Association of *Salmonella* Typhimurium effector protein SteA with host cellular proteins and its role in cell death mechanisms

Manisha Kalsain

MS16096

A dissertation submitted for the partial fulfilment of

BS-MS dual degree in Science

Indian Institute of Science Education and Research Mohali

May 2021

Certificate of Examination

This is to certify that the dissertation titled "**Association of *Salmonella* Typhimurium effector protein SteA with host cellular proteins and its role in cell death mechanisms**" submitted by Ms. Manisha Kalsain (Reg. No. MS16096) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Samarjit Bhattacharyya Dr. Kausik Chattopadhyay Dr. Arunika Mukhopadhaya

(Supervisor)

Dated: May 19, 2021

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Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Arunika Mukhopadhaya at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other University or Institute. Whenever contributions of others are involved, every effort is made to indicate that clearly, with due acknowledgment of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Manisha Kalsain

(Candidate)

Dated: May 19, 2021

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Arunika Mukhopadhaya

(Supervisor)

Acknowledgment

I wish to express sincere appreciation to my supervisor, Dr. Arunika Mukhopadhaya for accepting me as a Master's thesis student and for always encouraging and guiding me. I am indebted to Mr. Shashi Prakash Yadav for teaching me the lab work and specially the infection experiments. I wish to show gratitude to Ms. Arpita Sharma for teaching me all the dos and don'ts of the lab work and always being there when the circumstances got difficult. She supported me like a pillar throughout this journey. I wish to acknowledge the support of Mr. Yogesh Saxena and Dr. Deepinder Kaur for their critical input in every experiment. I would like to thank all the lab members for helping me sail through this wonderful journey. This work would have been impossible without the support of my family and friends. Lastly, I wish to pay special regards to IISER Mohali for providing me this opportunity.

Manisha Kalsain

MS16096

List of Figures

Notations

SteA	*Salmonella* translocated effector A
SPI	*Salmonella* Pathogenicity Islands
PCNA	Proliferating cell nuclear antigen
PRR	Post replication repair
FA	Fanconi Anemia
TLS	Translesion Synthesis
TS	Template switching
ISG15	Interferon-stimulated gene 15
FEN1	Flap endonuclease I
DHFR	Dihydrofolate reductase
FANCD2	Fanconi Anemia group D2 protein
Rab-7a	Ras related protein
Rac-2	Ras-related botulinum toxin substrate 2

Contents

Abstract

*Salmonella enteric*a serovar Typhimurium is a major food-borne pathogen which causes self-limiting gastroenteritis in humans and typhoid-like diseases in mice. It gets transmitted through contaminated food, poultry, meat etc. and leads to 155,000 deaths each year. *Salmonella* Typhimurium has large gene cassettes in its chromosome called the *Salmonella* pathogenicity islands (SPIs) which encode/regulate various virulence factors required for successful pathogenesis. SteA is an effector protein which is regulated by both SPI-1 and SPI-2. Under SPI-1 condition SteA suppresses the proinflammatory responses of the host cell. To find other functions associated with SteA, following GST-pull down mass spectrometry data analysis was done using bioinformatics' tools which suggested that SteA might be involved cell death mechanisms and could modulate the DNA damage repair pathways. It was also observed that SteA is involved in *Salmonella* Typhimurium-induced cell death in RAW264.7 murine macrophages and hinders the poly-ubiquitination of PCNA. Co-immunoprecipitation studies suggested the interaction of SteA with Rab-7 and Rac-2. Further, experiments can help reveal the complex interplay of the DNA repair pathways involved in response to infection of *Salmonella* Typhimurium.

Chapter 1

Introduction

1.1 Theory

1.2 Experimental materials and methods

Introduction

1.1 Basic Theory

- *Salmonella enterica* serovar Typhimurium
- *Salmonella* Pathogenicity Islands
- SteA – *Salmonella* translocated effector A
- DNA damage and post replication repair
- PCNA and its role in DNA damage repair
- Mono-ubiquitination vs poly-ubiquitination of PCNA
- Cross-talk between Fanconi Anemia and Translesion synthesis pathway
- Role of Rab-7 and Rac-2 of host cell in pathogenesis of *Salmonella*
- Objectives of this study

1.2 Experimental Materials and Methods

1.1 Basic theory

Salmonella enterica serovar Typhimurium

Salmonella enterica serovar Typhimurium is a pathogenic, rod shaped bacteria belonging to the family Enterobacteriaceae. This Gram negative, facultative anaerobe is a leading cause of gastroenteritis in humans and typhoid-like diseases in mice. It is a common food borne pathogen and gets transmitted through poultry, dairy and contaminated food. Each year around 93.8 million cases of non-Typhoidal gastroenteritis occur, leading to around 155,000 deaths. Of these, 80.3 million are foodborne (Majowicz et al., 2010). *S.* Typhimurium infects the gastrointestinal epithelium and invades the non-phagocytic cells. Further, it survives and replicates inside modified phagosomes known as *Salmonella* containing vacuoles (SCVs). Invasion of *Salmonella* results in the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) both of which can cause DNA damage in the form of lesions (Rhen, 2019, Nakad and

Schumacher, 2016). These lesions can stall the replication as well as transcription processes.

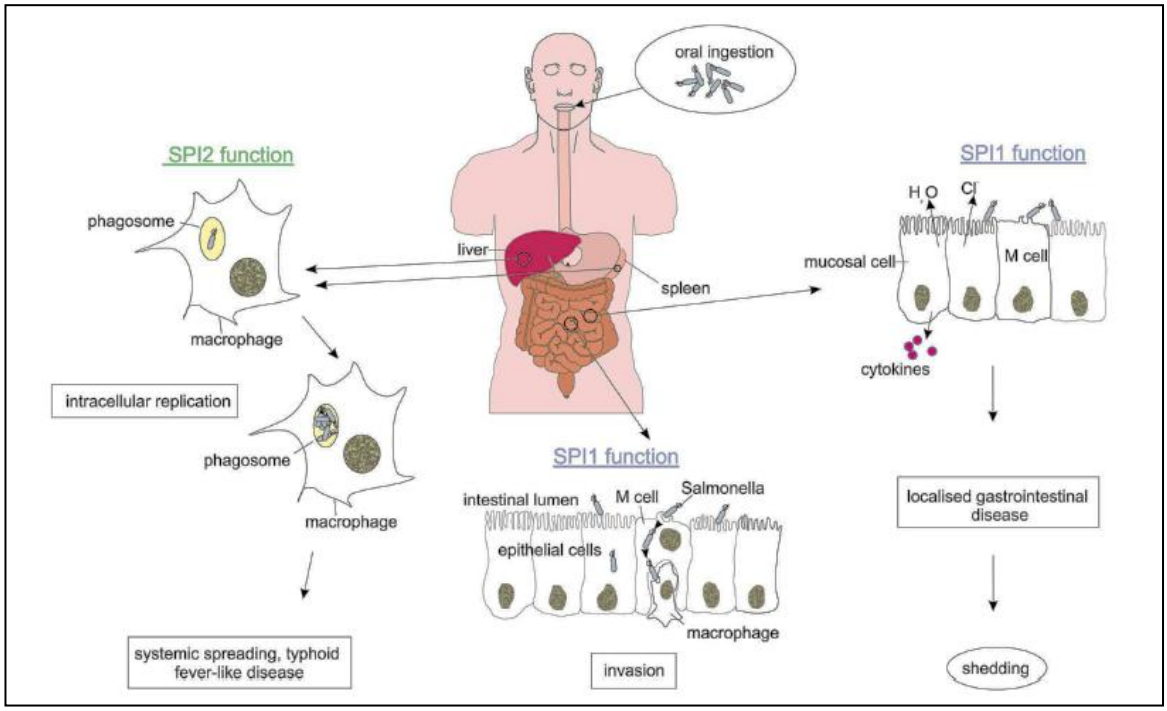


Figure 1: Schematic representation of host-pathogen interaction during *Salmonella* infection (Hansen-Wester, 2001)

Salmonella pathogenicity islands

For successful pathogenesis of the host, *Salmonella* requires multiple genes, most of which are found in the small stretches of chromosomes known as *Salmonella* pathogenicity islands (SPIs). They encode/regulate various virulence factors required to establish specific interactions with the host. So far, a total of five SPIs have been identified. Out of these five, SPI-1 and SPI-2 are critical players in the invasion and replication of *Salmonella* respectively (Marcus et al., 2000). Both SPI-1 and SPI-2 encode type-III secretion systems (T3SSs) which are injectosome-like structures used for translocating bacterial proteins into the host cytoplasm. T3SS-1 delivered effectors are mainly involved in early phases of infection such as invasion and immune modulation of the host whereas effectors delivered through T3SS-2 maintain the *Salmonella* containing vacuole (SCVs) and thus aid in the survival and replication of *Salmonella* Typhimurium.

Some of the effectors such as SteA, are secreted by both T3SS-1 and T3SS-2 suggesting its role in different phases of infection (Gulati et al., 2019).

Salmonella translocated effector A

SteA is important for *Salmonella* replication within the SCV in epithelial cells as well as vacuolar replication and survival in macrophages (McQuate et al., 2017). It has also been shown to contribute to the membrane dynamics of the SCVs (Domingues et al., 2014). Further, it has been shown that in SPI-I condition SteA interferes with the IκB degradation and suppresses the host proinflammatory responses (Gulati et al., 2019). Preliminary data shows that in SPI-II condition SteA can affect DNA damge and cell death.

DNA damage and Post replication repair

Under optimal conditions, replicative DNA polymerases such as Pol α, Pol δ, and Pol ε work in concerted fashion to ensure the efficient and accurate replication of DNA (Burgers, 2009). However, cells are constantly under many exogenic and endogenic stresses which include: ultraviolet (UV) and ionizing radiations, ROS production, alkylating agents etc. These genotoxic insults result in the formation of lesions which can stall the transcription and replication process and might even be lethal for the cell. To protect and maintain the genetic stability and integrity the cells possess an array of DNA repair mechanisms.

Post replication repair (PRR) mechanisms allow the replication forks to progress through the lesion on the damaged template. Two major PRR mechanisms are Translesion DNA synthesis (TLS) pathway and Template switching mechanism (Sale et al., 2012). TLS pathway employs damage tolerant, low-fidelity polymerases which are able to accommodate damaged or distorted templates in their active sites whereas Template Switching mechanism utilizes the newly synthesized sister chromatid as a replication template and it is relatively “error-free” in comparison to the TLS pathway.

The activation and functioning of PRR mechanisms is dependent on PCNA (proliferating cell nuclear antigen) which triggers the replacement of replicative polymerases with the TLS polymerases such as polymerase-η.

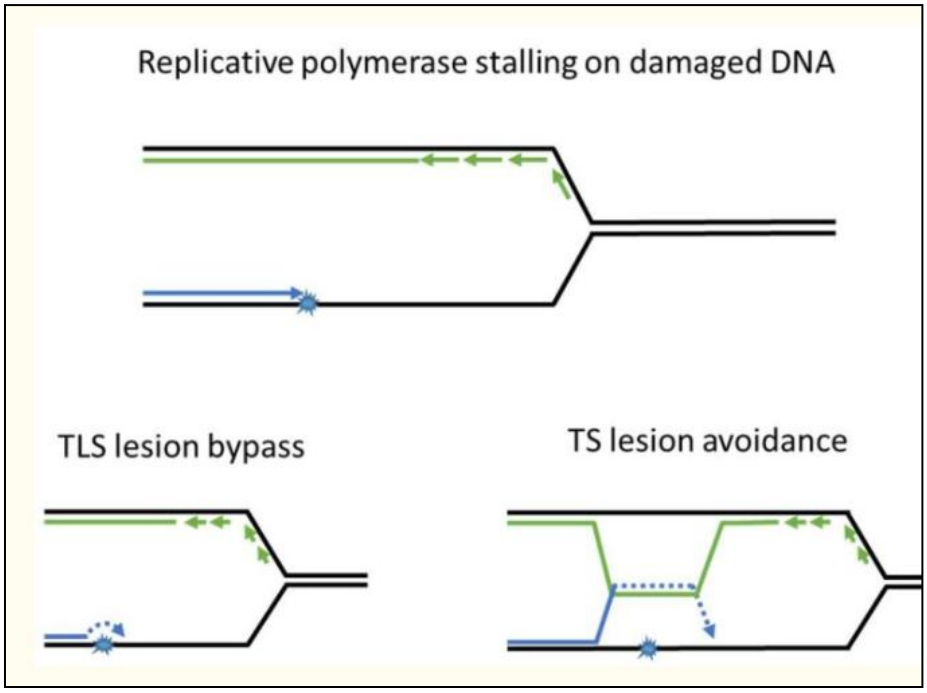


Figure 2: Schematic representation TLS and TS pathway upon lesion encounter (Gao et al., 2017)

PCNA and its role in DNA damage repair

PCNA is a member of the DNA sliding clamp family, which also includes the *E.coli* DNA polymerase (pol) III β-subunit and the phage T4 gene45 protein (Jónsson et al., 1997). With its high processivity, it is able to recruit polymerase δ for the duplication of the DNA. PCNA is a critical player in DNA damage response and has many interacting partners responsible for regulating a number of functions inside the cell such as regulation of cell cycle, DNA replication and damage response pathways. Most of the molecules interacting with PCNA have a conserved binding motif called the PCNA interacting motif or the PIP-box (Maga et al., 2003).

PCNA is known to undergo modifications like mono-ubiquitination, poly-ubiquitination and also SUMOylation upon DNA damage (Zhu et al., 2014). The type of modification decides the distinct repair pathways that would be chosen for the repair of DNA damage.

Mono-ubiquitination versus poly-ubiquitination of PCNA

Monoubiquitination of PCNA at Lys164, catalyzed by the RAD6-RAD18 E2-E3 complex, triggers the activation TLS polymerases which use unrepaired DNA strand as template and incorporate incorrect nucleotides leading to mutations in the genome. This lesion by-pass is mostly error-prone (Chen et al., 2011).

On the other hand, poly-ubiquitination of PCNA signals the Template switching mechanism which involves Rad5 and Ubc13-Mms2 complex and it uses newly synthesized sister chromatids as template. This lesion avoidance is mainly error-free (Tsui et al., 2006, Chui et al., 2005, Moldovan et al., 2006). This mechanism usually comes into play if the TLS pathway fails.

Cross-talk between Fanconi anemia pathway and Translesion synthesis pathway

The Fanconi anemia (FA) pathway is a DNA-repair pathway for DNA interstrand crosslinks (ICLs) Patients with FA exhibit birth defects, bone marrow failure and a lifelong predisposition to cancer, such as acute myeloid leukemia and head and neck cancer (Chen et al., 2015). The FA proteins FANCA, B, C, E, F, G, L and M form an upstream core complex with putative E3-ligase activity, whose target is the FANCD2-FANCI complex. FANCD2 is a critical component of the FA pathway, the mono-ubiquitination of which is essential for the DNA-repair focus formation and the repair of the interstrand crosslinks (Gregory et al., 2003).

PCNA and FANCD2 interact via PCNA-FANCD2 interacting peptide box (PIP-box) and it has been shown that mutation in this interacting motif hinders the FANCD2 mono-ubiquitination (Howlett et al., 2009). FANCD2 also interacts with polymerase-η and recruits it to the ICL sites.

Role of Rab-7 and Rac-2 of the host cell in pathogenesis of *Salmonella*

Mass spectrometry data analysis hints towards the interaction of SteA with Rab-7 and Rac-2.Rab-7a is a small GTPase which plays a key role in endo-lysosomal trafficking and also regulates pathogen induced phagosomes (vacuoles). Several survival strategies of microbial pathogens such as *Salmonella* employs Rab-7a. It has also been reports that Rab-7 regulates SCV biogenesis during the phase characterized by rapid acquisition of lysosomal proteins (Méresse et al., 1999, Harrison et al., 2004, Mohapatra et al., 2019).

Rac-2 or Ras-related C3 botulinum toxin substrate-2 is also a small GTPase which is implicated in the lamellipodia extension and actin remodulation. It has been reported that Rab and Rac GTPases work in a concerted manner in *Salmonella* pathogenesis (Troger et al., 2013).

Background:

Previous lab studies have shown that in the absence of SteA there is a decrease in cell death in RAW264.7 murine macrophages. Also, mass spectrometry data analysis hints towards the involvement of SteA in DNA damage response pathways. The emphasis of the study is towards the role of SteA in cell death mechanisms. To approach this, the first part of the study was to find out which host cellular proteins are interacting with SteA. Since ubiquitination of PCNA is a critical modification for the activation of damage response pathways, the second objective was to check the effect of SteA on PCNA ubiquitination upon DNA damage.

Objectives of the study:

- To find out the host cellular proteins interacting with SteA of *Salmonella* Typhimurium.
- To check the effect of SteA on ubiquitination of PCNA upon DNA damage.

Hypothesis -1

SteA probably affects PCNA-ISG15-FEN1 Translesion DNA synthesis pathway and hinders the mono-ubiquitination of PCNA. As a result the damage tolerant polymerase-η is unable to bind to PCNA and facilitate the activation of TLS pathway (Figure 3).

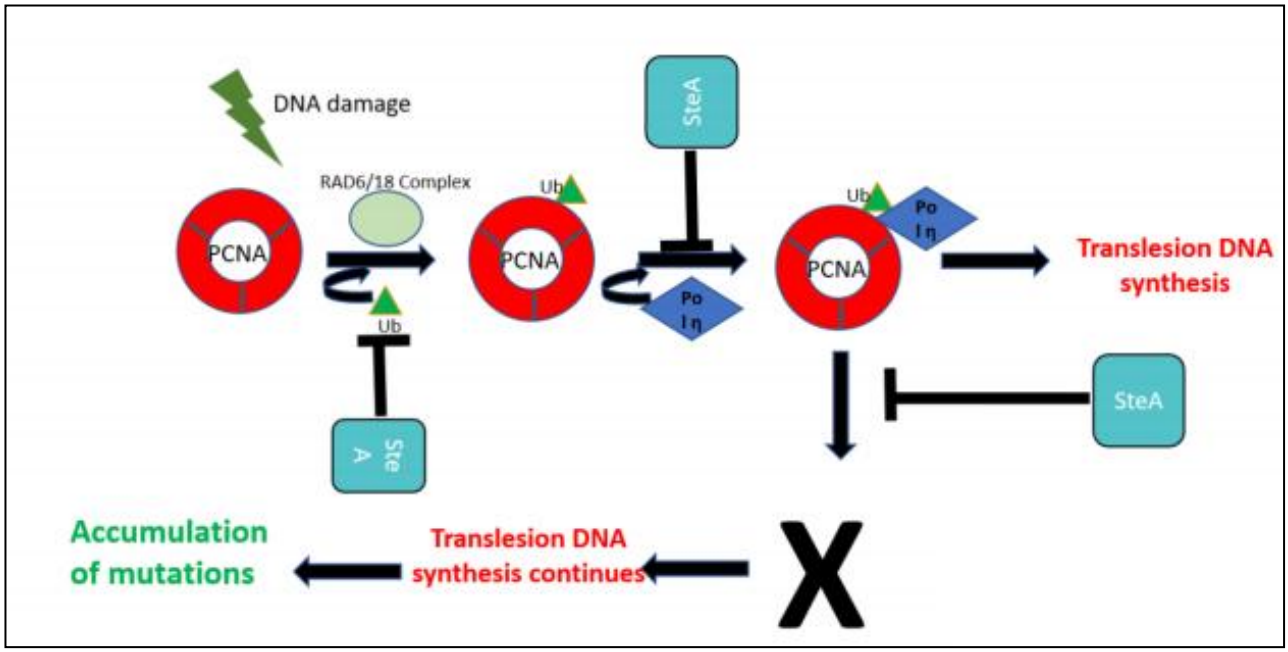


Figure 3: SteA hinders the activation of Translesion DNA synthesis pathway

Hypothesis-2

Mass spectrometry data analysis hints towards the interaction of SteA with FANCD2 and it might be possible that SteA through the selected set of proteins (shown below) hinders the mono-ubiquitination of FANCD2 which is critical for the FA pathway. Since, FANCD2 and PCNA have direct physical interaction, that might also be affected. Also FANCD2 can also modulate the function of polymerase-η of the TLS pathway (Park et al., 2010). This could lead to increase in cell death upon *Salmonella* Typhimurium invasion (Figure 4).

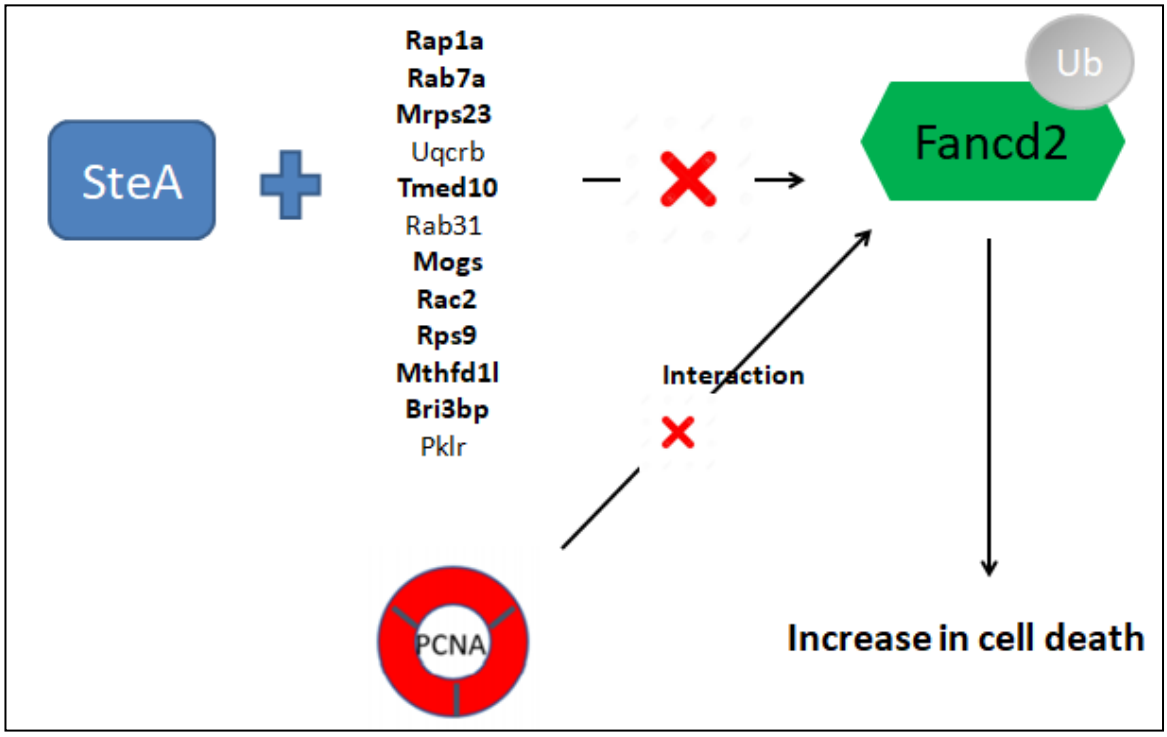


Figure 4: SteA interferes with the mono-ubiquitination of FANCD2 and its interaction with PCNA and polymerase-η

1.2 Experimental materials and methods

Bacterial culture

WT and mutant Δ*steA* strain of *Salmonella enterica* Typhimurium-SL1344 was cultured in Luria Bertani media (HIMEDIA) with appropriate antibiotics. SPI-2 condition was induced to check SPI-2 mediated role of SteA.

Antibiotics used (HIMEDIA): Streptomycin (50μg/ml) was used for both WT and Δ*steA* and Kanamycin (50μg/ml) for ΔSteA cultures.

SPI-2 inducing condition

Bacteria from LB cultures were washed and diluted in 1:100 with low-phosphate, low-magnesium minimal media (LPM) at pH 5.8 and incubated at 37℃ overnight.

Composition of Low- phosphate low-magnesium minimal medium (LPM)

1. 80mM 2-(N-morpholino) ethanesulfonic acid (pH 5.8)

2. 5M KCl

3. 7.5mM $(NH4)_2SO_4$

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 $MgCl_2$

0.22μm filter (Millipore Millex) was used to sterilize the media.

Cell Culture

RAW 264.7 macrophages (a murine macrophage cell line was obtained from National Centre of Cell Science Pune, India and HEK 293 (a human kidney epithelial cell line) was obtained from American Type Culture Collection, USA. Both were cultured in RPMI 1640 and DMEM media supplemented with 10% (v/v) FBS and 100 μg/ml of penicillin,

and 100 µg/ml of streptomycin (Invitrogen, Life Technologies, USA) at 37°C and 5% CO_2.

Invasion Assay in RAW 264.7 cells

RAW 264.7 cells were seeded at a density of $0.5x10^6$ /ml in a 24-well plate overnight at 37℃ and 5% CO_2 and infected with stationary phase bacteria at a multiplicity of infection (MOI): 20. The plate was centrifuged at 200 × g for 2 min followed by incubation for 30 min so that bacteria can invade the cells. After removing the media containing bacteria, fresh RPMI 1640 was added supplemented with 100 µg/ml of gentamicin. After 1 h, media were removed and all wells except the ones in which invasion was to be checked, were supplemented with 20µg/ml of gentamicin. In all the invasion assay experiments, the invasion was checked 30 min post infection by lysing the cells with 0.1% (v/v) Triton-X-100 (HIMEDIA) in PBS. Invasion was checked by enumerating the colony forming unit (CFU).

Cell Cytotoxicity Assay (LDH release)

Cell cytotoxicity assay was done using LDH release assay kit (Promega). Briefly, 25 µl of 10X lysis buffer was added in the positive control and incubated for 30 min. Supernatants were collected and centrifuged at 3500 rpm for 10 min. 50 µl of each sample was added (done in triplicates) to a 96 well plate. 50 µl of lactate dehydrogenase substrate was added to the samples and kept in dark for 7-10 min or till the colour developed. OD was measured at 490 nm using a spectrophotometer (BIO-RAD).

Plasmid isolation

LB media supplemented with ampicillin (50 µg/ml) was inoculated with bacterial culture and grown overnight at 37℃. Next day, the bacterial culture was centrifuged at 6000 rpm for 7-8 minutes. Plasmid was isolated using plasmid isolation kit (Qiagen®) using manufacture's protocol. Concentration of isolated plasmid was checked using a micro-volume spectrophotometer (Genova Nano) keeping Sigma water as blank.

Transfection of HEK 293 cells

HEK cells (7×10^6) were plated in DMEM in a 100 mm petri plate for the preparation of whole cell lysate. Cells were transfected with 3 μg of pcDNA3.1(+) empty plasmid and pcDNA 3.1(+) *steA* using polyethyleneimine (PEI) at a ratio of 1:3 (DNA/PEI). Media was changed after 8 h of transfection. After 18 h of transfection, hydroxyurea (0.5 mM) treatment was given (as a trigger for DNA damage response) for 12 h followed by whole cell lysate preparation.

Whole-cell lysate preparation

After 18 h of transfection, HEK 293 cells were washed with 1X PBS twice, scraped and transferred to 1.5 ml microcentrifuge tubes followed by centrifugation at 3500 rpm for 5 min. Supernatant was carefully removed and pellet was resuspended in 200 μl of whole cell lysis buffer containing 2μl of mammalian proteasomal inhibitor (1:100). It was then sonicated at 10Å for 15s (3 pulse of 5s each). Then, it was centrifuged at 16,000×g for 30 min at 4 ℃. The supernatant obtained was the whole-cell lysate.

Western blotting

Sample preparation: 5μl of loading dye was added to the samples and heated at 95℃ for 15 min.

SDS PAGE was done and proteins were transferred onto a PVDF membrane using wet transfer. After the transfer, blot was kept in blocking buffer (5% BSA in 1X TBST+1%Tween 20) for 1 h. Ubiquitinated-PCNA was probed using anti-PCNA antibody and Ubiquitin was probed using Ubiquitin antibody. ECL substrate (Cyanagen) was used to develop the blot in ImageQuant® LAS4000 Imager.

Antibodies used: Anti-human/mouse PCNA antibody (BioLegend®), Ubiquitin antibody (Cell Signalling Technologies®), anti-Mouse IgG Peroxidase antibody (Sigma®).

Co-immunoprecipitation studies

Whole-cell lysates were incubated with 2 µg anti-HA antibody for 3 h on a mini rotor at 4℃ for continuous low-speed shaking. Then 20 µl of A/G beads were added (Santa Cruz Biotechnologies, USA) and kept overnight on a rotor at 4 ℃. Next day, it was washed twice with whole-cell lysis buffer at 6000×g for 5mins. The beads were centrifuged at 6000×g for 5mins. The supernatant was carefully removed and resuspended in SDS-loading dye and heated at 95 ℃ for 10 min. The samples were then run on SDS-PAGE and subjected to immunoblotting.

Antibodies used: Rabbit mAb Rab-7 and Rac-2 (Cell Signalling Technologies®), anti-Rb IgG Peroxidase antibody (Sigma®).

Softwares used for Mass spectrometry analysis

Enrichr and Toppgene were used for the enrichment of the protein list and understand the different functions, interactions and pathways involved.

Enrichr:

- Enrichr was accessed from (https://maayanlab.cloud/Enrichr/enrich#).
- From the raw data file (i.e. Label-Free quantification data was obtained from Harvard Medical School), GST-SteA proteins were selected.
- Protein list was entered into Enrichr.
- **Gene Ontology** was selected to interpret the data and get an idea about the biological and molecular processes associated with the selected proteins.
- **Enrichr - Pathways** was selected to get a better understanding of the different kinds of pathways involved. A number of Pathway collections can be accessed in Enrichr. KEGG, Elsevier and Panther collection of pathways were chosen to observe the data.

Toppgene Suite Analysis:

Toppgene is another platform used for the functional enrichment of the protein. It was accessed using (https://toppgene.cchmc.org/).

ToppFun is a platform of Toppgene which allows you to detect the functional enrichment of the gene list based on Transcriptome, Proteome, Regulome (TFBS and miRNA), Ontologies (GO, Pathway), Phenotype (human disease and mouse phenotype), Pharmacome (Drug-Gene associations), literature co-citation etc.

- Selected proteins were entered into ToppFun.
- Keeping all the other parameters constant the input was entered.
- **GO enrichment analysis**: was done to know which proteins are over-represented under certain conditions.
- **GO Biological process**: was used to know the different biological processes involved with the given set of proteins.
- **GO Molecular proces**s: gave the different molecular processes of the proteins.
- **Interaction function** of ToppFun was used to know the potential interacting partners of SteA

Chapter 2

Results and Discussion

2.1. Results

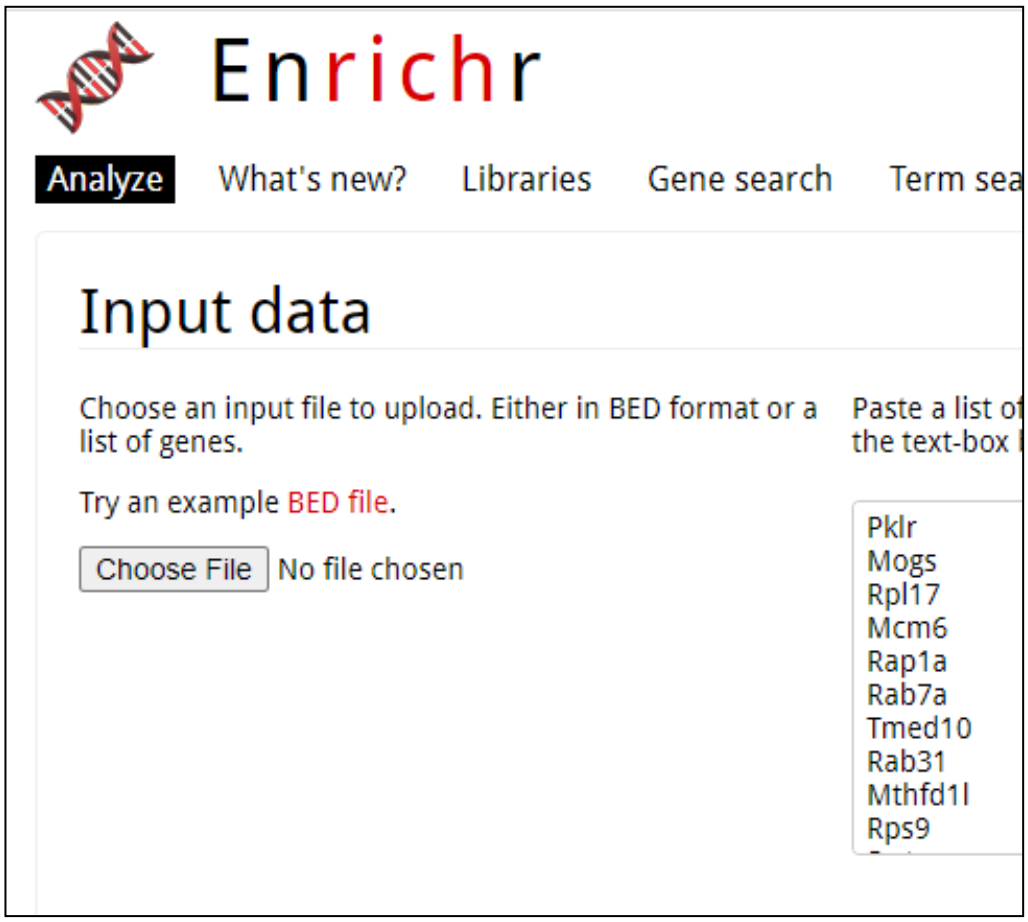
2.2. Discussion

Chapter 2

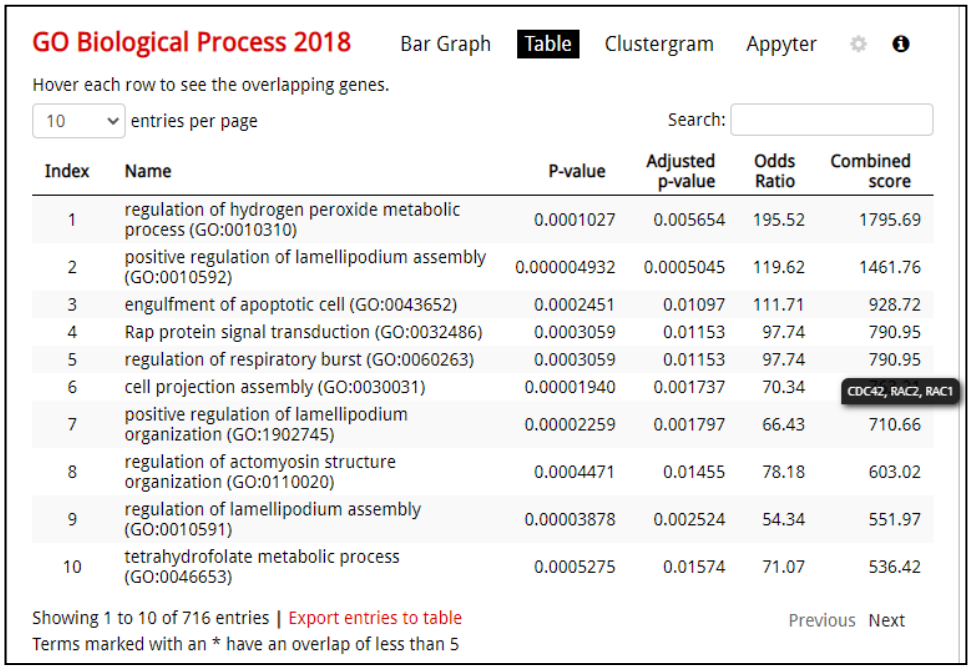
2.1 Results

(a)Analysis of the mass spectrometry data

Output of Enrichr software:



GO Biological and Molecular function results:



GO Biological Process 2018 Bar Graph **Table** Clustergram Appyter

Hover each row to see the overlapping genes.

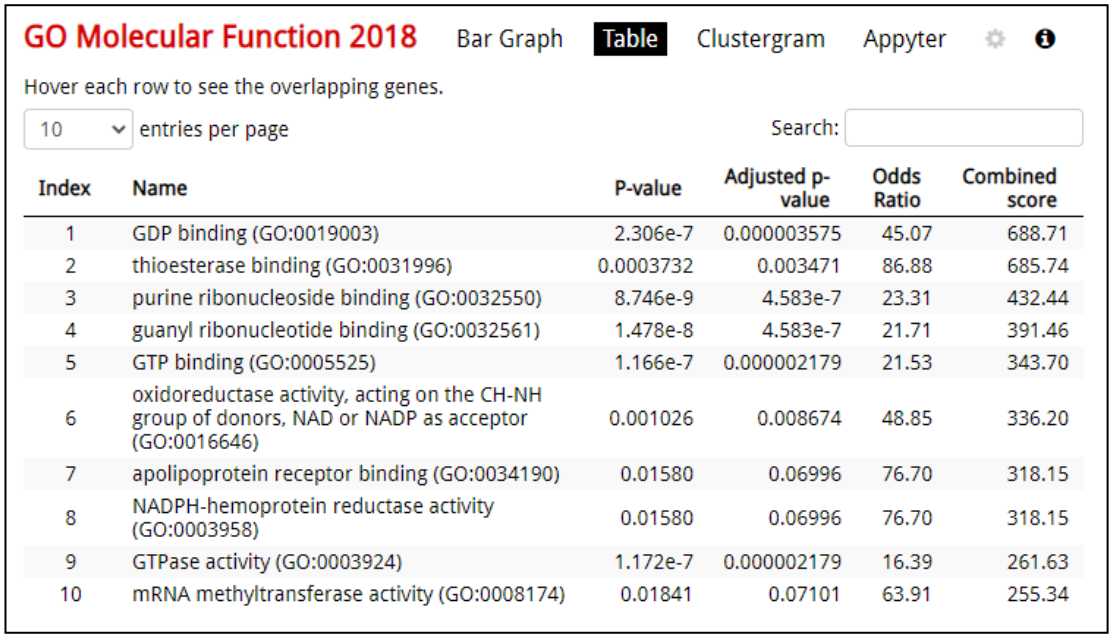
10 entries per page Search:

Index	Name	P-value	Adjusted p-value	Odds Ratio	Combined score
1	regulation of hydrogen peroxide metabolic process (GO:0010310)	0.0001027	0.005654	195.52	1795.69
2	positive regulation of lamellipodium assembly (GO:0010592)	0.000004932	0.0005045	119.62	1461.76
3	engulfment of apoptotic cell (GO:0043652)	0.0002451	0.01097	111.71	928.72
4	Rap protein signal transduction (GO:0032486)	0.0003059	0.01153	97.74	790.95
5	regulation of respiratory burst (GO:0060263)	0.0003059	0.01153	97.74	790.95
6	cell projection assembly (GO:0030031)	0.00001940	0.001737	70.34	CDC42, RAC2, RAC1
7	positive regulation of lamellipodium organization (GO:1902745)	0.00002259	0.001797	66.43	710.66
8	regulation of actomyosin structure organization (GO:0110020)	0.0004471	0.01455	78.18	603.02
9	regulation of lamellipodium assembly (GO:0010591)	0.00003878	0.002524	54.34	551.97
10	tetrahydrofolate metabolic process (GO:0046653)	0.0005275	0.01574	71.07	536.42

Showing 1 to 10 of 716 entries | Export entries to table Previous Next

Terms marked with an * have an overlap of less than 5

- Biological processes such as positive regulation of lamellipodium assembly, actomyosin structure organization and tetrahydrofolate metabolic process can be linked to different phases of *Salmonella* infection.



GO Molecular Function 2018 Bar Graph **Table** Clustergram Appyter

Hover each row to see the overlapping genes.

10 entries per page Search:

Index	Name	P-value	Adjusted p-value	Odds Ratio	Combined score
1	GDP binding (GO:0019003)	2.306e-7	0.000003575	45.07	688.71
2	thioesterase binding (GO:0031996)	0.0003732	0.003471	86.88	685.74
3	purine ribonucleoside binding (GO:0032550)	8.746e-9	4.583e-7	23.31	432.44
4	guanyl ribonucleotide binding (GO:0032561)	1.478e-8	4.583e-7	21.71	391.46
5	GTP binding (GO:0005525)	1.166e-7	0.000002179	21.53	343.70
6	oxidoreductase activity, acting on the CH-NH group of donors, NAD or NADP as acceptor (GO:0016646)	0.001026	0.008674	48.85	336.20
7	apolipoprotein receptor binding (GO:0034190)	0.01580	0.06996	76.70	318.15
8	NADPH-hemoprotein reductase activity (GO:0003958)	0.01580	0.06996	76.70	318.15
9	GTPase activity (GO:0003924)	1.172e-7	0.000002179	16.39	261.63
10	mRNA methyltransferase activity (GO:0008174)	0.01841	0.07101	63.91	255.34

Lists of functions using different pathway collections:

Elsevier Pathway Collection Bar Graph **Table** Clustergram Appyter

Hover each row to see the overlapping genes.

10 entries per page Search:

Index	Name	P-value	Adjusted p-value	Odds Ratio	Combined score
1	T-Cell Receptor -> ATF/CREB Signaling	0.000001495	0.0007163	58.07	778.94
2	FrizzledR -> JUN/PAX2 Signaling	0.0005275	0.007219	71.07	536.42
3	TLR1/2/6 -> NF-kB Signaling	0.0005275	0.007219	71.07	536.42
4	CD157/ITGB2 Signaling in Myeloid Cell	0.00004376	0.004849	51.98	521.67
5	Adherens Junction Assembly (Nectin)	0.00004913	0.004849	49.81	494.14
6	FibronectinR -> AP-1/ELK/SRF/SREBF Signaling	0.000007203	0.001725	37.79	447.43
7	SOD1 Mutation	0.00006115	0.004849	45.97	446.02
8	Macrophage M2-Related Phagocytosis	0.00007494	0.004849	42.68	405.44
9	AngiopoietinR -> AP-1 Signaling	0.00007494	0.004849	42.68	405.44
10	AGER -> CREB/SP1 Signaling	0.0009135	0.009944	52.11	364.68

KEGG 2019 Human Bar Graph **Table** Clustergram Appyter

Hover each row to see the overlapping genes.

10 entries per page Search:

Index	Name	P-value	Adjusted p-value	Odds Ratio	Combined score
1	Non-alcoholic fatty liver disease (NAFLD)	5.243e-9	6.711e-7	24.97	476.13
2	Fc gamma R-mediated phagocytosis	0.000004215	0.0002698	24.06	297.74
3	One carbon pool by folate	0.001270	0.008126	43.42	289.56
4	Pancreatic cancer	0.00004646	0.001487	22.85	228.00
5	Leukocyte transendothelial migration	0.00001166	0.0004975	19.31	219.40
6	Salmonella infection	0.00007945	0.002034	19.78	186.69
7	VEGF signaling pathway	0.0005144	0.005487	21.31	161.39
8	Viral myocarditis	0.0005144	0.005487	21.31	161.39
9	Shigellosis	0.0006834	0.006428	19.24	140.25
10	Epithelial cell signaling in Helicobacter pylori infection	0.0007797	0.006509	18.35	131.34

Panther 2016 Bar Graph **Table** Clustergram Appyter

Hover each row to see the overlapping genes.

10 entries per page Search:

Index	Name	P-value	Adjusted p-value	Odds Ratio	Combined score
1	Axon guidance mediated by Slit/Robo Homo sapiens P00008	0.00001394	0.0001673	79.73	891.42
2	Axon guidance mediated by netrin Homo sapiens P00009	0.00006782	0.0004069	44.27	424.90
3	Adenine and hypoxanthine salvage pathway Homo sapiens P02723	0.01318	0.02326	95.88	415.06
4	Formyltetrahydroformate biosynthesis Homo sapiens P02743	0.01318	0.02326	95.88	415.06
5	Axon guidance mediated by semaphorins Homo sapiens P00007	0.0009135	0.002557	52.11	364.68
6	Integrin signalling pathway Homo sapiens P00034	1.751e-7	0.000004201	20.22	314.58
7	Cytoskeletal regulation by Rho GTPase Homo sapiens P00016	0.00003540	0.0002832	24.59	252.02
8	Pyruvate metabolism Homo sapiens P02772	0.02101	0.03361	54.78	211.61
9	B cell activation Homo sapiens P00010	0.0004647	0.002231	22.10	169.62
10	Ras Pathway Homo sapiens P04393	0.0008137	0.002557	18.07	128.58

A number of interesting hits related to NFκB signalling, cancer, Tetrahydrofolate synthesis pathway, *Salmonella* infection etc. were found.

Output of Toppgene Suite analysis

Given below are the biological and molecular processes of the given data set.

2: GO: Biological Process [Display Chart] 51 input genes in category / 2393 annotations before applied cutoff / 20148 genes in category

	ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
1	GO:0048284	organelle fusion		2.635E-9	3.911E-6	3.269E-5	6.307E-6	16	1032
2	GO:0006887	exocytosis		7.660E-9	3.911E-6	3.269E-5	1.833E-5	15	953
3	GO:0140029	exocytic process		7.660E-9	3.911E-6	3.269E-5	1.833E-5	15	953
4	GO:0099500	vesicle fusion to plasma membrane		7.660E-9	3.911E-6	3.269E-5	1.833E-5	15	953
5	GO:0043299	leukocyte degranulation		8.432E-9	3.911E-6	3.269E-5	2.018E-5	12	546

1: GO: Molecular Function [Display Chart] 51 input genes in category / 332 annotations before applied cutoff / 19248 genes in cat

	ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
1	GO:0005525	GTP binding		7.482E-8	7.699E-6	4.915E-5	2.484E-5	10	397
2	GO:0032550	purine ribonucleoside binding		1.156E-7	7.699E-6	4.915E-5	3.839E-5	10	416
3	GO:0032549	ribonucleoside binding		1.236E-7	7.699E-6	4.915E-5	4.104E-5	10	419
4	GO:0001883	purine nucleoside binding		1.264E-7	7.699E-6	4.915E-5	4.196E-5	10	420
5	GO:0001882	nucleoside binding		1.506E-7	7.699E-6	4.915E-5	4.999E-5	10	428

Output of Interaction function in ToppFun:

	ID	Name	Source	pValue	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
1	int:PHB	PHB interactions		4.374E-17	1.523E-13	1.355E-12	1.788E-13	23	940
2	int:EED	EED interactions		7.450E-17	1.523E-13	1.355E-12	3.046E-13	26	1365
3	int:FANCD2	FANCD2 interactions		3.670E-16	5.002E-13	4.449E-12	1.501E-12	29	1955
4	int:AGR2	AGR2 interactions		5.509E-14	5.632E-11	5.008E-10	2.253E-10	20	898
5	int:ITGA4	ITGA4 interactions		2.672E-13	2.185E-10	1.943E-9	1.092E-9	16	521

FANCD2 interactions; int:FANCD2 - Google Chrome

toppgene.cchmc.org/showQueryTerms.jsp?userdata_id=ec82a778-1dfb-42c9-a601-b60803197180&feature=it&row=2

FANCD2 interactions; int:FANCD2

Enrich these genes with ToppFun

Export these genes with mouse gene symbols to GUDMAP.org

Export these genes to EMAGE

	Entrez Gene ID	Gene Symbol	Gene Name	Original Symbol
1	51593	SRRT	serrate, RNA effector molecule	Srrt
2	23435	TARDBP	TAR DNA binding protein	Tardbp
3	16	AARS1	alanyl-tRNA synthetase 1	Aars
4	5906	RAP1A	RAP1A, member of RAS oncogene family	Rap1a
5	22931	RAB18	RAB18, member RAS oncogene family	Rab18
6	5912	RAP2B	RAP2B, member of RAS oncogene family	Rap2b
7	55832	CAND1	cullin associated and neddylation dissociated 1	Cand1
8	23450	SF3B3	splicing factor 3b subunit 3	Sf3b3
9	5788	PTPRC	protein tyrosine phosphatase receptor type C	Ptprc
10	7841	MOGS	mannosyl-oligosaccharide glucosidase	Mogs
11	140707	BRI3BP	BRI3 binding protein	Bri3bp
12	25902	MTHFD1L	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1 like	Mthfd1l
13	10291	SF3A1	splicing factor 3a subunit 1	Sf3a1
14	1973	EIF4A1	eukaryotic translation initiation factor 4A1	Eif4a1
15	6203	RPS9	ribosomal protein S9	Rps9
16	51649	MRPS23	mitochondrial ribosomal protein S23	Mrps23
17	7879	RAB7A	RAB7A, member RAS oncogene family	Rab7a
18	4175	MCM6	minichromosome maintenance complex component 6	Mcm6
19	7514	XPO1	exportin 1	Xpo1
20	10972	TMED10	transmembrane p24 trafficking protein 10	Tmed10
21	3939	LDHA	lactate dehydrogenase A	Ldha
22	998	CDC42	cell division cycle 42	Cdc42
23	55272	IMP3	IMP U3 small nucleolar ribonucleoprotein 3	Imp3
24	54888	NSUN2	NOP2/Sun RNA methyltransferase 2	Nsun2
25	3689	ITGB2	integrin subunit beta 2	Itgb2
26	10992	SF3B2	splicing factor 3b subunit 2	Sf3b2
27	5879	RAC1	Rac family small GTPase 1	Rac1
28	5880	RAC2	Rac family small GTPase 2	Rac2
29	6139	RPL17	ribosomal protein L17	Rpl17

29 proteins are interacting with FANCD2. Though other important molecules were also there but the focus of the study was towards cell death mechanism and DNA damage pathways, therefore FANCD2 was selected for further studies.

- Given below is the list of top 20 proteins from both the sets and the common proteins between them (12 proteins).
- 9 out of 12 proteins were found to be interacting with FANCD2.

Rap1a	Pklr
Rab7a	Mogs
Mrps23	Rpl17
Uqcrb	Mcm6
Tmed10	Rap1a
Rab31	Rab7a
Mogs	Tmed10
Rab18	Rab31
Rac2	Mthfd1l
Xpo1	Rps9
Rac1	Srrt
Atp5l	Sdf2l1
Rps9	Lyz2
Mthfd1l	Rac2
Atp6v1a	Uqcrb
Ptprc	Mrps23
Bax	Bri3bp
Bri3bp	Ndufa2
Glg1	Atp5i
Pklr	Arpc3

FANCD2 interactions; int:FANCD2 - Google Chrome

toppgene.cchmc.org/showQueryTerms.jsp?userdata_id=ab0c07ab-4ed3-4c21-9021-ae6e24...

FANCD2 interactions; int:FANCD2

Enrich these genes with ToppFun

Export these genes with mouse gene symbols to GUDMAP.org

Export these genes to EMAGE

	Entrez Gene ID	Gene Symbol	Gene Name	Original Symbol
1	51649	MRPS23	mitochondrial ribosomal protein S23	Mrps23
2	7841	MOGS	mannosyl-oligosaccharide glucosidase	Mogs
3	140707	BRI3BP	BRI3 binding protein	Bri3bp
4	7879	RAB7A	RAB7A, member RAS oncogene family	Rab7a
5	25902	MTHFD1L	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1 like	Mthfd1l
6	5906	RAP1A	RAP1A, member of RAS oncogene family	Rap1a
7	5880	RAC2	Rac family small GTPase 2	Rac2
8	6203	RPS9	ribosomal protein S9	Rps9
9	10972	TMED10	transmembrane p24 trafficking protein 10	Tmed10

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(b) To check the role of SteA in cell death and apoptosis

RAW264.7 macrophages were infected with wt and mutant *ΔsteA* of *Salmonella* Typhimurium for 30 mins at a multiplicity of infection (MOI):20. Invasion was checked by enumerating the CFU.

Invasion Assay

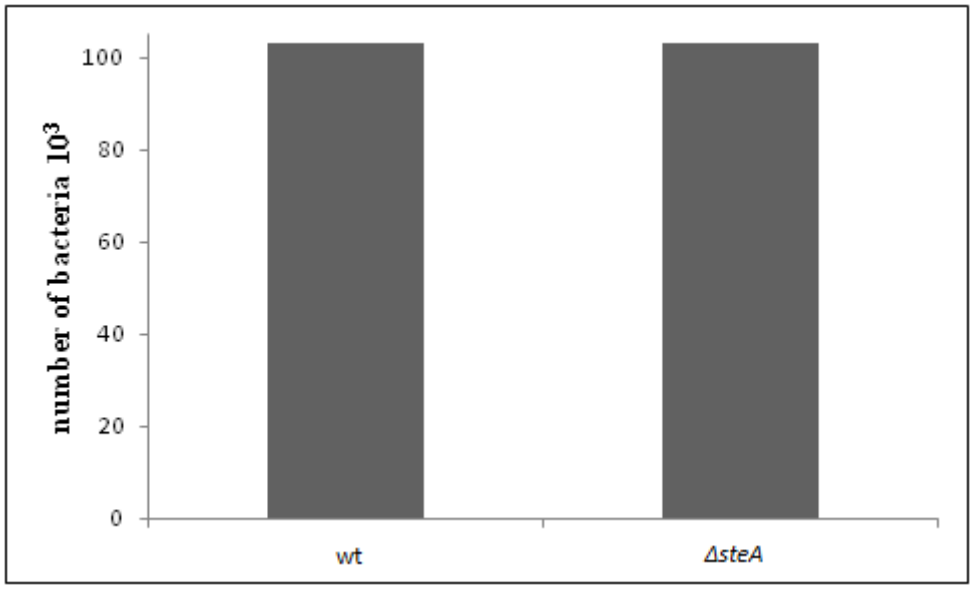
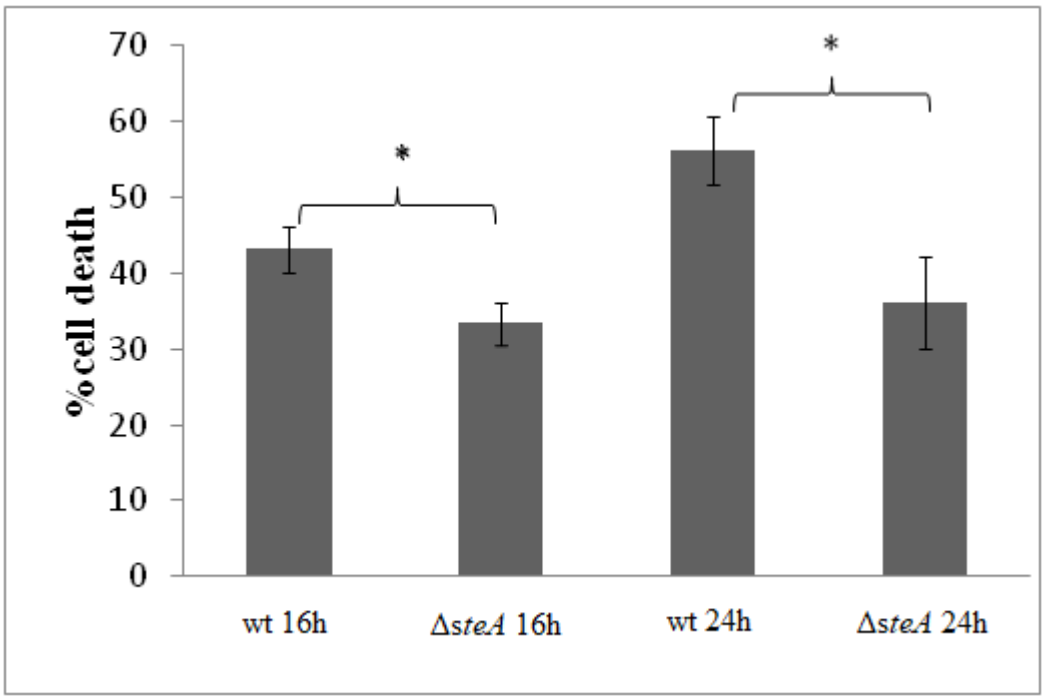


Figure 5: Invasion assay to compare the bacterial invasion of wt and Δ*steA* strain of *S.* Typhimurium

Inference: It was observed that CFU count was approximately equal in wt and *ΔsteA* mutant of *S.* Typhimurium.

Decrease in cell death due to absence of SteA

To check the cell death induced in response to wt and *ΔsteA* in SPI-2 conditions, LDH release assay was performed using the supernatant from the same infected cells which were used in invasion assay (Figure 6).



p-Value < 0.05 = * significant

Figure 6: LDH release assay of wt and *ΔsteA* infected RAW 264.7 cells

Inference: It is observed that there is decrease in cell death in mutant Δ*steA* mutant treated cells as compared to the wt treatment at both 16 hours and 24hours time points. Therefore it can be concluded that SteA is involved in *S.* Typhimurium induced cell death.

(c) Decrease in PCNA ubiquitination after endogenous expression of SteA

Towards checking the effect of SteA on the ubiquitination of PCNA, HEK 293 cells were transfected with pcDNA3.1(+)empty plasmid, pcDNA3.1(+) *steA*-HA and L307 *snx1*-HA (irrelevant protein for our study) for 18h followed by treatment of 0.5 mM Hydroxyurea for 12h (Figure 7).

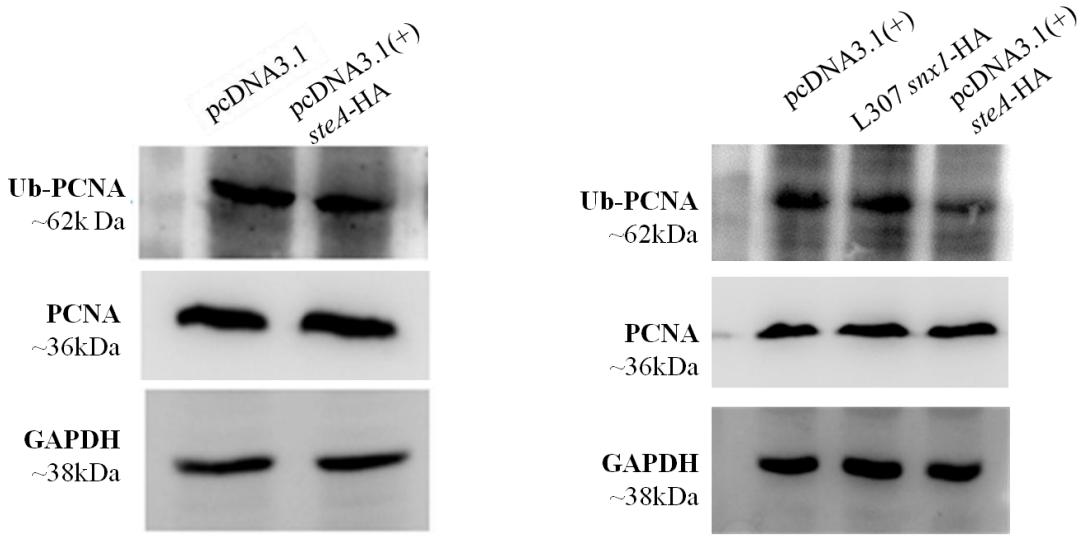


Figure 7: SteA decreases poly-ubiquitination of PCNA in response to Hydroxyurea

Inference: It is observed that there is decrease in poly-ubiquitinated PCNA level in presence of SteA as compared to Snx1 or when SteA is absent. It suggests that SteA hinders the poly-ubiquitination of PCNA in response to DNA damage.

(d) SteA interacts with Rac-2 and Rab-7 of the host cell

Towards checking the interaction of SteA with Rac-2 and Rab-7, co-immunoprecipitation was performed using anti-HA antibody followed by immunoblotting (Figure 8) and (Figure 9).

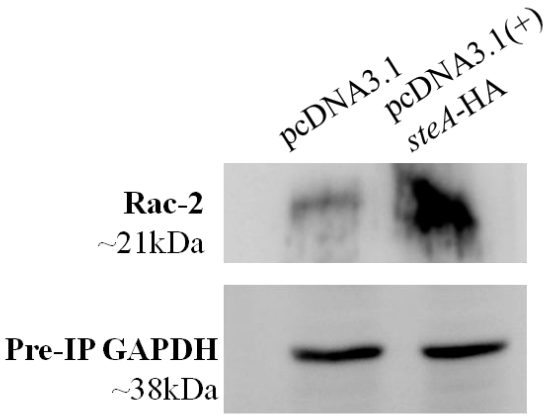


Figure 8: SteA interacts with Rac-2 of the host cell

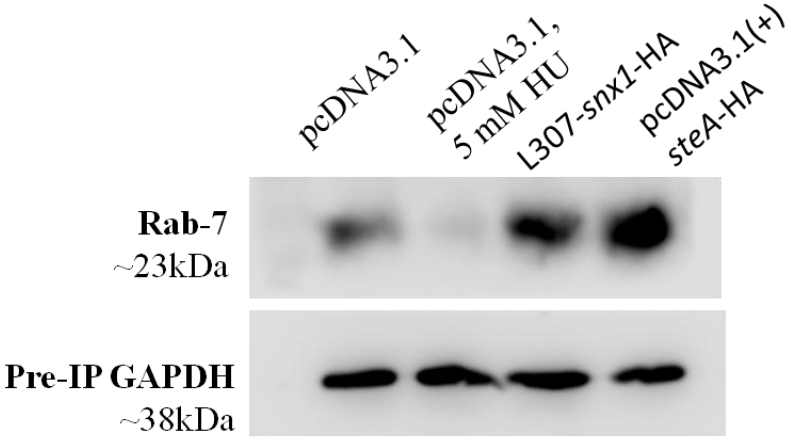


Figure 9: SteA interacts with Rab-7 of the host cell

(All samples shown in figure 7 have treatment of 0.5 mM hydroxyurea for 12h except sample in lane 2 which has 5 mM of hydroxyurea for 2h)

Inference: It was observed that there is enrichment of Rac-2 and Rab-7 with the expression of SteA. It can be inferred that SteA interacts with both these molecules of the host cell.

(e) Decreased ubiquitination in absence of Hydroxyurea

HEK 293 cells were transfected with pcDNA3.1 (+) for 18h followed by pre-treatment of 20 nM of MG132 for 3h and treatment of 5mM hydroxyurea for 2h (Figure 8).

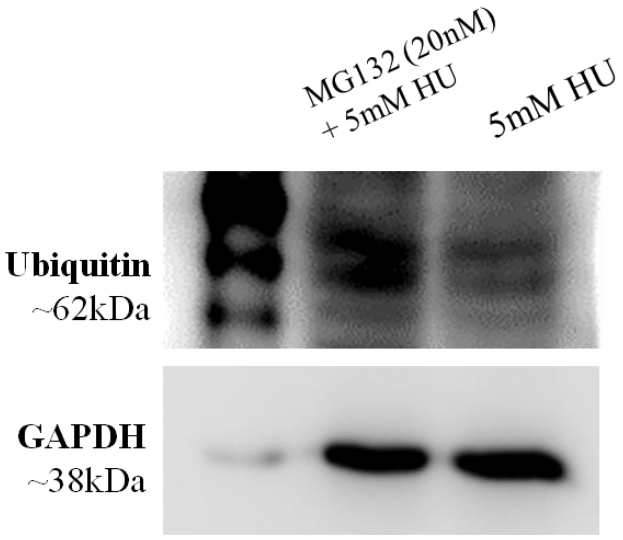


Figure 10: Decreased ubiquitination in absence of Hydroxyurea

Inference: Decreased ubiquitination is observed in absence of MG132 showing that MG132 prevents degradation of ubiquitin.

2.2 Discussion

Analysis of the mass spectrometry data using bioinformatics' tools such as Enrichr and Toppgene gave several interesting hits related to involvement of SteA in functions like DNA repair pathway, cancer, *Salmonella* infections, NFκB signalling, Tetrahydrofolate synthesis pathway. Also, it has been shown from the previous lab studies that SteA suppresses the proinflammatory responses of the by interfering with the IKB degradation (Gulati et al., 2014). It was observed that there is decrease in cell death in mutant ΔSteA as compared to WT *S*.Typhimurium. As a result, the focus was towards the cell death and DNA damage related aspects. Initially, the hypothesis was that SteA probably affects PCNA-ISG15-FEN1 Translesion DNA synthesis pathway. The critical step in this pathway is the monoubiquitination of PCNA which triggers its activation by recruiting the damage tolrerant polymerases such as polymerase-η (Chen et al., 2011). However, the experimental observations showed that SteA affects the poly-ubiquitination of PCNA suggesting that probably TLS pathway is not involved. Also, co-immunoprecipitation studies suggest the interaction of SteA with Rab-7 and Rac-2 consolidating the mass spectrometry analysis.

It was also interesting to note that out of 54 selected proteins that were entered in Toppgene software 29 proteins were interacting with FANCD2 which is an important part of the Fanconi Anemia pathway (involved in repair of ICLs). It is hypothesized that SteA through the shortlisted set of proteins hinders the monoubiquitination of FANCD2 and also the interaction with PCNA and Polymerase-η, thus affecting the cross-talk between these two DNA repair pathways.

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