Indian Journal of Biochemistry & Biophysics
Vol. 60, July 2023, pp. 525-544
DOI: 10.56042/ijbb.v60i7.1296

Studies on the molecular mechanisms involved in apoptosis triggered by Canine Distemper Virus (CDV)

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Received 09 May 2023; *revised 28 July 2023*

Apoptosis is a form of natural or stress induced cell death that plays a pivotal role in many cellular processes. Virus induced apoptosis is of significant importance since many viruses/viral proteins have been reported to induces apoptosis in different cell types. The present study was carried out to identify genes and pathways to explain the mechanisms involved in CDV induced apoptosis. For this, HCT-15 cells were infected with CDV-SH, a Snyder Hill strain of canine distemper virus, at different time points. Viability and apoptosis studies were performed using MTT and TUNEL assays, respectively. The qPCR arrays were custom designed to study the expression profile of apoptotic genes in HCT-15 cells after 6, 12, 24 and 48 h of CDV infection. Results showed viability of cells as 100%, 88.18%, 78.34% and 74.62% at 6, 12, 24 and 48 h after infection. Expression studies revealed increased expression of TNF-α, TRAIL, Calpain, IFN-β and RIG-1 genes and down-regulation of 21 genes in all the groups. Ingenuity Pathway Analysis (IPA) showed activation of apoptosis signaling pathway in CDV infected HCT-15 cells. Immuno-cytochemistry studies using antibodies against apoptotic protein (caspase-8, caspase-9, caspase-3, MAVS, IRF-3, cytochrome C, MKK7) showed only basal level of expression indicating no dysregulation in the expression of these genes which was in consistence with the qPCR array results. In qPCR & IPA analysis, although up-regulation of caspase 8, 9 and 3 was not observed, we hypothesized that CDV induced apoptosis in HCT-15 cell line might have occurred through caspase independent pathways (like Calpain or other molecule mediated).

Keywords: Apoptosis, Cancer cells, CDV, HCT-15 cell line, Infection, IPA

Apoptosis is a regulated form of cell death which occurs during physiological conditions. It plays a critical role in the homeostasis of multicellular organisms, and constitutes a common pathway for cell replacement, tissue remodeling, damaged cell removal and elimination of cancer cells. Cells undergoing apoptosis present typical morphological characteristics, including membrane blebbing, chromatin condensation, cell shrinkage and apoptotic body formation[1]. Apoptosis is triggered by sequential activation of caspases, a group of cysteine proteases, and proceeds primarily through two pathways. The extrinsic pathway involves activation of caspase-8 and is initiated by ligand interaction with death receptors, while the intrinsic pathway is activated by an imbalance between proapoptotic and anti-apoptotic proteins from Bcl-2 family in mitochondria[2]. Many viral proteins can influence the cellular pathways that control cell proliferation and apoptosis. Some viral proteins trigger apoptotic cell death, and this may be important in host defense and viral spread.

Canine distemper virus (CDV) is an enveloped virus with a single stranded RNA genome belonging to the family *Paramyxoviridae*. The genome encodes eight proteins, two of which (V and C) are non-structural proteins and are alternatively translated from the RNA[3]and six structural proteins (large protein, nucleoprotein, haemagglutinin protein, fusion protein and matrix protein). It has been observed that CDV infection induces apoptosis of cancer cells characterized by changes in cellular morphology and biochemical features, including DNA fragmentation, cytoplasm vacuolation, plasma membrane blebbing, and apoptotic body formation[4].

A recent study showed that a replication-incompetent hemagglutinating virus of Japan (HVJ, also known as Sendai virus) envelope (HVJ-E) induces the apoptotic demise of human cancer cell lines, including prostate cancer PC3 and DU145 cells, mammary carcinoma MDA-MB-231 cells and lung cancer A549 cells, but not of non-transformed cells such as prostate epithelial

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PNT1 and PNT2 cells as well as primary human fibroblasts[5]. The actual mechanism of apoptosis of cancer cells is not yet been properly studied in case of CDV infection. Some studies have shown that CDV induces apoptosis in Hela cells, triggering apoptosis by the intrinsic pathway[6]. Other authors have demonstrated that CDV activates the extrinsic pathway in infected Vero cells at 24 h post-infection (*p.i.*) with activation of caspase-8 and caspase-3 and gene expression of Fas death receptor[7]. There are few recent reports on the mechanism of apoptosis in CDV analogues like Measles virus (Human paramyxovirus) and Newcastle disease virus (Avian paramyxovirus). Nucleoprotein in Measles virus[8] and Matrix protein in Newcastle disease virus[9] are responsible for apoptosis.So far, there is no report in which a detailed molecular expression study has been carried out to explain CDV induced apoptosis in cancer cells. Therefore, the present study aimed at elucidating the detailed mechanism, gene(s)/protein(s) and pathways involved in CDV induced apoptosis in the cancer cells.

Materials and Methods

Culture of Cell lines and Infection with Canine Distemper Virus (CDV)

HCT-15 cells (cancerous) were procured from the National Centre for Cell Sciences (NCCS, Pune) and were grown in RPMI-1640 media (Himedia) supplemented with 10% FBS, 2X Antibiotic-Antimyotic solution (Himedia) kept at 37°C with 5% CO_2.HCT-15 cells at 60-70% confluence were infected with CDV (Snyder Hill strain) of passage-2 (ATCC, USA)at an MOI of 0.5.

After 7-8 days' post infection, the cells were visualized for any observable cytopathic effects and were harvested. The harvested culture was used for confirmation of CDV growth by Reverse Transcriptase-PCR using diagnostic primers designed in the lab against the L-gene of CDV.

Detection of apoptosis

Cell viability assessment

The cell viability/survival percentage assay was performed using MTT assay. HCT-15 cells were grown in 96-well cell culture platesin 5 replicates and were infected with CDV-SH (MOI = 0.5) at specific hourly intervals (6, 12, 24 & 48 h) along with non-infected controls and plain media as blank. After the specific time interval of CDV infection (*i.e.*, 6, 12, 24 & 48 h), 20 µL of yellow MTT (5 mg/ml in PBS) was added to each well including control and blank, wrapped in aluminium foil and kept in incubator at 37°C for 4 h. After 4 h, media with MTT was removed from the cells; 200 µL of acidic isopropanol (containing 0.04N HCl) was added to wells including controls and pipetted up and down to mix the purple formazoncyrstals. The plate was kept at 37°C for 10 min. The absorbance was measured at 540 nm wavelength using 630 nm as the reference wavelength in BioTek microplate reader.

DNA Fragmentation Assay (DNA laddering)

HCT-15 cells were grown in 6-well cell culture plates and upon reaching 60-70% confluence, the cells were infected with CDV-SH (p-2, MOI = 0.5)at different time intervals (6, 12, 24 & 48 h) keeping one well as non-infected control. After the specific time interval, the cells were harvested by pipetting and then centrifuged at 1500 rpm for 10 min ina swinging bucket rotor at room temperature. The cells were washed twice with 1x PBS and cell lysis buffer(IM Tris-HCl, 500 mM EDTA, 10% SDS, 5 M NaCl) along with Proteinase K was added and incubated overnight at 56°C. 500 µL of Phenol: Chloroform:Isoamyl alcohol (PCI) was added and mixed properly followedby centrifugation at 10000 rpm for 10 min at 4°C. The aqueous phase was collected in a fresh microcentrifuge tube and 500 µL of chloroform was added. The suspension was again centrifuged at 10000 rpm for 10 min at 4°C. DNA was precipitated with 3 M sodium acetate along with 500 µL of Isopropanol by keeping overnight at –20°C and then centrifuged at 10000 rpm for 10 min at 4°C. DNA pellets obtained were washed with 70% ethanol and air-dried and dissolved in 50 µL of TE buffer (1 M Tris-HCL, 0.5 M EDTA). The DNA was run on a 1.5% agarose gel at 50V for 2-3 h. DNA ladders were visualized under UV trans illuminator and photographed in a Gel documentation Unit (Biorad).

TUNEL Assay

Terminal deoxynucleotidyl transferase-mediated (Tdt) nick end labelling (TUNEL) assay was performed using Click-iT® TUNEL Alexa Fluor® Imaging Assay (C10245, Invitrogen, USA). HCT-15 cells grown in 5 wells of 8-well cell culture chamber slides and infected with CDV-SH (MOI = 0.5) at different time intervals (6, 12, 24 & 48 h) were fixed using 4% paraformaldehyde in PBS for 15 min. Permeabilization was done using 0.5% Triton X-100 for 4-5 min, after which the cells were washed twice with PBS for 2-3 min. Next, 100 µL of Tdt reaction buffer was added and the cells were incubated for

10 min. Tdt reaction cocktail (94 µL of Tdt reaction buffer, 2 µL EdUTP, 4 µL of Tdt) was added to the cells and incubated for 60 min at 37°C or can be left overnight. Washing was done twice with 3% BSA in PBS for 2 min. Next, 100 µL of Click-iT® reaction cocktail (97.5 µL of Click-iT® reaction buffer, 2.5 µL of Click-iT® reaction buffer additive) was addedto the chamber slides and the cells were incubated for 30 min at room temperature. The reaction was protected from light by wrapping the chamber with aluminium foil. The Click-iT® reaction cocktail was removed and the cells were washed with 3% BSA in 1X PBS for 5 minutes. After washing, the DNA of the cells were stained with 100 µL of 1X Hoechst 33342 (1: 5,000 dilution) and were incubated for 30 min at room temperature. The reaction was protected from light by wrapping the chamber slide with aluminium foil. The Hoechst 33342 solution was removed and the cells were washed twice with PBS. Hoechst 33342 bound to DNA of the stained cells were observed at wavelength - $350_{Ex}/460_{Em}$ under a fluorescent inverted microscope (Nikon Eclipse Ti Series).

Real time RT-PCR Array

For studying expression of apoptotic genes up-regulated or down-regulated in HCT-15 cells upon CDV infection, a qPCR array (Qiagen) [48 × 2 format] was designed with the list of cellular genes (43 in numbers plus 5 controls = 48 in total) (Table 1). Roughly HCT-15 cells were grown in duplicate wells of five 6-well culture plates and upon reaching 70-80% confluence, the growth media was removed and a gentle washing was given using RPMI-1640 maintenance media. The cells in duplicate wells in each plate were infected using CDV-SH (p-2, 100 µL, MOI = 0.5) for the different time intervals (6, 12, 24 & 48 h) while one plate was kept as non-infected controls. RNA was isolated from the harvested cultures after appropriate time intervals using RNeasy Mini kit (Qiagen). RNA quality was checked and quantified using Nanodrop (Thermo Scientific). RNA corresponding to 1 µg (for each sample) was used for the synthesis of cDNA using RT^2 First Strand Kit (Qiagen) by following the manufacturer's protocol. Real time PCR was carried out according to manufacturer's recommendations and following the MIQE guidelines in a total volume of 25 µL dispensed into each well of the 96-well array format (1350 µL of 2x RT^2 SYBR Green Mastermix, 102 µL of cDNA synthesis reaction and 1248 µL of RNase-free water). Reactions were run on a Step One Plus (Applied Biosystems, ABI) using standard thermal cycling parameters (95°C for 10 min, 40 cycles of 15s at 95°C, 1 min at 60°C to perform fluorescence data collection and continued melting curve analysis). The results were exported using the ABI software and the results in the form of Ct values were organized in Microsoft Excel for further analysis.

Table 1 — List of genes for apoptotic study in HCT-15 cells following CDV infection

96-Well Custom PCR Array Template

S. No	Gene Symbol	Gene Ref Seq #
1	Fas	NM_000043
2	FasLG	NM_000639
3	FADD	NM_003824
4	Bid	NM_001196
5	Bax	NM_004324
6	Bak1	NM_001188
7	Bad	NM_004322
8	Bcl-2	NM_000633
9	Cytochrome C (CYC1)	NM_018947
10	APAF1	NM_001160
11	PARP1	NM_001618
12	MAVS	NM_020746
13	Caspase-8 (CASP8)	NM_001228
14	Caspase-9 (CASP9)	NM_001229
15	Caspase-3 (CASP3)	NM_004346
16	JNK1 (MAPK8)	NM_002750
17	JNK2 (MAPK9)	NM_002752
18	MKK7 (MAP2K7)	NM_145185
19	PI3k (PIK3CA)	NM_006218
20	mTOR	NM_004958
21	IFN-α (IFN-α1)	NM_024013
22	IFN-β (IFN-β1)	NM_002176
23	RIG-1 (RARRES3)	NM_004585
24	IRF-3	NM_001571
25	PTEN	NM_000314
26	AKT1	NM_005163
27	TRAIL (TNF-SF10)	NM_003810
28	Smac (DIABLO)	NM_019887
29	TNF-α	NM_000594
30	VDAC1	NM_003374
31	TRAF2	NM_021138
32	Noxa (PMAIP1)	NM_021127
33	Puma (BBC3)	NM_014417
34	Calnexin (CANX)	NM_001746
35	Calreticulin (CALR)	NM_004343
36	CHOP (DDIT3)	NM_004083
37	Calpain (CAPNS2)	NM_032330
38	ATF6	NM_007348
39	PERK (EIF2AK3)	NM_004836
40	IRE1 (ERN1)	NM_001433
41	p53	NM_000546
42	CAD (DFFB)	NM_004402
43	Foxo3a	NM_001455
44	GAPDH	NM_002046
45	ACTB	NM_001101
46	HGDC	
47	RTC	
48	PPC	

Results and Discussion

Culture of HCT-15 cells and Infection with CDV

Laboratory available HCT-15 (Human adenocarcinoma cell line) was selected and grown in 25cc flasks in presence of RPMI-1640 growth media supplemented with 10% FBS. At 70% confluence, the HCT-15 cells were infected with 100 µL of CDV-SH, observed for 7 days, harvested, freeze thawed 2 times and subjected to 2 more subsequent passages. CDV induced morphological changes found in HCT-15 cell line was in the form of cell rounding, detachment and degeneration but no characteristic CPE like syncitia formation was observed (Fig. 1A & B). RT-PCR was carried out from the total RNA extracted from the harvested CDV-SH passages in HCT-15 cells. Diagnostic RT-PCR could detect an amplicon size of 267 bp confirming CDV growth (Fig. 2).

MTT assay

MTT assay was carried out in HCT-15 cells at 6, 12, 24, 48 h post CDV-SH infection. HCT-15 cells showed cell viability of 127%, 112%, 99.5% & 94.78% in MTT assay at 6, 12, 24 and 48 h post CDV infection, respectively (converted into a ratio below 100% in the figure)(Fig. 3). It was clearly observed that with the increase in time period the damaging effect of CDV was also seen increasing in cells (HCT-15 tumour cells).Liao *et al* reported decline of ovarian cancer cells survival following infection with live-attenuated measles vaccine virus in both time and dose dependent manner[10]. The Measles virus vaccine resulted in 2.7%, 4.3% and 17.6% cell death at 12, 24 and 48 h post infection respectively in SKOV-3 cells by MTT assay[11]. Even in case of Newcastle disease virus, 50% of cell death was observed in tumour cell line after 72 h of viral treatment by MTT assay[10].

Lanes	Samples
Lane 1	100bp plus marker
Lane 2	Passage 1
Lane 3	Passage 2
Lane 4	+ve control
Lane 5	NTC

Fig. 2 — RT-PCR detection of CDV growth in HCT-15 cell lines at subsequent passages

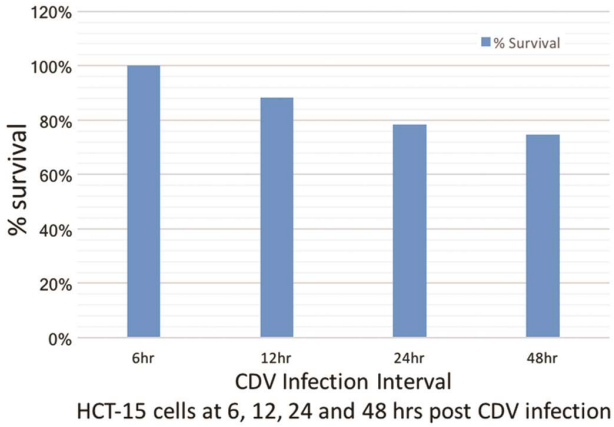


Fig. 3 — MTT assay for detection of cell viability in HCT-15 cell viability following CDV infection (6, 12, 24 and 48 h post infection)

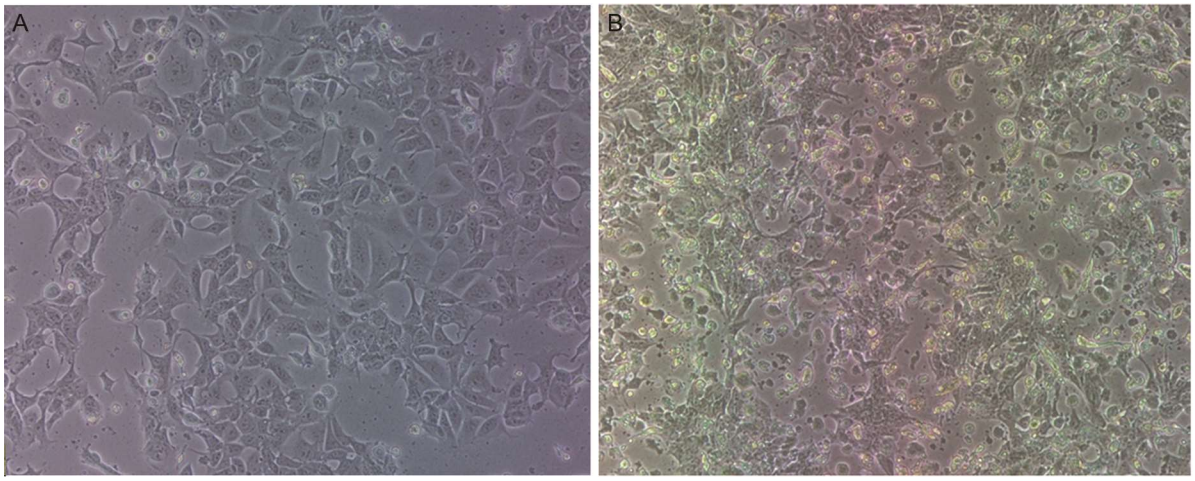


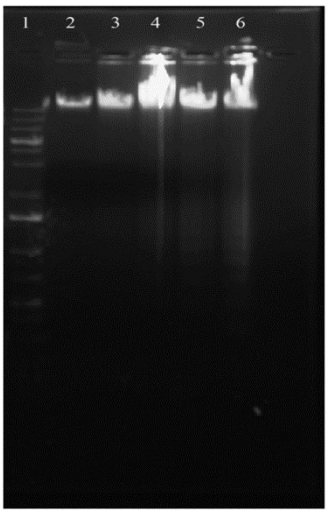
Fig. 1 — (A) HCT-15 cells in culture post 24 h post CDV infection; and (B) CDV infected HCT-15 cells at 7 days' post infection

DNA Laddering assay

DNA isolated from CDV infected HCT-15 cells at 6, 12, 24 and 48 h post infection were electrophoresed in agarose gel at low voltage and a very faint laddering pattern of DNA was observed. This result was indicative that CDV could induce minimal DNA fragmentation following 12, 24 and 48 h post infection in HCT-15 cells demonstrating its apoptotic potentials (Fig. 4). Kerr *et al* explained earlier that the nucleosomal DNA fragmentation takes place which is considered to be the hallmark of apoptosis[4]. A significant increase in DNA fragmentation was observed which formed a ladder like pattern of DNA fragments in agarose gel consisting of multiples of ~150-200 bp in CDV infected HeLa cells after 24 h of post infection[10]. Similarly, Zhao *et al* (2013) showed DNA laddering that revealed the consistent internucleosomal DNA fragmentation in case of Measles virus infected lung cancer cells[12]. Ali *et al* (2011) analyzed the DNA isolated by agarose gel electrophoresis from the brain tumor cells which were treated with Newcastle disease virus strain for 24, 48 and 72 h and observed DNA fragments of lower molecular weight that consists of multimers of 180-200 bp, the characteristic pattern of nucleosomal laddering specific to the mechanism of apoptosis[13].

Detection of Apoptosis induced by CDV by TUNEL Assay

In TUNEL assay, identification of DNA break sites is achieved through emission of green fluorescence due to the presence of Alexa Fluor® 488 dye labelled anti-BrdU antibody. HCT-15 cells infected with CDV at 24 and 48 h emitted green fluorescence in FITC filter and fragmented nuclei of the cells were clearly visible in DAPI filter (Fig. 5A-E). The emission of green fluorescence in CDV infected HCT-15 cells and nuclear fragmentation clearly indicates the occurrence of apoptosis. Phuong *et al* (2003) observed a significant increase in the number of apoptotic cells by the presence of green fluorescent nuclei after TUNEL assay of glioma cell lines infected with Measles virus at 48 h[14]. Zhao *et al* (2013) observed apoptotic cells within tumorous lung tissues when evaluated by TUNEL assays, showed an apparent increase in the number of glowing apoptotic cells when treated with higher multiplicity of infection (MOI) of the Measles virus[12].



Lanes	Samples
Lane 1	100bp plus marker
Lane 2	Control cells
Lane 3	6hr PI
Lane 4	12hr PI
Lane 5	24hr PI
Lane 6	48hr PI

Fig. 4 — Agarose gel electrophoresis detection of DNA laddering pattern in HCT-15 cells following CDV infection at 6 h, 12 h, 24 h & 48 h post infection

qPCR array for detection of apoptotic gene expression in HCT-15 cells following CDV infection

A panel of 48 genes associated with different apoptotic pathways listed in (Table 1), were selected to study their expression profile following CDV infection in HCT-15 cells. Briefly, we assayed the expression of genes from the extrinsic pathway (5), genes related to the intrinsic pathway (10), genes from the MAVS-MKK7-JNK2 pathway (4), genes from RIG-1/MAVS pathway (4), execution pathway genes (3), genes from ER stress induced apoptosis pathway genes (4) and genes from PTEN induced pathway (2). The list of selected genes was sent to Qiagen for custom designing of the qPCR arrays. For apoptotic gene expression studies, HCT-15 cells infected with CDV-SH were harvested at different time intervals (6), 12, 24 & 48 h) (Fig. 6A-E). RNA was extracted and cDNA was prepared. A diagnostic RT-PCR was first carried out to detect CDV growth in the harvested samples which showed a 267bp amplicon in agarose gel electrophoresis confirming the virus growth (Fig. 7). Then the cDNAs synthesized from different samples were used to perform the qPCR array to study the apoptotic gene expression profile. The qPCR was performed in duplicates for each target gene and the mean Ct values obtained were recorded for analysis of the test samples (infection intervals) against the control sample (non-infected cells). The hourly infection intervals (6, 12, 24 & 48 h) were named into groups (group 1, 2, 3 & 4), respectively. The qPCR data was checked for quality control to check the PCR array reproducibility which shows that if the average PPC (PCR positive control) Ct is 20±2

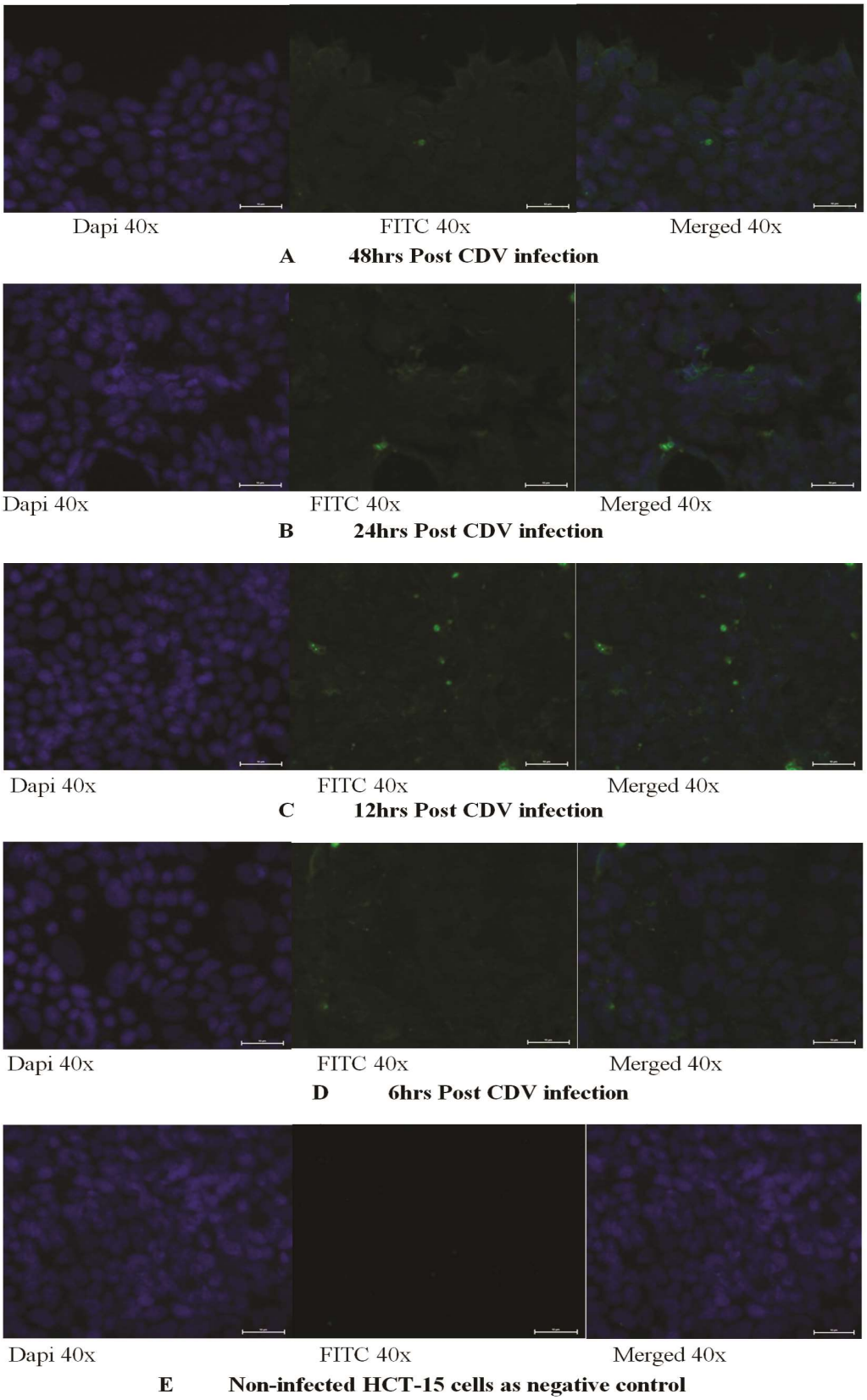


Fig. 5 — Detection of Apoptosis in HCT-15 cells by TUNEL assay

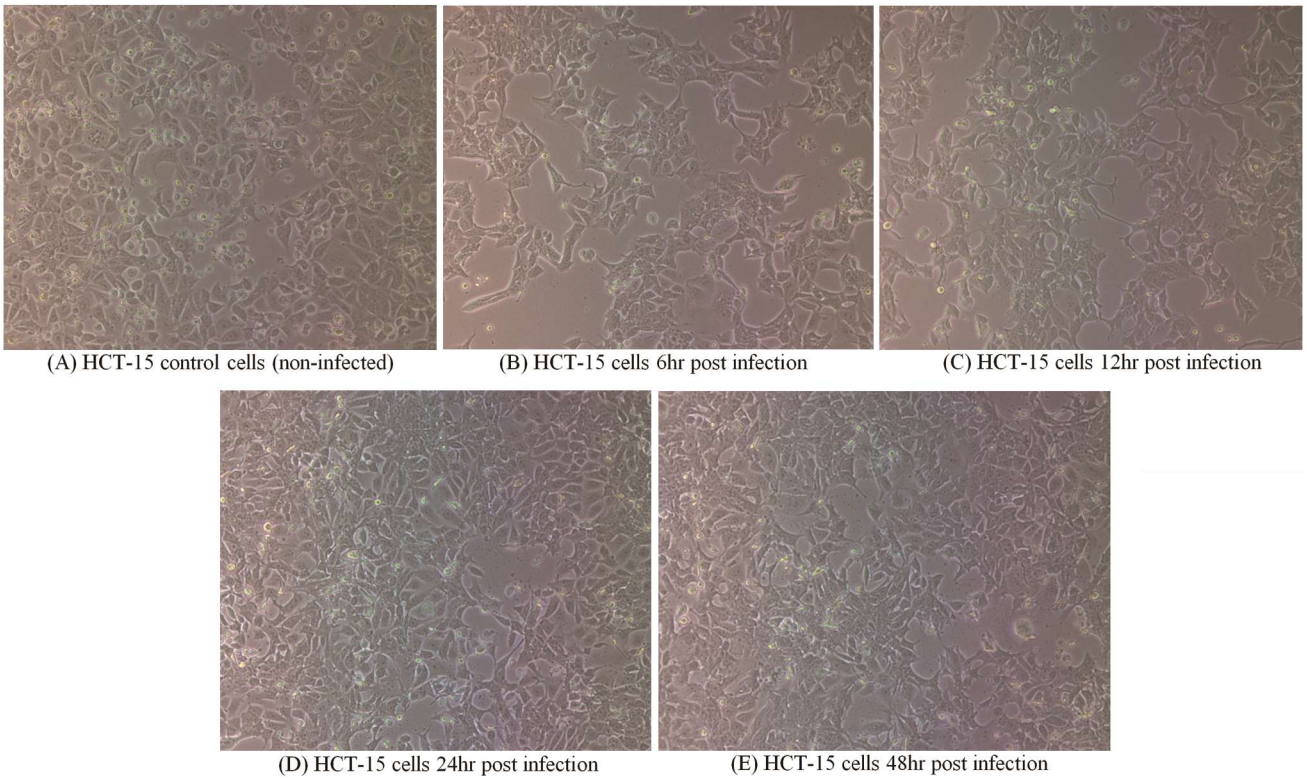
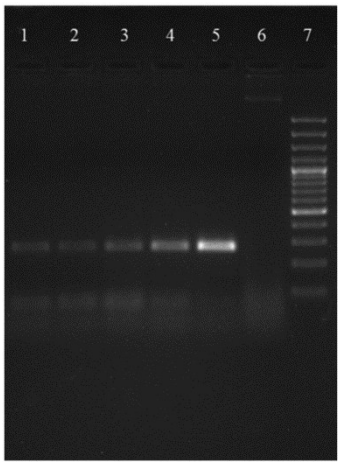


Fig. 6 — Morphological changes in HCT-15 cells following 6, 12, 24, 48 h post CDV-SH infection



Lanes	Samples
Lane 1	6hr PI
Lane 2	12hr PI
Lane 3	24hr PI
Lane 4	48hr PI
Lane 5	+ve control
Lane 6	NTC
Lane 7	100bp plus marker

Fig. 7 — RT-PCR detection of CDV-SH growth in HCT-15 cells at 6, 12, 24, 48 h post infection

and no two arrays have average PPC Ct that are > 2 away from one another then the sample and group pass. CT values were exported to an Excel file to create a table of CT values. This table was then uploaded on to the data analysis web portal at http://www.qiagen.com/geneglobe. Samples were assigned to controls and test groups. CT values were normalized based on a/an Manual Selection of reference genes.

The p values were calculated based on a Student's t-test of the replicate 2^ (- Delta CT) values for each gene in the control group and treatment groups. The fold change (cut off set to 2) was calculated for the up-regulated and down-regulated genes against the control (non-infected sample). The gene expression data was used to generate scatter plots of each group as compared to the control group (non-infected group). The scatter plot compares the normalized expression of every gene on the array between the two selected groups by plotting them against one another to quickly visualize large gene expression changes. The central line indicates unchanged gene expression. The dotted lines indicate the selected fold regulation threshold. Data points beyond the dotted lines in the upper left and lower right sections meet the selected fold regulation threshold (Fig. 8A-D).

Expression profile

In the group 1 (6 h PI), Calpain & TNF-α were over-expressed and 8 genes were under-expressed (TRAF2, RIG-1, Puma, TRAIL, CHOP, IRE1, Calnexin and APAF-1). Similarly, in group 2 (12 h PI), 3 genes (TNF-α, IFN-β & Calpain) were over-expressed and 8 genes (RIG-1, Puma, PERK, Bax, Caspase-8, CHOP, Bcl-2 and APAF-1) were under-

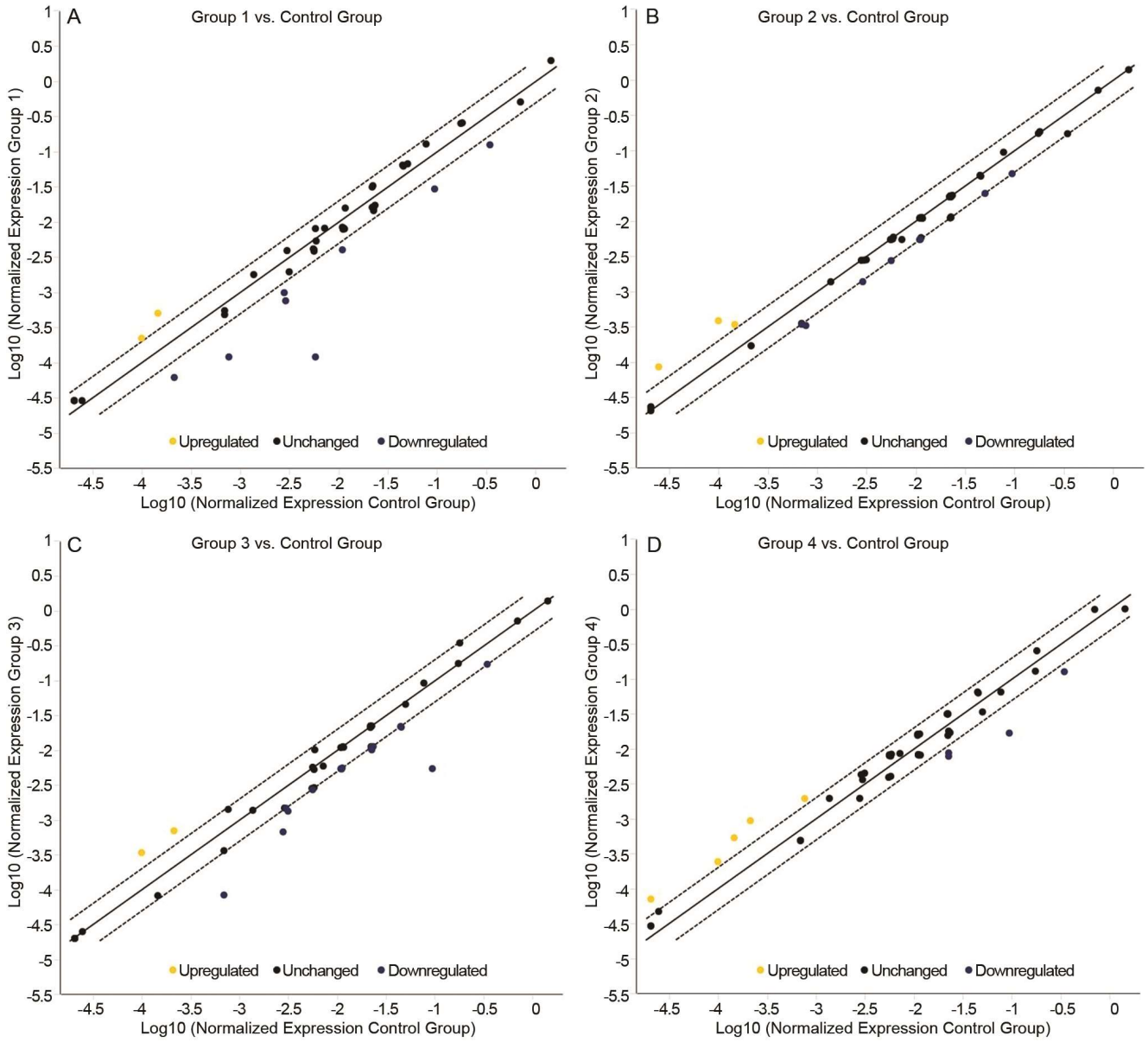


Fig. 8 — Scatter plot for expression profile of apoptotic genes in (A) Group 1; (B) Group 2; (C) Group 3; and (D) Group 4 as compared to non-infected controls of CDV infected HCT-15 cells

expressed. In group 3 (24 h PI), 2 genes (TNF-α & TRAIL) were over-expressed and 17 genes (CHOP, Bcl-2, IRE1, MAVS, Fas, IRF-3, PERK, PTEN, Noxa, JNK2, Caspase-8, ATF6, Bak1, Caspase-3, APAF-1 & Calnexin) were under-expressed. In group 4 (48 h PI), 5 genes (TRAIL, TNF-α, IFN-α, RIG-1 & Calpain) were over-expressed and 4 genes (CHOP, Caspase-3, Calnexin & Fas) were under-expressed. The fold change expression values for all the genes in the study have been indicated in (Table 2).The detail of each over-expressed genein CDV infected HCT-15 cells has been presented in (Table 3), while the under-expressed genes have been presented in (Table 4). The gene expression data was used to generate a heat map with cluster analysis of CDV infected HCT-15 cells. The cluster-gram in (Fig. 9) shows the qPCR array gene expression profile of CDV infected HCT-15 cells which shows group clustering analysis of differentially expressed genes identified in control versus CDV infected HCT-15 cells at time specific time intervals (6, 12, 24 & 48 h).

Table 2 — Fold change expression values for the genes involved in the study (*indicating positive fold change)

S. No.	Gene Symbol	Fold change			
		Group 1 (6 h PI)	Group 2 (12 h PI)	Group 3 (24 h PI)	Group 4 (48 h PI)
1	Fas	0.6468	0.5002	0.4529	0.3915
2	FasLG	1.405	1.108	0.9763	1.4251
3	FADD	0.9151	1.0018	1.7331	1.4297
4	Bid	1.3506	0.495	0.9197	0.6799
5	Bax	0.737	1.0067	1.0213	1.4509
6	Bak1	1.3165	0.9362	0.495	1.2164
7	Bad	0.7211	0.9782	1.0232	1.4305
8	Bcl-2	0.6985	0.4975	0.1216	0.7064
9	Cytochrome C (CYC1)	0.7923	0.5104	0.5252	0.7077
10	APAF1	0.368	0.4993	0.4979	1.4882
11	PARP1	1.3559	0.9386	0.9506	0.702
12	MAVS	0.6211	0.895	0.4251	1.4277
13	Caspase-8 (CASP8)	0.715	0.4956	0.493	0.7405
14	Caspase-9 (CASP9)	0.7562	0.9986	0.5188	0.7161
15	Caspase-3 (CASP3)	0.713	0.5049	0.4968	0.3472
16	JNK1 (MAPK8)	1.4813	1.0214	0.5006	0.7054
17	JNK2 (MAPK9)	0.731	0.5008	0.4889	1.4273
18	MKK7 (MAP2K7)	1.317	1.0103	1.0169	1.4392
19	PI3k (PIK3CA)	1.1449	0.7669	0.8329	1.2059
20	mTOR	1.435	1.0046	0.9702	1.4393
21	IFN-α (IFN-α1)	1.405	1.1323	0.9763	*3.4533
22	IFN-β (IFN-β1)	1.1636	*3.4483	1.0102	1.8979
23	RIG-1 (RARRES3)	0.1587	0.4335	1.8746	*2.5699
24	IRF-3	1.3781	0.9586	0.4755	1.4015
25	PTEN	0.737	0.9845	0.483	0.7278
26	AKT1	0.7004	0.9813	0.9863	1.4311
27	TRAIL (TNF-SF10)	0.2907	0.804	*3.3086	*4.4295
28	Smac (DIABLO)	0.7739	1.0058	1.0072	1.4374
29	TNF-α	*2.2431	*3.8909	*3.4285	*2.4509
30	VDAC1	1.445	0.9998	1.0096	0.741
31	TRAF2	0.0207	0.957	0.5023	1.3636
32	Noxa (PMAIP1)	0.7322	1.0074	0.487	0.7533
33	Puma (BBC3)	0.2614	0.474	0.5109	1.4803
34	Calnexin (CANX)	0.3629	0.5019	0.4989	0.3694
35	Calreticulin (CALR)	1.4438	1.0395	1.9507	1.426
36	CHOP (DDIT3)	0.3125	0.4959	0.0577	0.1785
37	Calpain (CAPNS2)	*3.4788	*2.3468	0.566	*3.6669
38	ATF6	0.7187	0.5125	0.4941	0.7273
39	PERK (EIF2AK3)	0.6827	0.4859	0.482	1.4623
40	IRE1 (ERN1)	0.3527	0.99	0.2409	0.6989
41	p53	0.6579	0.9686	0.9841	0.794
42	CAD (DFFB)	1.396	0.9811	0.9192	0.7019
43	Foxo3a	0.7225	1.0197	0.5084	1.4337
44	GAPDH	0.7242	1.0189	1.0247	1.414
45	ACTB	1.3808	0.9815	0.9758	0.7072
46	HGDC	1.405	0.994	0.9763	1.4251
47	RTC	1.4543	1.0075	0.4969	1.4789
48	PPC	1.6837	1.2358	1.2078	0.8542

Table 3 — Genes over-expressed in CDV infected HCT-15 cells compared with non-infected control cells as identified by qPCR array with fold change values

S. No.	Group 1 (6 h)	Group 2 (12 h)	Group 3 (24 h)	Group 4 (48 h)
1	TNF-α	TNF-α	TNF-α	TNF-α
2	Calpain (CAPNS2)	Calpain (CAPNS2)	TRAIL (TNF-SF10)	Calpain (CAPNS2)
3		IFN-β (IFN-β1)		IFN-α (IFN-1)
4				RIG-1 (RARRES3)
5				TRAIL (TNF-SF10)

Table 4 — Genes underexpressed in CDV infected HCT-15 cells compared with non-infected control cells as identified by qPCR array

S. No.	Group 1 (6 h)	Group 2 (12 h)	Group 3 (24 h)	Group 4 (48 h)
1	TRAF2	RIG-1 (RARRES3)	CHOP (DDIT3)	CHOP (DDIT3)
2	RIG-1 (RARRES3)	Puma (BBC3	Bcl-2	Caspase-3 (CASP3)
3	Puma (BBC3)	PERK (EIF2AK3)	IRE1 (ERN1)	Calnexin (CANX)
4	TRAIL (TNF-SF10)	Bid	MAVS	Fas
5	CHOP (DDIT3)	Caspase-8 (CASP8)	Fas	
6	IRE1 (ERN1)	CHOP (DDIT3)	IRF-3	
7	Calnexin (CANX)	Bcl-2	PERK (EIF2AK3)	
8	APAF1	APAF1	PTEN	
9			Noxa (PMAIP1)	
10			JNK2 (MAPK9)	
11			Caspase-8 (CASP8)	
12			ATF6	
13			Bak1	
14			Caspase-3 (CASP3)	
15			APAF1	
16			Calnexin (CANX)	

Expression Analysis of Over-expressed genes in CDV infected HCT-15 cells

In this study, qPCR expression studies showed that from the list of over-expressed genes, TNF-α was found to be up-regulated in all the groups (6, 12, 24 & 48 h). TNF-α is a member of TNF ligand and receptor family that are involved in immune regulation. Various members of the TNF super-family are able to mediate survival, proliferation and apoptosis. TNF-α is able to induce apoptotic cell death by binding to receptor TNF-R1 and TNF-R2 forming trimers which causes a conformational change to occur enabling them to bind with adapter protein TRADD. TRADD further binds to FADD, recruiting the protease caspase-8, which further leads to the activation of executioner caspases, finally leading to cell death. TNF family can also activate the MAPK pathways which induce a strong activation of stress-related JNK group of kinases. The JNK pathway is involved in cell proliferation, differentiation and is mostly pro-apoptotic. Next, in the study, the expression of TNF-related apoptosis-inducing ligand (TRAIL) was observed to be over-expressed in group 3 (24 h) & group 4 (48 h). TRAIL, a member of the tumor necrosis factor (TNF) family functions as a ligand which induces cell death and apoptosis through engagement of its death receptors. TRAIL is known to induce apoptosis in a number of tumor cells which has received great interest of its use in anti-cancer therapy[15]. TRAIL induces apoptosis by binding to its receptors, DR4 and DR5 resulting in their trimerization and leading to the formation of the death inducing signaling complex (DISC), which further leads to the recruitment of FADD. This leads to the activation of caspase-8, which then cleaves caspase-3 and downstream death substrates leading to cell death. The death-receptor mediated apoptosis is an important mechanism by which certain viruses can induce cell death, leading to progeny dissemination, which also plays a role in viral pathogenesis and targeting therapeutic potential. up-regulation of death receptors or their ligands is an important process by which viruses regulate the death receptor mediated apoptosis. Most viruses encode proteins that regulate death receptor mediated apoptosis for *e.g*. Newcastle disease virus (NDV) infection causes an up-regulation of TNF-α & TRAIL which induces the extrinsic apoptosis[16]. Reovirus infection makes different types of cancer cell lines prone to TRAIL-mediated apoptosis in a caspase-8 dependent manner[17,18]. Sindbis virus infection induces TNF-α-mediated apoptosis in PC-12 cells[19]. It has been investigated that since human TRAIL promoters contain interferon regulatory elements and is activated by interferons, TRAIL is the earliest gene induced by interferons.

Further, our study showed up-regulation of IFN-β in group 2 (12 h) whereas an up-regulation of IFN-α was observed in group 4 (48 h). IFNs are cytokines that communicate between cells which help to trigger protective defenses that aid in eradicating pathogens. Interferons specifically have the ability to interfere with viral replication, thus protecting the cells from virus infection. It has been observed that many immune cells increase TRAIL expression by proinflammatory cytokines such as interferons that are mainly produced during viral infection. For *e.g*. IFN-α/β induced modulation of the TRAIL system enhances the NK cell-mediated apoptotic killing of murine cells infected with encephalomyocarditis virus[20]. Zeng *et al* (2002) observed that New Castle

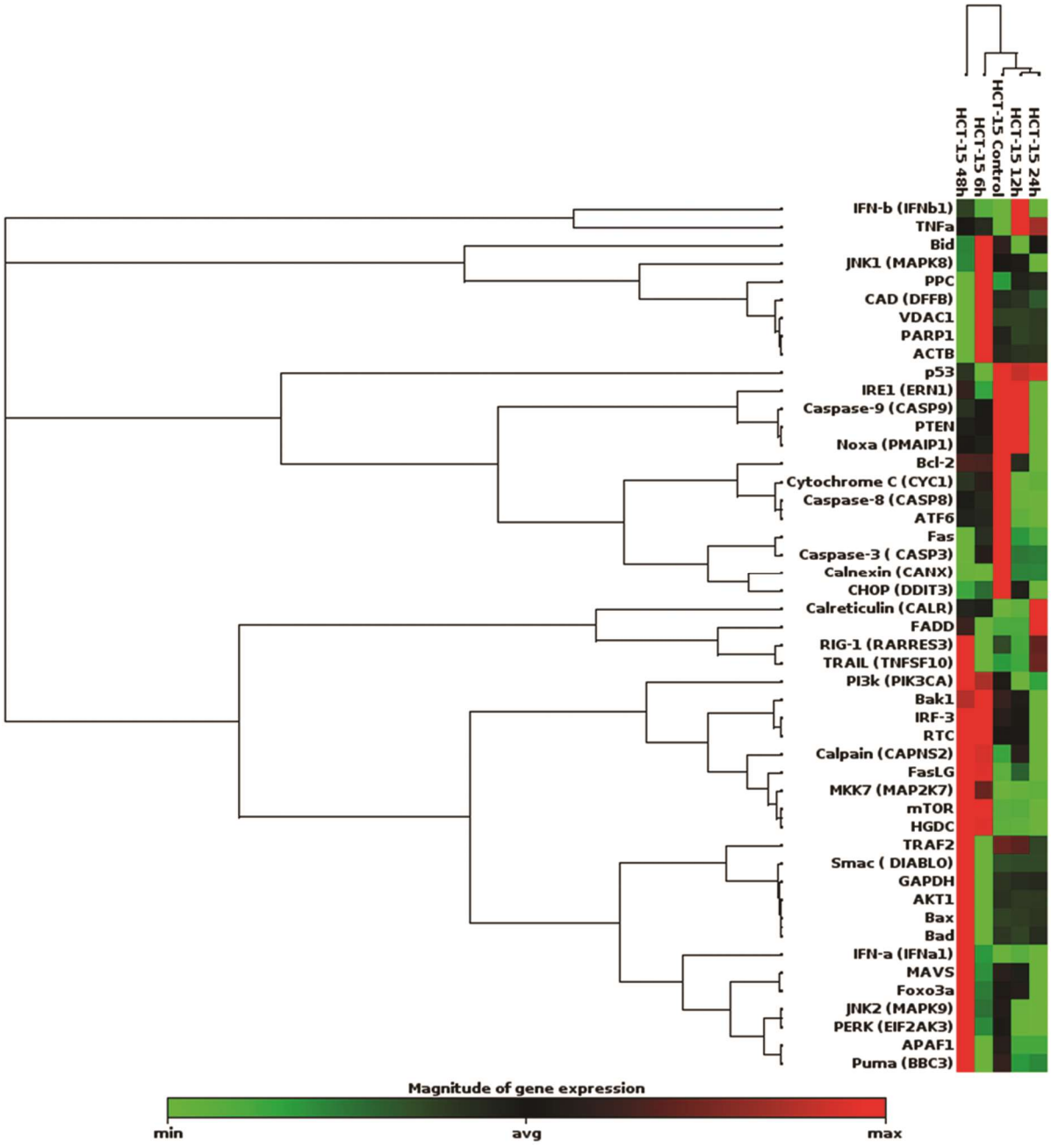


Fig. 9 — Clustergram for expression analysis of apoptotic genes in HCT-15 cells following CDV infection. Group clustering analysis of differentially expressed genes identified in control versus CDV infected HCT-15 cells at specific time intervals (6, 12, 24 & 48 h)

disease virus (NDV) could trigger IFN-α and TRAIL induction in BHK-21 cells and the HN protein of NDV induce TRAIL and IFN-α in PBMCs. It has also been reported that HN protein of NDV upregulates the expression of signal molecules such as IFN-α, TNF-α and TRAIL[21,22]. Rajmani *et al* (2015) showed that the mRNA expression levels of TNF-α, IFN-α & TRAIL in HN-infected Hela cells were found to be up-regulated as compared to the control groups[23]. Further in our study, Calpain expression was seen to be up-regulated in group 1, 2 & 4. Calpains are a family of calcium dependent cysteine proteases which are expressed in mammals. Under physiological and stress conditions, a generalized influx in the calcium levels leads to the activation of calpains, which through signal transduction pathways catalyses the proteolytic cleavage of many target proteins. Shirley *et al* (2002) provided evidence that ionomycin-

activated calpain, due to the calcium release, triggers apoptosis in lung cell carcinoma cell line with a decrease in Bcl-2 and Bid[24]. The decrease in Bid and Bcl-2 causes downstream release of cytochrome c, caspase activation and PARP cleavage. *Lu et al* (2013) showed that calpain activation via Ca^{2+}flux plays an essential role in eliciting an AIF-mediated, caspase-independent apoptotic pathway in EV71-infected cells[25]. In group 4, there was an up-regulation of Retinoic Acid Inducible gene I (RIG-I), which is a RIG-I like receptor dsRNA helicase enzyme, which functions as a pattern recognition receptor that is a sensor for viruses such as sendai virus, influenza virus etc. RIG-I can detect intracellular viral nucleic acids and helps in α/β-IFN signaling in infected cells. However, it has been observed that RIG-I induces apoptosis in B16 cells and this pro-apoptotic effect was dependent upon interferon signaling[26]. Kubler *et al* (2010) showed that after transfection with pppRNA or poly I: C, RIG-I gets activated and induces apoptosis in epithelial ovarian cells (EOC)[27].

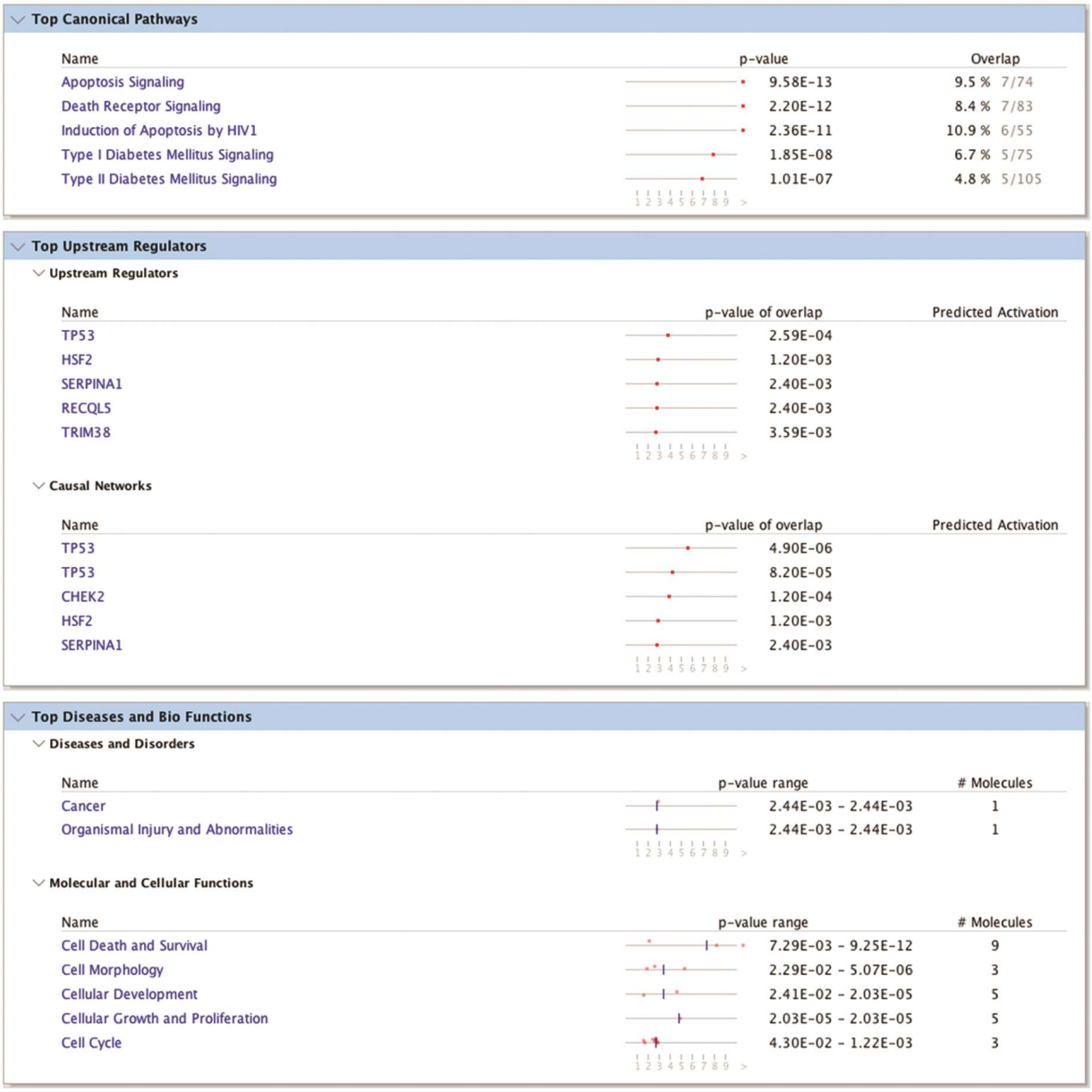
Ingenuity Pathway Analysis (IPA)

Analysis of CDV infected HCT-15 cells

To investigate the possible biological interactions of the differently expressed genes upon CDV infection in HCT-15 cells, all the datasets as described in (Tables 2 & 3) with expression fold change values analyzed after qPCR array studies were imported into the IPA tool. The list of differentially expressed genes analyzed by IPA revealed 12 significant networks and among them the top three networks identified by IPA were Apoptotic signaling; Death receptor signaling and Induction of apoptosis by HIV-1 comprising of 27 focused molecules and significance score of 19 (Fig. 10). Further, the IPA analysis also shows groups of differently expressed genes into biological mechanisms that are related to Cell Death and Survival $7.29E^{-03}$, Cellular Development $2.41E^{-02}$, Cellular Growth and Proliferation $9.09E^{-07}$, Cell Morphology $2.29E^{-02}$ and Cell to Cell Signaling and Interaction $8.50E^{-03}$(Fig. 10). In group 1 (6 h PI) fold change, the most important canonical pathway that was found was of the apoptotic signaling (p-value – 9.57E-13) with a z-score of 1.89 and a ratio of 0.095 consisting of seven up-regulated molecules (BAK1, BID, FASLG, MAP2K7, MAPK8, TNF & PARP1). A graphical representation of the pathway with the molecules involved in apoptotic signaling was generated by IPA (Fig. 11). In group 2 (12 h PI) fold change, the apoptotic signaling was found to be significant (p-value – 8.08E-11) with a z-score of 2.44 and a ratio of 0.081 consisting of six up-regulated molecules (BAX, FASLG, MAP2K7, MAPK8,
TNF & DIABLO).Figure 12 shows a graphical representation of the pathway with the molecules involved in apoptotic signaling generated by IPA. In group 3 (24 h PI) fold change, the apoptotic signaling was found to be significant (p-value – 6.25E-09) with a z-score of 2.23 and a ratio of 0.068 consisting of five up-regulated molecules (BAD, BAX, MAP2K7, TNF & DIABLO).Figure 13 shows a graphical representation of the pathway with the molecules involved in apoptotic signaling generated by IPA. In group 4 (48 h PI) fold change, the significant canonical pathway was the apoptotic signaling (p-value – 1.63E-12) with a z-score of 2.82 and a ratio of 0.108 consisting of eight up-regulated molecules (APAF1, BAD, BAK1, BAX, DIABLO, FASLG, MAP2K7 & TNF). A graphical representation of the pathway with the molecules involved in apoptotic signaling was generated by IPA (Fig. 14).As the CDV infection progressed from 6 h to 12 h, two more genes (Bax& Diablo) were found to be up-regulated, whereas Bak1, Bid & PARP1 did not show up-regulation as observed in group 1 (6 h). Similarly, in group 3 (24 h) & group 4 (48 h), BAD & APAF1 were found to be up-regulated, respectively indicating the sequential progress of apoptotic signaling pathway. However, in this study, the up-regulation of initiator caspases (caspase 8 & 9) and execution caspase 3 was not observed which are major players in the classical extrinsic and intrinsic apoptotic pathways.

Further, the upstream regulator analysis showed that TP53 is the most important upstream regulator in all the groups with a z score of (Group1 – 0.750; Group2 – not detected; Group3 – not detected; Group 4 – 1.453) and possible targets as shown by evidence for effects.

The analysis of possible genes also revealed many downstream effects which correlate with diseases or functions that are induced upon CDV infection of HCT-15 cells. The pattern of apoptotic gene up-regulation observed in this study was found to be consistent with that of “Apoptosis of colorectal cancer cell lines” as showed by the IPA predicted activation state under the diseases or functions annotation parameter with higher p-values and z-scores. On



Top Canonical Pathways

Name	p-value	Overlap
Apoptosis Signaling	9.58E-13	9.5 % 7/74
Death Receptor Signaling	2.20E-12	8.4 % 7/83
Induction of Apoptosis by HIV1	2.36E-11	10.9 % 6/55
Type I Diabetes Mellitus Signaling	1.85E-08	6.7 % 5/75
Type II Diabetes Mellitus Signaling	1.01E-07	4.8 % 5/105

Top Upstream Regulators

Upstream Regulators

Name	p-value of overlap	Predicted Activation
TP53	2.59E-04	
HSF2	1.20E-03	
SERPINA1	2.40E-03	
RECQL5	2.40E-03	
TRIM38	3.59E-03	

Causal Networks

Name	p-value of overlap	Predicted Activation
TP53	4.90E-06	
TP53	8.20E-05	
CHEK2	1.20E-04	
HSF2	1.20E-03	
SERPINA1	2.40E-03	

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value range	# Molecules
Cancer	2.44E-03 - 2.44E-03	1
Organismal Injury and Abnormalities	2.44E-03 - 2.44E-03	1

Molecular and Cellular Functions

Name	p-value range	# Molecules
Cell Death and Survival	7.29E-03 - 9.25E-12	9
Cell Morphology	2.29E-02 - 5.07E-06	3
Cellular Development	2.41E-02 - 2.03E-05	5
Cellular Growth and Proliferation	2.03E-05 - 2.03E-05	5
Cell Cycle	4.30E-02 - 1.22E-03	3

Fig. 10 — Top 5 canonical pathways, upstream regulators and diseases and functions showed by IPA in CDV infected HCT-15 cells

further downstream effect analysis, a greater number of apoptotic genes were found to beinvolved asthe CDVinfection progressed. In group 1, the downstream effects analysis showed that 5 genes (FasLG, Bid, TNF, MAPK8 and BAK1) have the measurement direction consistent with the increase in Apoptosis of colorectal cancer cell lines. In group 2, the downstream effects analysis showed that 8 genes (FADD, DIABLO, FasLG, FOXO3, BAX, TNF, PMAIP1 and MAPK8) have the measurement direction consistent with the increase in Apoptosis of colorectal cancer cell lines and 8 genes (FADD, DIABLO, FasLG, FOXO3, BAX, TNF, PMAIP1 and MAPK8) have the measurement direction consistent with the increase in Cell death of colorectal cancer cell lines. In group 3 the downstream effects analysis showed that 5 genes (FADD, DIABLO, TNF-SF10, BAX and TNF) have the measurement direction consistent with the increase in Apoptosis of colorectal cancer cell lines. In group 4, the downstream effects analysis showed that 10 genes (FADD, DIABLO, FasLG, FOXO3, BAX, TNF, BBC3, TNF-SF10, APAF1 and BAK1) have the measurement direction consistent with the increase in Apoptosis of colorectal

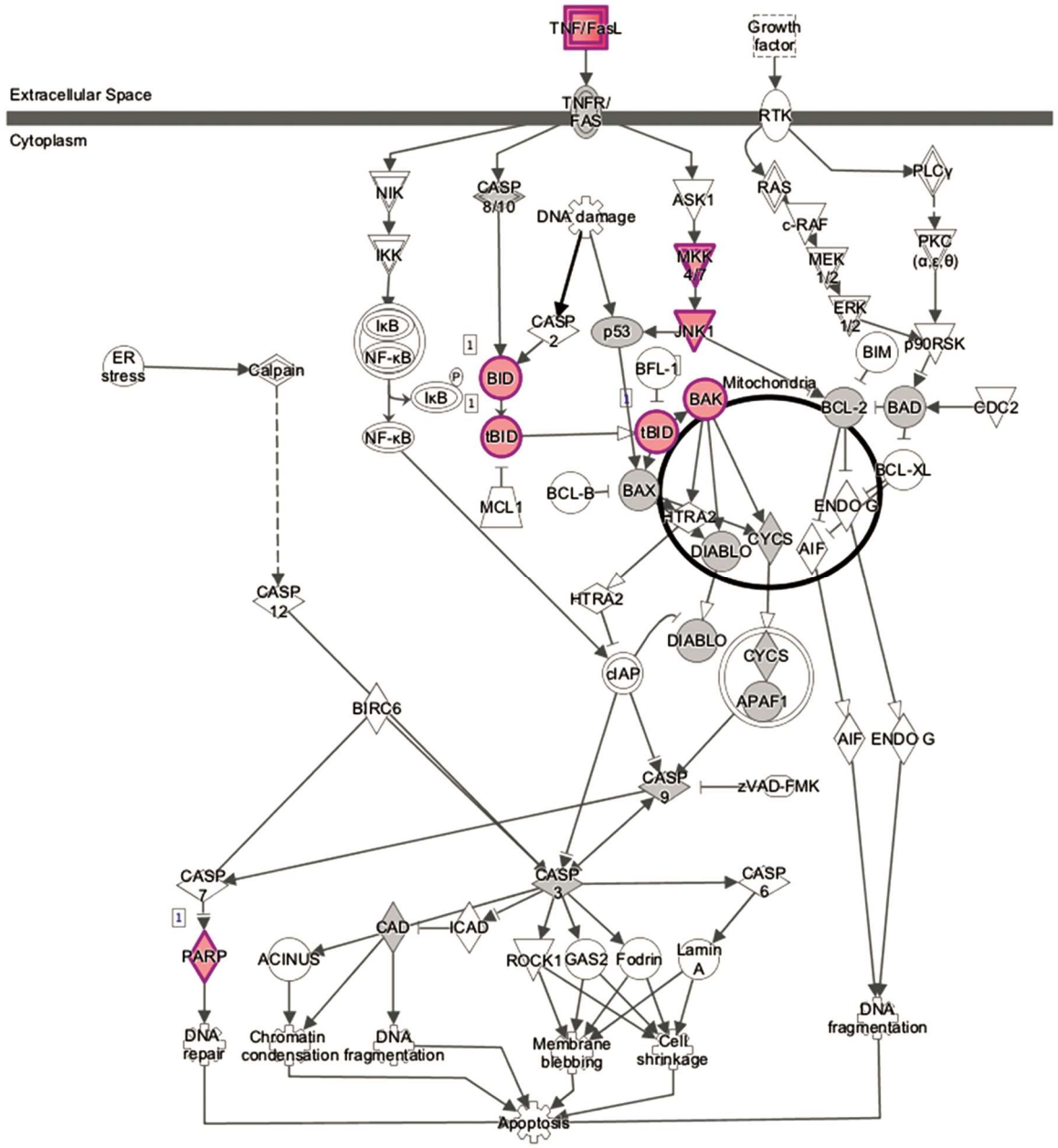


Fig. 11 — Apoptotic signaling pathway showing up-regulated genes in CDV infected HCT-15 cells (6 h pi); Pink - activated molecules; Grey - involved in pathway but did not pass fold change cutoff value & White - no change in activity

cancer cell lines. As described by IPA, the most highly rated network as shown in (Fig. 15) is the "Apoptosis of colorectal cancer cell lines" with different genes that are predicted to increase and regulate the process of apoptosis.

Prediction of apoptotic pathway

As described by the gene expression analysis by qPCR array and Ingenuity Pathway database, it was found that in the group 1 (6 h PI), TNF-α overexpression was observed which acts as a ligand for binding to the death receptor TNF-R1. Downstream of the death receptor activation, MAP2K7 and JNK1 was up-regulated describing their role in apoptosis activation. In response to external stimulus *e.g.*, stress or virus infection, MAP2K7 or MKK7 activates JNKs only which is a specific process unlike MKK4 which can activate both p38MAPK and JNKs. Both anti-apoptotic and pro-apoptotic proteins and signals they induce activation

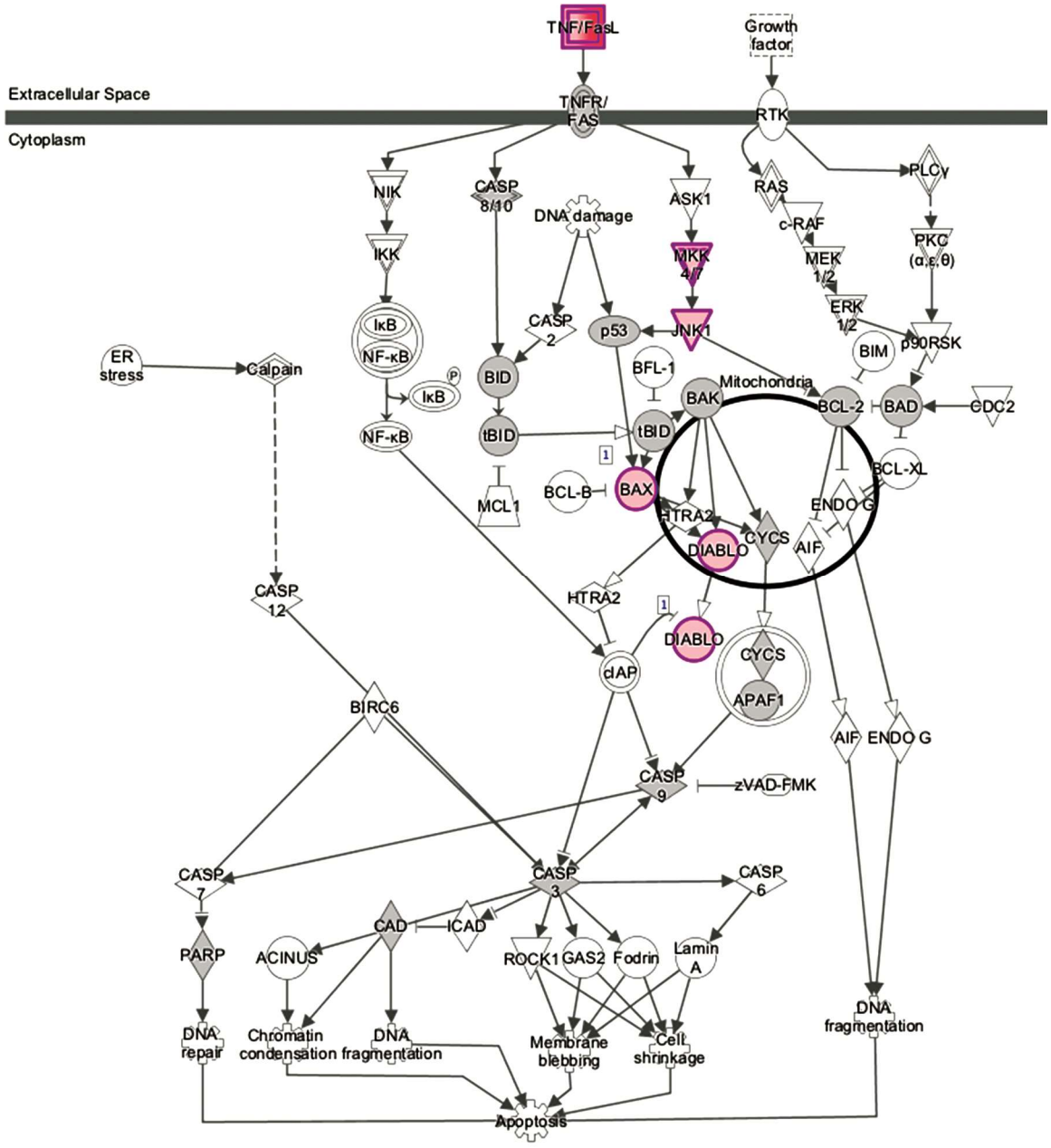


Fig. 12 — Apoptotic signaling pathway showing up-regulated genes in CDV infected HCT-15 cells (12 h pi); Pink - activated molecules; Grey - involved in pathway but did not pass fold change cutoff value & White - no change in activity

of MKK7-JNK signaling node. JNKs further activate the apoptotic signaling either by activation of certain transcription factors or through up-regulation of pro-apoptotic genes or by directly inducing the mitochondrial pro and anti-apoptotic protein activities through phosphorylation[28]. On the contrary, Bid, a BH3 only pro-apoptotic protein was found to be up-regulated with the activation of tBid. It is known that following the death receptor pathway Bid is cleaved by caspase-8, this cleavage results in the formation of a truncated protein tBid. It is further suggested that tBid is capable of activating mitochondrial outer membrane permeabilization (MOMP) as a result of the imbalance in the pro and anti-apoptotic proteins that enable it to occur. Thus, this caspase-8 cleavage of Bid into tBid forms a link between the extrinsic and the intrinsic pathway of apoptosis and thus leads to cross talk between the two pathways[29,30]. However, in our study we did not see up-regulation or activation of caspase-8, so this could be attributed to the

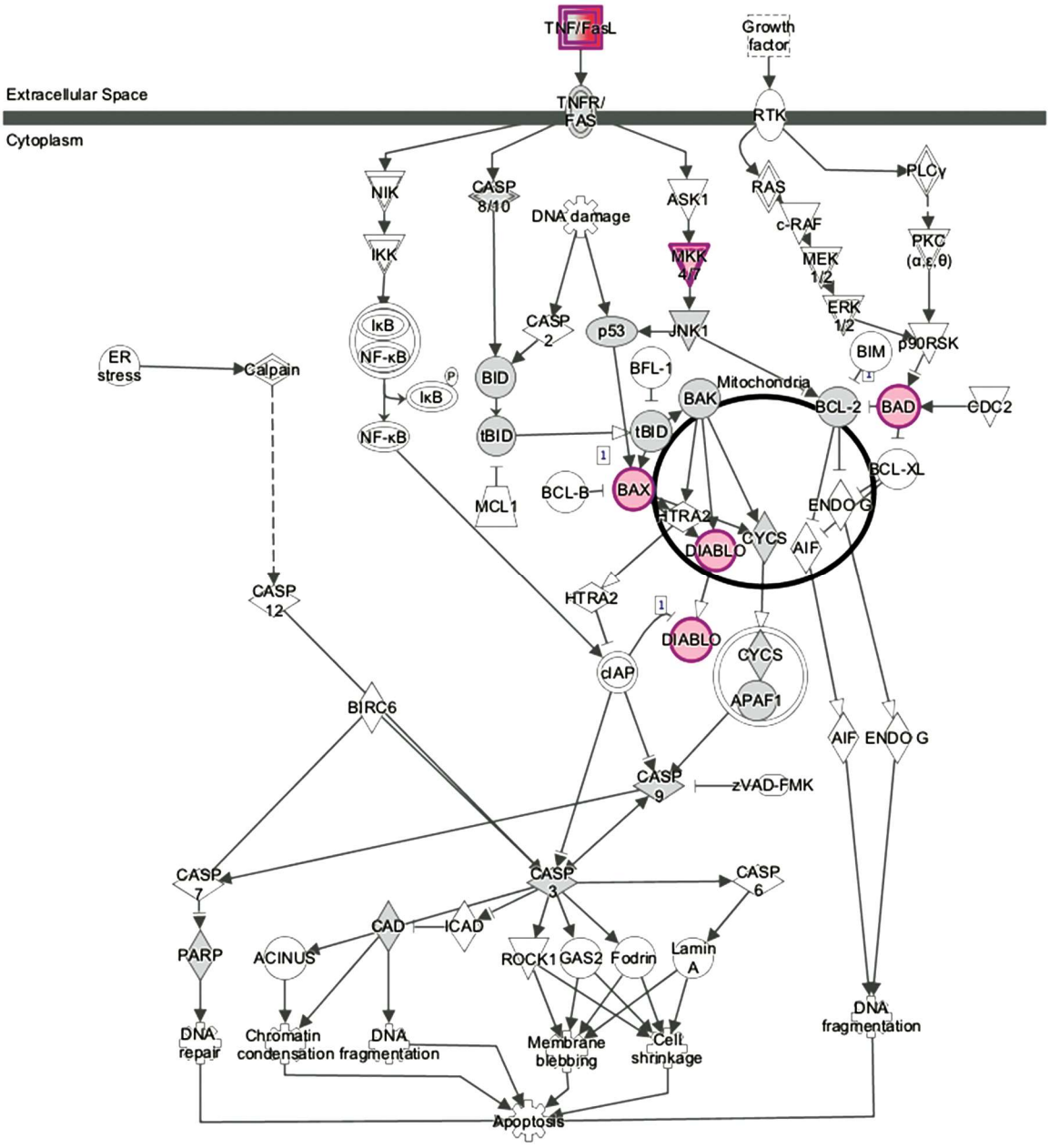


Fig. 13 — Apoptotic signaling pathway showing up-regulated genes in CDV infected HCT-15 cells (24 h pi); Pink - activated molecules; Grey - involved in pathway but did not pass fold change cutoff value & White - no change in activity

cleavage of Bid by other proteases such as cathepsins, calpains, granzyme B, caspase-3 which have been shown to cleave Bid and hence activating Bid[31-33]. Next, Bak activation was proposed in the pathway, which is Bcl-2 pro-apoptotic protein which localizes to the mitochondria and functions to induce apoptosis. The Bcl-2 proteins are a crucial checkpoint in the apoptosis at the mitochondria[43]. During apoptosis, the BH3s such as tBid activate Bak and Bax, thus inducing cytochrome C release and finally leading to caspase activation[34-36]. Further in the pathway analysis, in group 2, the expression of Bax and Diablo was up-regulated downstream of tBid and Bak. Upon apoptotic stimuli, it is believed that Bax and Bak get activated upon upstream signals and get oligomerized at the mitochondrial outer membrane (MOM) and causes its permeabilization, leading to initiation of downstream apoptotic cascades. Thus, Bax and Bak work at appropriate levels to facilitate the release of cytochrome C and Smac/Diablo into the

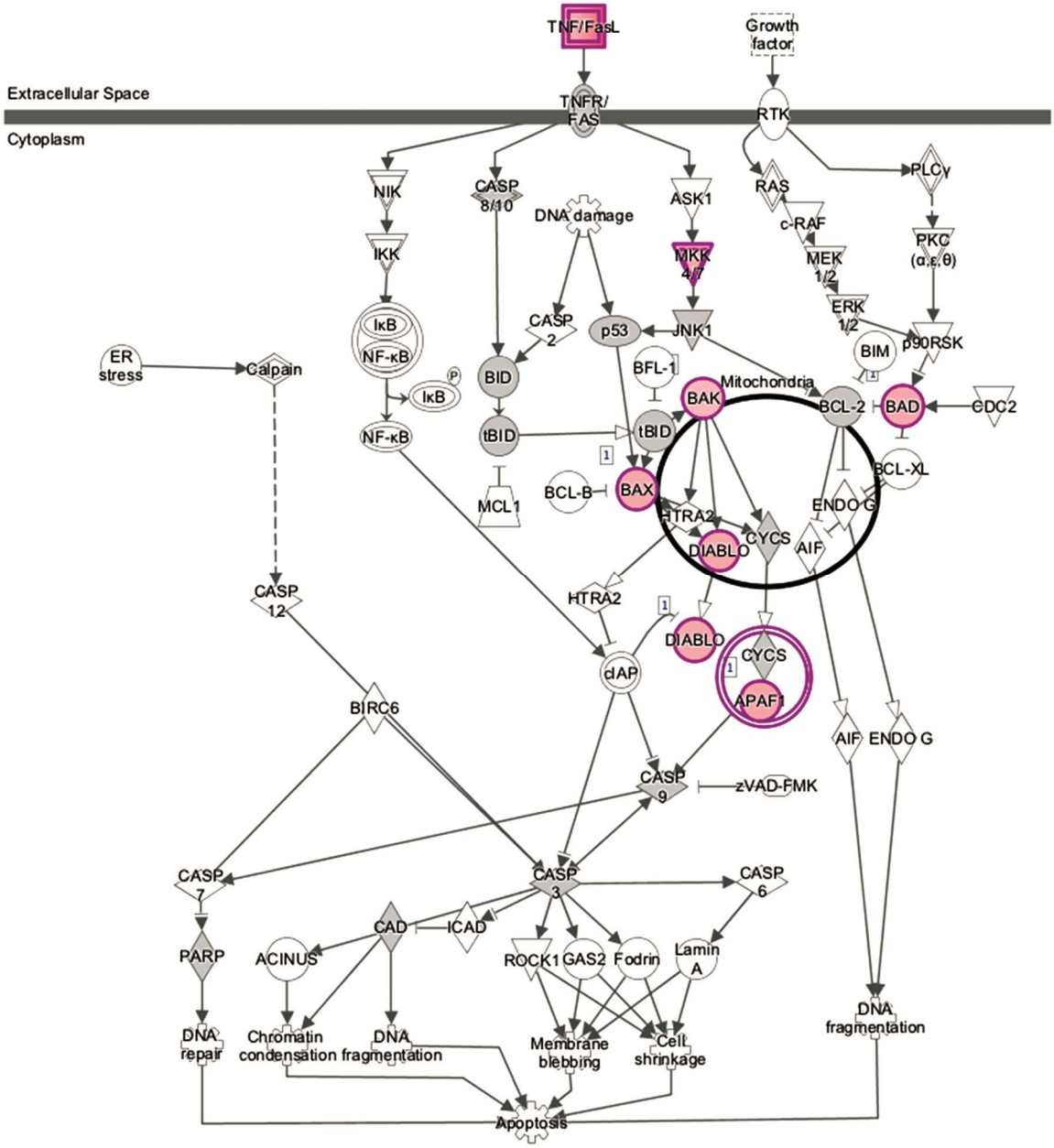


Fig. 14 — Apoptotic signaling pathway showing up-regulated genes in CDV infected HCT-15 cells (48 h pi); Pink - activated molecules; Grey - involved in pathway but did not pass fold change cutoff value & White - no change in activity

intermembrane space[37,38]. Direct IAP Binding Protein with low isoelectric point (Diablo) release from the mitochondria has been shown to repress the inhibitor of apoptosis proteins and further activates caspase-9. However, in this study up-regulation of caspase-9 could not be detected. In group 3 (24 h), the pathway shows the activation of Bad, Bcl-2 associated death promoter, which induces apoptosis by inhibiting anti-apoptotic proteins Bcl-2 &Bcl-xl. Also, similar to the group 2, the expression of Diablo and Bax was seen to be up-regulated which describes the permeabilization of mitochondria with the release of Diablo. Finally, in group 4, with the over-expression of proapoptotic proteins Bax, Bad &Bak, the release of Diablo from the mitochondria, there was up-regulation of Apoptotic protease activating factor (APAF1) which serves as a key molecule in the intrinsic apoptosis, which is known to oligomerize upon Cytochrome C release from the mitochondria resulting in the formation of a complex called the apoptosome[39].

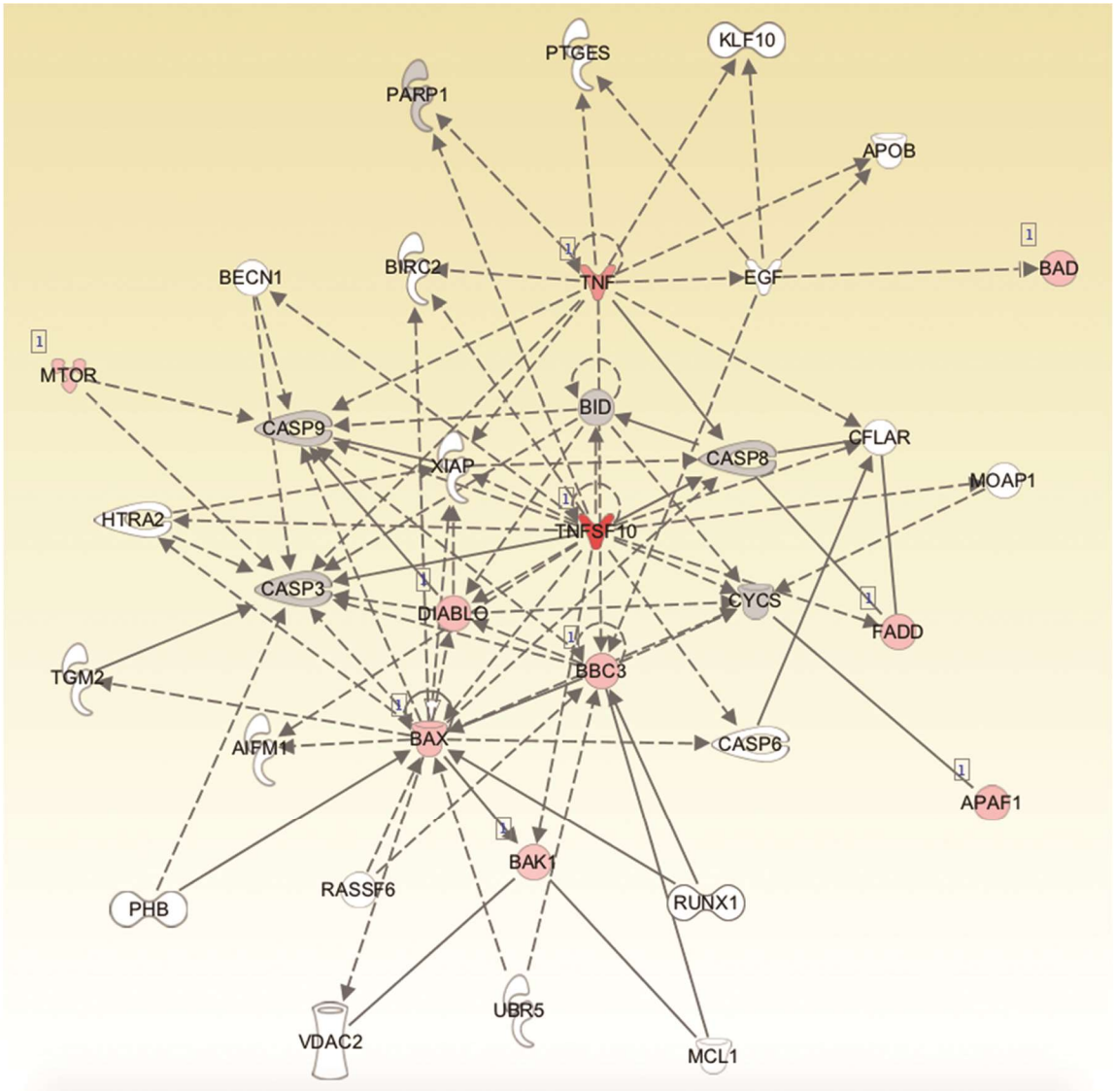


Fig. 15 — Graphical representation of the most highly rated network in the IPA analysis of CDV infected HCT-15 cells. The genes that are shaded were determined to be activated, with the solid line representing a direct interaction between the two genes and the dotted line indicating an indirect interaction

Since, Cytochrome C binding leads to the activation of APAF1, it is hypothesized that since the expression of Cytochrome C as detected by the fold change values did not pass the cut off in IPA analysis, it is assumed that Cytochrome C release did happen but in lower concentrations that lead to the activation of APAF1. Alternatively, APAF1 activation may also be due to some other upstream regulator. Usually, the activated APAF1 binds to the procaspase 9 to form the apoptosome complex through monomer interactions, which leads to the dimerization and formation of catalytically active caspase 9. Active caspase 9 leads to the cleavage and activation of the executioner caspases 3, 6 & 7 resulting in chromatin condensation, DNA fragmentation, cell shrinkage and finally apoptosis[40-42]. IPA revealed the up-regulation of major apoptotic proteins (Bak1, Bid, FasLG, MAP2K7, MAPK8, TNF, Bax, Diablo, Bad, APAF1, PARP1) from both extrinsic and intrinsic pathway of apoptosis along with important upstream regulator and downstream regulators. However, in this study, up-regulation of caspase 8, 9 and 3 was not detected which play major roles in the classical extrinsic and intrinsic apoptotic pathways. Therefore, it is hypothesized that CDV induced apoptosis occurs by some caspase independent pathway such as calpain or granzyme A mediated.

Conclusion

The present study suggests that CDV growth in HCT-15 cell line could be detected by RT-PCR, although no characteristic CPE was observed except cell rounding, death & degeneration following 6-7 days post infection. Apoptosis could be detected in HCT-15 cell line, as revealed by DNA laddering & TUNEL assays following 24 h onwards post CDV infection. 3 major canonical pathways *i.e*., Apoptotic signaling, Death receptor signaling and Induction of apoptosis by HIV-1, were involved in CDV induced apoptosis in HCT cell lines as revealed by IPA tool. A total of 11 apoptotic genes (out of 43) were found up-regulated following CDV infection of HCT-15 cell along with predicted major upstream and downstream regulators, similar to that of "Apoptosis of colorectal cancer cell lines". Although up-regulation of caspase 8, 9 and 3 was not observed it is hypothesized that CDV induced apoptosis in HCT-15 cell line might have occurred due to the involvement of some caspase independent pathways (like Calpain or other molecule mediated). In immunocytochemistry, a basic level of expression of the target proteins/markers (Caspase 3, Caspase 8, Caspase 9, Bax, Cytochrome C, MAVS, IRF-3, MKK7) could be detected in CDV infected HCT-15 cells which is consistent with the qPCR array results. Further, in-depth expression analysis is required to delineate the cellular processes that regulate apoptosis during CDV infection in cancerous cells.

Acknowledgement

We thank technical team Dr. Durgesh Kumar Singh & Jyoti Malik (Qiagen, India) for assistance with qPCR array experiment, data analysis and for providing the trial licence for IPA.

Conflict of interest

All authors declare no conflict of interest.

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