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An Investigation of Endoplasmic Reticulum-Associated Proteins Implicated in Efficient

Murine Polyomavirus Infection

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Abstract

Polyomaviruses are nonenveloped DNA tumor viruses that begin infection by trafficking through the endoplasmic reticulum (ER) en route to the nucleus. However, the mechanism by which the virus escapes the ER prior to nuclear entry is poorly understood. Previous research with Simian Virus 40 identified several ER-resident proteins involved in the Endoplasmic Reticulum Associated protein Degradation (ERAD) pathway as being necessary for efficient infection. In an attempt to better elucidate this potential mechanism and its possible similarity to SV40 infection, mouse A31-3T3 cells were transduced with lentiviral shRNAs targeted against the previously identified proteins and were then infected with murine polyomavirus (MuPyV). The results of these experiments suggested that several DNAJ molecular co-chaperones, two Derlin family retrotranslocons, and one ER sorting protein, BAP31, are indeed necessary for efficient MuPyV infection in mouse cells. Further studies of these proteins will provide insight into the molecular mechanisms of general polyomavirus infection and ER quality control function.

Introduction

Polyomaviruses are small, nonenveloped, DNA tumor viruses that infect a variety of animals, including: monkeys, humans, parakeets, hamsters, rats, and mice (1). Affecting the lattermost group is the murine polyomavirus (MuPyV), which typically spreads to the kidneys and brain in mice, and often results in latent infections that can be reactivated when the host's immune system is compromised or suppressed (2). Due to its similarities to the BK and JC viruses, MuPyV provides a model for human infections and, consequently, is the subject of much research.

MuPyV capsids are symmetrical icosahedrons approximately 50nm in diameter. The outer shell of each virion is composed of 360 copies of the major capsid protein VP1 grouped into 72 pentamers. Each pentamer is then attached to one copy of the minor capsid protein VP2 or VP3 (on the interior-facing side). Packaged inside the capsid is the viral genome, a 5.3kb circular, double-stranded DNA molecule (3).

Cellular entry begins when a virion's VP1 protein comes in contact with sialic acid residues of the GD1a gangliosides on the plasma membrane of the host cell (4). This action is soon followed by internalization of the entire virus via receptor-mediated endocytosis. The virus then travels through an early endosome, which matures to a late endosome, and the low pH induces conformational changes that facilitate later remodeling reactions (5). The virus is subsequently sorted to the ER by its functional receptor GD1a (5). Once inside the ER, the capsid's disulfide bonds are reduced and the protein coat is rearranged/partially disassembled by ERp29—an ER resident protein of the Protein Disulfide Isomerase (PDI) family—and possibly other residential factors (5). At this point, it is believed that the virus must then exit the ER to the cytosol before proceeding to the

nucleus. The mechanism by which this occurs is poorly understood. However, recent

publications suggest that the virus accomplishes this via host cell machinery.

The Endoplasmic Reticulum Associated protein Degradation (ERAD) pathway is a

quality control mechanism common to all eukaryotic cells whereby ER-resident proteins

recognize and eject misfolded proteins from the ER. The ERAD pathway is quite complex

and requires the synchronization of many different ER proteins, such Derlin-1, Derlin-2,

BAP31, and those of the DNAJ family. Derlin proteins are retrotranslocons embedded in

the ER membrane that are involved in the physical ejection of misfolded peptides. BAP31,

or B-cell receptor Associated Protein 31, binds to translocon-associated components and

interacts with newly synthesized membrane proteins as a quality control factor during

folding and assembly (9). DNAJ proteins are a class of Hsp40 co-chaperones that associate

with (and stimulate ATPase activity of) Hsp70 chaperones, such as BiP.

Recent research supports the hypothesis that polyomaviruses utilize the ERAD

pathway to escape the ER. In 2007, Schelhaas et al. identified Derlin-1 as being necessary

for infection by Simian Virus 40 (SV40), a polyomavirus that infects monkeys (8). Then, in

2011, multiple DNAJ proteins (namely DNAJB11, DNAJB12, DNAJB14, and DNAJC18) and

BAP31 were identified as also being necessary for efficient SV40 infection by Goodwin et al.

and Geiger et al., respectively (9, 10). These researchers have therefore postulated that the

partially disassembled viral particle mimics a misfolded protein in the ER and is ejected to

the cytosol via this pathway.   While these proteins have been shown to inhibit infection by

SV40, to date there has been little investigation into the role they may play with MuPyV.

However, findings presented in this paper suggest that these proteins are also necessary

for efficient MuPyV infection, with some being more vital than others.

Materials and Methods

Cell Culture: A31 3T3 mouse fibroblast cells were grown in D10C media: Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 10% bovine calf serum (BCS; HyClone) and 1% Penicillin-Streptomycin solution (P/S; Sigma). Cells were incubated at 37°C in a humidified incubator with an atmosphere of 5% CO_2.

Lentiviral Transduction: MISSION shRNA Lentiviral Particles were ordered from Sigma-Aldrich (Table 1) and created in the University of Colorado Functional Genomics Facility. A31 3T3 cells were incubated for 18-20 hours with 250μL of D10C, 1μL of Hexadimethrine Bromide (8mg/mL), and 750μL of lentiviral particle solution. The cells were then allowed to grow for 24 hours in D10C before selection with puromycin at a concentration of 6μM.

Gene	TRC Number	Sequence (5' → 3')
DNAJB11	TRCN0000009589	CCGGGCTGCCCAACTTTGATAACAACTCGAGTTGTTATCAAAGTTGGGCAGCTTTTT
	TRCN0000009590	CCGGCCCTAATGTCAAATTAGTGAACTCGAGTTCACTAATTTGACATTAGGGTTTTT
	TRCN0000009591	CCGGGGTTCCGAATCAAAGTTGTCAACTCGAGTTGACAACTTTGATTCGGAACTTTTT
DNAJB12	TRCN0000009559	CCGGGCCACAGTGATGGACTGTATACTCGAGTATACAGTCCATCACTGTGGCTTTTT
	TRCN0000009560	CCGGCGACCAGTTTGGTGATGACAACTCGAGTTGTCATCACCAAACTGGTCGTTTTT
	TRCN0000009561	CCGGGTGGAACGGAATGTAGAGGATCTCGAGATCCTCTACATTCCGTTCCACTTTTT
	TRCN0000009562	CCGGCAAGTTCCATCCAGACAAGAACTCGAGTTCTTGTCTGGATGGAACTTGTTTTT
	TRCN0000009563	CCGGGTACCACAGAGCACAGAAGATCTCGAGATCTTCTGTGCTCTGTGGTACTTTTT
DNAJB14	TRCN0000342056	CCGGCTACGAAGTCCTCGGAGTTACCTCGAGGTAACTCCGAGGACTTCGTAGTTTTTG
	TRCN0000341981	CCGGCGTGACTAATATTCGAAATAACTCGAGTTATTTCGAATATTAGTCACGTTTTTG
	TRCN0000341982	CCGGCAAAGTCTGGAGAGGATTATCCTCGAGGATAATCCTCTCCAGACTTTGTTTTTG
	TRCN0000341983	CCGGTCCCGAAGACCTGTTCAATATCTCGAGATATTGAACAGGTCTTCGGGATTTTTG
	TRCN0000341984	CCGGTACGTCAGTAAGGACTTTAAACTCGAGTTTAAAGTCCTTACTGACGTATTTTTG
DNAJC18	TRCN0000039175	CCGGCGCTATGATGAATATGGAGATCTCGAGATCTCCATATTCATCATAGCGTTTTT
	TRCN0000039176	CCGGCCTGGAGAAGACAATAGAGAACTCGAGTTCTCTATTGTCTTCTCCAGGTTTTT
	TRCN0000039178	CCGGCCCATATAGTCTGTTCTATAACTCGAGTTATAGAACAGACTATATGGGTTTTT
DERLIN1	TRCN0000126849	CCGGGGCTCAGCTTATCACTAAATAACTCGAGTTATTTAGTGATAAGCTGAGCTTTTT
	TRCN0000126851	CCGGCCTGTTACTTACCTTGGGTTACTCGAGTAACCCAAGGTAAGTAACAGGTTTTT
	TRCN0000126852	CCGGGCAGACTATTTATTCATGCTTCTCGAGAAGCATGAATAAATAGTCTGCTTTTT
DERLIN2	TRCN0000174518	CCGGCGTCGATTTAAGTTTCTTCAACTCGAGTTGAAGAAACTTAAATCGACGTTTTTTG
	TRCN0000174667	CCGGGACAGCAGACTTTGTATTTATCTCGAGATAAATACAAAGTCTGCTGTCTTTTTTG
BAP31	TRCN0000012418	CCGGCCATGGCTTATAGATCATTATCTCGAGATAATGATCTATAAGCCATGGTTTTT
	TRCN0000012420	CCGGGGCTCAGAGGAATCTCTATATTCTCGAGAATATAGAGATTCCTCTGAGCTTTTT
	TRCN0000012421	CCGGCCTGAAGAATGACCTGAGGAACTCGAGTTCCTCAGGTCATTCTTCAGGTTTTT
Control	SHC002V	Does not target any genes; confers ampicillin and puromycin resistance

Table 1: List of lentiviral transduction particles. TRC numbers are unique identifiers assigned by Sigma-Aldrich and used in this experiment to name the resultant cell lines.

RNA Extraction and cDNA Synthesis: Cells grown in monolayer were lysed with TRIzol reagent (1mL / 10cm^2) and left to incubate for 5 minutes at room temperature (RT). Cell lysate was mixed vigorously with chloroform (0.2mL per 1mL TRIzol) and left to incubate again for 3 minutes at RT. Samples were then spun at 12,000 x g for 15 minutes at 4°C. Following centrifugation, the aqueous phase was removed and washed over columns from the RNeasy Mini Kit (Qiagen). RNA extracts were then tested for purity via nanodrop to ensure A_{260}:A_{280} and A_{260}:A_{230} ratios were higher than 1.8. cDNA was then made from the resultant RNA following the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen).

Knockdown Assessment via qPCR: cDNA samples were first diluted with ddH$_2$O to a 1:5 ratio for target gene primers and 1:50 ratio for reference gene primers. 5µL of each sample was loaded into individual wells of 96-well plates along with 12.5µL SYBR Fast Master Mix (2X; ABI), 2µL primer mix at 12.5µM (Table 2), and 5.5µL ddH$_2$O. Additionally, RNA used to make the cDNA was also prepared in this manner to ensure no genomic DNA contamination was present during cDNA synthesis. Plates were then sealed, vortexed, centrifuged, and analyzed via Applied Biosystems' 7500 Fast Real-Time PCR System. Gene expression was evaluated using the ΔΔCt method with GAPDH as the reference gene.

Gene	Direction	Sequence (5' → 3')	Gene	Direction	Sequence (5' → 3')
DNAJB11	Forward	GGAGAAGGTGAGCCGCATGTGGA	DERLIN1	Forward	TGGGAGTGATGAGGGTTAGG
	Reverse	AGCCTCAACCAGTGAGACGGTCA		Reverse	CTGTGTCAGGAGCAACTCCA
DNAJB12	Forward	CCTGAGCCCGAGACCGTCAGT	DERLIN2	Forward	GAAAGATGGCGTACCAGAGC
	Reverse	TGAGGCTGGAGCCCGTGTACTC		Reverse	GACGCAGGCGGTAGTATAGG
DNAJB14	Forward	GCGCGCGCGTTATTGGAAATA	BAP31	Forward	AGAACATGCCAAACTGCAGGCAT
	Reverse	CCCAGCACCAGAAGTGCCGT		Reverse	GGTCAGGTAGAAGCCAGCGGC
DNAJC18	Forward	AGAGCGCTGGACCCAAGCTTAC	GAPDH	Forward	TGTGTCCGTCGTGGATCTGA
	Reverse	GCAGTTACAGCAGCCGCAAGGA		Reverse	CCTGCTTCACCACCTTCTTGA

Table 2: Primers used for qPCR analysis
Each primer set was validated and found to be nearly 100% efficient (±5%) using mouse DNA serial dilutions.

MuPyV Infection: Virus stock used was #NG59RA (date: 11/30/2007) courtesy of Dr.

Thomas Benjamin's lab at Harvard Medical School. Approximately 2 x 10^5 cells were plated

onto acid-washed, poly-L Lysine-coated coverslips in six-well plates with D10C and allowed

to adhere overnight. All cells were then serum-starved in DMEM supplemented with 0.5%

BCS for 18 hours. Virus stock was prepared at an MOI of 15 in adsorption buffer (1X

Hank's Buffer, 1% BCS, 10mM Hepes, pH 5.6-6.2) and spread over the coverslips,

500µL/well. Plates were incubated as previously described for 90 minutes and rocked

every 15 minutes to ensure even coverage. The virus solution was then aspirated and

replaced by D10C.

Immunofluorescence: At 28hpi, cells were washed three times with ice-cold PBS then

fixed with 4% PFA in PBS for 15 minutes at RT. Cells were again washed with ice-cold PBS

then with ice-cold cytoskeleton (CSK) buffer. Plates were incubated on ice for 5 minutes

with ice-cold CSK buffer containing 0.5% Triton X100. Cells were washed three times with

ice-cold PBS then blocked with PBS containing 10% BCS for 10 minutes at RT. Rat antibody

against T-Ag was diluted (1:5000) in PBS containing 2% BCS and applied to the cells, which

were then placed in a humidified 37°C incubator for 45 minutes. Cells were then washed

three times with PBS at RT and rocked for 5 minutes each time. Anti-rat Alexa 488

antibody was diluted (1:2000) in PBS containing 2% BCS and applied to the cells, which

were again placed in a humidified 37°C incubator for 45 minutes. Cells were then washed

three times with PBS at RT and rocked for 5 minutes each time. Coverslips were mounted

on slides with DAPI (4',6-diamidino-2-phenylindole) Prolong Anti-Fade Gold (Invitrogen)

mounting medium and allowed to dry overnight. Once dry, coverslips were sealed and

imaged on a confocal microscope.

Infection quantification: Knockdown (KD) cell lines were infected in duplicate alongside an A31 control cell line that had only puromycin resistance, created with MISSION Control SHC002V (Table 1). Approximately 150 cells per slide were counted (sampled at random locations). The percentage of infected A31 control cells was calculated during each infection and used to establish a baseline level of infection. All KD infection percentages were then normalized against this benchmark.

Results

DNAJB11: Cell lines 90 and 91 were identified as expressing the relatively least amounts of DNAJB11 RNA transcripts, down to 38% and 29.5% of the control A31 line, respectively. When presented with MuPyV, neither cell line appeared to greatly inhibit infection, reducing the relative level of infection by only 20%.

DNAJB12: Cells lines 59 and 62 were identified as expressing the relatively least amounts of DNAJB12 RNA transcripts. Line 59—whose DNAJB12 RNA transcript level was reduced to 15.8%—greatly inhibited infection, nearly a 75% reduction when compared against the A31 control. Line 62, however, did not perform as well. Despite having a reduction in gene transcripts down to 26%, it only reduced the relative level of infection down to 76.8%. Line 63 was also tested to see if perhaps a direct correlation could be drawn between KD level and infection level. Of all five DNAJB12 cell lines, 63 had the highest level of gene transcripts (64.7%) and hardly inhibited infection (Fig 1).
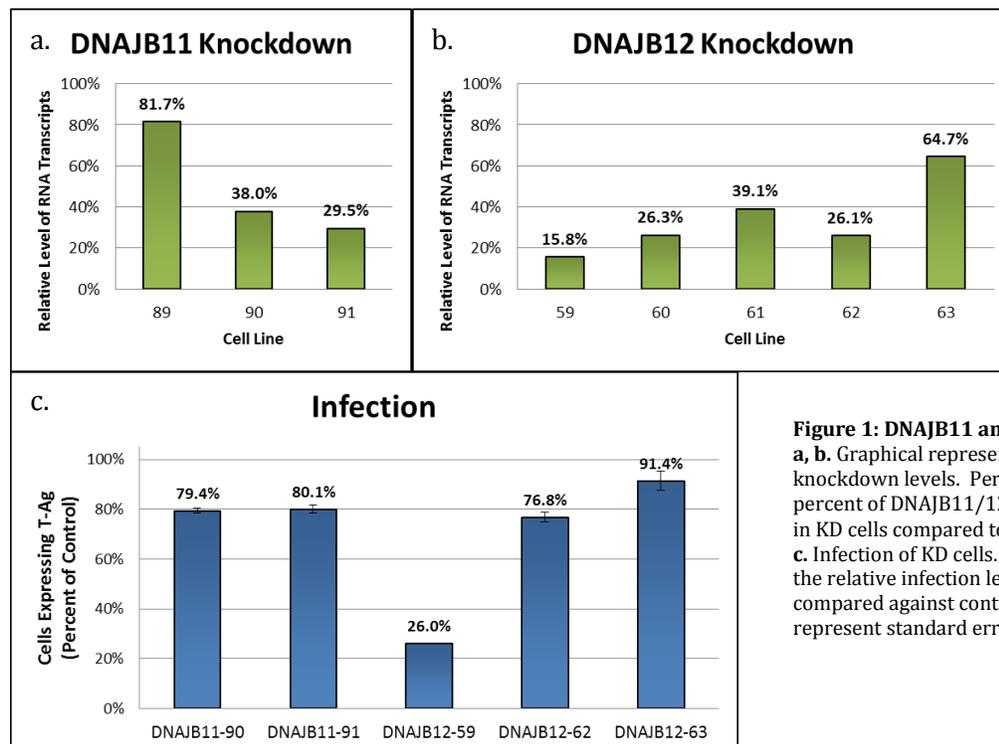

Figure 1: DNAJB11 and DNAJB12
a, b. Graphical representation of gene knockdown levels. Percentages represent the percent of DNAJB11/12 RNA transcripts present in KD cells compared to control A31 cells.
c. Infection of KD cells. Percentages represent the relative infection levels of the KD cells, compared against control A31 cells. Error bars represent standard error of the mean.

DNAJB14: Cell lines 56 and 84 showed the greatest reduction in RNA transcripts—5.8%

and 14.8% of the control, respectively. When presented with the virus, 56 inhibited

infection more so than 84 (a reduction to approximately 42% versus 62%).

DNAJC18: Of all the KD cell lines created in this experiment, those made against the

DNAJC18 gene showed the greatest reduction. The two best lines, 75 and 76, both

exhibited a decrease in RNA transcripts to about 5%. However, despite having nearly

equivalent KD level, each line resulted in rather different relative infection levels. Line 75

reduced infection to 31.3%, whereas 76 reduced infection to only 48.7% (Fig 2).
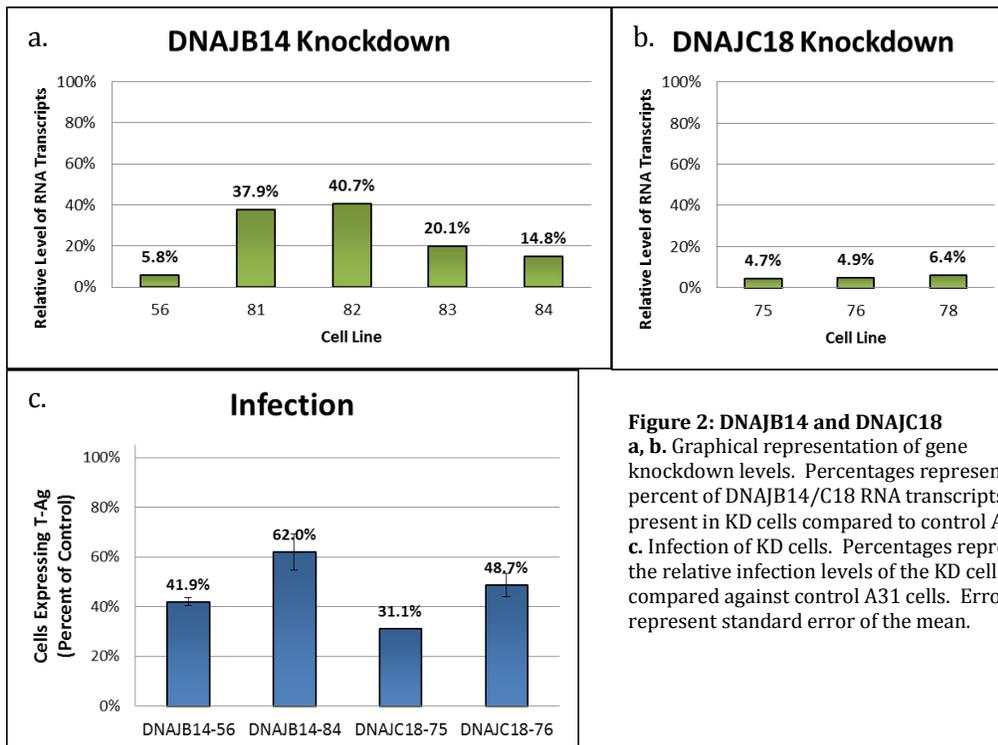

Figure 2: DNAJB14 and DNAJC18
a, b. Graphical representation of gene knockdown levels. Percentages represent the percent of DNAJB14/C18 RNA transcripts present in KD cells compared to control A31 cells. **c.** Infection of KD cells. Percentages represent the relative infection levels of the KD cells, compared against control A31 cells. Error bars represent standard error of the mean.

Derlin-1: Cell lines 49 and 52 showed the strongest KD levels, at 11.1% and 6.9%, respectfully. Despite this difference, however, both cell lines inhibited infection to similar levels—49 reduced infection to 43.1% and 52 reduced infection to 46.7%.

Derlin-2: Only two KD cell lines were created to suppress Derlin-2. Line 18 had the greater level of RNA transcripts, 63.7%, which had virtually no impact on infection. Line 67 was considerably knocked down (to 10.1%), and reduced relative infection level down to 31%.

BAP31: Cell lines 18 and 20 were identified as showing the best knockdown effect at 8.0% and 15.1%, respectfully. When presented with the virus, these two cell lines inhibited infection more than any other in the entire experiment. Cell line 18 reduced infection to 15.4% and 20 reduced infection to 8.9%. Note: BAP31 KD analysis was performed by Angelique Hill, a graduate student rotating through the lab (Fig 3).
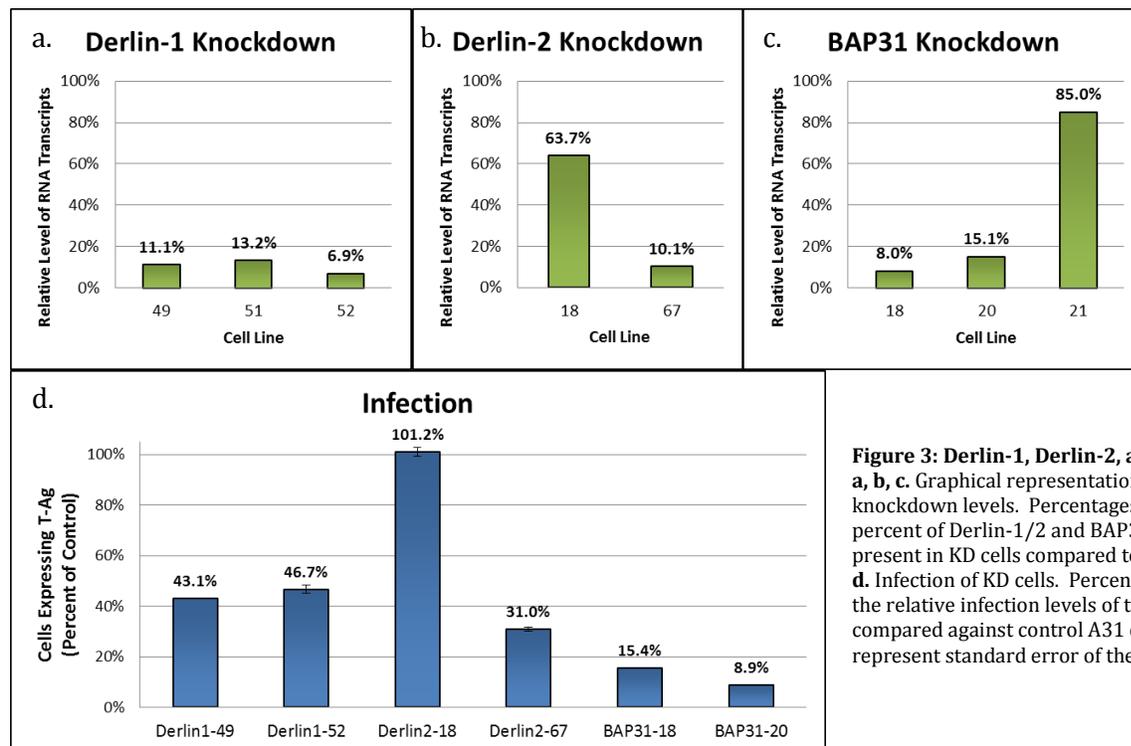

Figure 3: Derlin-1, Derlin-2, and BAP31
a, b, c. Graphical representation of gene knockdown levels. Percentages represent the percent of Derlin-1/2 and BAP31 RNA transcripts present in KD cells compared to control A31 cells.
d. Infection of KD cells. Percentages represent the relative infection levels of the KD cells, compared against control A31 cells. Error bars represent standard error of the mean.

Discussion

DNAJ Series: Research conducted by Goodwin et al. found that suppression of DNAJB11 in HeLa/E6 cells inhibited SV40 infection down to 18% compared against non-repressed cells. However, here it was found that suppression of DNAJB11 in mouse cells hardly inhibited MuPyV infection. Similarly, Goodwin et al. observed drastic results when repressing DNAJB12 and DNAJB14. They found that knocking down these two genes in HeLa/E6 cells greatly inhibited SV40, dropping the relative infection level to 2% each. While knocking down these genes in mouse cells did inhibit MuPyV infection, the results were not as extreme. Here, relative infection was only reduced to 26% and 42% by knockdown of DNAJB12 and DNAJB14, respectively. The disparities between these findings could suggest that these three genes are not quite as necessary to MuPyV infection in mouse cells as they are to SV40 infection in HeLa/E6 cells. However, it is also possible that the necessary level of knockdown was not achieved to have an extreme effect on infection. For instance, DNAJB11 here was only knocked down to 30% compared to normal A31 cells. Thus, a stronger knockdown may bring relative infection levels down to those observed by Goodwin et al., assuming that levels beneath 30% are not lethal. Still, knockdown of one DNAJ protein in particular, DNAJC18, did reduce infection to a level comparable to that seen with SV40. Goodwin et al. observed that suppression of DNAJC18 reduced SV40 infection to approximately 28%, and here, too, relative MuPyV infection was reduced down to 31%. However, despite having 95% less DNAJC18 than the control cells, infection was not greatly inhibited, suggesting that the protein may not be that important to infection.

Derlin series: Research performed by Schelhaas et al. found that suppression Derlin-1 in HeLa/E6 cells reduced the relative infection level by SV40 to 45%. Similarly, here it was observed that mouse A31 cells with Derlin-1 knocked down reduced relative MuPyV infection levels to 43%. These findings suggest perhaps similar importance of Derlin-1 for infection by both MuPyV and SV40. However, in a study conducted by Lilley et al., knockdown of Derlin-1 in rat C6 cells did not inhibit infection by MuPyV. Thus, it seems that Derlin-1 has a species-specific role regarding MuPyV infectivity. The same conjecture cannot be made for Derlin-2, though. When Lilley et al. knocked down Derlin-2 in rat C6 cells, MuPyV infection was reduced to approximately 27% compared to their control. Similar results were seen here with mouse A31 cells, where it was observed that suppression of Derlin-2 reduced MuPyV infection to 31%.

BAP31: When Geiger et al. knocked down BAP31 in both HeLa and mouse 3T6 cells and infected them with SV40 and MuPyV, respectively, they observed a reduction in viral infection to 16% by MuPyV and 24% by SV40. Here, when BAP31 was knocked down in A31 mouse cells, a drastic reduction in MuPyV infection was observed—down to 8% compared against the control. Suppression of BAP31 had the greatest impact on relative infection levels in the entire study presented here, suggesting that it has great importance to the MuPyV infection pathway in mouse cells. Table 3 below summarizes the analysis of DNAJ, Derlin, and BAP31 proteins compared against other similar research.

Protein	Relative Infection Levels		
	MuPyV	MuPyV (past research)	SV40 (past research)
DNAJB11	80%	—	18%[3]
DNAJB12	26%	—	2%[3]
DNAJB14	42%	—	2%[3]
DNAJC18	31%	—	28%[3]
Derlin-1	43%	100%[1]	45%[4]
Derlin-2	31%	27%[1]	—
BAP31	8%	16%[2]	24%[2]

Table 3: Summary of current and past research. Percentages listed are relative infection levels observed when the corresponding protein in that row was knocked down (as compared against a control, which would be 100%). The column titled only "MuPyV" lists findings found in this paper, and the percentages correlate to the cell line with the lowest relative infection level. Columns under "past research" list findings from other research in the field. [1] Lilley et al., 2006. (Rat C6 cells) [2] Geiger et al., 2011 (Mouse 3T6 cells) [3] Goodwin et al., 2011 [4] Schelhaas et al., 2007

Overview and future directions: Regardless of the individual infection percentages, these findings suggest that all seven proteins (with the possible exception of DNAJB11) play some role—however large or small—in efficient MuPyV infection. With the exception of BAP31, no single knockdown reduced infection below 25%. Therefore, it is possible that perhaps the virus uses alternate proteins when one isn't available (e.g. DNAJB14 in the absence of DNAJB12). However, further research needs to be conducted before any definitive statements can be made, as there were some limitations with this experiment.

Due to time constraints, the infection studies and knockdown analysis presented here could only be performed essentially once (although there was internal duplication). Thus, repetition of the experiments in triplicate would lend more fidelity to the data. Also, there was some difficulty in knocking down certain genes (such as DNAJB11 and DNAJB12) to very low levels. This could be due to inefficiency of the individual shRNAs or perhaps the potential lethality of not having that particular protein present in the cell. Further

investigation should be done with alternate shRNAs to see if a lower knockdown level is achievable. Additionally, the A31 control cell line that had puromycin resistance from SHC002V proved to be somewhat difficult to infect—i.e., only ~25% of the control cells in each round were infectable. This baseline level of infection is rather low compared to studies in other publications (near 50%), and thus the relative infection numbers presented here may actually be lower. Lastly, visually counting infected cells on coverslips restricts the sample size and, consequently, the overall precision of the data. This issue could perhaps be mitigated in the future by using a Flow cytometer to quantify infection level with a larger number of cells.

In the future, it would be interesting to study the effects that a single knockdown has on the regulation of other genes—e.g., if knocking down one DNAJ protein causes an upregulation in other isoforms. It might also be interesting to study the infected knockdown cells with electron microscopy in order to visually observe the precise location in the cell where the virus is being bottlenecked. Further research could even be conducted investigating the signal transduction pathway between ERAD and the Unfolded Protein Response (UPR) pathways, as polyomavirus-induced upregulation may exist. These suggested experiments and more will help elucidate not only the infection pathway of polyomaviruses, but also shed new light on the complex activity of several ER proteins.

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