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Direktor: Prof. Dr. med. Stefan Frantz

Molecular mechanisms underlying Woodhouse-Sakati syndrome: characterization of DCAF17 with specific, polyclonal antibodies.

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Joanna Blocka

aus Heidelberg

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Referent:	Priv.-Doz. Dr. med. Dr. rer. nat. Matthias Kroiß
Korreferent:	Prof. Dr. med. Thomas Haaf
Dekan:	Prof. Dr. med. Matthias Frosch

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1 INTRODUCTION

1.1 A previously undescribed multisystemic disease

1.1.1 First report and clinical characteristics of Woodhouse-Sakati syndrome

In 1983, Nicholas J. Y. Woodhouse and Nadia A. Sakati of the King Faisal Specialist Hospital in Saudi Arabia described six patients with similar clinical features: hypogonadism (of either hyper- or hypogonadotropic nature), alopecia, diabetes mellitus, intellectual impairment, sensorineural deafness, and ECG abnormalities[1]. The parents were phenotypically normal relatives, which indicated a presence of a genetic syndrome with an autosomal-recessive inheritance. In the following years, more patients with Woodhouse-Sakati syndrome (WSS) from various countries were reported in the medical literature and additional features of the syndrome described. Those symptoms included dysarthria[2], bilateral keratoconus[3], limited mobility of upper extremities[4], idiopathic thrombocytopenic purpura and camptodactyly[5], cerebral white matter lesions, extrapyramidal features (dystonia, chorea), seizures, polyneuropathy, thyroid dysfunction, and progery of facial skin[6,7] as well as anodontia[7] and edentulism[8]. The laboratory tests revealed low IGF-I and C-peptide as well as an elevated LDL in serum. Figure 1 A and B shows a typical appearance of patients with WSS[9].

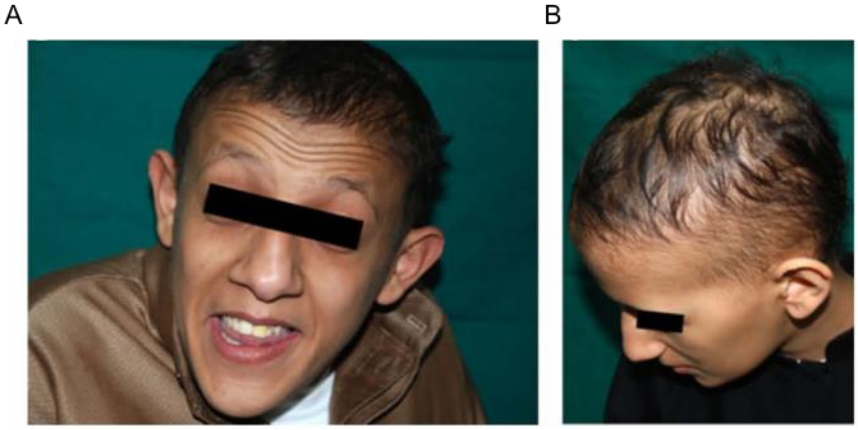


Figure 1. Clinical features of patients with WSS. Siblings from a consanguineous Bedouin family from Kuwait, a 19-year-old man (A) and a 15-year-old girl (B) present a typical appearance of patients with WSS: triangular-shaped face, large, low-set ears, fronto-temporal alopecia, sparse eyebrows (and eyelashes) as well as muscular dystonia. From Nanda et al.[9]

To date, approximately 80 patients with WSS from more than 30 families have been documented. The families are mostly of Middle Eastern origin, although patients of European heritage have also been reported. The founder mutation is thought to have arisen approximately 55 generations ago[10].

1.1.2 Molecular background of the disease

Mutations in C2orf37/DCAF17 gene cause WSS.

In 2008, Alazami et al. identified a loss-of-function mutation (single base pair deletion) of the *C2orf37* gene, located on chromosome 2q31.1 in the examined patients using linkage analysis followed by DNA sequencing[10]. To date, eleven mutations in *C2orf37* are known, including frameshift and nonsense mutations as well as splice-site ablations[4-8,10-15]. All these mutations result in a truncated protein (Table 1).

Table 1. Mutations in *C2orf37* gene in patients with WSS. The table presents different types of *C2orf37* mutations, such as frameshift and nonsense mutations as well as splice-site ablations, as cause of WSS[4-8,10-15]. All mutations result in a truncated protein. * = exact country of origin was not specified; ** = compound heterozygosity[15].

Family origin	***Site of mutation***	***Type of mutation***
Saudi Arabian[10], Qatari[12], Tunisian[13]	c.436delC[10,12-13]	frameshift[10,12-13]
Croatian[4,10]	c.50delC[4,10]	frameshift[4,10]
Middle Eastern*[6,10]	c.1091+6T>G[6,10]	splice-site[6,10]
Indian[5,10,15]	c.1422+5G>T[5,10]	splice-site[5,10]
	c.459-7_499del**[15]	splice-site[15]
	c.1238delA**[15]	frameshift[15]
Pakistani[8,14]	c.321+1G>A[8]	splice-site[8]
	c.270delA[14]	frameshift[14]
Turkish[11]	c.127-3delTAGinsAA[11]	splice-site[11]
Italian[7,11]	c.906G>A[7,11]	nonsense[7,11]
	c.341C>A[11]	
French Gypsy[11]	c.387G>A[11]	nonsense[11]

There is no known correlation between the genotype (i.e. site of mutations) and patient phenotype. Thus, it is believed that an intact, full-length protein is a condition *sine qua non* for a healthy phenotype. However, an important role of disease modifiers is suspected because of the phenotype variability among members of the same family[7].

Consensus nomenclature now refers to the *c2orf37* gene as *DCAF17*, as explained below.

WSS protein is the product of c2orf37/DCAF17 gene.

In his work, Alazami isolated at least 30 mRNA isoforms encoding the WSS protein. The two major WSS protein isoforms, α and β, are expressed in equal abundance. The α isoform is a 240-amino-acid, C-terminal part of the β isoform (520 amino acids). Immunohistochemistry on mouse embryonic tissues using a polyclonal antibody raised against the amino-acid sequence 412-426, which belongs to both α- and β-WSS, revealed an enhanced expression in the skin, liver, and brain (confirmed through in situ hybridization and mRNA-expression profile of a mouse ortholog of c2orf37a) with a nucleolar staining (Figure 2)[10].

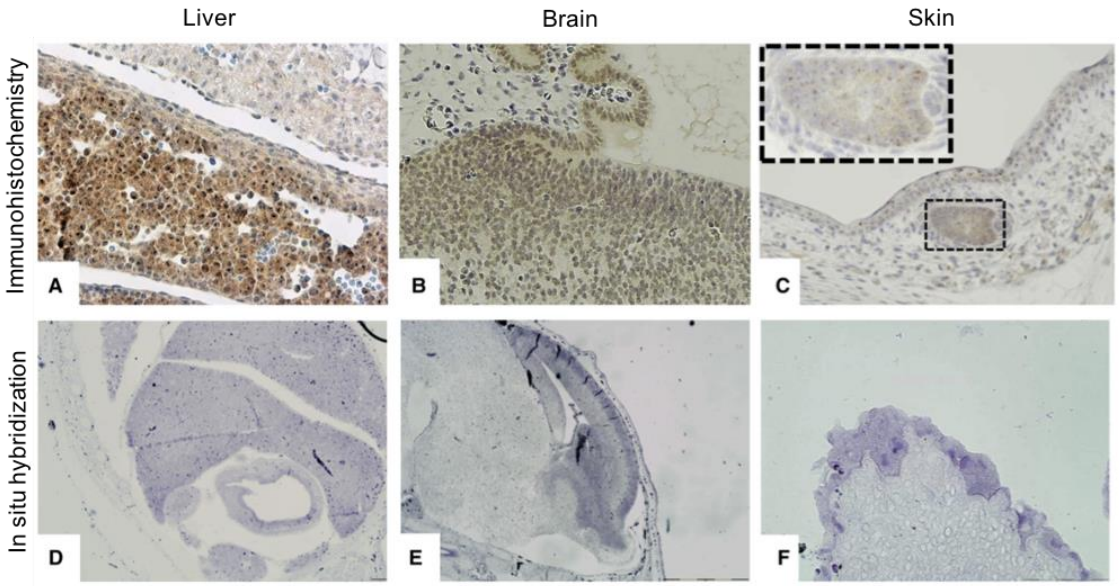


Figure 2. Immunohistochemistry and in situ hybridization on mouse embryonic tissues. Immunohistochemistry (A-C) showing nucleolar staining in liver (A) (heart tissue is pictured in the upper right corner), brain (B), and skin (C), including a hair follicle (inset); in situ hybridization (D-F), showing RNA expression of 4833418A01Rik (mouse ortholog of C2orf37) in liver (D), brain (E), and skin (F). From Alazami et al.[10]

In line with these findings, immunohistochemistry using the above-mentioned antibody performed on human embryonic kidney (HEK) 293 cells also showed a strong nucleolar and only a weak cytoplasmic signal[10]. Transfection of HEK293 cells with a GFP-tagged *C2orf37* construct and subsequent expression of the fusion protein led to disruption of the nuclear membrane and aggregation of the fusion protein with displacement of the nucleus. However, immunofluorescence using anti-C2orf37(412-426) antibody in HEK293 cells expressing α- and β-C2orf37 without any tag demonstrated a nucleolar localization of both isoforms, consistent with the immunohistochemistry in mice. The nucleolar localization was also shown in untransfected lymphoblasts of WSS patients as well as in the control group, confirmed through a colocalization of endogenous C2orf37 with B23 (nucleophosmin), which is a known granular component of the nucleoli. Further, HEK293

cells as well as patient and control lymphoblasts were subjected to low and high doses of actinomycin D. This enzyme at low dose selectively blocks the nucleolar RNA polymerase I, whereas at high dose it inhibits completely the transcription in the cell. In his experiments, Alazami showed that the lower dose of actinomycin D caused a complete diffusion of B23 into the nucleoplasm of HEK293 cells, whereas C2orf37 mostly retained its nucleolar localization. At higher doses of actinomycin D, a minority of cells still retained C2orf37 in the nucleoli. These results imply that the WSS protein is only partially dependent on active transcription, in contrast to B23, which is involved in processes such as ribosome biogenesis and susceptible to transcriptional blockade. In WSS patient lymphoblasts, even low doses of actinomycin D caused a translocation of both B23 and C2orf37 into the nucleoplasm, whereas in control lymphoblasts these proteins retained its nucleolar compartmentalization. These findings suggest a hypersensitivity of patient lymphoblasts to transcriptional blockade[10].

Despite their ubiquitous expression, a deficiency of nucleolar proteins frequently appears to clinically affect a limited number of organs. This phenomenon has previously been reported in other syndromes and may suggest a redundancy of nucleolar proteins in the phenotypically unaffected tissues[16]. Nousbeck et al. described a syndrome with alopecia, neurological symptoms, and endocrinopathy (ANE syndrome), caused by a decreased expression of RNA-binding motif 28 (RBM28)- a nucleolar protein associated with ribosome biogenesis[16].

However, it is unclear whether the increased sensitivity to actinomycin D treatment is related to a pathologic ribosome biogenesis as the underlying cause of WSS. RNA polymerase I not only blocks the rRNA transcription but also inhibits the fusion of prenucleolar bodies (PNBs) and nucleolar organizer regions (NORs). Other nucleolar functions, such as snRNA processing, mRNA transport, cell cycle regulation, cell aging, and apoptosis may subsequently be disrupted by RNA polymerase I blockade and therefore underly the pathogenesis of WSS[10].

DCAF17 is connected to the ubiquitin pathway through DDB1/Cul4 E3 ligase.

In 2006, Angers et al. conducted experiments to investigate functional features of DDB1-CUL4-ROC1 E3, a ubiquitin ligase complex containing damage-specific DNA binding protein 1 (DDB1), cullin 4A (CUL4A), regulator of cullins-1 (ROC1), and E3 ligase enzymes. Through a tandem affinity purification of human DDB1 and CUL4A, they identified a group of 16 proteins, which bound directly to DDB1 and served to recruit

substrates for E3. As no other function of these proteins was known at the time, they named them DDB1-CUL4A-associated factors (DCAFs)[17]. Soon after that, more groups took interest in this novel protein family, investigating their function as well as discovering other family members[18,19]. Among those, the protein product of *C2orf37* gene, DCAF 17, was identified.

1.2 The aim of this project

Although the *C2orf37/DCAF17* gene product has been found to be associated with DDB1-CUL E3 ligase, many aspects remain unexplored. Various intriguing questions arise, such as: what is the functional link between the mutations in the *c2orf37* gene, resulting in a truncated DCAF17, and the clinical manifestation of WSS? Which subcellular processes(es) is/are disrupted in order to evoke such diverse phenotype features? And, finally, what is the exact function of the wild-type protein, which appears to play a crucial role in numerous tissues and how is the phenotype related to the relatively high expression in the liver, skin, and brain? The experimental approaches of this work are presented in Figure 3. First, I aimed to develop- as a tool for subsequent experiments- specific, polyclonal, anti-peptide antibodies which would react with distinct epitopes of the WSS protein. Second, by means of these antibodies, it was my goal was to determine the distribution of the WSS isoforms in different cellular compartments (using cell fractionation and immunofluorescence); to analyze biochemical features of these proteins, including salt sensitivity and attachment to the nuclear envelope as well as connection to DNA/RNA. Third, I investigated an 80-kDa protein which was detected with the anti-WSS antibodies in immunoblot experiments. In particular, I approached the question whether this protein is a posttranslationally modified WSS isoform.

WSS protein analysis

Intracellular localization	Biochemical features	Posttranslational modification/80-kDa protein
•Cell protein fractionation •Immunofluorescence	•Salt sensitivity (attachment to nuclear envelope) •DNase and RNase digestion (attachment to DNA/RNA)	•Deglycosylation •Dephosphorylation

Figure 3. Three main experimental approaches of this work in order to investigate the WSS protein. Methods used for each approach are listed in the corresponding columns. Cell protein fractionation and immunofluorescence were performed to investigate the intracellular redistribution of α- and β-WSS. Treatment with varying concentrations of NaCl and digestion with DNase/RNase were conducted to examine the attachment of the WSS isoforms to the nuclear envelope and nucleic acid, respectively. Furthermore, the possibility of a posttranslational glycosylation/phosphorylation of the WSS protein, resulting in the appearance of an 80-kDa band on Western blot, was investigated by means of deglycosylation and dephosphorylation of nuclear extract.

2 Materials and methods

2.1 Bioinformatics

- *BioDocAnalyze* software (Analytik Jena, Jena) was used for UV-gel analysis
- *Eurofins Genomics* (Ebersberg) performed gene sequencing of the DNA subcloned in plasmid vectors
- *http://nebiocalculator.neb.com* was used to convert masses to moles of DNA
- *NanoDrop* software was used to measure DNA and RNA concentrations in obtained samples using NanoDrop spectrophotometer
- *PepSlide Analyzer* program was used for image analysis and interpretation of peptide microarray data (performed by Sicasys, Heidelberg)

2.2 Devices

- *Avanti J-25I*, equipped with JA-10 rotor (Beckman Coulture, Krefeld)
- *Axioplan 2*, equipped with standard filter sets for FITC, TRITC and DAPI (Carl Zeiss Microscopy, Göttingen)
- *Biofuge Fresco* (Heraeus, Hanau/ Thermo Fischer Scientific, Dreieich)
- *BioPhotometer D30* (Eppendorf, Wesseling-Berzdorf)
- *Dri-Block Heater DB-2D* (Techne, Staffordshire, UK)
- *ECOMAX Film Processor* (Protec, Oberstenfeld)
- *Forma Series 11 3111 Water Jacketed CO2 Incubator* (Thermo Fischer Scientific, Dreieich)
- *IBI UVT 400-M Ultraviolet Transilluminator* (IBI Scientific, Peosta, Iowa, USA)
- *inoLab pH 720* (WTW, Weilheim)
- *Joule Box* (Stratagene, La Jolla, California, USA)
- *Mastercycler gradient* (Eppendorf, Wesseling-Berzdorf)
- *Megafuge 1.0 R* (Heraeus, Hanau/Thermo Fischer Scientific, Dreieich)
- *Mini-PROTEAN 3 Multi-Casting Chamber* (Bio-Rad Laboratories, Munich)
- *Minishaker MS1* (IKA, Staufen)
- *Mini Trans-Blot Electrophoretic Transfer Cell* (Bio-Rad Laboratories, Munich)
- *MS-3000 High speed magnetic stirrer* (Biosan, Riga, Latvia)
- *MSH-300 Magnetic Stirrer with hot plate* (Biosan, Riga, Latvia)
- *NanoDrop 2000c Spectrophotometer* (Thermo Fischer Scientific, Dreieich)

- *Optima L-60 Ultracentrifuge* (Beckman Coulture, Krefeld)
- *Polymax 1040 incubating model* (Heidolph, Schwabach)
- *PowerPac 200 Power Supply* (Bio-Rad Laboratories, Munich)
- *PowerPac Basic Power Supply* (Bio-Rad Laboratories, Munich)
- *Quick Cookmate* (Deawoo Electronics, Eschborn)
- *Rotanta RPC* (Hettich, Tuttlingen)
- *Rotary shaker KS10 swip + TH10* (Edmund Bühler, Hechingen)
- *SBA 32 precision balance* (Scaltec Instruments, Göttingen)
- *Spectrafuge Mini* (Labnet, Edison, New Jersey, USA)
- *Sprout Mini Centrifuge* (Heathrow Scientific, Vernon Hills, Illinois, USA)
- *Thriller Thermoshaker Incubator* (Peqlab Biotechnologie/VWR Life Science Competence Center, Erlangen)
- *Uni Equip HF Generator Type Sonopuls GM 70 HD with 2kHz* (UniEquip, Planegg)
- *Vortex Mixer* (Heidolph, Schwabach)

2.3 Disposable utensils

- Disposable gravity flow columns (Thermo Fischer Scientific, Dreieich)
- *Whatman Cellulose Filter Paper* (Sigma Aldrich, Taufkirchen)

2.4 Chemicals and reagents

Sterile, deionised water (B. Braun, Melsungen) was used as diluent. Chemicals were purchased from Merck (Darmstadt), Roth (Karlsruhe), and Sigma-Aldrich Chemie (Taufkirchen).

Enzymes

- *Antarctic Phosphatase, Protein Phophatase 1 (PP1),* and *T-cell protein tyrosine phosphatase (TC PTP) Kits* (New England Biolabs, Frankfurt am Main)
- *DNase I and DNase I Reaction Buffer (New England BioLabs, Frankfurt am Main)*
- *NdeI*, *NotI*, *EcoRI* restriction enzymes (New England Biolabs, Frankfurt am Main)
- *peqGold Pwo DNA Polymerase Kit* (Peqlab Biotechnologie, Erlangen) was used

for preparative PCR

- *peqGold Taq All Inclusive Kit* (Peqlab Biotechnologie, Erlangen) was used for colony PCR
- *Protein Deglycosylation Mix* (New England Biolabs, Frankfurt am Main)
- *RNase A (Qiagen, Hilden)*
- *Subcellular Protein Fractionation Kit* (Thermo Fisher Scientific, Dreieich)
- *T4 DNA Ligase Kit* (New England Biolabs, Frankfurt am Main)

Commercial antibodies

- *Goat Anti-Mouse IgG H&L (Alexa Fluor 488)* and *Goat Anti-Mouse IgG H&L (Alexa Fluor 594)* antibodies (Abcam, Cambridge, UK)
- *Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey)* (GE Healthcare Europe, Freiburg)
- *Anti-Mitochondria (MTCO2), Anti-alpha 1 Sodium Potassium ATPase, Anti-Lamin A, Anti-Fibrillarin, Anti-Calreticulin,* and *Anti-Actin* mouse monoclonal antibodies (Abcam, Cambridge, UK)
- *Goat Anti-Mouse HRP (IgG H&L)* antibody (Abcam, Cambridge, UK)

Other reagents

- *6x Gel Loading Dye* and *DNA ladders* (New England Biolabs, Frankfurt am Main)
- *10 mM dNTP Mix* (Life Technologies, Darmstadt; New England Biolabs, Frankfurt am Main; Peqlab Biotechnologie, Erlangen)
- *200x Protease Inhibitor Cocktail III* (Calbiochem/ Merck, Darmstadt)
- *BCA Protein Assay Kit* (Novagen/Merck Millipore, Darmstadt)
- *BenchMark Unstained Protein Ladder, BenchMark Pre-stained Protein Ladder, and MagicMark XP Western Protein Standard* (Life Technologies, Darmstadt)
- *CNBr-Activated Sepharose 4B* (GE Healthcare Europe, Freiburg)
- *GeneRuler 1 kb, 100 bp, and 50 bp DNA ladders* (Life Technologies, Darmstadt)
- *NHS-Activated Sepharose 4 Fast Flow* (GE Healthcare Europe, Freiburg)
- *Ni-IDA Agarose* (Cube Biotech GmbH, Mannheim; Macherey-Nagel, Düren)
- *Oligonucleotides* (biomers.net, Ulm)
- *pET28a (+) vector* (Novagen/Merck Millipore, Darmstadt)
- *Roti-Lumin Ultra Chemiluminescence Substrate (1) and Enhancer (2) Solutions*

(Roth, Karlsruhe)

- *SulfoLink Immobilization Kit for Peptides* (Thermo Fisher Scientific, Dreieich)
- *VECTASHIELD Mounting Medium with DAPI* (Vector Laboratories, Burlingame, California, USA)

DNA purification kits

- *NucleoSpin Gel and PCR Clean-up Kit* (Macherey-Nagel, Düren)
- *NucleoSpin Plasmid/Plasmid (NoLid) DNA Purification Kit* ((Macherey-Nagel, Düren)
- *NucleoBond Xtra Midi Plasmid DNA Purification Kit* (Macherey-Nagel, Düren)
- *NucleoBond Xtra Maxi Plasmid DNA Purification Kit* (Macherey-Nagel, Düren)

Bacterial strains

E. coli strains DH5α and Rosetta 2 (Merck) were transformed with genetically engineered expression plasmids for propagation of plasmids and recombinant protein expression, respectively.

2.5 Molecular biology methods

Rationale: establishment of specific, polyclonal antibodies: anti-WSS(493-520), -WSS(80-88), -WSS(412-423), and -WSS(493-511)

In order to establish epitope-specific, polyclonal antibodies against distinct parts of the WSS protein, epitopes for WSS antibody needed to be identified. The epitope identification with PEPperCHIP Peptide Microarray was performed by PEPperMAP Service on behalf of my lab mate and colleague, Dr. Christoph Roscher, and the results have already been presented in his thesis. For a better understanding of my experiments, I will shortly illustrate the principle of the peptide array: During the experiment, β-WSS amino-acid sequence, in form of short (ca. 15 amino acids), overlapping peptides, was immobilized on a glass matrix. After application of the serum of a rabbit immunized with the WSS protein and a subsequent incubation with a fluorescent antibody, specific, „high-affinity“ binding sites were revealed. Peptide array of WSS-β protein showed eight overlapping epitopes[20]. I selected four of these epitopes on the basis of their location and different coupling chemistry for generation of peptide-specific antibodies and chose to

follow two different approaches of antigen preparation suitable for affinity purification of antibodies from a polyclonal serum.

First, a high-affinity epitope WSS(493-520) was recombinantly expressed in *E. coli* cells in order to purify antibodies directed towards the C-terminal part of the WSS protein. WSS(493-520) is part of both α- and β-WSS.

Second, three epitopes were chosen for a commercial peptide synthesis: 1) WSS(80-88), which is a high-affinity epitope located within β- but not α-WSS; 2) WSS(412-423), which is part of α- and β-WSS, and was the epitope Alazami et al. used for their experiments without the last three C-terminal amino acids; 3) WSS(493-511), which is a high-affinity epitope and part of α- and β-WSS as well. Additional cysteine was added to WSS(80-88) and WSS(412-423) to provide a functional group for SulfoLink column coupling, whereas a lysine residue present in WSS(493-511) permitted its coupling to NHS-Activated Agarose resin. Table 2 presents key features of these peptides, including their amino-acid sequence, location within the WSS protein, reactive groups as well as type of resin used for affinity purification of specific polyclonal antibodies.

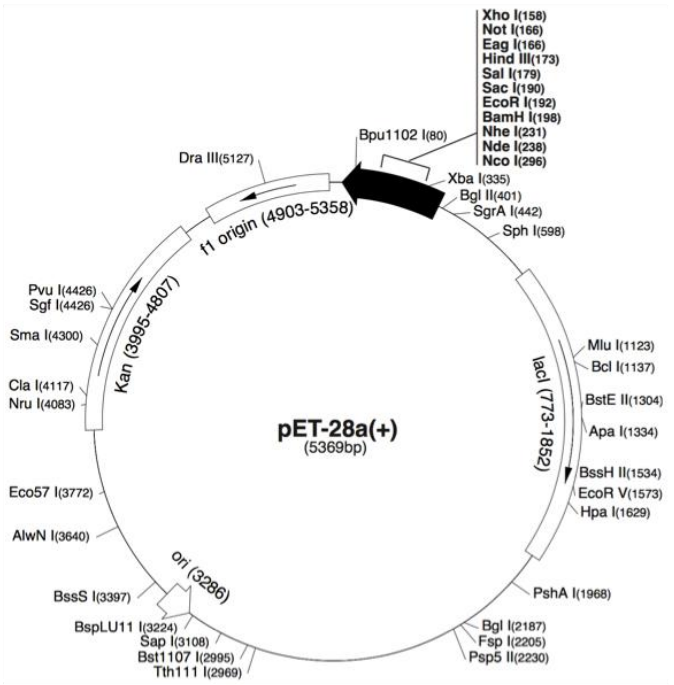
Table 2. Synthetic, chemically modified peptides used for affinity purification of specific polyclonal antibodies. The table depicts three peptides synthesized according to the results of the epitope mapping for anti-WSS antibody. Cysteine (C), containing the -SH group, was added to WSS(80-88) and WSS(412-423) for coupling to SulfoLink column, whereas lysine (K), containing the -NH_2 group, was already present in WSS(493-511) for coupling to NHS-Activated Agarose column. The added amino acids are underlined. WSS(80-88) is located at the N-terminus of the WSS protein and is therefore part of β- but not α-WSS, while WSS(412-423) and WSS(493-511) are part of both α- and β-WSS.

Synthetic peptide	***Part of WSS***	***Amino acid sequence***	***Functional group***	***Affinity purification column***
WSS(80-88)	β	<u>C</u>SEPRKLYEM	-SH	SulfoLink
WSS(412-423)	α and β	<u>C</u>FNLLDDDPEQET	-SH	SulfoLink
WSS(493-511)	α and β	<u>K</u>PNRVFSCYVYQMICDTGE	-NH_2	NHS-Activated Agarose

WSS(493-520) is situated at the C-terminus of WSS-β protein and the longest of all epitopes revealed by the epitope mapping for anti-WSS antibody on PEPperCHIP microarray using WSS-β from a serum of a pre-immunized rabbit. As part of the experiments, the cDNA sequence of a specific anti-WSS epitope was to be subcloned in a plasmid vector, followed by expression of the peptide in *E. coli* and subsequent affinity purification of specific polyclonal anti-peptide antibodies. The epitope WSS(493-520), coding for the amino-acid sequence KPNRVFSCYVYQMICDTGEEEETINRSC, was chosen for the recombinant expression and not commercial peptide synthesis because of its relative length. This sequence is part of both α- and β-WSS. I decided to subclone the coding sequence of *WSS(493-520)* into pET28a(+) vector. pET28a(+) is a plasmid which

carries a coding sequence for an N-terminal His-tag/thrombin/T7-tag, a C-terminal His-tag sequence, a lac operon site, and a kanamycin-resistance coding sequence. It has multiple restriction sites (Figure 4). Only a few of them, however, are located solely once within the multiple cloning sites of the vector[21]. To create the WSS(493-520):pET28a(+) construct, restriction sites for NdeI (location 238) and NotI (166) were used.

A



B

T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites	
(*Bam* H I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

C

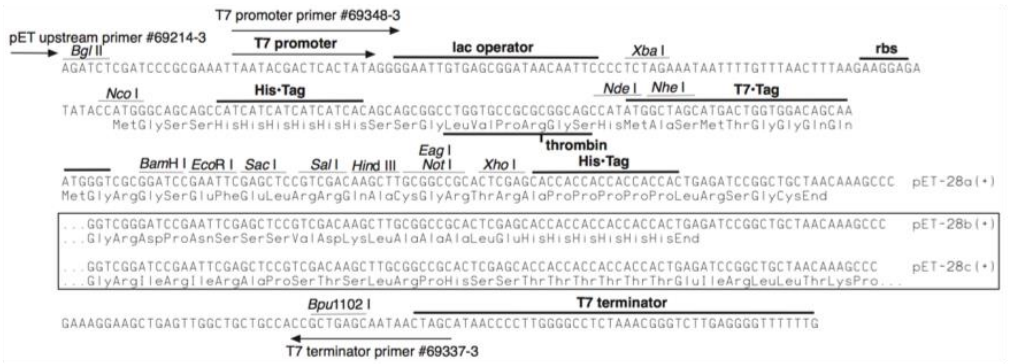


Figure 4. pET-28a(+) with its cloning and control sites. The figure shows pET-28a(+), a 5369-bp plasmid (A), with its landmark sequences (B) as well as the nucleobase and amino-acid sequence of the cloning/expression region (C). Restriction sites for NdeI (location 238) and NotI (166) were used for cloning of *WSS(493-520)* into pET-28a(+).
From http://www.helmholtz-muenchen.de/fileadmin/PEPF/pET_vectors/pET-28a-c_map.pdf.

DNA fragments, expression vectors, primer sequences, and enzyme restriction sites used in subcloning and protein expression experiments are listed in Table 3.

Table 3. Amplified DNA fragments, vectors, primer sequences, and restriction sites used for subcloning and protein expression experiments. The restriction sites within the primer sequences are underlined.

Amplified DNA fragment	*Vector*	*5'-primer sequence, restriction site*	*3'-primer sequence, restriction site*
WSS-β	pGEX5x1	CATGGAATTCATGGGCCCGAC CCGGAAG, *EcoRI*	CTAGCGGCCGCTTAACAGCTTC TGTTTATGGTTTCTTCTTC, *NotI*
WSS-α	pGEX5x1	TAGAATTCATGCCACCACTGC TCTTTGAGGTG, *EcoRI*	CTAGCGGCCGCTTAACAGCTTC TGTTTATGGTTTCTTCTTC, *NotI*
WSS(493-520)	pET28a(+)	TACATATGCTACACATAGAGC AGAAACCC, *NdeI*	CTAGCGGCCGCTTAACAGCTTC TGTTTATGGTTTCTTCTTC, *NotI*

Subcloning of α- and β-WSS in pGEX5x1, subsequent expression of recombinant α-WSS-Glutathione-S-Transferase(GST)-tag and β-WSS-GST-tag in Rosetta 2 cells, and affinity purification of anti-WSS(α) and anti-WSS(β) antibodies had previously been performed by Roscher[20].

Preparative polymerase chain reaction

Amplification of DNA sequences was performed using Pwo DNA Polymerase. Pwo (*Pyrococcus woesei)* polymerase is a 90-kDa, heat stable enzyme obtained through recombinant expression in *E. coli* cells. It is a 5'→3' DNA polymerase with additional 3'→5' (= proofreading) activity.

A typical setup for 50-µl reaction volume was composed as follows:

Table 4. PCR sample (50 µl) for Pwo DNA polymerase

Components	*50-µl sample*
dNTP Mix (10 mM)	1 µl
Downstream primer	1 µl
Upstream primer	1 µl
Template	100-500 ng
Reaction Buffer Complete (10x)	5 µl
Pwo DNA Polymerase (1 U/µl)	1-3 µl
Sterile, distilled H_2O	ad 50 µl

Sequential PCR steps are shown in Table 5:

Table 5. Sequential PCR steps for Pwo DNA polymerase. Time, temperature, and number of repeats are specified for each step.

PCR step	*Time*	*Temperature*	*No. of repeats*
Initial denaturation	2 minutes	95 °C	1x
Denaturation	30 seconds	95 °C	30x
Annealing	30 seconds	50 °C	
Elongation	2 minutes	68 °C	
Resting	∞	4 °C	-

The PCR products were purified with NucleoSpin Gel and PCR Clean-up Kit and their concentration was measured with NanoDrop spectrophotometer.

DNA gel electrophoresis

Depending on the length of a DNA fragment, 1.5-3 % agarose gels were prepared. PCR products were diluted with 6x Loading Dye and placed into gel pockets. Electrophoretic separation of DNA fragments was conducted under 15 mA/cm^2 in TAE Buffer.

TAE Buffer: 40 mM Tris
(1x, pH 8.0) 20 mM acetic acid
1 mM EDTA

Following electrophoresis, gel blocks were incubated for 15 min in ethidiumbromide solution (1μg/ml) and subsequently visualized under UV light. Data analysis was performed using BioDoc Analyze Software for UV gel analysis. Separated DNA fragments were extracted from agarose gel using NucleoSpin Gel and PCR Clean-up Kit. Concentration of DNA in the purified samples was measured with NanoDrop spectrophotometer.

DNA hydrolysis with restriction endonucleases

Double digestion was performed, using a buffer recommended by the manufacturer for the specific enzyme pair. A 50-μl reaction sample for DNA hydrolysis was composed as follows:

Table 6. DNA hydrolysis with restriction enzymes (50-µl sample)

Components	*50-µl sample*
Restriction enzyme I	1 µl (10 U)
Restriction enzyme II	1 µl (10 U)
DNA	1 µg
10x reaction buffer (NEB or SmartCut)	5 µl
Sterile, distilled H_2O	ad 50 µl

The components were mixed by pipetting the reaction mixture up and down or by "flicking" the reaction tube. Digestion took place for 1 h at 37 °C. In case of a plasmid DNA restriction, 1 µl Antarctic Phosphatase was added to the reagents following the enzyme restriction and incubated for 30 min at 37 °C. Antarctic Phosphatase is an enzyme which dephosphorylates the endonucleatically cut plasmid ends, preventing them from realignment and thus keeping the plasmid "open" for insertion of a cDNA-fragment. Finally, the digested DNA was purified with NucleoSpin Plasmid/ Plasmid (NoLid) DNA Purification Kit and its concentration was measured using NanoDrop spectrophotometer.

Ligation of DNA fragments

Prior to the ligation, the cDNA fragments and plasmid vectors were digested with the same type of restriction endonucleases.

DNA ligase is an enzyme that catalyzes the formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini in duplex DNA and RNA. It is capable of joining both blunt and cohesive ends as well as repairing single-stranded nicks in duplex DNA, RNA, and DNA/RNA hybrid[21]. A typical setup for a 20-µl DNA ligation sample was composed as follows:

Table 7. Ligation of insert and vector DNA with T4 Ligase (20-µl sample). Molar ratio vector:insert = 1:3.

Components	*20-µl sample*
Insert DNA	0.09 pmol (example)
Vector DNA	0.03 pmol (example)
T4 Ligase	1 µl
T4 Ligase Buffer (10x)	2 µl
Nuclease-free H_2O	ad 20 µl

The addition of reagents was performed on ice, with T4 Ligase added as the last component. The ligation substrates were used in the molar ratio 1:3 (vector:insert),

calculated with NEBioCalculator. The reagents were incubated at 16 °C overnight and subsequently chilled on ice before transformation of competent bacterial cells with the constructs.

Transformation of bacterial cells with plasmid constructs

E. coli DH5α (plasmid propagation experiments) and Rosetta 2 (protein expression experiments) cells were chemically prepared with the Inoue method[22] for a high-efficiency transformation. The bacterial cells, stored at -80 °C, were thawed on ice and joined with the plasmid DNA, followed by an incubation on ice for 30 min. Subsequently, the bacteria were placed in a heating block and subjected to a heat shock at 42 °C for 90 s, followed by a suspension in 500 µl lysogeny broth (LB) per sample and a 2-min incubation on ice.

LB: 10 g bactotryptone/peptone from caseine
5 g yeast extract
10 g NaCl
ad 1000 ml H_2O
The solution was autoclaved, cooled down to a room temperature, and stored in -4 °C.

In the last step, the transformed cells were shaken at 37 °C for 45 min and subsequently spread using the streak method on agar plates containing an antibiotic in the concentration 1:1000, followed by a cultivation overnight at 37 °C.

Agar plates: 3.75 g agar
250 ml LB
The solution was autoclaved and cooled down to 70 °C. An antibiotic (1:1000) was added.

PGEX5x1 carries an ampicillin-resistance, while pET28a(+) a kanamycin-resistance coding sequence. DH5α cells alone do not contain an antibiotic-resistant plasmid. Thus, an antibiotic for cultivation of DH5α cells was chosen based on the resistance of the plasmid they had been transformed with. Rosetta 2 cells carry a compatible chloramphenicol-resistant plasmid. Therefore, for expression experiments with Rosetta 2 cells, chloramphenicol and either ampicillin (for pGEX5x1) or kanamycin (for pET28a(+)) was used.

Colony PCR of transformed DH5α E. coli clones

This experiment was performed using Taq DNA polymerase. It is a heat-stable 5'→3'-polymerase with a weak 5'→3'-exonuclease activity.

The colonies grown on agar plates were transferred with different pipette tips each into PCR tubes, pre-filled with colony PCR Primer Mix. The pipette tips with some adherent cells where then placed in Falcon tubes pre-filled with 10 ml LB and 10 µl antibiotic each. Addition of Master Mix to the PCR tubes followed. A typical 25-µl setup for a Taq DNA polymerase reaction was composed as follows:

Table 8. Colony PCR sample (25 µl) for Taq DNA polymerase

Components	***25-µl sample***
Primer Mix	*12.5 µl*
5'-primer	0.5 µl
3'-primer	0.5 µl
ddH_2O	ad 12.5 µl
Master Mix	*12.5 µl*
Template-DNA	variable
dNTP-Mix (10 mM each)	0.5 µl
Reaction Buffer (10x)	2.5 µl
Enhancer Solution P (5x)	5 µl
Taq DNA Polymerase	0.25 µl
Sterile, distilled H_2O	ad 12.5 µl

Colony PCR of individual clones was performed. The sequential colony PCR steps are pictured below:

Table 9. Sequential colony PCR steps for Taq DNA polymerase.
Time, temperature, and number of repeats are specified for each step.

PCR step	***Time***	***Temperature***	***No. of repeats***
Initial denaturation	2 minutes	95 °C	1x
Denaturation	30 seconds	95 °C	30x
Annealing	30 seconds	50 °C	
Elongation	2 minutes	72 °C	
Prolonged Extension	4 minutes	72 °C	1x
Resting	∞	4 ° C	-

Positive clones, i.e. those which had successfully incorporated the vector, revealed corresponding bands under UV light after separation on an agarose gel.

DH5α clones were cultured overnight at 37° C in a bacteria shaker as liquid minicultures.

Miniculture: transformed bacterial cells
10 ml LB
antibiotic (1:1000)

The following day, plasmid DNA from the PCR-positive clones was purified from the minicultures using either NucleoSpin Plasmid DNA Purification Kit or NucleoBond Xtra Midi Plasmid DNA Purification Kit. DNA concentration was measured with NanoDrop spectrophotometer.

Ultimately, the purified PCR-positive clones were subjected to a sequence analysis, performed by Eurofins Genomics. The successfully transformed clones were then further cultivated in form of liquid maxicultures in a bacteria shaker at 37°C overnight:

Maxiculture: 5 ml miniculture
500 ml super broth (SB)
antibiotic (1:1000)

SB: 32 g bactotryptone/peptone from caseine (3.5 %)
20 g yeast extract (2 %)
5 g NaCl (0.5 %)
ad 1000 ml H_2O
The solution was autoclaved, cooled down to a room temperature, and stored in -4 °C.

Finally, plasmid DNA was purified from the maxicultures using NucleoBond Xtra Maxi Plasmid DNA Purification Kit. DNA concentration was measured with NanoDrop spectrophotometer.

2.6 Protein analysis

2.6.1 General methods of protein examination

Expression of recombinant WSS(493-520)/His-tag in Rosetta 2 cells

For the recombinant protein expression in Rosetta 2 cells, minicultures of the transformed clones grown on the selective agar plates were incubated in a bacteria shaker overnight at 37 °C. The following day, a maxiculture (500 ml) was established and subsequently incubated in a bacteria shaker overnight at 37 °C.

The next day, the maxiculture of Rosetta 2 cells transformed with WSS(493-520):pET28a(+) and enriched with chloramphenicol (1:1000) and kanamycin (1:1000)

was cultivated at 37°C in a shaker until the optical density of 0.6-0.8 (OD_{600}) was reached. A 1-ml sample („0h“) was taken, followed by its 1-min centrifugation at 12000 rpm and a disposal of the supernatant. 200 µl urea and 50 µl SDS buffer were added to the sample, followed by an incubation in a heat block (95 °C) for 5 min.

SDS buffer:	50 mM Tris
(5x, pH 6.8)	2 % SDS
	84 mM β-mercaptoethanol
	20 % glycerine
	0.04 % bromophenol blue

0.5 ml 1M Isopropylthiogalactoside (IPTG) was added to the remaining maxiculture to initiate protein expression. The culture was subjected to shaking for 4 h and a 1-ml sample of the culture was obtained every hour. The solution was then cultivated overnight at 37 °C, after which the last, „overnight“, sample was taken. The bacterial culture was centrifuged for 15 min at 4 °C and 4000 rpm in Beckman Avanti centrifuge equipped with a JA 10 rotor and the bacterial pellet was subsequently stored at -80 °C. All protein samples were electrophoretically separated on Tris-Tricine polyacrylamide gel.

SDS-PAGE protein separation

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to separate proteins according to their molecular weight (MW). Protein samples were prepared for the electrophoretic separation by diluting a 10-20 µg protein sample with 5 µl SDS buffer and distilled H_2O ad 20 µl, and incubating the solution at 95 °C for 5 min. Depending on the MW of the proteins that were to be separated, different gel types were used. Antibodies and full-length WSS-α and -β proteins were separated on a 10 % Bis-Tris gel system. For the recombinant WSS(493-520) peptide, Tris-Tricine gels were used. The electrophoresis was conducted in Mini-PROTEAN chamber in a suitable buffer (see below) for 30-40 min at 80 mA and 50 W per gel.

An exemplary setup for one 10% Bis-Tris gel was composed as follows:

Table 10. 10 % Bis-Tris gel for SDS-PAGE. Components for one gel, consisting of the separating and stacking part.

Components	*Separating gel*	*Stacking gel*
1.25 M Bis-Tris, pH 6.5-6.8	1.425 ml	0.565 ml
30:0.8 Acrylamide:Bisacrylamide Solution	1.665 ml	0.3325 ml
Sterile, distilled H_2O	1.865 ml	1.0675 ml
Ammonium persulfate (APS)	33.25 µl	10 µl
Tetramethylethylenediamine (TEMED)	8.25 µl	5 µl

Bis-Tris buffer: 1.25 Bis-Tris (65.39 g)
(3.5x, pH 6.5-6.8) ad 250 ml H_2O
pH was adjusted with 32 % HCl

Low MW Running Buffer (Low MWRB) was used for proteins of MW between 2 and 50 kDa, High MW Running Buffer (High MWRB) for proteins > 20 kD. Both buffers are appropriate for proteins of MW between 20 and 50 kDa.

Low MWRB: 500 mM MES (48.8 g)
(10x, 500 ml) 500 mM Tris base (30.3 g)
10 mM EDTA (3.0 g)
1.0 % SDS (5 g)
ad 500 ml H_2O
no pH adjustment necessary

High MWRB: 500 mM MOPS (52.2 g)
(10x, 500 ml) 500 mM Tris-base (30.3 g)
10 mM EDTA (3.0 g)
5 mM sodium bisulfite
1 % SDS (5 g)
ad 500 ml H_2O
no pH adjustment necessary

An exemplary setup for one Tris-Tricine gel was composed as follows:

Table 11. Tris-Tricine gel for SDS-PAGE. Components for one gel, consisting of the separating and stacking part.

Components	*Separating gel*	*Stacking gel*
3M Tris, pH 8.5	6.65 ml	1.5 ml
Sterile, distilled H_2O	8.3 ml	3.84 ml
40 % 29:1 Acrylamide:Bisacrylamide Solution	5 ml	600 µl
10 % APS	50 µl	20 µl
TEMED	25 µl	80 µl

The electrophoresis was performed in Cathode and Anode buffers.

Cathode buffer:	484 g Tris base
(10x)	716 g tricine
	40 g SDS
	ad 4 l H_2O
	no pH adjustment needed
Anode buffer:	968 g Tris base
(10x, pH 8.9)	ad 4 l H_2O
	pH was adjusted with concentrated HCl

Gel staining with standard Coomassie method

Following the gel electrophoresis, the gels were stained with Standard Coomassie Staining Solution (SCSS) for 15-30 min, followed by a 40-60-min incubation in Standard Coomassie Destaining Solution (SCDS).

SCSS:	40 % methanol
	10 % acetic acid
	0.1 % Coomassie Blue R250
SCDS:	40 % methanol
	10 % acetic acid

Affinity purification of WSS(493-520)/His-tag fusion protein on Ni-IDA Agarose

An effective way to purify a distinct protein from a multi-protein extract is to attach a “tag” to one of its termini, which would serve as an anchor, “trapping” it in a matrix, while all other proteins are able to flow through unbound. The polyhistidine (His) tag is one of the most commonly used affinity tags due to its small size (6-10 amino acids) and low MW (1 kDa), weak immunogenicity, and utility under difficult conditions, such as denaturation and presence of detergents. This tag was fused with WSS(493-520) peptide and expressed in Rosetta 2 cells. The polyhistidine tags non-covalently bind to transition metal ions. I used agarose with the chelating group iminodiacetic acid (IDA). Binding of WSS(493-520) was based on the interaction between the His-tag (its imidazole rings, specifically) of the recombinant protein and immobilized Ni^{2+} ions. Imidazole, which was the eluting agent in the Ni-IDA-based affinity purification process, was used to compete with the polyhistidine-tagged proteins for their binding to the matrix and elute the fusion peptide.

Bacterial pellets of WSS(493-520)/His-tag-expressing Rosetta 2 cells were thawed on ice and resuspended in 30 ml Buffer 1 each.

Buffer 1: 20 mM Tris (pH 8.0)
250 mM NaCl
5 mM β-mercaptoethanol (β-ME)

The resuspended bacterial cells were disrupted by 6 cycles of a 30-s sonication with a 30-s cooling period after each burst, followed by a 30-min centrifugation at 25000 rpm at 4 °C. After the centrifugation, the soluble target protein was found in the supernatant.

500 µl Ni-IDA Agarose beads were resuspended in 50 ml Buffer 1 and placed in a 50-ml Falcon tube. The suspension was centrifuged for 5 min at 4500 rpm and room temperature.

The supernatant of the bacterial cells lysate was added to the agarose matrix and incubated for 2 h in 4 °C on an end-over-end shaker. A 20-µl "Input" sample was taken before the suspension was transferred into a disposable gravity flow column with a capped bottom outlet. After removal of the bottom cap, flow-through was collected and a 20-µl "Flow-Through" sample was obtained. The resin was subsequently washed three times with 10 ml Buffer 1, followed by the final protein elution with Buffer 2 (10 samples á 500 µl).

Buffer 2: 20 mM Tris (pH 8.0)
250 mM NaCl
5 mM β-ME
300 mM imidazole

20 µl of each eluted sample as well as "Input" and "Flow-Through" were separated on Tris-Tricine gels and examined after staining with the Coomassie method.

Protein dialysis

This step was performed whenever detergents and/or salts needed to be removed from a protein fraction. A cellulose sleeve, pre-heated in a water bath, was used as a membrane for the dialysis of eluted proteins. The sleeve, filled with previously mixed WSS(493-520) elution samples, was incubated in dialysis buffer overnight at 4 °C.

Dialysis buffer: 0.1 M $NaHCO_3$
(pH 8.3) 0.5 M NaCl
ad 4.5 l H_2O

The affinity purified antibodies (see below) were dialysed in a similar way. In this case, however, PBS was used as dialysis buffer.

PBS: 0.1 M Na_3PO_4
(pH 7.2) 0.15 M NaCl

Coupling of WSS(493-520) peptide to CNBr-Activated Sepharose

The goal of this experiment was to covalently immobilize WSS(493-520) protein in order to later purify anti-WSS(493-520) antibody from serum of a previously WSS-immunized rabbit.

Cyanogen bromide (CNBr) in base reacts with -OH groups on agarose to form cyanate esters or imidocarbonates, which in turn react with $-NH_2$ groups of a ligand, enabling its covalent coupling to the agarose matrix.

To prepare the matrix, 300 mg sepharose powder were suspended in 15 ml 1 mM HCl, followed by a 15-min incubation in an end-over-end shaker, a subsequent 5-min centrifugation at 4500 rpm and a disposal of the supernatant. This washing procedure (beginning with the addition of 15 ml HCl) was repeated twice.

The dialysed WSS(493-520) peptide was added to the CNBr-Activated Sepharose beads and incubated overnight at 4 °C in an overhead rotator. Subsequently, the mixture was washed three times with the dialysis buffer (see above). The beads were resuspended in 15 ml 0.1 M Tris (pH 8.0) and incubated at room temperature for 2 h. To remove the excess of uncoupled ligand and make sure that no free substrate remained ionically bound to the immobilized ligand, the matrix suspension was washed alternately with low- (Buffer A) and high-pH (Buffer B) solutions three times each. After disposal of the supernatant, the beads were suspended in 15 ml 20 % ethanol for an overnight storage.

Buffer A: 0.1 M $NaC_2H_3O_2$ (NaOAc)
(pH 4.0) 0.5 M NaCl
Buffer B: 0.1 M Tris
(pH 8.0) 0.5 M NaCl

Affinity purification of anti-WSS(493-520) antibodies

The following day, 10 ml rabbit immunoserum, previously thawed in a 37 °C water bath, were placed in a 15-ml Falcon tube and centrifuged for 15 min at 4000 rpm and room temperature. A 10-µl "Input" supernatant sample was obtained before resuspension of 10 ml supernatant in 30 ml PBS.

The WSS(493-520)/CNBr-Activated Sepharose matrix was centrifuged for 2 min at 1000 rpm and room temperature and the supernatant was removed. The beads were washed twice in 10 ml PBS, transferred into a 50-ml Falcon tube, and incubated with the rabbit serum overnight at 4 °C in an end-over-end shaker. The following day, the serum-beads mixture was centrifuged for 2 min at 1000 rpm and room temperature and the unbound rabbit serum removed, followed by two cycles of alternate washing in 10 ml PBS and 2-min centrifugation at room temperature. The matrix was transferred into a gravity flow plastic column. A 20-µl "Flow through" sample was collected. Elution was performed by addition of 1 ml acidic (pH 2.7) 0.2 M glycine solution to the resin. The change in pH altered the degree of ionization of charged groups at the binding sites. The affinity purified antibodies were collected in Eppendorf tubes, pre-filled with 100 µl basic (pH 8.0) 1 M Tris-KCl. "Input", "Flow-through", and eluted antibody samples were electrophoretically separated on a 10 % Bis-Tris gel and examined after staining with the Coomassie method. Reactivity of the affinity-purified specific antibodies was tested in a Western blot procedure.

Coupling of WSS(80-88) and WSS(412-423) peptides to SulfoLink Coupling Resin

These experiments were conducted in order to affinity purify anti-WSS(80-88) and anti-WSS(412-423) antibodies. However, in contrast to the elution of anti-WSS(493-520) antibodies, chemically synthesized (by Peptide Specialty Laboratories, Heidelberg) epitope peptides were used. These peptides contained an additional N-terminal cysteine residue for immobilization on a functionalized, 6 % crosslinked beaded agarose (SulfoLink). Only free sulfhydryls react specifically with iodoacetyl groups of SulfoLink Coupling Resin at pH 7.5-9.0. A selective reduction of peptides and other disulfide-containing molecules, without interference with iodocetyl coupling, can be achieved by addition of Tris(2-carboxyethyl)phosphine (TCEP)[23].

The experiment was performed according to SulfoLink Immobilization Kit for Peptides protocol. The kit also contained L-Cysteine*HCl, Bond-Breaker TCEP Solution (stabilized aqueous 0.5 M Tris(2-carboxyethyl)phosphine (TCEP); pH neutral), BupH Phosphate Buffered Saline Pack (0.1 M phosphate, 0.15 M NaCl; pH 7.2 when dissolved in 500 ml H_2O), and diverse Column Accessories. In order to prepare samples for coupling, 1 mg of each peptide was dissolved in 2 ml Coupling Buffer.

Coupling Buffer:	50 mM Tris
(pH 8.5)	5 mM EDTA-Na

0.1 ml TCEP was added to the solution to reduce the sulhydryl groups and a 20-µl "Input" sample was taken, followed by a 30-min incubation of the solution at room temperature. After a removal of top and bottom caps, the SulfoLink Column was centrifuged for 1 min at 1000 x g and room temperature in a 15-ml collection tube to remove storage solution, and subsequently equilibrated with 6 ml PBS. Three cycles of addition of 2 ml Coupling Buffer with a subsequent 1-min centrifugation at 1000 x g were conducted, followed by a replacement of the bottom cap. 2 ml peptide solution were transferred into the resin. After a replacement of the bottom and top caps, the peptide-loaded resin was incubated in an end-over-end shaker at room temperature for 15 min with a subsequent 30-min incubation in an upright position at room temperature without mixing. The top and bottom caps were removed again before placing the column into a new tube with a subsequent 1-min centrifugation at 1000 x g and collecting the unbound (flow-through) fraction. A 20-µl "Flow-through" sample was obtained before four-times washing the column with 2 ml Wash Solution and centrifugation for 1 min at 1000 x g after each cycle.

Wash Solution: 1.0 M NaCl

0.05 % NaN_3

Subsequently, the column was washed twice with 2 ml Coupling Buffer, with centrifugation after each cycle. In order to block nonspecific binding sites, 15.8 mg L-Cysteine*HCl were dissolved in 2 ml Coupling Buffer and applied to the column, after replacing its bottom cap. A 15-min incubation while rocking with a subsequent 30-min incubation without mixing, both at room temperature, followed. The top and bottom caps were removed in order to allow the column to drain.

Affinity purification of anti-WSS(80-88) and -WSS(412-423) antibodies

Serum of a WSS-immunized rabbit was thawed in a 37 °C water bath and subsequently centrifuged for 15 min at 4000 rpm and room temperature. A 20-µl "Input" sample was taken, followed by a dilution of 5 ml serum in 45 ml PBS and a successive addition of the solution to the sulfhydryl-peptide coupled resin. After a replacement of bottom and top caps, a 1-h incubation at room temperature while rocking took place. In the next step, the top and bottom caps were removed and the column was centrifuged for 1 min at 1000 x g and room temperature. Without changing the collection tubes, 1 ml PBS was added and the column was centrifuged again the same way with an obtainment of a 20-µl "Flow-through" sample. Subsequently, the resin was washed four times in 2 ml PBS with centrifugation (see above) after each cycle. Elution of the specific anti-

peptide antibodies was performed with 2 ml 0.2 M glycine*HCl (pH 2.7) into a centrifuge tube containing 100 µl 1M Tris*HCl (pH 8.5). The eluted, neutralized sample was centrifuged for 1 min at 1000 x g and room temperature, and retained. The elution step was repeated three times.

Coupling of WSS(493-511) peptide to NHS-Activated Sepharose

During this experiment, the synthetically produced WSS(493-511) peptide which contained an additional N-terminal lysine was coupled to the matrix through an amide bond of the lysine's amine (-NH_2) group. NHS-Activated Sepharose 4 Fast Flow is a pre-activated 4 % agarose matrix. Because of its spacer arm, it is well suited for coupling of small proteins and peptides. N-hydroxysuccinimide (NHS) binds primary amino groups of the ligands by creating amide bonds[24].

1-2 mg peptide was dissolved in 4-5 ml PBS, followed by an obtainment of a 25-µl "Input" sample. After washing the NHS-Activated Speharose Column with 10 to 15 volumes of cold HCl (immediately before use), the peptide solution was added directly to the resin and incubated by rotating in an upside-down position for 2 h at room temperature or overnight at 4 °C. After opening of the column outlet, the flow-through was collected and a 25-µl "Flow-through" sample was taken. Subsequently, the resin was washed twice with 5 ml PBS. In order to block possible unbound groups on the medium, 4 ml 1 M ethanolamine (pH 7.4) were added to the column and incubated for 20 min at room temperature by rotating in an upside-down position. Finally, the column was washed twice with 5 ml PBS.

Affinity purification of anti-WSS(493-511) antibody

In order to affinity purify the specific anti-WSS(493-511) antibody, 5 ml WSS-immunized rabbit serum (previously thawed in a 37 °C water bath) were dissolved in 45 ml PBS and applied successively to the WSS(493-511)-coupled column. Elution of the specific anti-peptide antibodies was performed with 2 ml 0.2 M glycine*HCl (pH 2.7) into a centrifuge tube containing 100 µl 1M Tris*HCl (pH 8.5), in analogy to the affinity-purification of the sulfhydryl-peptides (see above). Samples of the "Input", "Flow-through" as well as eluted fractions were examined with SDS-PAGE and Coomassie-method staining.

BCA-Assay-based measurement of protein concentration

The BCA Protein Assay is a method based on the Biuret reaction, a reduction of cupric (Cu^{2+}) to cuprous (Cu^{1+}) ions as well as a concentration-dependent detection of Cu^{1+}. Bicinchoninic acid (BCA) is a chromogenic, chelating agent that reacts with the reduced copper, producing a purple-coloured complex with a strong absorbance at 562 nm.

Prior to the protein concentration measurement, Bovine Serum Albumin (BSA, 2 mg/ml) standards as well as BCA working reagent (BCAWR) needed to be prepared. PBS was used as a diluent for both standard and measured samples. The standards for the BCA Assay were composed as follows (a 400-600-µl setup each):

Table 12. Standard samples for BCA protein assay

Standard	*BSA volume*	*PBS volume*	*Final BSA concentration*
1	250 µl 2 mg/ml solution	250 µl	1000 µg/ml
2	250 µl standard 1	250 µl	500 µg/ml
3	250 µl standard 2	250 µl	250 µg/ml
4	300 µl standard 3	300 µl	125 µg/ml
5	100 µl standard 4	400 µl	25 µg/ml
6	0 µl	400 µl	0 µg/ml

BCA Solution was provided in the BCA Protein Assay Kit and contained bicinchoninic acid, sodium carbonate, sodium tartrate, and 0.1 M NaOH; pH 11.25.

BCAWR: 1 ml BCA Solution
20 µl 4 % cupric sulfate

Hela/HEK cells were thawed on ice and suspended in 1:10 ratio in 50 µl Radio-Immunoprecipitation Assay (RIPA) buffer.

RIPA buffer: 150 mM NaCl
(pH 8.0) 1.0 % IGEPAL CA-630
0.5 % sodium deoxycholate
0.1 % SDS
50 mM Tris

A disruption of the cells with 10 cycles (15 s each) of sonication with a 15-min cooling period after each burst followed. 5 µl of each standard/measured sample were diluted 1:10 with 50 µl PBS and transformed to 1.5-ml Eppendorf tubes. 1.02 ml BCAWR were added to each sample. The solutions were incubated for 30 min at 37 °C while gently shaking, followed by a photometric measurement of protein concentration in 0.7-ml microcuvettes at the absorbance OD A_{562}.

Western blot

Electrophoretically separated proteins were transferred from polyacrylamide gels to polyvinylidene fluoride (PVDF) membranes using Mini Trans-Blot Electrophoretic Transfer Cell system. The following „layers“ were prepared and subsequently placed, stacked one over another, in a gel holder cassette:

1. 3 x Whatman paper, wetted with Blotting Buffer
2. PVDF-membrane, pre-moistened with methanol to enhance its sucking capacity
3. Polyacrylamide gel
4. 3 x Whatman paper, wetted with Blotting Buffer

The protein transfer was conducted under 0.8-1.5 mA per cm^2 of gel for 2 h in Blotting Buffer.

Blotting Buffer: 25 mM Tris (pH 8.3)
192 mM Glycine
20 % Methanol
0.1 % SDS

Staining of PVDF membranes with Amido Black method

Following the immunoblot procedure, the membranes were incubated for 15 min in Amido Black Staining Solution (ABSS) while rocking gently, with a subsequent 15-min de-staining in Membrane De-Staining Solution (MDSS) (with an eventual De-staining Solution exchange until clear) while shaking.

ABSS: 10 % methanol
2 % acetic acid
0.2 % Amido Black dye

MDSS: 10 % methanol
2 % acetic acid

Application of primary and secondary antibodies to PDVF membranes

The PDVF membranes where washed once with Washing Buffer und subsequently incubated in Blocking Solution for 1 h at room temperature while rocking, in order to block unspecific protein-binding sites.

Washing Buffer: PBS
0.1 % Tween 20

Blocking Solution: 5 % skim milk powder
PBS with 0.1 % Tween 20

Washing of the membranes three times with Washing Buffer followed.

Anti-(493-520), -(480-488), -(412-423), and -(493-511) antibodies, diluted in various concentrations with Blocking Solution, were used as primary antibodies. The membranes were incubated with the diluted antibody for 1 h at room temperature or overnight at 4 °C while rocking gently. Subsequently, the membranes were washed three times with Washing Buffer, followed by their incubation- in analogy to the primary antibody- with a secondary antibody coupled to horseradish peroxidase. This antibody enabled, due to its peroxidase activity, a detection of the reaction between the primary antibody and the desired protein, in form of bands on an X-ray film. Following the incubation with the secondary antibody, the membranes were washed three times with Washing Solution.

Development of X-ray films

In order to prepare the membranes for developing in a dark chamber, Roti-Lumin Ultra Chemiluminescence Substrate (1) and Enhancer (2) Solutions were applied successively, followed by a 1-min incubation to let the solutions mix and react. The membranes were placed in a transparent plastic sheet inside a developing case, followed by a development of the X-ray film in the dark chamber.

Subcellular protein fractionation

Examination of the reaction pattern of different anti-WSS antibodies with certain subcellular protein fractions was aimed to gain insight into localization and distribution of the major WSS isoforms within the cell.

Subcellular Protein Fractionation Kit was used for separation of diverse subcellular protein fractions. The principle of this method is a stepwise preparation of these fractions by addition of a distinct agent (extraction buffer) to the pellet with subsequent incubation and centrifugation of the sample. As a result, the extracted fraction is found in the supernatant, whereas the pellet is subjected to a next fractionation step with a different extraction buffer.

The experiment was conducted in line with the protocol provided by the manufacturer. The incubation and centrifugation steps were performed at 4 °C unless specified otherwise. A pellet of 2 x 10^6 HeLa cells, pre-washed twice with ice-cold PBS,

served as the source of protein extract. The following buffers were provided with the kit and applied in a stepwise manner to the pellet: Cytoplasmic Extraction Buffer (CEB) to release soluble cytoplasmic contents by causing selective cell membrane permeabilization; Membrane Extraction Buffer (MEB), which dissolved plasma, mitochondria, and endoplasmic reticulum/Golgi membranes; Nuclear Extraction Buffer (NEB), yielding soluble nuclear fraction (SNF) (nuclear membranes)*;* Micrococcal Nuclease (≥ 100 U/µl), which, in combination with 100 mM $CaCl_2$ and NEB, extracted chromatin-bound nuclear fraction (CBNF), and Pellet Extraction Buffer (PEB) to isolate cytoskeletal proteins. 100x Halt Protease Inhibitor Cocktail was added to each extraction buffer in volume ratio of 1:100 immediately before use. This step was required to prevent proteolysis during handling. Volume ratios of the cells and above-mentioned buffers are given in Table 13.

Table 13. Cell and buffer volume ratios for subcellular protein fractionation

Components	Packed cell volume	CEB	MEB	NEB	NEB+ $CaCl_2$+ MNase	PEB
Volume ratio	1	10	10	5	5	5

Protein amount in the cell fractions was determined by performing BCA Assay.

To examine if the subcellular fractionation had been successful, control Western blots of the obtained fractions with specific antibodies against different cellular structures were conducted. The following antibodies, diluted with Blocking Solution, were used: antibody against the mitochondrially encoded cytochrome c oxidase II (MTCO2) (1:1000) for the cytoplasm; anti-alpha 1 sodium/potassium-ATPase (1:1000) and anti-calreticulin (1:1000) antibodies for the plasma and endoplasmic reticulum membranes; anti-lamin A antibody (1:1000) for the SNF; anti-fibrillarin antibody (1:400) for the CBNF; and anti-actin antibody (1:1000) for the cytoskeleton. As 2° antibody, Goat Anti-Mouse IgG H&L (HRP) antibody, diluted 1:5000 with Blocking Solution, was used. Western blots of the protein fractions with different anti-WSS antibodies, diluted with Blocking Solution: anti-WSS(493-520) (1:100), anti-WSS(80-88) (1:25), anti-WSS(412-423) (1:50), and anti-WSS(493-511) (1:25) were performed in order to examine the subcellular redistribution of α- and β-WSS. Amersham ECL Rabbit IgG, HRP-linked antibody (from donkey) was used as 2° antibody in dilution of 1:1000. 10 % Bis-Tris gel pockets were loaded with 15 µg protein, 5 µl SDS buffer and H_2O ad 20 µl each.

Isolation of nuclear extract

HeLa cells were harvested from cell culture media by centrifugation for 15 min at 1000 rpm and room temperature. The supernatant was discarded. The pellets (approximately 2 x 10^8 cells per pellet) were resuspended in five volumes of ice-cold, sterile PBS each and centrifuged for 15 min at 1000 rpm and 4 °C. Subsequently, the pellets were resuspended in five volumes of Buffer A, followed by a 15-min incubation on ice.

Buffer A:	10 mM HEPES
(pH 7.9, 4 °C)	1.5 mM $MgCl_2$
	10 mM KCl
	0.1 mM EDTA
	0.5 mM DTT

The pellets were collected again by centrifugation and resuspended in two volumes of Buffer A. Cell disruption was performed by pipetting the suspension up and down through a 27 G needle at least ten times, with a subsequent 15-min centrifugation at 800 rpm and 4 °C in a 15-ml Corex glass. The pellet contained the nuclear fraction. The supernatant (i.e. the cytoplasmic fraction) was transferred into a clean Corex glass tube and, after an obtainment of a 250-µl sample, centrifuged for 30 min at 25000 rpm and 4 °C. The resulting supernatant was frozen in liquid nitrogen at -70 °C in aliquots. The pellet was resuspended in 550 µl RIPA buffer with protease inhibitors and stored at -70 °C. Protein amount in each of the obtained fractions was measured using BCA Assay.

Isolation of nuclear envelopes

The pelleted nuclei were centrifuged for 2 min at 4000 rpm and 4 °C and the supernatant discarded. 1 ml freshly prepared Lysis Buffer was added dropwise to the pellet, while constantly vortexing the tube.

Lysis Buffer:	0.1 mM $MgCl_2$
	1 mM DTT
	1x protease inhibitors (leupeptin/pepstatin A, phenylmethylsulfonyl fluoride (PMSF), and aprotinin)
	5 µg/ml DNase I (volume ratio 1:100)
	5 µg/ml RNase A (volume ratio 1:100)

The suspension was immediately transferred into a rounded 15-ml Falcon tube, followed by a dropwise addition of 4 ml cold Extraction Buffer while vortexing the tube.

Extraction Buffer: 10 % (w/v) sucrose
(pH 8.5) 20 mM triethanolamine
0.1 mM $MgCl_2$
1 mM DTT
1x protease inhibitors

The protease inhibitors and DTT were added immediately before use. A 15-min incubation at room temperature followed, with a subsequent 5s-sonication of the extract eight times with a 10-s cooling period after each burst. Subsequently, the sonicated extract was underlayed with 4 ml ice-cold Sucrose Cushion Solution (SCS) and centrifuged for 15 min at 4000 rpm and 4 °C in a 15-ml Corex glass tube.

SCS: 30 % (w/v) sucrose
(pH 7.5) 20 mM Triethanolamine
0.1 mM $MgCl_2$
1 mM DDT
1 x protease inhibitors

The protease inhibitors and DDT were added immediately before use.

The resulting supernatant (i.e. the fraction above the sucrose cushion) contained the nucleoplasmic extract. The pellet (i.e. the fraction beneath the sucrose cushion) comprised the nuclear envelopes stripped of ribosomes and contaminating chromatin.

Protein amount in the obtained fractions was measured using BCA Assay. The nuclear envelopes pellet was resuspended in 200 µl RIPA buffer and frozen at -70 °C for storage.

Isolation of nucleoli

All steps were performed on ice or at -4 °C to prevent destruction of the nucleoli. The nuclear pellet was resuspended in 3 ml S1 Solution by pipetting up and down.

S1 Solution: 0.25 M sucrose
10 mM $MgCl_2$

Subsequently, the suspension was layered over 3 ml S2 Solution and centrifuged for 5 min at 2500 rpm, followed by a resuspension of the resulting “clear nuclear” pellet in 3 ml S2 Solution.

S2 Solution: 0.35 M sucrose
0.5 mM $MgCl_2$

The nuclear suspension was then subjected to six cycles of sonication of 10 s each with a 10-s interval after each burst. Subsequently, the sonicated extract was layered over 3 ml S3 Solution, followed by a 10-min centrifugation at 3500 rpm.

S3 Solution: 0.88 M sucrose
0.5 mM $MgCl_2$

The retained pellet contained the nucleoli, whereas the supernatant corresponded to the nucleoplasmic fraction. The nucleolar pellet was resuspended in 0.5 ml S2 Solution, followed by a 5-min centrifugation at 2500 rpm. The resulting pellet contained highly purified nucleolar extract and was used for further experiments or resuspended in 0.5 ml S2 Solution and stored at -80 °C.

2.6.2 Specific biochemical methods of protein characterization

Detachment of α- and β-WSS from nuclear envelopes with NaCl and urea

The results of the cell protein fractionation and immunofluorescence suggested that the WSS protein is part of or connected to the nuclear envelope. Following this hypothesis, I examined the biochemical properties of this possible attachment.

The nuclear envelopes were extracted following the protocol described above, resuspended in 1 ml Hypotonic Lysis Buffer (HLB), with a subsequent 10-min incubation on ice, and aliquoted á 250 µl into eight Eppendorf tubes.

HLB: 5 mM HEPES
(pH 7.4) 10 mM $MgCl_2$
1 mM KCl

The tubes were centrifuged for 1 min at 12000 rpm and 4 °C, followed by a disposal of the supernatant. The retained pellets were suspended in 100 µl following buffers each (PB = phosphate buffer):

Buffer 0:	PB	Buffer 4:	PB
	Ø NaCl/urea		250 mM NaCl
Buffer 1:	PB	Buffer 5:	PB
	50 mM NaCl		500 mM NaCl
Buffer 2:	PB	Buffer 6:	PB
	100 mM NaCl		1 M NaCl
Buffer 3:	PB	Buffer 7:	PB
	150 mM NaCl		8 M urea

A 15-min incubation at 4 °C while shaking, with a subsequent centrifugation for 15 min at 15000 x g and 4 °C, followed. The supernatants were retained after taking 20-µl samples of each ("SUP 0-7"), whereas the pellets were resuspended in 1:1 ratio in RIPA Buffer with protease inhibitors. All fractions were analyzed by immunoblotting with anti-WSS(493-520) (1° antibody) diluted 1:100 with Blocking Solution and Amersham ECL Rabbit IgG, HRP-linked whole antibody (from donkey) (2° antibody) diluted 1:1000. As a quality control, Western blot of the same fractions was performed with anti-lamin A (nuclear envelope marker) (1:250) and anti-fibrillarin (nucleolar marker) (1:100) antibodies. As 2° antibody, Goat Anti-Mouse IgG H&L (HRP) antibody diluted 1:5000, was used. Prior to the Western blot, 10 % Bis-Tris gels were loaded with 10 µg protein/lane.

DNase and RNase digestion of nuclear and nucleolar extract

In order to examine the connection of WSS to DNA and RNA, the nuclear and nucleolar pellets were digested with 1 µl DNase I (2 U/µl), 1µl RNase A (4 mg/ml), or both. For the hydrolysis with DNase I, 10 µg nuclear or nucleolar extract were resuspended in 1x DNase I Reaction Buffer ad 100 µl. 1 µl DNase was added to the sample, followed by an incubation for 10 min at 37 °C and a 15-s vortexing. 1 µl 0.5 M EDTA was added to a final concentration of 5 mM. A 10-min heat inactivation at 75 °C followed. For the hydrolysis with RNase A, 2 µl ready-to-use RNase A solution were mixed thoroughly with 10 µg protein extract and sterile, distilled H_2O ad 100 µl. An incubation for 10 min at 37° C and a 15-s vortexing followed. Finally, the RNase was inactivated by a 10-min incubation in a heat block at 75°C (Table 14).

Table 14. Hydrolysis of nuclear/nucleolar extract with DNase I (101-µl sample) and RNase A (102-µl sample)

Components	***Mass or volume***
DNase hydrolysis	*101 µl*
Nuclear/nucleolar extract	10 µg
1x DNase I Reaction Buffer	ad 100 µl
DNase I (2 U/µl)	1 µl
RNase hydrolysis	*102 µl*
Nuclear/nucleolar extract	10 µg
RNase A solution (4 mg/ml)	2 µl
Sterile, distilled H_2O	ad 100 µl

For the combined hydrolysis, both enzymes were added simultaneously at the protein and enzyme proportions described above. Following the hydrolysis and heat inactivation of DNase/RNase, a centrifugation for 15 min at 15000 x g and room temperature was performed. The pellets were resuspended 1:1 in RIPA buffer with protease inhibitors. The pellet suspensions and supernatants were loaded on 10 % Bis-Tris gels (10 µg protein/lane) and examined by immunoblotting with anti-WSS(493-520) antibody (1° antibody) diluted 1:100 with Blocking Solution and Amersham ECL Rabbit IgG, HRP-linked antibody (from donkey) (2° antibody) diluted 1:1000. As a quality control, Western blot of the pellets and supernatants with Anti-Lamin A (1:250) and Anti-Fibrillarin (1:100) antibodies was performed. As 2° antibody, Goat Anti-Mouse IgG H&L (HRP) diluted 1:5000, was used.

Analysis of a posttranslational modification of the WSS protein: deglycosylation and dephosphorylation

To test whether the 80-kDa protein is a posttranslationally modified DCAF17, I performed dephosphorylation and deglycosylation of the nuclear extract.

Deglycosylation

Glycosylation is one of the most common post-translational modifications of proteins. Glycans can be attached to asparagine residues (N-linked glycosylation) or serine/threonine residues (O-linked glycosylation) of proteins. Enzymatic methods of removing the oligosaccharides from glycoproteins include deamination of the asparagine residue to aspartic acid with Peptide N-glycosidase F and removal of the O-linked glycans by Endo-α-N-Acetylgalactosaminidase.

For the enzymatic deglycosylation of nuclear envelopes, Protein Deglycosylation Mix was used. The mix was provided in 50 mM NaCl, 20 mM Tris-HCl (pH 7.5 at 25°C), and 0.1 mM Na2EDTA. It contained following enzymes:

Peptide N-glycosidase F (PNGase F, 500000 U/ml, glycerol free, MW: 36 kDa), which cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins unless α(1-3) core is fucosylated;

Endo-α-N-acetylgalactosaminidase (O-glycosidase, 40000000 U/ml, MW: 147 kDa), which catalyzes the removal of core 1 and core 3 O-linked disaccharides from glycoproteins;

Sialidase (neuraminidase, 50000 U/ml, MW: 43 kDa), which catalyzes the hydrolysis of terminal, non-reducing α2,3, α2,6, and α2,8 linked N-acetylneuraminic acid residues from glycoproteins and oligosaccharides. Without removal of the sialic acid residues, the action of the O-Glycosidase would be blocked, as any modification of the core structures interferes with the function of this enzyme.

β1-4 galactosidase (8000 U/ml, MW: 94 kDa), which catalyzes the hydrolysis of terminal, non-reducing β1-4 linked D-galactopyranosyl residues from oligosaccharides and glycoproteins. Without the removal of those residues the action of O-glycosidase would be blocked.

β-N-acetylglucosaminidase (4000 U/ml, MW: 71 kDa), which catalyzes the hydrolysis of terminal, non-reducing β-N-acetylglucosamine residues from oligosaccharides and glycoproteins[25].

As a substrate control, 0.5 mg α2-HS glycoprotein (fetuin, 10 mg/ml) was used. α2HS is a glycoprotein which contains sialylated N- and O-linked glycans that can be removed by digestion with deglycosylating enzymes. The mix was supplied with 10x Glycoprotein Denaturing Buffer, 10x GlycoBuffer 2, and 10 % NP-40.

In the first step, 100 µg samples of the nuclear extract were dissolved in 2 µl 10x Glycoprotein Denaturing Buffer and H_2O ad 20 µl each, followed by protein denaturation by means of a 10-min incubation in a heating block at 100 °C. For the fetuin A deglycosylation, 2 µl fetuin A (10 mg/ml) were diluted in 1 µl 10x Glycoprotein Denaturing Buffer (GDB) (0.5% SDS*;* 40 mM DTT) and 5 µl H_2O. Subsequently, the samples were chilled on ice and centrifuged for 10 s at full speed. A 20-µl sample for the first step of deglycosylation was composed as follows:

Table 15. Step 1 of deglycosylation of nuclear extract (20-µl sample) and fetuin (control) (8-µl sample)

Components	***Mass or volume***
Deglycosylation of nuclear extract	*20 µl*
Nuclear extract	100 µg
10x GDB	2 µl
Steril, distilled H_2O	ad 20 µl
Deglycosylation of fetuin (control)	*8 µl*
Fetuin (10 mg/ml)	2 µl
10x GDB	1 µl
Sterile, distilled H_2O	ad 8 µl

GDB: 0.5 % SDS

(10x) 40 mM DTT

In the second step, following reagents were added to the reaction samples:

Table 16. Step 2 of deglycosylation of nuclear extract (50-µl sample) and fetuin (control) (11-µl sample)

Components	*Volume*
Deglycosylation of nuclear extract	*50 µl*
Step 1 deglycosylation sample with nuclear extract	20 µl
10x GlycoBuffer 2	5 µl
10 % NP40	5 µl
Deglycosylation Enzyme Cocktail	5 µl
Sterile, distilled H_2O	ad 50 µl
Deglycosylation of fetuin	*11 µl*
Step 1 deglycosylation sample with fetuin	8 µl
10x GlycoBuffer 2	1 µl
10 % NP40	1 µl
Deglycosylation Enzyme Cocktail	1 µl

GlycoBuffer 2: 50 mM sodium phosphate

(pH 7.5, 25 °C)

The samples were mixed gently by pipetting up and down and subsequently incubated for 4 h (nuclear extract) or 1 h (fetuin A) at 37 °C. The native as well as deglycosylated samples were analyzed by immunoblotting with anti-WSS(493-520) (1° antibody) diluted 1:100 with Blocking Solution and Amersham ECL Rabbit IgG, HRP-linked whole antibody (2° antibody) diluted 1:1000. The deglycolysation products (initial sample volume 50 µl, containing 100 or 200 µg protein, 5 µl 10x GlycoBuffer 2, 5 µl 10 % NP40, 2.5/5.0/7.5 µl Deglycosylation Enzyme Cocktail, and H_2O ad 50 µl) were loaded on 10 % Bis-Tris gels. Per lane, either 10 µl (20 µg protein) or 20 µl (40 µg protein) solution were loaded. In case of the negative control, the reaction sample volume was 20 µl, containing 100 µg nuclear extract, 2 µl 10x Glycoprotein Denaturing Buffer, and H_2O ad 20 µl. It was diluted 1:10 with H_2O so that 10 µg protein were loaded per gel lane.

Dephosphorylation

A second widespread type of posttranslational protein modification is addition of phosphate groups to peptide chains (phosphorylation). There are various methods of protein dephosphorylation. Some of the common dephosphorylating enzymes were used in this experiment:

Antarctic Phosphatase (AP), which removes 5' phosphates from DNA/RNA/rNTPs/dNTPs and is used to prevent recircularization of cloning vectors. It was obtained from an *E. coli* strain that carried the *TAB5 AP* gene, originally cloned in plasmid pNI and recloned in plasmid pEGTAB7-4.1[21]. An exemplary 20-µl sample for dephosphorylation of nuclear extract is shown in Table 17.

Table 17. Dephosphorylation of nuclear extract (20-µl sample)

Components	*Mass or volume*
Nuclear extract	100 µg
10x AP Buffer	2 µl
Antarctic Phosphatase	0 (negative control) or 1 µl
Sterile, distilled H_2O	ad 20 µl

AP Buffer: 50 mM Bis-Tris-Propane-HCl
(10x, pH 6, 25 °C) 1 mM $MgCl_2$
0.1 mM $ZnCl_2$

Antarctic Phosphatase was added as last component. The samples were incubated for 15 min at 37 °C.

Protein Phophatase 1 (PP1), which is a Mn^{2+}-dependent 330-amino-acid catalytic subunit of the PP1 α from rabbit skeletal muscle that removes phosphate groups from phosphorylated serine, threonine, and tyrosine residues in proteins. The source of the purchased enzyme was an *E. coli* strain that carried the coding sequence for rabbit skeletal muscle PP1 under the control of the trp-lac hybrid promoter[26]. Instead of performing the p-nitrophenyl phosphate assay, I empirically used 1 µl of PP1 for a 50-µl reaction sample:

Table 18. Dephosphorylation of nuclear extract with PP1 (50-µl sample). *= NEBuffer for Protein MetalloPhosphatases.

Components	*Mass or volume*
Nuclear extract	100 µg
Protease inhibitors	5 µl
10x NEB for PMP*	5 µl
$MnCl_2$	5 µl
PP1	1µl
Sterile, distilled H_20	ad 50 µl

NEB for PMP: 50 mM HEPES
(10x, pH 7.5, 25 °C) 10 mM NaCl

2 mM DTT

0.01% Brij 35

The sample was incubated for 10 min at 30 °C.

T-cell protein tyrosine phosphatase (TC PTP), which is a truncated form of the human TC PTP (residues 1-317) that lacks a C-terminal regulatory domain and can be used to release phosphate groups specifically from phospho-tyrosine residues in proteins. The purchased enzyme was isolated from a strain of *E. coli* which carried a clone expressing the T-cell protein tyrosine phosphatase under the control of the T7 promoter[27]. The contents of a 50-µl sample for TC PTP dephosphorylation of nuclear extract are listed in Table 19. In analogy to dephosphorylation with PP1, 1 µl of TC PTP was used for the 50-µl sample.

Table 19. Dephosphorylation of nuclear extract with TC PTP (50-µl sample). *= NEBuffer for Protein Tyrosine Phosphatases.

Components	***Mass or volume***
Nuclear extract	100 µg
Protease inhibitors	5 µl
10x NEB for PTP*	5 µl
TC PTP	1 µl
Sterile, distilled H_20	ad 50 µl

The sample was incubated for 10 min at 30 °C.

NEB for PTP: 50 mM Tris-HCl

(10x, pH 7.5, 25 °C) 100 mM NaCl

2 mM Na_2EDTA

5 mM DTT

0.01 % Brij 35

The native as well as dephosphorylated nuclear extract samples were analyzed by immunoblotting with anti-WSS antibodies. The AP reaction sample (20 µl) contained 100 µg nuclear extract, 2 µl 10x AP Buffer, 1 µl AP, and H_20 ad 20 µl. For Western blot, it was diluted 1:10 with H_2O and 10 µg protein were loaded per gel lane. The PP1 reaction sample (50 µl) contained 100 µg nuclear extract, 5 µl 1x protease inhibitors, 5 µl 10x NEBuffer for Protein MetalloProteinases, 5 µl $MnCl_2$, 1 µl PP1, and H_20 ad 50 µl. The sample was diluted 1:10 with H_2O and 10 µg protein were loaded per gel lane. The TC PTP reaction sample (50 µl) contained 100 µg nuclear extract, 5 µl 1x protease inhibitors, 5 µl 10x NEBuffer for Protein Tyrosine Phosphatases, 1 µl TC PTP, and H_20 ad 50 µl.

After 1:10 dilution with H_2O, 10 µg protein were loaded per gel lane. The negative control samples (100 µg nuclear extract and H_20 ad 20 µl) were diluted 1:10 with H_2O (10 µg protein) for gel electrophoresis and Western blot. Anti-WSS(493-520) diluted 1:100 with Blocking Solution was used as 1°, whereas Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) diluted 1:1000, as 2° antibody.

2.6.3 Immunofluorescence microscopy

The goal of this experiment was to examine the intracellular localization of DCAF17 protein, using the self-established anti-WSS(493-520) antibody, marker-antibodies specific for distinct subcellular structures as well as fluorescent antibodies, which enabled a later examination using fluorescent microscope. The marker- and fluorescent antibodies were centrifuged for 2 min at 13000 rpm and room temperature prior use, in order to remove precipitated antibody conjugate that would disturb their reaction pattern. Supernatants were subsequently diluted at different ratios with PBS + 0.5 % BSA.

In the first step, cell medium was removed and the cells (HeLa, HEK, or HepG2) were washed twice with a sterile filtered 3-Morpholinopropansulfonic Acid (MOPS) Buffer.

MOPS Buffer:	200 mM MOPS
(10x, pH 7.0)	50 mM NaOAc
	10 mM EDTA (pH 8.0)

Subsequently, the cells were overlayed with 4 % paraformaldehyde, followed by a 12-min incubation. After washing the cells twice with PBS, 40 mM glycine (pH 7.4) was applied to the cells with a subsequent 12-min incubation. Cell membrane permeabilization for 10 min with 0.25 % Triton-X followed. Finally, the cells were washed with PBS, followed by an addition of 400 µl of the first antibody, anti-WSS(493-520), diluted 1:5, 1:10, 1:25, and 1:50 with PBS + 0.5 % BSA, into each of the four chambers, and an incubation in a dark wet chamber overnight at 4 °C. The following day, the cells were washed three times with PBS, followed by an addition of 450 µl of the first fluorescent antibody, Alexa Fluor 594, diluted 1:400 with PBS + 0.5 % BSA, and a subsequent 2-h incubation at room temperature in the dark wet chamber. Finally, the cells were washed six times with PBS. An addition of 475 µl marker antibody, Anti-Fibrillarin, diluted 1:400 with PBS + 0.5 % BSA and a subsequent incubation overnight at 4 °C in the dark wet chamber followed. The following day, the cells were washed three times

with PBS and subsequently overlayed with 240 µl of the second fluorescent antibody, Alexa Fluor 488, diluted 1:200 with PBS + 0.5 % BSA, and incubated for 2 h at room temperature in the dark wet chamber. Subsequently, the cells were washed six times with PBS. The plastic chambers were removed from the slides and the fixed cells were embedded by a dropwise addition of VECTASHIELD Mounting Medium with DAPI and covered with transparent slides. The edges of the slides were sealed with a nail polish and left to dry in a dark drawer. Finally, the cells were examined under the Zeiss Axioplan 2 microscope equipped with standard filter sets for FITC, TRITC, and DAPI.

3 RESULTS

3.1.1 Cloning and expression of WSS(493-520) in pET28a(+) with subsequent affinity purification of anti-WSS(493-520) antibodies

Cloning of WSS(493-520) into pET28a(+)

The WSS(493-520) gene sequence was cloned into the pET28a(+) plasmid, using *NdeI* and *NotI* restriction sites. *WSS(493-520)* was inserted next to the N-terminal His-tag sequence of pET28a(+) (Figure 5). This enabled the later expression of the recombinant WSS(493-520)/His-tag protein in *E. coli* cells.

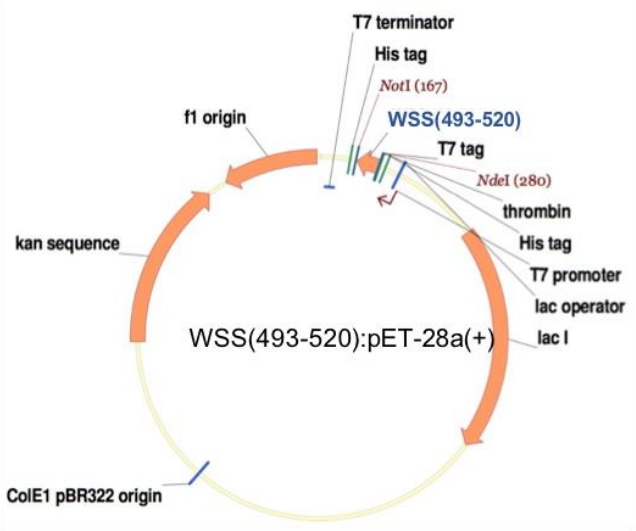


Figure 5. WSS(493-520):pET-28a(+). WSS(493-520) coding sequence was cloned into pET28a(+) juxtapositionally to His-tag coding sequence, enabling a later expression of the recombinant WSS(493-520)/His-tag protein in *E. coli*. Both the insert and the plasmid were cut with Nde I and Not I restriction enzymes.

Expression of WSS(493-520)/His-tag in E. coli

The liquid culture of Rosetta 2 *E. coli* cells transformed with WSS(493-520):pET28a(+) was monitored hourly for expression of the recombinant WSS(493-520)/His-tag peptide (Figure 6). After the addition of 0.5 ml 1M isopropylthiogalactoside (IPTG) to the cell culture to initiate protein expression, an over-time increase in expression of a 5-kDa protein was observed. 5 kDa correspond to the MW of WSS(493-520)/His-tag peptide.

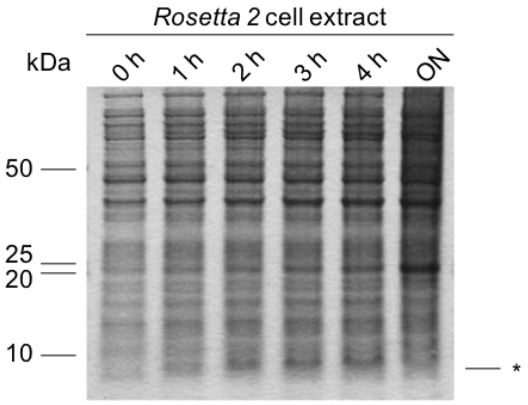


Figure 6. WSS(453-520)/His-tag expression in *E. coli*. Tris-Tricine polyacrylamide gel stained with Coomassie dye shows expression of the recombinant WSS(493-520)/His-tag peptide (*, 5 kDa) in a liquid *E. coli* Rosetta 2 cell culture enriched with chloramphenicol and kanamycin. The gel depicts an absent WSS(493-520)/His-tag expression at 0h as well as an initiation of protein expression after the addition of 1M IPTG (from 1h) with an expression increase over time (1h - overnight (ON)). Loading mass: 15 µg protein/lane. Ladder: PageRuler Unstained Protein Ladder.

Coupling and affinity purification of WSS(493-520)/His-tag on Ni-IDA agarose

Figure 7 shows the WSS(493-520)-peptide fractions, affinity-purified on Ni-IDA Agarose. The input (IP) contained the suspension of supernatant of the WSS(493-520):pET28a(+)-transformed Rosetta 2 cell lysate and Ni-IDA agarose beads, whereas the flow-through (FT) comprised the fraction unbound to the Ni-IDA agarose, obtained after transferring the peptide-beads suspension into a gravity-flow column. Electrophoretic protein separation on a Tris-Tricine polyacrylamide gel revealed bands at 5 kDa in the input and eluted fractions, which correspond to MW of the WSS(493-520)/His-tag peptide.

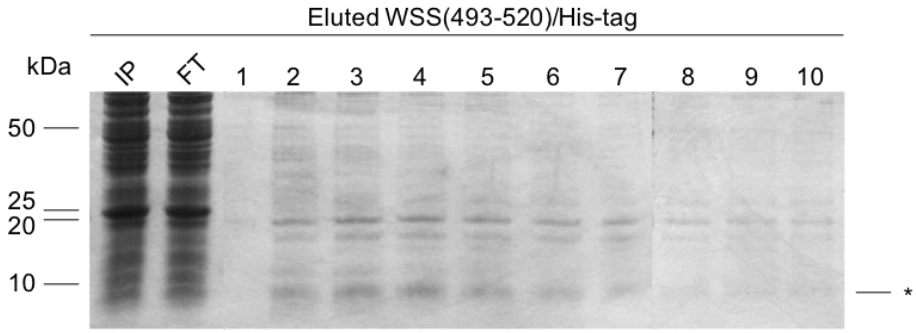


Figure 7. Affinitity purification of recombinant WSS(493-520)/His-tag peptide on Ni-IDA Agarose column. Tris-Tricine polyacrylamide gel stained with Coomassie dye shows input to the resin (IP), flow through/unbound proteins (FT) as well as the eluted WSS(493-520)/His-tag peptide (*, 5 kDa) in fractions 2-10. No protein was detected in fraction 1. Loading mass: 15 µg protein/lane. Ladder: PageRuler Unstained Protein Ladder.

Affinity purification of anti-WSS(493-520) antibodies

The WSS(493-520)/His-tag peptide was covalently coupled to the CNBr-Sepharose 4B column and used for affinity purification of specific anti-WSS(493-520) antibodies from a serum of a previously WSS-immunized rabbit. Figure 8 depicts the following fractions, electrophoretically separated on a 10 % Bis-Tris polyacrylamide gel: input (IP), which contained serum of a WSS-immunized rabbit; flow-through (FT), which comprised the unbound fraction after incubation of the rabbit serum with the WSS(493-520)/CNBr-Sepharose beads and transfer of the serum/WSS(493-520)/CNBr-Sepharose suspension into a gravity-flow column; as well as eluted anti-WSS(493-520) antibody fractions (1-4). The highest amount of eluted antibodies was present in fractions 3 and 4. The 25-kDa bands correspond to antibody light chain monomers, whereas the 50-kDa bands correlate with heavy chain monomers. Other bands seen on the gel may represent common plasma proteins, such as α1-antitrypsin (54 kDa, strongest signal in fractions 3 and 4), albumin (67 kDa, strongest signal in fractions 1 and 2), and transferrin (80 kDa, strongest signal in fractions 2 and 3).

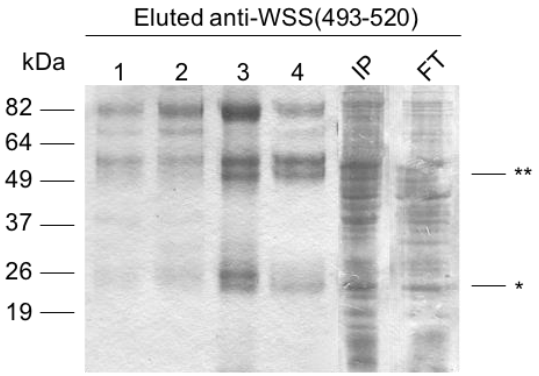


Figure 8. Affinity purification of anti-WSS(493-520) antibody on CNBr-Activated Sepharose column. The figure shows the eluted anti-WSS(493-520) antibody (fractions 1-4) as well as input to the resin (IP), i.e. serum of a WSS-immunized rabbit and flow through (FT), i.e. unbound protein, separated on a 10 % Bis-Tris polyacrylamide gel stained with Coomassie dye. The 25-kDa bands (*) correspond to antibody light chain monomers, the 50-kDa bands (**) to antibody heavy chain monomers. Fractions 3 and 4 contain the highest amount of the eluted antibody. Other bands seen on the gel could represent plasma proteins, such as α1-antitrypsin (54 kDa), albumin (67 kDa), and transferrin (80 kDa). Loading mass: 15 µg protein/lane. Ladder: BenchMark Pre-Stained Protein Standard.

The specificity of the newly eluted polyclonal anti-WSS(493-520) antibodies was examined by immunoblot (Western blot) (Figure 9). Christoph Roscher investigated WSS expression in different cell lines by real-time PCR and detected the highest expression of the WSS protein in HeLa cells. Based on these results, I used HeLa cells to examine the specificity of anti-WSS peptide antibodies. As previously mentioned, the estimated MW of β-WSS is 65 kDa, whereas the α-WSS-isoform has an expected MW of 27 kDa. The WSS(493-520) epitope is part of both α- and β-WSS. Therefore, in case of anti-WSS(493-520) antibody, I expected an appearance of the signal at 27 and 65 kDa. Both isoforms were best seen on immunoblots with anti-WSS(493-520) antibody concentration of 1:100. Interestingly, in our experiments, including the Western blot pictured below, I repeatedly noticed a strong reaction signal between the anti-WSS antibodies and an 80-kDa protein. The origin of this protein is unclear. I hypothesized that it might be a previously unknown third major WSS isoform or a posttranslationally modified α- or β-WSS. In the further chapters of this work, I will describe different approaches to investigate this 80-kDa protein.

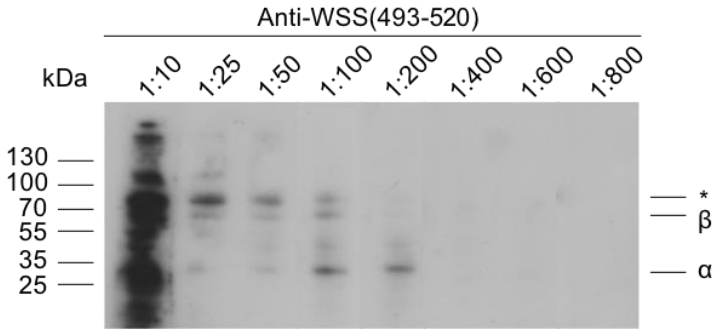


Figure 9. Western blot of HeLa cell protein extract with anti-WSS(493-520). The Western blot shows an unspecific signal at primary antibody (1° Ab) concentration of 1:10; a specific signal at 27 kDa (α-WSS) at 1° Ab concentrations of 1:25 and 1:50 (both weak) as well as 1:100 and 1:200; a specific signal at 65 kDa (β-WSS) at 1° Ab concentrations of 1:25, 1:50, 1:100, and (very weak) 1:200. Additionally, a signal at 80 kDa (possibly a posttranslationally modified WSS protein or a third major WSS isoform) was detected at 1° Ab concentrations of 1:25, 1:50, 1:100, and (very weak) 1:200. No signal was detected at 1° Ab concentrations of 1:400, 1:600, and 1:800. 1° Ab: anti-WSS(493-520) at varying concentrations; secondary (2°) Ab: Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) (1:1000). Loading mass for a 10 % Bis-Tris gel: 15 µg protein/lane. Ladder: PageRuler Plus Prestained Protein Ladder.

3.1.2 Affinity purification of anti-WSS(80-88), -(412-423), and -(493-511) antibodies

At variance with the recombinant WSS(493-520) peptide, three other epitopes: WSS(80-88), WSS(412-423), and WSS(493-511) were purchased as synthetic peptides with cysteine added to (WSS(80-88) and WSS(412-423)) and lysine present at (WSS(493-511)) their N-termini, which allowed their coupling to affinity columns.

In analogy to the procedure with the WSS(493-520) peptide, anti-WSS(80-88), -(412-423), and -(493-511) antibodies were affinity purified from a serum of a WSS-immunized rabbit.

Affinity purification of anti-WSS(80-88) and -WSS(412-423) antibodies on SulfoLink Couplin Resin

As mentioned before, the sulfhydryl group of the additional N-terminal cysteine allowed coupling of WSS(80-88) and WSS(412-423) peptides to the SulfoLink Column. Figure 10 A and B shows following protein fractions, electrophoretically separated on a 10 % Bis-Tris polyacrylamide gel: eluted anti-WSS(80-88) and -WSS(412-423) antibodies (1-4); input (IP), containing serum of a WSS-immunized rabbit; and flow-through (FT), comprising the unbound fraction after transfer of the WSS-immunized rabbit serum into the peptide-bound Sulfolink Column. The 25-kDa bands (*) correspond

to antibody light chain monomers, whereas the 50-kDa bands (**) to heavy chain monomers. Fractions 3 and 4 of both elutions contained the majority of the purified antibody. Bands at ~67 kDa, with the strongest signal in fractions 1 and 2 of the eluted anti-WSS(80-88) and fractions 3 and 4 of anti-WSS(412-423), may correspond to albumin, whereas those at ~54 kDa (strongest signal in fractions 3-4 of anti-WSS(80-88) and anti-WSS(412-423)) to α1-atitrypsin and at ~80 kDa (strongest signal in fractions 3 and 4 of both antibody elutions) to transferrin.

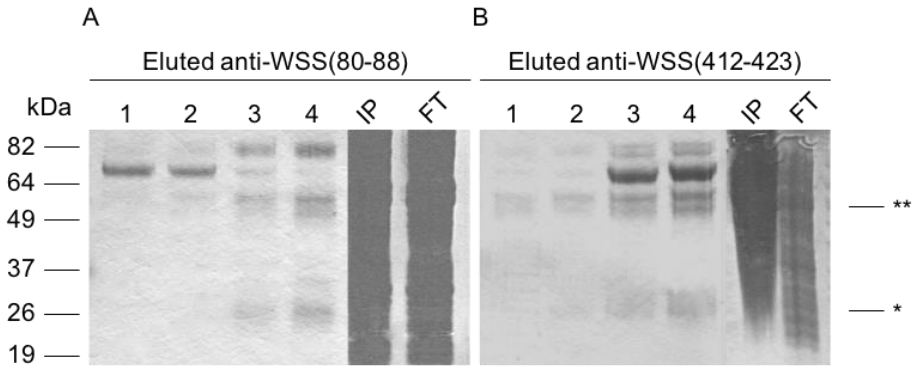


Figure 10. Affinity purification of anti-WSS(80-88) (A) and anti-WSS(412-423) (B) antibodies on SulfoLink Coupling Resin. The figure shows the eluted antibodies (fractions 1-4) as well as input to the resin (IP), i.e. serum of a WSS-immunized rabbit and flow through (FT), i.e. unbound protein, separated on a 10 % Bis-Tris polyacrylamide gels stained with Coomassie dye. The 25-kDa bands (*) correspond to antibody light chain monomers, the 50-kDa bands (**) to antibody heavy chains monomers. Fractions 3 and 4 contain the highest amount of the eluted antibodies. Other bands seen on both gels could represent plasma proteins, such as α1-antitrypsin (54 kDa), albumin (67 kDa), and transferrin (80 kDa). Loading mass: 15 µg protein/lane. Ladder: BenchMark Pre-Stained Protein Standard.

The affinity purified antibodies were further examined regarding their specificity. In analogy to anti-WSS(493-520), I used HeLa cell extract for Western blot experiments with anti-WSS(80-88) and -(412-423). As WSS(80-88) is part of β- but not α-WSS, an appearance of a 65-kDa but not 27-kDa band was expected. Indeed, in contrast to anti-WSS(412-423), a signal at 65 kDa but not at 27 kDa was apparent (Figure 11 A). As expected, anti-WSS(412-423) revealed a signal at both 65 and 27 kDa, WSS(412-423) being part of α- and β-WSS (Figure 11 B). In case of both antibodies, an appearance of the 80-kDa band was notable. Anti-WSS(80-88) revealed a strong signal for β-WSS and the 80-kDa protein at the concentrations of 1:10 and 1:25 as well as a weaker signal at the concentration of 1:50; whereas anti-WSS(412-423) revealed a strong signal for α- and β-WSS at the concentrations of 1:10-1:100 as well as a very weak signal at 1:200 and 1:400, and a strong signal at 80 kDa at the concentrations of 1:10 and 1:25 as well as a weaker signal at 1:50 and 1:100. The signal at ~33 kDa at anti-WSS(412-423)

concentrations of 1:10-1:100 (as well as very weak at 1:200 and 1:400) may be due to remaining anti-GST antibodies from α-WSS/GST-immunized rabbit serum.

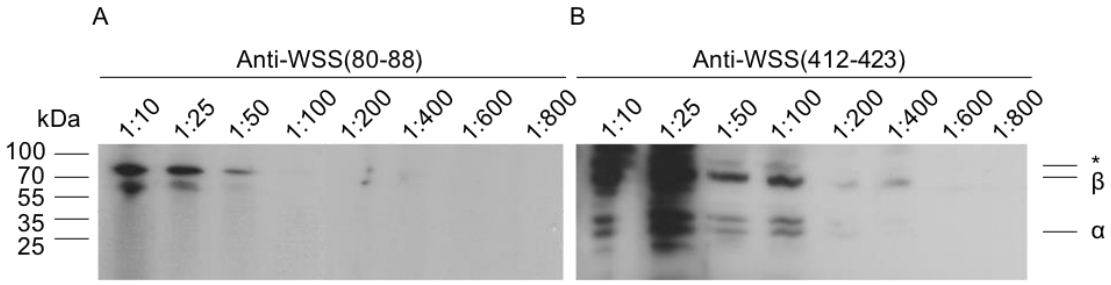


Figure 11. Western blots of HeLa cell protein extract with anti-WSS(80-88) (A) and anti-WSS(412-423) (B) antibodies. The Western blot with anti-WSS(80-88) antibody (A) shows a specific signal at 65 kDa (β-WSS) at primary antibody (1° Ab) concentrations of 1:10, 1:25, and (weak) 1:50. As WSS(80-88) is not part of α-WSS, no signal at 27 kDa (α-WSS) was detected. Signal at 80 kDa was detected at 1° Ab concentrations of 1:10, 1:25, and 1:50. No signal was detected at 1° Ab concentrations of 1:100, 1:200, 1:400, 1:600, and 1:800. The Western blot with anti-WSS(412-423) antibody (B) shows an unspecific signal at 1° Ab concentrations of 1:10 and 1:25; a specific signal at 27 kDa at 1° Ab concentrations of 1:50, 1:100 as well as (very weak) 1:200 and 1:400; a specific signal at 65 kDa at 1° Ab concentrations of 1:50, 1:100 as well as (weak) 1:200 and 1:400, and (very weak) 1:600. Additionally, a signal at 80 kDa was detected at 1° Ab concentrations of 1:50 and 1:100. Another signal, possibly of anti-GST antibodies, was detected at ~33 kDa at 1° Ab concentrations of 1:10, 1:25, 1:50, 1:100 as well as (vey weak) 1:200 and 1:400. 1° Ab: anti-WSS(80-88) and anti-WSS(412-423) at varying concentrations; secondary (2°) Ab: Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) (1:1000). Loading mass for a 10 % Bis-Tris gel: 15 µg protein/lane. Ladder: PageRuler Plus Prestained Protein Ladder.

Affinity purification of anti-WSS(493-511) antibodies on NHS-Activated Agarose

In order to purify the anti-WSS(493-511) antibody, NHS-Activated Agarose matrix was used, which bound the peptide through the amino-group of the additional, N-terminal lysine.

Figure 12 shows the eluted anti-WSS(493-511) antibodies (1-4); input (IP), containing serum of a WSS-immunized rabbit; and flow-through (FT), comprising the unbound fraction after transfer of the WSS-immunized rabbit serum into the peptide-bound NHS-Activated Agarose column. In analogy to the affinity purification of anti-WSS(493-520), -(80-88), and -(412-423) antibodies, the 25-kDa bands represent the antibody light chain monomers, whereas the 50-kDa bands correspond to heavy chain monomers. Fraction 3 contained the largest amount of eluted antibody.

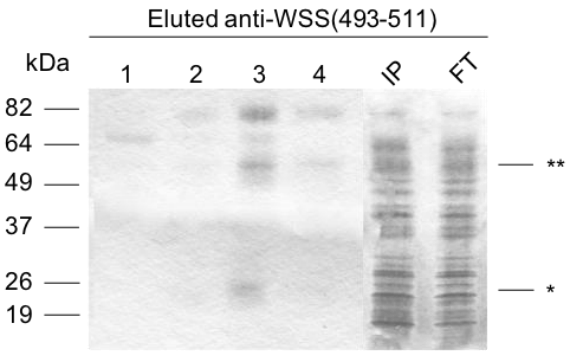


Figure 12. Affinity purification of anti-WSS(493-511) antibody on NHS-Activated Agarose resin. The figure shows the eluted anti-WSS(493-511) antibody (fractions 1-4) as well as input to the resin (IP), i.e. serum of a WSS-immunized rabbit and flow through (FT), i.e. unbound protein, separated on a 10 % Bis-Tris polyacrylamide gel stained with Coomassie dye. The 25-kDa bands (*) correspond to antibody light chain monomers, the 50-kDa bands (**) to antibody heavy chains monomers. Fraction 3 contained the highest amount of the eluted antibody. Other bands seen on the gel could represent plasma proteins such as albumin (67 kDa) and transferrin (80 kDa). Loading mass: 15 µg protein/lane. Ladder: BenchMark Pre-Stained Protein Standard.

Next, I performed a Western blot of HeLa cell extract with the anti-WSS(493-511) antibody to examine its specificity and assess its optimal concentration for further experiments. Figure 13 shows the α-WSS signal at the antibody concentrations of 1:10 and 1:25 as well as β-WSS and 80-kDa signals at anti-WSS(493-511) concentrations of 1:10-1:50. Other, unspecific signals of unknown origin were detected at 40 and 50 kDa at antibody concentrations of 1:10 and 1:25 as well as at 100 and 110 kDa at concentrations of 1:10-1:50. This antibody proved to cause the largest amount of unspecific signals from all purified anti-peptide antibodies.

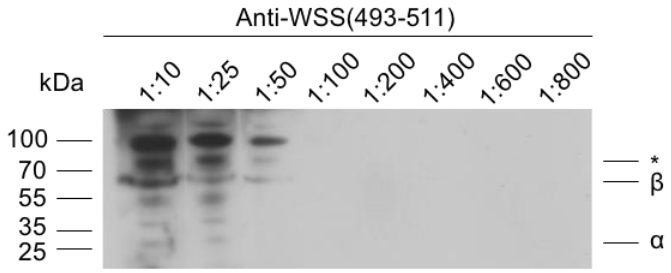


Figure 13. Western blot of HeLa cell protein extract with anti-WSS(493-511). The Western blot shows a signal at 27 kDa (α-WSS) at primary antibody (1° Ab) concentrations of 1:10 and 1:25 as well as a signal at 65 kDa (β-WSS) at 1° Ab concentrations of 1:10, 1:25, and 1:50. The 80-kDa band was detected at 1° Ab concentrations of 1:10, 1:25, and 1:50. Other, most likely unspecific signals were notable at 1° Ab concentrations of 1:10 and 1:25 at ~40, 50 as well as (also at 1° Ab concentration of 1:50) 100 and 110 kDa. No signal was detected at 1° Ab concentrations of 1:100, 1:200, 1:400, 1:600, and 1:800. 1° Ab: anti-WSS(493-511) at varying concentrations; secondary (2°) Ab: Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) (1:1000). Loading mass for a 10 % Bis-Tris gel: 15 µg protein/lane. Ladder: PageRuler Plus Prestained Protein Ladder.

3.2 Determination of the subcellular WSS protein localization and redistribution of its isoforms

3.2.1 Immunofluorescence

Anti-WSS(493-520) antibody shows a strong nucleolar and a dispersed nuclear signal.

As previously mentioned, Alazami et al. reported on a nucleolar localization of the WSS protein, as shown in their immunohistochemistry, in situ hybridization, and immunofluorescence experiments[10]. I performed immunofluorescence on several cell lines (HeLa, HepG, HEK), using anti-WSS(493-520), -(80-88), -(412-423), and -(493-511) antibodies in varying concentrations. The strongest signal was observed using HeLa cells and anti-WSS(493-520) antibody in the concentration of 1:25. Figure 14 shows HeLa cells co-stained with 4',6-diamidino-2-phenylindole (DAPI, blue) (A), which binds to adenine-/thymine-rich DNA; anti-fibrillarin antibody (green) (B); and anti-WSS(493-520) antibody (red) (C), which shows a dispersed nuclear signal but most prominent intensity in the nucleoli. The merged picture (D) demonstrates the co-localization of WSS with fibrillarin in the nucleoli. The additional presence of a more diffuse, non-nucleolar staining suggested that the localization of the WSS protein might not be limited solely to the nucleolus. This suspicion was further investigated through biochemical experiments (see Chapter 3.2.2).

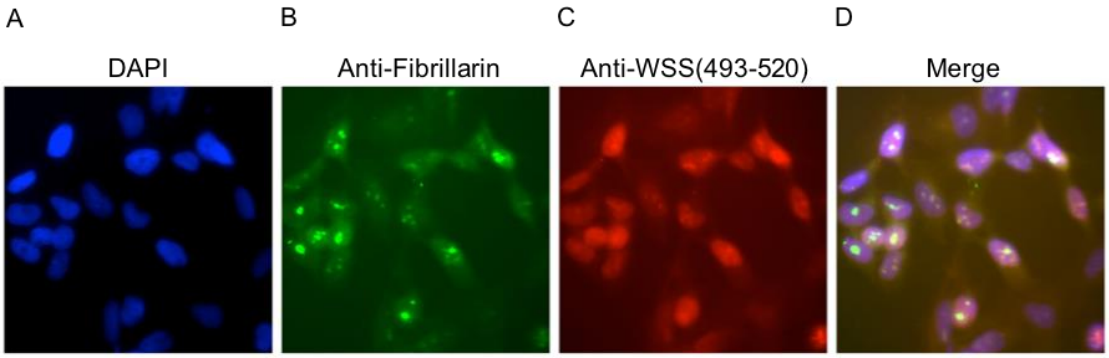


Figure 14. Immunofluorescence on HeLa cells with anti-WSS(493-520) and anti-fibrillarin antibodies. The figure shows a fluorescent microscopy picture of nuclei (blue) after staining with DAPI (A); nucleoli (green) after staining with anti-fibrillarin antibody (Ab) (B); WSS(493-520) (red) after staining with anti-WSS(493-520) (C); and a merge picture of all of the above (D). WSS(493-520), which is part of both α- and β-WSS, shows a significant colocalization with fibrillarin as well as a diffuse signal in the nucleus. 1st Ab: anti-WSS(493-520) (1:25); 2nd Ab: Alexa Fluor 594 (1:400); 3rd Ab: Anti-Fibrillarin (1:400); 4th Ab: Alexa Fluor 488 (1:200).

3.2.2 Biochemical methods of cell fractionation and characterization of DCAF17

WSS protein is localized predominantly in the soluble nuclear fraction.

The cell fractionation experiments were conducted to examine the localization of the WSS isoforms in different cellular compartments.

To ensure an adequate cell fractionation, a control Western blot with antibodies against different cellular structures was performed (Figure 15). Antibody against the mitochondrially encoded cytochrome c oxidase II (MTCO2), which is a part of the mitochondrial respiratory chain, in particular cytochrome c on the mitochondrial inner membrane, revealed a signal in the cytoplasm and membrane fractions at ~26 kDa. Anti-alpha 1 sodium/potassium ATPase (located within the plasma membrane) and anti-calreticulin (located in the lumen of the endoplasmic reticulum and in the cytoplasm) antibodies showed signals in the membrane fraction at ~112 kDa and 63 kDa, respectively. Further, anti-lamin A (constituent of the lamina on the inner side of the nuclear membrane) antibody revealed a signal in the SNF at ~74 kDa, whereas anti-fibrillarin (nucleolar methyltransferase) antibody showed a signal in the CBNF at ~34 kDa. Anti-actin (protein located in the cytoplasm and cytoskeleton, involved in the cell motility) antibody revealed a signal in the cytoplasm and cytoskeleton at ~42 kDa.

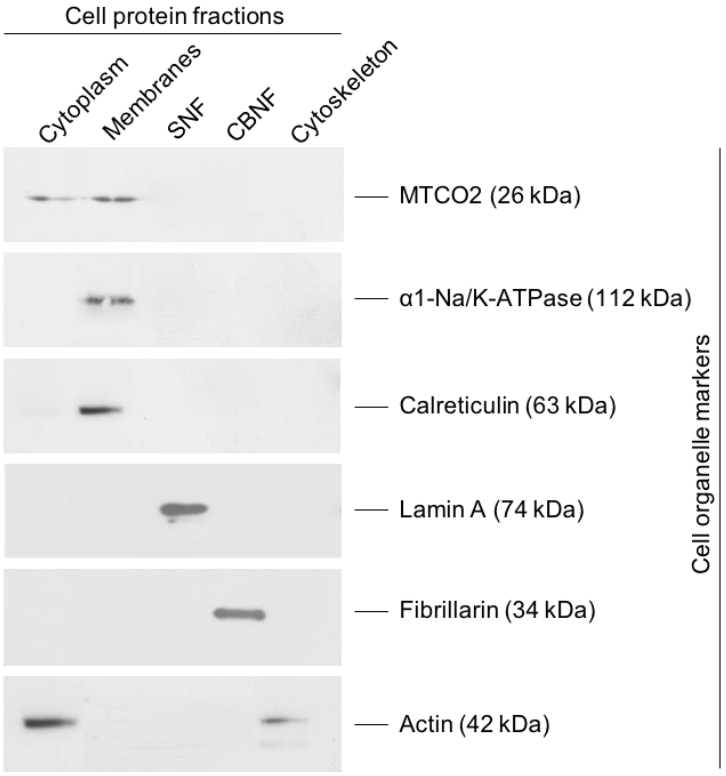


Figure 15. Western blot of cell protein fractions with antibodies against cell organelle markers. Antibody (Ab) against the mitochondrially encoded cytochrome c oxidase II (MTCO2) (1:1000) produced a signal at 26 kDa in the cytoplasm and membranes; anti-Na/K-ATPase Ab (1:1000) at 112 kDa in the membranes; anti-calreticulin Ab (1:1000) at 63 kDa in the membranes and (weak) in the cytoplasm; anti-lamin-A Ab (1:1000) at 74 kDa in the soluble nuclear fraction (SNF); anti-fibrillarin Ab (1:400) at 34 kDa in the chromatin-bound nuclear fraction (CBNF); anti-actin Ab (1:1000) at 42 kDa in the cytoplasm and cytoskeleton (as well as a weak, most likely unspecific band at ca. 40 kDa in the cytoskeleton). Secondary Ab: Goat Anti-Mouse IgG H&L (HRP) (1:5000). Loading mass for a 10 % Bis-Tris gel: 15 µg protein/lane. Ladder: PageRuler Plus Prestained Protein Ladder.

Using the self-established anti-WSS(493-520) antibody as well as the antibodies affinity-purified by means of the commercially obtained peptides (anti-WSS(80-88), -(412-423), and -(493-511)) allowed me to compare the reaction of antibodies directed against an epitope of β- but not α-WSS (WSS(80-88)) as well as against both isoforms (the remaining three antibodies) at the subcellular level. Immunoblot of cell protein fractions with anti-WSS(493-520) antibody showed a β-WSS signal in the CBNF as well as either β-WSS or 80-kDa signal (~70-80 kDa) in the cytoplasm. α-WSS was present mainly in the SNF but also in the membranes fraction and, to a very small amount, in the cytoplasm. Neither of the WSS isoforms nor the 80-kDa protein were detected in the cytoskeleton. Western blot of the cell protein fractions with anti-WSS(80-88) antibody revealed β-WSS and the 80-kDa protein presence in the SNF, CBNF, cytoplasm, and, to a smaller degree, membranes. There was no signal in the cytoskeleton. As expected, no

α-WSS was detected in any of the fractions. Western blot of the cell protein fractions with anti-WSS(412-423) showed β-WSS and the 80-kDa signal predominantly in the SNF, but also in the cytoplasm and, to a small degree, CBNF and (the 80-kDa protein) membranes (very weak signal). α-WSS was present in the cytoplasm fraction. Again, none on the proteins were detected in the cytoskeleton. Western blot of the cell protein fractions using anti-WSS(493-511) antibody showed presence of β-WSS mainly in the SNF but also in the CBNF and membranes, whereas the 80-kDa protein was found in the SNF. α-WSS signal was detected in the membranes fraction, SNF, and CBNF. Similarly to the other three specific antibodies, anti-WSS(493-511) did not show any signal in the cytoskeleton fraction.

It is of note, that the cytoplasm fraction showed weak, probably unspecific, signals in the anti-WSS(493-520) samples at ~20 and 35 kDa, in the anti-WSS(80-88) samples at ~100 kDa, and in the anti-WSS(412-423) samples at ~20 kDa. Other unspecific signals were seen in the anti-WSS(493-520) samples in the membranes fraction at ~35 kDa and in the SNF at ~20 kDa (Figure 16 A and B).

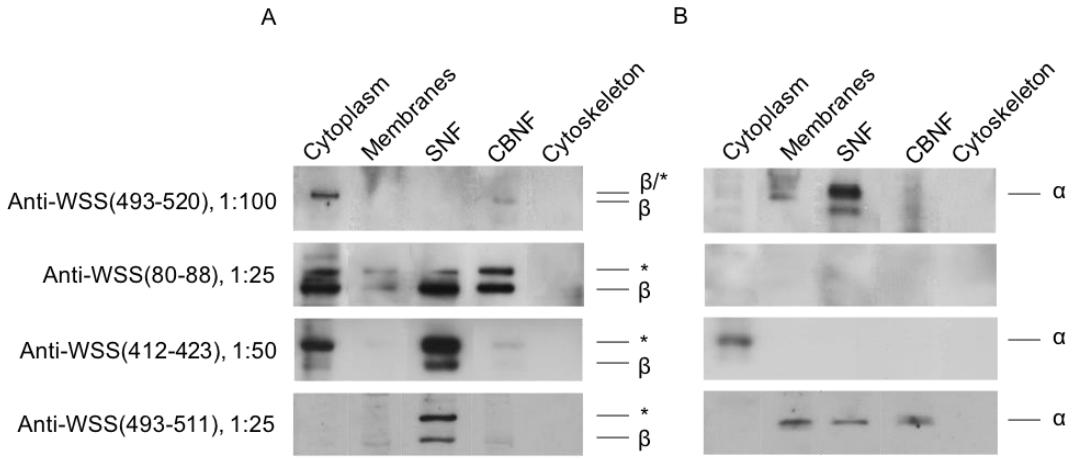


Figure 16. Western blots of cell protein fractions with specific polyclonal anti-peptide antibodies. The blots show a redistribution of WSS isoform β (65 kDa) and the 80-kDa band (*) (A) as well as isoform α (27 kDa) (B) in cell fractions. β-WSS was present in the cytoplasm of the anti-WSS(80-88) and (weak signal) anti-WSS(412-423) samples as well as, possibly (either β-WSS or the 80-kDa band), in the anti-WSS(493-520) sample; in the membranes fraction of the anti-WSS(80-88) and (weak signal) -WSS(493-511) samples; in the soluble nuclear fraction (SNF) of the anti-WSS(80-88), -WSS(412-423), and WSS(493-511) samples; in the chromatin-bound nuclear fraction (CBNF) of the anti-WSS(80-88) as well as (weak signals) anti-WSS(493-520), -WSS(412-423), and -WSS(493-511) samples. α-WSS was present in the cytoplasm of the anti-WSS(412-423) and (weak signal) -WSS(493-520) samples; in the membranes fraction of the anti-WSS(493-520) and -WSS(493-511) samples; in the SNF of the anti-WSS(493-520) and -WSS(493-511) samples as well as in the CBNF of the anti-WSS(493-511) sample. The 80-kDa band was notable in the same fractions and on the same immunoblots as β-WSS except for the cytoplasm of the anti-WSS(493-520) sample, where it is unclear whether the band at ~70-80 kDa represent β-WSS or the 80-kDa protein as well as the CBNF of the anti-WSS(493-511) sample, where the 80-kDa protein was absent. No α-, β-WSS, or 80-kDa signal was detected in the cytoskeleton. It is of note, that the signals in the cytoplasm and membranes were rather unspecific (not shown). Additionally, unspecific signals at ~100 kDa in the cytoplasm of the anti-WSS(80-88) and at ~20 kDa in the SNF of the anti-WSS(493-520) samples were seen. The primary antibodies (Ab) were diluted in ratios shown in the figure. Secondary Ab: Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) (1:1000). Loading mass for a 10 % Bis-Tris gel: 15 µg protein/lane. Ladder: MagicMark XP Western Protein Standard.

Altogether, the cell fractionation experiments revealed, as expected, a WSS localization mainly in the nuclear fractions. However, the predominant location of α- and β-WSS as well as the 80-kDa protein was the soluble, not chromatin-bound nuclear fraction. The presence of the WSS protein isoforms in the CBNF is in favor of the nucleolar localization of WSS, the hypothesis which had also been raised by Alazami et al. The strong signal in the SNF, however, has previously not been reported and prompted me to investigate the possibility of a WSS localization within or its attachment to the nuclear envelope.

WSS-α binds stronger than WSS-β to the nuclear envelope.

Based on the results of the subcellular protein fractionation, which showed the presence of WSS not only in the chromatin-bound, but also (predominantly) in the non-chromatin bound, so called soluble nuclear cell fraction, I decided to investigate the possibility of a WSS localization in the nuclear envelope. I intended to examine whether WSS is present in the nuclear envelope and, if so, how strongly it is attached to it. To this end, nuclear envelopes had been isolated from the nuclear extract of HeLa cells. I used an increasing concentration of NaCl and 8 M urea in phosphate buffer to attempt the detachment of WSS from the nuclear envelope.

As a result, α-WSS was partially detached from the nuclear envelope with 500 mM and 1 M NaCl as well as 8 M urea. A partial detachment of β-WSS was noticed after treatment with 100 mM-1M NaCl, whereas a complete detachment and mobilization into the supernatant was present after treatment with 8 M urea. The 80-kDa protein was detached from the nuclear envelope partially with 50 mM-1 M NaCl and completely with 8 M urea (Figure 17).

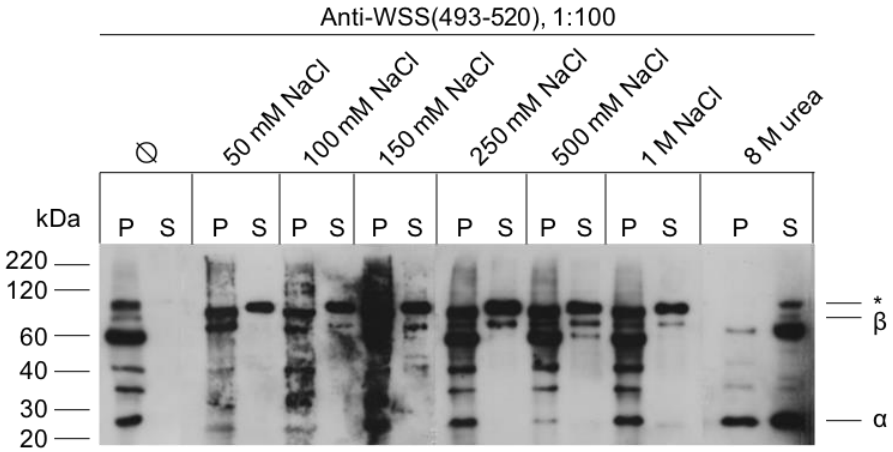


Figure 17. Detachment of WSS protein from nuclear envelopes after treatment with NaCl and urea. The Western blot shows the nuclear envelopes (NE) treated with varying NaCl concentrations as well as 8 M urea. The supernatant (S) of the negative control sample (⃠), treated only with phosphate buffer without NaCl or urea, showed an absence of anti-WSS(493-520) signal, while signals at 27 kDa (α-WSS), 65 kDa (β-WSS), 80 kDa (*) as well as most likely unspecific signals at 37, 40, and 60 kDa were detected in the pellet (P). These unspecific signals were seen in all P as well as in the S of the sample treated with 8 M urea. Additionally, the 60-kDa band was seen in the S of the 500 mM NaCl sample. α-WSS (27 kDa) was partially detached from NE after treatment with 500 mM NaCl (very weak signal in S), 1 M NaCl (weak signal in S), and 8 M urea. β-WSS (65 kDa) was partially detached from the NE after treatment with 100 mM-1 M NaCl and completely mobilized from the P to S after treatment with 8 M urea. The 80-kDa band (*) was partially detached from the NE after treatment with 50 mM-1 M NaCl. 8 M urea caused a complete disappearance of the 80-kDa band from the P and its mobilization into the S. Primary antibody (Ab): anti-WSS(493-520) (1:100); secondary Ab: Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) (1:1000). Loading mass/volume for a 10 % Bis-Tris gel: 15 µg NE pellet protein or 15 µl supernatant/lane. Ladder: MagicMark XP Western Protein Standard.

In order to prove the purity of the nuclear envelope fractions as well as to reassure that the supernatants contained only the detached proteins and not the nuclear envelope, a control Western blot with anti-lamin A and anti-fibrillarin antibodies of all of the examined samples was performed (Figure 18). The results confirm that the isolation of the nuclear envelopes from the nuclear extract was successful, i.e. the pellets contained nuclear envelopes (positive signal of anti-lamin A antibody at 74 kDa) and no nucleoli (no anti-fibrillarin antibody signal at 34 kDa). Furthermore, no anti-lamin A signal was detected in the supernatants at NaCl-concentrations of 0-500 mM. At 1 M NaCl, a beginning anti-lamin signal was seen in the supernatant. This signal was even stronger in the fraction treated with 8 M urea. This indicates that, at NaCl-concentrations of 0-500 mM, WSS (and possibly other proteins) was detached from the nuclear envelope but at the highest (1 M) NaCl concentration as well as after treatment with 8 M urea, the nuclear envelope fraction was disrupted and partially displaced into the supernatant.

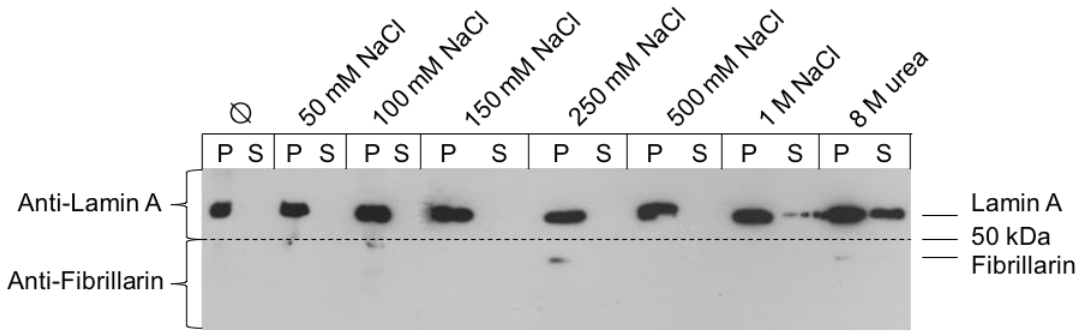


Figure 18. Western blot of nuclear envelopes, treated with NaCl and urea, with anti-lamin-A and anti-fibrillarin antibodies. The blot shows nuclear envelopes (NE) treated with varying NaCl concentrations as well as 8 M urea. Prior to the antibody (Ab) application, the PVDF-membrane was cut in two at ~50 kDa height. Anti-lamin A Ab was then applied to the upper, whereas anti-fibrillarin Ab to the lower membrane part. In the negative control (∅) as well as samples treated with 50-500 mM NaCl, anti-lamin-A signal (74 kDa) was detected in the pellets (P) but not supernatants (S), confirming the detachment of the washed proteins from the NE, while the NE extract remained entirely within the P. After treatment with 1M NaCl and 8 M urea, a partial mobilization of the NE from the P to S occured (positive anti-lamin-A signal in the P and S). No signal of the anti-fibrillarin antibody (34 kDa) was detected except for weak signals in the P of the samples treated with 250 mM NaCl and 8M urea. This indicates that the P indeed contained pure NE extract, except for the two latter samples, in which a mild contamination with nucleoli is suspected. Primary Ab: Anti-Lamin-A (1:250) and Anti-Fibrillarin (1:100). Secondary Ab: Goat Anti-Mouse IgG H&L (HRP) (1:5000). Loading mass/volume for a 10 % Bis-Tris gel: 15 µg P protein or 15 µl S/lane. Ladder: MagicMark XP Western Protein Standard.

Taken together, these results implicate that both the α and β isoform as well as the 80-kDa protein are attached to the nuclear envelope but binding of α-WSS to the nuclear envelope may be stronger than of β-WSS. Although still not knowing the origin of the 80-kDa protein, I could determine its easier (i.e. with a lower concentration of NaCl) detachment from the nuclear envelope than in case of α- and β-WSS.

WSS protein is connected to DNA rather than RNA within the nucleus.

Knowing that the WSS protein is located in the nucleoli and, according to my experiments, is part of or at least attached to the nuclear envelope as well, I further examined this protein by investigating its connection to the nucleic acid.

First, I digested nuclear extract of HeLa cells with DNase, RNase, and both (Figure 19). A protein detected at the apparent MW of ~70 kDa, which can correspond to β-WSS or the 80-kDa protein, was partially mobilized to the supernatant after treatment with DNase and DNase + RNase, but not with RNase alone (due to an air bubble inclusion, the signal at 70 kDa and 27 kDa (α-WSS) in the non-digested nuclear pellets was not distinguishable). No detachment of α-WSS from the nuclear pellet was noticed after digestion with DNase and DNase + RNase. There was also a most likely unspecific, weaker signal detected at ~100 kDa in the pellet of the DNase + RNase sample as well as in the supernatants of the DNase (very weak) and DNase + RNase samples.

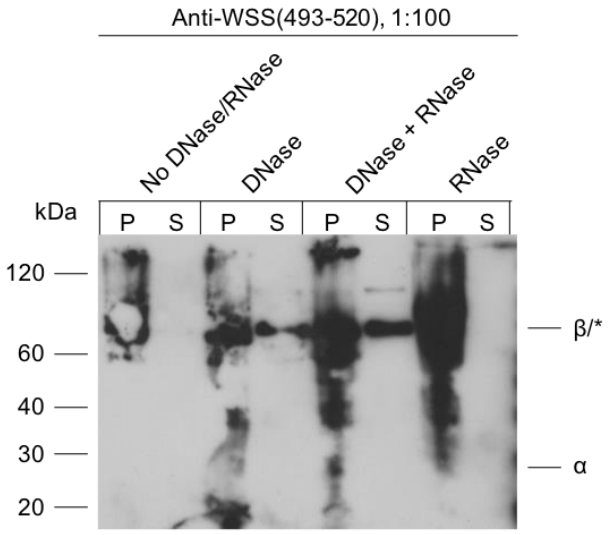


Figure 19. Western blot of nuclear extract, digested with DNase and RNase, with anti-WSS(493-520) antibody. The blot shows the nuclear extract samples treated with DNase, RNase, and both. α-WSS (27 kDa) presence was not evaluable in the pellet (P) and supernatant (S) of the negative control („No DNase/ RNase“) due to an air inclusion. It was detected in all remaining P but in none of the S. A protein at ~70 kDa (β/*), corresponding to either β-WSS (65 kDa) or the 80-kDa protein, was detected in all P as well as in the S of samples digested with DNase and DNase + RNase, suggesting its connection to the DNA in the nuclear extract P and its partial mobilization to the S after the digestion with DNase. A weak, probably unspecific signal was detected at ~100 kDa in the P of the DNase + RNase sample as well as the S of the DNAse (very weak) and DNase + RNase samples. Primary antibody (Ab): anti-WSS(493-520) (1:100). Secondary Ab: Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) (1:1000). Loading mass/volume for a 10 % Bis-Tris gel: 15 µg P protein or 15 µl S/lane. Ladder: MagicMark XP Western Protein Standard.

A quality-control Western blot of the DNase- and RNase-treated nuclear extract was performed with anti-lamin A and anti-fibrillarin antibodies (Figure 20). The anti-lamin A and anti-fibrillarin signal was present in the pellets but not supernatants, indicating that a) the isolation of the nuclei was successful, b) the hydrolysis and centrifugation of the nuclear extract did not cause a mobilization of nuclear envelopes or nucleoli into the supernatant (except for some positivity for lamin A in the supernatant of the sample treated with DNase).

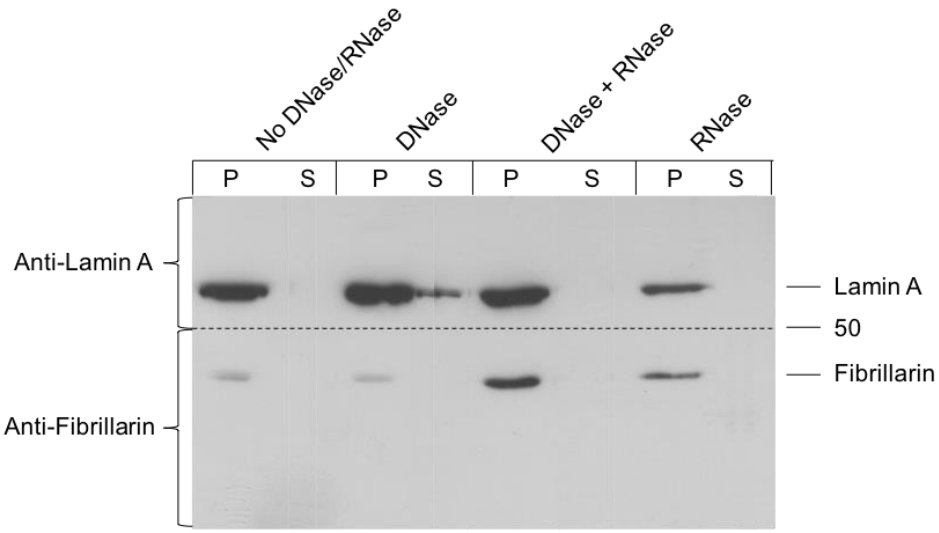


Figure 20. Western blot of nuclear extract, digested with DNase and RNase, with anti-lamin A and anti-fibrillarin antibodies. The blot shows the nuclear extract samples treated with DNase, RNase, and both. Lamin A is a nuclear envelope marker, whereas fibrillarin is a protein located in the nucleolus. Prior to the antibody application, the PVDF-membrane was cut in two at ~50 kDa height. Anti-lamin A antibody (Ab) was then applied to the upper, whereas anti-fibrillarin Ab to the lower membrane part. In the samples not treated with DNase/RNase, anti-lamin A (74 kDa) and anti-fibrillarin (33 kDa) signals were detected in all pellets (P). Some anti-lamin signal was detected in the supernatant (S) of the sample treated with DNase, indicating a partial mobilization of the nuclear envelopes to the S. Neither the anti-lamin A nor the anti-fibrillarin signal was detected in the S of other samples, confirming the absence of the nuclear envelopes and nucleoli in the S. Primary Ab: Anti-Lamin A (1:250) and Anti-Fibrillarin (1:100). Secondary Ab: Goat Anti-Mouse IgG H&L (HRP) (1:5000). Loading mass/volume for a 10 % Bis-Tris gel: 15 µg P protein or 15 µl S/lane. Ladder: MagicMark XP Western Protein Standard.

In order to examine the connection of the WSS protein to DNA/RNA at the subnuclear level, I performed DNase and RNase hydrolysis of the nucleoli. No nuclease digestion of isolated nuclear envelope extract was conducted since it had already been performed as part of the isolation procedure and quality controls with anti-fibrillarin and anti-lamin A antibodies of isolated nucleoli and nuclear envelopes were carried out.

Figure 21 shows a digestion of the nucleolar extract with DNase, RNase, and both. β-WSS and the 80-kDa protein were partially detached from the nucleoli after digestion with DNase and DNase + RNase, but not RNase alone. In contrast to the nuclear extract, where a signal at ~70 kDa, which could correspond to either β-WSS or the 80-kDa protein, was seen in the pellets and supernatants of the DNase- and DNase + RNase-digested fractions, two distinguishable signals in the same fractions of the nucleolar extract were detected. The 80-kDa protein was present in higher amount in the supernatant than β-WSS. Furthermore, α-WSS was partially mobilized to the supernatant after treatment with DNase + RNase.

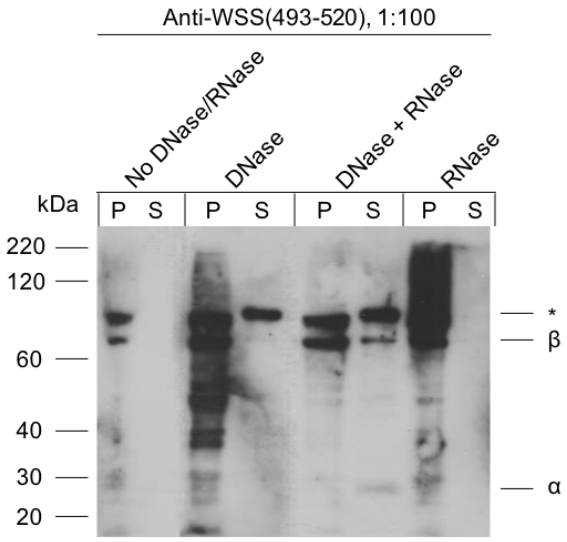


Figure 21. Western blot of nucleolar extract, digested with DNase and RNase, with anti-WSS(493-520) antibody. The blot shows the nucleolar extract samples treated with DNase, RNase, and both. α-WSS (27 kDa) was detected all pellets (P) as well as the supernatant (S) of the sample treated with DNase + RNase. β-WSS (65 kDa) was detected in all P as well as the S of samples digested with DNase (very weak signal) and DNase + RNase. These results suggest a partial detachment of α- and β-WSS from the nucleolar extract P after its digestion with DNase. The 80-kDa band (*) was detected in all P as well as the S of the samples treated with DNase and DNase + RNase. Primary antibody (Ab): anti-WSS(493-520) (1:100). Secondary Ab: Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) (1:1000). Loading mass/volume for a 10 % Bis-Tris gel: 15 µg P protein or 15 µl S/lane. Ladder: MagicMark XP Western Protein Standard.

In analogy to the nuclear extract, I performed a control Western blot of the nucleolar samples with anti-lamin A and anti-fibrillarin antibodies (Figure 22). Anti-fibrillarin but not anti-lamin A signal was present in the pellets, ruling out a contamination of the nucleolar samples with nuclear envelopes. Furthermore, no anti-fibrillarin signal was observed in the supernatants, excluding a disruption of the nucleoli and their mobilization from the pellets to supernatants.

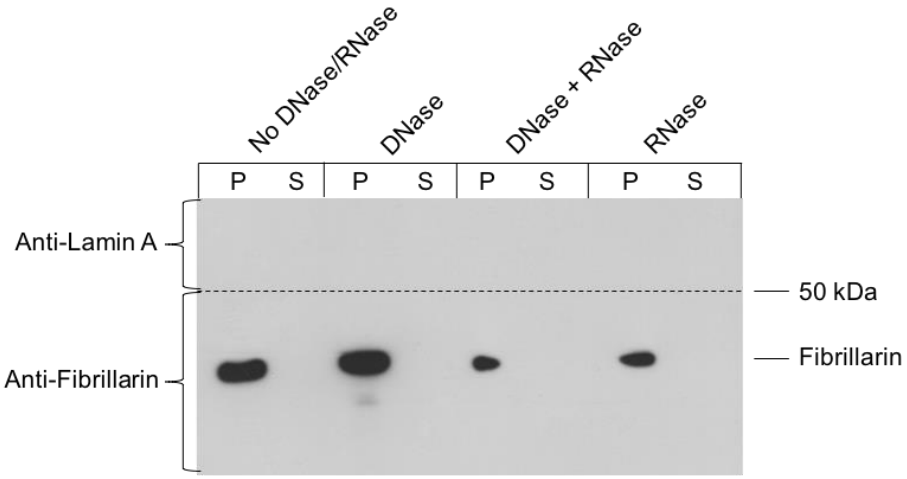


Figure 22. Western blot of nucleolar extract, digested with DNase and RNase, with anti-lamin A and anti-fibrillarin antibodies. The blot shows the nucleolar extract samples treated with DNase, RNase, and both. Prior to the antibody (Ab) application, the PVDF-membrane was cut in two at ~50 kDa height. Anti-lamin A Ab was then applied to the upper, whereas anti-fibrillarin Ab to the lower membrane part. Anti-lamin A (74 kDa) signal was not detected in any of the samples, excluding a contamination with nuclear envelopes. Anti-fibrillarin (33 kDa) signals were detected in all pellets (P) but not in the supernatants (S), ruling out a mobilization of the nucleoli from the P to S. Primary Ab: anti-lamin A (1:250); anti-fibrillarin (1:100). Secondary Ab: Goat Anti-Mouse IgG H&L (HRP) (1:5000). Loading mass/volume for a 10 % Bis-Tris gel: 15 µg P protein or 15 µl S/lane. Ladder: MagicMark XP Western Protein Standard.

In conclusion, the hydrolysis of the nuclear and nucleolar extract with DNase, but not RNase, caused a partial detachment of WSS and the 80-kDa protein from the extract. These results imply that WSS and the 80-kDa protein are connected to DNA rather than RNA. However, some differences were noticed between the hydrolysis of the nuclear and nucleolar extract. First, a dissociation of α-WSS from the pellet was seen in case of the nucleolar but not the nuclear extract. Second, a partial dissociation of a protein at ~70 kDa was seen in case of the nuclear extract. This protein could either represent β-WSS (expected MW 65 kDa) or the 80 kDa-protein. On the other hand, the hydrolysis of the nucleolar extract caused mainly a dissociation of the 80-kDa band and, to a smaller amount, also β-WSS. This might reflect a difference in the redistribution of the WSS isoforms on the subnuclear level. A mainly nucleolar localization of the 80 kDa-protein and α-WSS and a possible redistribution of β-WSS within other subnuclear departments are conceivable.

3.3 Analysis of a posttranslational WSS modification

The 80-kDa protein might be a posttranslationally modified or previously undescribed WSS isoform.

As presented in the experiments above, all of the specific anti-WSS antibodies showed a strong signal at ~80 kDa apparent MW. It was present in grossly the same cell protein fractions as the WSS isoforms, was detached from the nuclear envelope with NaCl and urea, similarly to the WSS protein, and was dissociated from the nucleoli and nuclei after digestion with DNase. Other properties of this protein remain unknown and it has not been reported as a major WSS isoform to date. There also have been no experiments analyzing a posttranslational modification of the WSS protein.

The most common types of posttranslational protein modification are glycosylation and phosphorylation. In this regard, I performed deglycosylation and dephosphorylation of the nuclear extract of HeLa cells to investigate the 80-kDa protein in more detail. It is important to know that the phosphorylation itself would not lead to a significant MW change of the WSS protein. However, the phosphate groups, although small in size, can considerably change the charge of proteins and therefore their migration pattern on electrophoresis.

The 80-kDa protein is probably not a glycosylated WSS isoform.

Prior to the deglycosylation of the nuclear extract, I performed a control deglycosylation of fetuin. It is a glycoprotein which containes sialylated N-linked and O-linked glycans and is therefore often used as a positive control for deglycosylation experiments. It was digested with Protein Deglycosylation Mix, containing peptide N-glycosidase F (PNGase F), endo-α-N-Acetylgalactosaminidase, sialidase (neuraminidase), β1-4-galactosidase, and β-N-acetylglucosaminidase. Figure 23 shows fetuin before (64 kDa) and after treatment with Protein Deglycosylation Mix (PDM) (45-50 kDa), separated on a 10 % Bis Tris polyacrylamide gel. In the “Fetuin + PDM” lane, some of the deglycosylating enzymes can also be seen: PNGase F (36 kDa), β-N-acetylglucosaminidase (71 kDa), and O-glycosidase (147 kDa). Neuraminidase (43 kDa) and β(1-4)galactosidase (94 kDa) were also present in the PDM, but are not seen on the gel.

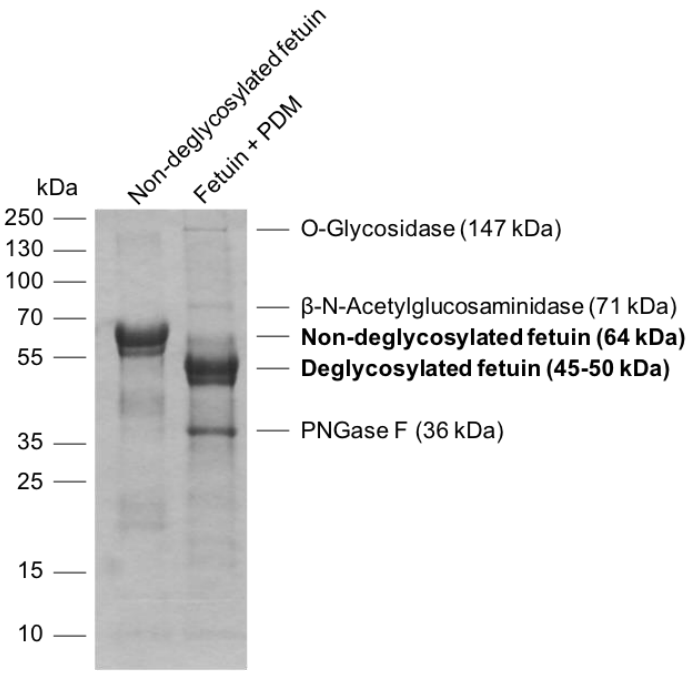


Figure 23. Deglycosylation of fetuin. The figure shows non-deglycosylated as well as deglycosylated fetuin and deglycosylating enzymes, separated on a 10 % Bis-Tris polyacrylamide gel stained with the Coomassie method. The apparent molecular weight (MW) of fetuin is 64 kDa. After treatment with Protein Deglycosylation Mix (PDM), the deglycosylated fetuin (45-50 kDa) as well as various deglycosylating enzymes were detected: peptide N-glycosidase F (PNGase F, 36 kDa), β-N-acetylglucosaminidase (71 kDa), and O-glycosidase (147 kDa). PDM also contained neuraminidase (43 kDa) and β(1-4)galactosidase (94 kDa), which are not depicted. Loading volume: 8 µl non-deglycosylated fetuin or 11 µl fetuin + PDM/lane. Ladder: PageRuler Plus Prestained Protein Ladder.

Figure 24 shows a Western blot with anti-WSS(493-520) antibody of deglycosylated HeLa cell nuclear extract. Varying concentrations of the Deglycosylation Enzyme Coctail (DEC) were used: 0, 2.5, 5.0, and 7.50 µl per 50 µl of reaction volume. 10 and 20 µl samples were loaded onto a 10 % Bis Tris gel. In none of the reaction samples, a change in abundance of the 80-kDa protein was observed. These results imply that the 80-kDa protein is not a glycosylated WSS isoform. However, I only focused on the most common protein glycosylation types, it is therefore possible that other, less common art of posttranslational glycosylation takes place.

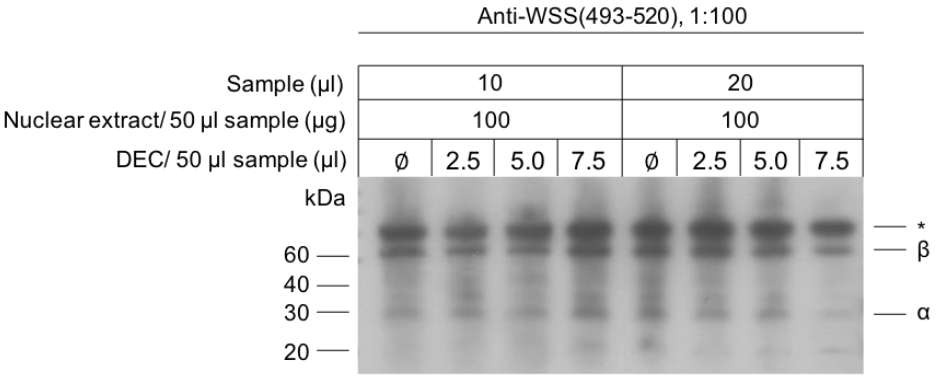


Figure 24. Deglycosylation of nuclear extract. The Western blot shows the nuclear extract samples treated with Protein Deglycosylation Mix. The initial reaction volume was 50 µl, 10 and 20 µl of which were loaded onto a 10 % Bis Tris gel, except for the negative controls (Ø), in case of which the initial sample volume was 20 µl. Ø contained 100 µg undigested nuclear extract, the remaining samples contained initially 100 µg denatured nuclear extract and were treated with 2.5, 5.0, or 7.5 µl Deglycosylation Enzyme Coctail (DEC). α- (27 kDa) and β-WSS (65 kDa) as well as the 80-kDa (*) signals were detected in Ø as well as the deglycosylated nuclear extract. None of the deglycosylating enzyme concentrations caused a disappearance of the 80-kDa band, implicating that this protein is not a glycosylated WSS. Primary antibody (Ab): anti-WSS(493-520) (1:100); secondary Ab: Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) (1:1000). Ladder: MagicMark XP Western Protein Standard.

The 80-kDa protein is probably not a phosphorylated WSS isoform.

In analogy to the deglycosylation experiments, I performed a dephosphorylation of the HeLa cell nuclear extract in order to investigate the origin of the 80-kDa protein. I used three common dephosphorylating enzymes: Antarctic Phosphatase (AP), Protein Phosphatase 1 (PP1), and T-cell Protein Tyrosine Phosphatase (TC PTP). 1 µl dephosphorylating enzyme was used for each sample volume (20 µl AP sample, 50 µl PP1 and TC PTP samples) (p-nitrophenol PNPP phosphatase activity assay was not performed).

Figure 25 shows a Western blot with anti-WSS(493-520) antibody after the dephosphorylation of the nuclear extract with AP, PP1, and TC PTP. None of the dephosphorylating enzymes caused a disappearance of the 80-kDa band from the extract. As mentioned above, the removal of phosphate groups does not significantly change the MW of proteins, but due to a change of their electrical charge, it can lead to a distinctly different migration pattern on gel electrophoresis. The results imply that the 80-kDa protein is not a phosphorylated WSS. However, I only used three common dephosphorylating enzymes. A postranslational phosphorylation of the WSS protein can therefore not completely be excluded.

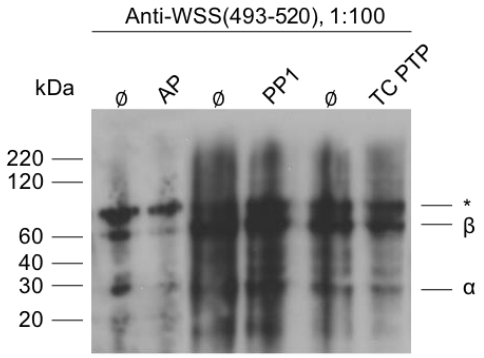


Figure 25. Dephosphorylation of nuclear extract. The Western blot shows the nuclear extract samples, treated with Antarctic Phosphatase (AP), Protein Phosphatase 1 (PP1), and T-Cell Protein Tyrosine Phosphatase (TC PTP). The initial reaction volume for the AP dephosphorylation sample was 20 µl, whereas for PP1 and TC PTP samples 50 µl. The negative controls (Ø) contained 100 µg undigested nuclear extract. The AP reaction sample (20 µl) contained 100 µg nuclear extract treated with 1 µl AP; the PP1 reaction sample (50 µl) contained 100 µg nuclear extract treated with 1 µl PP1; the TC PTP reaction sample (50 µl) contained 100 µg nuclear extract treated with 1 µl TC PTP. α- (27 kDa) and β-WSS (65 kDa) as well as the 80-kDa (*) signals were detected in Ø as well as the dephosphorylated nuclear extract. None of the dephosphorylating enzymes caused a disappearance of the 80-kDa band, implicating that this protein is not a phosphorylated WSS. Primary antibody (Ab): anti-WSS(493-520) (1:100); secondary Ab: Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) (1:1000). Ladder: MagicMark XP Western Protein Standard.

4 Discussion

4.1 Scientific background

Woodhouse-Sakati syndrome encompasses, despite its rarity, some of the most common and heavily researched clinical phenotypes, such as diabetes mellitus, hypogonadism, alopecia, and cognitive impairment. Similar symptoms have previously been described in other autosomal recessive syndromes, such as Alström's syndrome (hypergonadotrophic hypogonadism, diabetes mellitus, mental retardation, sensorineural deafness, obesity, retinitis pigmentosa), Laurence-Moon-Biedl syndrome (hypothalamic hypogonadism, diabetes mellitus, mental retardation, sensorineural deafness, obesity, retinitis pigmentosa), Sohval-Soffer syndrome (hypergonadotrophic hypogonadism with skeletal abnormalities), and Crandall syndrome (hypogonadism, alopecia, sensorineural deafness)[1]. Although only approximately 80 cases have been reported in the literature to date, WSS may be considered as a differential diagnosis in various medical areas, such

as dermatology, endocrinology, peadiatrics, and neurology. It is possible that migration events will increase the rate of this disease in the Western countries.

Some of the molecular aspects of WSS have already been investigated. Alazami et al. identified the gene, *C2orf37*, mutated in WSS and different types of underlying mutations, all of them resulting in a truncated protein. They also examined the wild-type WSS protein in murine and human cells. Immunohistochemistry and in situ hybridization of murine tissues performed by Alazami's group revealed a ubiquitous WSS protein expression with a significantly higher production in the liver, skin, and brain. Further, the group performed immunofluorescence on HEK-cells and human lymphoblasts, which showed a colocalization of the WSS protein with B23, a nucleolar marker. In addition to these findings, Alazami suggested that the wild-type WSS is only partially dependent on active transcription whereas the lymphoblasts of WSS patients display a hypersensitivity to transcriptional blockade. He subjected HEK cells to low and high doses of actinomycin D (RNA polymerase I inhibitor) and noticed a complete dislocation of B23 to the nucleoplasm even at low doses of actinomycin D, while C2orf37 mostly retained its nucleolar localization at a lower dose and dislocated to the nucleoplasm at a higher dose of actinomycin D. In the patient lymphoblasts, the WSS protein (and B23) mobilized into the nucleoplasm even at a lower dose of actinomycin D, whereas a higher dose was required for a dislocation of C2orf37 in the control lymphoblasts.

The protein product of *C2orf37* was identified as part of the DDB1-CUL4 ubiquitin ligase complex. The proteins in this complex are bound directly to DDB1 and serve to recruit substrates for DDB1-CUL4-ROC1 E3 ubiquitin ligase (therefore the name DCAFs = DDB1-CUL4A-associated factors)[17].

Little has been reported about DCAF17 itself. Recently, Ali et al. identified a novel single base pair deletion in Exon 3 of *C2orf37* in a Pakistani family[14]. They also used homology modeling (based on DCAF10) to predict 3D models of the wild-type and mutated DCAF17. Through a molecular docking analysis, they suggested 22 amino acids responsible for binding of DCAF17 to DDB1, 12 of them belonging to DCAF17 and 10 to DDB1. In the mutated DCAF17, this interaction is restricted to only nine amino acids (five of DCAF17 and four of DDB1). This- possibly inefficient- interaction may disturb a normal functioning of the DDB1-CUL4A ubiquitin ligase complex.

Moreover, my colleague Dr. Christoph Roscher undertook approaches to characterize the WSS protein by means of a polyclonal, non-specific anti-WSS antibody. Through rabbit immunization with inclusion bodies containing α- and β-WSS

recombinantly expressed with attached His or GST tag in *E. coli* cells and affinity purification of the pre-immunized serum on CNBr-Activated-Sepharose-bound α-WSS, he obtained antibodies against α-WSS, which he used for further experiments[20].

4.2 Rationale for the applied methods

Peptide-specific antibodies

I chose to use peptide-specific antibodies against distinct WSS epitopes in order to further characterize the WSS protein. These antibodies warrant a high-affinity bond to short (ca. 10-20 amino acids) fragments of the protein. This provides a stronger signal as well as reduces the amount of cross reactions. Furthermore, this approach allowed me to compare the signals of antibodies against both isoforms (anti-WSS(493-520), -(412-423), and -(493-511)) with the signal of anti-WSS(β) but not -(α) (anti-WSS(80-88)).

Based on the successful recombinant expression of WSS(412-426) and subsequent affinity-purification of anti-WSS(412-426) antibody by Alazami[10], I decided to a) affinity purify an anti-peptide antibody against another high-affinity epitope, recombinantly expressed in bacteria cells, to compare my results to those of Alazami's, b) purify three other high-affinity peptide antibodies using commercially produced epitope peptides (revealed in the PepperChip Assay) in order to compare the reaction patterns and sensitivity/specificity of both types of antibodies. One of the commercially produced epitopes was the WSS(412-423) peptide, similar to the peptide used by Alazami (without the three C-terminal amino acids). For the recombinant expression in *E. coli* cells, I chose the longest of all high-affinity epitopes, WSS(493-520), for an easier purification on the Ni-IDA agarose and detection on SDS-PAGE.

Recombinant expression tag

Among many tags available for recombinant protein expression and affinity purification of the recombinant peptide, I opted for the polyhistidine (His) tag for various reasons. First, its small MW (1 kDa) and size (6-10 amino acids) do not distort the migration pattern of the recombinant protein on SDS-PAGE. Second, due to its weak immunogenicity, the risk of anti-His-tag antibody reaction on Western blot (as it was observed in case of the GST tag in Dr. Roscher's work) is minimized. Third, some tags, such as the GST tag, might alter protein functionality due to a larger size and strong

immunogenicity, and their proteolytic removal may be very difficult, as it was in case of the GST but not His tag in the recombinant WSS protein (Roscher[20]).

Bacterial expression system

I chose the bacterial system for the recombinant expression of WSS(493-520)/His-tag based on following considerations: a) the amount of the expressed protein is usually high[28], b) the cultivation is relatively simple and quick when compared to other expression systems such as yeast, insect, or mammalian cells[20], c) the protein translation is easily controllable. Among different bacterial cell lines, *E. coli* cells have been widely used and approved as an easily manageable cell culture system with a high-yield protein expression. However, I am aware that *E. coli* is not the "natural" expression system for the WSS protein. cDNA of β-WSS, in this case a heterologous protein, was compared with the tRNA population of *E. coli* and resulted in a codon adaption index of 0,62 (Roscher[20], based on [29-32]). A comparison of different *E. coli* strains showed the highest α-WSS expression in *Rosetta 2*, compared to *BL21 pLysS* and *BL21 pRare*[20]. Another difference between the pro- and eukaryotic system is that processes such as posttranslational modification, taking place in the eukaryotes including yeast, do not occur or take place to a much lower extent in the prokaryotes[33]. Building of inclusion bodies, which had occurred in the experiments of Dr. Christoph Roscher[20], was not the case in my experiments, likely due to the smaller size of the expressed epitope peptides than of α- and β-WSS.

Cloning vector

For the optimal plasmid choice, I was looking for following features: a known compatibility with *E. coli* cells, a His-tag coding sequence for recombinant peptide expression, a well controllable promoter, an operon sequence for expression induction, and an antibiotic resistance sequence other than that of *Rosetta 2* cells for a selective cultivation of successfully transformed cells. A plasmid that fulfilled the above-mentioned criteria was pET28a(+). It is a very effective vector used for recombinant protein expression in *E. coli* cells. Its coding sequences encompass N-terminal His-tag, T7 promoter, lacI operon (enabling expression induction with IPTG), and kanamycin resistance sequences. Furthermore, I was trying to avoid vectors with GST tag for reasons mentioned above *("Recombinant expression tag"*) and with GFP tag sequence, which was used by Alazami for recombinant WSS-GFP expression in HEK 293 cells and led to

disruption of the nuclear membrane and aggregation of the fusion protein with displacement of the nucleus.

Immunofluorescence

Among various commercially available human cell lines, HeLa cells were shown to yield the highest amount of expressed WSS protein (Roscher[20]) and were therefore used for determination of the subcellular/subnuclear redistribution of the WSS isoforms and investigation of the posttranslational modification in my experiments.

As mentioned before, the occurrence of a diffuse nuclear WSS protein signal (additionally to a strong signal in the nucleoli) prompted me to investigate the possibility of WSS protein ocurrence in/connection to other subnuclear departments. I initially aimed at confocal microscopy to further investigate the subnuclear distribution of the WSS protein. This was however hampered by a pronounced bleaching and a relatively low intensity of the anti-WSS signal. Other suitable method would have been electron microscopy with immunogold labeling; however, this would have required an excellent antibody and optimization experiments that were beyond the scope of this work.

Subcellular protein fractionation

Motivated by the results of the immunofluorescence, I performed a protein fractionation of HeLa cell extract to investigate the subcellular and subnuclear WSS redistribution in more detail. Surprisingly, Western blot with specific anti-WSS antibodies showed a substantial amount of both WSS isoforms in the SNF, which represented the non-chromatin-bound nuclear fraction. According to the results of this work, both isoforms were more abundant in the SNF than CBNF. The WSS protein presence in CBNF might confirm the nucleolar localization, described by Alazami[10], whereas the significant amount of WSS in SNF implies that this protein is also located in other subnuclear fractions which are not directly attached to chromatin. The (less abundant) presence of the WSS protein in the cytoplasmic and membranes fraction can be explained by the fact that the outer nuclear membrane is continuous with the endoplasmic reticulum (see below). The differences in reactivity of the different antibodies used for detection are difficult to explain. One may consider a different accessibility of the epitopes as a cause, however I could not completely solve this issue.

The application of the detergent-based subcellular fractionation has the disadvantage that it cannot discriminate between organelles. However, it is relatively easy

to use and fast as well as avoids tedious optimization procedures. I knew from the immunofluorescence data as well as experiments published by Alazami et al. that the nucleus with certain subnuclear structures are a key location of the WSS protein. Hence, I decided to use a detergent extraction with the respective kit and perform a control cell fractionation with marker proteins. Strategies such as free flow electrophoresis appeared dispensable for the above-mentioned reasons.
To investigate the subnuclear redistribution of the WSS protein in more detail, isolation of nuclei, nuclear envelopes, and nucleoli was performed.

Detachment from the nuclear envelope

The presence of the WSS protein in the SNF and the existence of various hereditary (both autosomal dominant and recessive) diseases caused by defects in the nuclear envelope or one of its associated proteins prompted me to investigate the attachment of DCAF17 to the nuclear envelope.
We know nowadays that the nuclear envelope is not merely a physical barrier between the gene expression department and the rest of the cell but also plays a role in different cellular processes and signaling cascades[34]. The nuclear envelope structure with some of its key components is shown in Figure 26[35]. It consists of an outer and inner nuclear membrane, joined at sites to form the nuclear pore complex[35], through which a selective, bidirectional transfer of molecules between the nucleus and cytoplasm takes place[36]. Nuclear lamina network underlies the inner nuclear membrane. It is a meshwork consisting primarily of A and B lamins (type V intermediate filament proteins)[35,37]. These proteins were initially thought to play a solely structural role in the nuclear envelope but the evidence has grown that they are functionally important in maintaining essential cellular processes[37]. A selective knock out of different lamin-encoding genes in mice had deleterious, in some cases lethal consequences caused by defects in myocardial, skeletal, and smooth muscle as well as neural system and connective tissue[37]. Number of diseases caused by defects in the nuclear envelope or proteins connected with it have been described in humans. For example, Emery–Dreifuss muscular dystrophy, characterized by wasting of the upper and lower limb muscles and consequent tendon contractures, is caused by mutations in an X-chromosome linked gene *emerin*. Pelger-Huet anomaly, a neutrophile differentiation disorder, is caused by heterozygous mutations in lamin-B receptor gene, while a lethal chondrodystrophy called Greenberg skeletal dysplasia is caused by homozygous mutations in the same gene. Mutations in the lamin A genes lead

to limb-girdle muscular dystrophies, dilated cardiomyopathy, lipodystrophies such as Dunnigan-type partial lipodystrophy and mandibuloacral dysplasia, axonal neuropathies like Charcot-Marie-Tooth disorder type 2, postnatal growth retardation with skeletal malformations as well as mottled skin pigmentation. Another lamin-A-defect-associated disease is Hutchinson-Gilford progeria syndrome, characterized by accelerated ageing, growth retardation, atherosclerosis, atrophy of the subcutaneous fat tissue, delayed dentition, and bone deformation. Atypical Werner syndrome is also a progeria caused by lamin A gene mutations. Interestingly, there are tissue-specific differences in the protein composition of the nuclear envelope, which underscores the role of the transmembrane protein compound as well as different signal transmission and gene expression regulators[38]. These findings could partly explain the complexity and clinical diversity of the "nuclear-envelope-based" diseases.

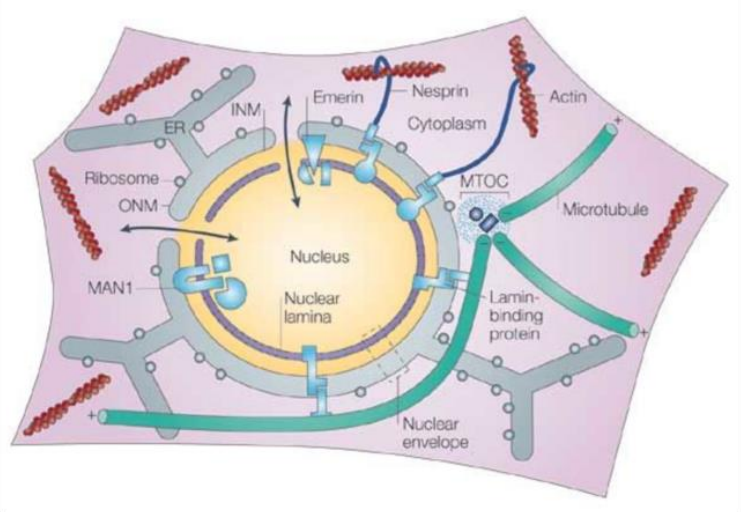


Figure 26. Nuclear envelope and its related structures. Schematic diagram represents the composition of the nuclear envelope (NE). The outer and inner nuclear membrane (ONM and INM, respectively) join to form pores, through which selective, bidirectional molecule transfer takes place. The ONM is continuous with the endoplasmic reticulum (ER). Microtubule organizing center (MTOC) is connected to the NE by lamin-dependent protein complexes (blue interlocking shapes). Nesprins, proteins located within the ONM, attach to actin filaments (red). Lamins (purple) are located on the innerside of the INM. Internal lamin network within the nucleus is not shown. Integral INM proteins form lamin-dependent complexes such as emerin (blue triangle) and MAN1 (blue U-shape). From Gruenbaum et al.[30]

According to the results of this work, the WSS protein is not only present in the nucleoli but indeed in the nuclear envelope as well. The previously mentioned tissue-specific composition of the nuclear envelope might therefore contribute to differences in the distribution of the WSS protein in HEK cells, human lymphoblasts (both of healthy controls and WSS patients), used by Alazami, and HeLa cells, used for my experiments. For future investigative approaches, it might also be helpful to use liver, skin, or brain

tissue, as these organs are affected in WSS and these tissues are known to overexpress the WSS protein. However, it is also possible that a relatively slight staining intensity of the WSS protein may appear as diffuse nuclear in usual transmission microscopy but actually be related to the nuclear envelope.

Since the immunocytochemistry indicated a location of the WSS protein in the nucleus, this was further investigated by applying biochemical fractionation. I also used an increasing ionic strength to determine the nature and stability of binding of the WSS protein to the nuclear envelope. This is a frequently used strategy to determine whether a particular protein is embedded into the membrane via trans-membrane domains or lipid modification such as glycosylphosphatidylinisotol (GPI) anchor or farnesylation, or not. Proteins that do not interact with the membrane through lipid interaction are expected to be washed from the membrane with saline and not only using detergents. Increasing ionic strength led to a partial detachment of a fraction of the 80-kDa protein from the nuclear envelope at concentrations inferior to those required to detach WSS-β and -α. Moreover, the 80-kDa protein remaining in the non-soluble fraction appeared at a slightly lower MW than the 80 kDa protein in solution. WSS-α was partially detached only at high urea concentrations indicating likewise a non-covalent, likely non-lipid interaction with the nuclear envelope. Together, these data indicate that there likely exists a multimeric organization of the WSS protein isoforms that possibly interact with each other. However, by means of these experiments, no further insight could be gained beyond the fact that all described isoforms of the WSS protein including the 80-kDa variant are non-covalently bound to the nuclear envelope.

Methods to study further study this topic would be recombinant expression of the WSS protein isoforms which, however, requires fine-tuning of expression levels to avoid aggregation as observed by Alazami et al. Purification of nuclear envelopes fractions enriched in WSS protein might allow binding experiments with recombinantly expressed e.g. S-35 labeled WSS isoforms. Electron microscopy and immunogold labeling as well as cryo-electron microscopy would be additional techniques once the location at nuclear envelopes is firmly established.

Nuclease digestion of nuclei and nucleoli

Apart from the presence in the nuclear envelope, I also noted a significant amount of the WSS protein in the CBNF and- on immunofluorescence- its co-localization with the nucleolar marker protein fibrillarin. Some (possibly tissue-specific) proteins within

the nuclear envelope play role as regulators of gene expression. Additionally, lamin-binding proteins contribute to chromatin organization, gene expression, and epigenetic regulation as well as influencing signaling cascades[39]. Heterochromatin is known to be connected to both nucleoli and lamins. The regions of direct connection between lamins and chromatin are thought to be of transcriptionally repressive nature but lamins are also supposed to play a role in DNA replication and repair. There are reports that lamins connect to chromatin of mitotic chromosomes either indirectly through histone cores or directly to DNA strands. Furthermore, lamin-binding proteins bind to DNA[40]. According to these reports, lamins seem to be connected- either directly or in an indirect manner- to DNA rather than RNA. The detailed functional significance of these connections is yet to be explored. Moreover, epigenetic changes, causing distorted chromatin organization, are also observed in patients with genetic defects affecting lamin expression[40].

There are no reports on direct connection of DCAF17 to chromatin. Given the intimate linkage of nucleoli with nuclear envelopes and the in part diffuse nuclear, in part nucleolar location of the WSS protein on immunofluorescence, I further investigated the connection of the WSS protein to chromatin by digesting HeLa cell nuclear and nucleolar extract with DNase and RNase. As a result, the WSS protein could partially be detached from the pellets by DNAse but not RNase hydrolysis. α-WSS signal was only detected in the supernatant of the nucleolar and not nuclear extract. This could imply that α-WSS is located primarily within the nucleoli (nucleolus makes up for up to 25 % of nuclear volume[41]) and the concentration of α-WSS in the nuclear extract or of the primary antibody was to low to evoke a signal. Furthermore, the pellets and supernatants of the nucleolar extract showed a band at ~70 kDa. It is unclear whether this protein corresponds to β-WSS (65 kDa) or the 80-kDa protein. Irrespective of this, the question arises about the appearance of only one band instead of both β-WSS and the 80-kDa one. The hypothesis might be the same as in case of α-WSS, where a primary location in the nucleoli is suspected. However, as the exact origin of the band is unknown (β-WSS versus the 80-kDa protein), further pursuing of this topic is not possible at this point. This may be contributed to technical difficulties encountered with immunoblotting of nucleic-acid-containing samples. It is nevertheless possible, that both isoforms show a different redistribution within the nucleus, i.e. β-WSS may have a higher concentration in nuclear structures such as the nuclear envelope whereas α-WSS (and possibly the 80-kDa protein) may be associated mostly with the nucleoli.

Deglycosylation and dephosphorylation

In the Western blot experiments using anti-WSS(493-520), -(80-88), -(412-423), and -(493-511) antibodies, I repeatedly observed an appearance of a strong signal at the apparent MW of ~80 kDa. This protein was present in the same subcellular fractions (i.e. soluble and chromatin-bound nuclear fractions, cytoplasm, nucleoli, nuclear envelope) as α- and β-WSS. It was also detached from the nuclear envelope at lesser ionic strength than α- and β-WSS and was dissociated from the nuclei and nucleoli after hydrolysis with DNase, but not RNase, similar to α- and β-WSS. I therefore hypothesized that this protein is either a posttranslationally modified WSS or a previously undescribed, third major WSS isoform.

Posttranslational protein modifications encompass chemical processes such as glycosylation, phosphorylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation, and proteolysis. They can occur at any stage of protein "life cycle" and greatly increase the functional complexity of the proteome. Through these modifications, protein interactions with other molecules and cell structures are influenced as well as activity and redistribution of proteins are regulated[42].

Phosphorylation and glycosylation are frequently occurring posttranslational protein modifications. I therefore performed deglycosylation and dephosphorylation experiments of nuclear extract with common deglycosylating and dephosphorylating enzymes. These approaches did not lead to an abundance change in/disappearance of the 80-kDa band from the nuclear extract, suggesting that it is not a glycosylated or a phosphorylated WSS protein. Although I performed a control deglycosylation of fetuin before deglycosylating the nuclear extract, in case of dephosphorylation, I opted for an empirical use of 1 µl dephosphorylating enzymes for 100 µg HeLa cell nuclear extract instead of performing the p-nitrophenyl phosphate assay. As mentioned above, there are other ways of posttranslational protein modification than glycosylation and phosphorylation which are yet to be investigated.

4.3 Role of the WSS protein in the E3 ubiquitin ligase complex

Considering the DCAF17 interaction with DDB1 in the DDB1-CUL4A E3 ubiquitin ligase complex and its disturbance in case of the mutated DCAF17[14], it is possible that the DDB1-CUL4A E3 ubiquitin ligase pathway is interrupted in patients with WSS. What are the functions of this complex in healthy individuals?

Ubiquitination is a process consisting of three sequential steps, performed by specific complexes: E1 = activating, E2 = conjugating, and E3 = ligase enzymes. The ubiquitin pathway plays a crucial role not only in protein degradation, but also other elementary subcellular processes such as cell cycle and transcription as well as regulation of molecule trafficking[43]. An increasing number of inherited diseases have been found to be caused by dysfunctions in ubiquitination. Mutation in the E3 (= ligase) enzyme or its substrate and subsequent substrate stabilization results in hereditary diseases such as von Hippel-Lindau disease, caused by mutations in *VHL* which lead to stabilization of HIF-1. On the other hand, both reversible and irreversible, anticipated inhibition of the ubiquitin-proteasome pathway found use in therapy of plasma cell diseases such as multiple myeloma and light-chain amyloidosis.

Knowing the functions of the ubiquitin pathway in general and trying to understand the mechanisms underlying WSS, the following question arises: what is the specific role of the DDB1-CUL4A E3 ubiquitin ligase complex? Some of the functions of this complex are already known and are closely associated with chromatin biology. These functions include regulation of cell proliferation and survival, genomic integrity (through ubiquitination of histones H3 and H4, DNA repair factor xeroderma pigmentosum complementation group C (XPC), and transcription-coupled repair factor Cockayne syndrome B protein (CSB) as well as through proliferating cell nuclear antigen (PCNA)-dependent destruction of Cdt1), and nucleotide-excision repair (both global genome and transcription-coupled repair)[17-19,44-47]. Thus, there are known associations between the disruption of DDB1-CUL4A E3 ubiquitin ligase pathway and hereditary diseases, such as Xeroderma pigmentosum and Cockayne syndrome[46].

4.4 Ideas for future approaches

The investigation of DCA17, as part of the ubiquitin pathway, opens various doors to understanding the physiology of the "daily" cell functioning, cell reaction and adaptation to stress factors, and consequences of disruption of these mechanisms. Diverse ideas for future experiments to understand this complex mechanism arise.

One of them is to further investigate the origin of the 80-kDa protein, repetitively appearing in Western blot experiments with anti-WSS antibodies. An interesting approach would be a siRNA-knock-down of the WSS protein expression and following analysis of immunoblot bands. The disappearance of the 80-kDa signal, together with

other WSS bands, would indicate that this protein is indeed either a modified, or, perhaps, a previously undescribed third major isoform of DCAF17.

Furthermore, other ways of posttranslational WSS modification than the more common deglycosylation and dephosphorylation are possible. The role of ubiquitin in a broad spectrum of intracellular processes and signaling as well as indices that some factors in the ubiquitin-ligase pathway undergo ubiquitination themselves rise the possibility that the 80-kDa protein might be a ubiquitinated WSS isoform. Thus, deubiquitination experiments of protein extract containing the WSS protein could shed light on the origin of the 80 kDa-band.

Although DCAF17 had previously been shown to be localized abundantly in the nucleoli[10], the results of this work suggest that a significant amount of the WSS protein is present in the SNF and is connected to or a part of the nuclear envelope. Further immunofluorescence experiments could help better characterize the subcellular localization of the WSS protein. Using immunofluorescence with antibodies against CUL4 and DDB1 could help clarify the question if DCAF17 is exclusively co-localized with the E3 ubiquitin ligase or has additional functions in the nucleoli.

Furthermore, an approach to estimate the size of potential protein complexes involving DCAF17 through gel filtration (= size exclusion) chromatography would be worth investigating.

The approaches undertaken in this project are only a minute step on a path to understand the molecular mechanisms of DCAF17 and thus the pathology of WSS. Regarding the multisystem and complex phenotype of the disease and assuming that it will be more common and spread across the Western countries due to migration and mixing of the population, I hope to have woken the interest of the future scientists in this disease and am looking forward to witnessing further approaches in order to understand its pathology.

5 Summary

Woodhouse-Sakati syndrome (WSS) is a multisystemic, autosomal recessive disease, first described in 1983. Approximately 80 patients, mainly from families of Middle-Eastern origin, have been reported to date. The underlying cause of WSS are mutations of *c2orf37* gene, which result in a truncated protein. Little is known about the function of C2orf37 (DDB1-CUL4A-associated factor 17, DCAF 17) apart from it being part of the DDB1-CUL4-ROC1 E3 ubiquitin ligase complex, specifically binding directly to DDB1 and serving as a substrate recruiter for E3. There are two major isoforms of DCAF17: β (65 kDa, 520 amino acids) and α (27 kDa, 240 amino acids), which is a C-terminal part of β. The intracellular localization of the WSS protein is thought to be primarily nucleolus. A murine ortholog protein was found to be expressed in all tissues with a relatively higher expression in the brain, liver, and skin.

The aim of this work was to investigate DCAF17 in HeLa cells in more detail, in particular the redistribution of both WSS isoforms on the subcellular and -nuclear level as well as their chemical features. For these experiments, I developed, through recombinant expression and affinity purification, a specific polyclonal antibody against a WSS-epitope 493-520. Furthermore, three other specific polyclonal antibodies were obtained through affinity purification with help of commercially produced high-affinity epitope peptides.

By means of these antibodies, I determined- through immunofluorescence and subcellular protein fractionation- that, apart from the redistribution of the WSS protein within the non-soluble = chromatin-bound nuclear fraction, a significant amount of both WSS isoforms is present in the soluble nuclear fraction. Indeed, treatment of purified nuclear envelopes with an increasing concentration of NaCl as well as urea confirmed a non-covalent binding of the WSS protein to the nuclear envelope with the detachment of β-WSS at a lower NaCl concentration than α-WSS. In regard to the chromatin-bound WSS protein, I performed hydrolysis of nuclear and nucleolar extract with DNase and RNase. The results indicate that the WSS protein is bound to DNA but not RNA, with α-WSS being possibly located more abundantly in the nucleolus, whereas β-WSS within other subnuclear departments. Furthermore, in all above-mentioned experiments, a presence of an 80-kDa protein, which specifically reacted with the polyclonal high-affinity antibodies and showed similar redistribution and chemical features as α- and β-WSS, was observed. In order to investigate whether this protein is a posttranslationally

modified WSS isoform, I performed deglycosylation and dephosphorylation of nuclear extract, which showed no disappearance or change in abundance of the 80-kDa band on Western blot. While other ways of poststranslational modification cannot be excluded as the cause of occurrence of the 80-kDa protein, an existence of a third, yet undescribed, major isoform is also conceivable.

Summarizing, this work contributed to a deeper characterization of the WSS protein, which can help future investigators in developing new experimental ideas to better understand the pathology of WSS.

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Lebenslauf

Persönliche Daten

Name:	Joanna Blocka
Geburtsdatum:	02.11.1987
Geburtsort:	Zielona Gora, Polen

Schulausbildung

09/1994-08/1999	Janusz-Korczak-Grundschule in Nowa Sol, Polen
09/1999-08/2002	Henryk-Sienkiewicz-Grundschule in Nowa Sol, Polen
09/2002-04/2005	Edward-Dembowski-Gymnasium in Zielona Gora, Polen
04/2005	Abitur
09/2003-07/2004	Arlington High School, Arlington, OR, USA

Studium

10/2005-09/2008	Studium der Humanmedizin an der Karol-Marcinkowski-University of Medical Sciences in Posen, Polen
10/2008-09/2009	Studium der Humanmedizin im Rahmen des Erasmus-Stipendienprogramms an der J.W.-Goethe-Universität in Frankfurt am Main
10/2009-9/2013	Studium der Humanmedizin an der Julius-Maximilians-Universität in Würzburg (Praktisches Jahr in Würzburg, Zürich und Boston)
10/2011-04/2012	Promotionsstipendium der Medizinischen Fakultät, Promotionsthema: „Molecular mechanisms underlying Woodhouse-Sakati syndrome: characterization of DCAF17 with specific, polyclonal antibodies.“ Medizinische Klinik und Poliklinik I: Endokrinologie und Diabetologie, Universitätsklinikum Würzburg (Priv.-Doz. Dr. med. Dr. rer. nat. M. Kroiß)
06/2014	Approbation als Ärztin, Julius-Maximilians-Universität Würzburg

Berufliche Tätigkeit:

Seit 04/2015	Assistenzärztin an der Medizinischen Klinik V: Hämatologie, Onkologie und Rheumatologie, Universitätsklinikum Heidelberg