Characterization of *Salmonella* effector protein SteA using MS-Proteomics analysis, Flow Cytometry and Western Blotting

Shubham Gajrani

A dissertation submitted for the partial fulfilment of
BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali

April 2020

Acknowledgment

I wish to express my sincere appreciation to my supervisor, Dr. Arunika Mukhopadhaya for encouraging and guiding me in both scientific and personal matters. I wish to wholeheartedly thank her for immense support and guidance to do the right thing even when the journey got tough. I am indebted to Ms. Deepinder Kaur for critical discussions as well as guidance in Flow cytometry experiments. I wish to show gratitude to Dr. Aakanksha Gulati for teaching me lab work and help in cloning experiments and Mr. Shashi Prakash Yadav for assistance in cell culture and infection experiments. I wish to acknowledge the support of Mr. Yogesh Saxena, Dr. GVR Krishna Prasad, Ms. Shraddha Gandhi, Ms. Vinica Dhar, Ms. Arpita Sharma, Ms. Pratima Verma, and Mr. Shivansh and all other Lab members in making this journey a wonderful experience. I wish to pay special regards to IISER Mohali for providing me this opportunity. This work wouldn't have been possible without the love and support of my family and friends.

Shubham Gajrani

List of Figures

List of Tables

Notation

SteA	*Salmonella* translocated effector A
SPI	*Salmonella* Pathogenicity Islands
FITC	Fluorescein Isothiocyanate
PI	Propidium Iodide
PCNA	Proliferating cell nuclear antigen
ISG15	Interferon-stimulated gene 15
FEN1	Flap endonuclease I
DHFR	Dihydrofolate reductase

Contents

Abstract

Salmonella enterica serovar Typhimurium is one of the major foodborne pathogens that causes Salmonellosis in humans and Typhoid-like disease in mice. It spreads through contaminated food, poultry, eggs and meat and causes around 93.8 million cases of nontyphoidal *Salmonella* gastroenteritis and 155,000 deaths each year. *Salmonella* Typhimurium invades, survives and replicates inside host cells by effector proteins expressed by gene clusters known as *Salmonella* Pathogenicity Islands (SPI). *Salmonella* Typhimurium SteA is an effector protein under the regulation of both SPI-1 and SPI-2. SteA under SPI-1 condition performs immunosuppressive roles interfering with NFκB activation pathway. To explore other possible functions of SteA, Mass spectrometry-based proteomics analysis was performed using Perseus platform. STRING network and PANTHER classification system were used to assimilate the results from Perseus analysis. The analysis indicated the possibility that SteA could be involved in host cell death pathway. Further, Initial Flow cytometry-based apoptosis detection experiments show that SteA deletion in *Salmonella* Typhimurium causes a decrease in apoptosis in RAW264.7 macrophages on infection. Further experiments can help consolidate results and identify targets and mechanism involved.

Chapter 1

Introduction

1.1 Basic Theory

1.1.1 *Salmonella enterica* serovar Typhimurium

Salmonella enterica Typhimurium is a rod-shaped Gram-negative facultative anaerobe, belonging to family Enterobacteriaceae. It causes salmonellosis in humans and Typhoid-like disease in mice. *Salmonella* is one of the most commonly occurring foodborne pathogens found in poultry, eggs, dairy products as well as unhygienic food. It has been estimated that each year around 93.8 million cases of non-Typhoidal *Salmonella* gastroenteritis occur, leading to around 155,000 deaths[1]. *Salmonella* is a truly a genius pathogen – once it has entered through contaminated food, it gains access to the non-phagocytic host cells by inducing its phagocytosis. *Salmonella*'s pathogenicity and mode of action are controlled by gene clusters known as *Salmonella* Pathogenicity Islands (SPI).

1.1.2 *Salmonella* Pathogenicity Islands

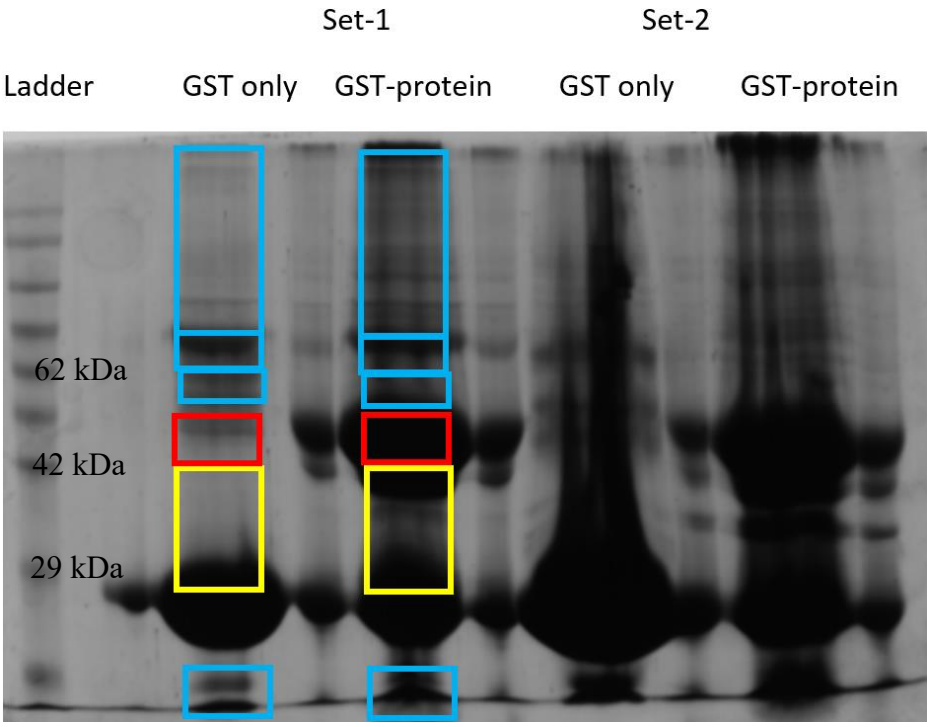
Salmonella Pathogenicity Islands (SPI) encode/regulate for various virulence factors as well as structures involved in the invasion and replication process[2]. SPI1 and SPI2 play a critical role in *Salmonella* invasion and replication respectively. One of the structures is the Type III secretion system (T3SS), which is a flagellum-like injectisome structure responsible for the transport of effectors across the cell membrane to host cells[3]. These complex nano-needles are composed of at least 20 subunits and on injecting bacterial effectors into the host cytoplasm, they cause signal transductions leading actin cytoskeleton remodulation. This causes cells to form an outward extension of the cell membrane and engulf the bacteria. Once *Salmonella* gets inside host cells, it causes the formation of *Salmonella*-containing vacuole (SCV) – which is made up of the host cell membrane. Then *Salmonella* injects other effectors into the vacuole which causes remodulation of the environment inside SCV to aide *Salmonella* intracellular survival as well as replication.

1.1.3 SteA – *Salmonella* translocated effector A

Salmonella translocated effector A or SteA is a secreted effector protein that is regulated by both SPI-1 and SPI-2[4]. To check which host proteins of the IkB degradation pathway directly interacts with the SteA, cloning was performed to obtain SteA with components of

the Ubiquitin E3 ligase complex as bait and prey clones for performing Yeast 2-Hybrid experiment [5]. To explore other possible associations/functions of SteA in host cells,

GST pulldown was performed by Aakanksha Gulati and SDS PAGE gel samples in Coomassie blue staining were sent to Harvard Medical School for Mass spectrometry – to identify target proteins that SteA probably binds to.



Sample1- blue boxes of GST only (set1) **Sample2**- blue boxes of GST- steA (set1)

Sample 3- yellow boxes of GST only (set-1) **Sample 4**- yellow boxes of GST- steA (set-1)

Sample 5- blue boxes of GST only (set-2) **Sample 6**- blue boxes of GST- steA (set-2)

Sample 7- yellow boxes of GST only (set-2) **Sample 8**- yellow boxes of GST- steA (set-2)

Label-Free quantification data was obtained from Harvard Medical School and Proteomics analysis was performed on the Perseus platform.

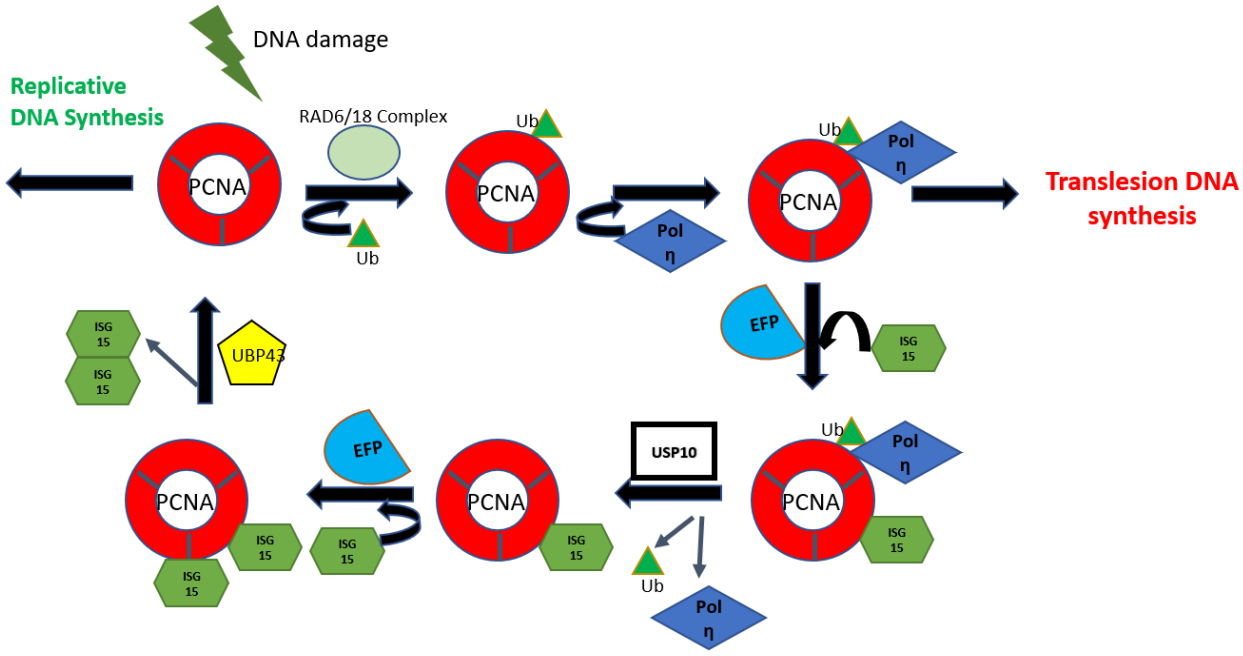
Further, the Mass spectrometry data were analyzed and indicated the interaction of SteA with PCNA and ISG15 of host cells.

1.1.4.1 Proliferating cell nuclear antigen (PCNA) and its role in Translesion DNA synthesis pathway

Proliferating cell nuclear antigen (PCNA) plays a central role in the decisions of life and death of the cell. *PCNA* gene is induced by p53. It is a ring-like protein belonging to the DNA sliding clamp family and plays critical roles in both DNA replication as well as DNA repair mechanisms[6]. Post-translational modifications observed in PCNA include monoubiquitination, polyubiquitination, SUMOylation, etc.[7]. Absence/low levels of functional PCNA leads to apoptosis in the cells.

Salmonella invasion causes the release of reactive oxygen species (ROS) as well as reactive nitrogen species (RNS), which in turn are reported to cause DNA damage in the form of lesions[8, 9]. DNA lesions can block genome transcription and replication. In response, cells possess a universal DNA damage tolerance mechanism known as Translesion DNA synthesis (TLS).

In response to DNA damage, PCNA undergoes mono-ubiquitination in the UBZ domain by the RAD6/18 complex, leading to triggering of DNA Translesion synthesis pathway, followed by recruitment of polymerase- η.



Monoubiquitinated PCNA shows a much higher affinity than unmodified PCNA for polymerase-η. Damage tolerant polymerase-η lacks proofreading activity and can lead to the introduction of incorrect nucleotides, opening Pandora's box of mutagenesis. Thus, after bypassing DNA lesion, error-prone TLS polymerases need to be replaced by replicative DNA polymerases. Failure of such exchange may lead to excessive mutagenesis and probably cell death.

However, it is unknown how DNA replication is resumed after TLS termination. But in TLS termination, the process of ISGylation plays a central role[10]. ISG15 (Interferon-stimulated gene 15) is a 17kDa ubiquitin-like protein that shares structural similarity with ubiquitin as it contains 2 tandem ubiquitin homology domains. ISGylation is catalyzed by :

- ISG15-activating E1 enzyme - UBE1L
- ISG15-conjugating E2 enzyme – UBCH8
- ISG15 E3 ligase – EFP

TLS Termination: EFP interacts with mono-ubiquitinated PCNA and leads to its ISGylation, which in turn leads to binding of USP10. USP10 leads to the removal of ubiquitin from PCNA and thus removal of the polymerase-η[10]. PCNA has two ISG15 acceptor sites (to form di-ISGylated PCNA) – Lys164 and Lys168. Removal of di-ISG15 tag from PCNA by UBP43 leads to the resumption of Replicative DNA synthesis.

1.1.4.2 ISG15 – cell's defense mechanism

ISG15 and its conjugation to targets i.e. ISGylation have been reported to be induced by infection, IFNα, IFNβ, DNA damage as well as aging. ISGylation acts as a cellular defense mechanism by inhibiting exosome secretion as well as inhibiting normal protein translation by modifying protein kinase R(PKR) – steps to prevent the spread of pathogens[11]. It has also been reported that p53, tumor necrosis factor (TNF), inducible nitric oxide synthase (iNOS), etc. stimulate the expression of ISG15 and its conjugating enzymes[11].

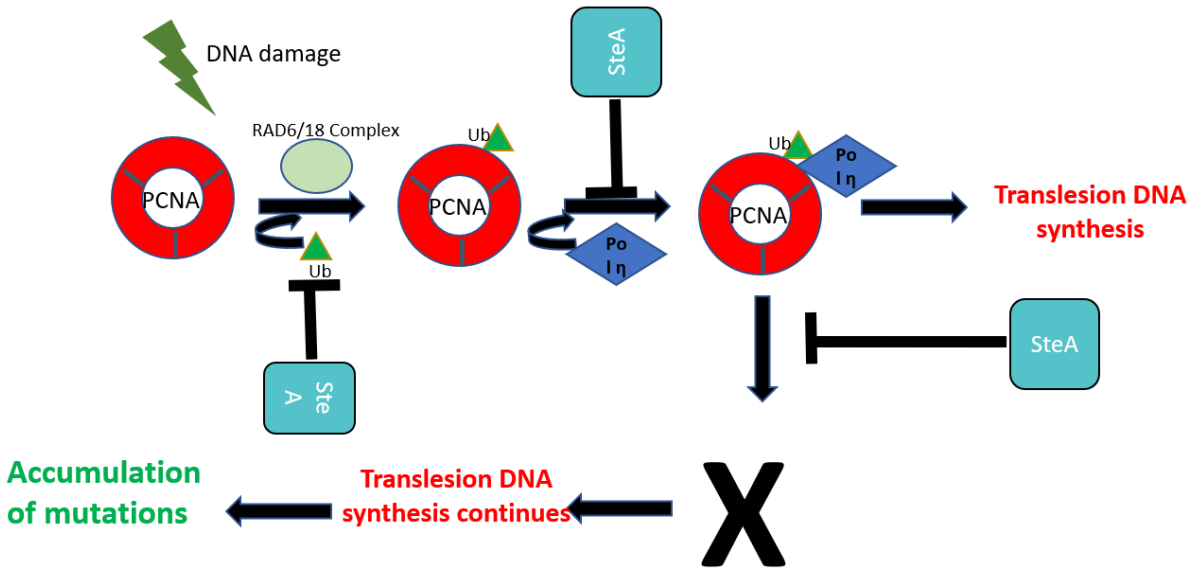
1.1.4.3 SIVA1-a critical regulator of PCNA monoubiquitination

SIVA1 protein consists of 175 amino acids and is known to induce apoptosis through multiple pathways[12]. RAD6-RAD18 ubiquitin-conjugating/ligase complex is responsible

for the monoubiquitination of PCNA and regulation of TLS repair. SIVA1 has been shown to play the role of critical regulator of PCNA monoubiquitination as it constitutively interacts with PCNA via conserved PCNA-interacting peptide motif[13]. It has also been shown that SIVA1-PCNA complex formation was DNA damage independent (in HeLa cells as well as in HEK293T cells)[3]. SIVA1 associated with RAD18 and PCNA through nonoverlapping regions.

Another study shows differential expression of *SIVA1* in HD11 Chicken macrophages apoptosis on infection with *Salmonella* post Selenium treatment[14].

1.1.4.4 Hypothesis



Data from mass spectrometry-based proteomics analysis reveals that SteA probably affects PCNA-ISG15-FEN1 Translesion DNA synthesis pathway. It has already been observed by other member (Shivansh) of the lab that deletion of SteA leads to decrease in cell death in RAW264.7 murine macrophages by SPI-2 induced *Salmonella* Typhimurium. The hypothesis of this study from mass-spectrometry data is, TLS pathway could be modulated by SteA and, potential targets could be:

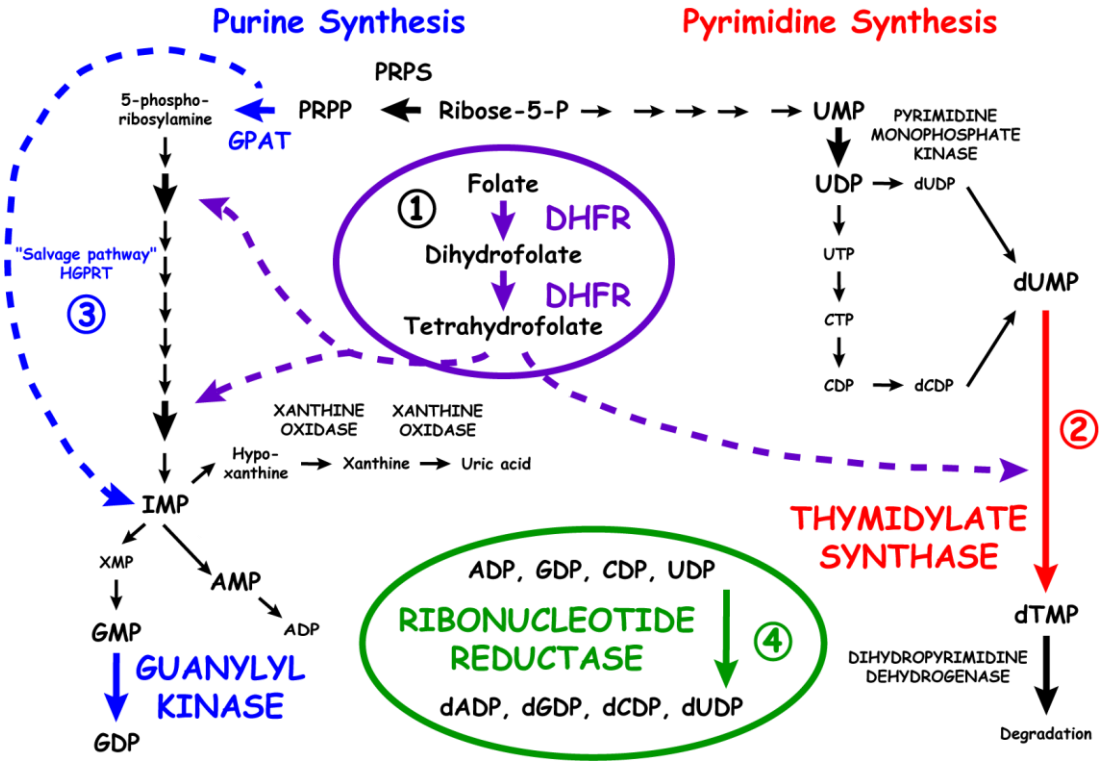
- Inhibition of PCNA monoubiquitination
- Inhibition of recruitment of polymerase-η

- Binding to PCNA-FEN-1 complex
- Inhibition of ISGylation

Towards this, the first step is to check if PCNA antibody [BioLegend anti-Mouse PCNA] binds to post-translationally modified forms of PCNA. This was performed using Hydroxyurea to induce DNA damage in RAW264.7 macrophages and western blotting using anti-PCNA antibody.

1.1.5.1 Dihydrofolate reductase

Mass-spectrometry also suggests that SteA could interact with dihydrofolate reductase (DHFR). DHFR is a key enzyme responsible for tetrahydrofolate (THF) synthesis, which catalyzes the reduction of folic acid into dihydrofolate[15]. Since DHFR is essential for the biosynthesis of purines, DHFR inhibition causes disruption in purine and thymidylate biosynthesis as well as DNA replication and repair and hence, induce cell cycle arrest or even apoptosis[16]. As DHFR knockdown causes lead to reduced cell proliferation and increased apoptosis, it has also been targeted in cancer treatments using Folate inhibitors like Methotrexate.



[19]

DHFR showed the highest significance in protein hits from mass spectrometry-based proteomics analysis of SteA. Therefore, cell death due to SteA could be due to interaction of SteA with DHFR.

1.1.5.2 Hypothesis

SteA probably binds to DHFR, hence disrupting THF synthesis leading to inhibition of DNA replication and repair, leading to increased apoptosis.

To get an indication of SteA in tetrahydrofolate synthesis, Folinic acid could be used to bypass the SteA-mediated suppression of DHFR as DHFR can be bypassed by supplementing its product – Folinic acid/Leucovorin externally to the cells. Folinic acid is used to reduce the toxic effects of Methotrexate in chemotherapy.

Experiment: Apoptosis check using FITC-PI assay and supplementing RAW264.7 macrophages with Folinic acid to bypass DHFR role and comparing apoptosis caused by wt and ΔsteA *Salmonella*. According to the hypothesis, if SteA represses DHFR role, cells infected by wt *Salmonella* would show decreased apoptosis on Folinic acid supplementation.

1.2. Experimental Material and Methods

1.2.1 Experimental Materials

Bacterial culture

Salmonella enterica Typhimurium SL1344 – wt and mutant ΔsteA were cultured using Luria Bertani medium (HIMEDIA) along with appropriate antibiotics. Since SteA is encoded by both SPI-1 and SPI-2, SPI-2 induction was performed to check SPI-2 mediated role of SteA.

SPI-2 inducing conditions

Bacterial LB cultures were washed and made into 1:100 dilution with LPM media at pH 5.8 and incubated overnight at 37°C with shaking. It is well established that acidic minimal media (LPM) causes SPI-2 gene expression.

Low-phosphate low-magnesium minimal medium (LPM)

LPM media consists of:

1. 80mM 2-(N-morpholino) ethanesulfonic acid (pH 5.8)
2. 5M KCl
3. 7.5mM $(NH_4)_2SO_4$
4. 0.5mM K_2SO_4
5. 0.1% Casamino acids
6. 38mM Glycerol
7. 337.5μM K_2HPO_4-KH_2PO_4 (pH 7.4)
8. 8μM $MgCl_2$

Media was filter sterilized with 0.22μm filter (Millipore Millex)

Antibiotics used

- Streptomycin (HIMEDIA) was used for both wt and ΔsteA *Salmonella* Typhimurium cultures at concentration of 50μg/mL.
- Kanamycin (HIMEDIA) was used for ΔsteA *Salmonella* Typhimurium cultures at concentration of 50μg/mL.

- Gentamicin (HIMEDIA) was used in Infection experiments at concentrations - 100µg/mL and 20µg/mL.

Cell culture

RAW264.7 cells (ATCC®) were cultured in RPMI media supplemented with 10% FBS and incubated in 5% CO_2 at 37°C.

Cloning

- Vectors used: PGAD-C1 prey vector, PGBD-C1 bait vector
- Restriction enzymes: BamHI [NEB] – Recognition sequence – 5'- GGATCC -3'
  Sal1 [NEB] – Recognition sequence – 5'- GTCGAC -3'
- Competent cells: *E. coli* TOP10 strain
- cDNA obtained from Reverse Transcriptase – PCR using RNA isolated from RAW264.7 murine macrophages

Bioinformatics analysis

- Genomic sequences were obtained from NCBI (https://www.ncbi.nlm.nih.gov/)
- Protein sequences and functional information was accessed through UniProt (https://www.uniprot.org/)
- Mass spectrometry-based proteomics analysis was performed on Perseus 1.6.0.2 platform (https://maxquant.net/perseus/)
- PANTHER Classification system was accessed through the website (http://www.pantherdb.org/)
- SMART sequence analysis was performed on the webpage (http://smart.embl-heidelberg.de/)

Western blotting

BioLegend® anti-Human/mouse PCNA antibody and Sigma® Anti-Mouse IgG Peroxidase antibody was used in Western blotting experiments.

Bacterial counts using Spectrophotometer

OD_{600} readings for bacterial counts in Infection experiments were taken using Jenway Genova Plus spectrophotometer.

Flow cytometry and post-acquisition analysis

Flow cytometry experiments were performed on BD Accuri® C6 Flow cytometer and fluorescent staining performed using BD Pharmigen™ FITC Annexin V Apoptosis detection kit and post-acquisition analysis was performed on FlowJo V10. Spectral compensation was performed using 'Compensation' tab in FlowJo to compensate for spectral overlap generated due to FITC emission spectrum spill. Staurosporine is a protein-kinase inhibitor used to induce apoptosis for positive controls in spectral compensation.

Folinic acid rescue experiment

Apoptosis detection using BD Pharmigen™ FITC Annexin V Apoptosis detection kit and flow cytometry was performed along with samples supplemented with 200µM Folinic acid [GETWELL Leucowel® 10mg/mL] post-infection.

1.2.2 Experimental Methods

1.2.2.1 Mass spectrometry-based proteomics data analysis using Perseus

Quantitative protein abundance analysis utilizing Label-free quantification (LFQ) intensity data was analyzed using Perseus software platform (from Max Planck Institute of Biochemistry). LFQ intensity data was provided by Harvard Medical School. Following steps were followed for analysis:

1. Matrix was generated using LFQ Intensity data (Load -> Generic matrix upload) and columns were rearranged.

Data

	Sum Intensity C#1	Sum Intensity C#2	Sum Intensity C#3	Sum Intensity C#4	Sum Intensity C#5	Sum Intensity C#6	Sum Intensity C#7	Sum Intensity C#8	T: Gene Symbol	T: referen...	T: Annota...
Type	Main	Main	Main	Main	Main	Main	Main	Main	Text	Text	Text
1	490000...	320000...	170000...	170000...	110000...	370000...	120000...	120000...		SjGST...	japonic...
2	640000...	950000...	1900000	6700000	450000...	790000...	800000	3300000	Hspa5	sp|P20...	78 kDa...
3	3600000	380000...	160000	350000	9500000	270000...	470000	930000	Hsp90...	sp|P08...	Endop...
4	200000	850000	3400000	8900000	2300000	3800000	720000	5200000	Atp5b	sp|P56...	ATP sy...
5	2700000	7500000	770000...	360000...	110000...	260000...	320000...	560000...	Actb	sp|P60...	Actin, c...
6	170000...	4400000	770000	3100000	9400000	260000...	420000	5100000	Pdia3	sp|P27...	Protein...
7	1400000	410000...	NaN	1500000	110000...	430000...	190000	NaN	Hsp90...	sp|P11...	Heat s...
8	2300000	8100000	26000	NaN	180000...	240000...	720000	2500000	Pkm	sp|P52...	Pyruva...
9	6900000	6400000	110000	760000	4000000	140000...	NaN	1400000	Hspd1	sp|P63...	60 kDa...
10	1900000	110000...	950000	210000...	270000...	4600000	700000	8300000	Atp5a1	sp|Q03...	ATP sy...
11	9900000	7500000	380000	170000	100000...	130000...	160000	NaN	Hspa9	sp|P38...	Stress...
12	NaN	120000...	NaN	NaN	220000	640000	NaN	NaN	Tfrc	sp|Q62...	Transf...
13	2300000	190000...	NaN	NaN	4900000	9800000	72000	580000	Hspa8	sp|P63...	Heat s...
14	6400000	1700000	120000	NaN	1100000	760000	NaN	NaN	Lcp1	sp|Q61...	Plastin...
15	NaN	4300000	NaN	160000	190000	1600000	NaN	NaN	Uba1	sp|Q02...	Ubiqui...
16	NaN	6700000	480000	170000...	580000	3200000	8200000	9500000	Gapdh	sp|P16...	Glycer...
17	960000	560000	1000000	1300000	6400000	3000000	1000000	2700000	Vim	sp|P20...	Vimen...
18	NaN	NaN	2100000	5100000	330000	NaN	1300000	3700000	Eno1	sp|P17...	Alpha-...
19	NaN	NaN	760000	2800000	NaN	440000	4400000	9900000	Pgk1	sp|P09...	Phosp...
20	NaN	100000...	NaN	NaN	330000	2800000	NaN	NaN	Trim28	sp|Q62...	Transc...
21	NaN	3900000	NaN	NaN	100000	530000	NaN	NaN	Lonp1	sp|Q8C...	Lon pr...
22	430000	320000...	2100000	130000...	3900000	110000...	4200000	4300000	Eef1a1	sp|P10...	Elonga...

2. Log2(x) transformation of data (Processing -> Basic -> Transform) to simplify further analysis.

3. Define groups based on replicates in Set1 and Set2 – Sum Intensity C#1 and Sum Intensity C#5 come under group 1 and so on. This step is performed through Processing -> Annot. Rows -> Categorical annotation rows).

4. The next step is Filtering – to filter out contaminants and non-specific interactions. The idea behind filtering is to rule out proteins that were identified in Set1 and not Set2 and vice-versa.

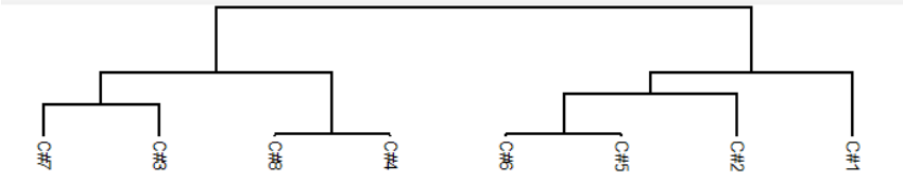
   This step is carried out using function (Processing -> Filter rows -> Filter rows based on valid values -> Minimum 2 valid values in each group). Due to filtering, out of 1039 hits, only 375 hits are left in the dataset.

Data | Clustering

	Sum Intensity C#1	Sum Intensity C#2	Sum Intensity C#3	Sum Intensity C#4	Sum Intensity C#5	Sum Intensity C#6	Sum Intensity C#7	Sum Intensity C#8	T: Gene Symbol	T: referen...	T: Annota...
Type	Main	Main	Main	Main	Main	Main	Main	Main	Text	Text	Text
Group1	1	2	3	4	1	2	3	4			
1	35.5121	34.8974	33.9848	37.3067	36.6787	35.1068	36.8042	36.8042		SjGST...	japonic...
2	25.9316	26.5014	20.8576	22.6757	25.4234	26.2353	19.6096	21.654	Hspa5	sp|P20...	78 kDa...
3	21.7796	25.1795	17.2877	18.417	23.1795	24.6865	18.8423	19.8269	Hsp90...	sp|P08...	Endop...
4	17.6096	19.6971	21.6971	23.0854	21.1332	21.8576	19.4576	22.3101	Atp5b	sp|P56...	ATP sy...
5	21.3645	22.8385	26.1984	28.4234	23.391	24.632	28.2535	29.0609	Actb	sp|P60...	Actin, c...
6	24.019	22.0691	19.5545	21.5638	23.1642	24.632	18.68	22.2821	Pdia3	sp|P27...	Protein...
7	20.417	25.2891	NaN	20.5165	23.391	25.3578	17.5356	NaN	Hsp90...	sp|P11...	Heat s...
8	21.1332	22.9495	14.6662	NaN	24.1015	24.5165	19.4576	21.2535	Pkm	sp|P52...	Pyruva...
9	22.7182	22.6096	16.7471	19.5356	21.9316	23.7389	NaN	20.417	Hspd1	sp|P63...	60 kDa...
10	20.8576	23.391	19.8576	24.3239	24.6865	22.1332	19.417	22.9847	Atp5a1	sp|Q03...	ATP sy...
11	23.239	22.8385	18.5356	17.3752	23.2535	23.632	17.2877	NaN	Hspa9	sp|P38...	Stress...
12	NaN	23.5165	NaN	NaN	17.7471	19.2877	NaN	NaN	Tfrc	sp|Q62...	Transf...
13	21.1332	24.1795	NaN	NaN	22.2243	23.2243	16.1357	19.1457	Hspa8	sp|P63...	Heat s...
14	22.6096	20.6971	16.8727	NaN	20.0691	19.5356	NaN	NaN	Lcp1	sp|Q61...	Plastin...
15	NaN	22.0359	NaN	17.2877	17.5356	20.6096	NaN	NaN	Uba1	sp|Q02...	Ubiqui...
16	NaN	22.6757	18.8727	24.019	19.1457	21.6096	22.9672	23.1795	Gapdh	sp|P16...	Glycer...
17	19.8727	19.0951	19.9316	20.3101	22.6096	21.5165	19.9316	21.3645	Vim	sp|P20...	Vimen...
18	NaN	NaN	21.002	22.2821	18.3321	NaN	20.3101	21.8191	Eno1	sp|P17...	Alpha-...
19	NaN	NaN	19.5356	21.417	NaN	18.7471	22.0691	23.239	Pgk1	sp|P09...	Phosp...
20	NaN	23.2535	NaN	NaN	18.3321	21.417	NaN	NaN	Trim28	sp|Q62...	Transc...
21	NaN	21.895	NaN	NaN	16.6096	19.0156	NaN	NaN	Lonp1	sp|Q8C...	Lon pr...

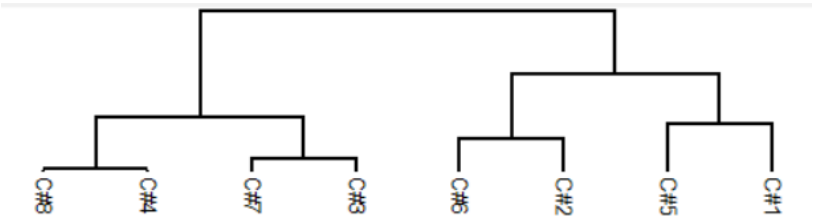
375 items

5. To check if data requires imputation or not, Hierarchical clustering is performed using function (Analysis -> Clustering -> Hierarchical clustering).



Due to missing data, C#5 is interpreted closer to C#6 rather than C#1 (C#1 and C#5 are replicates). Thus, imputation is required.

6. To replace missing data from the normal distribution, the following function is used: (Processing -> Imputation -> Replace missing values from normal distribution) Hierarchical clustering is performed again after imputation.



Now, clustering displays replicates and respective groups correctly – experiment and data processing worked correctly.

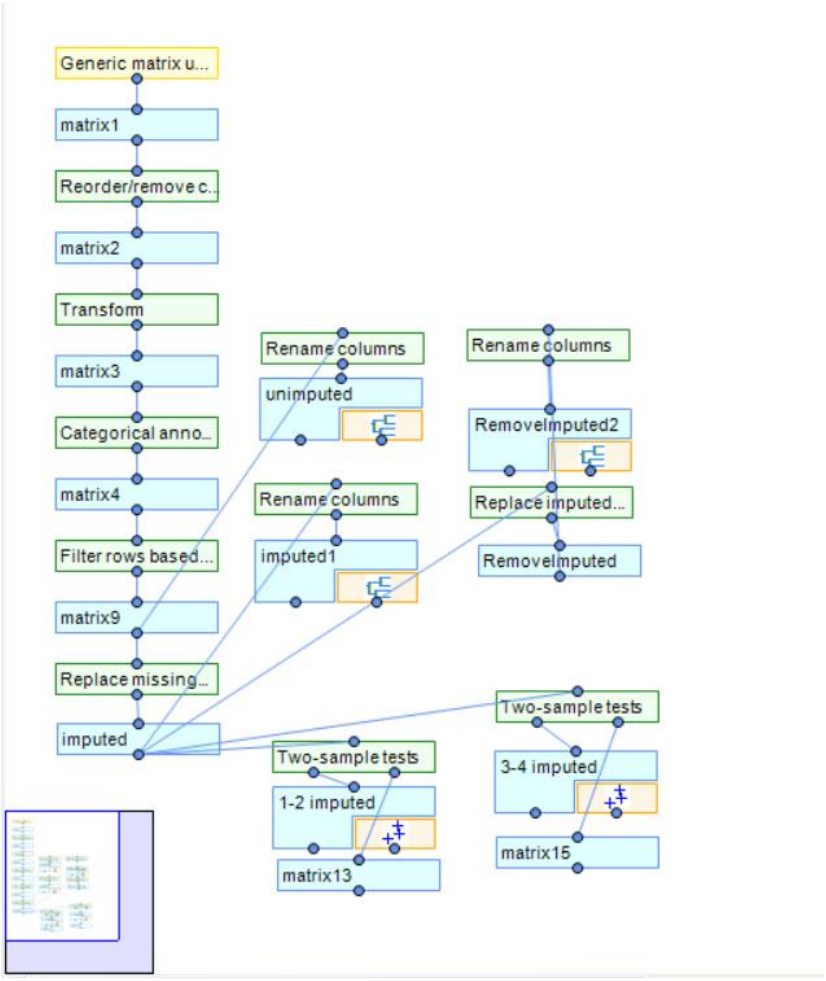
7. Two sample t-tests were performed using the function (Processing -> Tests -> Two-sample tests). Tests are performed in Group 1 vs Group 2 and Group 3 vs Group 4. Significant proteins are identified with a separate column with a + sign.

Data | Scatter plot

	Sum Intensity C#1	Sum Intensity C#2	Sum Intensity C#3	Sum Intensity C#4	Sum Intensity C#5	Sum Intensity C#6	Sum Intensity C#7	Sum Intensity C#8	C: Studen... T-test	C: Studen... T-test	N: -Log Studen... T-test	N: Studen... T-test	N: Studen... T-test	N: Studen... T-test	T: Gene Symbol	T: refer
Type	Main	Main	Main	Main	Main	Main	Main	Main	Catego...	Catego...	Numeric	Numeric	Numeric	Numeric	Text	Text
Group1	1	2	3	4	1	2	3	4								
1	12.9626	20.3101	12.5855	16.3733	13.9657	21.6971	13.5532	14.5434	+	1_2	1.89817	0.0450...	-7.53946	-8.80915	Pklr	sp|P
2	14.3811	19.417	11.3214	14.3698	14.5166	18.5731	13.7093	15.0219	+	1_2	2.05944	0.0551...	-4.54621	-10.6381	Ndufa2	sp|Q
3	13.1763	16.1155	13.5416	14.9122	13.8644	16.9882	12.9801	13.6481	+	1_2	1.49497	0.05984	-3.03147	-5.45587	Rnmt	sp|Q
4	13.676	15.8238	11.5084	15.6954	13.0148	15.3233	13.9638	14.4414	+	1_2	1.48253	0.0534...	-2.22815	-5.3744	Cotl1	sp|Q
5	17.68	19.417	12.3275	17.5163	17.8113	19.2419	12.8045	13.6468	+	1_2	2.3244	0	-1.5838	-14.4761	Snu13	sp|Q
6	14.7814	17.4576	12.3101	15.8722	14.1875	17.8113	14.0952	13.5328	+	1_2	1.92712	0.0496	-3.15001	-9.11337	Ndufa5	sp|Q
7	13.2994	19.5356	12.9825	15.1183	12.7115	19.0156	11.839	14.5036	+	1_2	2.40963	0	-6.27018	-15.9788	Sdf2l1	sp|Q
8	16.7471	21.5165	12.6743	15.3056	15.5258	21.1946	14.244	15.2763	+	1_2	1.84379	0.0714...	-5.21912	-8.26394	Rps29	sp|P
9	14.0912	17.5356	12.4965	15.3287	13.9642	17.3752	12.9636	14.5542	+	1_2	3.05084	0	-3.4277	-33.5065	Dhfr	sp|P
10	13.819	16.283	15.2877	15.2886	13.9134	16.0102	18.0951	19.5545	+	1_2	2.39982	0	-2.2804	-15.7982	Hnrnpdl	sp|Q
11	13.9331	15.16	13.1672	15.2137	13.9658	15.392	15.2137	17.1946	+	1_2	2.11285	0.062	-1.32656	-11.3214	Actr3b	sp|Q
12	18.1457	15.3902	12.6597	14.5367	18.9601	14.5451	12.1312	13.9237	+	1_2	1.58908	0.0434...	3.58526	6.10972	Isca2	sp|Q
13	18.9601	15.3008	12.1606	15.6536	18.9601	13.6492	12.9947	15.9752	+	1_2	1.49122	0.0554...	4.48514	5.43123	Isg15	sp|Q
14	13.0214	17.1946	12.3625	15.3579	13.3501	18.2419	14.195	13.6447	+	1_2	1.84321	0.0666...	-4.53248	-8.25832	Cdc42	sp|P
15	13.7077	19.6628	13.6998	16.003	11.8855	20.7796	14.301	14.6842	+	1_2	1.69695	0.05	-7.42456	-6.94784	Rpl17	sp|Q
16	20.6971	21.9316	12.8542	14.3743	20.5165	21.5638	14.6993	16.1643	+	1_2	1.51207	0.0416...	-1.14089	-5.56973	Rps28	sp|P
17	13.5854	18.4972	12.2337	16.1605	14.6135	18.4972	12.8142	15.8619	+	1_2	1.87328	0.0413...	-4.39773	-8.5555	Arpc3	sp|Q
18	14.021	18.3752	13.5764	14.155	15.0732	18.0426	13.7987	14.8165	+	1_2	1.65835	0.0476...	-3.66181	-6.63635	B4galnt1	sp|Q
19	14.3264	21.417	13.7104	15.8775	13.6455	19.714	13.9665	14.8421	+	1_2	1.72404	0.0555...	-6.5795	-7.17466	Rab31	sp|Q
20	15.1791	17.6096	14.7508	14.1899	15.8238	18.3321	14.8379	15.2158	+	1_2	1.43947	0.04675	-2.46942	-5.10079	Rps15	sp|P
21	13.4115	15.6662	13.1196	18.1457	13.8991	15.8238	18.7796	17.4576	+	1_2	1.83265	0.0625	-2.08966	-8.15632	Tardbp	sp|Q

8. Scatter plot [t-test difference vs -log t-test p value] is generated with the resulting matrix using the function (Analysis -> Visualization -> Scatter plot) and the significant protein category is selected and displayed in red font.

9. Complete workflow:



1.2.2.2 STRING: functional protein association networks

- STRING was accessed through the webpage (https://string-db.org/).
- 'Multiple proteins' tab from the left panel was selected and protein identifiers from Perseus analysis were copied in the appropriate format.
- In the 'Organism' tab, *Mus musculus* was selected as GST pulldown was performed using RAW264.7 Murine macrophages.
- Click on Search and desired protein identifiers from the confirmation page were selected and clicked on 'Continue'.

- STRING network was generated. Node colors, Interaction color codes were accessed through the 'Legend' tab and network image saved using the 'Exports' tab.

1.2.2.3 PANTHER Classification system

- PANTHER Classification system was accessed through the webpage (http://www.pantherdb.org/)
- Gene list analysis tab was selected and in the ID list dialogue box, hits from Perseus analysis were entered in the appropriate format.
- Under the 'Organism' tab, *Mus musculus* was selected.
- Under the 'Analysis' tab, 'Functional classification viewed in graph chart' was selected and the query was submitted.
- Desired graph charts were generated and analyzed along with the STRING network.

1.2.2.4 Studying Anti-PCNA antibody binding by Western blotting

- Sample preparation: Loading dye was added to lysates and heated at 95°C for 15 mins.
- SDS-PAGE gel was run and proteins was transferred on activated PVDF membrane.
- After the transfer of proteins on the PVDF membrane, blot was kept in blocking buffer (5% BSA in 1X TBST) overnight at 4°C.
- Primary antibody anti-mouse PCNA was added in 1:1000 dilution in 5% BSA and incubated for 3 hours at room temperature on a rocker.
- After incubation, the primary antibody was removed and the blot was washed 3 times with 1X TBST after every 15 min.
- Secondary antibody anti-mouse IgG peroxidase was added in 1:5000 dilution in 1X TBST and incubated for 1 hour at room temperature.
- After incubation, the secondary antibody was removed and the blot was washed 3 times with 1X TBST after every 15 min.
- Blot was developed using ECL substrate and ImageQuant® LAS4000 Imager.

1.2.2.5 Studying role of SteA in Salmonella Typhimurium SPI-2 mediated apoptosis by Annexin-FITC/PI Assay.

Infection:

- Bacterial stocks of *Salmonella enterica* Typhimurium SL1344 (wt and ΔsteA) were obtained and streaked on LA + Strep and LA + Strep-Kan plates respectively and grown overnight at 37°C.
- Primary cultures (3mL each) of bacterial strains were grown in: LB + Strep for wt and LB + Strep-Kan for ΔsteA at 37°C overnight with shaking.
- To prepare LPM culture for SPI-2 induction: 500µL of LB culture was taken in 1.5mL MCT and centrifuged at 10,000 rpm for 3mins at room temperature. After carefully removing the supernatant, 500µL of LPM media was added in MCT. Further, 3.6mL of LPM was taken in 15mL polypropylene tube and antibiotics (Streptomycin and Kanamycin) were added in wt and ΔsteA. Further, 400µL of LPM-culture was added from MCT to 15 mL polypropylene tube – making the final volume 4mL. The culture was grown at 37°C overnight with shaking.
- **Infection of RAW264.7 macrophages:** 0.5 million cells/well were plated 10 hours before infection in 24 well plate. Initial OD_{600} was measured for both LPM cultures using a spectrophotometer. The volume required for OD_{600} of 1 was calculated and divided into 2 mct for each culture and then centrifuged for 3mins at 10,000 rpm at room temperature. The supernatant was carefully removed and 100µL was left to keep pellet undisturbed and then resuspended in sterile 1x PBS for the final volume of 1mL. Then using 1x PBS as blank, OD_{600} was measured in a spectrophotometer using the same cuvette. OD_{600} was made equal for both cultures till the second decimal place. Since at 1 OD_{600}, 1mL bacterial culture contains approximately $2.5x10^8$ cells, calculations were performed for volume required to ensure MOI of 20 for 0.5 million RAW264.7 macrophages. 20µL Bacterial culture was added in 280µL RPMI + 10% FBS (without antibiotics) for each well and added to macrophages in 24 well plate. Then the plate was centrifuged for 2mins at 500g and incubated at 37°C for 30 mins. After 30 mins, the media was carefully removed and fresh RPMI media with 100µg/mL Gentamicin was added and incubated at 37°C for 1 hour.
- Post 1hour treatment, media was carefully removed and Fresh RPMI media with 20µg/mL Gentamicin was added and plate kept in an incubator at 37°C and 5% CO_2.

Invasion assay:

- To check the equal invasion of both wt and ΔsteA, media is removed from well and cells are harvested inside the well with 450µL 1x PBS. Then 1% Triton X-100 is added and incubated for 30mins at 37°C.
- After 30 mins, cells are harvested in 1.5mL mct and serially diluted to 10^4 and plated on LB agar plates and incubated at 37°C overnight.
- The next day, Bacterial CFU count is performed to check for the equal invasion in both wt and ΔsteA *Salmonella* infected RAW 264.7 macrophages.

Annexin-FITC/PI assay:

- 12hr post-infection, cells were harvested and centrifuged at 1800rpm for 5mins at 4°C. Then cells were washed with 1x PBS twice.
- 1x Binding buffer was prepared from 10x Binding buffer using Milli-Q water. Cells were resuspended in 350µL 1x Binding buffer for each well.
- For Flow cytometric analysis, 100µL of samples were added to each FACS tube and the volume of each dye (Annexin-FITC and PI) to be used in each tube is 2.5µL.
- Samples were prepared as mentioned in the table:

	UNSTAINED	**Single Stained FITC**	**Single Stained PI**	**Double Stained FITC + PI**
wt infected				✔
ΔsteA infected				✔
Staurosporine		✔	✔	✔
Untreated	✔			✔

- Dyes were added according to the sample table and incubated at room temperature for 15 mins in dark.
- 300µL Binding buffer was added to each tube and analyzed in BD Accuri® C6 Flow cytometer.
- Spectral compensation was performed in FlowJo V10 using function ‘Compensation’.

Chapter 2

Result & Discussions

2.1 Experimental Results

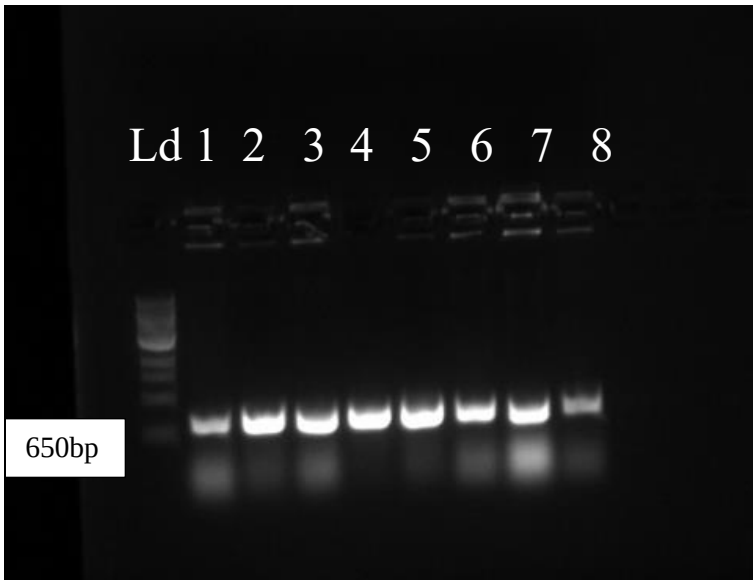
2.2 Discussion

2.3 Future outlook

2.1 Experimental Results

2.1.1 Cloning for Yeast-2-hybrid

- Colony PCR for SteA-BD clones – SteA PCR +ve for all 8 colonies.

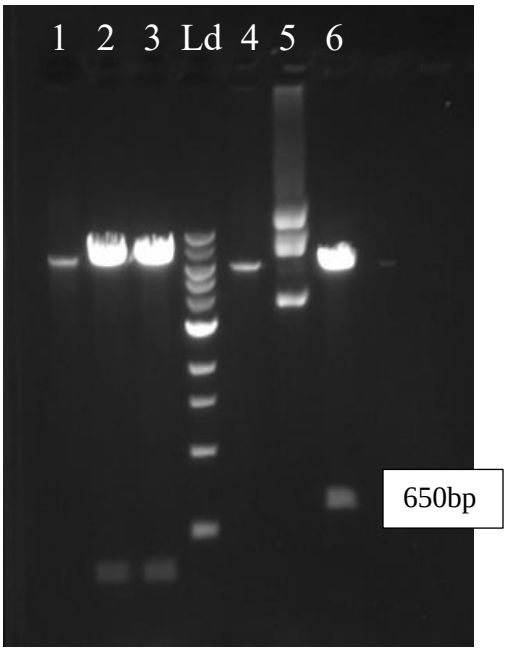


- Double digestion of SteA-BD Plasmids from +ve colonies using BamHI and SalI

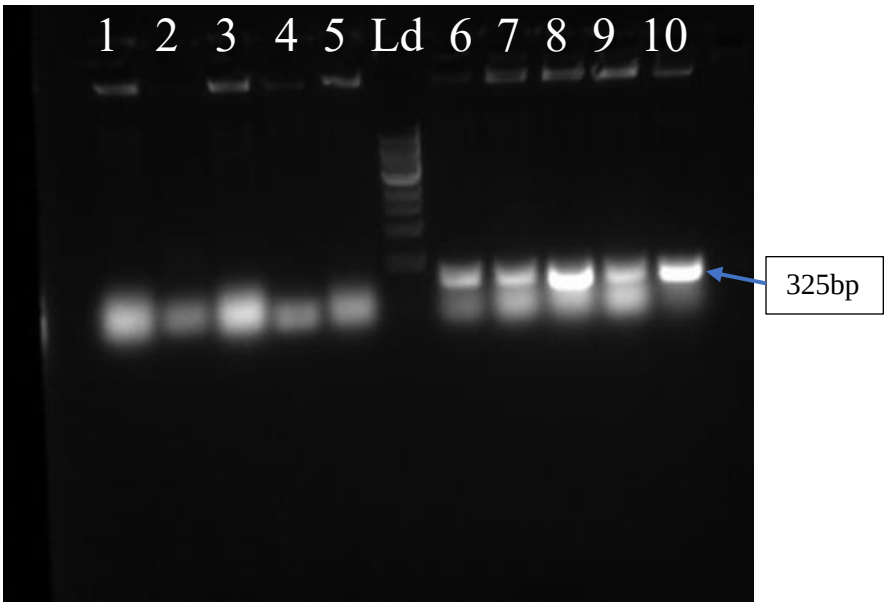
  Lane 4 – BD digested Size- **5.9Kb**

  Lane 5 – SteA-BD digested colony 1 clone✓

  Lane 6 - SteA-BD digested colony 2 clone✓



- Colony PCR for Rbx1-AD clones – Colony 6,7,8,9,10 Positive

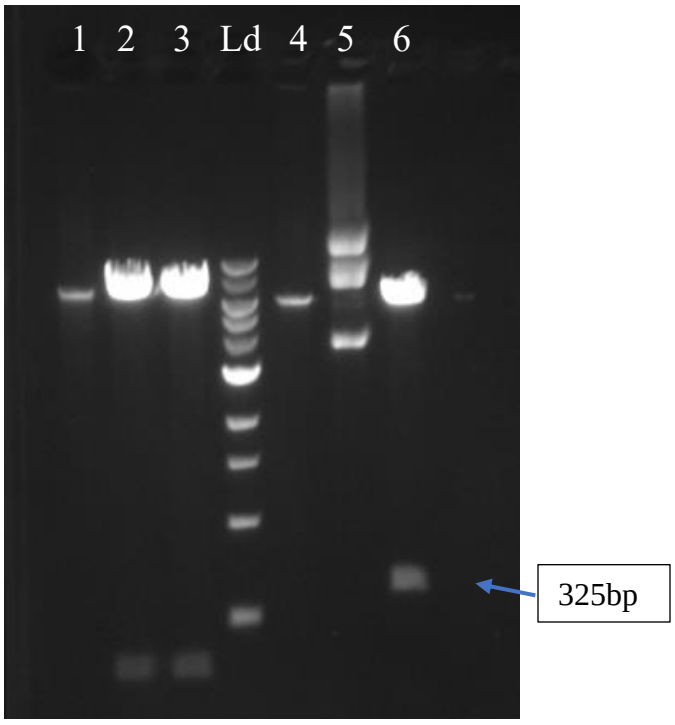


- Double digestion of Rbx1-AD Plasmids from +ve colonies using BamHI and SalI

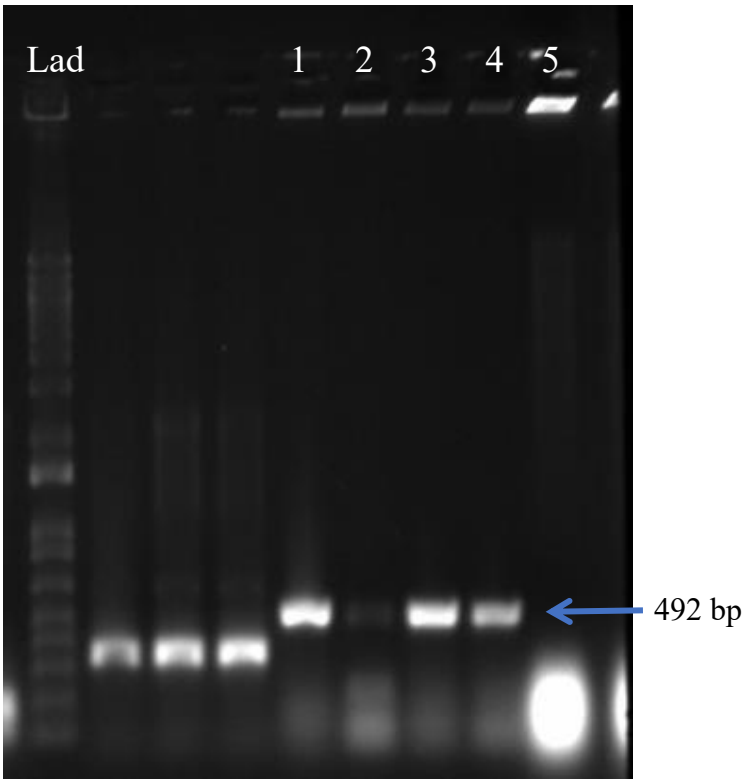
  Lane 1 – AD digested – Size **6.6Kb**

  Lane 2 – RBX1-AD digested colony8 clone✓

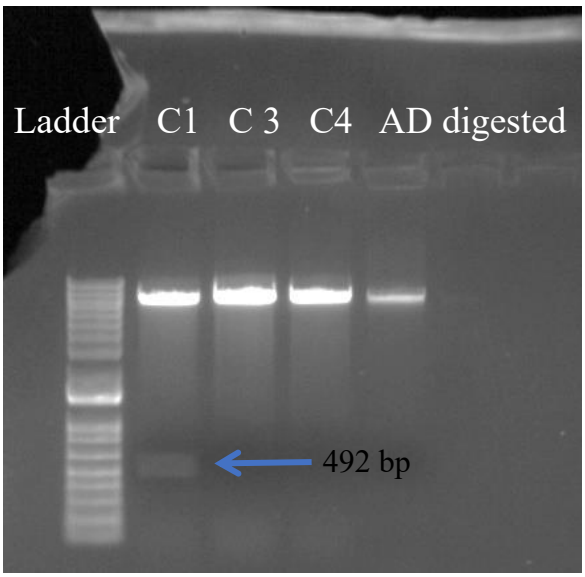
  Lane 3 – RBX1-AD digested colony10 clone✓



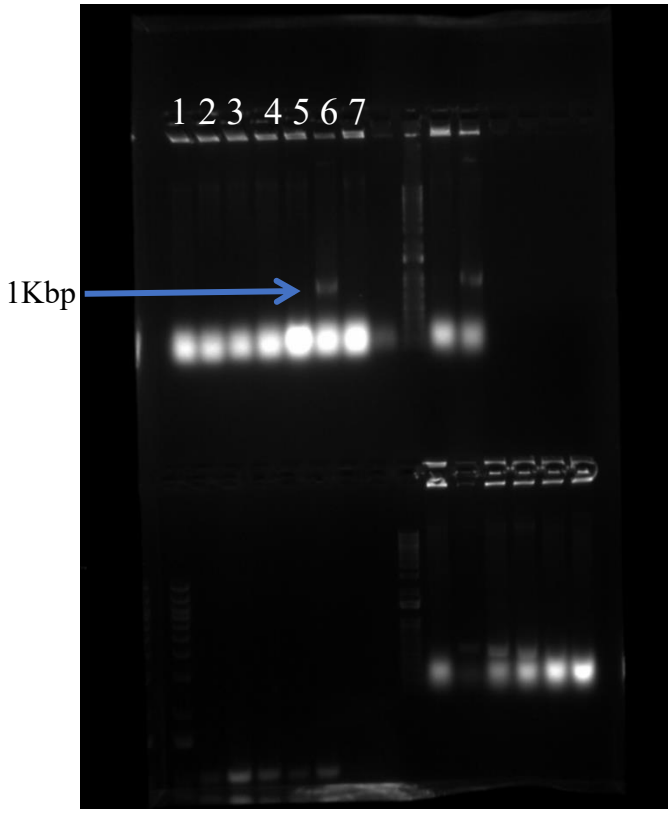
- Colony PCR for Skp1-AD clones – colonies in Lane 1,3,4 positive



- Double digestion of Skp1-AD plasmids from +ve colonies using BamHI and SalI Lane C1 – Skp1-AD clone✓

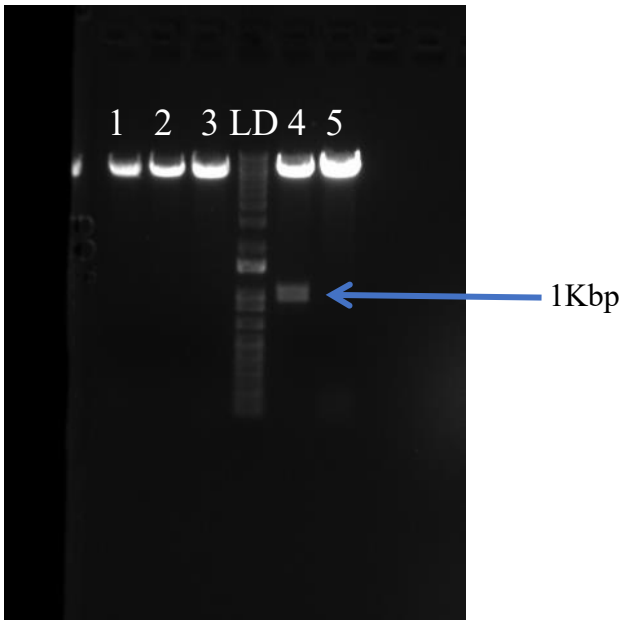


- Colony PCR for IκB-AD clones - Colony 6 positive



- Double digestion of IκB-AD plasmids from +ve colony using BamHI and SalI

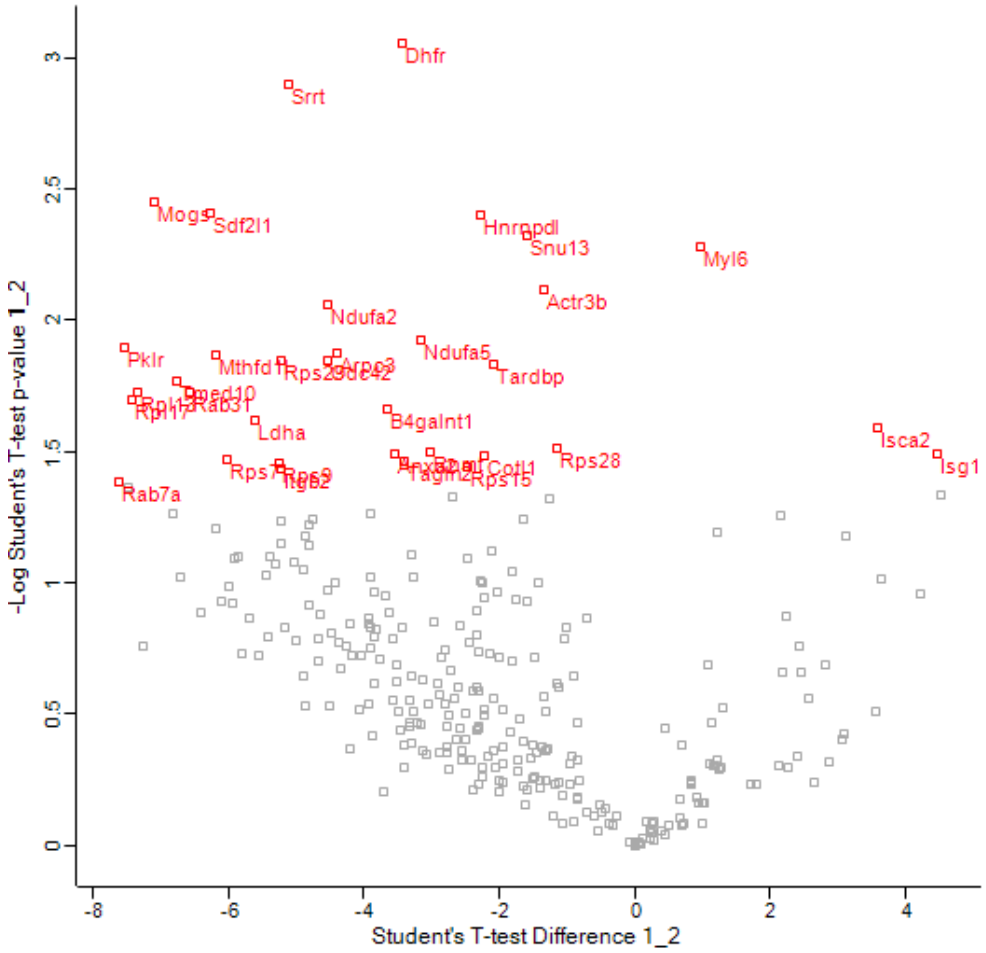
  Lane 4 – Colony 6 - IκB-AD clone✓



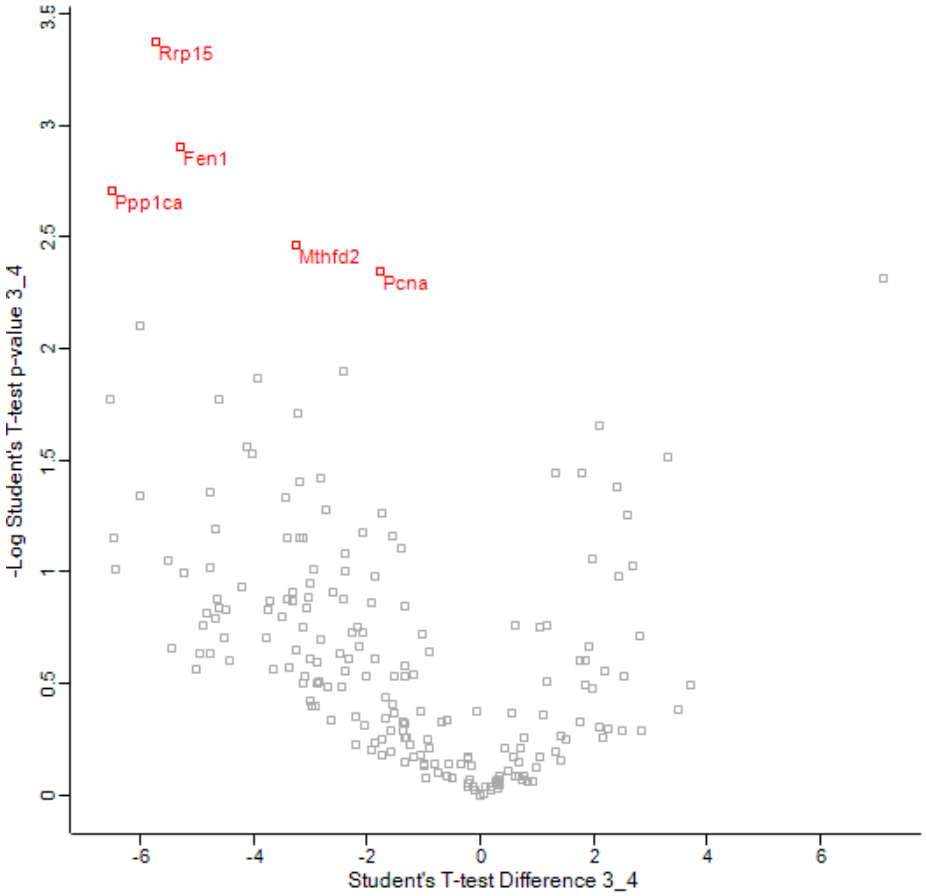
Conclusion: Above results showed that we got right clone for SteA-BD, Rbx1-AD, Skp1-AD and IkB-AD. Further, sequencing results confirmed that they are of correct sequence and there is no mutation.

2.1.2 Significant hits from Mass spectrometry-based proteomics analysis using Persues

Scatter plots (t-test difference vs -Log t-test p-value) for group 1 vs 2 and group 3 vs 4 respectively:



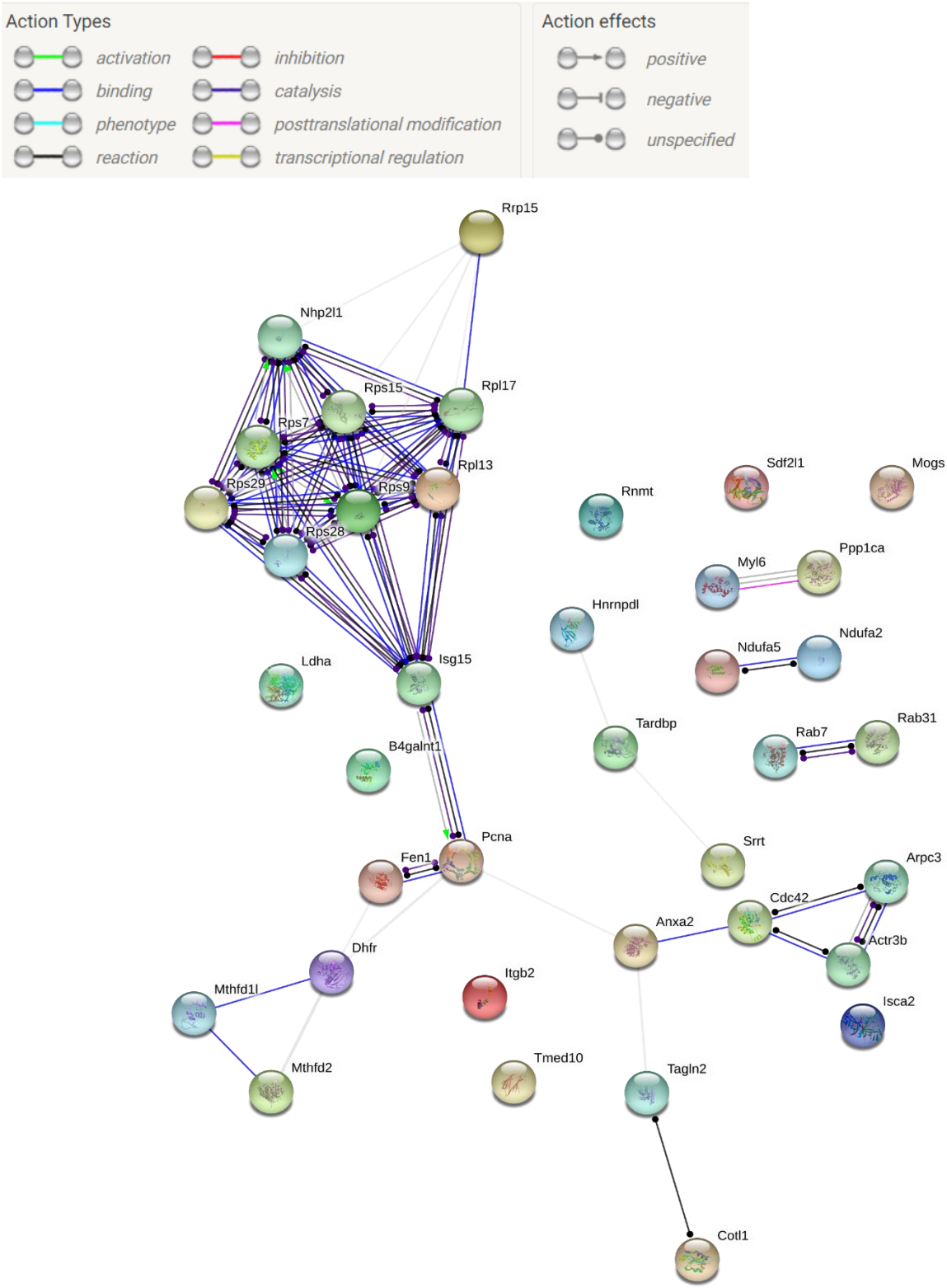
Significant hits from Proteomics analysis using Perseus are displayed in Red font. The X-axis depicts fold-change of protein observed in GST only vs GST-SteA pulldown, while Y-axis depicts the significance of a particular hit.



List of Significant proteins observed in GST-SteA pulldown (Orange for Group 1 v 2) and (Blue for Group 3 v 4), arranged according to significance:

1. DHFR	8. ACTR3B	15. CDC42	22. LDHA	29. RPS7	1.RRP15
2. SRRT	9. NDUFA2	16. TARDBP	23. ISCA2	30. TAGLN2	2.FEN1
3. MOGS	10. NDUFA5	17. TMED10	24. RPS28	31. RPS9	3.PPP1CA
4. SDF2L1	11. PK1R	18. RAB31	25. RNMT	32. RPS15	4.MTHFD2
5. HNRNPDL	12. ARPC3	19. RPL13	26. ANXA2	33. ITGB2	5.PCNA
6. SNU13	13. MTHFD1L	20. RPL17	27. ISG15	34. RAB7A	
7. MYL6	14. RPS29	21. B4GALNT1	28. COTL1		

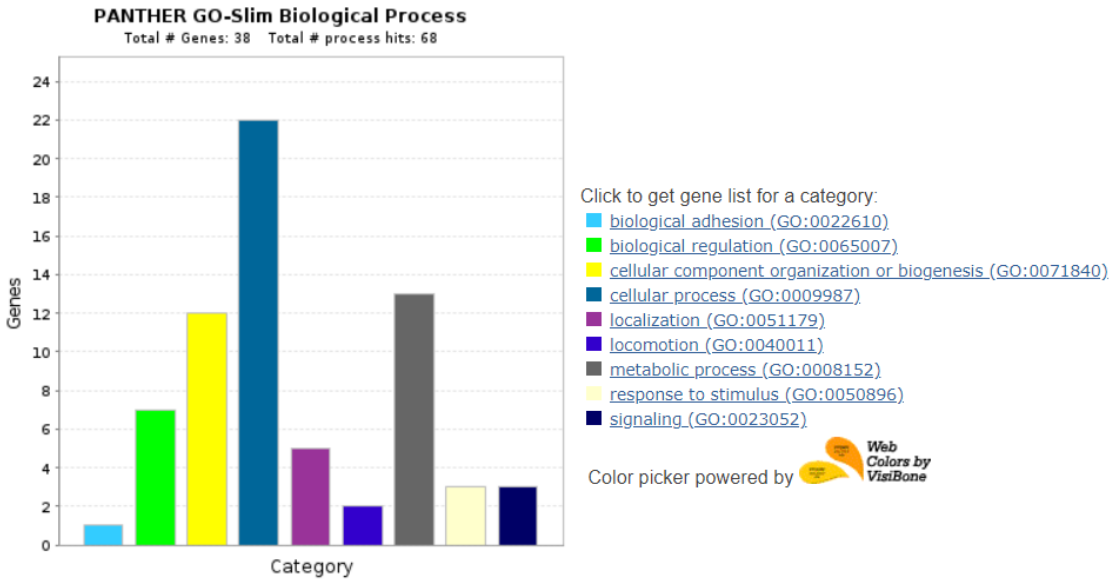
2.1.3 STRING network



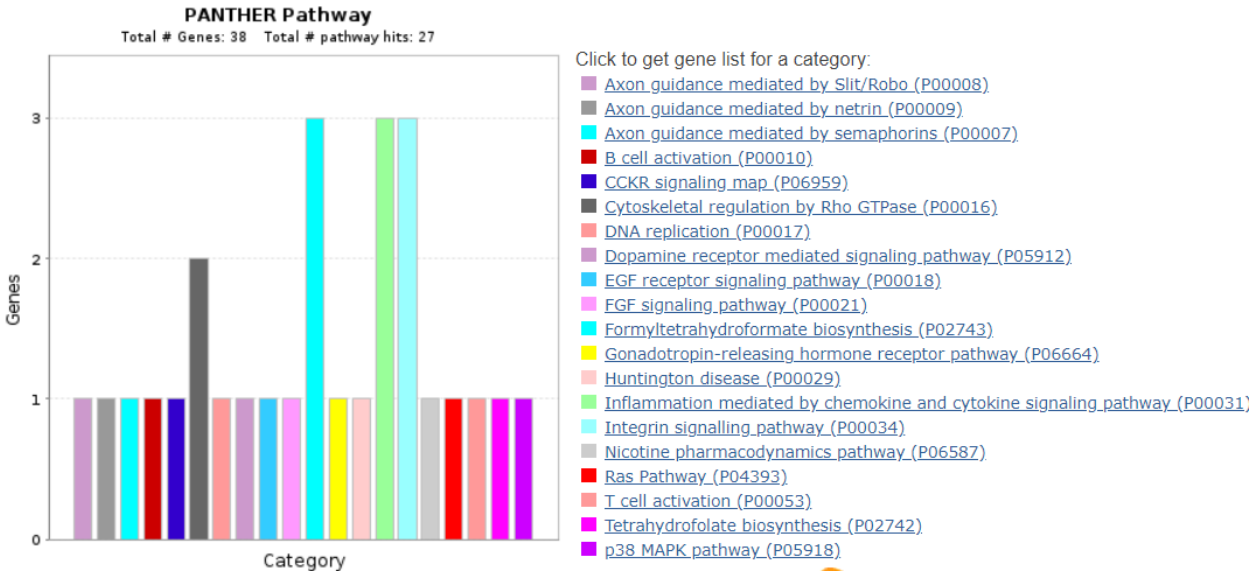
STRING network[17] was generated using significant hits from Perseus analysis and along with PANTHER classification data, pathways in which SteA probably acts are identified.

2.1.4 PANTHER Classification system

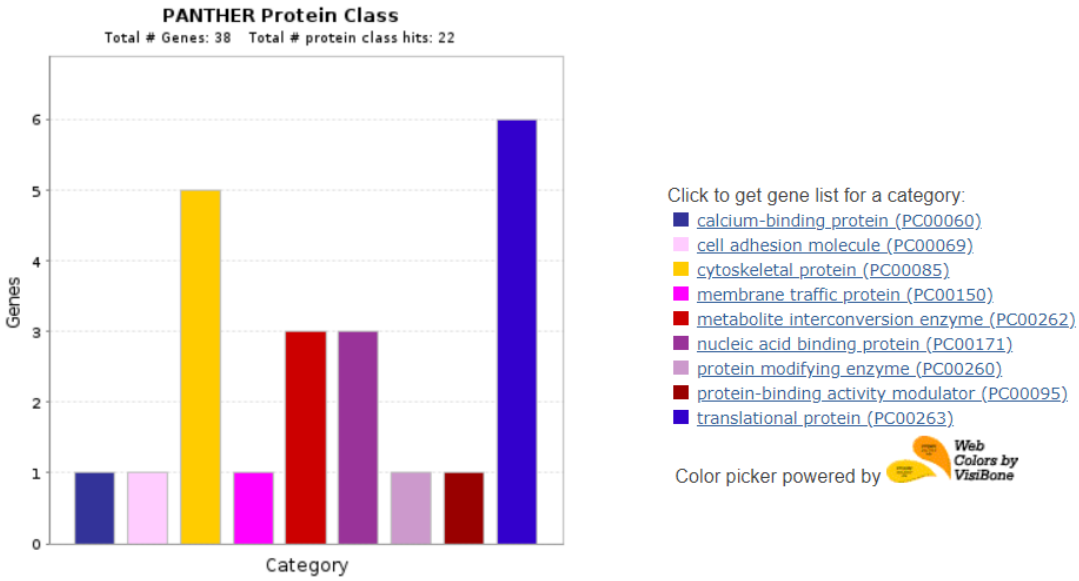
- PANTHER[18] GO-Slim Biological Process depicting biological roles of proteins probably interacting with SteA.



- PANTHER Pathway depicting pathways in which *Salmonella* effector SteA could probably be playing role in.



- PANTHER Protein class depicting protein classes of probable interactors with SteA.



Conclusion: PANTHER Classification system along with the STRING network played an important role in helping classify probable interaction groups of SteA, which could be further studied through experimental techniques. Selected groups for further study are:

- PCNA – ISG15 – FEN1: DNA Translesion synthesis (TLS) pathway
- MTHFD2 – DHFR – MTHFD1L: Formyltetrahydrofolate biosynthesis pathway

2.1.5 SMART Sequence analysis

No confidently predicted domains, repeats, motifs, and features could be detected

2.1.6 Anti-PCNA antibody binding to modified forms of PCNA

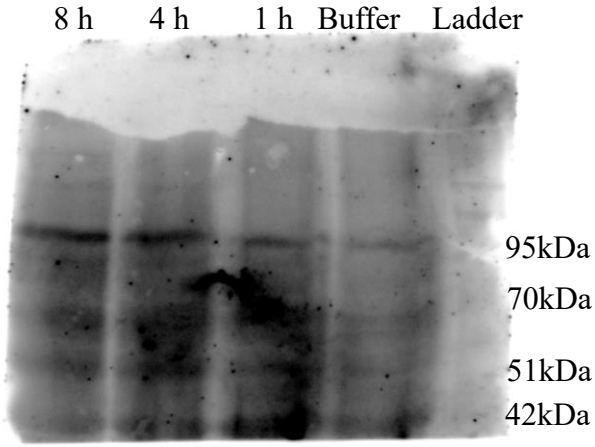
Towards, checking whether SteA interferes with PCNA activity by interacting with it

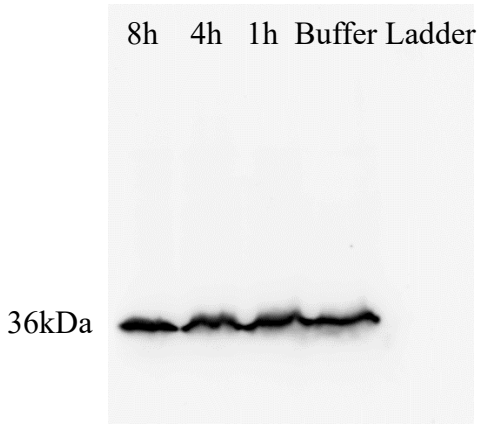
First step-whether we could observe all the forms of PCNA with anti-PCNA antibody.

- SET 1:

  Parameters: 5mM Hydroxyurea (as positive control to obtain all the modified forms of PCNA)

  Time points: 1 h, 4 h, 8 h





Observation: Various modified forms of PCNA were observed as:

Mono-ISGylated PCNA 54kDa

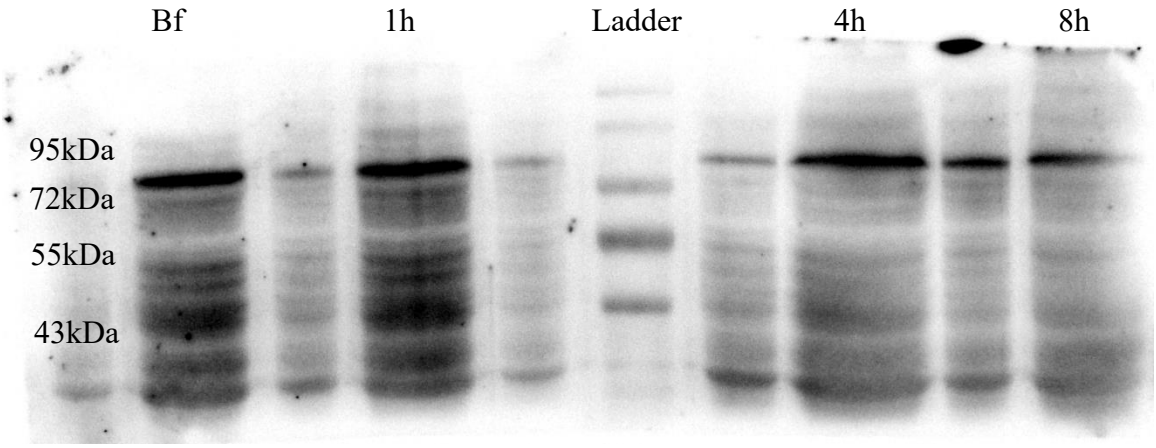
Di-ISGylated PCNA 69kDa

Mono-Ub PCNA 43kDa

- SET 2:

  Parameters: 7mM Hydroxyurea

  Time points: 1hr, 4hr, 8hr



Conclusion: Similar bands were observed at higher concentrations of Hydroxyurea. The intensity of modified PCNA bands was not considered as β-actin protein load was not set. Since anti-PCNA antibody detects modified forms of PCNA, the next step would be Infection of RAW264.7 macrophages with wt and ΔsteA *Salmonella*

Typhimurium and then Co-Immunoprecipitation with anti-PCNA antibody and western blotting with anti-Ubiquitin and anti-ISG15 antibodies respectively.

2.1.7 Flow cytometry-based apoptosis detection experiment

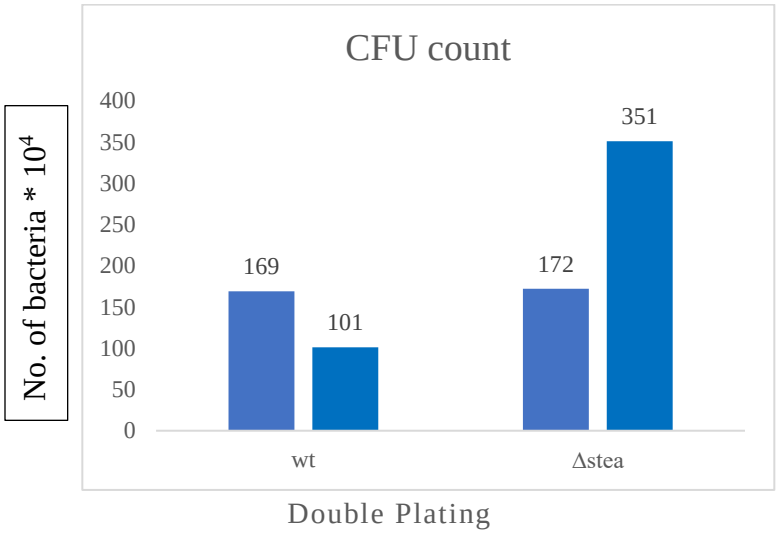
Towards probing whether SteA has any effect on cell death and apoptosis we have treated the cells with wild type (wt) as well as steA-deleted (ΔsteA) *Salmonella* Typhimurium strain.

- Experiment 1:

Parameters – Multiplicity of Infection (MOI) – 50

12 h post-infection

1μM Staurosporine (positive control)

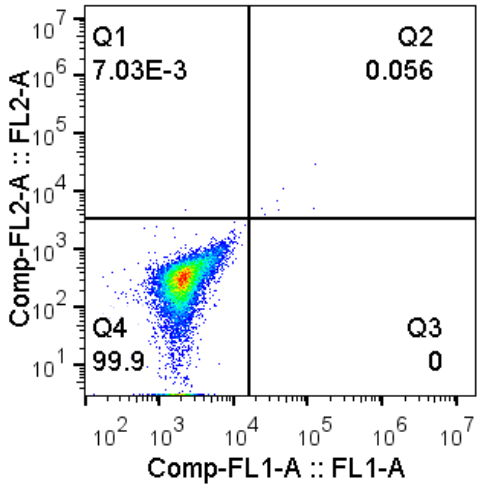


Double Plating

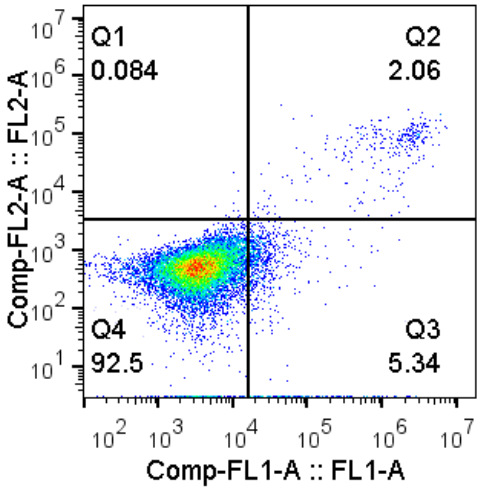
Conclusion: Infected cells from both the samples after 2 h of infection were lysed and plated on an agar plate with dilution and incubated for 24 h. CFU were enumerated to check the invasion. From the result it appears that there is a difference in the initial infection experiment therefore, instead of equal invasion in ΔsteA-infected cells, the invasion was more.

To check whether there is any difference in the membrane flipping (as during apoptosis due to membrane flipping Phosphatidylserine comes to the outer membrane) in wt- and ΔsteA-treated cells, following infection we stained the cells with Annexin-V FITC and PI. Annexin-V binds to the Phosphatidylserine into the outer leaflet and PI intercalates with the DNA of damaged cells.

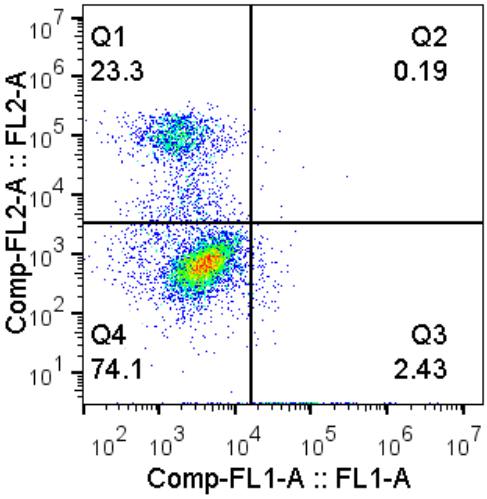
Flow cytometry – FL1 vs FL2 plots: Annexin-V-FITC vs PI



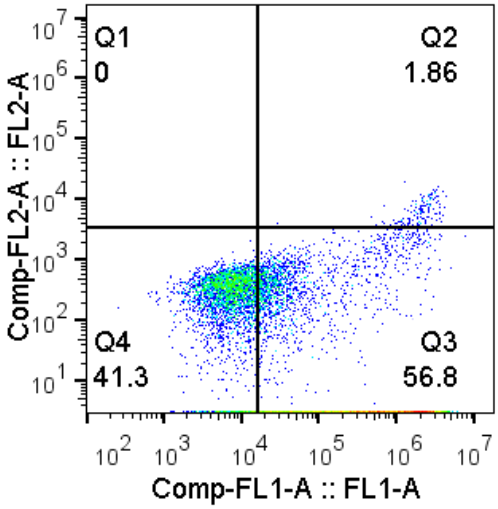
Uninfected - unstained



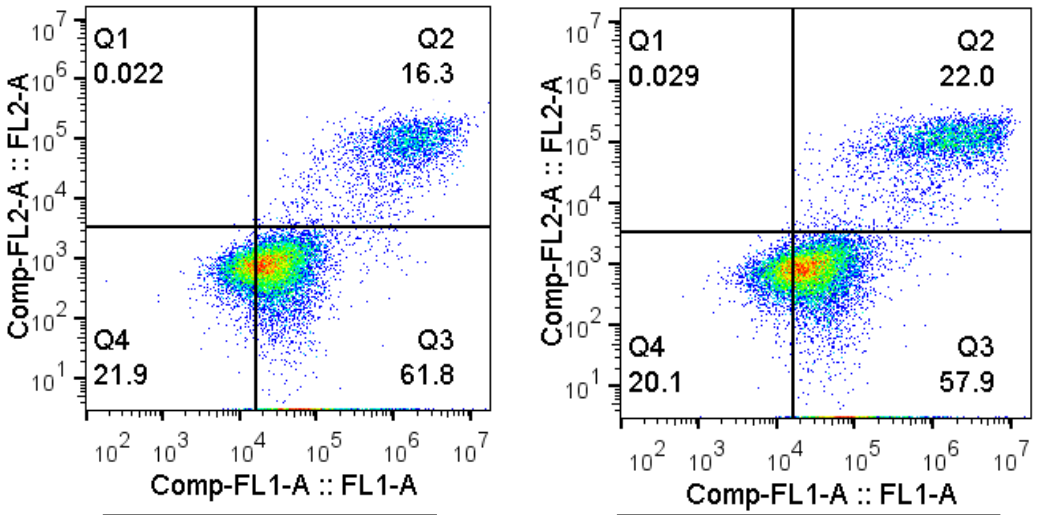
Uninfected – Double stained



Staurosporine - PI



Staurosporine - FITC



wt *Salmonella* infected

ΔsteA *Salmonella* infected

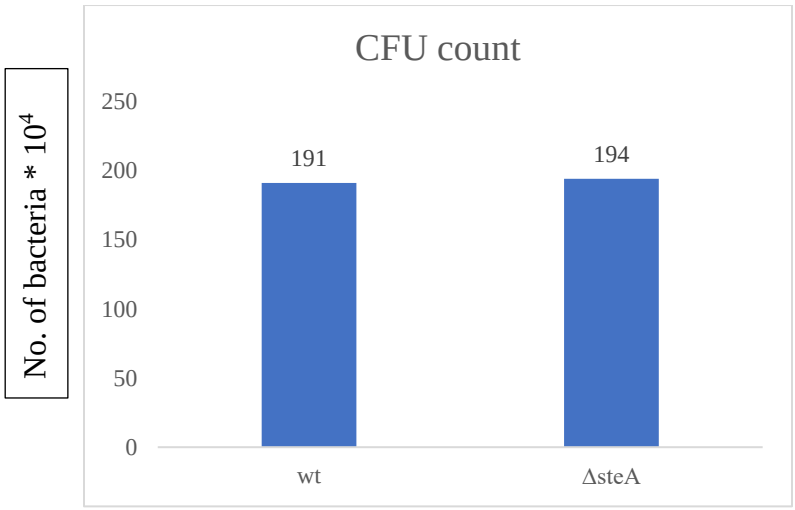
Conclusion: FITC is detected on the FL1 channel while PI is detected on the FL2 channel. Despite the higher average invasion of bacteria in the case of ΔsteA infection, the early apoptotic population (Q3) is lesser than wt *Salmonella* infected. We thought may be the difference in the early apoptotic population will be more pronounced if we decrease the MOI of the infection.

- Experiment 2: Similar experiments were done again with decrease in MOI

  Parameters – MOI – 20

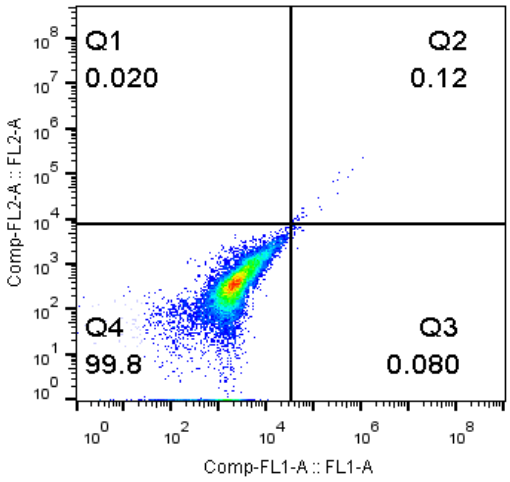
  12 h post-infection

  1μM Staurosporine (as positive control for apoptosis)

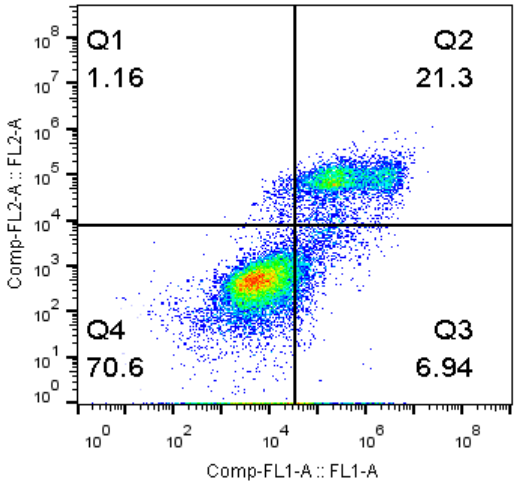


Conclusion: There is similar invasion/infection for wt and ΔsteA-infected cells

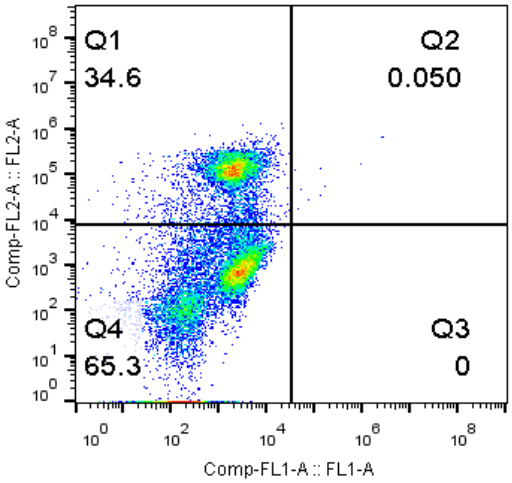
Flow cytometry – FL1 vs FL2 plots: Annexin V-FITC vs PI



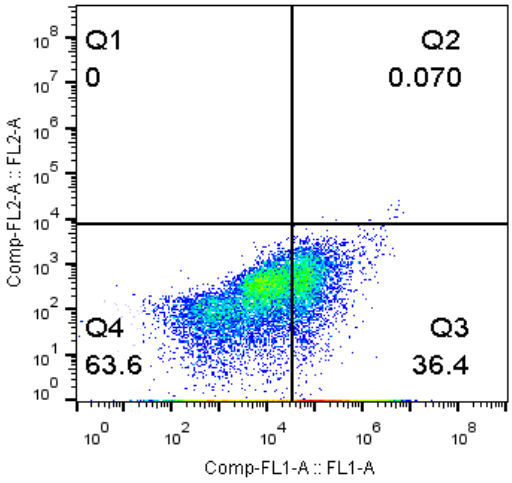
Uninfected - unstained



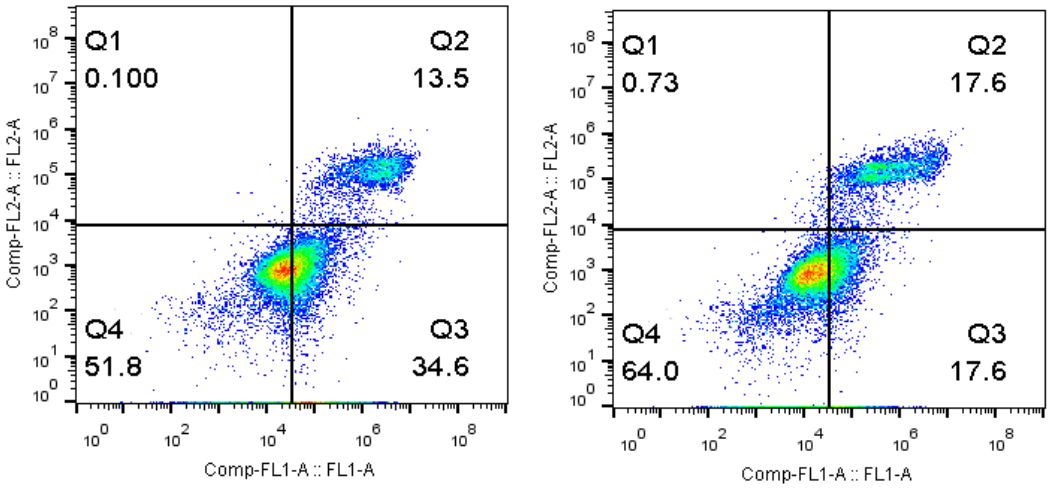
Uninfected – Double stained



Staurosporine - PI



Staurosporine - FITC



wt *Salmonella* infected

ΔsteA *Salmonella* infected

Conclusion: Early apoptotic populations (Q3) are almost halved in ΔsteA *Salmonella* infected RAW264.7 macrophages as compared to wt *Salmonella* infected macrophages. This result indicated that SteA play a role in induction of apoptotic cell death during *S.* Typhimurium infection.

Further, we wanted to check whether folinic acid could rescue the cell death in ΔsteA-treated cell

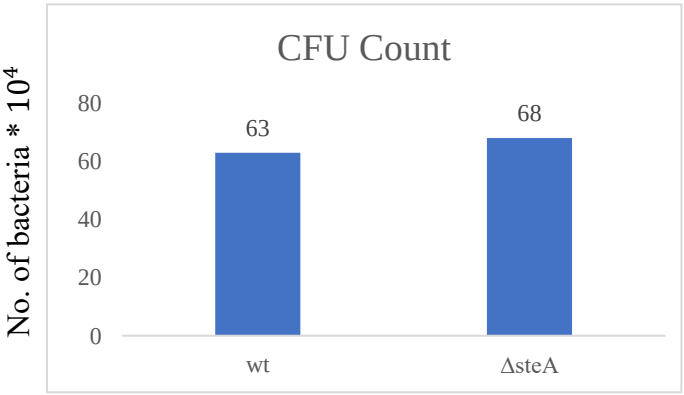
2.1.8 Folinic acid rescue experiment

Parameters – MOI – 20

24 h post-infection

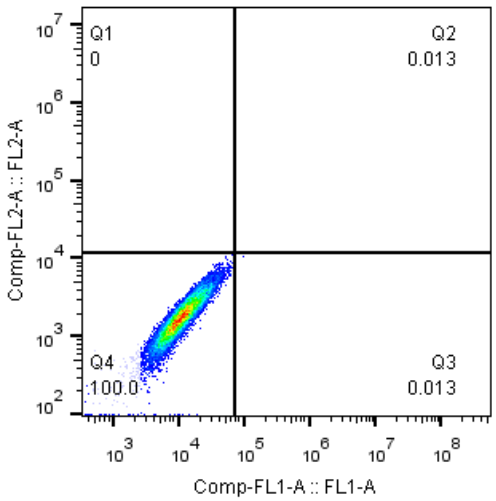
1μM Staurosporine, 200μM Folinic Acid

Invasion assay:

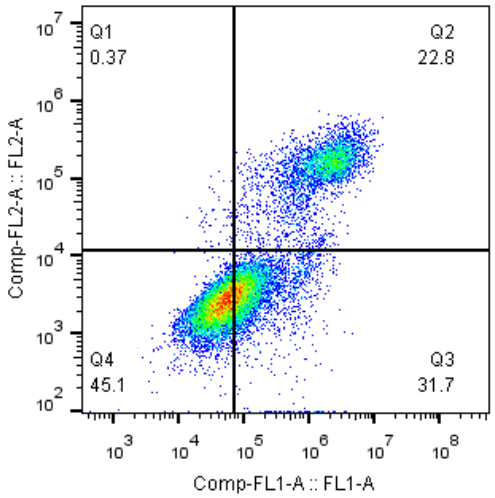


Conclusion: The invasion is equal in both the infections.

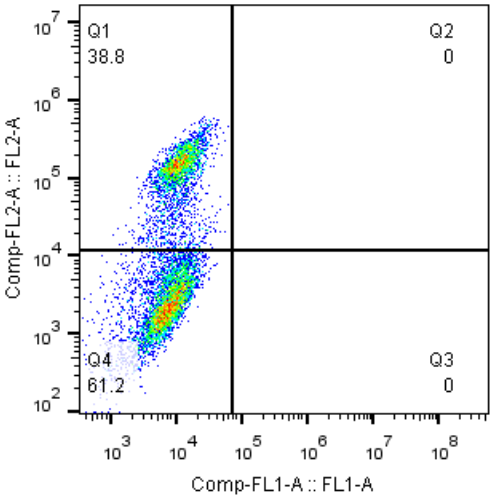
Flow cytometry – FL1 vs FL2 plots: Annexin-V FITC vs PI



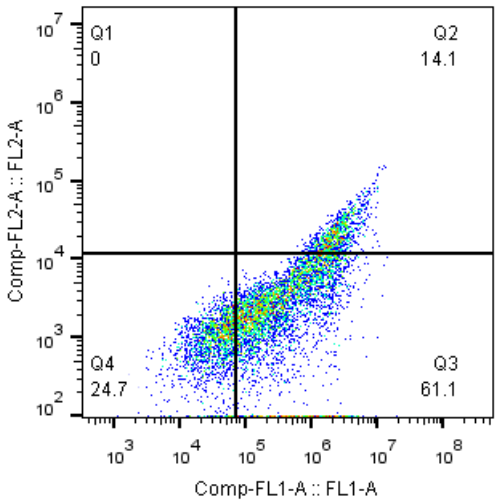
Uninfected - unstained



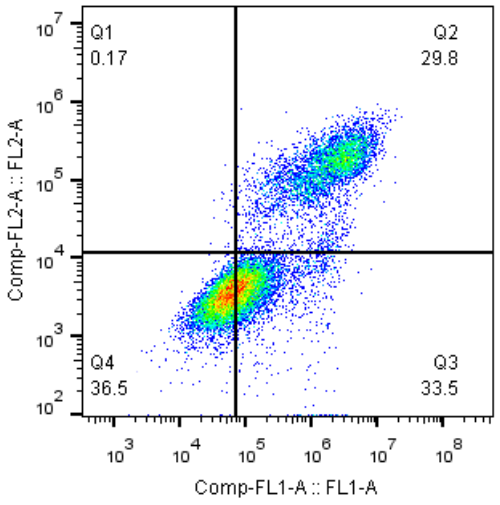
Uninfected – Double stained



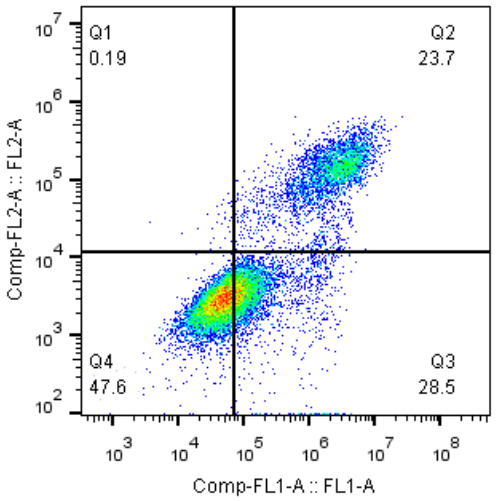
Staurosporine - PI



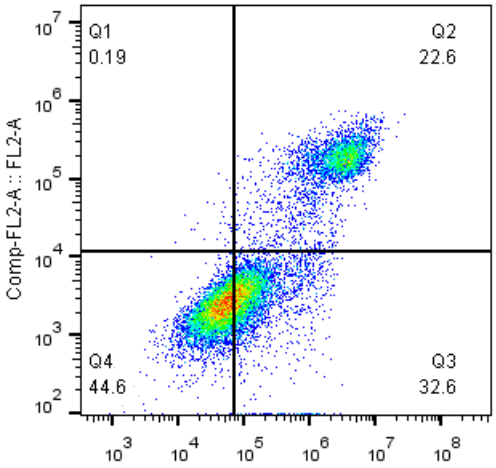
Staurosporine - FITC



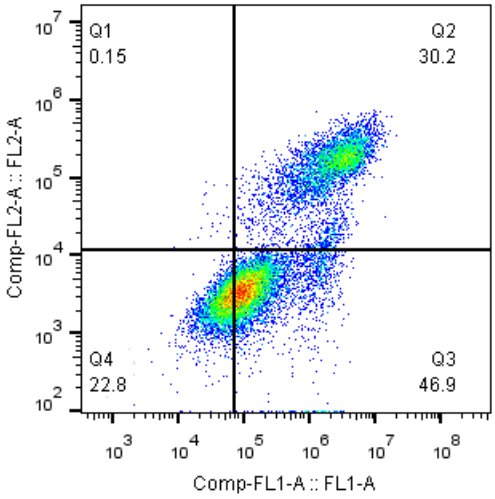
wt *Salmonella* infected



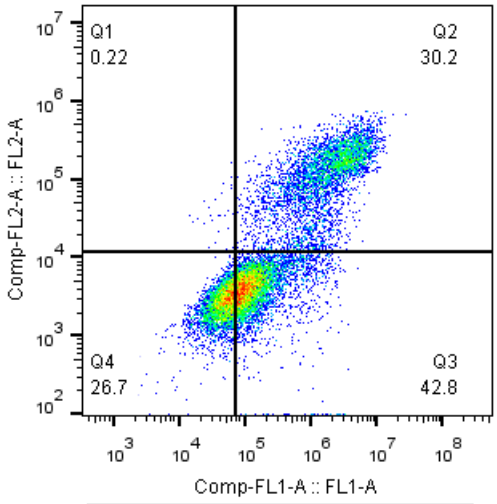
ΔsteA *Salmonella* infected



Uninfected + 200μM Folinic Acid– Double stained

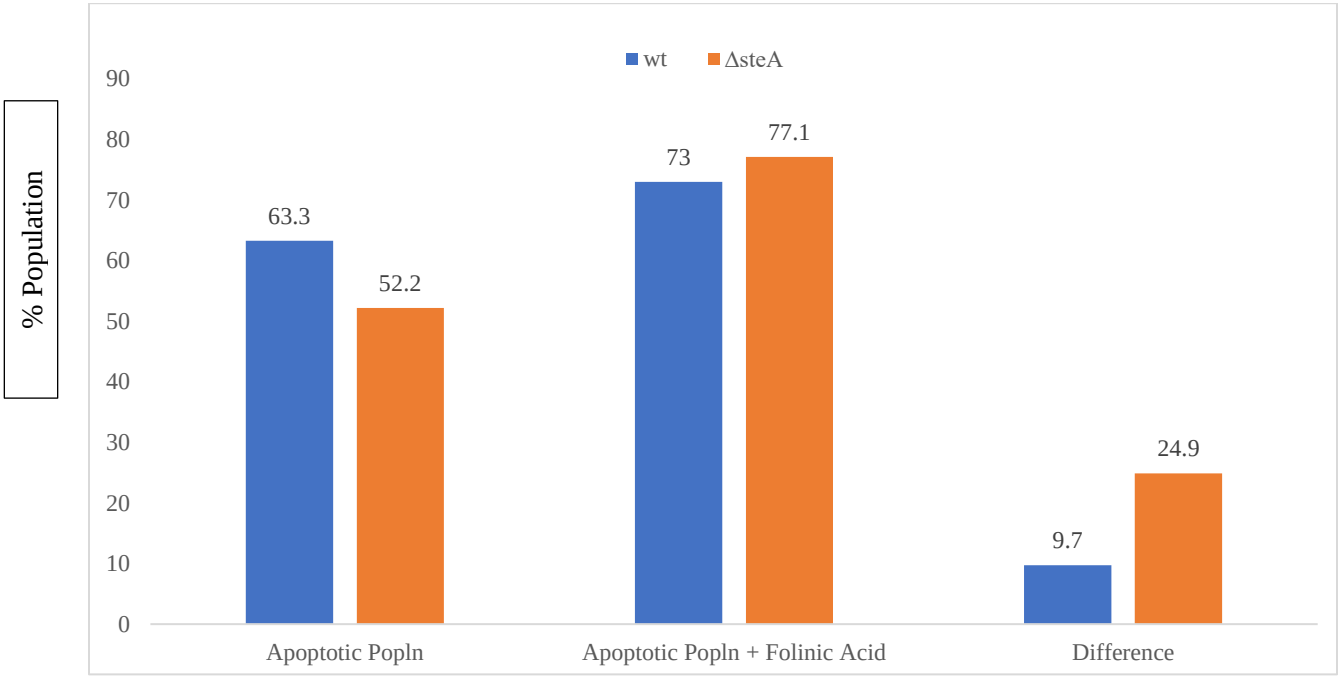


wt *Salmonella* infected
+ 200μM Folinic acid



ΔsteA *Salmonella* infected
+ 200μM Folinic acid

Total apoptotic population (Q2+Q3 i.e., 2nd quadrant +3rd quadrant of the plot or early apoptotic plus late apoptotic) difference:



Conclusion: The addition of Folinic acid resulted in greater apoptosis in both wt and ΔsteA *Salmonella* infected macrophages. This could probably be due to the availability of more Folinic acid for *Salmonella* present inside SCV of infected macrophages. However, the increase in apoptosis in ΔsteA *Salmonella* infected macrophages is much higher on Folinic acid supplementation as compared to wt *Salmonella* infected macrophages. Supplementation to bacteria could be solved by Transfection experiments. *Apoptosis in uninfected was probably observed due to a higher number of passages in RAW264.7 cells.

2.2 Discussion and conclusion:

Bait and prey clones (SteA-BD, Skp1-AD, Rbx1-AD, IκB-AD) for performing Yeast 2-hybrid assay were successfully cloned and correct sequence was observed from sequencing results. To identify other functions performed by *S.* typhimurium effector protein SteA, mass spectrometry-based proteomics analysis using Perseus was performed and, several protein hits were generated which probably interact with SteA. Bioinformatics tools such as STRING network and PANTHER classification system were used to identify pathways from hits, in which SteA might modulate/interfere to aid *Salmonella* pathogenesis. Mass spectrometry analysis revealed several interesting hits – including 40S Ribosomal proteins indicating SteA might play a role in modulating host protein translation. Flow cytometry-based apoptosis detection experiments suggested that SteA might play a role in inducing apoptotic cell death during S. typhimurium infection. Two pathways chosen to further experiments were:

- PCNA – ISG15 – FEN1: DNA Translesion synthesis (TLS) pathway
- MTHFD2 – DHFR – MTHFD1L: Formyltetrahydrofolate biosynthesis pathway

To identify mechanism involved, first step was to check if anti-PCNA antibody binds to modified forms of PCNA. Using western blotting, it was shown to be true. Further, Co-Immunoprecipitation experiment couldn't be performed to check levels of mono-ubiquitinated PCNA after infection with wt and ΔsteA *S.* tyhimurium. Another pathway that could probably be targeted by SteA was tetrahydrofolate synthesis pathway. To check if SteA represses DHFR function, Folinic acid was supplemented to bypass DHFR function. Although overall increase in apoptotic cell death was observed (probably due to increased availability of Folinic acid for *S.* tyhimurium), increase in apoptosis in wt infected cells was much lesser as compared to ΔsteA infected cells suggesting that SteA might play a role in repression of tetrahydrofolate synthesis pathway leading to defects in DNA replication as well as repair – pushing cells into apoptosis. Transfection experiments could help rule out increased bacterial function due to Folinic acid supplementation.

2.3 Future Outlook

Biological replicates need to be performed to consolidate SPI-2 induced SteA's role in apoptosis in macrophages as well as other mammalian cell lines. Future experiments could include:

- Infection of RAW264.7 macrophages with wt and ΔsteA *Salmonella* Typhimurium and Co-Immunoprecipitation with anti-PCNA antibody and western blotting with anti-Ubiquitin and anti-ISG15 antibodies respectively.
- Infection of RAW264.7 macrophages with wt and ΔsteA *Salmonella* Typhimurium and EMSA assay to check binding of PCNA with polymerase-η.
- Transfection of RAW264.7 macrophages with pcDNA3.1(+) carrying HA epitope tagged SteA gene and supplementation with Folinic acid to check difference in apoptosis.

SteA's immunosuppressive role has already been shown by Gulati et. al. (2019) and if its role in DHFR repression is found, it can probably act as a better anti-cancer treatment as compared to traditional anti-folates like Methotrexate.

Bibliography

1. Majowicz, S.E., et al., *The global burden of nontyphoidal Salmonella gastroenteritis.* Clin Infect Dis, 2010. **50**(6): p. 882-9.
2. Zhang, S., et al., *Molecular pathogenesis of Salmonella enterica serotype typhimurium-induced diarrhea.* Infect Immun, 2003. **71**(1): p. 1-12.
3. Ramos-Morales, F., *Impact of Salmonella enterica Type III Secretion System Effectors on the Eukaryotic Host Cell.* ISRN Cell Biology, 2012. **2012**: p. 1-36.
4. Domingues, L., D.W. Holden, and L.J. Mota, *The Salmonella effector SteA contributes to the control of membrane dynamics of Salmonella-containing vacuoles.* Infect Immun, 2014. **82**(7): p. 2923-34.
5. Gulati, A., R. Shukla, and A. Mukhopadhaya, *Salmonella Effector SteA Suppresses Proinflammatory Responses of the Host by Interfering With IkappaB Degradation.* Front Immunol, 2019. **10**: p. 2822.
6. Paunesku, T., et al., *Proliferating cell nuclear antigen (PCNA): ringmaster of the genome.* Int J Radiat Biol, 2001. **77**(10): p. 1007-21.
7. Zhu, Q., et al., *Post-translational modifications of proliferating cell nuclear antigen: A key signal integrator for DNA damage response (Review).* Oncol Lett, 2014. **7**(5): p. 1363-1369.
8. Rhen, M., *Salmonella and Reactive Oxygen Species: A Love-Hate Relationship.* J Innate Immun, 2019. **11**(3): p. 216-226.
9. Nakad, R. and B. Schumacher, *DNA Damage Response and Immune Defense: Links and Mechanisms.* Front Genet, 2016. **7**: p. 147.
10. Park, J.M., et al., *Modification of PCNA by ISG15 plays a crucial role in termination of error-prone translesion DNA synthesis.* Mol Cell, 2014. **54**(4): p. 626-38.
11. Villarroya-Beltri, C., S. Guerra, and F. Sanchez-Madrid, *ISGylation - a key to lock the cell gates for preventing the spread of threats.* J Cell Sci, 2017. **130**(18): p. 2961-2969.

12. Py, B., et al., *Siva-1 and an alternative splice form lacking the death domain, Siva-2, similarly induce apoptosis in T lymphocytes via a caspase-dependent mitochondrial pathway.* J Immunol, 2004. **172**(7): p. 4008-17.
13. Han, J., et al., *SIVA1 directs the E3 ubiquitin ligase RAD18 for PCNA monoubiquitination.* J Cell Biol, 2014. **205**(6): p. 811-27.
14. Liu, Z., et al., *Selenium Treatment Enhanced Clearance of Salmonella in Chicken Macrophages (HD11).* Antioxidants (Basel), 2019. **8**(11).
15. Sun, S., et al., *Dihydrofolate reductase is required for the development of heart and outflow tract in zebrafish.* Acta Biochim Biophys Sin (Shanghai), 2011. **43**(12): p. 957-69.
16. Llado, V., et al., *Pivotal role of dihydrofolate reductase knockdown in the anticancer activity of 2-hydroxyoleic acid.* Proc Natl Acad Sci U S A, 2009. **106**(33): p. 13754-8.
17. Szklarczyk, D., et al., *STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets.* Nucleic Acids Res, 2019. **47**(D1): p. D607-D613.
18. Mi, H., et al., *PANTHER version 14: more genomes, a new PANTHER GO-slim and improvements in enrichment analysis tools.* Nucleic Acids Res, 2019. **47**(D1): p. D419-D426.
19. https://www.d.umn.edu/~jfitzake/Lectures/DMED/Antineoplastics/DNASynthesisInhibitors/NucleotideBiochemistry.html

Index