CELLULAR TARGETS INVOLVED IN REOVIRUS-INDUCED ONCOLYSIS OF RAS-TRANSFORMED CELLS

by

JUNG HWA CHUNG

Presented to the Faculty of the Graduate School of

The University of Texas at Arlington in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE IN BIOLOGY

THE UNIVERSITY OF TEXAS AT ARLINGTON

December 2007

ACKNOWLEDGEMENTS

I would like to thank Dr. Michael Roner for his support and guidance throughout my graduate career and specifically for his insight and direction during my research and thesis preparation. I would also like to thank my additional committee members, Dr. Pawel Michalak and Dr. Thomas Chrzanowski for investing their time to take part in assisting with both the completion and defense of my thesis. In addition, I would like to acknowledge Dr. Michelle Badon for her role as a mentor during my time as one of her graduate teaching assistants. Lastly, I wish to specially thank my friends and family for their infinite support during the many hours I dedicated to achieving this milestone in my education.

November 26, 2007

ABSTRACT

CELLULAR TARGETS INVOLVED IN REOVIRUS-INDUCED ONCOLYSIS OF RAS-TRANSFORMED CELLS

Publication No. _____

Jung Hwa Chung, M.S. in Biology

The University of Texas at Arlington, 2007

Supervising Professor: Dr. Michael R. Roner

Reovirus is a ubiquitous virus with demonstrated oncolytic properties. In this study, we investigate the pathways which reovirus produces a lytic infection in SV-40 transformed WI-38 cells and the noncytocidal infection of normal WI-38 cells using microarray technology. The analysis revealed that reovirus infection induced changes in 178 genes, of which 112 genes were up regulated and 66 genes down regulated. Of the 112 up regulated genes, 13 of the WI-38 2RA genes were shown to be involved in apoptosis or inhibition of cellular DNA synthesis. Of the 66 genes down regulated, seven of the WI-38 genes displayed effects involved in apoptosis or inhibition of cell cycle progression, suggesting that inhibition of these genes prevented death in these

cells. Additionally, five of the 62 up regulated WI-38 genes were found to be involved in cell survival. Reovirus caused lysis of transformed cells by over inducing a multitude of cellular targets.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Reovirus is a small, nonenveloped icosahedral virus with double-stranded RNA composed of discrete segments which are divided among large (L), medium (M), and small (S) size classes. The 10 genome segments include three large (L1, L2, L3), three medium (M1, M2, M3), and four small (S1, S2, S3, S4) segments (reviewed in reference 1). Mammalian reovirus is a ubiquitous virus that can infect a wide variety of mammalian species, with isolates obtained from, but not limited to humans, pigs, dogs, and cats (review in reference 2). Although human infection occurs frequently, reovirus has not been associated with any serious human disease and oftentimes goes unnoticed (2). This absence of association to any human diseases has allowed reovirus to be termed as an orphan virus with infections being isolated to the gastrointestinal system and respiratory tract of animals. Hence, the name Reovirus was obtained – REO standing for Respiratory Enteric Orphan virus (2).

An interesting characteristic of reovirus is its natural ability to be oncolytic. Through various methods of cellular destruction, such as inhibition of DNA synthesis, induction of apoptosis, and interferon induction, reovirus currently serves as an excellent tool in understanding the processes involved in the lysis of transformed cells (1, 2).

When reovirus infections occur, one consequence can be the inhibition of cellular DNA synthesis. When L cells are infected with reovirus type 3, inhibition of DNA synthesis is detected within 8 to 12 hours and continues to occur more rapidly as the MOI is increased (review in reference 1). Furthermore, inhibition of DNA synthesis has been suggested to involve a block in the G1/S transition of the cell cycle (1).

In addition to inhibition of DNA synthesis, reovirus has shown characteristics of inhibition of cellular RNA and/or protein synthesis. For the inhibition of cellular RNA and/or protein synthesis to occur, viral replication is required within the cell and is dependent on MOI (1). In order to replicate, viruses must take up the host cell protein synthesis machinery. To counteract the synthesis of viral proteins over cellular proteins, the host cell releases interferons in infected and nearby uninfected cells, which triggers a signaling cascade that activates the expression of the interferon-induced, dsRNA-activated protein kinase, PKR (56). PKR blocks the cellular translational machinery which stalls the synthesis of viral proteins and prevents the spread of more virus (56). The σ3 protein, which is encoded by the S4 genome segment, regulates the activation of PKR, thereby influencing the level of translation. Activation of PKR results in phosphorylation of the alpha-subunit of the translation initiation factor eIF2 and inhibition of translation initiation. Evidence has shown that σ3 is found throughout the cytoplasm in cells where cellular translation in not inhibited, whereas in cells in which cellular translation is inhibited, σ3 is localized to perinuclear viral factories (reviewed in reference 1). This suggests that σ3 down-regulates PKR thus sparing viral protein synthesis and inhibiting cellular protein synthesis in reovirus infected cells (3).

Another effect that reovirus has on host cells is the induction of interferons. A previous study determined that interferon induction is dependent on the M1, S2, and L2 genome segments of reovirus which encode the core proteins μ2, σ2, and λ2, respectively (4). Through a variety of mechanisms, interferons have shown to inhibit reovirus replication. For instance, interferon α/β appears to inhibit the translation of early reovirus mRNAs. Cells treated with interferon α/β contained an 2',5'-oligo(A)-dependent endoribonuclease (Rnase L) which cleaves reovirus mRNA and may facilitate the antiviral mechanism (reviewed in reference 1). As previously mentioned, interferon-induced expression of PKR causes inhibition of viral protein synthesis. Interferon-γ, on the other hand, is a poor inducer of PKR, thereby having no major effect on viral protein synthesis, yet decreasing the yield of progeny virions.

Reovirus invades a cell by binding to the ubiquitous cell surface receptor sialic acid, which accounts for its ability to infect most mammalian cells (5). However, simply binding to its cell surface receptor does not ensure a productive infection which suggests that reovirus infection is dependent on specific internal downstream events (5). In an earlier study, Hashiro *et al.* (1977) observed that efficient reovirus infection occurred in certain virally and spontaneously transformed murine cell lines, whereas normal human cells and other mammalian cells were not (6). Shortly after this discovery, Duncan *et al.* (1978), while working with normal and SV-40 transformed WI-38 cells, found that reovirus entry and primary transcription was shown to be equivalent in both normal and transformed cells, but the translation of viral proteins was blocked in the normal cells. Therefore, reovirus sustained a lytic infection in the

transformed cells and a noncytocidal infection in the normal WI-38 cells (7). This led to the idea that the efficiency of reovirus infection is correlated to the transformed state of the cell.

Although reovirus displays oncolytic characteristics, this ability has shown to be limited to ras-transformed cell lines (8). Ras is a small guanosine triphosphatase (GTPase) that can □ransducer signals across the cell and plays a central role in the control of cell differentiation, proliferation, and motility (9). Inactivation and activation of Ras proteins are regulated by the GDP/GTP exchange, respectively, and are regulated by guanine nucleotide exchange factors (GEFs). When upstream molecules such as receptor tyrosine kinases are activated, GEFs are stimulated to convert the inactive GDP-bound form of Ras to the active GTP-bound form (10). Activated ras, along with cooperative activation of many downstream effectors, is a powerful inducer of cellular transformation. The Ras-GTP complex stimulates and signals through more than 18 effectors, such as Raf kinases and phosphatidylinositol 3-kinase (PI-3 kinase), which collaborate to contribute to tumorigenesis (9). Activating mutations in ras genes has been linked to >30% of all human cancers and the frequency of ras mutations is found to be the highest among any genes involved in human cancer. With the pervasiveness of ras-mediated oncogenesis and the ability of reovirus to replicate in ras-transformed cells, reovirus demonstrates a potential value as a Ras pathway-directed cancer therapy.

In the present study, mammalian reovirus serotype 3 was used to infect SV-40 transformed human embryonic fibroblasts, WI-38 2RA cells and normal WI-38 cells.

The gene expression of cellular targets were obtained through microarray analysis, and the data from infected and noninfected transformed cells were compared to those of infected and noninfected normal cells to determine the particular functions of genes involved in reovirus-induced oncolysis.

CHAPTER 2

MATERIALS AND METHODS

SV-40 transformed human embryonic fibroblasts, WI-38 2RA cells and normal WI-38 cells were mock infected and reovirus ST3 infected. 4 individual tubes containing 60µg of the following total RNA: WI-38 2RA (control), WI-38 2RA infected with reovirus ST3 (test), WI-38 (control), and WI-38 infected with reovirus ST3 (test) were sent to Geneka Biotechnology Inc. for cDNA sample labeling, microarray pre-hybridization, hybridization, washing, scanning, and microarray data analysis. The concentrations of each tube were 1.10 µg/µl, 1.40 µg/µl, 2.23 µg/µl, and 1.73 µg/µl, respectively (refer to Table 2.1).

Geneka's P.R.O.M.™ (Proteomic Regulators Oligonucleotides Microarray) platform focuses on human genes coding for gene expression regulators, such as transcription factors, co-activators and corepressors, and other proteins directly involved in gene expression. The P.R.O.M.™ slide contains approximately 1500 oligo-probes which hybridize specifically to gene expression regulators.

Table 2.1 Samples sent to Geneka Biotechnology Inc.

Conditions	*# of Tubes*	*Quantity (µg)*	*Concentration (µg/µl)*
2RA Control	1	60	1.10
2RA Test	1	60	1.40
WI-38 Control	1	60	2.23
WI-38 Test	1	60	1.73

2.1 cDNA sample labeling

Lyophilized *Agamous* primer-RNA mix and lyophilized 18S primer were resuspended in 25 µL of DEPC-treated H_2O and kept on ice. A 300 µL RT mix was prepared in a tube with the following, and kept on ice: 140 µL of the first strand buffer 5X, 68 µL of 100 mM DTT, 3 µL of 100 mM dATP, 3 µL of 100 mM dGTP, 3 µL of 100 mM dTTP, 0.6 µL of 100 mM dCTP, and 82.4 of DEPC H_2O. A 14 µL RNA mix was prepared in a tube with the following, and kept on ice: 12 µL total volume of 10 µg of RNA and 2 µg of oligo dT added to DEPC-treated H_2O, 1 µL of Agamous primer-RNA mix, and 1 µL of 18S primer. The RNA mix was denatured by incubating for 10 minutes at 70ºC and immediately put on ice for 1 minute. The sample was spun down and the following was added to the RNA mix: 15 µL of RT mix, 3 µL of Cy3-dCTP or Cy5-dCTP 1 mM, and 2 µL of Superscript II RT (200U/µL) to give a final volume of 34 µL. The mixture was vortexed and incubated without light for 2 hours at 42ºC. After incubation, the tubes were spun down and the reaction was stopped by adding 1.5 µL of 20 mM EDTA and 1.5 µL of 500 mM NaOH. The remaining RNA was degraded by

heating up the tubes at 70ºC for 10 minutes and the reaction was neutralized by adding 1.5 µL of 500 mM HCl. The fluorescent labeled cDNA samples were column-purified using QIAquick PCR Purification Kit from Qiagen according to the manufacturer's procedures (QIAquick PCR Purification Kit Protocol). The column was eluted with 2 X 100 µL of Buffer EB. The Cy3 and Cy5 labeling reactions were pooled for a total volume of approximately 200 µL.

2.2 Pre-hybridization

250 µL of SDS 10% was added to 25 mL of the provided Blocking solution and pre-heated at 45ºC until further use. 50 mL of washing solution 1 (4X SSC, 0.1% SDS) was prepared and kept at 45ºC. The Proteomic Regulators Oligonucleotide Microarray (P.R.O.M.™) slide was incubated in the diluted Blocking solution at 45ºC for 20 minutes and rinsed twice with Nanopure deionized water. Then, the P.R.O.M.™ slide was washed in washing solution 1 at 45ºC for 30 minutes and rinsed twice with Nanopure deionized water. The slide was centrifuged at 127g at room temperature for 5 minutes.

2.3 Hybridization

The lyophilized 18S competitor was resuspended in 25 µL of H_2O and kept on ice. The hybridization chamber was attached to the P.R.O.M.™ slide by aligning the array, label face-up, to the template and removing the clear adhesive strip from the chamber. The wooden stick was used to ensure that all of the chamber borders were well sealed to the array. 0.5 µL of the 18S competitor was added to the fluorescent-labeled cDNA samples, then heat-denatured at 95ºC for 3 minutes and put on ice for 1 minute. The samples were spun down and kept at 45ºC to avoid any renaturation. 200 µL of the labeled cDNA samples were mixed with 200 µL of the pre-hybridization solution by

pipetting up and down. 400 µL of the mixture was applied to the P.R.O.M.™ slide through the holes in the hybridization chamber. Then, ~200 µL of diluted hybridization solution (1:1 (v/v), Hybridization sln to H_2O) was added and a bubble was left in the chamber for better agitation. The hybridization chamber was carefully sealed by gently pressing the two self-adhesive port seals on the holes and incubated at 45ºC overnight in a rotating hybridization oven or an agitating bath in a light-protected area.

2.4 Washes

For each slide, the following washing solutions were prepared: washing solution 2: 4X SSC, (50 mL), washing solution 3: 2X SSC, 0.1% SDS, (400 mL), 45˚C, washing solution 4: 0.2X SSC, (200 mL), and washing solution 5: 0.1X SSC, (200 mL). The hybridization chamber was removed from the P.R.O.M.™ slide starting by the corners, and rinsed in 50 mL of washing solution 2. The P.R.O.M.™ slide was washed in the following order: two times in 200 mL of washing solution 3 for 15 minutes at 45˚C, one time in 200 mL of washing solution 4 for 15 minutes at room temperature, and one time in 200 mL of washing solution 5 for 15 minutes at room temperature. Then, the P.R.O.M.™ slide was centrifuged at 127g for 5 minutes at room temperature. The array was protected from light and was ready to be scanned.

2.5 Scanning

The two fluorescent signals of the hybridized P.R.O.M.™ were scanned using GenePix 4000A dual laser scanning system (Axon Instruments). Each wavelength (red: 635 nm, green: 532 nm) was scanned and the ratios of the emitted fluorescence were calculated for each gene. The reference sample labeled with Cy3 was scanned at 532 nm and the second sample labeled with Cy5 was scanned at 635 nm. The ratios were

calculated using the following formula: (Median feature intensity at 635 nm – Median of background intensity at 635 nm) / (Median feature intensity at 535 nm – Median of background intensity at 532 nm).

2.6 Statistical Analysis

The data generated by the GenePix software were sorted in Microsoft Excel and a list of down regulated and up regulated genes were produced for each assay. Genes demonstrating ratios above 2 were considered up regulated whereas those with ratios less than 0.5 were considered down regulated. Intensities in both wavelengths (635 nm or 532 nm) were considered significant when the intensity of the spot was 3 times higher than the local background (B635 nm or B532 nm).

CHAPTER 3

RESULTS

3.1 P.R.O.M.™ analysis

In order to eliminate bias in the analysis due to mRNA having a labeling preference for one of the two cyanines (Cy3 or Cy5), 4 slides were performed where all the samples were labeled with both dyes and co-hybridized on the same slide. For instance, the slide 22467-13, which has the 2RA control labeled in Cy3 and Cy5 and labeled cDNAs were co-hybridized on the same slide. In result, a ratio of 1.0 for most genes should be obtained. Genes with ratios of medians higher or equal to 2.0 and lower or equal to 0.5 are genes that may have a preference for one of the two dyes. Therefore, those genes were eliminated from the analysis.

Controls and standards include slides 13-16 for labeling and hybridization differences and β-actin, 18S-rRNA, and GAPDH ratios for ssRNA quality (refer to Table 3.2).

Table 3.1 Co-Hybridization

Slides	***Cy3-dCTP labeled***	***Cy5-dCTP labeled***
22467-13	2RA Control	2RA Control
22467-14	2RA Test	2RA Test
22467-15	WI-38 Control	WI-38 Control
22467-16	WI-38 Test	WI-38 Test
22467-33	2RA Control	2RA Test
22467-34	WI-38 Control	WI-38 Test
22467-35	2RA Control	2RA Test
22467-36	WI-38 Control	WI-38 Test

Table 3.2 Average ratio of medians for each control in every experiment

Slides	***22467-13***	***22467-14***	***22467-15***	***22467-16***	***22467-33***	***22467-34***	***22467-35***	***22467-36***
Average of Agamous ratio	1.46	1.18	1.42	1.64	2.86	1.73	1.89	1.09
Average of B-actin ratios	1.10	0.85	1.16	1.27	0.95	1.06	0.74	1.07
Average of 18S RNA ratios	0.74	1.43	1.34	0.56	2.27	1.09	2.64	1.44
Average of GAPDH ratios	0.96	0.88	1.08	1.10	0.79	1.10	0.74	1.18

Table 3.3 Up regulated genes in experiment slide 22467-33 for 2RA test/control samples

Gene	Samples 2RA test/control
BRAHMA	3.825 (3.76 to 3.890)
BRN-4	2.189 (2.017 to 2.36)
BTEB	2.027 (1.989 to 2.065)
BTF3A	2.05 (2.046 to 2.054)
CBF(5)	2.378 (2.209 to 2.547)
CDX2	2.160 (2.047 to 2.273)
CDX4	2.022 (1.917 to 2.128)
CEBPA	2.603 (2.321 to 2.886)
CEBPD	2.334 (2.314 to 2.354)
CEZANNE	2.11 (2.065 to 2.154)
CHFR	3.853 (3.786 to 3.919)
CIITA-8 MHC CLASS II	3.753 (3.496 to 4.010)
C-MAF	2.355 (2.321 to 2.389)
c-myb	10.783 (0.99 to 10.783)
CREBL2	2.088 (1.987 to 2.19)
CRX	3.084 (2.817 to 3.350)
CSPG4	3.207 (3.085 to 3.328)
CSX	2.678 (2.231 to 3.126)
DELTAMAX	2.163 (2.124 to 2.201)
DLC1	2.282 (2.111 to 2.452)
ERCC2	2.818 (0.257 to 5.385)
ERT	2.228 (2.159 to 2.298)
FLJ11340	2.27 (2.269 to 2.270)
FLJ22252	5.617 (5.278 to 5.957)
GFI1	2.667 (2.611 to 2.723)
GOS24	2.329 (2.277 to 2.380)
HAP2	4.052 (4.017 to 4.086)
HLTF	6.214 (6.127 to 6.301)
HOX11L2	2.969 (2.884 to 3.054)
HOXC4	2.092 (2.080 to 2.104)
IRF2	3.503 (0.849 to 6.164)
IRX-3	2.731 (2.664 to 2.798)
JUN-D	2.823 (2.748 to 2.898)
KIAA0071	2.628 (2.482 to 2.773)
KIAA0352	2.155 (1.987 to 2.324)
KIAA0360	2.558 (2.532 to 2.584)
KIAA0595	2.124 (2.097 to 2.152)
KIAA1106	2.130 (2.011 to 2.25)
KIAA1528	5.078 (5.038 to 5.118)
KID	4.276 (4.208 to 4.344)
KPNA2	2.127 (1.997 to 2.262)
LIMK	2.222 (2.181 to 2.262)
LOC51591	3.036 (3.032 to 3.039)
MYT2	2.771 (2.483 to 3.059)
N143	3.060 (2.872 to 3.249)

Table 3.3 – continued

NCOR1	2.293 (2.274 to 2.313)
NF-1A	4.881 (4.822 to 4.941)
NR4A2	3.457 (3.344 to 3.570)
NSEP1	2.523 (2.492 to 2.553)
NUCLEAR FACTOR I-B2	3.204 (2.568 to 3.840)
OAS	2.262 (2.160 to 2.364)
OTF3C	2.059 (1.986 to 2.133)
PAR1	2.319 (2.275 to 2.363)
PC3B	2.584 (2.536 to 2.631)
PPARGC1	2.074 (1.759 to 2.388)
PREB	3.74 (3.735 to 3.745)
PRX2	2.206 (2.036 to 2.375)
PVT GENE	2.840 (2.836 to 2.844)
RBL1	2.266 (2.262 to 2.270)
RLF	2.671 (2.647 to 2.695)
RORALPHA2	3.255 (3.195 to 3.315)
RPGRIP1	2.115 (2.069 to 2.161)
RRNA 18S	2.567 (1.151 to 3.47)
SIM2	6.097 (5.996 to 6.199)
SIRT6	3.972 (3.752 to 4.193)
SLUG	3.509 (3.483 to 3.534)
SRCAP	3.422 (3.404 to 3.440)
STAT1	7.611 (7.213 to 8.01)
SUPT3H	2.516 (2.394 to 2.639)
TAF(II)135	5.046 (4.811 to 5.281)
TAF-172	2.311 (2.252 to 2.369)
TBX3	2.597 (2.569 to 2.625)
TBX6	6.439 (5.554 to 7.324)
TEL-2	2.094 (1.688 to 2.501)
THG-1	3.897 (3.658 to 4.137)
TIS11D	4.84 (4.787 to 4.892)
TP73	5.798 (5.554 to 6.042)
TR2/D15	6.786 (6.169 to 7.403)
TRIAD1 TYPE I	2.023 (2.003 to 2.044)
WUGSC:H_DJ525N14.1	2.011 (1.994 to 2.027)
ZFP37	2.043 (2.017 to 2.069)
ZNF C2H2	2.014 (1.985 to 2.043)
ZNF DP	3.353 (3.252 to 3.455)
ZNF131	2.537 (2.190 to 2.884)
ZNF157	2.668 (2.651 to 2.685)
ZNF216	2.608 (1.038 to 4.349)
ZNF232	2.269 (0.198 to 4.651)
ZNF259	2.042 (2.027 to 2.056)
ZRP-1	2.09 (2.072 to 2.107)

Table 3.4 Up regulated genes in experiment slide 22467-34 for WI-38 test/control samples

Gene	Samples WI-38 test/control
BCL6	2.113
BRAHMA	3.617 (3.612 to 3.622)
BTEB	2.076 (1.951 to 2.201)
CEBPA	2.217 (2.114 to 2.320)
CHFR	3.108 (3.038 to 3.177)
CIITA-8 MHC CLASS II	2.753 (2.561 to 2.945)
C-MAF	2.877 (2.871 to 2.884)
c-myb	5.225 (0.976 to 5.468)
CREBL2	2.097 (2.068 to 2.125)
CRX	3.44 (3.320 to 3.559)
CSPG4	3.153 (3.147 to 3.159)
DELTAMAX	2.022 (1.963 to 2.082)
DJ1043E3.2	2.176 (1.947 to 2.405)
DLC1	2.104 (1.891 to 2.316)
ERCC2	2.338 (0.208 to 4.518)
FLJ11340	2.363 (2.327 to 2.399)
FLJ22252	3.873 (3.777 to 3.969)
GFI1	3.070 (2.796 to 3.345)
HDAC4	2.095 (1.979 to 2.212)
HDAC6	2.512 (2.487 to 2.537)
HLTF	4.583 (4.553 to 4.612)
HOX11L2	2.551
KIAA0262	2.702 (2.551 to 2.853)
KIAA0360	2.183 (1.964 to 2.401)
KIAA1528	4.469 (4.425 to 4.513)
LIMK	2.225 (2.073 to 2.378)
LOC51591	2.416 (2.384 to 2.448)
MAP4	2.181 (1.97 to 2.392)
MYF-4	3.78 (3.746 to 3.813)
MYT2	2.138 (2.104 to 2.172)
N143	2.353 (2.309 to 2.398)
NCOR1	2.421 (2.188 to 2.654)
NF-1A	4.469 (4.432 to 4.506)
NR4A2	3.746 (3.649 to 3.844)
NSEP1	2.478 (2.421 to 2.534)
NUCLEAR FACTOR I-B2	3.408 (3.38 to 3.436)
OTF3C	2.296 (2.129 to 2.462)
PHOSPHORIBOSYL TRANSFERASE	2.001 (1.921 to 2.081)
PPARGC1	2.093 (1.809 to 2.378)
PREB	2.947 (2.668 to 3.226)
RORALPHA2	2.224 (2.205 to 2.243)
SIM2	4.765 (4.722 to 4.808)
SIRT6	4.37 (4.342 to 4.398)

Table 3.4 – continued

SLUG	3.808 (3.710 to 3.907)
SOX11	2.323 (2.271 to 2.376)
SRCAP	3.512 (3.368 to 3.657)
STAT1	8.571 (8.344 to 8.797)
TBX6	2.441 (2.366 to 2.516)
TEF-4	2.48 (2.444 to 2.516)
THG-1	3.741 (3.581 to 3.901)
TP73	2.207 (2.202 to 2.212)
TR2/D15	2.305 (2.241 to 2.369)
ZNF C2H2	2.101 (2.022 to 2.18)
ZNF131	2.068 (1.908 to 2.227)
ZNF157	2.557 (2.518 to 2.597)
ZNF216	2.679 (1.204 to 4.241)
ZNF74	2.142 (1.968 to 2.316)
ZRP-1	2.265 (2.243 to 2.288)

Table 3.5 Up regulated genes in experiment slide 22467-35 for 2RA test/control samples

Gene	**Samples 2RA test/control**
AML2	2.087 (2.003 to 2.171)
ASCL2	2.696 (2.556 to 2.836)
BRAHMA	3.614 (3.531 to 3.697)
BRN-4	2.961 (2.448 to 3.473)
BTF3A	2.051 (2.004 to 2.098)
CBF(5)	2.346 (2.183 to 2.508)
CDX4	2.163 (2.143 to 2.183)
CEBPA	2.273 (2.214 to 2.332)
CEBPD	2.929 (2.786 to 3.073)
CEZANNE	2.246 (2.198 to 2.294)
CHFR	3.292 (3.241 to 3.342)
c-myb	6.453 (6.186 to 6.720)
CRX	3.834 (3.168 to 4.500)
CSPG4	3.259 (3.214 to 3.304)
CSX	3.02 (2.596 to 3.443)
DLC1	2.479 (2.452 to 2.505)
ERCC2	3.389 (3.333 to 3.445)
ERT	2.347 (2.328 to 2.365)
FLJ11340	2.421 (2.365 to 2.477)
FLJ22252	5.613 (5.498 to 5.727)
GFI1	2.22 (2.152 to 2.287)
HAP2	4.239 (4.041 to 4.438)
HLTF	5.760 (5.752 to 5.780)
HOX11L2	3.067
HOXC4	2.038 (2.001 to 2.074)

Table 3.5 – continued

IRF2	4.871 (0.664 to 9.063)
IRX-3	2.279 (1.796 to 2.761)
JUN-D	2.434 (2.248 to 2.619)
KIAA0071	2.869 (2.225 to 3.513)
KIAA0352	2.052 (1.859 to 2.246)
KIAA0360	2.458 (2.162 to 2.755)
KIAA0595	3.410 (3.232 to 3.588)
KIAA1528	5.050 (4.901 to 5.199)
KID	6.369 (6.238 to 6.501)
LOC51591	2.647 (2.334 to 2.96)
MRG1	2.014 (1.816 to 2.212)
MTF-1	2.004 (0.754 to 4.358)
MYT2	2.489 (2.453 to 2.525)
N143	3.418 (3.165 to 3.671)
NCOR1	2.076 (1.899 to 2.252)
NF-1A	6.819 (6.77 to 6.868)
NR4A2	3.618 (3.517 to 3.718)
NSEP1	2.526 (2.238 to 2.815)
NUCLEAR FACTOR I-B2	3.035 (3.031 to 3.039)
OAS	2.367 (2.355 to 2.379)
OTF3C	2.224 (2.151 to 2.297)
PAR1	2.277 (2.186 to 2.368)
PREB	3.949 (3.733 to 4.165)
PVT GENE	2.520 (2.271 to 2.769)
RLF	3.168 (2.808 to 3.528)
RORALPHA2	3.171 (2.863 to 3.478)
ROX	2.056 (1.989 to 2.124)
RPGRIP1	2.180 (2.164 to 2.197)
RRNA 18S	3.330 (1.553 to 5.278)
SIM2	5.969 (5.812 to 6.125)
SIRT6	3.867 (3.128 to 4.606)
SLUG	3.000 (2.949 to 3.051)
SRCAP	3.61 (3.487 to 3.732)
STAT1	6.496 (6.259 to 6.734)
SUPT3H	2.609 (2.536 to 2.682)
TAF(II)135	5.952 (5.876 to 6.028)
TAF-172	2.034 (1.882 to 2.186)
TBX3	2.265 (2.24 to 2.289)
TBX6	4.999 (4.973 to 5.026)
TEL-2	2.005 (1.183 to 2.826)
THG-1	3.606 (3.186 to 4.026)
TIS11D	4.080 (3.480 to 4.680)
TP73	5.397 (5.370 to 5.424)
TR2/D15	4.740 (4.080 to 5.400)
TTF-1	2.259 (1.999 to 2.520)
WUGSC:H_DJ525N14.1	2.032 (2.017 to 2.048)
ZFP37	2.145 (2.088 to 2.202)
ZHX1	2.072 (1.988 to 2.156)

Table 3.5 – continued

ZNF DP	3.626 (3.622 to 3.630)
ZNF131	2.134 (2.016 to 2.252)
ZNF142	2.064 (1.897 to 2.231)
ZNF157	2.493 (2.262 to 2.724)
ZNF232	2.173 (0.347 to 4.662)
ZRP-1	2.077 (2.053 to 2.102)

Table 3.6 Up regulated genes in experiment slide 22467-36 for WI-38 test/control samples

Gene	Samples WI-38 test/control
BRAHMA	3.067 (2.839 to 3.295)
CEBPA	2.291 (2.094 to 2.487)
CHFR	3.375 (3.341 to 3.410)
C-MAF	2.589
c-myb	3.822 (0.701 to 3.927)
CRX	3.233 (2.649 to 3.818)
CSPG4	3.626 (3.575 to 3.676)
DJ1043E3.2	2.251 (2.218 to 2.283)
DLC1	2.470 (2.387 to 2.553)
EGR2	2.175 (2.045 to 2.304)
EHF	2.015 (1.97 to 2.06)
ERCC2	2.977 (0.344 to 5.700)
FLJ11340	2.585 (2.571 to 2.6)
FLJ22252	3.120 (3.015 to 3.226)
GFI1	2.332 (2.209 to 2.454)
HAP2	2.87 (2.789 to 2.951)
HDAC6	2.373 (2.246 to 2.501)
HLTF	3.668 (3.629 to 3.707)
HOX11L2	2.962 (2.865 to 3.059)
HSIM2	2.080 (1.793 to 2.367)
IRF7	2.24 (0.649 to 3.872)
IRX-3	2.009 (1.902 to 2.116)
KIAA0360	2.604 (2.365 to 2.842)
KIAA1247	2.014 (1.994 to 2.034)
KIAA1528	4.496 (4.28 to 4.713)
KID	2.97 (2.944 to 2.996)
LOC51591	2.414 (2.368 to 2.460)
MRG1	2.099 (2.065 to 2.132)
MYF-4	3.311 (3.284 to 3.339)
N143	2.512 (2.338 to 2.685)
NCOR1	2.570 (2.516 to 2.624)
NF-1A	3.992 (3.938 to 4.046)

Table 3.6 – continued

NR4A2	3.734 (3.7 to 3.768)
NSEP1	2.606 (2.555 to 2.657)
NUCLEAR FACTOR I-B2	2.785 (2.526 to 3.045)
OTF3C	2.594 (2.561 to 2.626)
PPARGC1	2.199 (2.116 to 2.282)
PREB	3.293 (3.269 to 3.317)
RLF	2.7 (2.665 to 2.735)
RORALPHA2	2.434 (2.316 to 2.553)
SIM2	4.726 (4.621 to 4.832)
SIRT6	2.422 (2.231 to 2.614)
SLUG	4.092 (4.069 to 4.115)
SOX11	2.112 (2.043 to 2.182)
SRCAP	3.621 (3.031 to 4.210)
STAT1	9.452 (9.145 to 9.759)
TAF(II)135	2.969 (2.873 to 3.065)
TBX6	2.088 (2.087 to 2.09)
TEF-4	2.667 (2.598 to 2.736)
THG-1	3.080 (2.955 to 3.206)
TRIAD1 TYPE I	2.152 (2.121 to 2.183)
ZHX1	2.653 (2.628 to 2.679)
ZNF C2H2	2.267 (2.200 to 2.333)
ZNF DP	2.007 (1.977 to 2.037)
ZNF131	2.017 (2.001 to 2.033)
ZNF157	2.626 (2.519 to 2.733)
ZNF216	2.104 (0.792 to 3.423)
ZNF74	2.678 (2.414 to 2.943)
ZRP-1	2.308 (2.261 to 2.355)

Table 3.7 Down regulated genes in experiment slide 22467-33 for 2RA test/control samples

Gene	**Samples 2RA test/control**
GTF2IP1	0.44 (0.293 to 0.586)

Table 3.8 Down regulated genes in experiment slide 22467-34 for WI-38 test/control samples

Gene	Samples WI-38 test/control
BZRP	0.364 (0.349 to 0.378)
CDKN1C	0.496 (0.492 to 0.499)
CER-D4	0.493 (0.486 to 0.500)
COL9A2	0.405 (0.404 to 0.405)
DKFZP434C151	0.442 (0.414 to 0.471)
DKFZP762E1112	0.3 (0.298 to 0.301)
DSIPI	0.462 (0.456 to 0.467)
E2A (E12)	0.422 (0.42 to 0.424)
E2F4	0.361 (0.266 to 0.457)
EIF4A1	0.481 (0.457 to 0.505)
ELONGATION FACTOR 1-ALPHA	0.366 (0.357 to 0.374)
ERM	0.45 (0.021 to 0.880)
ERR2	0.312 (0.308 to 0.315)
EVX-1	0.309 (0.274 to 0.343)
FIR	0.488 (0.482 to 0.495)
FREAC2	0.480 (0.160 to 0.817)
FTL	0.448 (0.390 to 0.506)
GABPA	0.484 (0.446 to 0.522)
GCN5	0.392 (0.367 to 0.417)
HCENP-B	0.371 (0.291 to 0.452)
HCRHP	0.221 (0.213 to 0.229)
HMG-1	0.492 (0.490 to 0.493)
HNF-6ALPHA	0.47 (0.425 to 0.514)
HOXB8	0.403 (0.372 to 0.434)
HPH1	0.447 (0.446 to 0.448)
HR	0.418 (0.379 to 0.457)
HSF1	0.494 (0.49 to 0.497)
IKAPPABR	0.419 (0.417 to 0.422)
IKBKG	0.475 (0.429 to 0.522)
K-ALPHA-1	0.406 (0.390 to 0.422)
KBP-1	0.379 (0.366 to 0.392)
KIAA0395	0.463 (0.453 to 0.473)
MAFK	0.481 (0.458 to 0.504)
MBP-2	0.401 (0.358 to 0.444)
MIZ-1	0.5 (0.497 to 0.502)
MLN 62 CART1	0.409 (0.407 to 0.410)
MYC	0.497 (0.449 to 0.545)
MZF-1	0.339 (0.336 to 0.341)
NDUFA6	0.477 (0.458 to 0.495)
NEUROD1	0.311 (0.307 to 0.315)
NKX-2.8	0.39 (0.389 to 0.390)
NPAS1	0.474 (0.423 to 0.525)
NRF	0.425 (0.413 to 0.436)

Table 3.8 – continued

NUCLEAR DEAF-1	0.185 (0.165 to 0.205)
OC-2	0.272 (0.238 to 0.306)
OCT-4B	0.387 (0.386 to 0.387)
P64 CLCP	0.485 (0.462 to 0.507)
P65	0.474
PEPD	0.492 (0.462 to 0.522)
PHOG	0.259 (0.247 to 0.272)
PU.1	0.499 (0.482 to 0.515)
RPL13A	0.355 (0.331 to 0.378)
RPS19	0.427 (0.426 to 0.429)
RREB-1	0.444 (0.436 to 0.452)
SALL1	0.395 (0.393 to 0.397)
SNX3	0.387 (0.374 to 0.399)
SOX30	0.447 (0.42 to 0.475)
TBX21	0.252
VAV	0.277 (0.267 to 0.288)
VAX-2	0.414 (0.411 to 0.416)
VIMENTIN	0.422 (0.422 to 0.423)
ZMPSTE24	0.44 (0.427 to 0.453)
ZNF198	0.323 (0.168 to 0.470)
ZNFN1A3	0.439 (0.437 to 0.440)

Table 3.9 Down regulated genes in experiment slide 22467-35 for 2RA test/control samples

Gene	Samples 2RA test/control
NDUFA6	0.484 (0.442 to 0.526)

Table 3.10 Down regulated genes in experiment slide 22467-36 for WI-38 test/control samples

Gene	Samples WI-38 test/control
COL9A2	0.488 (0.476 to 0.501)
DKFZP762E1112	0.452 (0.434 to 0.469)
E2F4	0.462 (0.301 to 0.622)
ELONGATION FACTOR 1-ALPHA	0.371 (0.352 to 0.389)
EVX-1	0.408 (0.359 to 0.457)

Table 3.10 – continued

FTL	0.498 (0.484 to 0.512)
HCRHP	0.375 (0.346 to 0.403)
HR	0.489 (0.459 to 0.519)
K-ALPHA-1	0.467 (0.462 to 0.472)
NEUROD1	0.460 (0.445 to 0.475)
NUCLEAR DEAF-1	0.350 (0.348 to 0.353)
OC-2	0.408 (0.394 to 0.422)
PHOG	0.470 (0.296 to 0.645)
RPL13A	0.407 (0.388 to 0.426)
TBX21	0.423 (0.397 to 0.45)
VAV	0.448 (0.430 to 0.466)
VHNF-1A	0.376 (0.375 to 0.378)
VIMENTIN	0.460 (0.452 to 0.468)

The P.R.O.M.™ analysis revealed that reovirus infection induced significant changes in a total of 178 genes, of which 112 genes were up regulated and 66 genes were down regulated. Information for each gene was obtained by utilizing a variety of databases such as OMIM, PubMed, Genecards, Genelynx, and others. After analysis of all the genes, it was determined that most of the up regulated genes were transcription factors required by the cell for normal cellular processes. However, 13 of the up regulated WI-38 2RA genes were determined to be involved in either the inhibition of DNA synthesis, induction of apoptosis, or interferon induction following reovirus infection. The following is a detailed description of the significantly up regulated WI-38 2RA genes that are involved in the inhibition of DNA synthesis, induction of apoptosis, and interferon induction. Refer to Table 3.11 and 3.12 for functions of all other up regulated WI-38 2RA genes and Table 3.13 for down regulated 2RA genes.

3.2 WI-38 2RA genes involved in inhibition of DNA synthesis, induction of apoptosis, and interferon induction

3.2.1 Runt-related transcription factor 3

AML2, also known as RUNX3, is a runt-related transcription factor 3 and is implicated as a tumor suppressor gene in gastric cancer. It interacts with the Forkhead transcription factor FoxO3a/FKHRL1 which is known to be an important regulator of apoptosis and the cell cycle. The expression of Bim, a proapoptotic BH3-only protein, occurs when RUNX3 interacts with FoxO3a/FKHRL1. Alone, neither RUNX3 nor FoxO3a could activate Bim and induce apoptosis, while the cooperation of RUNX3 and FoxO3a could activate and induce both (11). RUNX3 is an important component of the

TGF-β-mediated signaling cascade. TGF- β responses are initiated by interacting with two cell surface receptors, TGF- β type I and type II transmembrane receptor serine/threonine kinases, which utilize Smad transcription factors (Smads) to transduce downstream signals. Type I receptor kinase directly phosphorylates Smad2 and Smad3, after which they bind with Smad4 and translocate to the nucleus. Once in the nucleus, they act as transcriptional regulators of target genes such as those involved in apoptosis, differentiation, and growth inhibition (12). In the Bim promoter, there is a potential Smad-binding element immediately downstream of the conserved Runx-binding element (RBE2) suggesting that expression of Bim may be regulated by cooperation of RUNX3 with either Smads or FoxO3a, depending on the cellular context (11).

3.2.2 CCAAT/enhancer binding protein alpha

CEBPA or CCAAT/enhancer binding protein alpha is a potent regulator of the cell cycle and is a strong inhibitor of cell proliferation when over expressed. The CCAAT/enhancer-binding protein family is composed of transcription factors that help to control cell proliferation and differentiation. CEBPA is the founding member of this family and is expressed primarily in post-mitotic cells. Its antiproliferative activity is displayed by blocking cell cycle progression at the G1-S boundary (13). This is accomplished by a variety of mechanisms, one of which involves the inhibition of cyclin-dependent kinase (CDK) activity. CEBPA targets both CDK2 and CDK4, which play key roles in regulating cell-cycle progression in the G1 phase by phosphorylating specific substrates, most importantly, the retinoblastoma (pRB) tumor suppressor. CDK4 activation occurs when it associates with type D cyclins. The cyclin D/CDK4

complex causes growth arrest at the G1 phase by phosphorylating pRB, which leads to the inactivation of this substrate, thereby causing its release from E2F transcription factors which activates S-phase genes required for DNA replication and mitotic-specific genes. CEBPA counteracts this process by binding to CDK2 and CDK4 in vitro, inhibiting their ability to phosphorylate substrates. Wang *et al.* (2001) discovered a region of homology among CDK2 and CDK4 called the T-loop region where CEBPA interacts with both kinases. The interaction of CEBPA with the T-loop region of CDK2 prevents or disrupts the formation of CDK2/cyclin complexes by forming inactive CEBPA-CDK2 complexes. This direct interaction of CEBPA with the T-loop region also occurs in CDK4, inhibiting the CDK4-Rb pathway through regulation of CDK4 activity (14).

Another model of CEBPA-induced growth arrest is the stimulation of the cyclin-dependent kinase inhibitor protein, p21. This inhibitor protein is induced by the p53 stress response pathway following DNA damage and may cause cell cycle arrest at G1 and G2, or S-phase. The carboxyl terminal region of p21 binds to and inhibits proliferating cell nuclear antigen (PCNA)-dependent DNA polymerase processitivity. Harris et al. showed that this site binds to CEBPA and increases the inhibition of CDK2 by p21, thus directly inhibiting the CDK enzymatic activity needed for cell cycle progression (15).

3.2.3 Cellular zinc finger anti-NFκB

Cezanne, or cellular zinc finger anti-NFκB is a cytoplasmic zinc finger that inhibits the activity of nuclear factor kappa-B (NFκB). Cezanne has shown a sequence

similarity to the deubiquitinating enzyme, A20 which is a member of the OTU (ovarian tumor protease) family composed of cysteine proteases that are identified by the presence of an OTU domain. Cezanne has also been identified as a novel deubiquitinating enzyme which may explain its inhibitory effect on NFκB (16).

3.2.4 Checkpoint with forkhead and ring finger domains

CHFR, or checkpoint with forkhead and ring finger domains, is a checkpoint protein that contains three separate domains, an NH2-terminal forkhead-associated (FHA) domain, a central ring finger (RF) domain, and a COOH-terminal cysteine-rich (CR) domain. Both the FHA and CR domains are required for the checkpoint function, whereas the RF domain function is currently unknown (17).

Progression from G2 to M is regulated by the activation of a mitosis promoting factor (MPF), Cdc2, and its regulatory subunit, cyclin B. Upon entry into prophase, accumulation of mitotic cyclin B leads to the formation of Cdc2-cyclin B complexes which is then transported to the nucleus. Within the nucleus, Cdc2 undergoes posttranslational modifications, such as phosphorylation and dephosphorylation, respectively, that control the activation of MPF. Cdc2 becomes activated once it is dephosphorylated, in turn allowing MPF to trigger chromosome condensation, breakdown of the nuclear envelope, and formation of the mitotic spindle, thus progression into M phase. CHFR delays entry into mitosis upon mitotic stress and this delay occurs by negatively regulating Cdc2 kinase activation at the G2-M transition (17).

In addition to the effects of entry into mitosis, CHFR is also a tumor suppressor that has shown to be lost in 20-50% of primary tumors. A deficiency in CHFR leads to chromosomal instability in embryonic fibroblasts and is frequently downregulated in human cancers, such as lung, colon, esophageal, and gastric cancer. Also, CHFR has been found in tumor cell lines of lung, colon, esophageal, brain, bone, breast, gastric, and hematopoietic origin. Progression into mitosis is regulated by many key mitotic kinases, such as the aforementioned Cdc2, PLK1, and Aurora kinases. Aurora A kinase, when overexpressed, induces chromosome segregation defects and cytokinesis failure which can lead to aneuploidy and may contribute to tumorigenesis. CHFR physically interacts with this kinase and ubiquitinates Aurora A both *in vitro* and *in vivo*. CHFR is an E3 ubiquitin ligase that is involved in protein degradation, and directly targets Aurora A for ubiquitination and degradation. CHFR maintains genomic stability by regulating the levels of Aurora A, and disruptions in the CHFR-Aurora A pathway encourage tumorigenesis (18).

3.2.5 Myeloblastosis oncogene

c-myb, or myeloblastosis oncogene, is a DNA binding transcription factor that regulates expression of specific genes in hematopoietic and other cell lines during development and differentiation. The product of the *c-myb* protooncogene, c-Myb protein, has latent transforming activity, and slightly minor changes can convert c-Myb from a regulator of normal proliferation and differentiation to a virulent transforming protein. Lei *et al*. showed that c-Myb forms stable complexes with CDKs in human T cells and the transcriptional activity of c-Myb is affected when cell cycle regulators are

expressed. A relatively large fraction of c-Myb and CDK6 exists in a stable complex in human T cells, suggesting that c-Myb is under direct control of the cell cycle process and the transcriptional activity of c-Myb changes during the G1-S transition when cyclin D1-directed kinases become active. c-Myb transcriptional activity is inhibited by cyclin D1 and CDKs and stimulated by the expression of CDK inhibitors, p16, p21, and p27. Thus, c-Myb activity is inhibited during the early part of G1 phase when cyclin D1-dependent CDKs levels are high, and increases as the levels decrease at the G1/S phase transition when the expression of CDK inhibitor proteins occur (19).

3.2.6 Myc-associated factor X

Deltamax, also called Max, is a member of the basic helix-loop-helix leucine zipper (bHLHZ) family of transcription factors that encodes a protein that interacts specifically with the Myc protein (20). Max does not posses an activation segment; however, it acts as an obligate, physiological heterodimerization partner for Myc. Myc is an oncoprotein involved in cell proliferation, differentiation, and apoptosis, but these functions require the heterodimerization of the Myc and Max bHLHZ domain before sequence specific DNA binding can occur. Myc-Max heterodimers recognize the Enhancer box (E box), which is a core hexanucleotide element (5'-CACGTG-3'), and activate transcription at promoters where E boxes are present (21). TRRAP, the ATM-related c-Myc binding protein, interacts with Myc-Max heterodimers to facilitate recruitment of histone acetyltransferases, thus causing the upregulation of gene expression (reviewed in reference 21). In addition, Max can also form homodimers and heterodimers with other family members, such as Mad and Mxi1. Mad, also known as

MAX-dimerizing protein, is found in growth arrested, and differentiated cells where Myc is not present. The Mad protein inhibits cell growth and interferes with the transforming function of Myc, implying that Mad-Max heterodimer acts as a transcriptional repressor (21). The recruitment of histone deacetylase, condensation of chromatin structure, and subsequently the reduced transcription levels are mediated by the recruitment of mSin3 corepressors to promoter DNA by Mad-Max heterodimers (reviewed in reference 21). Therefore, competition between the two transcription factor pairs, Myc-Max or Mad-Max heterodimers for their common E box targets appears to control cell fate, determining whether a cell will divide and proliferate or differentiate and become quiescent, respectively (21).

3.2.7 Oncogene JUN-D

JUND is the most broadly expressed member of the JUN family of basic region leucine zipper (bZIP) DNA-binding proteins (22). It is also part of the AP1 transcription factor complex which plays an important role regulating cell cycle progression, proliferation, oncogenic transformation, and apoptosis in response to stimuli or stress (23). JunD appears to be a negative regulator of the cell cycle and Ras-mediated transformation. When JunD is overexpressed in immortalized fibroblasts, proliferation of the cells slow down and accumulation of the cells in the G1 phase of the cell cycle occurs (29). It may act as a negative regulator of cell growth by causing quiescence within the cell. Additionally, JunD is a weak positive activator of cyclin D1, a major regulator of the G1/S transition, whereas its family member c-Jun is a strong transcriptional activator of cyclin D1. This displays the potentially opposite

effects that these two family members have on the progression of the cell cycle. Furthermore, JunD and c-Jun continue to show antagonistic roles in the regulation of Ras-transformation. Ras-transformed fibroblasts display higher levels of c-Jun, while JunD protein level decreases dramatically (29). c-Jun is found to cooperate with Ras to promote transformation (29) by increasing the cyclin D1 protein levels which increases the G1/S transition by shortening the G1 phase. Excess JunD functions to moderately suppress the transformed phenotype (29) and antagonize proliferation by regulating cell cycle regulators such as cyclin D1. Thus, the down regulation of JunD in transformed cells correlates with the idea that JunD functions as an inhibitor of cell proliferation and works antagonistically with c-Jun to decrease its harmful effects (23).

3.2.8 2', 5' oligoadenylate synthetase

OAS, or 2', 5' oligoadenylate synthetase is an interferon-induced protein that has the ability to catalyze the synthesis of 2', 5' oligomers of adenosine. The family of 2', 5' oligoadenylate synthetases is part of the 2-5A system which is an RNA degradation pathway induced by interferons. This system acts as a host response mechanism against viral infection in cells and occurs in response to interferon induction. Double-stranded RNA, derived from viral replication intermediates, activates OAS resulting in the synthesis of 2',-5'-linked oligoadenylates (2-5A) from ATP (25). 2-5A activates the 84 kDa Ribonuclease L (RNase L), which is normally latent (14). RNase L contains a C-catalytic domain that appears to be responsible for the cleavage of mRNA at the 3' side of UpNp sequences (26). The activation of RNase L leads to the inhibition of cellular protein synthesis and prevention of viral replication.

Moreover, RNase L cleaves 28S and 18S ribosomal RNA (rRNA), causing ribosomal inactivation and translational inhibition (27).

3.2.9 B-cell translocation gene 4

PC3B, also known as B-cell translocation gene 4 (BTG4), is a member of the PC3/BTG/TOB family of growth inhibitory genes involved in the negative regulation of the cell cycle. The main characteristic of this family is the BTG/Tob homology domain, which is a highly conserved N-terminal region present in each member. This homology domain contains two highly homologous regions, termed the A and B boxes. Of these two regions, the A box, or GR region is suggested to be involved in the antiproliferative role of this family (30, 31). Buanne *et al.* (2000) found that the GR region is highly conserved between PC3B and other PC3/BTG/TOB family members that have antiproliferative functions, specifically the ability to induce G1-arrest. They observed that the expression of PC3B in NIH3T3 cells induced a marked increase of the cells in the G1 phase of the cell cycle, along with a complementary decrease of cells in the S and G2/M phases, thus identifying PC3B as a cell cycle inhibitor (31).

3.2.10 Retinoblastoma-like 1

RBL-1, also called p107 is a member of the family of pocket proteins involved in the negative regulation of the cell cycle. Pocket proteins are characterized by the presence of a bipartite pocket structure called the A/B domains which interact with E2F transcription factors and viral oncoproteins. When p107 is overexpressed in fibroblasts,

CDK2 activation is inhibited causing a delay of S phase entry. This occurrence is correlated to the accumulation of the CDK inhibitor protein, p27, and its tight binding to cyclin E-Cdk2 complexes. Furthermore, p107 stabilizes p27 by directly down-regulating the expression of the F box protein Skp2 (32) which specifically binds phosphor-(Thr187) p27 and is needed for ubiquitin-mediated degradation of p27 (33). During G0/early G1, the levels of Skp2 are undetectable or low and increase gradually during progression to S and G2 phases, but when p107 is overexpressed, it partially suppresses the increase in Skp2 mRNA as the cells progress into G1 phase (32). This suggests that p107 inhibits cell proliferation by controlling Skp2 expression and stabilizing p27, in turn inhibiting CDK2 activation. (refer to Fig. 2)

In addition, p107 is part of a complex containing Smad3 and the transcription factors E2F4/5 and DP1 that preexists in the cytoplasm. Smad3 becomes phosphorylated in response to TGF-β, allowing the complex to associate with Smad4 and translocate from the cytoplasm into the nucleus. Once inside the nucleus, the complex recognizes a composite Smad-E2F site on c-*myc* causing repression. Transcriptional repression of c-*myc* removes the growth-promoting functions and eases the induction of cdk inhibitors. p107 remains bound to E2F4/5 transcription factors which act primarily as transcriptional repressors, and prevents expression of E2F1-3 target genes which encode activities required for DNA synthesis and are essential for cell proliferation, thus, inhibiting cell cycle progression (34). (refer to Fig. 3)

3.2.11 MAX-interacting protein

Rox, also called MAX-interacting protein (MNT) is a novel basic helix-loop-helix leucine zipper (bHLHZ) protein that is part of the Myc and Mad family of proteins that bind DNA at the E box (CANNTG) consensus by heterodimerizing with Max. Members of this family are involved in transcriptional regulation and regulate cell differentiation and proliferation. Rox also heterodimerizes with Max and weakly homodimerizes (35). Rox-Max heterodimers act as transcriptional repressors and effectively repress transcription at Myc-Max E box sites. Rox-Max heterodimers are able to bind the preferred Myc-Max binding sequence at the E box and both Rox and Myc bind Max with comparable affinity and is coexpressed with Myc in various proliferating cell types. Moreover, Rox contains a 13-amino acid sequence within the amino-terminal helical region that displays homology to the Sin3 interacting domain (SID) found in all Mad family proteins. This SID region of Rox is essential for transcriptional repression function as deletion of this region converts Rox from a suppressor of transformation to a collaborating oncogene. Rox and Myc display antagonistic roles and relative levels of each may be important in the regulation of cell proliferation (36).

3.2.12 Signal transducer and activator of transcription 1

STAT1, or signal transducer and activator of transcription 1 is a member of the family of transcription factors known as the STAT protein family. This family of proteins is involved in mediating the effects of various cytokines, polypeptide growth factors, hormones, and oncoproteins. STAT transcription factors also regulate the

expression of target genes, subsequently regulating a multitude of functions such as cell growth and survival, differentiation, and development (37). STAT proteins are important mediators of interferons. When type I IFN binds to cell surface receptors, activation of STAT1 occurs via phosphorylation of Tyr^{701} by Janus protein kinases (JAKs) which leads to STAT1 dimerization through its Src homology 2 (SH2) domains. The STAT dimer associates with ISGF3G/IRF-9 forming a complex termed ISGF3 transcription factor and translocates into the nucleus. ISGF3 binds to the IFN stimulated response element (ISRE) which causes activation of the transcription of interferon stimulated genes. This drives the cell to an antiviral state. Conversely, if type II IFN binds to cell surface receptors, STAT1 is activated via phosphorylation of both Tyr^{701} and Ser^{727} to form a homodimer termed IFN-gamma-activated factor (GAF) which enters the nucleus. Once in the nucleus, GAF binds to the IFN gamma activated sequence (GAS) which activates the expression of target genes, consequently inducing an antiviral state (38).

3.2.13 Tumor protein 73

TP73, or tumor protein 73 is a homologue of the tumor suppressor gene p53 which is the most frequently mutated gene in human cancer. p73 is composed of three domains, the N-terminal transactivation domain, the sequence-specific DNA binding domain, and the tetramerization domain (39). p73 binds to p53 binding sites and activates the promoters of a multitude of p53-responsive genes such as BAX, p21, MDM2, and GADD45. Like p53, p73 is activated in response to DNA damage leading to either cell cycle arrest or apoptosis. p53 mediates p21 cyclin-dependent kinase

inhibitor which prevents CDK-cyclin activity and subsequently induces G1/S cell cycle arrest. This allows DNA repair to occur prior to replication. If the DNA is irreparable, p53 activation will eliminate the damaged cells via apoptosis. The final outcome of p53 activation is dependent on a variety of factors and is regulated by the pattern of downstream effector genes transactivated by p53. p53 must rapidly accumulate in order to perform its cellular functions of either cell cycle arrest or apoptosis. Post-translational modifications such as phosphorylation, acetylation, and addition of SUMO polypeptides at specific sites regulate the rapid accumulation of p53. Recent studies have shown that in response to DNA damage, p73 also accumulates and is tyrosine phosphorylated and is dependent on the activation of the nuclear c-Abl tyrosine kinase (40).

Table 3.11 WI-38 2RA Housekeeping Genes

Gene	Accession No.	Gene Function	Ratio
Brn-4	X82324	Brain 4; POU Domain, Class 3, Transcription factor 4	2.189
BTEB	D31716	GC box binding protein	2.027
BTF3A	M90352	Basic transcription factor 3a	2.05
CEZANNE	NM_020205	Zinc finger protein Cezanne	2.11
CHFR	NM_018223	Checkpoint with forkhead and ring finger domains	3.853
C-MAF	AF055376	Short form transcription factor C-MAF	2.355
DELTAMAX	X60287	MAX protein	2.163
HAP2	M59079	CCAAT-binding protein	4.052
HOXC4	NM_014620	Homeobox C4	2.092
IRF2	NM_002199	Interferon regulatory factor 2	3.503
IRX-3	U90305	Iroquois-class homeodomain protein	2.731
KID	D38751	Kinesin-like DNA binding protein	4.276
LIMK	D26309	LIM domain kinase	2.222
MRG-1	AF109161	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	2.014
MYT2	NM_003871	Myelin transcription factor 2	2.771
PPARGC1	NM_013261	Peroxisome proliferative activated receptor, gamma, coactivator 1	2.074
RLF	NM_012421	Rearrange L-myc fusion sequence	2.671
RPGRIP1	NM_020366	Retinitis pigmentosa GTPase regulator interacting protein 1	2.115
TAF(II)135	Y11354	TATA box binding protein (TBP)-associated factor , RNA polymerase II, C1, 130kD	5.046
TP73	NM_005427	Tumor protein 73	5.798

Table 3.11 - continued

TR2/D15	AJ245600	Hypothetical protein	6.786
TRIAD1 TYPE1	AF099149	TRIAD1 type 1	2.023
TTF-1	U43203	Thyroid transcription factor 1	2.259
ZFP37	NM_003408	Zinc finger protein homologous to Zfp37 in mouse	2.259
ZHX1	NM_007222	Zinc fingers and homeoboxes 1	2.072
ZNF DP	AF153201	Zinc finger protein dp	3.626
ZNF 216	NM_006007	Zinc finger protein 216	2.608

Table 3.12 WI-38 2RA Specific Genes

Gene	Accession No.	Gene Function	Ratio
AML2	Z35278	PEBP2aC1 acute myeloid leukemia	2.087
ASCL2	NM_005170	Achaete-scute complex homolog-like 2	2.696
CBF(5)	M37197	CCAAT-box-binding factor	2.346
CDX2	Y13709	Caudal-type homeobox protein 2	2.16
CDX4	NM_005193	Caudal-type homeobox transcription factor 4	2.163
CEBPA	NM_004364	CCAAT/enhancer binding protein alpha	2.603
CEBPD	NM_005195	CCAAT/enhancer binding protein delta	2.929
CIITA-8 MHC ClassII	U18259	MHC class II transactivator CIITA	3.753
CREBL2	NM_001310	cAMP-responsive element binding protein-like 2	2.088
CSX	NM_004387	Cardiac-specific homeobox	3.02
ERT	AF017307	ets-related transcription factor	2.347

Table 3.12 - continued

GOS24	M92844	Zinc finger transcriptional regulator	2.329
JUN-D	X56681	Jun D proto-oncogene	2.823
KIAA0071	NM_015156	KIAA0071 protein	2.869
KIAA0352	NM_014830	KIAA0352 gene product	2.155
KIAA1106	AB029029	KIAA1106 protein	2.13
KIAA0595	AB011167	KIAA0595 protein	3.41
KPNA2	NM_002266	Karyopherin alpha 2 (RAG cohort 1; importin alpha 1)	2.129
MTF-1	AJ362992	Heavy metal-responsive transcription factor	2.004
OAS	AJ225089	2'-5'-oligoadenylate synthetase	2.367
PAR1	AF084645	Orphan nuclear receptor	2.319
PC3B	AJ271351	BTG family, member 4	2.584
PRX2	NM_016307	Paired related homeobox protein	2.206
PVT Gene	M34428	Pvt-1 (murine) oncogene homolog, MYC activator	2.84
RBL-1	NM_002895	Retinoblastoma-like 1 (p107)	2.266
rRNA 18S	X03205	rRNA 18S	3.33
ROX	X96401	ROX protein	2.056
SUPT3H	NM_003599	Suppressor of Ty (*S. cerevisiae*) 3 homolog	2.609
TAF-172	AJ001017	TBP-associated factor 172	2.311
TBX3	AF140240	T-box 3 (ulnar mammary syndrome)	2.597
TEL2	NM_016135	ets transcription factor TEL-2b	2.094
TIS11D	U07802	Tis11d protein	4.84
WUGSCH_ DJ525N14.1	AC002086	Similar to zinc finger 5 protein from *Gallus gallus*	2.032

Table 3.12 - continued

ZNF142	NM_005081	Zinc finger protein 142 (clone pHZ-49)	2.064
ZNF232	NM_014519	Zinc finger protein 232	2.269
ZNF259	NM_003904	Zinc finger protein 259	2.042

Table 3.13 WI-38 2RA down regulated genes

Gene	Accession No.	Gene Function	Ratio
GTF2IP1	AF036613	General transcription factor 2-I	0.44
NDUFA6	NM_002490	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 6 (14kD, B14)	0.484

CHAPTER 4

DISCUSSION

Reovirus has displayed the ability to induce apoptosis, inhibit DNA synthesis, and induce interferons upon infection of transformed cells, but not normal cells. Through the use of microarray technology, we have identified a multitude of cellular targets that could possibly explain the cellular processes that occurs post reovirus infection, thus preventing the proliferation of transformed cells while having little effect on normal cells. SV-40 transformed human embryonic fibroblasts, WI-38 2RA cells and normal WI-38 cells were mock infected and reovirus ST3 infected and sent to Geneka Biotechnology, Inc. for microarray analysis. The data from the P.R.O.M.™ analysis was obtained and cellular targets involved in apoptosis, cell cycle progression, and interferon induction were identified. Of the 178 genes that incurred significant changes after reovirus infection, only 13 of the 2RA genes were found to be involved in either apoptosis, inhibition of DNA synthesis, or interferon induction.

4.1 Reovirus-induced alterations of WI-38 2RA genes involved in apoptosis

Reovirus infection of the WI-38 2RA cells induced changes in a variety of pro-apoptotic genes, such as AML2, OAS, and TP73. Up regulation in each of the pro-apoptotic genes suggests that reovirus causes apoptosis of 2RA cells through a variety of pathways. When reovirus enters the cell, the viral dsRNA activates 2'5' oligo (A) synthetase (OAS) which converts ATP to 2-5A. 2-5A activates RNase L which leads to

degradation of cellular mRNA and inhibition of translation, or ribosomal inactivation and cell death (refer to Fig. 4.1).

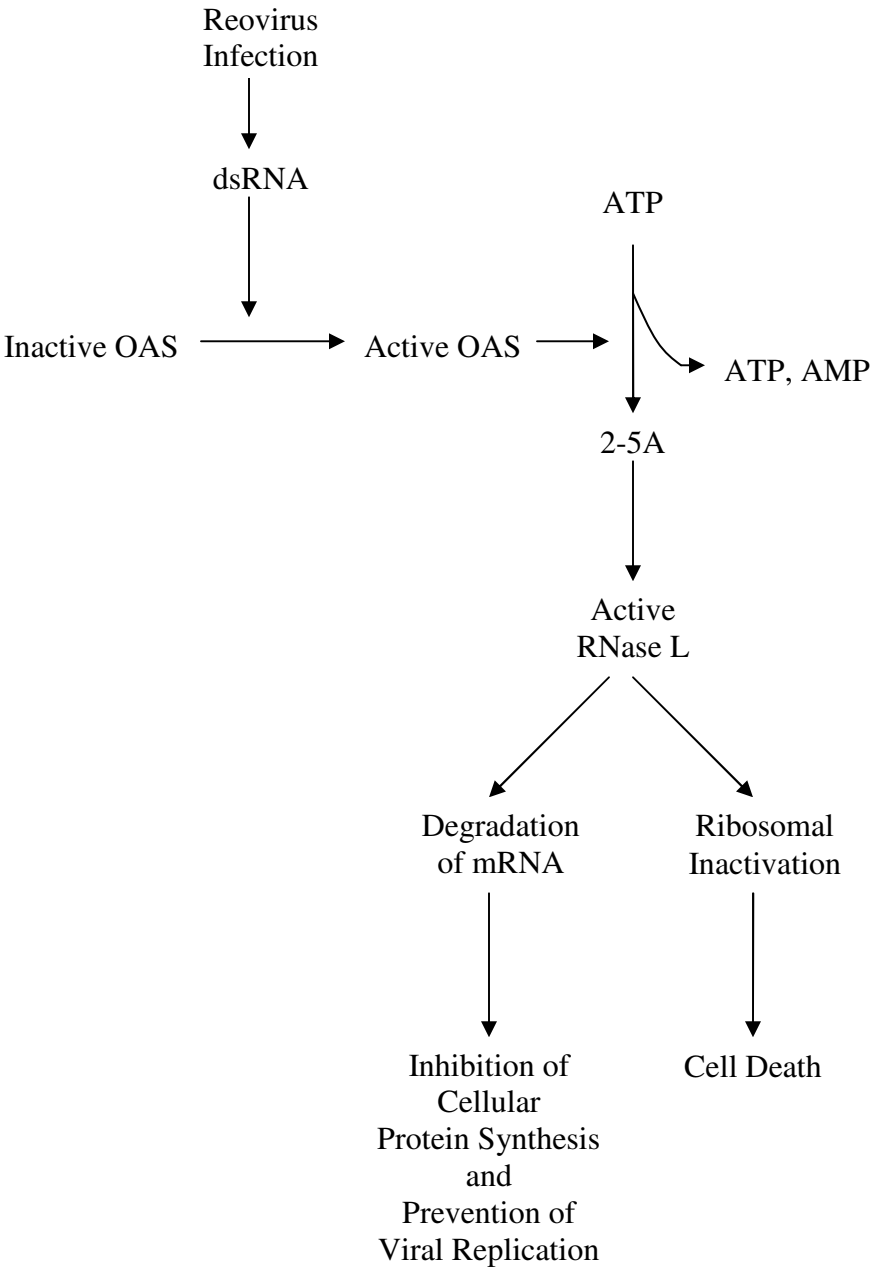

Figure 4.1 Proposed model for the inhibition of translation and induction of apoptosis by Reovirus-activated 2-5A system.

The runt-related transcription factor 3 (Runx3 or AML2) was also found to be up regulated in the 2RA cells. Reovirus infection induces the expression of AML2 which interacts with FoxO3a/FKHRL1 and activates the pro-apoptotic gene, Bim, which induces apoptosis by binding to, and antagonizing the apoptosis repressor, BCL2. Bim resides in the cytosol and translocates to the mitochondria following death signaling to promote the release of cytochrome c and activate CASP3, subsequently causing apoptosis of the cell (refer to Fig. 4.2) (41). In addition, AML2 interacts with TGF-β type I transmembrane receptor kinase which directly phosphorylates Smad2 and Smad3, which then binds Smad4 and translocates to the nucleus where they act as transcriptional regulators of genes involved in apoptosis.

Another method of reovirus-induced apoptosis was displayed by the over-expression of tumor protein 73 (TP73). TP73 is a homologue of the tumor suppressor gene p53 and binds to p53-specific binding sites to activate promoters of several p53-responsive genes, specifically, BAX. BAX, like Bim, is a pro-apoptotic protein that induces cell death by antagonizing BCL2, and by acting on the mitochondria (41) (refer to Fig. 4.2).

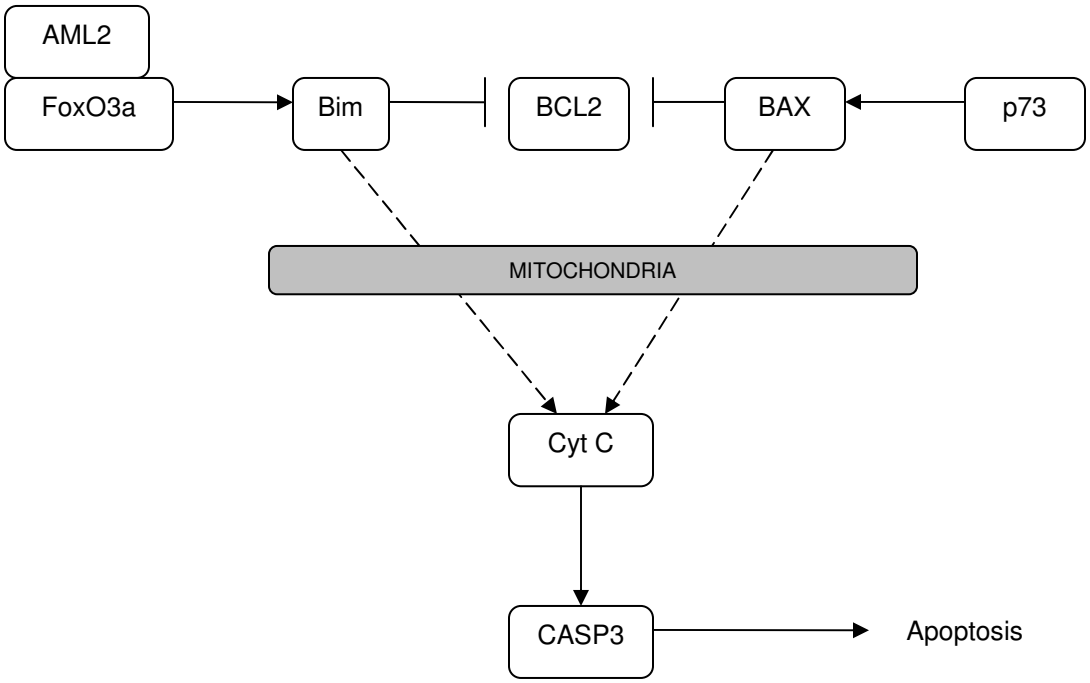

Figure 4.2 Proposed model for reovirus-mediated cell death by over expression of AML2 and TP73

4.2 Reovirus-induced alterations of WI-38 2RA genes involved in cell cycle progression

Oncogene JunD was found to be over expressed in the 2RA cells post reovirus infection. Over expression of JunD causes proliferation of the cells to slow down and leads to accumulation in the G1 phase of the cell cycle. Given that the 2RA cells were ras-transformed, we should expect to see high expression levels of c-Jun, but c-Jun was not identified as an up regulated gene in the 2RA cells. Instead, JunD, which antagonizes the effects of c-Jun by regulating cyclin D1 was found to be highly up-regulated in the 2RA cells.

B-cell translocation gene 4, PC3B, was shown to be up regulated in the 2RA cells. The GR region on PC3B has antiproliferative properties, specifically the ability to induce G1-arrest. Overexpression of PC3B causes cells to accumulate in the G1 phase of the cell cycle while greatly decreasing the number of cell in S phase.

Another gene found to be up regulated in only the 2RA cells was the retinoblastoma-like 1 (RBL-1 or p107) gene. p107 is a member of the family of pocket proteins involved in the inhibition of cell cycle progression. Reovirus infection in the 2RA cells causes over expression of p107 which has been shown to cause CDK2 inactivation and delay into S phase of the cell cycle. This occurs by accumulation of the CDK inhibitor protein, p27, which is stabilized by p107's ability to directly down regulate Skp2. p27 tightly binds to cyclin E-Cdk2 complexes and prevents progression into S phase (refer to Fig. 3). In addition, p107 is part of the Smad3-E2F4/5-DP1 complex that preexists in the cytoplasm. The complex becomes phosphorylated by TGF-β and associates with Smad4 and translocates into the nucleus. The complex recognizes a composite Smad-E2F site on c-*myc* causing repression and removing growth-promoting functions of c-*myc*. This allows for the induction of cdk inhibitors, thus inhibiting cell cycle progression (refer to Fig 4). Over expression of p107 is essential for the repression of transcription as it must be bound to E2F4/5 to prevent the expression of E2F1-3 target genes which act as transcriptional activators.

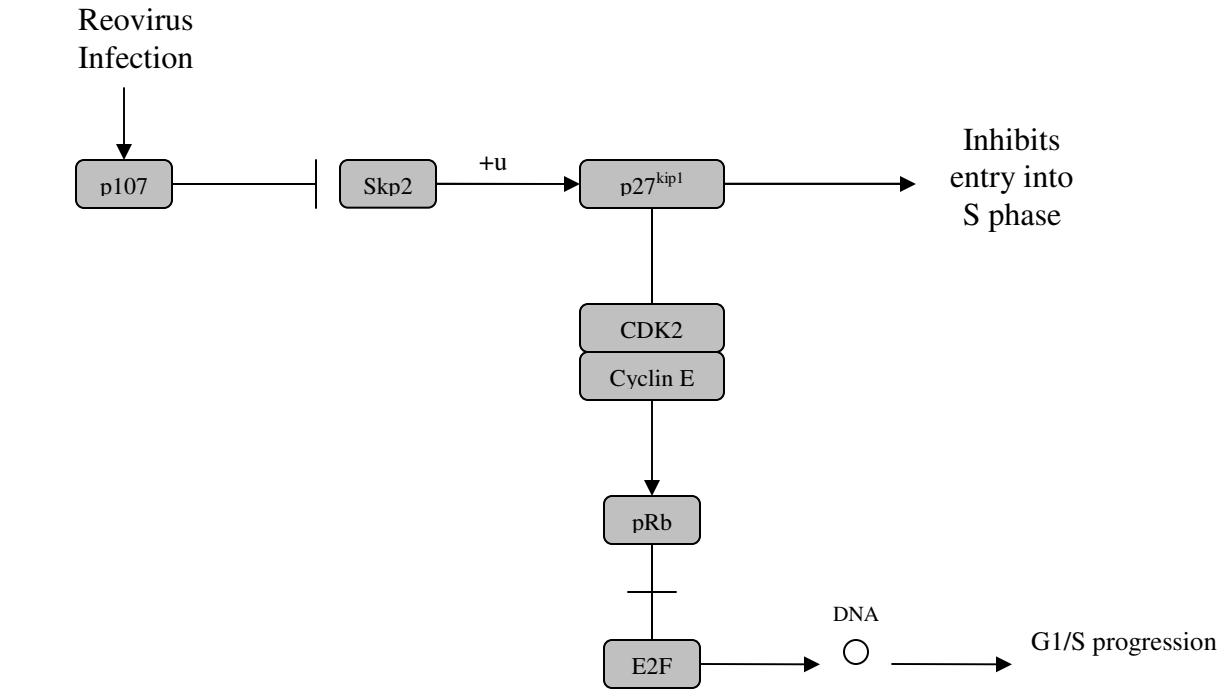

Figure 4.3 Proposed model for the Inhibition of G1/S phase progression by negative regulation of Skp2 by p107 due to reovirus infection

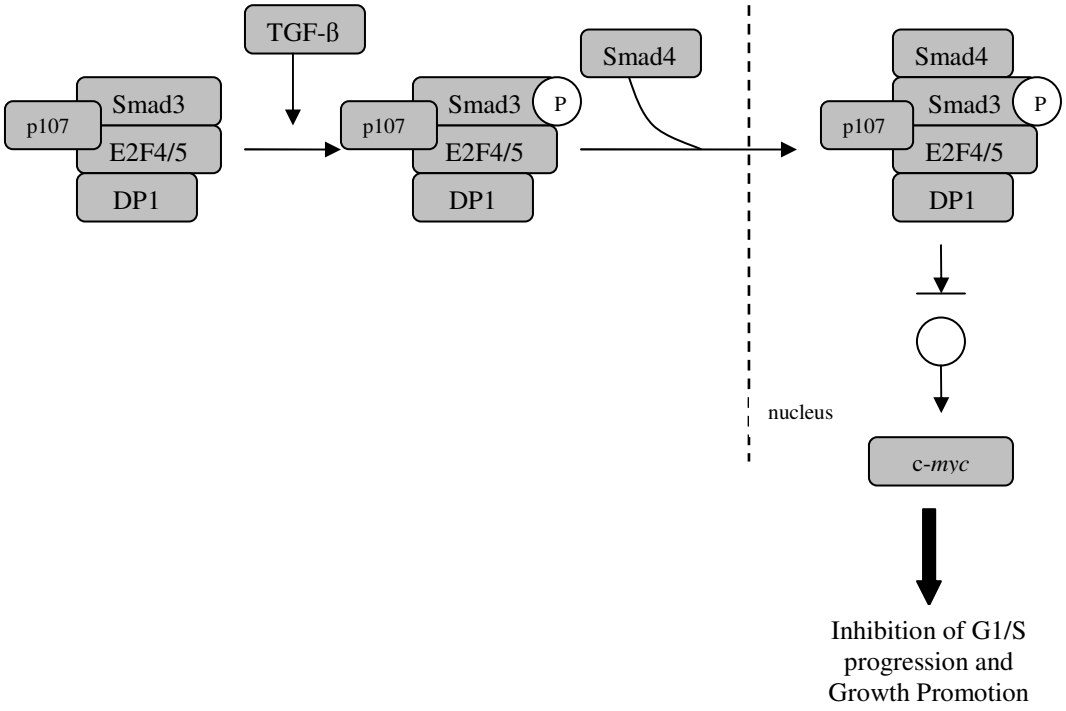

Figure 4.4 Proposed model for the Inhibition of G1/S phase progression by repression of c-*myc* due to reovirus infection

Myeloblastosis oncogene, c-myb, was shown to be significantly over expressed in reovirus infected 2RA cells, displaying a ratio of 10.783. c-myb is placed in a pathway downstream of CDK inhibitors, such as p21, p27, cyclin D1, and CDK6 that regulates important genes required for G1/S progression. Given that c-myb is stimulated by CDK inhibitors, such as p16, p21, and p27, this vast over expression of c-myb suggests that reovirus infection causes an accumulation of CDK inhibitors, which ultimately leads to the inhibition of DNA synthesis by preventing progression into S phase.

CEBPA was up regulated in both normal WI-38 cells and 2RA cells, but the average ratio was slightly higher in the 2RA than the WI-38 cells, 2.438 to 2.254, respectively. Reovirus infection causes over expression of CEBPA, which is a potent regulator of the cell cycle and a strong inhibitor of cell proliferation. CEBPA inhibits the ability of both CDK2 and CDK4 to phosphorylate substrates necessary for S phase progression. CEBPA directly interacts with the T-loop region of CDK2 to form inactive CEBPA-CDK2 complexes. This also occurs in CDK4, preventing the CDK4-Rb pathway. Additionally, over expression of CEBPA stimulates the CDK inhibitor, p21, which binds to and inhibits PCNA-dependent DNA polymerase processitivity and increases the inhibition of CDK2/4 by p21 (refer to Fig. 5).

Checkpoint with forkhead and ring finger domains (CHFR) was shown to be up regulated in both WI-38 cells and 2RA cells, but the average ratio was higher in the 2RA than the WI-38 cells, 3.517 to 3.334, respectively. Over expression of CHFR by reovirus delays entry into mitosis by negatively regulating Cdc2 kinase activation at the G2-M transition.

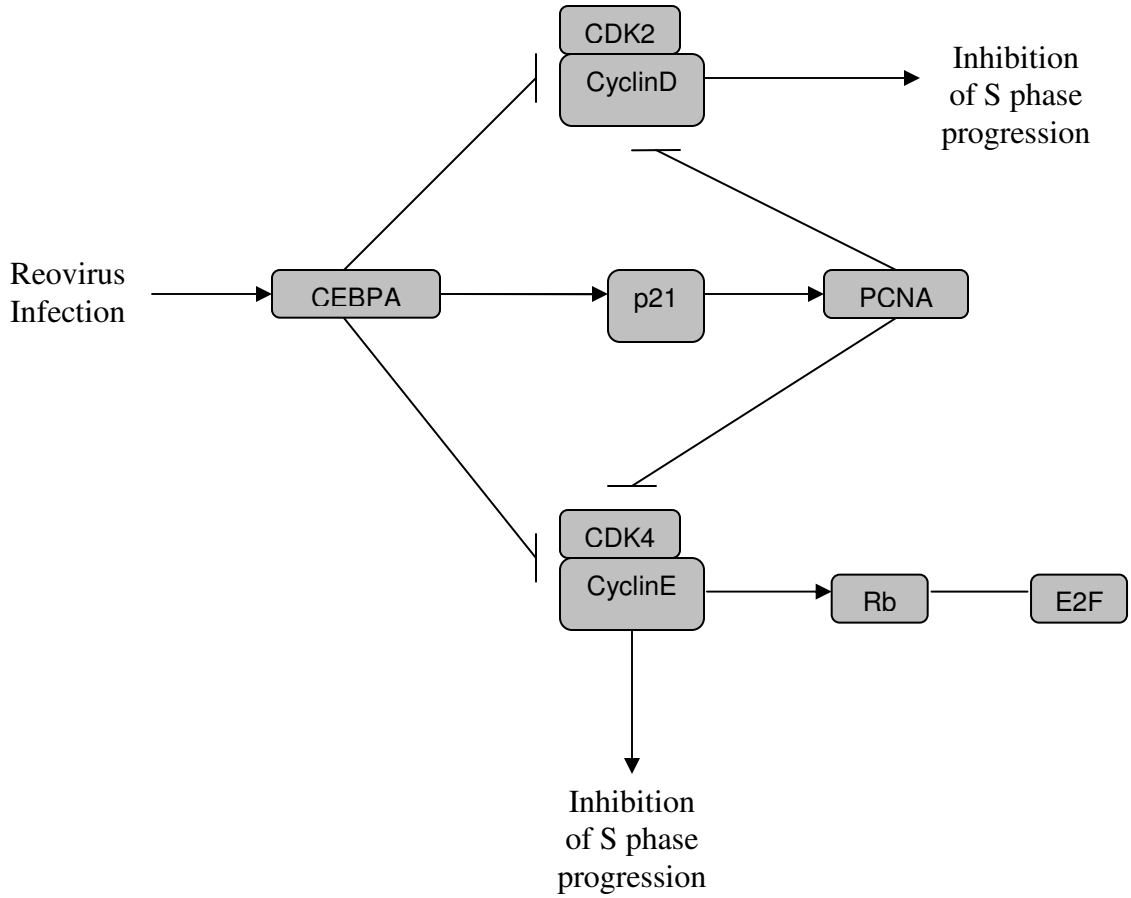

Figure 4.5 Proposed model for the inhibition of S phase progression by CEBPA due to over expression by Reovirus infection

Another method of reovirus-induced checkpoint arrest was shown by the up regulation of p73. As previously mentioned, p73 activates the promoters of various p53-responsive genes such as BAX which can lead to apoptosis. p73 can also activate cell cycle regulatory genes, such as p21 and GADD45. Activation of p21 cyclin-dependent kinase inhibitor leads to the inhibition of CDK-cyclin activity, thus preventing cell cycle progression into S phase. p73-mediated activation of GADD45 leads to the inhibition of

cdc2 kinase activity by dissociating it from cyclin B, which results in G2/M cell cycle arrest (42).

4.3 Reovirus-induced transcriptional repression in WI-38 2RA cells

Reovirus-induced transcriptional repression was displayed by the over expression of both Myc-associated factor X (Max) and Max-interacting protein (Rox). Max is a member of the bHLHZ family that encodes a protein that interacts specifically with Myc, which is an oncoprotein that is involved in cell proliferation, differentiation, and apoptosis. When Max heterodimerizes with Myc, it recognizes the Enhancer box and activates transcription to up regulate gene expression. Alternatively, Rox can also heterodimerize with Max and act as transcriptional repressors to effectively repress transcription at the Myc-Max E box sites.

4.4 Cellular targets involved in the prevention of reovirus-induced apoptosis or inhibition of DNA synthesis in WI-38 cells

Unlike ras-transformed cells which undergo lysis via a pro-apoptotic pathway or through inhibition of DNA synthesis, normal cells undergo a noncytocidal infection where cellular destruction does not occur. The use of microarray technology has given us a better understanding of what cellular targets may be involved in the prevention of apoptosis and inhibition of DNA synthesis in normal WI-38 cells post reovirus infection. After analysis of the microarray data, it was determined that the cellular targets involved in the prevention of apoptosis and inhibition of DNA synthesis were mostly down regulated. Of the 178 genes that incurred significant changes after reovirus infection, only 7 of the down regulated WI-38 genes were found to be involved in either apoptosis or the cell cycle. In addition, 5 up regulated WI-38 genes were found to be involved in cell survival.

4.4.1 Cellular targets down regulated in WI-38 cells

Cyclin-dependent kinase inhibitor (CDKN1C), also known as p57, is an inhibitor of cyclin dependent kinases 2 and 4 (CDK2, CDK4) which are involved in G1/S phase progression (43). Reovirus infection in the WI-38 cells caused a down regulation of CDKN1C, displaying a ratio of 0.496. Normally, CDKN1C would inhibit necessary cyclin/CDK complexes and cause G1 arrest in cells, subsequently inhibiting DNA synthesis. However, in the WI-38 cells, CDKN1C was observed to be down regulated which allowed normal cyclin/CDK complexes to form, thus allowing S phase progression to occur.

Transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47) is a bHLH protein that binds to the E box sequence (CANNTG). E2A has been shown to cause growth arrest by up regulating cyclin dependent kinase inhibitor p21 (44). After reovirus infection, the WI-38 cells showed a ratio of 0.361, suggesting that inhibition of DNA synthesis was prevented by down regulating an important protein that mediates a potent cell cycle inhibitor.

E2F4 transcription factor is part of the E2F/retinoblastoma tumor suppressor (pRB) complexes which regulate cell cycle progression. E2F4 was shown to be down regulated in the WI-38 cells after reovirus infection. Unlike other E2F family members (E2F1, E2F2, and E2F3) which act as transcriptional activators, E2F4 acts as a transcriptional repressor and preferentially binds the pocket proteins, p130 and p107 (45). p107 negatively regulates cell cycle progression by stabilizing cyclin dependent kinase inhibitor p27 which inhibits CDK2 activation. Therefore, reovirus-induced down

regulation of E2F4 permits S phase progression to occur by preventing the binding to p107.

MLN 62 CART1, also known as TRAF4, is a member of the tumor necrosis factor receptor (TNFR) –associated factor family of proteins that is involved in cell survival and destruction. When TRAF4 is over expressed, apoptosis is induced in cells. Additionally, TRAF4 may play a role in p53-mediated proapoptotic signaling pathway under cellular stress (47). TRAF4 was found to be down regulated in the WI-38 cells suggesting one method in which apoptosis may be prevented post reovirus infection.

Nuclear deaf-1 was shown to be down regulated in the WI-38 cells. Reovirus infection limits the expression of nuclear deaf-1 which is transcription factor that acts as an inhibitor of cell proliferation by causing cell cycle arrest in G0 or G1 phases (48). As a result, WI-38 cells promote cell cycle progression by down regulating nuclear deaf-1 after reovirus infection.

Prevention of apoptosis and cell cycle progression was displayed by the down regulation of both MYC and MIZ-1 in the WI-38 cell after reovirus infection. MYC is a protooncogene that is highly overexpressed in a number of cancers, and MIZ-1 is a MYC-interacting zinc finger protein-1 that interacts specifically with the helix-loop-helix domain of MYC. Alone, MIZ-1 is a potent inducer of cellular growth arrest, but interaction between MYC and MIZ-1 inhibits MIZ-1-induced growth arrest. Furthermore, when DNA damage occurs, activation of p53 induces either cell cycle arrest or apoptosis. MYC is the primary determinant of which p53-mediated pathway will occur. MIZ-1 directly recruits MYC to the promoter of cyclin dependent kinase inhibitor p21 which blocks the induction of p21 by p53. This causes MYC to switch from

cytostatic to apoptotic by inhibiting the activation of p21, thus favoring the initiation of apoptosis and influencing the p53 response to end in apoptosis (49). Consequently, the limited expression of both these genes plays a suggestive role in the prevention of apoptosis and progression of the cell cycle in the WI-38 cells after infection with reovirus.

4.4.2 Cellular targets up regulated in WI-38 cells

Among the up regulated genes of WI-38 cells, 5 targets were found to be involved in cell survival. c-Maf is a member of the basic-leucine zipper family of transcription factors and was found to be up regulated in the WI-38 cells after reovirus infection. c-Maf is a protooncogene that plays an important role in the regulation of cell differentiation (50). It has been shown to transactivate the promoter of cyclin D2, thus promoting cell cycle progression. Over expression of c-Maf in myeloma cells causes increased cellular division and DNA synthesis by causing an increase in cyclin D2 levels within the cell (51). Reovirus infection caused a slight increase in the regulation of c-Maf in WI-38 cells by a ratio of 2.677, which suggests that this may be one method in which normal cells overcome viral infection and avoid a lytic infection.

Microtubule-associated protein 4 (MAP4) was shown to be up regulated in the WI-38 cells and is found to be involved in microtubule assembly. It is also known to interact with the cell cycle protein cyclin B. MAP4 serves as a cyclin B binding site causing it to translocate the active cyclin-dependent kinase/cyclin B complex to the mitotic spindle. Moreover, over expression of MAP4 has been shown to cause increased microtubule amounts in both interphase and mitosis, which suggests that it has the potential to regulate all phases of the cell cycle. Reovirus causes up regulation of

MAP4, subsequently allowing WI-38 cells to survive the viral infection by stabilizing microtubule assembly during the cell cycle (52).

Interferon regulatory factor 2 and 7 (IRF2, IRF7) are part of the interferon regulatory transcription factor (IRF) family and were found to be up regulated in the WI-38 cells after reovirus infection. IRFs are transcriptional activators that bind to the interferon-stimulated response element (ISRE) in the promoters of IFNs. IRF's are activated in response to viral infection and act as a molecular switch for antiviral activity (53). IRF7 demonstrates a role in viral-dependent signaling, whereas IRF2 is required for proper IFN-dependent gene expression. In addition, IRFs regulate ISRE-containing genes by use of the Jak-STAT pathway which stimulates type I or II IFN, resulting in the phosphorylation of STAT1 and STAT2. STAT1 was also shown to be highly up regulated in the WI-38 cells (54). Once phosphorylated, STAT family members form homo- or heterodimers and translocate to the nucleus where they act as transcriptional activators. STATs regulate the expression of a multitude of cellular genes responsible for cell viability in response to pathogens or different stimuli (55). The over expression of IRF2, IRF7, and STAT1 clearly suggests that reovirus infection induces the anti-viral mechanism within the WI-38 cells, thus preventing cellular destruction.

This study suggests that through various cellular targets, reovirus induces oncolysis of transformed cells either by undergoing apoptosis or inhibiting DNA synthesis. Reovirus was able to induce transcription of various pro-apoptotic genes and genes involved in the inhibition of cell cycle progression within the transformed cells. We have identified a multitude of potential pathways in which both apoptosis and inhibition of DNA synthesis may occur. In addition, potential cellular targets of normal

WI-38 cells have been identified that help to clarify possible methods in which normal cells are able to evade a lytic infection after being introduced to reovirus. This study, along with further analysis of the recognized genes should facilitate in determining the precise pathway in which reovirus kills transformed cells.

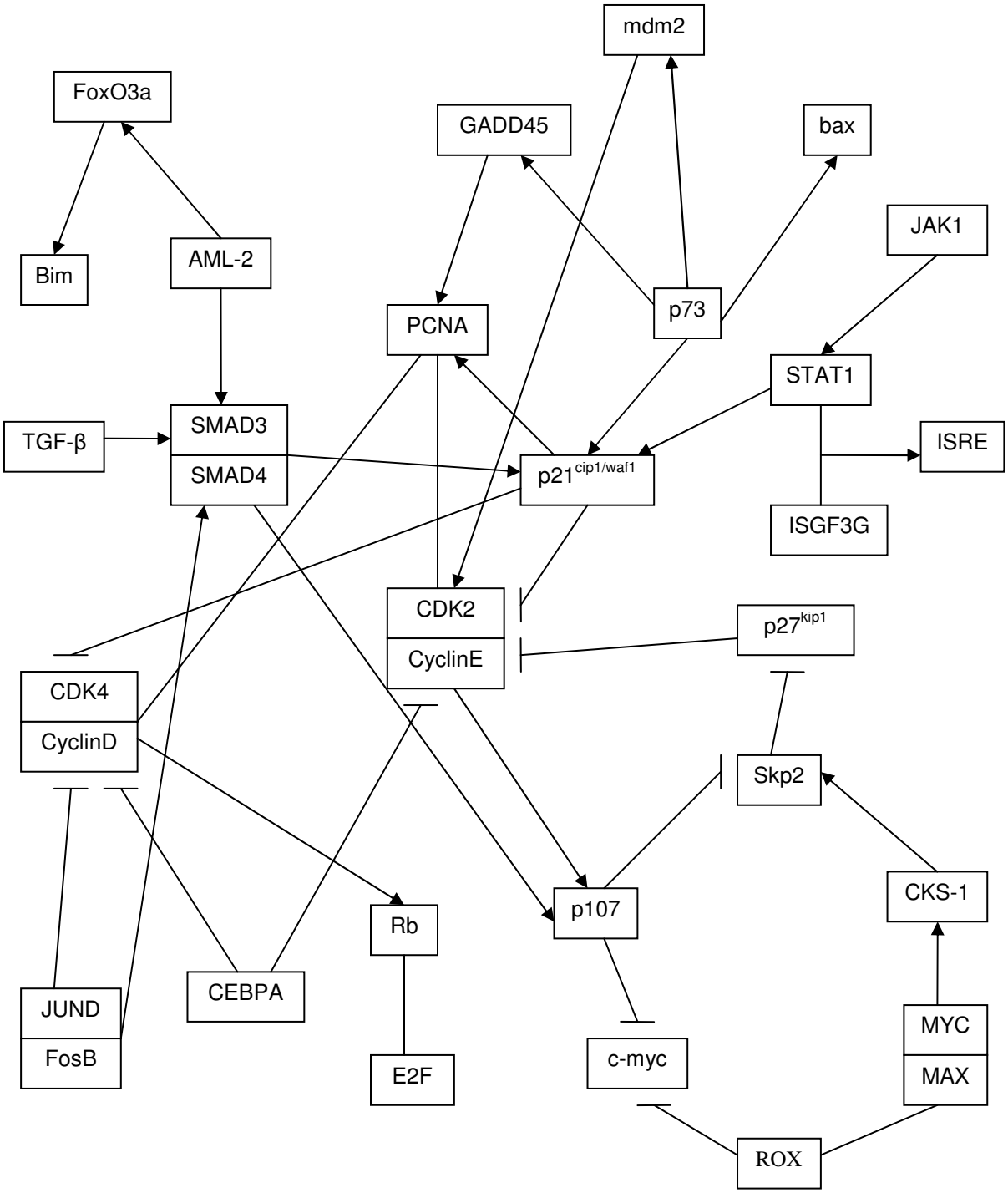

Figure 4.6 Proposed model for the interactions between WI-38 2RA cellular targets of reovirus

Table 4.1 Description of interactions between genes

AML-2 → FoxO3a	RUNX3 (AML2) physically interacts with the Forkhead transcription factor FoxO3a which induces apoptosis by activating Fas ligand expression and Fas-Fas L apoptosis pathway
AML-2 → SMAD3/SMAD4	AML-2 associates with SMADS to activate $p21^{cip1/waf1}$ promoter
FoxO3a → Bim	RUNX3-induced apoptosis depends on the expression of Bim, a proapoptotic BH3-only protein, and both RUNX3 and FoxO3a/FKHRL1 are required for induction of Bim expression
TP73 → bax	TP73 activates promoters of the p53-responsive gene bax which is a proapoptotic protein that induces cell death by acting on the mitochondria
TP73 → $p21^{cip1/waf1}$	TP73 activates promoters of the p53-responsive gene $p21^{cip1/waf1}$ which inhibits CDK's activities which prevents Rb phosphorylation
TP73 → GADD45	TP73 activates promoters of the p53-responsive gene GADD45 which stimulates DNA excision repair in vitro and inhibits entry of cells into S phase
GADD45 → PCNA	GADD45 binds to PCNA, or proliferating cell nuclear antigen, a normal component of cyclin-dependent kinase complexes and a protein involved in DNA replication and repair
$p21^{cip1/waf1}$ → CDK2/CyclinE	$p21^{cip1/waf1}$ inhibits CDK2 activity
$p21^{cip1/waf1}$ → CDK4/CyclinD	$p21^{cip1/waf1}$ inhibits CDK4 activity by preventing Rb phosphorylation
$p21^{cip1/waf1}$ → PCNA	$p21^{cip1/waf1}$ directly interacts with PCNA to block DNA synthesis by DNA polymerase δ
PCNA → CDK2/cyclinD	PCNA binds CDK2/cyclinD
PCNA → CDK4/cyclinE	PCNA binds CDK4/cyclinE
TGF-β → SMAD3/SMAD4	SMADS potentiate TGF-beta mediated cell death

Table 4.1 – continued

SMAD3/SMAD4 → $p21^{cip1/waf1}$	SMADS activate $p21^{cip1/waf1}$ promoter in the TGF-beta signaling for cell growth inhibition
SMAD3/SMAD4 → p107	p107 (RBL-1) is part of SMAD3 complex that binds SMAD4
JAK1 → STAT1	After type I IFN binding to cell surface of receptors, JAK1 is activated leading to tyrosine phosphorylation of STAT1
STAT1 → binds ISGF3G	Phosphorylated STAT1 dimerizes and associates with ISGF3G/IRF-9
STAT1/ISGF3G → ISRE	STAT1/ISGF3G dimmer forms a complex termed ISGF3 transcription factor that enters the nucleus and binds to ISRE, IFN stimulated response element, to activate the transcription of interferon stimulated genes, which drives the cell in an antiviral state
STAT1 → $p21^{cip1/waf1}$	STAT1 can promote apoptosis through upregulation of the apoptotic regulatory genes $p21^{cip1/waf1}$
CDK4/cyclinD → Rb	Cdk4-cyclin D can phosphorylate the retinoblastoma gene product Rb, which sequesters transcription factors, to allow the progression into S phase
Rb → E2F	Rb function depends in part on interactions with E2F family of DNA-binding transcription factors; E2F sites are found in the promoters of many genes that are important for cell cycle progression, and Rb appears to repress transcription of these genes through its interaction with E2F
CEBPA → CDK2/cyclinE	CEBPA interacts with the T loop region of CDK2 and competes for binding with cyclins to inhibit CDK2 by disruption or prevention of CDK2/cyclinE complexes by forming CEBPA/CDK2 complex

Table 4.1 – continued

CEBPA → CDK4/cyclinD	CEBPA interacts with the T loop region of CDK4 and inhibits CDK4-Rb pathway through regulation of CDK4
JUND → CDK4/cyclinD	Increase JUND levels causes a decrease in the cyclinD levels thereby decreasing the availability of cyclin D to complex with CDK4 inhibiting progression to S phase
JUND/FosB → SMAD3/SMAD4	JUND-FosB interacts with SMADS to potentiate TGF-beta mediated cell death
p107 → Skp2	p107 (RBL-1) down-regulates expression of F-box protein Skp2
Skp2 → $p27^{kip1}$	Skp2 causes accumulation of $p27^{kip1}$, which is a CDK inhibitor
$p27^{kip1}$ → CDK2	Accumulation of $p27^{kip1}$ leads to CDK2 inactivation and inhibits progression into S phase
p107 → c-myc	p107 (RBL-1) is part of SMAD3 complex that binds SMAD4 to repress myc
ROX → c-myc	ROX is known as a MYC-antagonist gene; levels of ROX and MYC are inversely proportional
ROX → MYC/MAX	ROX is a member of MYC and MAD family and binds DNA at E box by forming heterodimer with MAX
MYC/MAX → CKS-1	MAX encodes a protein that interacts specifically with MYC
CKS-1 → Skp2	CKS-1 associates with the F-box protein Skp2 and is essential for the recognition of $p27^{kip1}$ ubiquitination activity

REFERENCES

1. Nibert, M.L., Schiff, L.A. Reoviruses and Their Replication. In: Knipe, D.M., Howley, P.M., editors-in-chief. Griffin, D.E., Martin, M.A., Lamb, R.A., Roizman, B., Straus, S.E., associate editors. Fields Virology. 4th ed. Vol. 2. Philadelphia: Lippincott Williams & Wilkins. 2001; 1679-1728.

2. Tyler, K.L. Mammalian Reoviruses. In: Knipe, D.M., Howley, P.M., editors-in-chief. Griffin, D.E., Martin, M.A., Lamb, R.A., Roizman, B., Straus, S.E., associate editors. Fields Virology. 4th ed. Philadelphia: Lippincott Williams & Wilkins. 2001; Vol. 2: 1729-46.

3. Schmechel, S., Chute, M., Skinner, P, et al. Preferential translation of reovirus mRNA by a σ3-dependent mechanism. Virology 1997; 232: 62-73.

4. Sherry, B., Torres, J., Blum, M.A. Reovirus induction of and sensitivity to beta interferon in cardiac myocyte cultures correlate with induction of myocarditis and are determined by viral core proteins. J. Virol. 1998; 72: 1314-23.

5. Strong, J.E., Coffey, M.C., Tang, D., Sabinin, P., Lee, P.W. The molecular basis of viral oncolysis: usurpation of the Ras signaling pathway by reovirus. EMBO J. 1998; 17: 3351-62.

6. Hashiro, G., Loh, P.C., Yau, J.T. The preferential cytotoxicity of Reovirus for certain transformed cell lines. Arch. Virol. 1977; 54: 307-15.

7. Duncan, M.R., Stanish, S.M., Cox, D.C. Differential sensitivity of normal and transformed human cell to reovirus infection. J. Virol. 1978; 28: 444-49.

8. Shmulevitz, M., Marcato, P., Lee, P.W.K. Unshackling the links between reovirus oncolysis, Ras signaling, translational control and cancer. Oncogene. 2005; 24: 7720-28.

9. Norman, K.L., Hirasawa, K., Yang, A.D., Shields, M.A., Lee, P.W.K. Reovirus oncolysis: The Ras/RalGEF/p38 pathway dictates host cell permissiveness to reovirus infection. PNAS 2004; 101: 11099-104.

10. Yamamoto, T., Shinichiro, T., Kaibuchi, K. Ras-induced transformation and signaling pathway. J. Biochem. 1999; 126: 799-803.

11. Yamamura, Y., Lee, L.L., Inoue, K., Ida, H., Ito, Y. RUNX3 Cooperates with FoxO3a to Induce Apoptosis in Gastric Cancer Cells. J. Biol. Chem. 2006; 281(8): S267-76.

12. Torquati, A., O'Rear, L., Longobardi, L., Spagnoli, A., Richards, W.O., Beauchamp, R.D. RUNX3 inhibits cell proliferation and induces apoptosis by reinstating transforming growth factor beta responsiveness in esophageal adenocarcinoma cells. Surgery 2004; 136(2): 310-16.

13. Johnson, P.F. Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. J. Cell Sci. 2005; 118: 2545-55.

14. Wang, H., Iakova, P., Wilde, M., Welm, A., Goode, T., Roesler, W.J., Timchenko, N.A. C/EBPα Arrests Cell Proliferation through Direct Inhibition of Cdk2 and Cdk4. Mol. Cell 2001; 8: 817-28.

15. Harris, T.E., Albrecht, J.H., Nakanish, M., Darlington, G.J. CCAAT/Enhancer-binding Protein-α Cooperates with p21 to Inhibit Cyclin-dependent Kinase-2 Activity and Induces Growth Arrest Independent of DNA Binding. J. Biol. Chem. 2001; 276(31): 29200-9.

16. Evans, P.C., Smith, T.S., Lai, M., Williams, M.G., Burke, D.F., Heyninck, K., Kreike, M.M., Beyaert, R., Blundell, T.L., Kilshaw, P.J. A Novel Type of Deubiquitinating Enzyme. J. Biol. Chem. 2003; 278(25): 23180-6.

17. Kang, D., Chen, J., Wong, J., Fang, G. The checkpoint protein Chfr is a ligase that ubiquitinates Plk1 and inhibits Cdc2 at the G2 to M transition. J. Cell Biol. 2002; 156(2): 249-59.

18. Yu, X., Minter-Dykhouse, K., Malureanu, L., Zhao, W., Zhang, D., Merkle, C.J., Ward, I.M., Saya, H., Fang, G., van Deursen, J., Chen, J. Chfr is required for tumor suppression and Aurora A regulation. Nat. Genet. 2005; 37(4): 401-6.

19. Lei, W., Liu, F., Ness, S.A. Positive and negative regulation of c-Myb by cyclin D1, cyclin-dependent kinases, and p27 Kip1. Blood 2005; 105(10): 3855-61.

20. Online Mendelian Inheritance in Man, OMIM (TM). Johns Hopkins University, Baltimore, MD. MIM Number: 154950: 09/27/2007. World Wide Web URL: http://www.ncbi.nlm.nih.gov/omim/

21. Nair, S.H., Burley, S.K. X-Ray Structures of Myc-Max and Mad-Max Recognizing DNA: Molecular Bases of Regulation by Proto-Oncogenic Transcription Factors. Cell 2003; 112: 193-205.

22. Weitzman, J.B., Fiette, L., Matsuo, K., Yaniv, M. JunD protects cells from p53-dependent senescence and apoptosis. Mol. Cell 2000; 6(5): 1109-19.

23. Mechta-Grigoriou, F., Gerald, D., Yaniv, M. The mammalian Jun proteins: redundancy and specificity. Oncogene 2001; 20: 2378-89.

24. Castelli, J., Wood, K.A., Youle, R.J. The 2-5A system in viral infection and apoptosis. Biomed Pharmacother 1998; 52: 386-90.

25. Dong, B., Silverman, R.H. 2-5A-dependent RNase molecules dimerize during activation by 2-5A. J. Biol. Chem. 1995; 270: 4133-37.

26. Floyd-Smith, G., Slattery, E., Lengyel, P. Interferon action: RNA cleavage pattern of a (2'-5') oligoadenylate-dependent endonuclease. Science 1981; 212: 1030-32.

27. Iordanov, M.S., Paranjape, J.M., Zhou, A., Wong, J., Williams, B.R.G., Meurs, E.F., Silverman, R.H., Maguyn, B.E. Activation of p38 mitogen-activated protein kinase and c-Jun NH2-terminal kinase by double stranded RNA and encephalomyocarditis virus: involvement of RNase L, protein kinase R, and alternative pathways. Mol. Cell Biol. 2000; 20: 617-27.

28. Kerr, I.M., Brown, R.E. pppA2'p5'A2'p5'A: an inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells. Proc. Natl. Acad. Sci. USA 1978; 75: 256-60.

29. Pfarr, C.M., Mechta, F., Spyrou, G., Lallemand, D., Carillo, S., Yaniv, M. Mouse JunD negatively regulates fibroblast growth and antagonizes transformation by ras. Cell 1994; 76(4): 747-60.

30. Sasajima, H., Nakagawa, K., Yokosawa, H. Antiproliferative proteins of the BTG/Tob family are degraded by the ubiquitin-proteasome system. Eur. J. Biochem. 2002; 269: 3596-3604.

31. Buanne, P., Corrente, G., Michell, L., Palena, A., Lavia, P., Spadafora, C., Lakshmana, M.K., Rinaldi, A., Banfi, S., Quarto, M., Bulfone, A., Tirone, F. Cloning of PC3B, a Novel Member of the PC3/BTG/TOB Family of Growth Inhibitory Genes, Highly Expressed in the Olfactory Epithelium. Genomics 2000; 68: 253-63.

32. Rodier, G., Makris, C., Coulombe, P., Scime, A., Nakayama, K., Nakayama, K.I., Meloche, S. p107 inhibits G1 to S phase progression by down-regulating expression of the F-box protein Skp2. J. Cell Biol. 2005; 168(1): 55-66.

33. Spruck, C., Strohmaier, H., Watson, M., Smith, A.P.L., Ryan, A., Krek, W., Reed, S.I. A CDK-Independent Function of Mammalian Cks1: Targeting of SCF^{Skp2} to the CDK Inhibitor $p27^{Kip1}$. Mol. Cell 2001; 7: 639-50.

34. Chen, C., Kang, Y., Siegel, P.M., Massague, J. E2F4/5 and p107 as Smad CoFactors Linking the TGFβ Receptor to c-myc Repression. Cell 2002; 110: 19-32.

35. Meroni, G., Reymond, A., Alcalay, M., Borsani, G., Tanigami, A., Tonlorenzi, R., Lo Nigro, C., Messali, S., Zollo, M., Ledbetter, D.H., Brent, R., Ballabio, A., Carrozzo, R. Rox, a novel bHLHZip protein expressed in quiescent cells that heterodimerizes with Max, binds a non-canonical E box and acts as a transcriptional repressor. EMBO J. 1997; 16(10): 2892-2906.

36. Hurlin, P.J., Queva, C., Eisenman, R.N. Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediats repression at Myc binding sites. Gene. Dev. 1997; 11: 44-58.

37. Battle, T.E., Frank, D.A. The Role of STATs in Apoptosis. Curr. Mol. Med. 2002; 2: 381-92.

38. GeneCards: encyclopedia for genes, proteins and diseases. Weizmann Institute of Science. Bioinformatics Unit and Genome Center. Rehovot, Israel. GeneCard Number: GC02M191542: 08/21/2007. World Wide Web URL: http://www.genecards.org

39. Fontemaggi, G., Kela, I., Amariglio, N., Rechavi, G., Krishnamurthy, J., Strano, S., Sacchi, A., Givol, D., Blandino, G. Identification of Direct p73 Target Genes Combining DNA Microarray and Chromatin Immunoprecipitation Analyses. J. Biol. Chem. 2002; 277(45): 43359-68.

40. Levrero, M., De Laurenzi, V., Costanzo, A., Sabatini, S., Gong, J., Wang, J.Y.J., Melino, G. The p53/p63/p73 family of transcription factors: overlapping and distinct functions. J. Cell Sci. 2000; 113: 1661-70.

41. GeneCards: encyclopedia for genes, proteins and diseases. Weizmann Institute of Science. Bioinformatics Unit and Genome Center. Rehovot, Israel. GeneCard Number: GC19P054149: 08/21/2007. World Wide Web URL: http://www.genecards.org

42. Poggioli G.J., DeBiasi, R.L., Bickel, R., Jotte, R., Spalding, A., Johnson, G.L., Tyler, K.L. Reovirus-Induced Alterations in Gene Expression Related t Cell Cycle Regulation. J. Virol. 2002; 76(6): 2585-94.

43. GeneCards: encyclopedia for genes, proteins and diseases. Weizmann Institute of Science. Bioinformatics Unit and Genome Center. Rehovot, Israel. GeneCard Number: GC11M002861: 08/21/2007. World Wide Web URL: http://www.genecards.org
44. Kim, W., Kook, S., Kim, D.J., Teodorof, C., Song, W.K. The 31-kDa Caspase-generated Cleavage Product of $p130^{cas}$ Functions as a Transcriptional Repressor of E2A in Apoptotic Cells. J. Biol. Chem. 2004; 279(9): 8333-42.

45. Balciunaite, E., Spektor, A., Lents, N.H., Cam, H., te Riele, H., Scime, A., Rudnicki, M.A., Young, R., Dynlacht, B.D. Pocket Protein Complexes Are Recruited to Distinct Targets in Quiescent and Proliferating Cells. Mol. Cell. Biol. 2005; 25(18): 8166-78.

46. Kikuchi, H., Takami, Y., Nakayama, T. GCN5: a supervisor in all-inclusive control of vertebrate cell cycle progression through transcription regulation of various cell cycle-related genes. Gene. 2005; 347(1): 83-97.

47. Sax, J.K., El-Deiry, W.S. Identification and Characterization of the Cytoplasmic Protein TRAF4 as a p53-regulated Proapoptotic Gene. 2003; 278(38): 36435-44.

48. GeneCards: encyclopedia for genes, proteins and diseases. Weizmann Institute of Science. Bioinformatics Unit and Genome Center. Rehovot, Israel. GeneCard Number: GC11M000634: 08/30/2007. World Wide Web URL: http://www.genecards.org

49. Online Mendelian Inheritance in Man, OMIM (TM). Johns Hopkins University, Baltimore, MD. MIM Number: 190080: 09/07/2007. World Wide Web URL: http://www.ncbi.nlm.nih.gov/omim/

50. Online Mendelian Inheritance in Man, OMIM (TM). Johns Hopkins University, Baltimore, MD. MIM Number: 177075: 09/07/2007. World Wide Web URL: http://www.ncbi.nlm.nih.gov/omim/

51. Hurt, E.M., Wiestner, A., Rosenwald, A., Shaffer, A.L., Campo, E., Grogan, T., Bergsagel, P.L., Kuehl, W.M., Staudt, L.M. Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. Cancer Cell. 2004; 5: 191-99.

52. Holmfeldt, P., Brattsand, G., Gullberg, M. Interphase and monoastral-mitotic phenotypes of overexpressed MAP4 are modulated by free tubulin concentrations. J. Cell Sci. 2003; 116(18): 3701-11.

53. GeneCards: encyclopedia for genes, proteins and diseases. Weizmann Institute of Science. Bioinformatics Unit and Genome Center. Rehovot, Israel. GeneCard Number: GC11M000602: 08/30/2007. World Wide Web URL: http://www.genecards.org
54. Ning, S., Huye, L.E., Pagano, J.S. Regulation of the transcriptional activity of IRF7 promoter by a pathway independent of interferon signaling. J. Biol. Chem. 2005; 280(13): 12262-70.

55. Online Mendelian Inheritance in Man, OMIM (TM). Johns Hopkins University, Baltimore, MD. MIM Number: 600555: 09/07/2007. World Wide Web URL: http://www.ncbi.nlm.nih.gov/omim/

56. Williams, B.R. PKR: a sentinel kinase for cellular stress. Oncogene 1999; 18: 6112-20.

BIOGRAPHICAL INFORMATION

Jung Hwa Chung earned a Bachelor of Science in Biology from The University of Texas at Arlington in 2005, after which she began graduate school immediately following graduation. During graduate school she worked as a graduate teaching assistant under the guidance of Dr. Michelle Badon and was also the lead GTA for the microbiology labs for multiple semesters. She joined Dr. Michael Roner's Laboratory of Molecular Virology and Tumor Biology upon entrance into graduate school and began her thesis project in January 2006. Recently, in August 2007 she began her career as a Microbiologist at a pharmaceutical company where she utilizes skills obtained during her schooling at UTA.