VILNIUS UNIVERSITY

MILDA NORKIENĖ

OPTIMIZATION OF POLYOMAVIRUS VP1 PROTEIN BIOSYNTHESIS IN *S.CEREVISIAE* CELLS AND APPLICATION OF RECOMBINANT VIRUS-LIKE PARTICLES FOR SEROLOGY

Summary of doctoral dissertation

Physical Sciences, Biochemistry (04 P)

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VILNIAUS UNIVERSITETAS

MILDA NORKIENĖ

POLIOMOS VIRUSŲ VP1 BALTYMŲ BIOSINTEZĖS YPATYBIŲ *S.CEREVISIAE* LĄSTELĖSE TYRIMAS BEI PANAUDOJIMAS SEROLOGINIAMS TYRIMAMS

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Contents

List of abbreviations

aa	Amino acid(s)
APyV	Avian polyomavirus 1 or Budgerigar Fledgling Disease virus
BKPyV	BK polyomavirus or Human polyomavirus 1
BVPyV	Blank vole polyomavirus
CPyV	Crow polyomavirus
CVPyV	Common vole polyomavirus
EGFP	Enhanced green fluorescent protein
EIA	Enzyme immunoassay; ELISA
EM	Electron microscopy
ER	Endoplasmic reticulum
FPyV	Finch polyomavirus
GHPyV	Goose haemorrhagic polyomavirus
HA	Hemagglutination assay
HaPyV	Hamster polyomavirus
HPyV	Human polyomaviruses
HRP	Horseradish peroxidase
JCPyV	JC polyomavirus or Human polyomavirus 2
JDP	J-domain proteins
KIPyV	KI polyomavirus or Human polyomavirus 3
MAb	Monoclonal antibodies
MCPyV	Merkel cell polyomavirus or Human polyomavirus 5
MWPyV	MW polyomavirus or Human polyomavirus 10
NAC	Nascent polypeptide-associated complex
NJPyV	New Jersey polyomavirus or Human polyomavirus 13
NLS	Nuclear localization signal
NTA	Nanoparticle tracking analysis
OD	Optical density
ORF	Open reading frame
PAb	Polyclonal antibodies
PCR	Polymerase-chain reaction
PEG	Polyethylene glycol
pRb	Retinoblastoma protein
RAC	Ribosome-associated complex
SD	Standard deviation
SDS	Sodium dodecylsulphate
STLPyV	STL polyomavirus or Human polyomavirus 11
TSPyV	Trichodysplasia spinulosa polyomavirus or Human polyomavirus 8
VLP	Virus-like particle
VP1	Virus protein 1
WB	Western blotting
WUPyV	WU polyomavirus or Human polyomavirus 4

Introduction

Polyomaviruses (PyVs) represent quickly growing group of viruses found in mammals, birds, or fish. Twelve new human polyomaviruses (HPyVs) have been identified in past ten years. PyVs are small, non-enveloped viruses that contain double-stranded, circular DNA genome about 5000 base pairs in length. The icosahedral viral capsids of approximately 45nm in diameter is composed of three proteins, major VP1, and two minor VP2, and VP3 (Imperiale and Major, 2007). When expressed in eukaryotic cells, VP1 proteins are able to self-assemble into virus-like particles (VLPs), which are used in assays to detect specific antibodies. In contrast to better-studied polyomaviruses JCPyV, BKPyV and partially MCPyV, the routes of infection, entry pathways and cell tropism of new HPyVs remain unknown.

HPyVs are part of the normal microbial flora and the infection of these viruses in their host is thought to be asymptomatic but, when host immunity is compromised the infection can cause acute systemic disease or tumor induction. These diseases include BK virus–related nephropathy, JC virus–related progressive multifocal leukoencephalopathy, trichodysplasia spinulosa (van der Meijden et al., 2010), HPyV6 or HPyV7-related epithelial hyperplasia (Ho et al., 2015) or Merkel cell carcinoma (Feng et al., 2008). Worldwide seroprevalence for HPyVs is reported to be approximately 80 % in adult individuals, as detected by anti-HPyV IgG antibodies (Abs) in human serum.

For the advance research on biology of the new HPyVs the development of cell culture systems is important though in some applications VLPs could be used instead, especially in cases when the wild-type virus cannot easily be cultivated in cell culture. Budding yeast *Saccharomyces cerevisiae,* as a natural host of their own indigenous viruses, is one of the most attractive and most widely used eukaryote microorganism for virus research and recombinant protein synthesis (Zhao, 2017). Many of the fundamental cellular functions such as cell proliferation, cell cycle regulation, cellular transport, programmed cell death or activity of molecular chaperones are highly conserved from yeasts to higher eukaryotes (Goloubinoff and De Los Rios, 2007; Zhao, 2017;). Yeast cell is an excellent host for the production of recombinant self-assembling VP1 VLPs. Synthesis of recombinant PyVs VP1 VLPs is essential for the study of their structure and interaction properties with other proteins, diagnostics of viral diseases and virus reactivation. VLPs resemble native virions in size, shape and antigenicity, and thus are useful as antigens (Ag) for immunoassays, seroprevalence or structural studies.

The aim of the dissertation was optimization and investigation of features of PyVs VP1 protein biosynthesis in *S. cerevisiae* cells, and application of purified HPyV VP1 VLPs for serology.

Tasks of the dissertation:

1. The investigation of the molecular chaperones impact on effectiveness of polyomavirus VP1 protein biosynthesis;
2. Characterization of the yeast flocculation phenomenon induced by polyomavirus VP1 biosynthesis.
3. Purification of novel human polyomaviruses capsid-forming VP1 proteins from yeast, and investigation of their immunogenic properties;
4. Testing the aptness of HPyV VP1 VLPs for use in serological assays.

Scientific novelty

Consistent elucidation of the requirement of yeast molecular chaperones for the optimization of recombinant polyomavirus VLPs biosynthesis was investigated for the first time. This study revealed the importance of Hsp90 chaperones for efficient polyomavirus biosynthesis (Valaviciute et al., 2016). Hornikova with colleagues confirmed the Hsp90 chaperone significance by showing their capability to assist PyV VP1 pentamers binding to microtubules in order to block the host cell cycle (Hornikova et al., 2017). Hopefully, Hsp90 chaperones might be a target fighting active polyomavirus infection and its complications in immunocompromised patients, for example, suffering from still incurable progressive multifocal leukoencephalopathy triggered by JC polyomavirus. This study determined that activity of Ssz1 and Zuo1 chaperones which form ribosome associated complex (RAC) were not beneficial for PyV VP1 and other viral proteins' biosynthesis in yeast. The connection between virus protein biosynthesis and RAC may be helpful for better understanding of protein synthesis processes.

In this work the cause of yeast flocculation induced by recombinant VP1 protein biosynthesis was investigated. It was demonstrated for the first time that APyV, MCPyV, MWPyV, STLPyV and BVPyV VP1 biosynthesis directly or indirectly restrain Cyc8 protein participation in Cyc8-Tup1 complex. Because of unavailability of Cyc8 the Cyc8-Tup1 complex is unable to repress *FLO1* gene and produced flocculins 1 causes yeast flocculation. It was shown that VP1

protein sequence, structure and its localization as well contributed to the induction of flocculation caused by APyV VP1 biosynthesis, and VP1 which had efficient NLS (not native) lost the ability to induce flocculation. Whereas yeast flocculation is related to a cell response to stress, and orthologs of Cyc8 in vertebrates are lysine demethylases, it can be assumed, that flocculation caused by synthesis of PyV VP1 proteins may be associated with pathogenic properties of VP1 proteins. Similarly, multifunctionality of mouse polyomavirus VP1 protein was demonstrated in mammalian cells when VP1 interaction with microtubules was responsible for cell cycle block at the G2/M phase (Hornikova et al., 2017).

The attempts of HPyV12 VP1 production in yeast were contributed to determination of the true position of HPyV12 VP1 protein translation initiation site. The synthesis of HPyV12 VP1 protein using the second methionine codon highly improved VP1 expression and VLP formation. These results from Norkiene et al., 2015 publication were used in the report of the 10th International Committee in Taxonomy of Viruses describing possible size of HPyV VP1 protein (Moens et al., 2017).

To our best knowledge, VP1 VLPs of the eleven new human PyV were generated and purified from yeast cells for the first time. Three of them (STLPyV, HPyV12 and NJPyV) were not ever synthesized in other expression system before. All new PyV VP1 VLPs were employed in hemagglutination assay (HA) using guinea pig or healthy human volunteer erythrocytes. The results revealed that only MCPyV, HPyV9, HPyV10 and HPyV12 VP1 VLPs can be used for detecting Abs against respective PyVs by HA assay.

To date, there is no commercially available serological test for HPyV, thus enzyme immunoassay (EIA) using all 13 HPyV VP1 VLPs for detection of Abs against HPyV VP1 was created. The serological study of the prevalence of all known HPyV was performed for the first time. According to our results, HPyVs are widespread in Lithuania population: 19-67 % healthy individuals' sera samples were positive (of 627 serum samples tested). Moreover, competitive EIA was generated using all 13 types of HPyV VP1 VLPs for preincubation with human sera samples in order to test cross-reactive anti-HPyV Abs activities. Competitive EIA results showed that due to possible cross-reactivity of anti-HPyV IgG Abs, some positive results in VP1 based indirect EIA detected for some PyVs (especially analyzing newest HPyV12 and NJPyV) can be false positive.

Defended propositions

1. VP1 proteins of novel human polyomaviruses assemble into VLPs in yeast's expression system;
2. The Hsp90, Hsp70, and Hsp40 chaperones are important for biosynthesis of recombinant PyV VP1 proteins in yeast;
3. Biosynthesis of VP1 VLPs of some of PyV in yeast cells creates deficiency of Cyc8 protein what triggers flocculin Flo1 dependent yeast flocculation;
4. The initiation of HPyV12 VP1 protein's biosynthesis using second translation iniciation site in the VP1-encoding ORF highly improves VP1 expression and VLP formation;
5. Recombinant HPyV VP1 protein VLPs are suitable for serological assays;
6. High content of anti-HPyV IgG antibodies was detected in the majority of tested human serum samples.

The contents of doctoral thesis. The doctoral thesis (in Lithuanian) contains the following parts: Introduction, Literature review, Material and Methods, Results and Discussion, List of references (344 citations), List of publications (5 positions), participation at conferences (5 positions), Figures (38), and Tables (13). 166 pages in total.

Materials and Methods

Reagents, enzymes and kits. All reagents used in this work were the highest quality available. All enzymes, their reaction buffers and kits were provided by UAB'Thermo Fisher Scientific Baltics' (Lithuania) and used according to the manufacturer's recommendations.

Oligonucleotides. Oligonucleotide primers for PCR were purchased from Metabion (Steinkirchen, Germany).

Bacterial, yeast strains and plasmids. All cloning procedures were performed in *E. coli* DH5α F^-gyrA96 (NaI^r) recA1 relA1 endA1 thi-1 hsdR17 ($r_k^- m_k^+$) glnV44 deoR Δ(lacZYA-argF)U169 [φ 80dΔ(lacZ)M15] or *E. coli* GM119 F^- dam-3 dcm-6 lacY1 galK2 galT22 tsx-78 glnV44 metB1 thi? fhuA? mtl? cells. Yeast Knock Out and YTHC collections were used for screening experiments (Thermo Fisher Scientific, Huntsville, USA). *S. cerevisiae* strains AH22 (*MATa leu2 his3*), AH22-214-Δ*pep4* (a, *his*4 *leu*2) and BY4741Δ*mnn10* were used for protein expression. Plasmids pFX7 (Sasnauskas et al., 1992), pFGG3 (Slibinskas et al., 2004), pFGPGK1 and pFGCNE1 (Ciplys et al., 2011) were used as *S. cerevisiae* expression vectors.

Bacterial, yeast media, transformation and growing conditions. *E. coli* strains were grown in LB medium at 37ºC. Transformations of *E. coli* were carried out by the $CaCl_2$-heat shock method (Bolivar and Backman, 1979). *S. cerevisiae* strains were grown at 30ºC in YEPD medium, transformed with plasmid DNA by LiAc/ssDNA/PEG method (Gietz et al., 1995). For selection and growing of *S. cerevisiae* transformants, YEPD medium was supplemented with 5 mM formaldehyde, for induction of recombinant gene expression YEPG medium (yeast extract 1%, peptone 2%, and galactose 3%) was used.

DNA preparation and manipulation. DNA amplification, DNA hydrolysis with restriction endonucleases, filling-inrecessed 3'-termini of double-stranded DNA, dephosphorylation of DNA 5'-termini and DNA ligation was performed using 'Thermo Fisher Scientific Baltics' (Lithuania) enzymes and kits, according to manufacturer's recommendations.

Flow cytometry. Samples were prepared by suspending harvested yeast cells in PBS containing 1 % BSA and 0.1 % NaN_3 after induction of recombinant protein synthesis. Intracellular proteins fused with enhanced green fluorescent protein (EGFP) levels were then analyzed using the CyFlow flow cytometry system with an excitation wavelength of 488 nm and a 512-542 nm emission filter on the FL1 channel. For each sample, the fluorescence of 10^4 live yeast cells was analyzed using FloMax 2.8 software (Partec GmbH, Germany).

Genomic yeast DNA extraction. Yeast cells walls were removed by Lyticase enzyme and isolation of yeast genomic DNA performed as described earlier (Johnston, 1994).

Preparation of whole yeast lysate. Yeast cells were suspended in Yeast Protein Extraction Reagent (Thermo Fisher Scientific, USA) or an equal volume of glass beads in destruction buffer and lysed by vortexing at room temperature for 20 min. The lysates were then mixed with 2× SDS-PAGE electrophoresis sample buffer (1 M Tris, pH 6.8, 50 % glycerol, 10 % SDS, 0.5 % bromophenol blue, 10 % β-mercaptoethanol) and boiled for 10 min. After boiling, equal amounts of protein sample were applied to an SDS-PAGE gel and run in SDS-Tris-glycine buffer.

SDS-PAGE, Western blotting (WB) and antibodies. SDS-PAGE and WB were performed according to standard protocols (Ausubel, 1999; Sambrook and Russel, 2001). Almost all mAb were produced in Department of Cell Biology and Immunology (head Dr. A. Žvirblienė).

VLPs purification from yeast cells and electron microscopy. Purification of recombinant PyV VP1 proteins and Electron microscopy were carried out as described previously (Norkiene et al., 2015).

Hemagglutination assay (HA) was performed as described earlier (Norkiene et al., 2015).

Indirect EIA. Flat-bottomed 96-well polystyrene microplates (Nerbe plus, Germany) were coated with the purified VLPs (2 µg/mL, 100 µL/well). The plates were washed twice with 10mM phosphate-buffered saline (PBS) containing 0.05 % Tween 20 (PBS-T) and then blocked with 150 µL of Blocking agent (Roti®-Block, ROTH, Germany) for 1 h at 37 °C. After three washes with PBS-T, each serum sample was serially diluted 1:100, 1:200 and 1:400 in PBS buffer and 100 µL was added per well at 37 ˚C for 1 h, followed by washing five times. The wells were incubated with 100 µL of anti-human IgG immunoglobulins conjugated to HRP (Thermo scientific, USA) diluted at 1:12000, at 37 ˚C for 60 min. After washing five times, TMB were used as the substrate to develop the color for 5 min. The reaction was stopped with 50 µL 10 % sulphuric acid per well. The absorbance of wells was measured at a 450 nm wavelength (TECAN infinite M200, Switzerland).

Competitive EIA. For competitive indirect EIA, the serum specimens were diluted at 1:100 and incubated with 5, 15, 25 mg/mL of each of competitive antigen at 37 °C for 60 min. These mixtures subjected to EIA in parallel with non-preadsorbed samples and further procedures were identical to those

described in indirect ELISA. The results were reported as % of OD reduction after preadsorption with VLPs calculated according to the following formula: [(OD non-preadsorbed — OD preadsorbed)/OD non-preadsorbed] × 100.

Statistical analysis. All data were analyzed using Microsoft Excel 2013 Analysis ToolPak program. Parental strain fluorescence was standardized to 100 %. At least six independent clones ($n < 6$) were used for the evaluation of protein expression in each strain. The data are presented as the mean and standard deviation (± SD) of at least six independent experiments. A two-tailed unpaired Student's t-test was used to determine significant differences in protein production levels.

Results

1. Optimization of PyV main capsid proteins synthesis in *S.cerevisiae*

Rationale of the experiments used for the targeted yeast genes requirement analysis for PyV capsid protein biosynthesis. Although great sequence identity of human PyV VP1 proteins' (van der Meijden et al., 2013), expression and purification of some of PyV VP1 in yeast were a struggle because of low protein yield, yeast flocculation, insufficient VLPs formation. In order to generate high quality PyV VP1 VLPs, first task was to investigate some features of PyV VP1 protein synthesis in yeast cells. First, we estimated the requirement for molecular chaperones during the biosynthesis of PyV capsid proteins in yeast. Second, reasons of yeast flocculation caused by expression of recombinant APyV VP1 protein were studied. HaPyV VP1-EGFP fusion protein as a recombinant viral protein model was used. VP1-EGFP was expressed in Yeast Knock out Collection strains derived from *S. cerevisiae* strain BY4147 with individual chaperone gene deletions. Differences in VP1-EGFP fluorescence levels were evaluated in live yeast cells by flow cytometry and were compared to VP1-EGFP fluorescence levels in parental BY4741 strain cells. VP1 and VP1-EGFP proteins form multi-molecular VLP structures in yeast; thus, to evaluate the requirement for general molecular chaperones during recombinant protein production, a soluble recombinant goat UK114 protein fused to EGFP at the C-terminus (UK114-EGFP) under the control of the constitutive PGK1 promoter was used as a non-viral protein control in parallel with VP1-EGFP (Colombo et al., 1998). To verify the results obtained by flow cytometry using the VP1-EGFP fusion, the production level of recombinant unmodified HaPyV VP1 protein was monitored in the yeast lysates by WB. For yeast flocculation analysis APyV VP1 gene was exploited as a flocculation-inducing protein.

The production of human PyV VP1-derived VLPs was analyzed in selected yeast mutant strains. For the biosynthesis of KIPyV-, WUPyV-, HPyV6-, HPyV7-, TSPyV-, HPyV9-, STLPyV-, and NJPyV-derived VLPs in yeast, codons in the VP1-encoding open reading frames (ORFs) were optimized for expression in *S. cerevisiae*. HPyV10 VP1 gene was synthesized according to the native virus gene sequence without codon optimization. MCPyV VP1 gene was amplified by PCR from the MCPyV virus genome cloned from a healthy volunteer skin swab. All 10 VP1-encoding genes were inserted in the same site in the expression vector, pFX7 (Sasnauskas et al., 1999), under the control of a galactose-inducible promoter. Plasmids with JCPyV and BKPyV VP1 genes were taken from collection of Department of Eukaryote Genetic Engineering

(Sasnauskas et al., 2002). Additionally, evaluation experiments of HaPyV VP1-EGFP and unmodified VP1 protein production levels in conjunction with the mild overexpression of ORFs encoding the chaperones or other selected proteins under the control of the *CNE1* promoter was performed. The *CNE1* promoter was chosen, as the expression of some chaperone genes under stronger PGK1 promoter was toxic to yeast cells. ORFs encoding the chaperones or other selected proteins were amplified by PCR from yeast genomic DNA.

Survey of molecular chaperone requirement for the biosynthesis of Polyomavirus VP1 capsid protein. Molecular chaperones play important role in cell life by preventing protein aggregation and participating in protein folding, both in normal cell growth or cell development and under stress conditions. The production of the VP1, VP1-EGFP, and UK114-EGFP proteins were affected in all of the yeast strains with single deletions of the Hsp104, Hsp90, and Hsp70 molecular chaperones compared to the parental BY4741 strain (Fig. 1). The yeast stress response chaperone Hsp104, had an apparent negative effect on HaPyV VP1 protein production levels. VP1-EGFP fluorescence levels were slightly higher in the Δ*hsp104* strain, but lower in the yeast strain that mildly overexpressed *HSP104* compared to the parental BY4741 strain (Fig. 1 B). In contrast, the biosynthesis of the HaPyV capsid protein was highly dependent on Hsp90 chaperones. The yeast Hsp90 family consists of two isoforms encoded by the genes *HSC82* and *HSP82* (Csermely et al., 1998). Deletion of *HSC82* and *HSP82* significantly reduced VP1-EGFP protein levels by 70 % and 50 %, respectively, compared to the parental BY4741 strain (Fig. 1 A). Likewise, the expression of *HSC82* and *HSP82* under the control of the *CNE1* promoter significantly increased the production of VP1-EGFP protein by 182 % and 166 %, respectively, compared to corresponding protein levels in the parental BY4741 strain (Fig. 1 B). These results were consistent with the observation that the deletion of *STI1*, which is a component of the Hsp90 chaperone complex required for its activity (Li et al., 2012), also decreased VP1-EGFP fluorescence levels by 68 % but did not affect UK114-EGFP expression (Valaviciute et al., 2016).

During the PyV infection Hsp70 chaperones may be important because the large T antigen of PyV also containing J-domen. The large T antigens of polyomavirus have been shown to stimulate Hsp70 protein expression in mouse fibroblasts cells (Cripe et al., 1995). In addition, Hsp70 chaperones interact with the C-terminal domain of VP1 to inhibit calcium-mediated capsid assembly in vitro (Chromy et al., 2003). In yeast, the Hsp70 chaperone family includes nine cytosolic members that are encoded by four *SSA* genes, two *SSB* genes, two *SSE* genes, and *SSZ1*. *LSH1* and *KAR2* encode ER Hsp70 chaperone family members.

According to considerable functional redundancy in the Hsp70 family, particularly in the SSA subfamily (Werner-Washburne et al., 1987), additional yeast strains with deletions of both *SSA*1 and *SSA2* and both *SSA3* and *SSA4* were used. In both double mutants VP1-EGFP and UK114-EGFP production levels were reduced (Fig. 1 A). Deletions of the other Hsp70 chaperone family members *SSE1*, *SSE2*, *SSB1*, *SSB2* and *LHS1* reduced VP1-EGFP levels by 50 %, whereas the levels of the UK114-EGFP protein were reduced by 25 % (Fig. 1 A). In contrast, from the mild overexpression of Hsp70 chaperones in yeast analysis, only *SSE1* significantly increased VP1-EGFP production (Fig. 1 B), which indicated the possible involvement of this chaperone in VP1 synthesis. Deletion of *SSZ1*, which encodes an atypical Hsp70, resulted in a 2.3-fold increase in VP1 levels compared to corresponding levels in the parental BY4741 strain (Fig. 1 A). The Ssz1 chaperone is a component of the ribosome-associated complex (RAC), which stimulates the specific ATPase activity of both Ssb chaperones and enhances the interaction of Ssb with newly synthesized polypeptide chains (Huang et al., 2005).

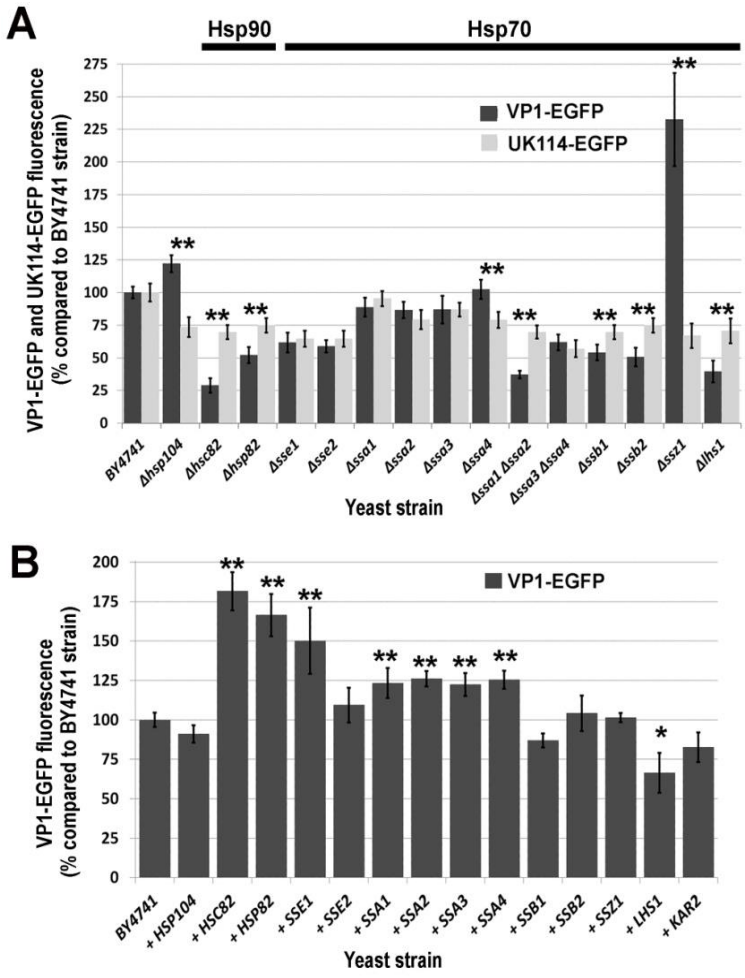

Fig. 1 VP1-EGFP and UK114-EGFP protein fluorescence analysis in Hsp104, Hsp90, and Hsp70 chaperone deletion (A) and mild overexpression (B) strains. The results are presented as % change relative to the fluorescence level in the parental yeast BY4741 strain and are from at least six independent transformants. Values shown are mean ± SD. The asterisks represent significant differences between VP1-EGFP and UK114-EGFP protein fluorescence:
(*) = $P < 0.05$,
(**) = $P < 0.01$.

The majority of yeast strains with single deletions of genes encoding J-domain family proteins (also known as Hsp40 co-chaperones) produced lower levels of the recombinant proteins of interest compared to the parental BY4741 yeast strain (Fig. 2). Hsp40 co-chaperones associate with Hsp70 chaperones and influence their function, in part by stimulating Hsp70 ATPase activity. VP1-EGFP levels were threefold greater in the Δ*zuo1* strain but decreased by more than 60 % in the Δ*jem1,* Δ*caj1*, and Δ*erj5* strains. Also, production of VP1-EGFP and VP1 in the yeast Δ*zuo1* strain was even higher than in its RAC partner *SSZ1* deletion strain (Figs. 1 A and 2). VP1-EGFP expression was restored to wild-type levels by the expression of exogenous *ZUO1* (data not shown). Moreover, these deletions did not have negative effect on virus like particle formation (Valaviciute et al., 2016), despite lower translation fidelity that is characteristic for these deletions (Huang et al., 2005; Chen et al., 2014). The mild overexpression of chaperones with a J domain in the parental BY4741 strain had variable effects on VP1 production. The overexpression of YDJ1 increased VP1 levels by 235 %, whereas the overexpression of SIS1, CAJ1, and ERJ5 increased VP1 levels to approximately 150 % (Fig. 3).

Effects of Hsp104, Hsp90, Hsp70 and Hsp40 chaperones on the production levels of VP1-EGFP, as determined by flow cytometry, was confirmed by WB using the unmodified VP1 protein (Fig. 3; Valaviciute et al., 2016).

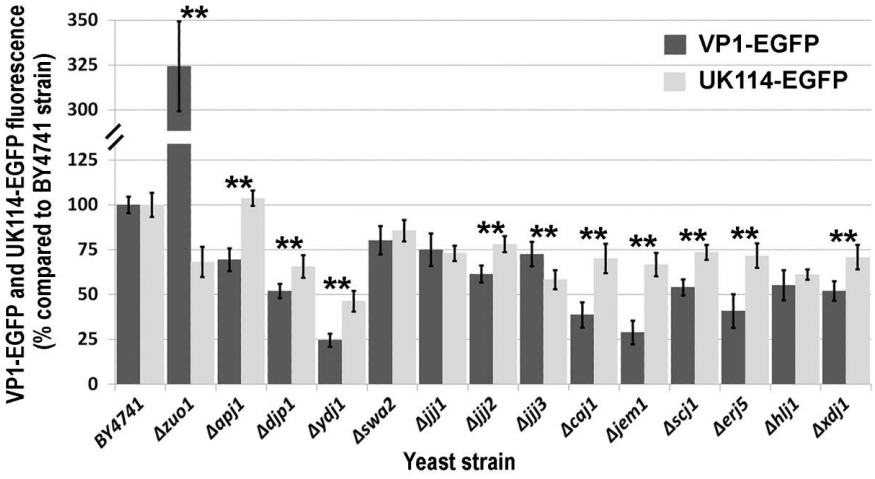

Fig. 2 Analysis of VP1-EGFP and UK114-EGFP protein fluorescence Hsp40 molecular chaperone deletion strains. The results are presented as % change relative to the fluorescence levels in the parental yeast BY4741 strain and are from at least six independent transformants. Values are shown as mean ± SD. The asterisks represent significant differences between VP1-EGFP and UK114- EGFP protein fluorescence: (*) = $P < 0.05$, (**) = $P < 0.01$.

It was discovered that *ssz1* and *zuo1* deletions increased viral protein expression levels of all 13 types HPyV VP1 investigated. Because of slow growth, *Δssz1* and *Δzuo1* mutants were not used for HPyV VP1 purification.

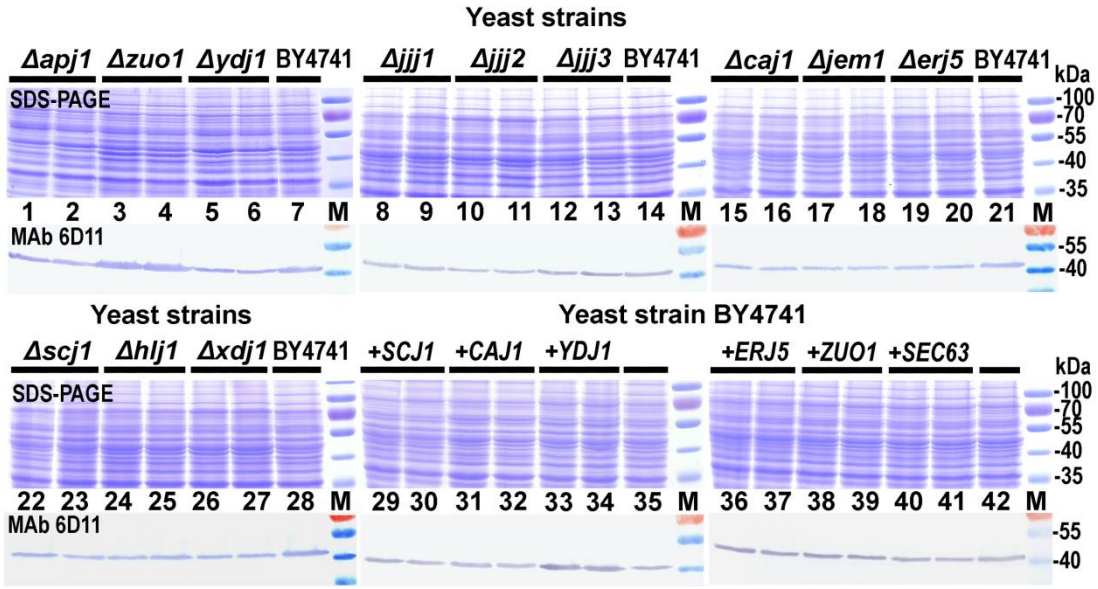

Fig. 3 Evaluation of VP1 production in Hsp40 chaperone deletion and mild overexpression strains by Western blot. Production of the unmodified VP1 protein was analyzed in yeast lysates. Coomassieblue-stained SDS-PAGEs and MAb 6D11 WBs. The lanes are as follows: lysates of VP1 produced in the Δ*apj1* (lanes 1–2), Δ*zuo1* (lanes 3–4), Δ*ydj1* (lanes 5–6), BY4741(lanes 7, 14, 21, 28, 35, and 42), Δ*jjj1* (lanes 8–9), Δ*jjj2* (lanes 10–11), Δ*jjj3* (lanes 12–13), Δ*caj1* (lanes 15–16), Δ*jem1* (lanes 17–18), Δ*erj5* (lanes 19–20), Δ*scj1* (lanes 22–23), Δ*hlj1* (lanes 24–25), and Δ*xdj1* (lanes 26–27) strains; lysates of VP1 produced by the co-expression of external SCJ1 (lanes 29–30), CAJ1 (lanes 31–32), YDJ1 (lanes 33–34), ERJ5 (lanes 36–37), ZUO1 (lanes 38–39), and SEC63 (lanes 40–41) under the control of the CNE1 promoter in the BY4741 strain. M, protein molecular weight marker (Thermo Fisher Scientific Baltics).

Analysis of yeast flocculation induced by synthesis of PyV VP1 proteins. Flocculation is a remarkable capacity of *S. cerevisiae* cells, which is conferred by a class of special cell wall lectin-like proteins, called flocculins. Flocculent yeast cells carry several different flocculins, essential for special adhesion to each other (El-Kirat-Chatel et al., 2015). Several signaling cascades tightly control synthesis of the different flocculins. Together, these pathways trigger flocculation in response to different kind of stress's or nutrient limitation (Claro et al., 2007). In addition, flocculins are subject to telomeric and subtelomeric epigenetic switching, resulting in stochastic expression patterns (Verstrepen and Klis, 2006). Biosynthesis of birds PyV (APyV, CPyV, FPyV, GHPyV) (Sasnauskas et al., 2002; Zielonka et al., 2006; 2012), voles PyV (CPyV, BVPyV) or some of human PyV VP1 proteins' in yeast results in yeast cell flocculation. In order to improve yield and quality of flocculation inducing PyV VP1 protein, we analysed the reason of this phenomena. First, we

determined that Flo1 floculine is responsible for flocculation induced by APyV VP1 biosynthesis as it was eliminated only in Δ*flo1* mutant strain but not in yeast strains where other flocculin's or its regulating genes were deleted. Whereas *FLO1* gene is localised in yeasts first chromosome's telomeric region and normally is repressed (Russell et al., 1980), we screened 163 selected yeasts mutants with deletions of telomeric silencing genes. However, nor 131 nonessential genes deletions, nor 21 of essential gene deletions, did have impact in APyV VP1 induced flocculation. Thus, further 11 mutants which flocculated by itself were analysed by expressing in mutant yeasts all 11 respective protein encoding genes amplified by PCR and inserted under *PGK1* promoter in the expression vector with APyV VP1 or HaPyV VP1 genes under the control of galactose-inducible promoter *GAL7*. *CYC8* gene expression not only compensated deficiency of Cyc8 protein in *Δcyc8* yeast strain, but also totally eliminated flocculation induced by biosynthesis of APyV VP1 in both *Δcyc8* and parental BY4741 yeast strains. Cyc8, APyV and HaPyV VP1 protein levels were confirmed in yeast lysates by WB (data not shown). In order to assure that Cyc8p is responsible also for human and vole PyV VP1 production caused flocculation, plasmids containing *CYC8* gene and one of MCPyV, HPyV10, STLPyV, BVPyV VP1 genes were generated. After the induction of MCPyV, HPyV10, STLPyV, BVPyV VP1 gene expression, yeast transformants with additional Cyc8p synthesis no longer flocculate. These results showed that yeast flocculation induced by some of PyV VP1 biosynthesis may be consequence of Cyc8p deficiency and restriction of it in participation in Cyc8-Tup1 complex. The deprivation of Cyc8-Tup1 complex leads to activated state of *FLO1* gene transcription and flocculation (Fleming et al., 2014).

Using a second approach we investigated which part of APyV VP1 protein might be responsible for flocculation induction. Ten truncated variants of APyV VP1 (Fig. 4) and three HaPyV VP1 with changed N-terminus (Table 1) encoding genes were generated and subcloned into pFX7 expression vector and used for induction of mutant VP1 genes expression. The levels of recombinant modified APyV and HaPyV VP1 proteins were monitored in the yeast lysates by WB (data not shown). Visual analysis of yeast transformants' flocculation showed that the truncation of APyV VP1 till 288N does not affected flocculation induction capability. This VP1 N288N mutant does not have C-terminal arm, but has intact globular domain and is still able to form pentamers.

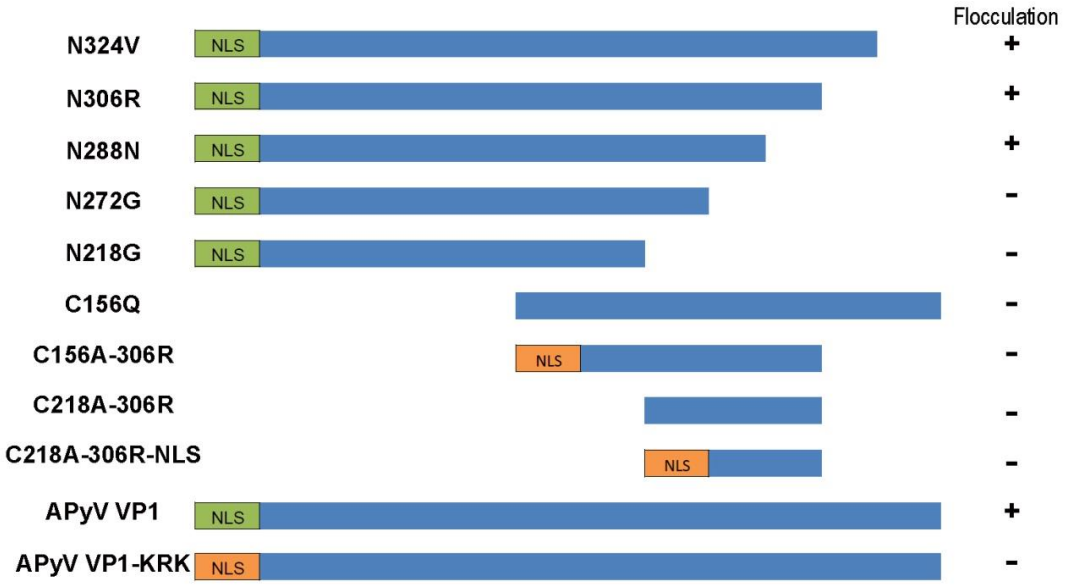

Fig. 4 Schematic representation of full or truncated APyV VP1 protein variants with native or changed NLS. The numbers and letters in the name of the proteins in the left indicate truncation position and last aa. Protein ability to cause flocculation marked + in the right. Native NLS marked in green colour, SV40 virus NLS - orange colour and protein's aa. sequence – blue colour.

Table 1. HaPyV and some of APyV VP1 proteins used for flocculation assay

Name	Protein sequence	aa. of NLS	Size, kDa
APyV VP1	Full lenght APyV VP1 protein (344 aa.)	**MSQKGK-GSCPR**	37,54
APyV VP1del	APyV VP1 protein with no NLS	**M------GSCPR**	36,99
HaPyV VP1	HaPyV VP1 protein	**MAPKRKSGASSR**	42,35
Ha-Nm1	HaPyV VP1 protein	**MAPKGKSGASSR**	41,6
Ha-Nm2	HaPyV VP1 protein without KRK aa.	**MAP---SGASSR**	41,03
Ha-Nm3	HaPyV VP1 protein with APyV VP1 N-terminus sequence	**MSQKGKG--SSR**	41,23

Further truncation of VP1 protein affects globular domain and are unable for yeast flocculation induction. Thus, correct APyV VP1 structure or/and ability to form pentamers is important for yeast flocculation induction. Another cause affecting floculiation induction by the VP1 biosynthesis might be its NLS. Typicaly PyV VP1 protein has conservative sequence of MAP(x)KRK aa which serves as NLS on its N-terminus, but APyV VP1 NLS sequence is different and inefficient at least in insect cells (Zielonka et al., 2012). The yield of APyV VP1del variant (Table 1) with deleted NLS and ability of flocculation induction in yeast was very similar to native APyV VP1 suggesting that native APyV VP1

NLS is also inefficient in yeast (data not shown). The biosynthesis of APyV VP1 with SV40 VP1 NLS (VP1-KRK protein) improved its yield and no longer induced yeast flocculation, VP1-KRK protein was transported to nucleus and possible no longer held Cyc8p in cytoplasm. On the other hand, native HaPyV VP1 protein as well as its mutant with no NLS (Ha-Nm2) or partial and full APyV VP1's NLS (Ha-Nm1 or Ha-Nm3) (Table 1) did not induce yeast flocculation. Interestingly, PyV VP1 proteins able to induce flocculation have atypically longer N-termini (MCPyV, HPyV10 and STLPyV VP1) or no typical NLS (APyV, GHPyV, FPyV, CPyV and BVPyV VP1). Moreover, human PyV VP1 proteins with atypical NLS (WUPyV, KIPyV, HPyV6 or HPyV7 VP1) formed mainly small size 25–35 nm VLP (Fig. 5) comparing to usual 45-50 nm size of PyV VLP.

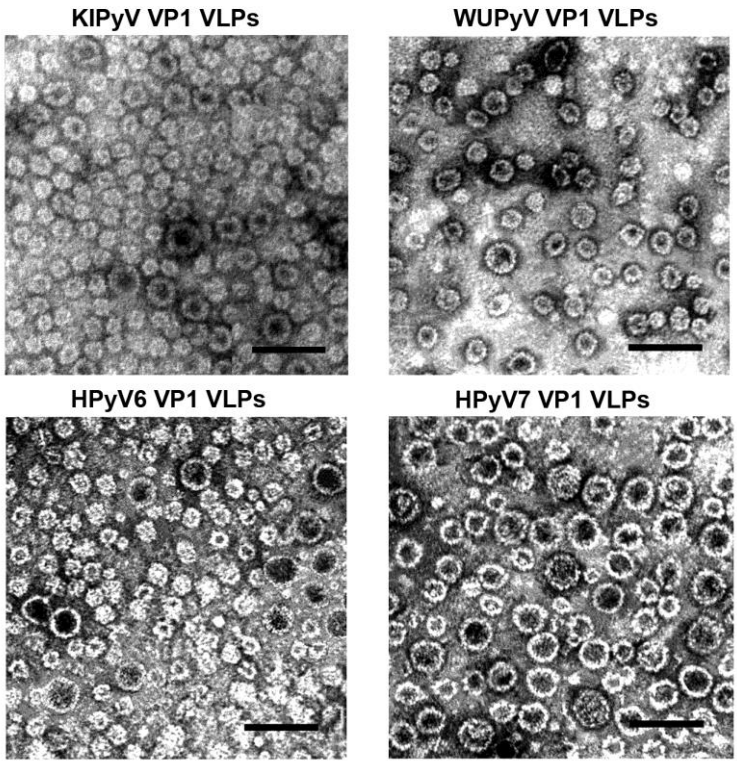

Fig. 5 Electron micrograph of human KIPyV, WUPyV, HPyV6 and HPyV7 VP1 proteins VLPs. Scale bar – 100 nm.

The impact of NLS in WUPyV VP1 VLPs yield and assembly quality. In order to analyze possible changes of VLPs size and/or VP1 protein yield modified WU VP1-KRK gene was used. The modified WU VP1-KRK protein had MAPTKRK aa sequence at N-terminus, instead of native for WU PyV VP1 sequence - MACTAR. Analysis of WU VP1 and V1-KRK protein expression levels in yeast cells lysates and in pelets collected after ultracentrifugation through 30 % sucrose cushion showed no differences (data not shown). Nevertheless, the yield of WU VP1-KRK protein was two-times greater than WU VP1, after purification by CsCl gradient centrifugation (data not shown). Examination of purified VP1 protein samples by negative staining microscopy

(EM) demonstrated that WU VP1-KRK formed typical VLPs with a diameter of 45-50 nm more efficiently than native VP1 protein (Fig. 6). Furthermore, we used nanoparticle tracking analysis (NTA using NanoSight LM10-HS apparatus) for the VLP size characterization of purified WU VP1-KRK and VP1 proteins. After NTA analysis we calculated the proportion of normal (41.5-50.5 nm) sized and small (31.5-40.5 nm) sized VLPs in both samples. These proportions were $3.6 \times 10^6 : 0.02 \times 10^6$ for WU VP1-KRK, and $9.5 \times 10^6 : 1.5 \times 10^6$ for WU VP1. Thus, NTA analysis revealed that NLS sequence modification of WU VP1-KRK protein increased efficiency of VLPs assembly up to 28 times.

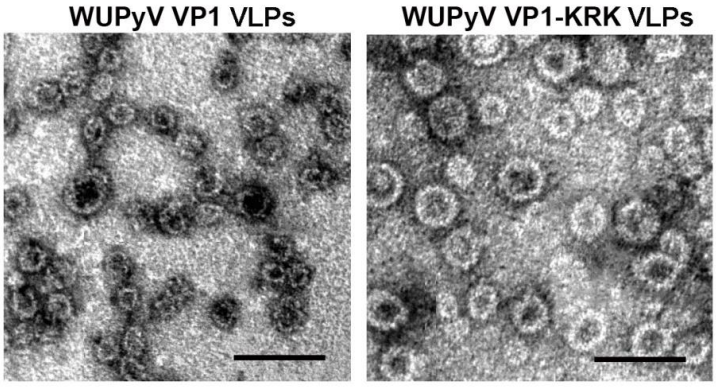

Fig. 6 Electron micrograph of human WUPyV VP1 and VP1-KRK proteins VLPs. Scale bar – 100 nm.

Determination of HPy12 VP1 translation initiation codone. After sequencing of HPyV12 genomic sequence it was assumed that VP1 gene encodes a 380-aa-long protein (GenBank:JX308829). For production of HPyV12-derived VP1 VLPs in yeast, we used both the native virus gene sequence (HPyV12 VP1**n**^{380}) and a version that was codon-optimized for *S. cerevisiae* expression (HPyV12 VP1**s**^{380}), synthesized by GenScript. Both VP1-encoding genes were cloned into the expression vector, pFX7 and used for expression in the yeast strain, AH22-214. Expression of HPyV12 VP1^{380}, analyzed using SDS-PAGE, was low using both versions of the gene (Fig. 7 A). Our attempts to purify HPyV12 VP1^{380} using sucrose and CsCl gradient centrifugation did not achieve complete purity of VP1 protein (Fig. 7 A, line 9). The yield of partially-purified VP1^{380} was very low: only 0.05 mg per 1 g of wet yeast biomass. Examination of HPyV12 VP1^{380} assembly into VLPs using negative staining EM demonstrated that the majority of the recombinant VP1^{380} formed atypical, small (20 nm in diameter) particles or aggregates (Fig. 8). Sequences alignment of VP1 proteins derived from different PyVs showed that the N-terminal end of the HPyV12 VP1 protein sequence overhangs in the alignment (Norkiene et al., 2015). On the other hand, the N-terminal arm encoded within a sequence starting from a second potential translation initiation

site in the VP1 gene appeared to be typical of PyV VP1. Based on these observations, we decided to express HPyV12 VP1 using a sequence that did not encode an atypical, hydrophobic, 16-aa-long N-terminal peptide. We used the native virus gene sequence (HPyV12 VP1**n**^{364}) and one that was codon-optimized for *S. cerevisiae* expression (HPyV12 VP1**s**^{364}), starting at the second translation initiation site. Production of the 364-aa-long HPyV12 VP1^{364} protein was analyzed using SDS-PAGE. The expression level of VP1^{364} was greatly improved compared with the longer version of the gene (Fig. 7 A and B).

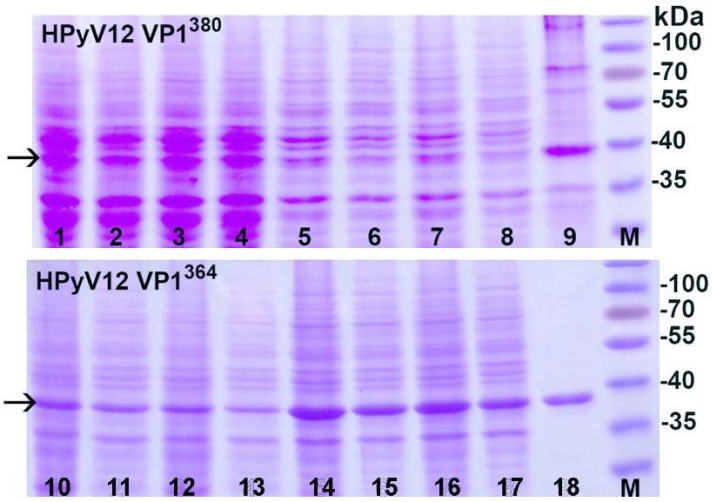

Fig. 7 Analysis of HPyV12-derived VP1 VLP production in yeast using Coomassie blue-stained SDS-PAGEs. Production of HPyV12 VP1^{380} protein was analyzed in yeast clones transformed with pFX7-HPyV12-VP1**n**^{380} plasmid (lines 1–4) and pFX7-HPyV12-VP1**s**^{380} plasmid (lines 5–8); production of HPyV12 VP1^{364} protein was analyzed in yeast clones transformed with pFX7-HPyV12-VP1**n**^{364} (lines 10-13) and pFX7-HPyV12-VP1**s**^{364} plasmid (lines 14-17). In lanes: 1, 3, 5, 7, 10, 12, 14 and 16 – yeast's whole lysates; 2, 4, 6, 8, 11, 13, 15 and 17 - the soluble fraction recovered after centrifugation of yeast's whole lysates; 9 and 18 - purified HPyV12-VP1^{380} protein, and purified HPyV12-VP1^{364} protein; M - Prestained protein weight marker.

HPyV12 VP1^{364} was purified and the yields of VP1^{364} were approximately 0.4 mg and 1 mg per 1 g of wet yeast biomass using the native gene and the codon-optimized gene, respectively. Thus, using codon-optimized HPyV12 VP1^{364} clearly increased expression and yield of the protein. Analysis of the purified HPyV12 VP1^{364} lacking the 16-aa-long N-terminal using negative staining EM showed that this protein efficiently assembled into VLPs that were 45–50 nm in diameter (Fig. 8). Collectively, these data suggest that the translation initiation site of VP1 was predicted incorrectly by the HPyV12 genome sequence annotated in GenBank. The second translation initiation site in the putative ORF encoding VP1 is more likely the true start site, resulting in

efficient translation of a 364-aa-long VP1 protein and it's self-assembly into VLPs.

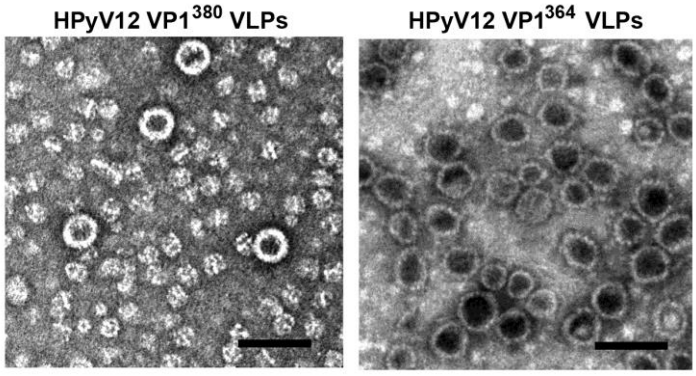

Fig. 8 Detection of purified HPyV12 derived VP1 VLPs using negative staining electron microscopy. Electron micrographs of HPy12 $VP1^{380}$ ir $VP1^{364}$ VLPs produced in yeast. Scale bar 100 nm.

2. Production and purification of recombinant VP1-derived virus-like particles from novel human PyVs in yeast

KIPyV, WUPyV, MCPyV, HPyV6, HPyV7, TSPyV, HPyV9, STLPyV, and HPyV12 VP1 proteins were produced and purified from AH22-214 yeast strain and HPyV10 and NJPyV VP1 proteins from AH22-214-Δ*pep4* mutant strain. To determine whether all 11 recombinant VP1 protein were expressed in yeast transformed with pFX7 carrying HPyV VP1-encoding ORFs, yeast lysates were analyzed using SDS-PAGE (Fig. 7 B lines 5 and 7; Norkiene et al., 2015). All 11 recombinant VP1 proteins stayed in the soluble fraction after the centrifugation of yeast lysates (Fig. 7 B lines 6 and 8; Norkiene et al., 2015) and the solubility of VP1 allowed for the efficient purification by sucrose and CsCl gradient centrifugation (Fig. 7 B line 9; Norkiene et al., 2015). The yield of purified VP1-derived VLPs ranged from 0.44 to 1.05 mg per 1 g of wet yeast biomass (Norkiene et al., 2015). The lower yields of purified VP1 from HPyV6, HPyV7, HPyV10, and NJPyV may have been the consequence of minor differences in expression level and solubility. During purification, KIPyV-, HPyV6-, and HPyV7-derived VP1 tended to degrade, therefore, additional bands in the SDS-PAGE gel were observed. Highly-soluble TSPyV- and HPyV9-derived VP1 were purified with yields as high as 1 mg per 1 g of wet yeast biomass. The ability of VP1 proteins to self-assemble into VLPs was examined using negative staining EM. All 11 purified VP1 proteins formed VLPs (Figs. 6, 7 and 9). MCPyV-, TSPyV-, and NJPyV-derived VLPs were found to be the most similar in size, with diameters of 45–50 nm (Fig. 9). Although the majority of VLPs in preparations from KIPyV, HPyV7, HPyV9, HPyV10, and STLPyV were 40–50 nm in diameter (Figs. 6 and 9), fractions of

smaller VLPs, 25–35 nm in diameter, predominated in samples from WUPyV and HPyV6 (Fig. 6).

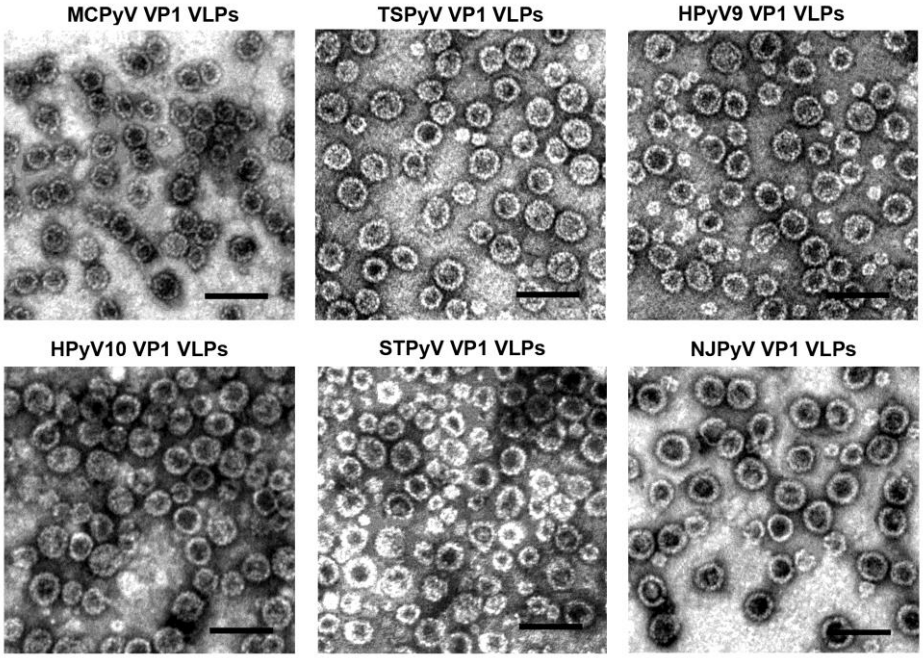

Fig. 9 Detection of purified HPyV-derived VP1 VLPs using negative staining electron microscopy. Electron micrographs of MCPyV, TSPyV, HPyV9, HPyV10, STPyV and NJPyV VP1 VLPs produced in yeast.

3. HPyV VP1 application for serology

Detection of purified HPyVs derived VP1 proteins by WB. The sequence alignment of VP1 proteins derived from different HPyVs showed 25–78 % identity in amino acid sequence (Moens et al., 2013). Therefore, the cross-reactivity of VP1 proteins allowed detection of all purified HPyV VP1 proteins by WB using only four polyclonal antibodies (PAb): anti-JCPyV, anti-HaPyV, anti-MCPyV, and anti-WUPyV VP1 PAb. PAb raised against JCPyV-VP1 protein cross-reacted with BKPyV VP1 protein (78 % identity) (Sasnauskas et al., 2002) as well as with TSPyV (52 % identity), HPyV12 (54 % identity), and HPyV13 (48 % identity) VP1 proteins (Fig. 10 B, lanes 3, 9, 13, 14). A high degree of cross-reactivity of HPyV VP1 proteins was observed with rabbit antiserum raised against HaPyV VP1 protein. This antiserum failed to detect only KIPyV (31 % identity), WUPyV (31 % identity), HPyV10 (47 % identity), and STLPyV (43 % identity) VP1 proteins (Fig. 10 C, lanes 4, 5, 11, 12). Anti-MCPyV VP1 polyclonal antibody cross-reacted with TSPyV (57 % identity) and HPyV10 (42 % identity) VP1 proteins (Fig. 10 D, lines 9 and 11). Anti-WUPyV VP1 polyclonal antibody showed a very strong cross-reactivity with KIPyV VP1

(66 % identity) but also detected HPyV7, HPyV10, and HPyV13 VP1 proteins identical to WUPyV VP1 only 41, 33, and 48 %, accordingly (Fig. 10 E, lines 4, 8, 11, and 14).

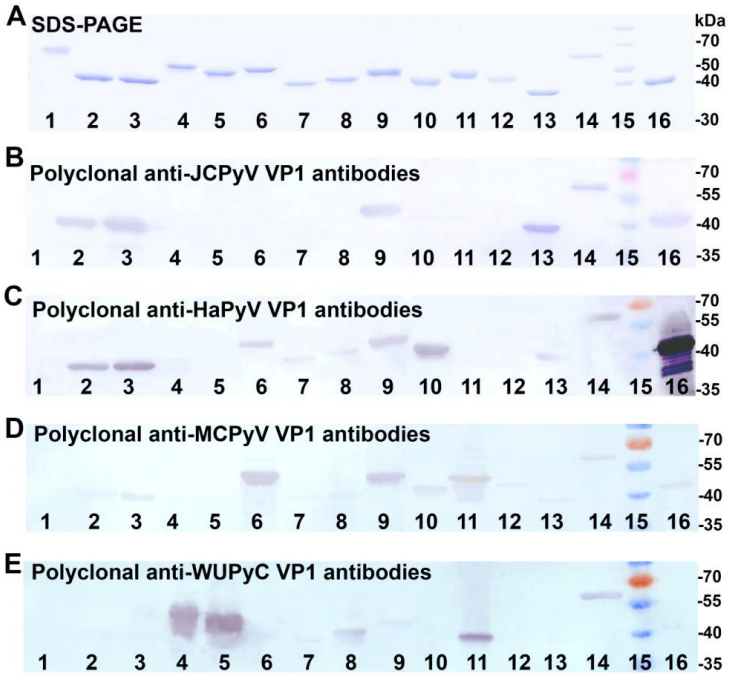

Fig. 10 Detection of purified HPyV-derived VP1 VLPs in Western blot with PAb. Coomassie blue-stained SDS-PAGE (**A**); Western blot with anti-JCPyV VP1 (B), anti-HaPyV VP1 (C), anti-MCPyV VP1 (D), and anti-WUPyV VP1 (E) PAb. The same samples of purified proteins were run on each gel. In lanes: 1 - HPyV16 L1, 2 - BKPyV VP1, 3 - JCPyV VP1, 4 - KIPyV VP1, 5 - WUPyV VP1, 6 - MCPyV VP1, 7 - HPyV6 VP1, 8 - HPyV7 VP1, 9 - TSPyV VP1, 10 - HPyV9 VP1, 11 - HPyV10 VP1, 12 - STLPyV VP1, 13 - HPyV12 VP1, 14 - NJPyV VP1 and 16 - HaPyV VP1 proteins. 15 - Protein weight marker (Thermo Fisher Scientific Baltics).

HA activity of VP1-derived VLPs from novel PyVs. All 11 purified VLPs were subjected to HA testing using guinea pig erythrocytes. JCPyV- and BKPyV-derived VP1 VLPs were used as positive controls and SV40-derived VP1 VLPs was used as negative control with no HA activity (Knowles et al., 2003). HPyV10-, and HPyV12-derived VP1 VLPs demonstrated strong HA activity, down to a concentration of 0.2 μg mL^{-1}. TSPyV and HPyV9-derived VP1 VLPs showed HA activity down to a concentration of 0.39 μg mL^{-1} and 0.78 μg mL^{-1}, respectively. WUPyV-, KIPyV-, HPyV6-, HPyV7-, STLPyV-, and NJPyV-derived VP1 VLPs did not show any HA activity (Fig. 11).

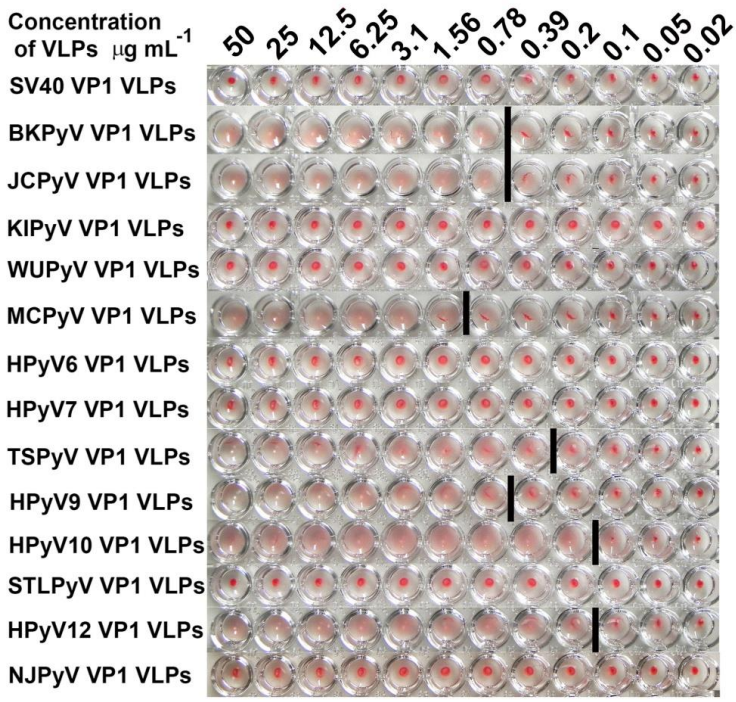

Fig. 11 HA activity of HPyV-derived VP1 VLPs. A 2-fold dilution series of purified KIPyV-, WUPyV-, MCPyV-, HPyV6-, HPyV7-, TSPyV-, HPyV9-, HPyV10-, STLPyV-, HPyV12-, and NJPyV-derived VP1 VLPs expressed in *S. cerevisiae* were subjected to a HA assay with 1 % guinea pig erythrocytes. The concentrations of VP1-derived VLPs in μg mL^{-1} are shown on the top. The bar indicates the highest VLP concentration at which HA of guinea pig erythrocytes was observed (HA titer).

Indirect EIA using VP1-derived VLPs from PyVs. Enzyme immunoassay (EIA) is the most widely used technique for HPyV Abs detection, which varies in performance, serum dilutions, empirically derived cut-offs and Ag preparations. EIA with VLPs as the coating antigen has been shown to be more sensitive and specific than VP1 in the monomer or pentamer form (Bodaghi et al., 2009). All 13 recombinant HPyV VP1 VLPs were employed to detect virus-specific antibodies in human serum specimens**.** Since commercial serologic test systems for PyV infection are still not available, it was not possible to surely identify anti-PyV positive and negative sera. In that case, an optical density (OD) cut-off value was conservatively determined separately for each antigen: the mean OD value plus 3 SD for 121 negative serum samples from immunocompetent children less than 5 years old (Table 2). Four control sera known to be positive or negative for appropriate antigen were tested on each plate. To confirm these results, samples with values within 10 % above the cut-off value were retested. Only samples with repeated positive results were considered reactive. Briefly, EIA analysis of the 1106 serum specimens from two different groups was performed. First (low risk) group of sera specimens (n=627) isolated from immunocompetent, healthy Lithuanians venous blood,

and the second (high risk) group of sera specimens (n=479) isolated from patients of dermal-venereal center (Bulgaria) venous blood.

Table 2. The cut-off OD values used in this study for PyVs VP1 based EIA analysis. Sera diluted 100×

HPyV VP1 VPD	Mean OD value	SD	Cut-off value
BKPyV	0.1416	0.0459	0.2796
JCPyV	0.1731	0.0691	0.3806
KIPyV	0.1779	0.0756	0.4048
WUPyV	0.1862	0.0744	0.4097
MCPyV	0.1807	0.0633	0.3708
HPyV6	0.2803	0.1149	0.625
HPyV7	0.2531	0.0946	0.537
TSPyV	0.1815	0.07	0.3918
HPyV9	0.2049	0.0949	0.4897
HPyV10	0.2691	0.1029	0.5778
STLPyV	0.1842	0.0675	0.3867
HPyV12	0.1459	0.041	0.2691
NJPyV	0.1447	0.049	0.2906

The determination of the seroprevalences in our study for each of the viruses, seropositivity was assessed by calculating the proportion of sera that displayed seroreactivity above the established cut-off value for each virus (Table 2). The results of indirect EIAs using as antigens all 13 HPyV VP1 VLPs for the human serum specimens were summarized in Table 3. On the basis of our calculations, overall seroprevalence in the first group for WUPyV, MCPyV, TSPyV and STLPyV were high (more than 50 %) and for BKPyV, KIPyV, MWPyV, HPyV7, HPyV9 quite high (about 30 %). For all new PyVs seropositivity in second (high risk) group was from 6 % to 30 % higher than in the first group (Table 3).

The majority of participants were seropositive for at least 1 PyV, and this is similar to results from other studies (Gossai et al., 2016). The mean number of different PyV tested positive in the same serum was approximately 2.5 for serum samples in the first group and 6 for serum samples in the second group (Fig. 12). Percentage of seronegative serum specimens, with no anti-PyV Ab detected for all 13 tested PyVs, was 7.97 % in low risk group and only 0.62 % in high risk (II) group (Fig. 12).

Table 3. The seroprevalence of HPyV-specific IgG Ab among two sera groups investigated

HPyV VP1 VLP	I group	II group
BKPyV	32,37%	31,73%
JCPyV	25.67%	16.49%
KIPyV	31,25%	44,46%
WUPyV	56,77%	84,34%
MCPyV	66,82%	88,72%
HPyV6	27,59%	57,62%
HPyV7	29,66%	44,25%
TSPyV	62,51%	75,78%
HPyV9	29,34%	35,49%
HPyV10	37,48%	63,46%
STLPyV	55,5%	61,37%
HPyV12	18,66%	47,39%
NJPyV	18,97%	48,43%

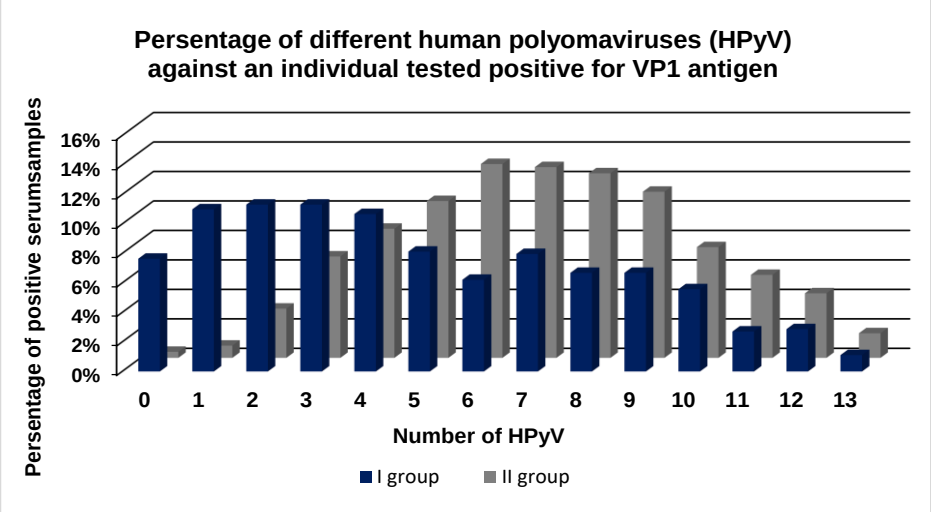

Fig. 12 The distribution of the number of different PyVs tested positive in the same serum among two groups of serum samples (1106 study participants). The cut-off values for the serologic assay were established as described earlier and displayed in Table 2

Competitive EIA using all 13 HPyV-derived VP1 VLPs. Based on shared VP1 aa sequence identity, cross-seroreactivity might be expected between phylogenetically closely related PyV. To explore this potential drawback, we performed competitive EIA using serum preincubation with all 13 different HPyV VP1 proteins. Serum preincubation eliminates cross-reactive IgG Abs, thus only specific Abs interacts with coated VP1 protein. Ten healthy human serum sample specimens, with moderately high titer of anti-HPyV Abs

according to results of EIA without competition were examined. The results of competitive EIA of serum 11 are shown in Fig. 13. Column graph represents the composition of anti-HPyV Ab in serum 11 defined by EIA and that OD was set as a maximum OD (100 %). In competitive EIA graphs below the change of OD (adsorption) due to the serum preincubation with different HPyV VP1 VLPs is expressed as a percentage. OD reduction to 50 % and more, according to control serum preincubated with analyzed VP1 VLP, shows cross-reactivity of anti-HPyV IgG Abs in human sera. The cross-reactivity of JCPyV and BKPyV VP1 proteins, and also, WUPyV and KIPyV VP1 proteins was determined in serum 11 (Fig. 13). We also detected cross-reactivity of HPyV6 and HPyV7, and also, HPyV9 and HPyV10, in other serum samples (data not shown). Similar results of cross-reactivity of JCPyV and BKPyV or WUPyV and KIPyV VP1 proteins were also observed in other studies (Hamilton et al., 2000; Viscidi et al., 2003; Kean et al., 2009; Nguyen et al., 2009). OD reduction from 70 to 50 % in competitive EIA, comparing to control, showed minor cross-reactive anti-HPyV IgG Abs activity. We detected plenty of minor cross-reactive anti-HPyV IgG Abs, using almost all different VLPs for preincubation. In some serum samples all detected anti-HPyV12 or anti-NJPyV VP1 Abs was cross-reactive with other HPyV VP1 VLPs (Fig. 13, graph of HPyV 12). Due to high diversity and antigenic similarity of HPyV, cross-reactivity of anti-HPyV Abs in VP1 based indirect EIA in some cases can lead to false positive results.

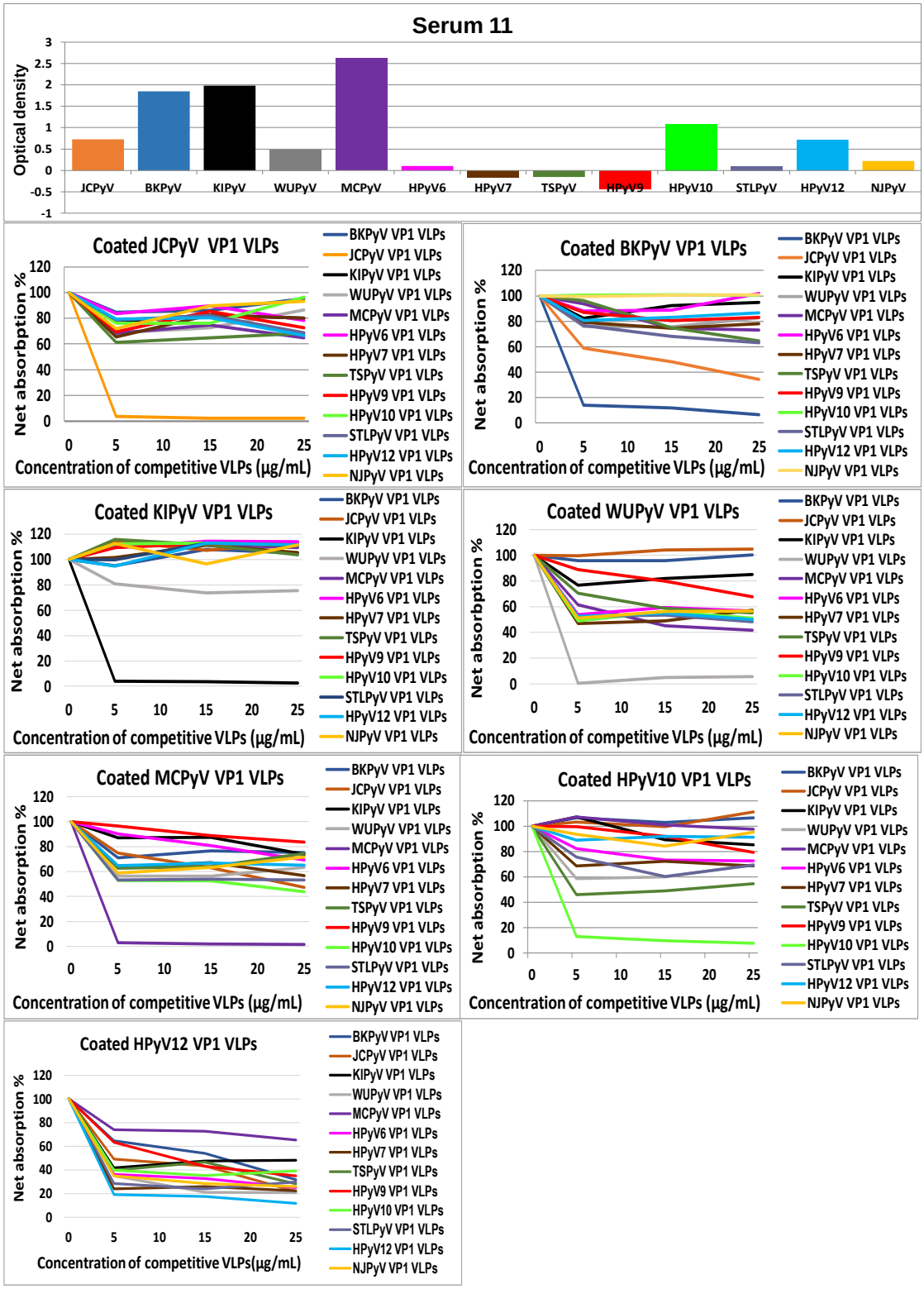

Fig. 13 VP1 specific competition of PyV seroresponses. The competitive EIA performed using all different types of HPyV VP1 protein, including the one investigated for serum preincubation

Discussion

In this study, a targeted genetic approach to optimize PyV VP1 proteins synthesis in *S. cerevisiae* cells was used. The requirement for molecular chaperones during biosynthesis of PyV capsid protein VP1 was evaluated, the reasons of yeast flocculation caused by expression of recombinant VP1 protein derived from some PyV (MCPyV, HPyV10 and STLPyV VP1) was investigated and the impact of NLS in WU VP1 N-termini for VLPs formation was analysed. To advance research on new HPyVs, development of cell culture systems will be important. However, in some applications, VLPs may be used instead, particularly in cases where the wild-type virus is not easily cultivated using cell cultures.

There are host specific differences of recombinant VLP assembly *in vivo* (Mach et al., 2006). The assembly may influence the structural, immunogenic and antigenic properties of VLPs. It has been shown that PyV VLPs expressed in yeast may harbor cellular nucleic acids (Palkova et al., 2000) and are more stable than bacterial VP1 VLPs with regard to disassembly and limited proteolysis (Simon et al., 2014), suggesting a role for host cell factors during intracellular assembly in yeast. Such cellular assembly 'helpers' might include the interaction of VP1 with cellular RNR and assembly of VLPs in the nuclei, cellular chaperones or protein isomerases, which can induce assembly of VP1 into VLPs *in vitro*, and cytoskeletal and nuclear matrix proteins, which have been shown to interact with VP1 during expression (Palkova et al., 2000). Even though the assembly of VLPs *in vitro* is a spontaneous process (Salunke et al., 1986), cellular factors might control and direct this process to increase the efficiency of assembly and the structural homogeneity of viral capsids.

The investigation of requirement for molecular chaperones during biosynthesis of PyV capsid protein which in yeast form multi-molecular VLP structures, as well as a small non-viral UK114-EGFP protein revealed that the deletion of most chaperone genes negatively influenced the production of both recombinant proteins by reducing expression levels by 10 to 50 %. Because molecular chaperones are involved in most cellular processes, including the folding and translocation of many proteins encoded in the genome of eukaryotic organisms and the assembly and/or disassembly of macromolecular structures, this finding suggests that generally, an intact physiological state is very important for the production of recombinant proteins in yeast cells. Nevertheless, some differences that were specific to viral protein production were detected. The existing knowledge about chaperone assistance in the synthesis of PyV capsid proteins primarily pertains to cytosolic and ER Hsp70

chaperones (Cripe et al., 1995; Chromy et al., 2003; Goodwin et al., 2011). To this end, our results confirmed the importance of Hsp70 chaperones for the production of the VP1 protein especially Ssa1, Ssa2, Sse1, Sse2, Ssb1 and Ssb2 chaperones. However, the most notable positive effect on VP1 biosynthesis was achieved by deletion of the Ssz1 chaperone and Zuo1, its RAC counterpart (Figs. 1, 2 and 3). In eukaryotes, two chaperone systems are associated with ribosomes. The first is the RAC, which functionally interacts with the Hsp70 chaperone (in yeast, with SSB family chaperones), and the second is the heterodimeric nascent highly conserved from yeast to humans - polypeptide-associated complex (NAC) (Gautschi et al., 2002; Huang et al., 2005). Deletion of any one of three NAC subunit genes or SSB family genes decreased VP1 production levels by 30 to 50 % (data not shown, Fig. 1). In contrast, the absence of any one of the RAC counterparts resulted in a two- to threefold increase in VP1-EGFP production (Figs. 1 and 2). Likewise, the absence of the RAC had a positive impact on the biosynthesis of viral protein VP1-EGFP (and VP1), but not on the production of UK114-EGFP (Figs. 1 A and 2). The RAC acts as a heterodimer and is formed from Ssz1 and Zuo1 proteins (Jaiswal et al., 2011), thus these results suggests an important role of RAC in viral protein production. Interestingly, a previous study of flock house virus RNA replication revealed similar results, in which a marked increase in viral RNA polymerase and viral RNA was detected in the *Δzuo1* and *Δssz1* yeast strains (Weeks et al., 2010). Our results showed that the quality of VP1 VLP assembly in the absence of a functional RAC in the Δ*zuo1* and Δ*ssz1* strains was not affected (Valaviciute et al., 2016). Therefore, the positive effect of deletion of *SSZ1* and *ZUO1* genes on VP1 biosynthesis is not likely to be due to less-sensitive cellular translational quality control mechanisms but instead is probably related to a still unknown function of these chaperones when they act independently from SSB family chaperones.

The deletion of all of the J-domain (JDP) Hsp40 chaperones, with the exception of Zuo1, decreased VP1 production to various extents (Figs. 2 and 3). Hsp40 co-chaperones are ubiquitous cellular proteins that stimulate the ATPase activity of a partner Hsp70 during protein translation, folding, assembly, or transport (Walsh et al., 2004). It should be noted that there might also be a special requirement for yeast JDP Hsp40 chaperones for recombinant VP1 VLP production related to the lack of large T antigen possessing a functional J-domain. The results showed that VP1 production was significantly decreased in Δ*ydj1* and Δ*caj1* strains (Figs. 2 and 3), pointing to the importance of these two chaperones. Yeast Ydj1 has broad substrate specificity so the need of Ydj1 chaperone could be expected. However, Caj1 might be the JDP chaperone that specifically contributed to the production of VP1. The location of Caj1 is thought to be the nucleus, and its function is predicted to be Ca^{2+}-dependent (Mukai et

al., 1994; Walsh et al., 2004). Because VP1 assembly into VLP structures could be mediated by Ca^{2+} (Stehle et al., 1994) and it is likely that in yeast at least some PyVs VLPs traffic to the nucleus, the apparent involvement of the Caj1 chaperone in VP1 biosynthesis appears very probable.

It has been established that PyVs capsids traffic through the ER during infection (Tsai et al., 2003; Gilbert and Benjamin, 2004; Qian et al., 2009); however, there is no data regarding the trafficking of VP1 or capsid proteins through the ER during or after translation. Nevertheless, although some negative effects on UK114-EGFP levels were apparent, VP1-EGFP levels were reduced 60–70% in *Δjem1*, *Δerj5* and *Δlsh1* strains, and approximately 50% in the other ER chaperone deletion strains including *Δscj1* and *Δhlj1* (Fig. 2), what suggests potential direct or indirect involvement of ER chaperones in VP1 protein biosynthesis.

Although recombinant protein synthesis induces stress conditions in host cells and VP1 forms multi-molecular VLP structures, Hsp104 chaperones were not needed for VP1-EGFP or for unmodified VP1 production in yeast cells. In contrast, the absence of the Hsp90 chaperones affected VP1 and VP1-EGFP production more drastically than UK114-EGFP production. The importance of Hsp90 chaperones was likewise demonstrated by the overexpression of the yeast *HSP82* and *HSC82* chaperone genes, which improved VP1 production (Fig. 1). Hsp90 molecular chaperones are highly abundant and are involved in many fundamental cellular processes (Li et al., 2012). The reduction in VP1-EGFP levels by more than half in the Δ*hsc82* strain (Fig. 1) suggested a specific requirement for Hsp90 chaperones during viral capsid protein synthesis. One explanation might be that Hsp90 chaperones aid in the stabilization of VLPs and another might be VP1 protein multifunctionality. Similar relationships between Hsp90 chaperones and viral proteins was demonstrated in previous studies, including the requirement for the Hsc82 chaperone for stability of the hepatitis C virus (HCV) nascent core in yeast cells (Kubota et al., 2012). The assistance of Hsp90 chaperones in the folding and assembly of hepatitis B virus (HBV) core proteins, HCV nonstructural protein NS3, and picornavirus capsid proteins has likewise been demonstrated. PyV VP1 as a protein with Hsp90 chaperones possesses regulatory functions affecting cell cycle progression (Hornikova et al., 2017), so inhibition of Hsp90 may be possible target fighting PyV infections in immunosuppressed patients.

Yeast flocculation induction was not beneficial for PyV VP1 production and purification. Investigation of the cause of flocculation during recombinant PyV VP1 protein synthesis in yeasts revealed that *FLO1* gene expression is responsible for it. Yeast mutant Δ*flo1* efficiently eliminates flocculation caused by MCPyV, HPyV10, STLPyV and BVPyV VP1, but Δ*flo1* yeast strain cannot

be used for VP1 biosynthesis because of slower growth and sensitivity. Our deeper analysis demonstrated that the deficiency of Cyc8p is responsible for yeast flocculation induced by PyV VP1 biosynthesis. According to other studies, Cyc8p mediates distinct protein-protein interactions that link the Cyc8-Tup1 co-repressor to DNA-binding proteins required for pathway-specific regulation (Tzamarias and Struhl, 1995). The prionization of Cyc8p may provide an additional level of control over the dynamics of chromatin remodeling (Patel et al., 2009) together with Hsp104 and Sis1 yeast chaperones which dissolve Cyc8p aggregates (Patel et al., 2009). Hsp104 was not needed for VP1-EGFP production in yeast cells; however, overexpression of *SIS1* chaperone genes improved VP1-EGFP production up to 150 % (Valaviciute et al., 2016). According to *Alliance of genome resources* database, orhtologs of yeast Cyc8p in *Homo sapiens* cells are lysine demethylases (KDM6A and KDM6B), which are responsible for dynamic gene regulation. Lysine demethylase's interactions with retinoblastoma protein's (pRb) and E2 factor target promoters illustrate their regulatory role in cell cycle progression and oncogenesis (Kang et al., 2017). Our results that VP1 biosynthesis induced deficiency of Cyc8p in yeast affects gene regulation by Tup1-Cyc8 complex might suggest involvement of lysine demethylases in similar process in human cells. Multifunctionality of mouse VP1 protein was already demonstrated (Hornikova et al., 2017), but the actual role of the interaction between VP1 VLPs and Cyc8p still has to be investigated. In this study it has been demonstrated that the sequence and structure PyV VP1 and its inefficient transport to the nucleus are essential for availability of Cyc8p, which is necessary for repressing Tup1-Cyc8 complex formation. Interestingly, KDM6B (ortholog of Cyc8 in human cells) also can be transported to the cytoplasm for interaction with pRb in senescent WI38 human fibroblasts (Zhao et al., 2015).

To advance research on new HPyVs, development of cell culture systems will be important. However, in some applications, VLPs may be used instead, particularly in cases where the wild-type virus is not easily cultivated using cell cultures. Recombinant VP1 proteins from previously discovered PyVs were successfully produced in *Escherichia coli*, yeast or baculovirus/insect cell expression systems (Teunissen et al., 2013). Yeast or baculovirus/insect cell expression systems were used for efficient production of self-assembled VP1 VLPs derived from BKPyV, JCPyV, HaPyV and other PyVs (Sasnauskas et al., 1999; 2002; Teunissen et al., 2013). However, the recombinant VP1 proteins produced in *E. coli* were found mainly as pentamers which self-assembled into VLPs during purification (Ou et al., 1999; Erickson et al., 2009). Only JCPyV- and HaPyV-derived VP1 were shown to form VLPs inside bacteria (Voronkova et al., 2007; Liew et al., 2010). HPyV6-, HPyV7-, TSPyV-, HPyV9-, and

MWPyV/HPyV10-derived VP1 VLPs have also been successfully produced in insect cells (Canganella et al., 1997; Nicol et al., 2013; 2014). In this study, we demonstrated that VP1-derived VLPs from 11 recently discovered HPyVs can be efficiently produced in the budding yeast, *S. cerevisiae*. The lowest yield (approximately 0.4 mg per 1 g of wet yeast biomass) of purified HPyV10- and HPyV12-derived VP1 protein was obtained using the native gene sequence and NJPyV-derived VP1 protein using gene optimized for expression in *S. cerevisiae*. The properties of recombinant proteins, stability, resistance to proteases, and solubility, in particular, may have also had an impact on purification efficiency and the final yield. Therefore, the optimization of genes for use in *S. cerevisiae* resulted in only slightly better yields of VP1 from KIPyV, WUPyV, HPyV6, HPyV7, and STLPyV (approximately 0.5–0.6 mg per 1 g of wet yeast biomass) compared to HPyV10-derived VP1. The yield of highly soluble TSPyV-, HPyV9-, and HPyV12-derived VP1 VLPs, produced using optimized genes, was almost two times greater (approximately 1 mg per 1 g of wet yeast biomass). On the other hand, the difference between the optimized versus native genes was obvious when a shorter HPyV12 VP1 gene version was used. In this case, utilization of optimized VP1 gene improved the yield of VP1**s**^{364} by a factor of nearly 2.5 (Fig. 7). As a result of our attempts to express HPyV12-derived VP1 VLPs in yeast and alignment of various PyV-derived VP1 N-terminal sequences (Norkiene et al., 2015), our data suggested that the true translation initiation site is the second translation site predicted by the VP1-encoding ORF (Korup et al., 2013). There are only a few such cases known in other PyV genomes. HaPyV-, JCPyV-, and SV40-derived VP1 are synthesized starting from the second of two potential translation initiation sites in their respective genes (Siray et al., 1999). The development of cell culture systems for the production of HPyV12 and determination of the N-terminal sequence of VP1 isolated from the virus capsid could be useful in confirming the true translation initiation start site.

HA activity using guinea pig erythrocytes can expand the number of potential applications for VP1-derived VLPs. They can be used as antigens, not only in enzyme immunoassays (EIA), but also in more specific HA inhibition assays to detect corresponding HPyV-specific antibodies in human sera (Knowles and Sasnauskas, 2003). On the other hand, the HA activity of isolated HPyV-derived VP1 VLPs could serve as an initial index of virus cell-binding properties, which are related to viral spreading, cell tropism, and pathogenicity (O'Hara et al., 2014). The variation in HA activity observed in this study among VP1-derived VLPs from different PyVs might reflect diversity in receptor recognition among PyVs. HA of guinea pig erythrocytes by TSPyV-, HPyV9-, HPyV10-, and HPyV12-derived VP1 VLPs suggests that these viruses might use

sialic acid residues to bind the virus particle to the host cell. In contrast, KIPyV-, WUPyV-, HPyV6-, HPyV7-, STLPyV-, and NJPyV-derived VP1 VLPs showed no HA activity and thus more likely use other receptor molecules. Indeed, this was demonstrated in some *in vitro* experiments with HPyV9-, HPyV6-, HPyV7-, WUPyV-, and KIPyV-derived VP1 proteins (Neu et al., 2011).

All 13 HPyV VP1 VLPs were used to develop EIA for detection of HPyVs specific Abs in two groups of sera samples (1106 samples). We demonstrated that seroprevalence of HPyV was 18-75 % in the first group of serum samples collected in Lithuania and up to 30 % higher in second group of serum samples collected from high-risk persons venous blood in Bulgaria (Table 2). The highest seropositivity determined in both serum sample groups was detected for WUPyV, MCPyV and TSPyV and confirmed results reported by some studies in other countries (Kean et al., 2009; Nicol et al., 2013). The seroprevalences some HPyVs showed slight differences comparing to the results other studies. Slight differences in seroprevalences between studies could represent true differences in seroprevalence in different countries, but could also reflect differences in composition of studied population, in techniques used for the detection of Abs, and in cutoff definitions. Indeed, anti-HPyV Abs was detected in 92 % of sera samples in first group and in all serum samples of second group collected from high risk persons (Fig. 12). The co-infection with multiple HPyVs is common and was showed in Gossai and colleagues study (Gossai et al., 2016). Typical for all HPyVs investigated so far is that primary infection occurs in early childhood and seroprevalence increases with age (Kean et al., 2009; Nicol et al., 2013; Gossai et al., 2016). Seroepidemiological studies frequently based on the presence of antibodies against the major capsid protein VP1 or VLPs indicate that most human adults have been exposed to many, if not all, human polyomaviruses (Moens et al., 2013). However, because of the high amino acid sequence identity between VP1 of some human polyomaviruses, cross-reactivity of antibodies is occasionally observed (Moens et al., 2013). In this study we found some degree of cross-reactivity between BKPyV and JCPyV, HPyV6 and HPyV7, HPyV9 and HPyV10, WUPyV and KIPyV VP1 VLPs. Moreover, analyzing the HPyV12 or NJPyV VP1VLPs in competitive EIA in some serum samples we detected cross-reactive anti-HPyV IgG Abs activities using almost all different VLPs for serum preincubation (Fig. 13, graph of HPyV 12). Some other studies did not detect cross-reactivity between MCPyV, TSPyV, HPyV9, and MWPyV VLPs or VP1s produced in bacteria (van der Meijden et al., 2013; Nicol et al., 2013, 2014). This may be explained by lower quality of antigen or the lower degree of amino acid identity between the loop domains of the VP1 protein. It can be assumed that there has been

evolutionary pressure for the immunogenic epitopes of the HPyVs to diverge so that several viruses can establish co-infections (Carter et al., 2003).

Conclusions

1. VP1 proteins of novel human polyomaviruses assembled into VLPs in yeast expression system.
2. Deletion of Hsp90, Hsp70 and Hsp40 chaperone genes negatively influenced the production of PyV VP1 proteins in yeast by reducing expression levels by 10 % to 50 %.
3. The VP1 protein sequence, structure and localization and the availability of Cyc8 protein, are responsible for yeast cell Flo1 dependent flocculation induced by some PyVs VP1 protein biosynthesis.
4. The employment of yeast expression system for the production of HPyV12 VP1 confirmed the true translation initiation start site.
5. The seroprevalence of HPyV was 18-67 % in the first group of serum samples collected in Lithuania and up to 30 % higher in second group of serum samples collected from high-risk persons' venous blood in Bulgaria.
6. From 10 % to 100 % anti-HPyV IgG antibodies in specimens of human serum samples were cross-reactive due to large number of different HPyV and VP1 sequence identity.

References

1. Ausubel Frederick BR, Kingston Robert, Moore David. Short Protocols in Molecular Biology : A Compendium of Methods from Current Protocols in Molecular Biology John Wiley & Sons; 1999.
2. Bodaghi S, Comoli P, Bosch R, Azzi A, Gosert R, Leuenberger D, Ginevri F, Hirsch HH. Antibody responses to recombinant polyomavirus BK large T and VP1 proteins in young kidney transplant patients. *J Clin Microbiol* 2009; 47: 2577-2585.
3. Bolivar F, Backman K. Plasmids of Escherichia coli as cloning vectors. *Methods Enzymol* 1979; 68: 245-267.
4. Canganella F, Paganini S, Ovidi M, Vettraino AM, Bevilacqua L, Massa S, Trovatelli LD. A microbiology investigation on probiotic pharmaceutical products used for human health. *Microbiol Res* 1997; 152: 171-179.
5. Carter JJ, Madeleine MM, Wipf GC, Garcea RL, Pipkin PA, Minor PD, Galloway DA. Lack of serologic evidence for prevalent simian virus 40 infection in humans. *J Natl Cancer Inst* 2003; 95: 1522-1530.
6. Chen DH, Huang Y, Liu C, Ruan Y, Shen WH. Functional conservation and divergence of J-domain-containing ZUO1/ZRF orthologs throughout evolution. *Planta* 2014; 239: 1159-1173.
7. Chromy LR, Pipas JM, Garcea RL. Chaperone-mediated in vitro assembly of Polyomavirus capsids. *Proc Natl Acad Sci U S A* 2003; 100: 10477-10482.
8. Ciplys E, Sasnauskas K, Slibinskas R. Overexpression of human calnexin in yeast improves measles surface glycoprotein solubility. *FEMS Yeast Res* 2011; 11: 514-523.
9. Claro FB, Rijsbrack K, Soares EV. Flocculation onset in Saccharomyces cerevisiae: effect of ethanol, heat and osmotic stress. *J Appl Microbiol* 2007; 102: 693-700.
10. Colombo I, Ceciliani F, Ronchi S, Bartorelli A, Berra B. cDNA cloning and Escherichia coli expression of UK114 tumor antigen. *Biochimica et biophysica acta* 1998; 1442: 49-59.
11. Cripe TP, Delos SE, Estes PA, Garcea RL. In vivo and in vitro association of hsc70 with polyomavirus capsid proteins. *J Virol* 1995; 69: 7807-7813.
12. Csermely P, Schnaider T, Soti C, Prohaszka Z, Nardai G. The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. *Pharmacol Ther* 1998; 79: 129-168.
13. Egli A, Infanti L, Dumoulin A, Buser A, Samaridis J, Stebler C, Gosert R, Hirsch HH. Prevalence of polyomavirus BK and JC infection and replication in 400 healthy blood donors. *J Infect Dis* 2009; 199: 837-846.
14. El-Kirat-Chatel S, Beaussart A, Vincent SP, Abellan Flos M, Hols P, Lipke PN, Dufrene YF. Forces in yeast flocculation. *Nanoscale* 2015; 7: 1760-1767.
15. Erickson KD, Garcea RL, Tsai B. Ganglioside GT1b is a putative host cell receptor for the Merkel cell polyomavirus. *J Virol* 2009; 83: 10275-10279.
16. Feng H, Shuda M, Chang Y, Moore PS. Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science* 2008; 319: 1096-1100.
17. Fleming AB, Beggs S, Church M, Tsukihashi Y, Pennings S. The yeast Cyc8-Tup1 complex cooperates with Hda1p and Rpd3p histone deacetylases to robustly repress transcription of the subtelomeric FLO1 gene. *Biochimica et biophysica acta* 2014; 1839: 1242-1255.
18. Gautschi M, Mun A, Ross S, Rospert S. A functional chaperone triad on the yeast ribosome. *Proc Natl Acad Sci U S A* 2002; 99: 4209-4214.

19. Gietz RD, Schiestl RH, Willems AR, Woods RA. Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* 1995; 11: 355-360.
20. Gilbert J, Benjamin T. Uptake pathway of polyomavirus via ganglioside GD1a. *J Virol* 2004; 78: 12259-12267.
21. Goloubinoff P, De Los Rios P. The mechanism of Hsp70 chaperones: (entropic) pulling the models together. *Trends Biochem Sci* 2007; 32: 372-380.
22. Goodwin EC, Lipovsky A, Inoue T, Magaldi TG, Edwards AP, Van Goor KE, Paton AW, Paton JC, Atwood WJ, Tsai B, DiMaio D. BiP and multiple DNAJ molecular chaperones in the endoplasmic reticulum are required for efficient simian virus 40 infection. *MBio* 2011; 2: e00101-00111.
23. Gossai A, Waterboer T, Nelson HH, Michel A, Willhauck-Fleckenstein M, Farzan SF, Hoen AG, Christensen BC, Kelsey KT, Marsit CJ, Pawlita M, Karagas MR. Seroepidemiology of Human Polyomaviruses in a US Population. *Am J Epidemiol* 2016; 183: 61-69.
24. Hamilton RS, Gravell M, Major EO. Comparison of antibody titers determined by hemagglutination inhibition and enzyme immunoassay for JC virus and BK virus. *J Clin Microbiol* 2000; 38: 105-109.
25. Ho J, Jedrych JJ, Feng H, Natalie AA, Grandinetti L, Mirvish E, Crespo MM, Yadav D, Fasanella KE, Proksell S, Kuan SF, Pastrana DV, Buck CB, Shuda Y, Moore PS, Chang Y. Human polyomavirus 7-associated pruritic rash and viremia in transplant recipients. *J Infect Dis* 2015; 211: 1560-1565.
26. Hornikova L, Fraiberk M, Man P, Janovec V, Forstova J. VP1, the major capsid protein of the mouse polyomavirus, binds microtubules, promotes their acetylation and blocks the host cell cycle. *FEBS J* 2017; 284: 301-323.
27. Huang P, Gautschi M, Walter W, Rospert S, Craig EA. The Hsp70 Ssz1 modulates the function of the ribosome-associated J-protein Zuo1. *Nat Struct Mol Biol* 2005; 12: 497-504.
28. Imperiale MJ, Major E.O. In Fields Virology. Philadelphia: Lippincott Williams and Wilkins; 2007.
29. Jaiswal H, Conz C, Otto H, Wolfle T, Fitzke E, Mayer MP, Rospert S. The chaperone network connected to human ribosome-associated complex. *Mol Cell Biol* 2011; 31: 1160-1173.
30. Johnston JR. Molecular Genetics of Yeast. A Practical Approach.: IRL Press at Oxford University Press; 1994.
31. Kang MK, Mehrazarin S, Park NH, Wang CY. Epigenetic gene regulation by histone demethylases: emerging role in oncogenesis and inflammation. *Oral Dis* 2017; 23: 709-720.
32. Kean JM, Rao S, Wang M, Garcea RL. Seroepidemiology of human polyomaviruses. *PLoS Pathog* 2009; 5: e1000363.
33. Knowles WA, Sasnauskas K. Comparison of cell culture-grown JC virus (primary human fetal glial cells and the JCI cell line) and recombinant JCV VP1 as antigen for the detection of anti-JCV antibody by haemagglutination inhibition. *J Virol Methods* 2003; 109: 47-54.
34. Korup S, Rietscher J, Calvignac-Spencer S, Trusch F, Hofmann J, Moens U, Sauer I, Voigt S, Schmuck R, Ehlers B. Identification of a novel human polyomavirus in organs of the gastrointestinal tract. *PLoS One* 2013; 8: e58021.

35. Kubota N, Inayoshi Y, Satoh N, Fukuda T, Iwai K, Tomoda H, Kohara M, Kataoka K, Shimamoto A, Furuichi Y, Nomoto A, Naganuma A, Kuge S. HSC90 is required for nascent hepatitis C virus core protein stability in yeast cells. *FEBS Lett* 2012; 586: 2318-2325.
36. Li J, Soroka J, Buchner J. The Hsp90 chaperone machinery: conformational dynamics and regulation by co-chaperones. *Biochimica et biophysica acta* 2012; 1823: 624-635.
37. Liew MW, Rajendran A, Middelberg AP. Microbial production of virus-like particle vaccine protein at gram-per-litre levels. *J Biotechnol* 2010; 150: 224-231.
38. Moens U, Calvignac-Spencer S, Lauber C, Ramqvist T, Feltkamp MCW, Daugherty MD, Verschoor EJ, Ehlers B, Ictv Report C. ICTV Virus Taxonomy Profile: Polyomaviridae. *J Gen Virol* 2017; 98: 1159-1160.
39. Moens U, Van Ghelue M, Song X, Ehlers B. Serological cross-reactivity between human polyomaviruses. *Rev Med Virol* 2013; 23: 250-264.
40. Mukai H, Shuntoh H, Chang CD, Asami M, Ueno M, Suzuki K, Kuno T. Isolation and characterization of CAJ1, a novel yeast homolog of dnaJ. *Gene* 1994; 145: 125-127.
41. Nainys J, Timinskas A, Schneider J, Ulrich RG, Gedvilaite A. Identification of Two Novel Members of the Tentative Genus Wukipolyomavirus in Wild Rodents. *PLoS One* 2015; 10: e0140916.
42. Neu U, Wang J, Macejak D, Garcea RL, Stehle T. Structures of the major capsid proteins of the human Karolinska Institutet and Washington University polyomaviruses. *J Virol* 2011; 85: 7384-7392.
43. Nguyen NL, Le BM, Wang D. Serologic evidence of frequent human infection with WU and KI polyomaviruses. *Emerg Infect Dis* 2009; 15: 1199-1205.
44. Nicol JT, Leblond V, Arnold F, Guerra G, Mazzoni E, Tognon M, Coursaget P, Touze A. Seroprevalence of human Malawi polyomavirus. *J Clin Microbiol* 2014; 52: 321-323.
45. Nicol JT, Robinot R, Carpentier A, Carandina G, Mazzoni E, Tognon M, Touze A, Coursaget P. Age-specific seroprevalences of merkel cell polyomavirus, human polyomaviruses 6, 7, and 9, and trichodysplasia spinulosa-associated polyomavirus. *Clin Vaccine Immunol* 2013; 20: 363-368.
46. Norkiene M, Stonyte J, Ziogiene D, Mazeike E, Sasnauskas K, Gedvilaite A. Production of recombinant VP1-derived virus-like particles from novel human polyomaviruses in yeast. *BMC Biotechnol* 2015; 15: 68.
47. O'Hara SD, Stehle T, Garcea R. Glycan receptors of the Polyomaviridae: structure, function, and pathogenesis. *Curr Opin Virol* 2014; 7: 73-78.
48. Ou WC, Wang M, Fung CY, Tsai RT, Chao PC, Hseu TH, Chang D. The major capsid protein, VP1, of human JC virus expressed in Escherichia coli is able to self-assemble into a capsid-like particle and deliver exogenous DNA into human kidney cells. *J Gen Virol* 1999; 80 (Pt 1): 39-46.
49. Palkova Z, Adamec T, Liebl D, Stokrova J, Forstova J. Production of polyomavirus structural protein VP1 in yeast cells and its interaction with cell structures. *FEBS Lett* 2000; 478: 281-289.
50. Patel BK, Gavin-Smyth J, Liebman SW. The yeast global transcriptional co-repressor protein Cyc8 can propagate as a prion. *Nat Cell Biol* 2009; 11: 344-349.
51. Qian M, Cai D, Verhey KJ, Tsai B. A lipid receptor sorts polyomavirus from the endolysosome to the endoplasmic reticulum to cause infection. *PLoS Pathog* 2009; 5: e1000465.

52. Russell I SG, Reader HP, Johnston JR, Martin PA. Revised nomenclature of genes that control yeast flocculation. *J Inst Brew* 1980; 86: 120-121.
53. Salunke DM, Caspar DL, Garcea RL. Self-assembly of purified polyomavirus capsid protein VP1. *Cell* 1986; 46: 895-904.
54. Sambrook JaR, D. W. Molecular Cloning: A laboratory manual. New York: Cold Spring Harbor Laboratory Press; 2001.
55. Sasnauskas K, Bulavaite A, Hale A, Jin L, Knowles WA, Gedvilaite A, Dargeviciute A, Bartkeviciute D, Zvirbliene A, Staniulis J, Brown DW, Ulrich R. Generation of recombinant virus-like particles of human and non-human polyomaviruses in yeast Saccharomyces cerevisiae. *Intervirology* 2002; 45: 308-317.
56. Sasnauskas K, Buzaite O, Vogel F, Jandrig B, Razanskas R, Staniulis J, Scherneck S, Kruger DH, Ulrich R. Yeast cells allow high-level expression and formation of polyomavirus-like particles. *Biol Chem* 1999; 380: 381-386.
57. Sasnauskas K, Jomantiene R, Januska A, Lebediene E, Lebedys J, Janulaitis A. Cloning and analysis of a Candida maltosa gene which confers resistance to formaldehyde in Saccharomyces cerevisiae. *Gene* 1992; 122: 207-211.
58. Simon C, Klose T, Herbst S, Han BG, Sinz A, Glaeser RM, Stubbs MT, Lilie H. Disulfide linkage and structure of highly stable yeast-derived virus-like particles of murine polyomavirus. *J Biol Chem* 2014; 289: 10411-10418.
59. Siray H, Ozel M, Jandrig B, Voronkova T, Jia W, Zocher R, Arnold W, Scherneck S, Kruger DH, Ulrich R. Capsid protein-encoding genes of hamster polyomavirus and properties of the viral capsid. *Virus Genes* 1999; 18: 39-47.
60. Slibinskas R, Samuel D, Gedvilaite A, Staniulis J, Sasnauskas K. Synthesis of the measles virus nucleoprotein in yeast Pichia pastoris and Saccharomyces cerevisiae. *J Biotechnol* 2004; 107: 115-124.
61. Stehle T, Yan Y, Benjamin TL, Harrison SC. Structure of murine polyomavirus complexed with an oligosaccharide receptor fragment. *Nature* 1994; 369: 160-163.
62. Teunissen EA, de Raad M, Mastrobattista E. Production and biomedical applications of virus-like particles derived from polyomaviruses. *J Control Release* 2013; 172: 305-321.
63. Tsai B, Gilbert JM, Stehle T, Lencer W, Benjamin TL, Rapoport TA. Gangliosides are receptors for murine polyoma virus and SV40. *EMBO J* 2003; 22: 4346-4355.
64. Tzamarias D, Struhl K. Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8-Tup1 corepressor complex to differentially regulated promoters. *Genes Dev* 1995; 9: 821-831.
65. Valaviciute M, Norkiene M, Goda K, Slibinskas R, Gedvilaite A. Survey of molecular chaperone requirement for the biosynthesis of hamster polyomavirus VP1 protein in Saccharomyces cerevisiae. *Arch Virol* 2016; 161: 1807-1819.
66. van der Meijden E, Bialasiewicz S, Rockett RJ, Tozer SJ, Sloots TP, Feltkamp MC. Different serologic behavior of MCPyV, TSPyV, HPyV6, HPyV7 and HPyV9 polyomaviruses found on the skin. *PLoS One* 2013; 8: e81078.
67. van der Meijden E, Janssens RW, Lauber C, Bouwes Bavinck JN, Gorbalenya AE, Feltkamp MC. Discovery of a new human polyomavirus associated with trichodysplasia spinulosa in an immunocompromized patient. *PLoS Pathog* 2010; 6: e1001024.
68. Verstrepen KJ, Klis FM. Flocculation, adhesion and biofilm formation in yeasts. *Mol Microbiol* 2006; 60: 5-15.

69. Viscidi RP, Rollison DE, Viscidi E, Clayman B, Rubalcaba E, Daniel R, Major EO, Shah KV. Serological cross-reactivities between antibodies to simian virus 40, BK virus, and JC virus assessed by virus-like-particle-based enzyme immunoassays. *Clin Diagn Lab Immunol* 2003; 10: 278-285.
70. Voronkova T, Kazaks A, Ose V, Ozel M, Scherneck S, Pumpens P, Ulrich R. Hamster polyomavirus-derived virus-like particles are able to transfer in vitro encapsidated plasmid DNA to mammalian cells. *Virus Genes* 2007; 34: 303-314.
71. Walsh P, Bursac D, Law YC, Cyr D, Lithgow T. The J-protein family: modulating protein assembly, disassembly and translocation. *EMBO Rep* 2004; 5: 567-571.
72. Weeks SA, Shield WP, Sahi C, Craig EA, Rospert S, Miller DJ. A targeted analysis of cellular chaperones reveals contrasting roles for heat shock protein 70 in flock house virus RNA replication. *J Virol* 2010; 84: 330-339.
73. Zhao L, Zhang Y, Gao Y, Geng P, Lu Y, Liu X, Yao R, Hou P, Liu D, Lu J, Huang B. JMJD3 promotes SAHF formation in senescent WI38 cells by triggering an interplay between demethylation and phosphorylation of RB protein. *Cell Death Differ* 2015; 22: 1630-1640.
74. Zhao RY. Yeast for virus research. *Microb Cell* 2017; 4: 311-330.
75. Zielonka A, Gedvilaite A, Reetz J, Rosler U, Muller H, Johne R. Serological cross-reactions between four polyomaviruses of birds using virus-like particles expressed in yeast. *J Gen Virol* 2012; 93: 2658-2667.
76. Zielonka A, Gedvilaite A, Ulrich R, Luschow D, Sasnauskas K, Muller H, Johne R. Generation of virus-like particles consisting of the major capsid protein VP1 of goose hemorrhagic polyomavirus and their application in serological tests. *Virus Res* 2006; 120: 128-137.

List of publications

1. Valaviciute M, **Norkiene M**, Goda K, Slibinskas R, Gedvilaite A. Survey of molecular chaperone requirement for the biosynthesis of hamster polyomavirus VP1 protein in *Saccharomyces cerevisiae*. 2016, 161(7):1807-19, Archives of Virology;
2. **Norkiene M**, Stonyte J, Ziogiene D, Mazeike E, Sasnauskas K, Gedvilaite A. Production of recombinant VP1-derived virus-like particles from novel human polyomaviruses in yeast. 2015, 15:68, BMC Biotechnology;
3. **Norkiene M**, Gedvilaite A. Influence of codon bias on heterologous production of human papillomavirus type 16 major structural protein l1 in yeast. 2012, 979218, ScientificWorldJournal.

Other publications

1. Lasickiene R, Gedvilaite A, **Norkiene M**, Simanaviciene V, Sezaite I, Dekaminaviciute D, Shikova E, Zvirbliene A. The use of recombinant pseudotype virus-like particles harbouring inserted target antigen to generate antibodies against cellular marker p16INK4A. ScientificWorldJournal. 2012, 10.1100/2012/263737;
2. Gedvilaite A, Jomantiene R, Dabrisius J, **Norkiene M**, Davis RE. Functional analysis of a lipolytic protein encoded in phytoplasma phage based genomic island. 2014, 169(5-6):388-94, Microbiological research.

Conference reports

1. 2016.06.28-30, „XIVth International Conference of The Lithuanian Biochemical Society“, Druskininkai, Lithuania. “Studies on the seroprevalence of human polyomaviruses using yeast-generated VP1-derived virus-like particles”, (poster).
2. 2015.06.8-10, Global Virus Network conference of virologists for the Scandinavia-Baltic-Ukraine region, Sweden. “Expression in yeast of novel human polyomaviruses VP1-derived virus-like particles, their purification and application in serology”, (oral presentation).
3. 2014.11.13-16, 16th European Congress on Biotechnology, Edinburgh, United Kingdom. Gedvilaitė A. Norkienė M, Lasickiene R, „The employment of a heterologous yeast expression system for production of VP1-derived virus-like particles originated from novel human polyomaviruses”, (poster).
4. 2012.06.28-30, XIIth International Confernence of Lithuanian Biochemical Society "50 years for Biochemistry in Lithuania", Kernavė, Lithuania. “HPV recombinant proteins application for serology”, (oral presentation).
5. 2012.04.11, V National Doctoral Scientific Conference „Science – Health“, Kaunas, Lithuania. „HPV L1 proteins formed VLPs application for serology“, (oral presentation).

Santrauka

Poliomos virusai (PyV) yra maži (40-45 nm skersmens), neturintys apvalkalėlio apie 5 kb dydžio dvigrandininės žiedinės DNR virusai, galintys infekuoti žinduolius, paukščius ir žuvis (Moens et al., 2017). Net dvylika naujų žmogaus poliomos virusų (HPyV) atrasta per pastarąjį dešimtmetį. Šiuo metu žinoma keturiolika žmogaus poliomos virusų.

Šiame darbe buvo atliktas PyV pagrindinių kapsidės VP1 baltymų sintezės optimizavimo ir ypatybių *S. cerevisiae* ląstelėse tyrimas, kuris apjungė nuoseklų mielių šaperonų poreikio poliomos virusų VP1 formuojamų į virusus panašių dalelių (VPD) biosintezei mielėse tyrimą, mielių ląstelių flokuliacijos, sukeltos poliomos virusų VP1 VPD biosintezės priežasčių bei baltymo N-gale esančios į branduolį nukreipiančios sekos įtakos VPD formavimosi efektyvumui bei išeigai tyrimą. Šiame tyrime pirmą kartą buvo pademonstruota, kad sėkmingai PyV sintezei svarbus Hsp90 šaperonų dalyvavimas, tačiau ją pagerina Ssz1 ir Zuo1 šaperonų genų pašalinimas. Pirmą kartą buvo nustatyta, kad už VP1 baltymo biosintezės sukeltą mielių flokuliaciją atsakinga *FLO1* geno aktyvacija, kurią sukelia Cyc8 represorinio baltymo trūkumas. Be to, VP1 baltymo sukeliamai flokuliacijai svarbi jo erdvinė struktūra ir/ar lokalizacija, nes sustiprinus į branduolį nukreipiančią VP1 baltymo signalinę seką, flokuliacija nebebuvo indukuojama.

Parinkus PyV VP1 sintezei optimalias sąlygas iš mielių ląstelių buvo išgryninti visų trylikos HPyV kapsidės VP1 baltymai. Vienuolika šių rekombinantinių VP1 baltymų pirmą kartą susintetinta mielių ląstelėse, o trys iš jų iki šiol nebuvo susintetinti jokioje kitoje raiškos sistemoje. Elektroniniu mikroskopu įsitikinta, kad išgryninti VP1 baltymai efektyviai susirenka į 45-55 nm dydžio VPD. Rekombinantinio žmogaus poliomos viruso 12 (HPyV12) VP1 baltymo biosintezė mielėse leido patikslinti, kad jo transliacijos iniciacija vyksta ne nuo pirmo, bet nuo antrojo metionino kodono.

Visų 13 išgrynintų HPyV VP1 baltymų VPD buvo panaudotos serologiniams tyrimams, kurie iki šiol dar niekada nebuvo atlikti Lietuvoje, taip pat ištirtos jų panaudojimo hemagliutinacijos testui galimybės. Netiesioginės imunofermentinės analizės (IFA) metodu išanalizavus 1106 kraujo serumų mėginius nustatyta, kad žmogaus poliomos virusai plačiai paplitę: antikūnai prieš įvairius HPyV buvo aptikti 18-75% sveikų asmenų ir 30% dažniau aukštos rizikos asmenų kraujo serumų mėginiuose. Be to, šis tyrimas parodė, kad HPyV koinfekcijos yra plačiai paplitęs reiškinys, nes sveikų asmenų grupėje viename kraujo serumo mėginyje vidutiniškai buvo Ak prieš 2,5 skirtingų tipų HPyV VP1 VPD, o aukštos rizikos grupėje - vidutiniškai prieš 6 skirtingas HPyV VP1 VPD.

Šiame darbe panaudojus konkurencinės IFA metodą parodyta, kad prieš skirtingus HPyV susidaro ir kryžmiškai reaguojančių antikūnų, todėl kai kurie netiesioginės IFA rezultatai gali būti klaidingai teigiami. Kadangi HPyV yra dalis mūsų natūralios mikrobiotos, šie tyrimai yra aktualūs, juo labiau, kad sutrikus imuninei sistemai jie gali sukelti ligas.

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