

Research Article



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Proteomics characterization of the adenovirus VA1 non-coding RNA on the landscape of cellular proteome

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Abstract

Objectives: Gene therapy using adenoviruses has shown tremendous promise in animal and human models in the past. The tumor responses achieved by these viruses were either because of their oncolytic properties or therapeutic genes expressed from the genome of the virus. We have previously shown enhanced viral replication in cell models that have lost or lower expression of the cyclin dependent kinases inhibitor (p21/Waf-1). Beside the early onset of many viral genes in the p21/Waf-1 knock out cell model, we observed a significantly higher copy number for the viral VA1 but non VA2 non-coding RNA transcripts. In this study we investigated the effect of adenoviral VA1 non-coding RNA on the landscape of cellular proteins.

Methods: Using a DU145 cell line as a model that was transiently transfected with a plasmid carrying the adenoviral VA1 non-coding RNA, we were able to study changes in the proteome and the cellular cascade of the cells. Using state of the art global proteomics analyses of the differentially expressed proteins between the VA1

overexpressed and control cells demonstrated how the early onset of VA1 transcript affected cellular machinery.

Results: Using a 1.5 fold cut off between the down-regulated or overexpressing proteins, we were able to demonstrate how the non-coding VA1 gene might be responsible for suppressing the proteasome degradation, the endocytic and lysosomal pathways. Similarly, overexpression of the non-coding VA1 transcript was responsible for the upregulation (1.5 fold) of approximately 40 different proteins in the spliceosome machinery.

Conclusions: These data demonstrated how the non-coding VA1 RNA functioned independently of any other viral proteins in modulating the cell signaling cascades to promote viral propagation. To our knowledge this is the first report to investigate the function of VA1 non-coding RNA on the cellular proteome.

Keywords: adenovirus; prostate cancer; proteomics; VA1 non-coding RNA.

Introduction

Adenoviruses are double standard, non-enveloped DNA viruses that have been shown to infect both the proliferative and non-proliferative cells [1, 2]. These characteristics together with methods to efficiently manipulate their genome made them popular in gene transduction technologies [3, 4]. The genome is a linear 36 Kb long DNA, where the terminal proteins are covalently linked to inverted terminal repeats (ITR) on either ends [5]. Genes are located on both strands of the DNA, which are transcribed in overlapping transcript units. The virus entry into the cell is dependent on the interaction between the fiber and the coxsackievirus B and adenoviral receptor (CAR) on the cell surface membrane [6, 7]. Once the attachment happens the exposed RGD motif on the penton interact with the alpha V integrin that triggers clathrin dependent endocytosis of the virion [8]. The acidic environment of the endosome induced virion escape within the cytoplasm. Utilizing the

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microtubules within the cells the virion made the journey and ultimately ended into the nucleus. The disassembly of the capsid at the nuclear pore complex allows for the import of the viral genome and the transcriptional regulation [9, 10]. The first viral transcript expressed in the infected cell is the E1A gene of the virus [11]. The E1A produces multiple mRNA and proteins products which are important for the immortalization of the host cells. During the infection cycle the E1A protein is responsible for the transactivation of other early transcription units in the viral genome (E1B, E2 and E3 and E4) [12]. The E1 and E3 gene are transcribed from the sense strand, however the E2 and E4 are made from antisense strand of the virus [13]. To overcome these challenges, the viruses has evolved the VA1 and VA2 non-coding RNA, which has been studied and known to saturate the cellular miRNA machinery [14, 15]. We have previously shown that the VA1 and VA2 non-coding RNA transcripts can be measured as early as 6hr after infection of the HCT116 cells [15]. The VA1 but not the VA2 RNA transcript was at least 25- fold higher when compared to the E1A mRNA in the first 6hr of the virus infection and reached more than 450 folds in the first 24 hr of infection cycle. In this study, we used a proteomics approach to explore the importance of the non-coding VA1RNA in the biology of virus life cycle. Using the global proteomics approach, we were able to identify several important cellular signaling pathways that was significantly affected by the VA1 non-coding RNA independent of viral infection.

Materials and methods

Cell lines and reagents

The DU145 cell line were previously obtained from American Type Culture Collection (Manassas, VA, USA) and were maintained in T-75 flasks in RPMI-1640. Majority of chemical reagents and compounds were ordered from Sigma Aldrich, unless otherwise specified. Aminolink resin, spin columns (snap cap), Zeba spin desalting columns were purchased from Life Technologies (Grand Island, NY). Trypsin gold Mass spectrometry grade was from Promega (Madison, WI). QE Orbitrap LC-MS (Thermo) was used for quantitative analysis of global peptides.

Transfection experiment

DU145 cells were plated in a T-25 flask, one day before the transient transfection experiment. Cells were transfected with 10ug of pVA1 or control plasmid using Lipofectamine 2000 (Invitrogen, CA) as per the manufacturer's protocol. Control plasmid was constructed using the *HindIII* HF restriction digest (New England Biolabs, Ipswich, MA, USA) of the parent plasmid (pVA1). Plasmid was self-ligated using T4 DNA

ligase (NEB) overnight. After transformation into the DH5 alpha six colonies were picked for the mini-prep DNA isolation using QIAGEN miniprep kit (Germantown, MD, USA). Correct clones were identified and confirmed by double restriction digesting using *HindIII* HF and *BamHI* HF enzymes. The transfection media was replaced with regular RPMI-1640 media containing 10% fetal bovine serum (FBS) and antibiotics 100 U of penicillin and 100 µg/mL of streptomycin. All transfection assays were performed in replicates. Cells were harvested at 48 h post transfection in 1 mL protein denaturing buffer (8 M urea and 0.4 M NH_4HCO_3) followed by a thorough sonicated on ice for global peptides isolation.

Peptide fractionation using basic RPLC

The high-pH RPLC (reverse-phase liquid chromatography) separation was performed on the 1,220 Infinity LC system with a Zorbax Extended-C18 analytical column containing 1.8 µm particles (Agilent Technologies, Inc. CA); flow rate was 0.2 mL/min. The mobile-phase A consisted of 10 mM ammonium formate (pH 10) and B consisted of 10 mM ammonium formate and 90% ACN (pH 10). 50 µg peptides were fractionated using the following linear gradient: from 0 to 2% B in 10 min, from 2 to 8% B in 5 min, from 8 to 35% B in 85 min, from 35 to 95% B in 5 min and then held at 95% B for an additional 15 min. Peptides were detected at 215 nm and ninety-six fractions were collected along with the LC separation in a time-based mode from 16 to 112 min. The separated peptides in 96 wells were concatenated into 24 fractions by combining four wells to one sample, such as 1, 25, 49, and 73 as fraction one; 2, 26, 50, and 74 as fraction two; and so on. The peptides were then dried in a Speed-Vacuum and stored at -80 °C until LC-MS/MS analysis.

iTRAQ labeling of global tryptic peptides from cell lines

Each iTRAQ (isobaric tags for relative and absolute quantitation) reagent was dissolved in 70 µL of ethanol. One mg of each tryptic peptide sample was added into 250 µL of iTRAQ dissolution buffer, then mixed with iTRAQ reagent and incubated for 1 h at room temperature. iTRAQ channel 119 and 121 was used to label DU145 cells transfected with pVA1 plasmid, iTRAQ 117 and 118 were used for labelling vector transfected control DU145 cells. The sets of tagged peptides were combined and purified by SCX column. Then, 10% of the labeled peptides were dried and re-suspended into 0.4% acetic acid solution prior to fractionation for mass spectrometry analysis. Samples were run on the QE executive in the positive mode. Raw data generated from the QE were subjected to proteome discovery software PD 1.4 (Thermo Scientific USA).

Protein expression data analysis

MS/MS data and differential expression analysis: We searched our tandem mass spectrometry derived raw data against the RefSeq protein database using the pyccloud search. We specified oxidation of methionine, carbamidomethylation of cysteine, N-terminal iTRAQ modification as fixed residue modifications. We specified lysine (K) and tyrosine (Y) iTRAQ modifications as dynamic modifications. Peptide identification false discovery rate (FDR) was specified as 0.01. Parsimonious protein grouping was specified to allow at least one peptide per protein. High confidence PSMs (i.e. peptide spectrum

matches better than pre-specified false discovery rate cut-off) were used for protein grouping. Peptide and protein quantifications were based on ratios of iTRAQ reporter ions. Our specified reporter ion quantification DU145 VA1/DU145 Ctr. All data were median intensity normalized.

As a second-level quality control measure, we have also filtered out peptide spectrum matching and their associated proteins with reporter ion ratios using a 30% cut off. Those proteins that had a coefficient of variations (CV) greater than 30% were removed from the list of the spectrum matching and associated proteins. All experiments data were performed in biological replicates. Statistical analyses on the data were performed using Microsoft Excel running on an IBM-PC compatible computer on the Windows 10 operating system. Statistical significance was defined as a p-value <0.05. The differentially expressed proteins with at least 1.5 fold changes were selected for bioinformatics analysis using Kyoto Encyclopedia of Gene and Genome (KEGG) enrichment analysis.

Results and discussion

The VA1 model to study the dysregulated proteome of the DU145 cell line

In order to understand the effect of the non-coding VA1 RNA on the biology of the cells, we transiently transfected the DU145 cells with pVA1 (from adenovirus type 2 VA1 non-coding RNA) or control plasmids (Gift from Dr. Göran Akusjärvi, Uppsala University [16]. Cells were scraped from the surface of the T-75 flask after 48 h in the urea buffer [15, 17]. Protein and peptide isolation were carried out using the Mass Spectrometry workflow as described in the material and method section. Equal amounts of the labelled peptides from the DU145-VA1 transfected and control cells after mixing were fractionated on HPLC followed by LC-MS/MS analysis. Data obtained from the QE were subjected to the PyCloud software [18]. A total of 610,019 peptides which corresponded to 5,201 proteins were identified from the search results (2 PSM per peptide and 2 peptides per protein). Using the differentially expressed protein between the DU145 VA1 and control cells (1.5 fold changed), a total of 2,281 proteins were identified to be dysregulated (Supplemental Table 1A, B and C). There were a total of 972 proteins that were over-expressed at least 1.5 fold or above and around 1,309 proteins that were down regulated between the two cell lines. These differentially regulated proteins were subjected to the KEGG Mapper pathway analysis. Beside the predominant involvement of the known metabolic pathways, we identified several

pathways that were dysregulated in the VA1 transfected DU145 cells. Some of these pathways are described below.

Proteasome and its subset proteins

The 26 S proteasome complex is responsible for the digests of the misfolded proteins that are marked for degradation by the ubiquitin protein [19]. The complex is comprised of the 20 S proteolytic core particle (CP), which is bounded by a cap at one or the other ends with a 19S regulatory particle Figure 1A. The 20S proteasome CP is the principal proteolytic structure consisting of two pairs of rings each containing of the seven subunits Figure 1B. Three of these subunits are crucial for proteolytic activity, the β 1 subunit with “caspase-like” activity, the β 2 subunit with trypsin-like activity, and the β 5 subunit with chymotrypsin-like activity. Because protein turnover is very important for the survival and program cell death, an intact ubiquitin-proteasome system is important for cellular homeostasis [20]. Based on our quantitative iTRAQ data, we identified 40 different protein subunits of the proteasome that were at least 1.5 fold downregulated in the VA1 transfected DU145 cells. The regulatory cap region, the 19 S region which comprise of the ten subunits of the lid (Rpn3, Rpn5, Rpn6, Rpn7, Rpn8, Rpn9, Rpn10, Rpn11, Rpn12 and Rpn15), the nine subunits from the 19S base (Rpn1, Rpn2, Rpn13, Rpt1, Rpt2, Rpt6, Rpt4, Rpt5, and Rpt3) Figure 1C identified in our proteomics data were downregulated in the DU145 VA1 cells (Figure 1; Supplementary Table S1B and S2). Similarly, the core particle (CP) of the 20S proteasome that are comprised of the alpha (1–7) and beta (1–7) where downregulated in VA1 transfected cells compared to the controls. These data demonstrated that during the viral life cycle VA1 RNA suppress the cellular proteasome pathway to overcome and escape the protein hemostasis for viral protein propagation.

Suppression of the endocytic machinery by the non-coding VA1 RNA

Adenovirus infection is initiated by the interaction of the fiber knob with the CAR receptors on the surface of cell membrane [6, 21]. The entry of the virus is mediated by the endocytic machinery which package the virus into a clathrin coated vesicles for cellular internalization followed by virion release from the vesicles and trafficking

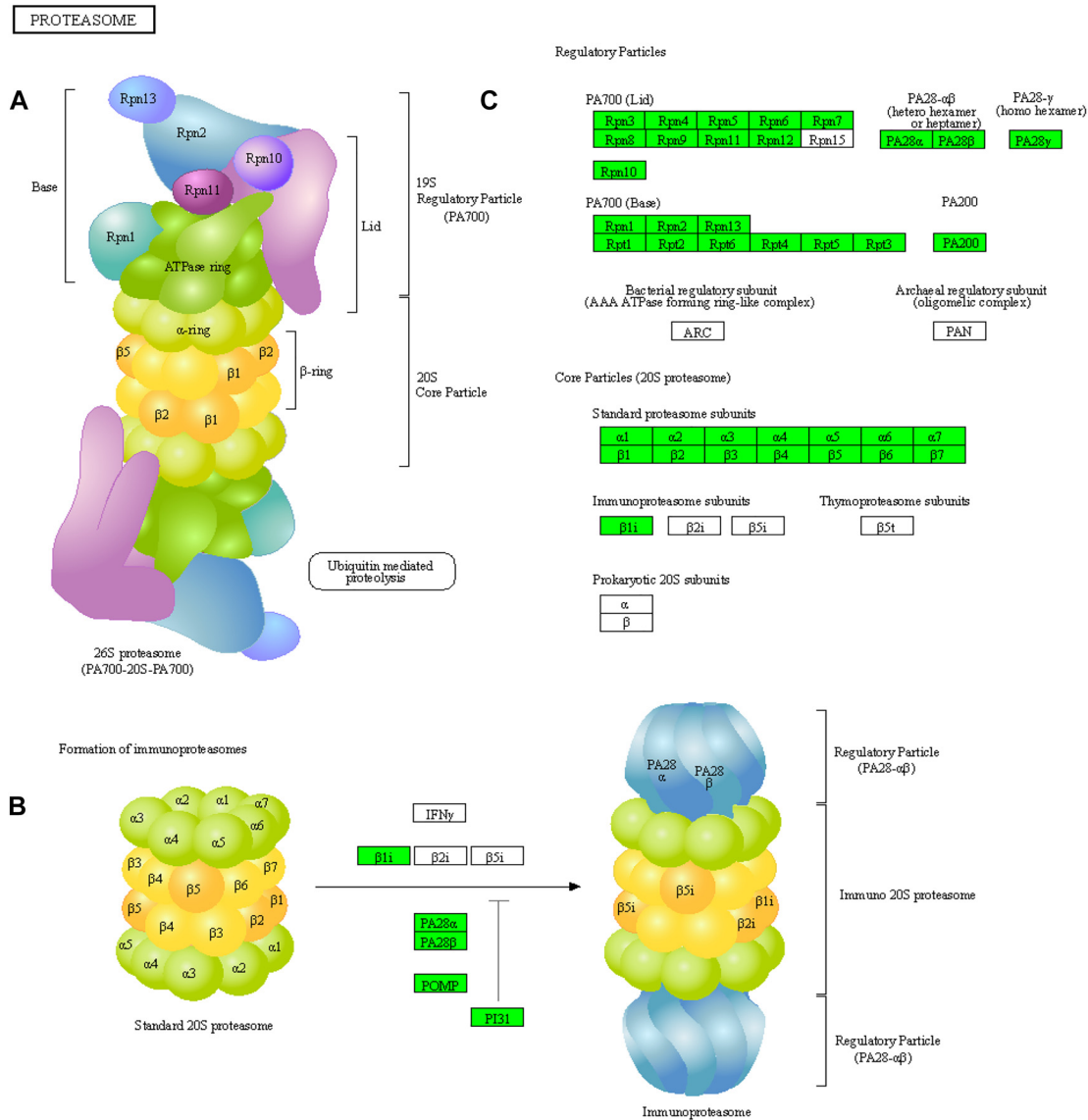


Figure 1: Overexpression of viral VA1 non-coding RNA suppressed the proteasomal degradation pathway.

Several downregulated (1.5-fold expression) subunits of 26S proteasome identified by LC-MS/MS are highlighted in gene. The 26S proteasome subunit, which comprise of the 19S regulatory particle and the 20S core particle (A, B). The lid is mainly composed of Rpn 3–9, 11, 12 and 15. The formation of immunoproteasome is facilitated by the interaction between alpha (α 1-7) of lid and base proteins with the beta (β 1-7) to form the standard 20S proteasome. The PI31 protein along with POMP, PA28 α and PA28 β proteins identified in our VA1 overexpressed DU145 cells inhibit the formation of immunoproteasome (C).

to the nuclear pore. However, during the process of viral infection and viral DNA replication, these viruses has evolved functions that suppress the entry of new virions into the cell by suppressing the endocytic machinery of the cells [14]. Using the VA1 overexpressing cells here we demonstrated the that non-coding VA1 RNA of Adenovirus was enough to suppress a total of 44 proteins (using 1.5 fold cut off) in the endocytic pathway (Figure 2, Supplementary Table S1B and S3) including the endophilin, clathrin, E3 ligase, the CAPZA and CAPZB, the early endosome VPS29,

VPS26 and VPS35 and the late endosome VPS37, Alix, CHAMP4 and CHAMP5. These data demonstrated a novel functional for the non-coding VA1 RNA in the adenovirus life cycle which can work independently of any other viral proteins in suppressing the endocytosis of the cells. The data support the notion, how virions process the cellular machinery in order to suppress the entrance of new infectious particles into the cells. Further studies will be required to confirm these observations. One way of confirming these data could be to utilize Adenovirus

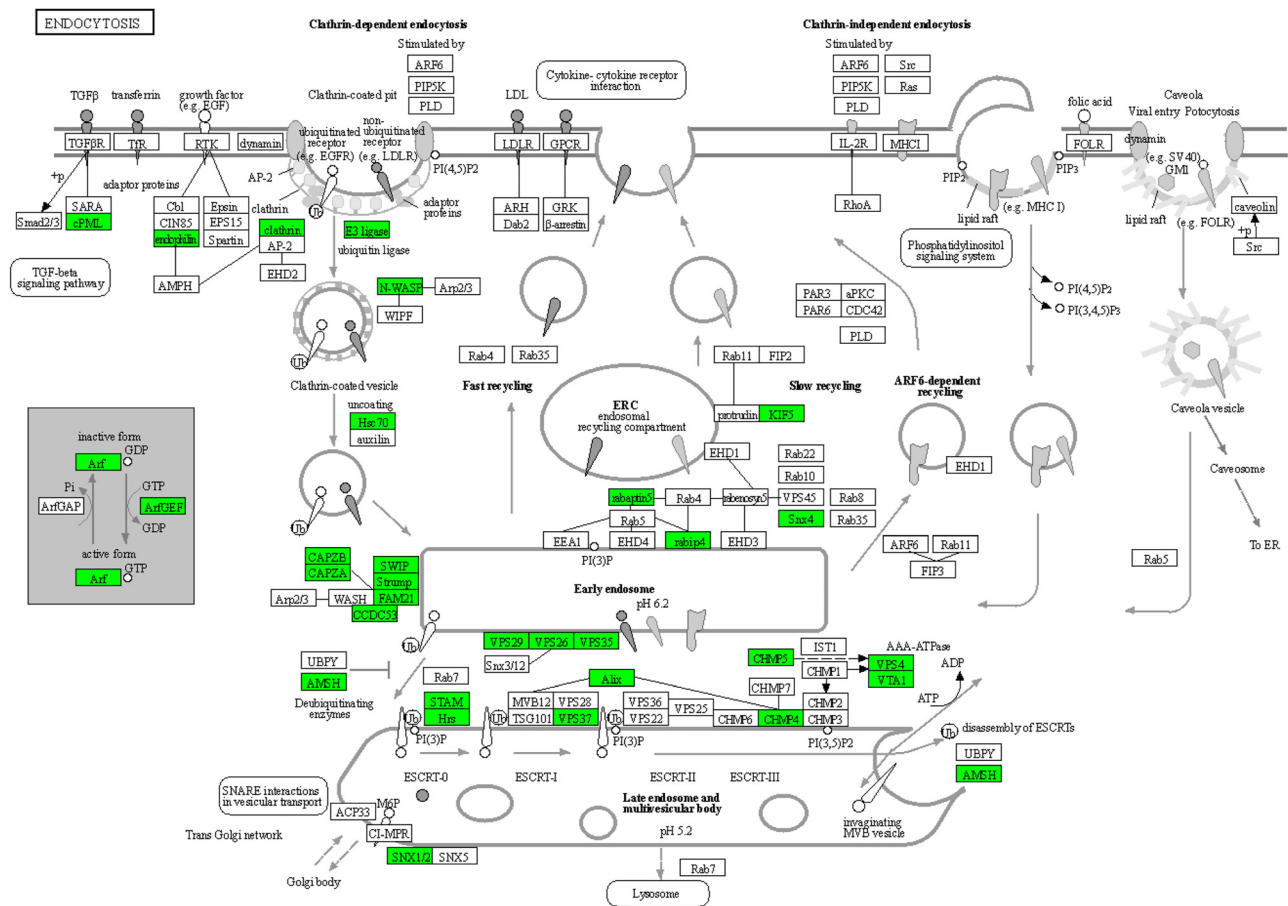


Figure 2: Overexpression of the Adenoviral VA1 non-coding RNA in DU145 cells suppress several host endocytic machinery specific proteins independent of any other viral genes.

particles that express different fluorescent proteins from the backbone followed by infection of cells at different time points using fluorescent microscopy.

VA1-non-coding RNA and the cellular apoptotic pathway

The adenoviral early protein “E1A and E1B” which has been shown to interact with tumor suppressors retinoblastoma (RB1) and Tumor suppressor P53 (p53). The E1A protein interacts with the RB to remove it from the RB/E2F complex, which in turn lead to the activation of the E2F and its targets gene to promote cellular proliferation. The E1B 55K on the other hand regulates p53 by different mechanisms that include the binding of the E1B55K to the N-terminus of the p53 causing inhibition of p53-dependent transcription. Similarly, the adenoviral early protein “E1B 19K” protein is known to suppress the Bcl-2 Associated X-protein (Bax) and Bcl-2 antagonist killer (Bak1) dimerization and in

tumor necrosis factor alpha (TNF-alpha) program cell death [10, 22]. We became interested to explore whether VA1 of the Adenovirus has anti-apoptotic function. The 1.5-fold differentially expressed proteins identified between the VA1 transfected and control DU145 cells were subjected to the KEGG pathway analysis. As shown in Figure 3, Supplementary Table S1B and S3, 22 different proteins in the TNF alpha signaling including the Bid, caspase-8, and caspase-3 were identified to be down-regulated in VA1 transfected cells.

Upregulation of spliceosome machinery in VA1 transfected cells

Adenovirus is a double standard DNA virus where many gene including the early E1A and E1B genes and the late gene under than major late promoter (MLU) is known to significantly undergo splicing during viral proteins expression [23]. The major late transcriptional unit (MLTU)

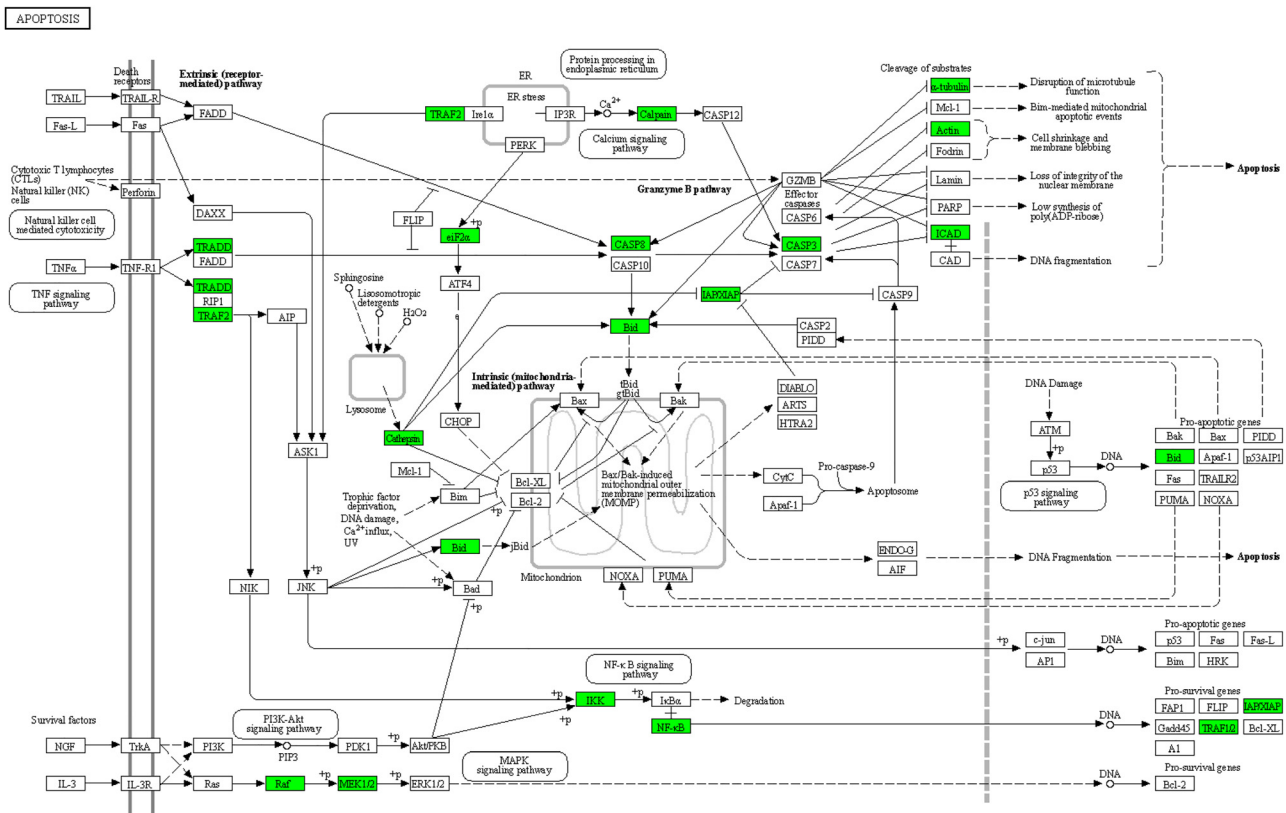


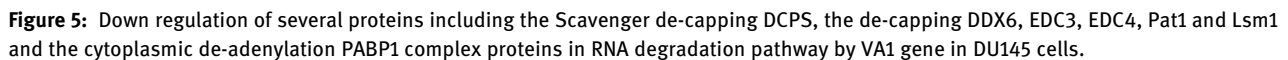
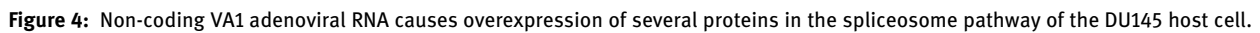
Figure 3: Overexpression of the VA1 gene in cells downregulate several pro-apoptotic proteins in order to create a milieu that is suitable for viral gene expression and progeny generation.

transcripts use alternative splicing to produce approximately 20 different mRNAs, therefore. While there is high demand for splicing within infected cells to translate viral genome, how the viruses overcome such challenges to efficiently translate its genome, we evaluated whether the VA1 RNA has any functions in mediating the RNA splicing machinery.

Using the differently expressed proteomics data between the VA1 transfected and mock transfected DU145 cells, using a 1.5 fold change as our cut of, we were able to identify a total of 56 upregulated proteins in the spliceosome machinery. As shown in Figure 4, Supplementary Table. S1C and S5, several of the identified spliceosome protein components including the proteins in U1 complex (CA150), the heterogeneous nuclear ribonucleoproteins (hnRNP) in the common components related protein were overexpressed in the VA1 transfected cells. These data further emphasis the importance of the non-coding VA1 RNA in the life cycle of the virus and reinforced the previous findings that adenovirus VA1 RNA is required for the splicing events of adenoviral transcripts and the translation enhancement function of viral genome [14].

RNA degradation and the VA1 non-coding RNA

Human adenovirus utilizes the host nucleus for the transcription and replication of its genome [23]. Adenovirus is a double standard DNA virus, which has been shown to encode for around 40 different polypeptides. Once the mature mRNA is formed it is released from the matrix and is transported to the cytoplasm for protein translation. In Ad5 infection evidence for the regulation of RNA transport from the nucleus into the cytoplasm comes from many genetic studies, where mutation in the E1B 55K or the E4 OrF6 proteins were associated with reduced level of late viral mRNA in the cytoplasm that led to lower viral titers [24]. In order to investigate if VA1 the non-coding RNA of the Adenoviruses play any role in RNA degradation and surveillance in the cells, we used the global quantitative proteomics data obtained from the iTRAQ labelled-VA1 transfected DU145 cells and evaluate the differentially regulated proteins between the control and VA1 transfected DU145 cells. This has been shown in Figure 5,



Supplementary Table S1B and S6. DU145 cells that were transiently transfected to carry the VA1 plasmid was responsible for the down regulation of many (eleven proteins using a 1.5 fold cut off) proteins in RNA degradation pathway including the proteins in the Scavenger de-capping of the 5' m7G RNA (DDX6, EDC3, EDC4 and Pat1). In the cytoplasmic de-adenylation complexes PABPC4; poly(A) binding protein cytoplasmic 4 and the LSM 1–7 complex (Lsm1) were down regulated in the VA1 overexpressed DU145 cells compared to the vector transfected control cells.

Adenovirus gene expression and replication kinetics have been previously studied in detail. In this study, we tried to understand the function of the non-coding VA1 RNA in transiently transfected cells with plasmid that carried the VA1 gene. The VA1 gene that is located between the late L2 and L3 genomic region of adenovirus has been shown to make millions of transcripts that can be detected as early as 6 h post viral infection [16, 18]. Because of such a high transcriptional rate, we became interested to evaluate if VA1 has functions other than saturating the XPO5 and miRNA processing machinery that is needed for the virus to avoid the cellular immune response and viral prorogation [25]. Using the quantitative proteomics approach, we were able to identify several pathways that were dysregulated by the transiently expression of the viral non-coding VA1 plasmid.

Adenovirus infection is facilitated by the interaction between the fiber and the CAR receptors present on the cell membrane of the permissive cells [26, 27]. The internalization of the virus is mediated through the clathrin-mediated endocytosis. Viruses have evolved many mechanisms in order to overcome cellular inhibitory mechanisms. Besides, targeting the tumor suppressor TP53 and RB proteins by the viral immediate early proteins E1A and E1B respectively [28], we observed in our data analysis that the VA1 non-coding RNA was responsible for the suppression of endocytosis which was independent of any viral protein. Interestingly, this mechanism of suppressed endocytosis by a non-coding VA1 RNA was previously recognized. During the viral infection cycle, VA1 gene is highly transcript. We suggest that the inhibition of the endocytosis is a crucial step to avoid further intake of the viruses by the cells that have already been infected. Similarly, using the differentially expressed proteomics data from the VA1 transfected and control cells, we observed overexpression (1.5 fold upregulation) of approximately forty different proteins in the spliceosome machinery representing how important the VA1 RNA is in the replication biology of the adenovirus. It has been reported that after infection of the adenovirus, an interferon induced

kinase becomes activated by double-stranded RNA which is thought to be derived from symmetrical transcription of the viral genome [29] the VA1-RNA directly interact with the kinase and inactivate it. This notion of double standard RNA duplexes has been recently evaluated by Price et al. [30], where they have shown that adenovirus suppress the dsRNA duplex formation by promoting efficient splicing and mRNA processing, thus avoiding host innate immune responses [30]. Our data was in agreement with the findings of Price et al. [30], where a significant numbers of protein (n=40) in the spliceosome machinery was upregulated by at least 1.5 fold or higher. The VAI-RNA has also been shown to enhance protein synthesis from non-viral genes in transient assays [31] by apparently similar mechanisms involving blockage of activation of an eIF2-specific protein kinase [32]. In this study, we have performed the global proteomics analysis on the VA1 transfected cells and presented evidence that the adenovirus VA1-RNA exerted a profound effect on the gene expression of several important cellular signaling pathways including the suppression of the proteasome, endocytosis, RNA degradation and cell apoptosis pathways. Importantly, these effects on the cellular proteome were independent of any other viral proteins.

While the study highlighted the importance of the VA1 non-coding RNA biology on the cellular proteome, some of the limitations of our study was the utilization of a single cancer cell line (DU145), it will be interesting to demonstrate other cell lines especially normal cell lines to have the same VA1-regulation mechanisms. In addition, our study was based on the transient transfection experiment using the VA1 gene. For proteomic analysis, a stably selected VA1 expressing cell line model might be a better way to approach the overall proteome changes affected by the VA1 non-coding RNA. We, however, believe that our study with the VA1 expression have identified several important cellular mechanisms including the over-expression in the spliceosome pathway, which is overexpressed during virus replication and propagation.

Conclusions

We demonstrated that the Ad2- VA1 non-coding RNA was capable to independently dysregulate cellular proteome, involved in different cellular signaling pathways in order to make the intracellular environment suitable for efficiently viral propagation. Further studies will be required in order to confirm our proteomics studies.

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Author contributions: Zafar Uddin carried out compilation work and data interpretation. Munazza Khan performed results compilation and data analysis. Naseruddin Höti performed project designing, supervision and provision of facilities. Jabbar Khan carried out the manuscript writing and project design. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests: Authors state no conflict of interest.

Informed consent: Not applicable.

Ethical approval: Not applicable.

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