletion still expressed the hSSB1 protein. Sequencing revealed a single amino acid deletion from the N-terminal, which

did not appear to impair the protein's expression. However, since no functional changes associated with this specific truncation have been reported, further investigation was not pursued.

We primarily confirmed that RPE1 cells express hSSB2 at the mRNA level based on exome sequencing data provided by our collaborative partners; however, we failed to detect hSSB2 by Western blot. The primary antibodies were also tested in HeLa cells, where they showed specific signals, suggesting that the most likely explanation is the low protein expression in RPE1 cells. Based on previous studies, the expression of OBFC2A, which encodes hSSB2, is regulated by specific miRNAs, with miR17 and miR21 being associated with OBFC2A depletion (Vernin et al., 2014). Examining the expression levels of these miRNAs in RPE1 cells could provide insights into the regulation of hSSB2 transcript levels.

The limitations of this study include the lack of an examination of potential Cas9 off-target effects beyond the amplified regions. While CRISPR-Cas9 is highly specific, off-target mutations in unintended regions of the genome may have occurred, and this was not assessed. The other challenges include the low protein expression level of hSSB2 in RPE1 cells, which prevented validation of the protein absence, as both wild-type and knockout cells produced non-visible results. As it is known that hSSB1 plays a distinct role in UVB damage repair (Boucher et al., 2021), UV treatment may potentially induce hSSB2 protein expression to a detectable level. This approach is being considered as a potential strategy to address this challenge.

The primary aim of this project was to create these cell lines as a foundational tool for subsequent experiments. Upcoming research will focus on the functional analysis of the knockout cells, with experiments needed to elucidate the biological consequences of these gene deletions. Given that hSSB1 and mtSSB are essential proteins, we expect these KO cells to have more dramatic phenotypic changes, such as point mutations, shorter cell lifespan, morphological differences, or slower proliferation. In addition to these expected short-term phenotypic effects, future studies should also investigate the long-term consequences, such as delayed cellular senescence, increased genomic instability, or altered DNA repair mechanisms over time. These anticipated changes have not yet been explored, but they will likely be critical for understanding the full impact of genetic disruptions in future studies. Investigating these aspects will provide a more comprehensive understanding of the role of SSB proteins in maintaining cellular homeostasis.

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