On the rise of TRIM8 as a multifunctional workhorse during the course of mitosis

Utsa Bhaduri

ACKNOWLEDGEMENT

As a child during the school age, I had the very first opportunity of my life to look at the typical text book illustration of mitotic cell cycle. Although this learning was a part of the 10th standard school level board examination, this left an ever-lasting imprint on my mind that has always worked as the secret motivation in my life towards developing a research interest to enter the fascinating world of biology. I must acknowledge Dr. Saugata Basu, my school age biology teacher, who inculcated me with a deep love for modern biology and thus motivated me to pursue a research career in this amazing branch of science. I am very much thankful to my parents who provided me with an extra-ordinary support to continue my academic journey with that interest. I don't think I would have been able to build a PhD research career without their love and continued support. At every step of life, my parents have always motivated and encouraged me to make contributions for the betterment of humanity. Between my school age in Kolkata and doctoral years in Italy and Europe, I had almost a decade that I spent in Bangalore, where I completed undergraduate and postgraduate studies, and thereafter spent three years as a Junior Research Fellow with Prof. M. R. S. Rao. I believe Prof. Rao has played a crucial role in my academic life, as I was introduced to the scientific research, particularly in the field of lncRNA biology, epigenetics, and cancer genomics, under his guidance. Now, I must give a special thanks to my best friend Manjistha Roy, who has always offered the most precious emotional strength to me in all ups and downs of life ever since I met her in Bangalore. After I moved to Italy for the first time in the November of 2019 to start PhD research, I came across many people who don't come in the periphery of scientific practice but they welcomed me in Italy as a member of their family. In this regard, I must mention the name of Kronos Bar, the cafeteria where I used to be a frequent visitor and that emerged as the lifeline of my Italian chapter as I spent time there not only with *cappuccino* and *cornetto al cioccolato bianco* but also in writing articles, reading papers, and in my deep world of thinking.

Coming back to the academic quarter, I am very much thankful to my university tutor Prof. Germana Meroni of University of Trieste, Italy and external advisor Prof. Pascual Sanz of Instituto de Biomedicina de Valencia, Spain for their valuable advice and humble support during my doctoral years. I must give a special thanks to Prof. Germana Meroni for helping me always in sorting out all university affairs. I am truly grateful and thankful to the generous financial support from the TRIM-NET ITN of the Marie Skłodowska-Curie Actions (MSCA) of European Commission that not only helped me to pursue PhD but also introduced me to the world of TRIM E3 ubiquitin ligases in the era of targeted protein degradation, a paradigm shift in drug discovery. I also take this opportunity to sincerely thank my alma maters, University of Trieste and Fondazione IRCCS Casa Sollievo della Sofferenza, for giving me the wonderful opportunity to carry out this doctoral thesis work.

I highly appreciate the kind support that I received from all members of MerLaB, the Laboratory of Regulatory and Functional Genomics led by Prof. Giuseppe Merla, at Fondazione IRCCS Casa Sollievo della Sofferenza and that has genuinely helped the progress of my doctoral research work. In this connection, I thank Dr. Gabriella Maria Squeo and Dr. Eleonora Di Venere for their technical support in managing the in-house RT-qPCR and microscopic facilities respectively. I would also like to acknowledge a former member of the MerLaB, Dr. Natascia Malerba, who helped me to settle down in the cell culture room where I was a beginner and also in Italy where I was a newcomer with no expertise in speaking the language of the land. I am very much thankful to Dr. Vincenzo Giambra of Fondazione IRCCS Casa Sollievo Della Sofferenza for his valuable advice, scientific inputs, and technical support towards designing and performing a high-throughput assay like BD Rhapsody single-cell RNA sequencing. I am also thankful to Dr. Francesco Tamiro from the lab of Dr. Giambra who gave me the hands-on-training in BD Rhapsody library preparation for Whole Transcriptomic Analysis (WTA). I thank Dr. Rossella Di Paola and Dr. Lucia Salvemini Fondazione IRCCS Casa Sollievo Della Sofferenza for providing the precious help to perform sequencing assays with Illumina NextSeq 500. I thank Dr. Edyta Kowalczyk of BD Rhapsody team for her continuous discussion with me in optimising the single-cell RNA sequencing analysis pipeline on SeqGeq™ platform. I thank Dr. Danilo Licastro of AREA Science Park, Trieste for his support in designing and performing statistical tests in both transcriptomic and proteomic data. I sincerely thank group of Dr. Maria Monti at the University of Naples Federico II for her collaboration to perform the differential proteomics study with LC-MS/MS facility and Dr. Giulia Guarguaglini of Sapienza University of Rome for generously gifting hTERT RPE-1 cells and antibodies against CEP170 for the co-localisation study.

Finally, I would like to thank my PhD supervisor Prof. Giuseppe Merla for giving me a great opportunity not only to pursue a PhD on Marie Curie Fellowship but also for allowing me to make a transition into experimental molecular biology with my background training in computational genomics and bioinformatics. Moreover, my school age dream to study cell cycle, as I mentioned in the beginning, has come to a reality with the support of Prof. Merla, and I am sincerely thankful to him in this regard. Although I have a long way to go, I strongly believe the opportunity Prof. Giuseppe Merla gave me in this important phase of my academic career path, will be an asset in my postdoctoral life and beyond in making contributions to science. Last but not least I am grateful to omnipresent and omnipotent almighty for giving the ability to dream and think.

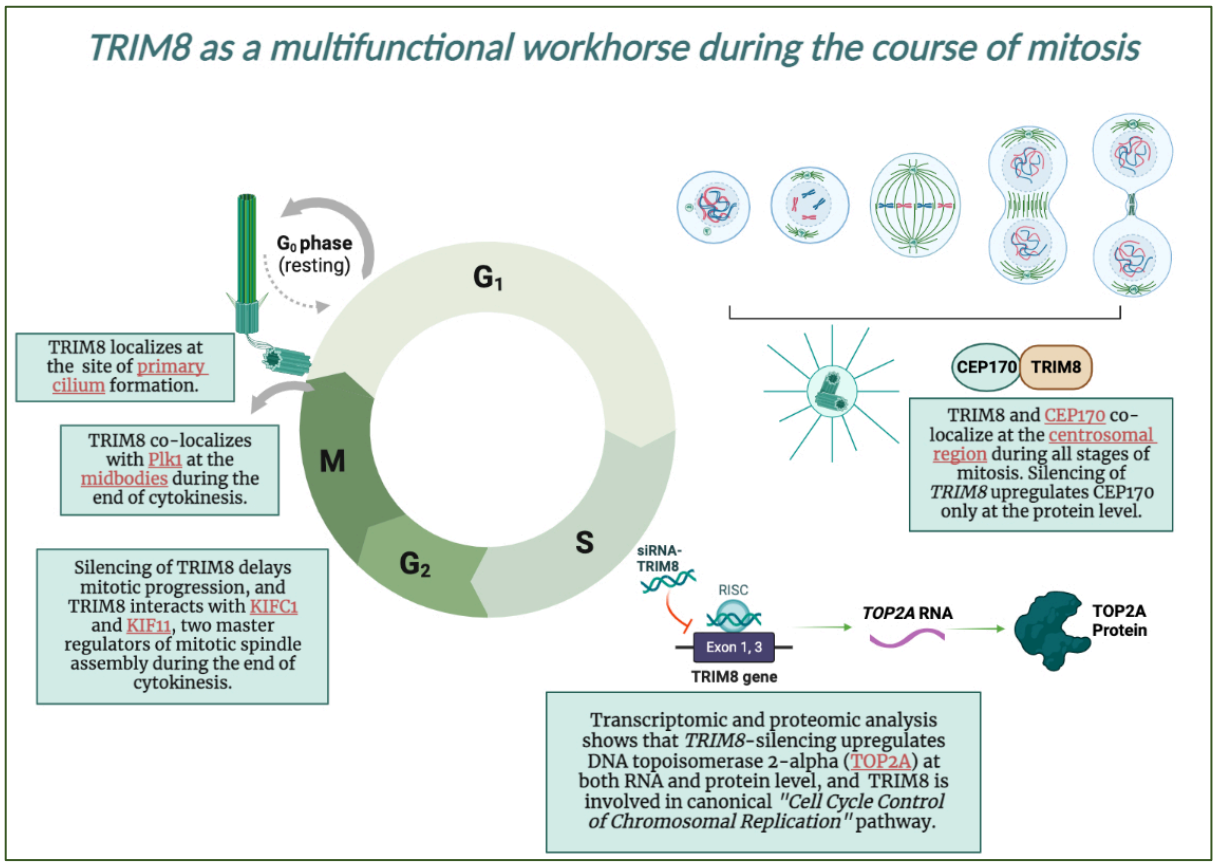
Utsa Bhaduri

Trieste, Italy
April 7, 2023

ABSTRACT

The human **TRI**-partite **M**otif containing protein 8 (TRIM8), that I named as a "molecule of duality (MoD)", can play a dual role of tumour suppressor and oncoprotein, and belongs to the TRIM family (earlier called as RBCC proteins) of E3 ubiquitin ligases. In my PhD research project, I attempted to understand the involvement of TRIM8 in mitosis in a cell cycle stage-specific manner with the aid of differential transcriptomic (single-cell RNA sequencing) and proteomic (LC-MS/MS) approaches along with the functional experimental and molecular studies. I standardised a pipeline based on already available machine-learning algorithms on the SeqGeq™ platform to divide a population of cells from a single cell line into three phases, G1, S, and G2/M. The importance of the pipeline is that without eliminating cell-cycle heterogeneity (which is typically removed in standard analysis of scRNA-seq data from tissues) we can compare the impact of a gene silencing in a mitotic stage-specific manner and can also evaluate the impact on cell cycle phase-specific marker genes without being biased by the cell-cycle heterogeneity of a single-cell RNA sequencing data from a single cell line. For instance, impact of *TRIM8*-silencing on the expression of *TOP2A*, a candidate marker of G2/M phase, has been compared in a stage specific manner with BD Rhapsody single-cell RNA sequencing data generated from both wild-type and *TRIM8*-silenced hTERT-immortalized retinal pigment epithelial cells (hTERT RPE-1 or RPE cells). Upon implementation of this new pipeline, I showed the impact of *TRIM8*-silencing in three different stages of mitosis (G1, S, and G2/M), and illustrated a very significant impact of *TRIM8*-silencing on the "Cell Cycle Control of Chromosomal Replication" pathway. Furthermore, based on the computational analysis of scRNA-seq data, important candidate genes of the pathway, such as *TOP2A, CDK6,* have been shown to be upregulated upon silencing of *TRIM8* by experimental validation with RT-qPCR. With the indication from transcriptomic data, differential proteomic data has also been generated upon silencing of *TRIM8* in RPE cells using bioanalytical method LC-MS/MS, and the "Cell Cycle Control of Chromosomal Replication" pathway is shown as the most significant hit at the protein level. From differential transcriptomic and proteomic studies, I showed that DNA topoisomerase 2-alpha (TOP2A), which is known to be essential for DNA replication and a key member of the aforementioned pathway, is upregulated at both RNA and protein level upon silencing of *TRIM8*. Furthermore, I showed that E3 ubiquitin ligase TRIM8 co-localises with centrosomal protein CEP170 during all phases of mitosis at the centrosomes in HeLa cells, and silencing of *TRIM8* upregulates CEP170 only at the protein level in RPE cells. And, it is also shown that TRIM8 localises at the site of primary cilium formation during G0 phase in RPE cells, and downregulation of *TRIM8* shows clear impact on two ciliary genes/proteins, *NEDD9* and *NEK7*, in the single-cell RNA sequencing analysis. Overall, my PhD thesis work, along with earlier published studies, brings out an impression of TRIM8 as a multifunctional workhorse during the course mitosis.

GRAPHICAL ABSTRACT



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Chapter 1. Introduction

The human TRIM8 was originally discovered as the Glioblastoma Expressed RING-finger Protein (GERP) and it is located on chromosome 10q24.32, a region which shows frequent loss of heterozygosity or deletion in glioblastoma.[1] TRIM8 belongs to the TRIM family of E3 ubiquitin ligases that has more than 80 members in humans, and that are known to be involved in a multitude of cellular processes and pathophysiological conditions ranging from cancers to neurological disorders to rare genetic diseases.[2, 3] TRIM family proteins (earlier called as RBCC proteins) do maintain a common structural feature by having a **tri**partite **m**otif (TRIM) that can be distinguished by the presence of three motifs or domains - RING finger domain (R), one or two zinc-finger motifs named B-box (B), and a coiled coil region (CC).[4] In case of TRIM8, the RING domain plays an important role in context of cell proliferation, as it is essential for TRIM8's activity to control stabilization and activation of tumour suppressor p53 (TP53).[5] The conserved B-box coiled-coil domain of TRIM8 is sufficient enough to interact with SOCS1,[6] another tumour suppressor gene, whereas the coiled-coil domain is required for homodimerization, and the deletion of the C-terminal region of TRIM8 leads to protein mis-localization.[4] Functionally, TRIM8 can work as both tumour suppressor and oncogenic protein. TRIM8 interacts with TP53 to induce its activity for directing the cell cycle arrest and plays the role of a tumour suppressor protein.[5] Interestingly, TRIM8 can also function as an oncoprotein by positively regulating NF-kB activation through K63-linked polyubiquitination of TAK1 that results in cell proliferation.[7] Because of this unique ability to function as both tumour suppressor and oncogenic molecule, at the beginning of my PhD, I reported TRIM8 as a "molecule of duality (MoD)" (Bhaduri et al., 2020).[2] More specifically, in the context of mitotic cell cycle, it was also known that TRIM8 interacts with master regulators of mitotic spindle machinery, including KIFC1 and KIF11, and silencing of *TRIM8* delays mitotic cell-cycle progression. Mass-spectrometry data indicated that TRIM8 interacts with centrosomal protein CEP170, and immunofluorescence study showed that TRIM8 co-localizes with serine/threonine-protein kinase Plk1 at the midbodies. Interestingly, CEP170 also happens to be a physiological substrate of Plk1.[8] Overall, although there was a preliminary indication that TRIM8 may play some important role during the course mitotic cell cycle, no study has investigated the involvement of TRIM8 in detail during the course of mitosis so far.

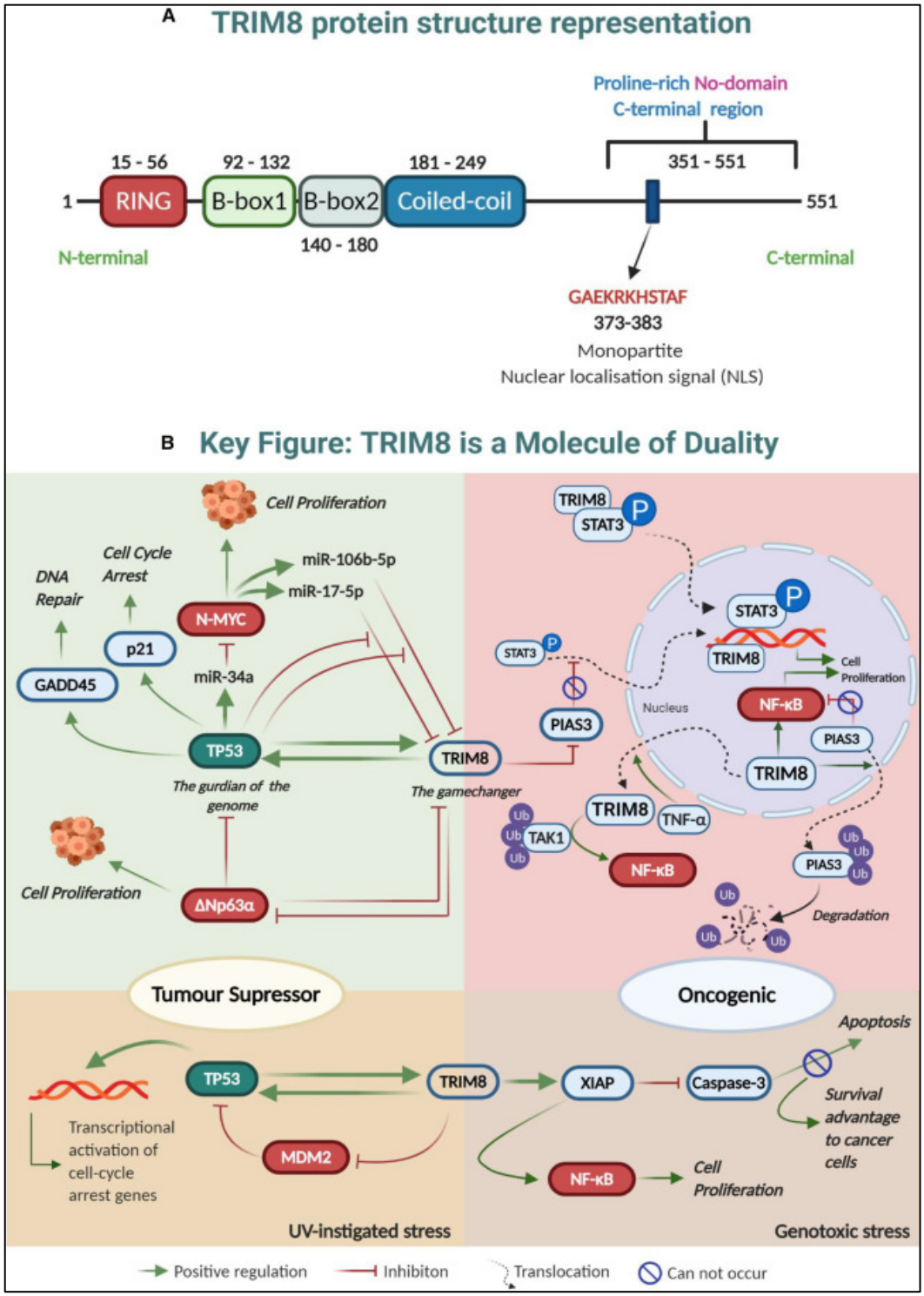
Chapter 2. Literature review: the background

In the first year of my PhD, I reviewed the E3 ubiquitin ligase TRIM8, and named it as the "molecule of duality (MoD)" for multiple reasons. Below, I incorporated an excerpt from my first review article titled "Rise of TRIM8: A Molecule of Duality", that I published as part of this PhD programme, to describe the duality of TRIM8 in multiple contexts.[2]

2.1. TRIM8 can act as both anti-proliferative and pro-oncogenic

It has been shown that TRIM8 can act as a tumour suppressor by inducing TP53-dependent cell cycle arrest. TRIM8, a direct target gene of TP53, induces TP53 expression and tumour suppressor activity through a positive feedback loop-forming mechanism with TP53 during UV-instigated stress conditions by augmenting CDKN1A (p21) and GADD45 expression.[5] TRIM8 is highly downregulated in clear cell renal cell carcinoma (ccRCC), and the recovery of TRIM8 expression can lead to the enhancement of efficacy of chemotherapeutic drugs by re-activating the TP53 pathway, suggesting that TRIM8 can be used as an enhancer of chemotherapy efficacy in a TP53 wild-type background.[9] Further, TRIM8 can restore the TP53 activity by blunting N-MYC activity in chemo-resistant tumors, like ccRCC and colorectal cancer (CRC), upon inhibition of miR-17-5p (also known as MIR17) and/or miR-106-5p. The inhibition of miR-17-5p and/or miR-106-5p leads to the recovery of TRIM8-mediated TP53 tumor suppressor activity and inhibits N-MYC-dependent cell proliferation through miR-34a upregulation.[10] Overall, these studies established an anti-proliferative image of TRIM8 during the course of cell proliferation and cancer, and, following the same trend, recent studies have also provided further evidence toward the anti-proliferative activity of TRIM8. It has recently been shown that TRIM8 can blunt the pro-proliferative action of oncogenic ΔNp63α in a TP53 wild-type background.[11] Altogether, based on the current knowledge, it can be stated that TRIM8 has the capacity to exercise its anticancer power in three ways: by inducing the TP53 tumor suppressor activity through a positive feedback loop formation, restoring TP53 functions by blunting N-MYC activity in chemo-resistant tumors, and quenching the ΔNp63α oncogenic activity by forming a negative feedback loop. However, it should not go unnoticed that, in all three cases, TRIM8's anti-proliferative property is subject to the TP53 functional or wild-type background, suggesting a strong demand for research on TRIM8 and its anti-cancer capacity in a TP53 mutant background, as most of the TP53-associated axis (e.g., TP53-MDM2 axis) based on E3 ubiquitin ligase-targeting drugs fail in this caveat. Indeed, it would be of high importance to look at the capacity of TRIM8 to restore the native conformation of TP53 mutants and reactivate the tumor-suppressor function. In contrast to the anti-proliferative activity, TRIM8 can also work as an oncogenic protein. TRIM8 serves as a critical regulator of tumor necrosis factor alpha (TNF-α)- and interleukin (IL)-1β-induced nuclear factor κB (NF-κB) activation by mediating K63-linked polyubiquitination of TAK1, and

overexpression of TRIM8 activates NF-κB and potentiates TNF-α- and IL-1β-induced activation of NF-κB, whereas knock down of TRIM8 brings opposite effects. Silencing of TRIM8 potentially inhibits TNFα gene expression in U937 cells (histiocytic lymphoma), indicating the oncogenic capacity of TRIM8.[7]



Literature Review Illustration 1. TRIM8, a Molecule of Duality. A summary figure that illustrates dual role of TRIM8 as both tumour suppressor and oncogenic molecule. **Credit**: Bhaduri, Utsa, and Giuseppe Merla. "Rise of TRIM8: A Molecule of Duality." Molecular therapy. Nucleic acids vol. 22 434-444. 2 Sep. 2020, doi:10.1016/j.omtn.2020.08.034.

Further, TRIM8 positively regulates the TNF-induced NF-κB pathway at the p65 level by inducing the translocation of PIAS3 (protein inhibitor of activated STAT-3) from the nucleus to the cytoplasm.[12] TRIM8 also regulates the clonogenic and migration ability of the cells through the NF-κB pathway, and knockdown of TRIM8 in the breast cancer MCF7 cell line significantly decreases the cell proliferation and clonogenic potential of cells.[12] Once activated, NF-κB promotes cell proliferation and protects cells from entering apoptosis. Thus, by positively regulating the TNF-induced NF-κB pathway, TRIM8 plays the role of an important oncogene that drives cell proliferation. Overall, from these two highly contrasting features, it can be stated that TRIM8 can function as both a tumour suppressor and an oncogenic molecule.

2.2. Duality of TRIM8 under stress conditions

The duality of TRIM8 comes out under different stress conditions, like UV-instigated stress or genotoxic stress. Under exposure to UV, TP53 induces the expression of TRIM8, which, in turn, stabilizes TP53, leading to cell cycle arrest and reduction in cell proliferation through upregulation of CDKN1A (p21) and GADD45. Notably, TRIM8 silencing prevents TP53 activation after UV radiation. Further, the overexpression of TRIM8 reduces the half-life of MDM2, the key negative regulator of TP53, without altering MDM2 mRNA expression, which in turn results in an increased TP53 protein expression. Finally, it has also been proven that the TRIM8-RING domain is essential to regulate TP53 and MDM2 stability and activity. Overall, it suggests that, under UV-instigated stress conditions, TRIM8 plays an important role as a tumor suppressor to dictate cell cycle arrest.[5] In contradiction to this, a recent study has reported that TRIM8 can provide a survival advantage to cancer cells by enhancing autophagy flux through lysosomal biogenesis during genotoxic stress conditions. The study has shown TRIM8-regulated autophagy degrades the cleaved Caspase-3 subunit to inhibit genotoxic stress-induced cell death. TRIM8 knockdown reduces the expression of X-linked inhibitor of apoptosis protein (XIAP), whereas the enhanced expression of TRIM8 stabilizes XIAP during genotoxic stress conditions. XIAP also strongly activates NF-κB via BIR (baculovirus inhibitor of apoptosis protein repeat) domain-mediated dimerization and binding to TGF-β-activated kinase 1 (MAP3K7) binding protein 1. This XIAP-mediated NF-κB activation also induces expression of genes involved in autophagy, like Beclin-1. Interestingly, during genotoxic stress, TRIM8-mediated XIAP stabilization can also initiate inactivation of Caspase-3, one of the primary executioners of apoptotic cascade. Therefore, TRIM8-mediated XIAP stabilization has the capacity to bring two important oncogenic outcomes during the course of tumorigenesis. First, TRIM8-mediated XIAP stabilization can activate NF-κB, leading to expression of genes involved in autophagy and cell proliferation. Second, TRIM8 mediated stabilized XIAP prevents activation of Caspase-3, leading to the suppression of apoptosis. Through this novel mechanism, TRIM8 prevents cell death during genotoxic stress and radiation therapy,[13] and this suggests TRIM8's highly potential oncogenic calibre can provide survival assistance to cancer cells.

2.3. TRIM8 can act in both an E3 ubiquitin ligase-dependent and -independent manner

Historically, TRIM8 is considered to be among E3 ubiquitin ligases due to the presence of RING domain. Although the mechanism of activation of its E3 ubiquitin ligase activity is not known yet, TRIM8 has been shown to function as an E3 ubiquitin ligase in several important biological pathways. It is demonstrated that TRIM8 mediates K63-linked polyubiquitination of TGF-β-activated kinase 1 (TAK1), triggered by TNF-α and IL-1β, and, through this, TRIM8 serves as a critical regulator of TNF-α- and IL-1β-induced NF-κB activation.[7] During Pseudomonas aeruginosa (PA)-induced keratitis infection, TRIM8 promotes K63-linked polyubiquitination of TAK1, leading to its activation and enhanced inflammatory responses.[14] Ye et al. [15] reported that TRIM8 can interact with Toll/IL-1 receptor domain-containing adaptor-inducing IFN-β (TRIF) and mediates its K6- and K33-linked polyubiquitination, which leads to the disruption of the TRIF-TANK-binding kinase-1 association. In general, it is known that K63-linked ubiquitination is involved in regulating proteasome-independent functions, including cellular processes, like endocytosis and inflammatory immune responses, innate immunity, protein trafficking, and NF-κB signaling, whereas K6-linked polyubiquitination is known to be associated with DNA damage response and Parkin-mediated mitophagy, and K33-linked polyubiquitination is associated with TCR signaling, post Golgi-trafficking, and AMPK-related kinase signaling.[16] Currently, it is experimentally well established that TRIM8 can perform K63-, K6-, and K33-linked polyubiquitination. TRIM8 also plays an important role in proteasomal degradation of SOCS1,[6] although it has not been proven yet whether it is through TRIM8-mediated K48-linked ubiquitination or in association with other protein complexes. Nevertheless, TRIM8's E3 ubiquitin ligase activity and its involvement in cancer and immunity lies much beyond doubt. But, against this running flow, recent studies have reported that TRIM8 can not only act in an E3 ubiquitin ligase-independent manner, but it can also protect phosphorylated IRF7 (pIRF7) from proteasomal degradation through an E3 ubiquitin ligase-independent path by preventing its recognition by the peptidyl-prolyl isomerase Pin1.[17]

2.4. Duality in localization: nucleus and cytoplasm

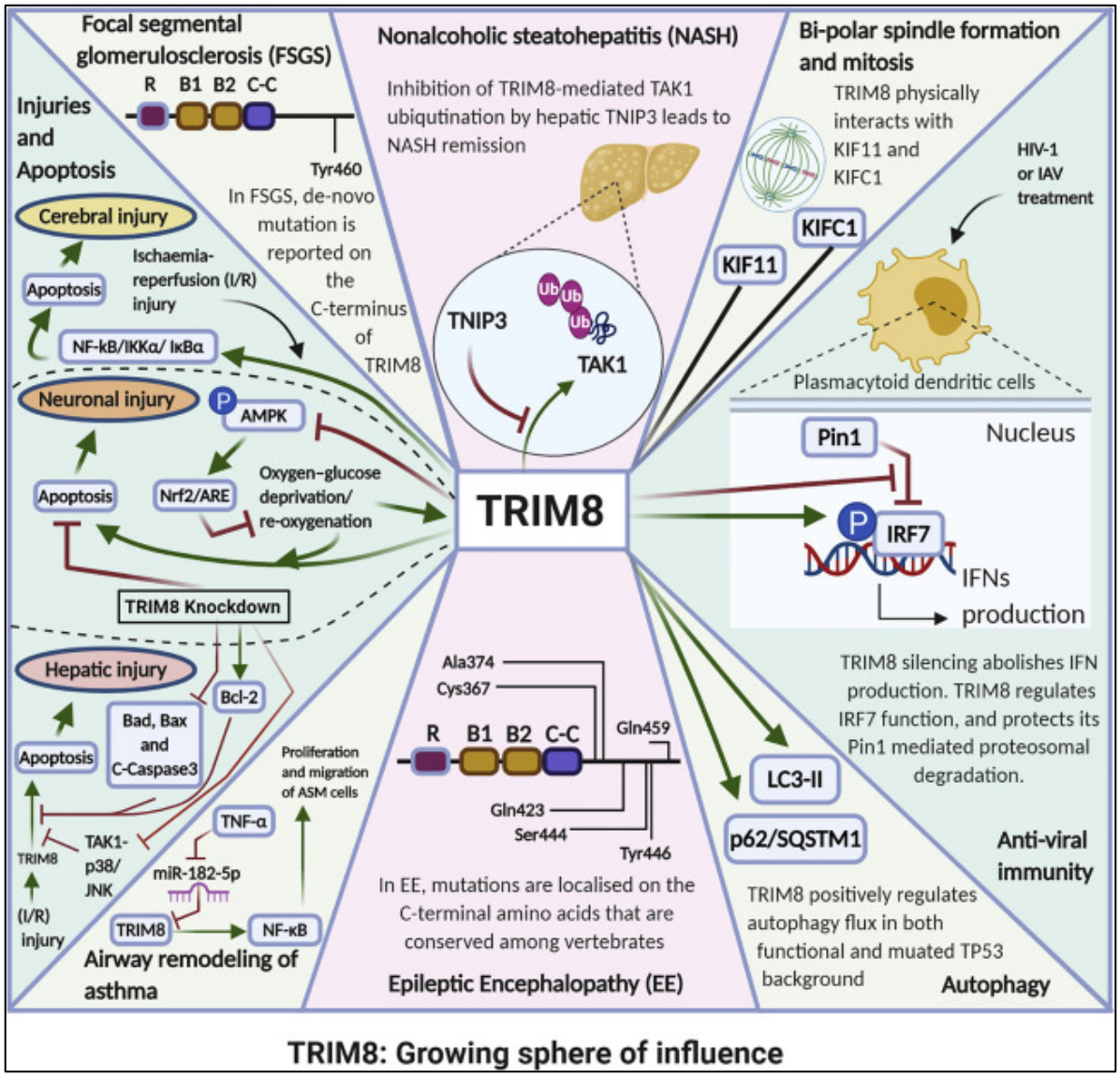
TRIM8 can function at two subcellular sites—nucleus and cytoplasm—to regulate NF-κB, one of the central signaling pathways that plays a critical role in carcinogenesis and inflammatory diseases. PIAS3 is known to negatively regulate the NF-κB pathway via its interaction with p65 in the nucleus. Expression of TRIM8 enhances NF-κB activity even in the presence of PIAS3, suggesting TRIM8 can inhibit PIAS3-mediated negative regulation of NF-κB.[12] But TRIM8's ability to positively regulate NF-κB activity is not limited within the nucleus. Li et al. observed earlier that TRIM8 positively regulates the NF-κB pathway by K63-linked polyubiquitination of cytoplasmic protein TAK1. Following this, Tomar et. al.[12] showed that TRIM8 can

also regulate NF-κB activity in the cytoplasm under the influence of TNF-α. The study revealed that TNF-α induces the translocation of TRIM8 from the nucleus to the cytoplasm, which positively regulates NF-κB. A time-course study for TRIM8 nucleo-cytoplasmic translocation upon TNF treatment showed that TRIM8 translocates to the cytoplasm within 15 min and re-translocates back to the nucleus after 12 h.[12] Another instance of TRIM8's nucleo-cytoplasmic translocation can be found in the regulation of STAT3 by PIAS3. In the cytoplasm, the ectopic expression of TRIM8 promotes proteasomal degradation of PIAS3, leading to the nuclear translocation of STAT3, whereas, in the nucleus, TRIM8 facilitates the recruitment of STAT3 to the STAT3-inducible element (SIE) regions of several brain- and cancer-related genes and binds to the SIE regions of the same genes.[18]

2.5. Duality of TRIM8 in glioblastoma

The regulation of STAT3 by TRIM8 via PIAS3 is extensively important in the context of glioblastoma. Zhang et al. reported that TRIM8 initiates STAT3 signaling to maintain stemness and self-renewal capacity in glioblastoma-like stem cells (GSCs) by suppressing the expression of PIAS3. Knockdown of TRIM8 reduces GSC self-renewal, whereas overexpression of TRIM8 leads to enhanced GSC self-renewal.[19] The study has further shown that STAT3 activation can also upregulate TRIM8 expression, even in the setting of hemizygous gene deletion in glioblastoma, and this bi-directional positive feedback mechanism facilitates stemness in GSCs. This suggests that TRIM8 acts as an oncogenic molecule by promoting the self-renewal capacity of GSCs. In contrast, we showed that the downregulation of TRIM8 in glioblastoma compared to its normal counterpart is indeed associated with a significant increase in the risk of disease progression in patients. Most importantly, TRIM8 overexpression and restored TRIM8 expression significantly reduce both cell proliferation and clonogenic potential in glioma cells, suggesting the anti-proliferative capacity of TRIM8 in glioma patients.[20] However, it is not at all clear how a molecule that helps the self-renewal capacity in GSCs can alter its mechanism to reduce the proliferation and clonogenic potential in glioma cells.

Finally, it can be said that TRIM8 is a unique molecule to possess duality in multiple functional aspects, and this interesting functional feature of TRIM8 compelled me to name it as a "molecule of duality (MoD)" as I summarized in the Literature Review Illustration 1.



Literature Review Illustration 2. Growing sphere of the influence of TRIM8 in multitude of biological processes. **Credit**: Bhaduri, Utsa, and Giuseppe Merla. "Rise of TRIM8: A Molecule of Duality." Molecular therapy. Nucleic acids vol. 22 434-444. 2 Sep. 2020, doi:10.1016/j.omtn.2020.08.034.

2.6. Emerging role of TRIM8 in mitosis

Over the last two decades, several studies reported the involvement of TRIM8 in different aspects of cell and disease biology (Literature Review Illustration 2). In a recent study it is shown that TRIM8 interacts with KIF11 (also known as EG5) and KIFC1, two master regulators of mitotic spindle assembly and cytoskeleton reorganization, and localizes at the mitotic spindle during the stages of bi-polar spindle formation. The study also suggested that TRIM8 is required for chromosomal stability and the control of chromosomal replication, as it is observed that silencing of TRIM8 caused aneuploidy and resulted in a significant increase of cells with less than 46 chromosomes. In particular, KIF11 inhibition and KIFC1 overexpression are known

to be sufficient enough to induce a monopolar spindle phenotype in mitotic cells, and thus the physical interactions of TRIM8 with KIF11 and KIFC1 strongly advocate for the plausible role of TRIM8 in coordinating cell polarity during mitosis. From this experimental data, some interesting clues are emerging. On one hand, TRIM8 localizes on centrosomes and colocalizes with Plk1 and directly interacts with CEP170-like centrosomal protein, and silencing of TRIM8 induces a delay of the mitosis progression with a cell accumulation in the G2/M phase.[8] Therefore, while reviewing the literature, I suggested that TRIM8 can play an important function during the course of centrosome duplication. On the other hand, TRIM8 is present throughout all phases of mitosis and physically interacts with some of the most important mitotic assembly proteins like KIF11, KIFC1, KIF20B, and KIF2C.

Altogether, it can be suggested that TRIM8 may play an important function in the bi-polar spindle formation from the very onset of centrosome duplication until the end of the division of one cell into two daughter cells, a signature process in eukaryotic life mediated by microtubules and kinesin family proteins. Or in short, it can also be said, at different stages of cell cycle TRIM8 has different roles to play. But so far there is no systematic study that has attempted to evaluate or to estimate the plausible role of TRIM8 or involvement in pathways during different phases of mitosis.

Chapter 3. Objectives of the thesis

In my PhD research project, I attempted to understand the possible involvement of E3 ubiquitin ligase TRIM8 in different stages of mitosis with the aid of differential transcriptomic (single-cell RNA sequencing) and proteomic (LC-MS/MS) approaches along with the cell and molecular biology techniques by integrating experimental biology with big data analysis and machine learning. Based on my literature review, as mentioned in the previous section, I raised three precise questions and set them as the objectives of the thesis. First, whether TRIM8 is involved in any of the mitotic cell cycle or cell cycle associated pathways. Second, if silencing of *TRIM8* can impact any of the key proteins that are involved in chromosomal replication or stability. And, third, whether TRIM8 has any possibility to play any role at the centrosomal region during the course of mitosis. The broad aim of this descriptive thesis is to perform a systematic study for elucidating the involvement or plausible role of TRIM8 in different biological processes/molecular pathways during the course of mitotic cell cycle, from its early onset till the very end.

Chapter 4. Materials and methods

4.1. Cell culture

hTERT-immortalized retinal pigment epithelial cells (hTERT RPE-1; hereafter RPE) and HeLa cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L Glucose with UltraGlutamineTM w/ sodium pyruvate (BE12-604F/U1), and 10% filtered Fetal bovine serum (FBS) and 1% Penicillin-Streptomycin Mixture (DE17-602F).

4.2. siRNA mediated silencing of TRIM8

Synthetics siRNA-mediated silencing of *TRIM8* was carried as per the instructions of Lipofectamine® RNAiMAX Transfection (Invitrogen™) protocol. $6x10^5$ RPE cells were cultured on a 10 cm dish. Lipofectamine® RNAiMAX Transfection Reagent was diluted in Opti-MEM™ Reduced-Serum Medium as per the manufacturer's instruction. Incubation time of transfection reagent was set 24 hours and the final amount of *TRIM8*-siRNA was 80 pmols for each 10 cm dish. After 24 hours of transfection, RPE cells were collected for further experimental studies.

4.3. RNA isolation, cDNA preparation, and quantitative reverse transcription PCR (RT-qPCR)

Total RNA from RPE cells transfected with a *TRIM8*-siRNA or with the siRNA negative control was isolated as per the instruction of QIAGEN RNeasy Mini Kit for purification of total RNA. Upon isolation, reverse transcription was done using the Quantitect Transcription kit (Qiagen), according to the manufacturer's instructions. DNA oligonucleotide primers for RT-qPCR were designed using the Applied Biosystems™ Primer Express™ software with default parameters. *GAPDH* and *EEF1A1* were used as reference genes in the experiment. The reactions were run in triplicate in 10 ul of final volume with 10 ng of sample cDNA, 0.3mM of each primer, and 1XPower SYBR Green PCR Master Mix (Thermo Fisher Scientific-Applied Biosystems). Reactions were set up in a MicroAmp™ Optical 384-Well Reaction Plate with Barcode and run in an ABI Prism7900HT (Thermo Fisher Scientific-Applied Biosystems) with default amplification conditions. Raw Ct values were taken from SDS 2.4 (Applied Biosystems). Normalisation and relative changes in gene expression were quantified using $2^{-\Delta\Delta Ct}$ method.

4.4. Differential proteomic study (LC-MS/MS): sample preparation and analysis

RPE cells transfected with a TRIM8-siRNA or with the siRNA negative control were collected for cell lysis treatment with 5% SDS, 50mM ammonium bicarbonate

(NH4HCO3). Cells were sonicated, centrifuged and protein extraction was recovered. Protein lysis was performed using S-trap cartridge and label-free proteomics data was analysed on MaxQuant software. Further detail is illustrated below.

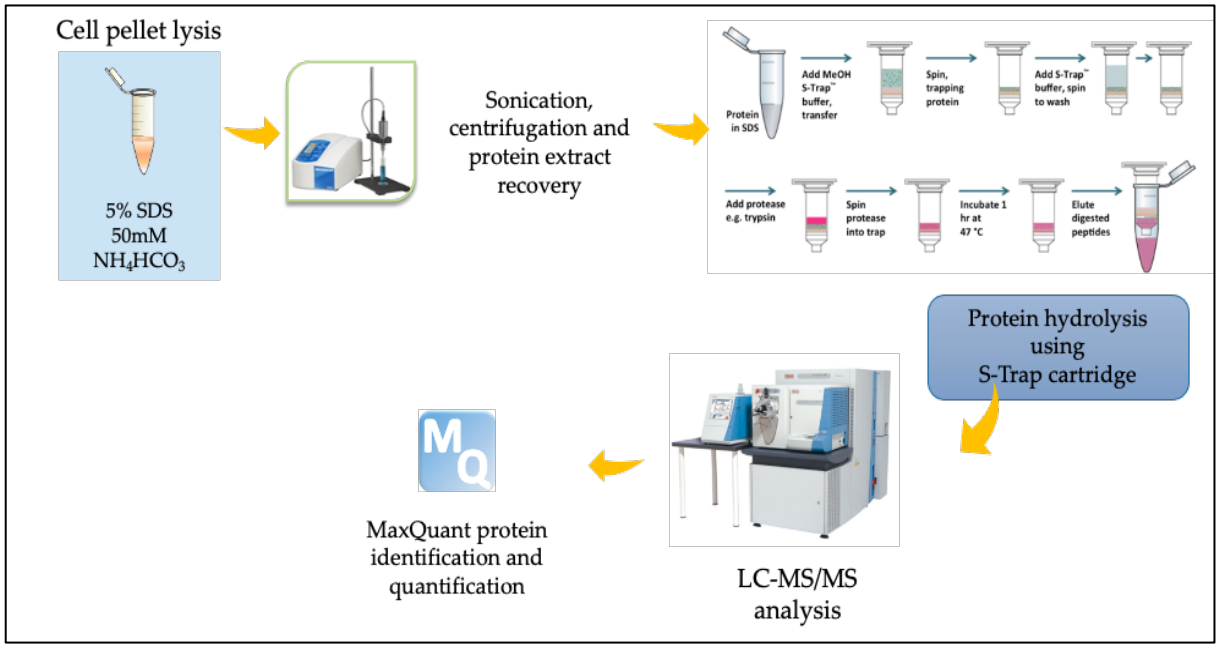


Illustration. Workflow for Liquid chromatography-tandem mass spectrometry (LC-MS/MS). **Courtesy**: Group of Dr. Maria Monti, University of Naples Federico II.

4.5. Library preparation for BD Rhapsody scRNA-seq

Sample preparation for mRNA Whole Transcriptome Analysis (WTA) was performed as per the guideline of BD Single-Cell Multiplexing Kit (Cat. No. 633781). To distinguish two conditions in the POOL of cell, Sample Tag 05 was used for siRNA negative control RPE cells and Sample Tag 06 was used for TRIM8-silenced RPE cells. A detailed illustration of BD Rhapsody protocol is given below.

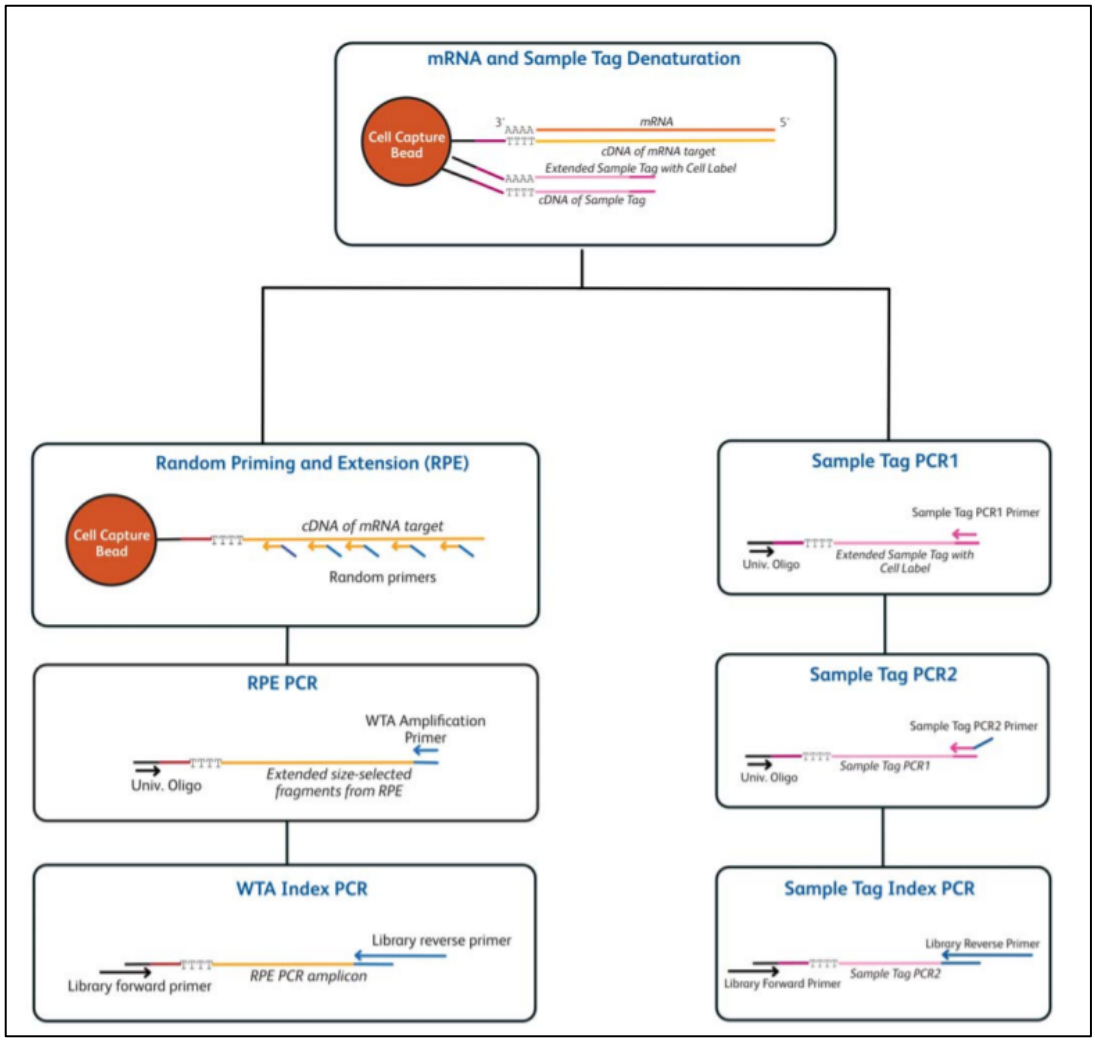


Illustration: BD Rhapsody™ mRNA whole transcriptome analysis (WTA) and sample tag library preparation protocol. **Credit:** BD Rhapsody™.

4.6. RNA sequencing with Illumina NextSeq 500

RPE POOL1 (4000 cells) and RPE POOL2 (4000 cells). Each POOL was a mixed-up sample of TRIM8-silenced and siRNA control for a respective cell line which is distinguishable by sample tags as mentioned in the BD Rhapsody Library Preparation. To maintain the rigour, each pool was run separately on Illumina NextSeq 500 (High Output Kit v2.5; paired-end 2x75 bp sequencing). In each of the two runs, 4000 cells were targeted to be achieved, with an attempt of 8000 cells in total for further analysis. Sequencing was performed on the in-house NextSeq®500 System as per the instructions from Illumina Technical Support in Europe.

4.7. Confocal microscopy and immunofluorescence study

HeLa cells were synchronized with 12h double block with thymidine 4mM. For immunofluorescence study, cells were fixed in ice cold methanol for 15′ at +4°C.

Blocking was done in PBS + 1% BSA for 1h at Room Temperature (RT), followed by incubation with primary antibody (1° Ab in PBS + 0.1% BSA; 2h RT), secondary antibody (2° Ab in PBS + 0.1% BSA 1h RT), and 200 nM DAPI for 5' at RT. Primary antibodies used in experiment were rabbit anti-CEP170, mouse anti-TRIM8 (sc-398878), rabbit anti-TRIM8 (HPA023561), and mouse anti-Y-tubulin (T-6557). Polyclonal rabbit antibodies used in this experiment against CEP170 were originally raised against N-terminal (aa 15–754) fragment of CEP170, as described in Guarguaglini, Giulia et al. (2005).[21]

4.8. Bioinformatic analysis of BD Rhapsody single-cell RNA-seq (scRNA-seq) data

Raw single cell RNA-seq data generated on Illumina NextSeq 500 was first checked on Sequencing Analysis Viewer. Thereafter, the FASTQ files were generated and quality control analysis was performed on FastQC. For the single cell analysis, FASTQ files were analysed on the standard BD Rhapsopy Pipeline available on the Seven Bridges platform. Data table containing molecules per gene per cell based upon RSEC error correction was generated and differential expression analysis was carried out at POOL level using BD Rhapsody pipeline on the SeqGeq platform. As dispersion is related to variance, and variance tends to correlate with the ability of a parameter to separate biologically relevant populations within data, the highly dispersed genes were selected to perform the Principal Component Analysis (PCA) on the total population of cells. PCA was performed to generate two distinct clusters for control and TRIM8-silenced condition using sample tags used in library preparation (Sample Tag 05 was used for siRNA negative control RPE cells and Sample Tag 06 was used for TRIM8-silenced RPE cells). Further, t-SNE plot was generated to preserve the local structure in the high dimensional data set. PhenoGraph machine learning was used to divide the population of cells into different subpopulations based on the 97 known cell cycle marker genes in humans (43 S-phase marker genes, 54 G2/M marker genes). A further in-depth differential analysis was performed again at different stages of cell cycle to see the real impact of TRIM8-silencing on cell cycle marker genes so that the analysis doesn't get biased by the cell cycle heterogeneity of single-cell data. Pathway analysis and z-score calculation was performed to mease the activation of biological function at both POOL level data and on the mitotic stage specific data (G0/G1, S, G2/M).

4.9. Sucrose gradient fractions study for translational activity

TRIM8-siRNA treated RPE cells and negative siRNA treated control RPE cells were collected upon transfection. Upon cell lysis, sucrose-gradient centrifugation was performed to get the different fractions and absorbance was measured on 254 nm to differentiate polysome and monosome bound RNAs and free RNAs. Polysome to monosome ratio was calculated to check the changes in translation activity upon silencing of *TRIM8*.

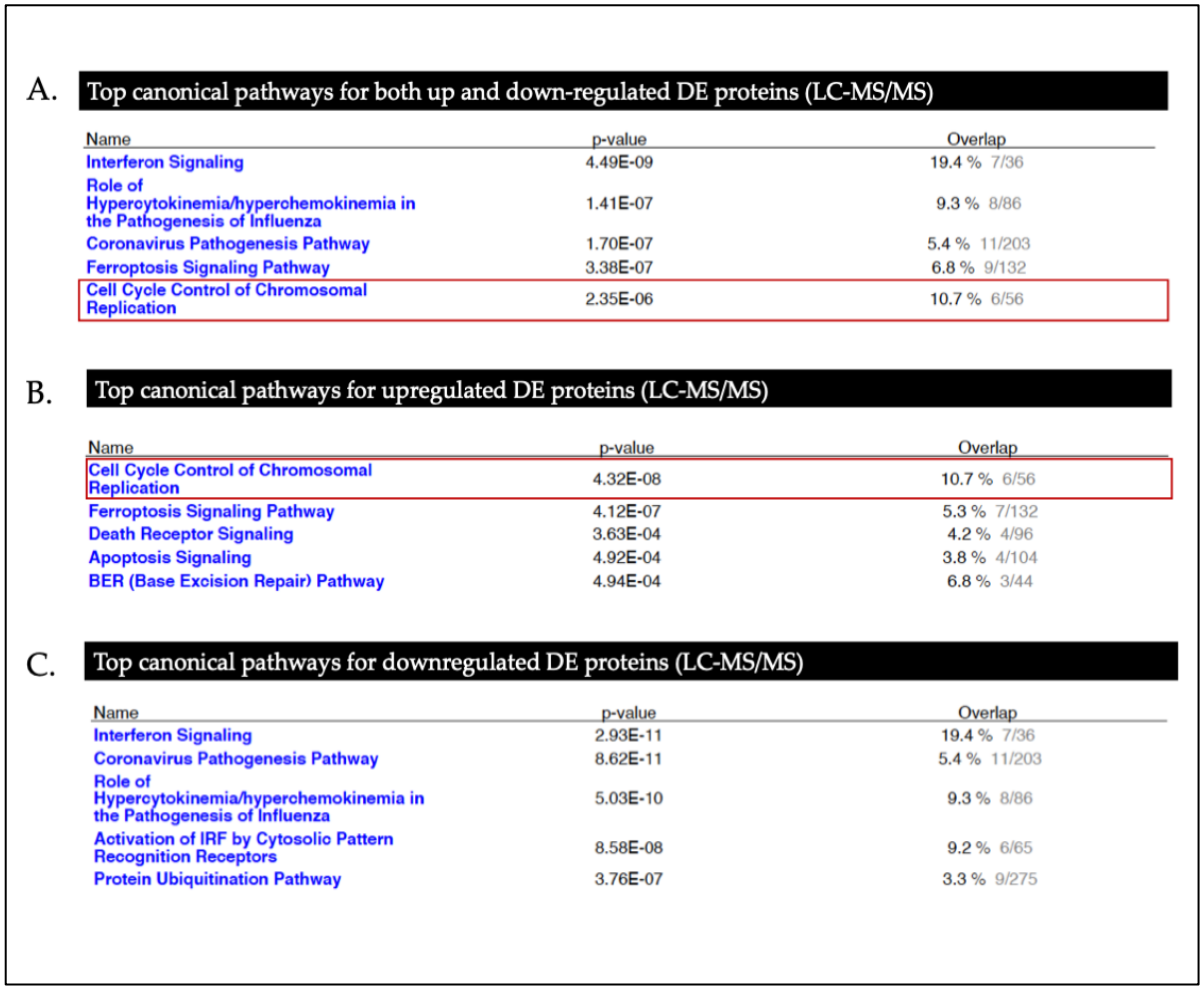
Chapter 5. Results

5.1. Differential proteomic study (LC-MS/MS) upon silencing of *TRIM8*

5.1.1. Identification of up- and down-regulated pathways

Analysis of differential proteomic expression study revealed 163 differentially expressed proteins upon silencing of TRIM8 in RPE cells, of which 83 were found to be upregulated and 80 downregulated. Top five most significant upregulated proteins were Tropomyosin alpha-1 chain (TPM1), Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1 (NUCKS1), Proliferation marker protein Ki-67 (MKI67), Sequestosome-1 (SQSTM1), DNA topoisomerase 2-alpha (TOP2A), Thymosin beta-4 (TMSB4X). Whereas top five most significant down regulated proteins were Interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), Interferon-induced protein with tetratricopeptide repeats 1 (IFIT1), Interferon-induced protein with tetratricopeptide repeats 2(IFIT2), Ubiquitin-like protein ISG15 (ISG15), Probable ATP-dependent RNA helicase DDX58 (DDX58). When all 163 up- and down-regulated proteins were fed together in Ingenuity Pathway Analysis (IPA), top five significant pathways were Interferon Signalling, Role of Hypercytokinemia /heyperchemokinemia in Pathogenesis of Influenza, Coronavirus Pathogenesis Pathway, Ferroptosis Signalling Pathway, Cell Cycle Control of Chromosomal Replication (Figure 1.A) . Interestingly, when the IPA pathway analysis was done only with the 83 upregulated proteins, Cell Cycle Control of Chromosomal Replication emerged as the top most hit with a p-value of 4.32E-08 and an overlap of 10.7%, followed by Ferroptosis Signalling Pathway, Death Receptor Signalling, Apoptosis Signalling, and Base Excision Repair pathway (Figure 1.B). This shows the high tendency of upregulated differentially expressed proteins upon silencing of *TRIM8* to be involved in important cell cycle and cell cycle associated pathways. When 80 downregulated proteins were analysed separately, Interferon Signalling pathway came as the top hit followed by Coronavirus Pathogenesis Pathway, Role of Hypercytokinemia /heyperchemokinemia in Pathogenesis of Influenza, Activation of IRF by Cytosolic Pattern Recognition Receptors, and Protein Ubiquitination Pathway (Figure 1.C). As TRIM8 is a distinguished member of the TRIM E3 ubiquitin ligases, emergence of Protein Ubiquitination Pathway in the top five downregulated pathways is quite expected. Whereas, Cell Cycle Control of Chromosomal Replication pathway as top most significant upregulated pathway in IPA analysis shows the importance of TRIM8 as an important cell cycle protein. Alongside the IPA analysis, the traditional STRING analysis of the upregulated proteins in humans also showed the "Cell Cycle, Mitosis" is the top hit (FDR: 1.19e-05) in the reactome pathways (Figure 2). Other than TOP2A which is essential for DNA replication, major MCM complex proteins (MCM3, MCM4, MCM5, MCM6, and MCM7), that are known to regulate once per cell cycle,[22] were also significantly upregulated in the cell cycle control of chromosomal replication pathway. Centrosomal protein CEP170, that was

previously shown in a published work to interreact with TRIM8,[8] is also upregulated in this present differential proteomics study.



A. Top canonical pathways for both up and down-regulated DE proteins (LC-MS/MS)

Name	p-value	Overlap
Interferon Signaling	4.49E-09	19.4 % 7/36
Role of Hyperoytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	1.41E-07	9.3 % 8/86
Coronavirus Pathogenesis Pathway	1.70E-07	5.4 % 11/203
Ferroptosis Signaling Pathway	3.38E-07	6.8 % 9/132
Cell Cycle Control of Chromosomal Replication	2.35E-06	10.7 % 6/56

B. Top canonical pathways for upregulated DE proteins (LC-MS/MS)

Name	p-value	Overlap
Cell Cycle Control of Chromosomal Replication	4.32E-08	10.7 % 6/56
Ferroptosis Signaling Pathway	4.12E-07	5.3 % 7/132
Death Receptor Signaling	3.63E-04	4.2 % 4/96
Apoptosis Signaling	4.92E-04	3.8 % 4/104
BER (Base Excision Repair) Pathway	4.94E-04	6.8 % 3/44

C. Top canonical pathways for downregulated DE proteins (LC-MS/MS)

Name	p-value	Overlap
Interferon Signaling	2.93E-11	19.4 % 7/36
Coronavirus Pathogenesis Pathway	8.62E-11	5.4 % 11/203
Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	5.03E-10	9.3 % 8/86
Activation of IRF by Cytosolic Pattern Recognition Receptors	8.58E-08	9.2 % 6/65
Protein Ubiquitination Pathway	3.76E-07	3.3 % 9/275

Figure 1. Ingenuity Pathway Analysis (IPA) for the differentially expressed proteins in differential proteomics study (LC-MS/MS). A) Top canonical pathways for both up and down-regulated DE proteins (LC-MS/MS). B) Top canonical pathways for upregulated DE proteins (LC-MS/MS). C) Top canonical pathways for downregulated DE proteins (LC-MS/MS).

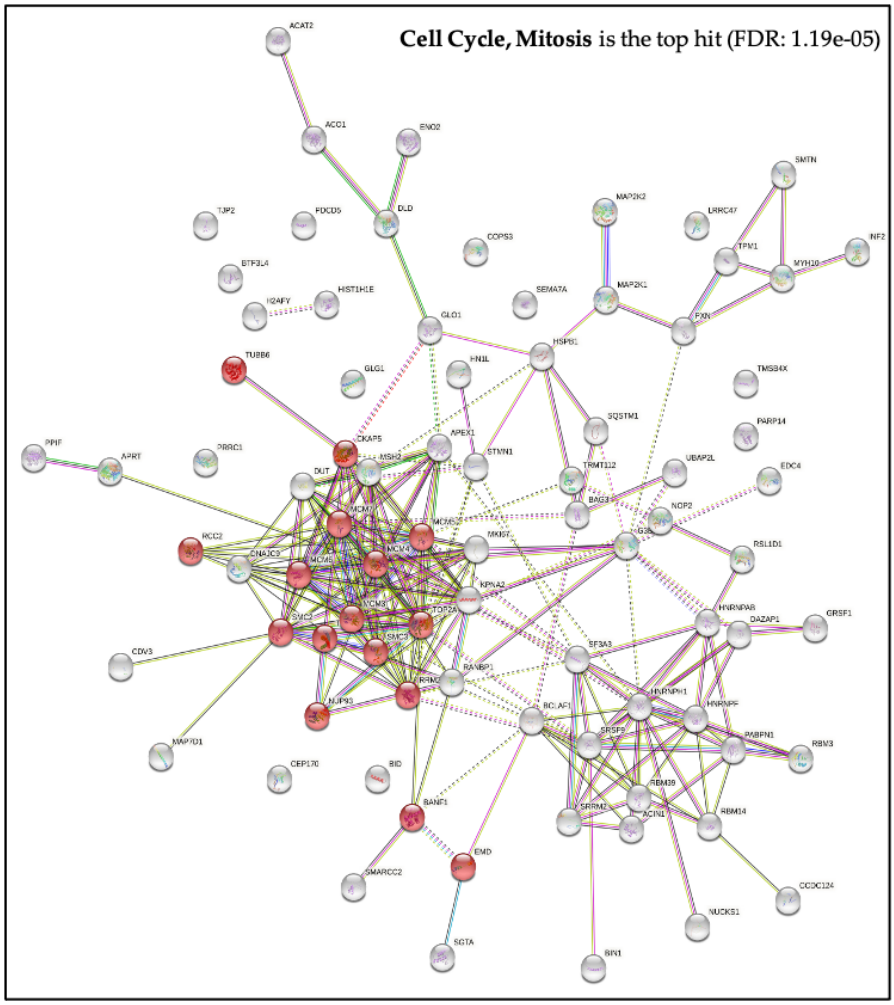


Figure 2. STRING analysis of the 83 upregulated proteins in differential proteomics study (LC-MS/MS). "Cell Cycle, Mitosis" is the top hit with FDR: 1.19e-05.

5.1.2. Identification of transcription factors among the up- and down-regulated proteins

Next, protein class identification of the differentially expressed proteins in the LC-MS/MS study was performed using the functional classification system of PANTHER database (http://www.pantherdb.org/). Protein modifying enzyme and metabolite interconversion enzyme were the two top most classification. Importantly, SMARCC2, a chromatin binding regulatory protein was identified that is also known to regulate transcriptional activity of certain genes that are involved in chromosomal stability (Figure 3). Publicly available ChIP-Seq data on UCSC Genome Browser shows that SMARCC2 also occupies the promoter region of TOP2A (Figure 4). Five other gene specific transcriptional regulators, STAT1, STAT2, IFI16, TF3L4, IFI35, were also identified among the differentially expressed proteins (Figure 3).

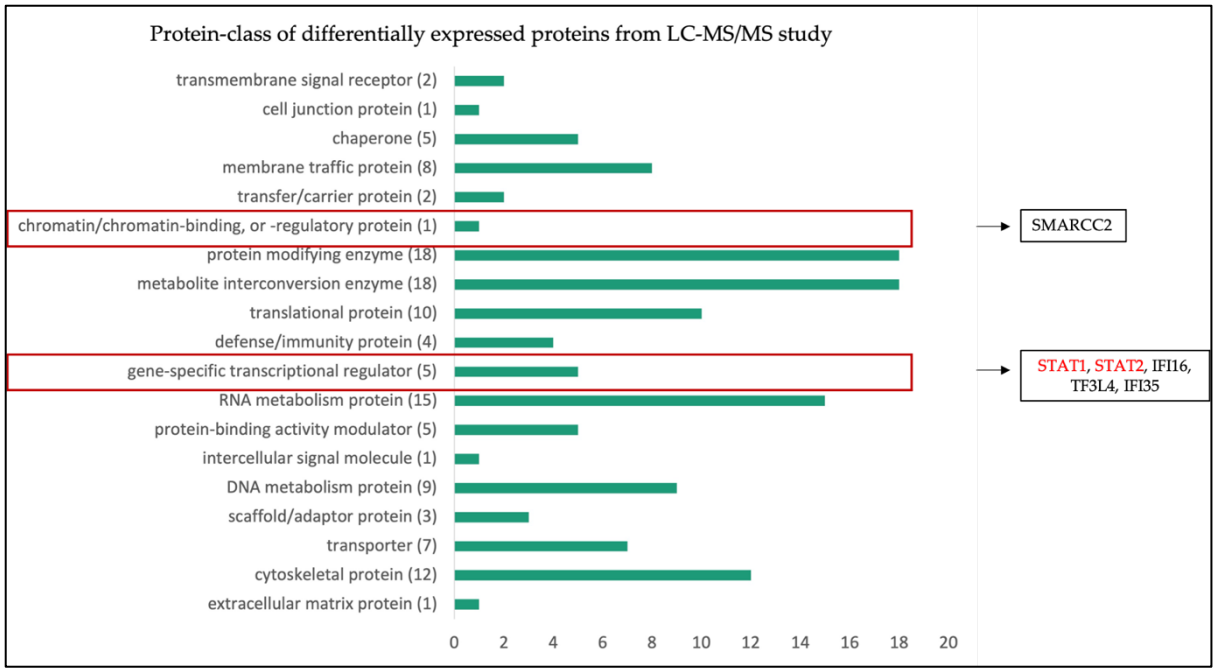


Figure 3. PANTHER protein-class identification of differentially expressed proteins in in LC-MS/MS study. Six transcriptional regulatory proteins SMARCC2, STAT1, STAT2, IFI16, TF3L4, IFI35 have been identified.

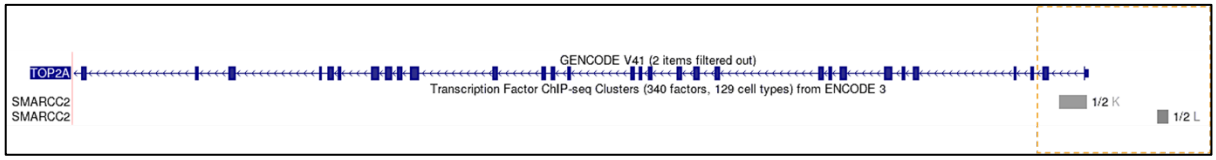


Figure 4. Visualisation of SMARCC2 ChIP-seq data on UCSC Genome Browser. SMARCC2 occupies the TOP2A promoter region.

5.1.3. z-score calculation predicts that TRIM8 activates the canonical "Cell Cycle Control of Chromosomal Replication" pathway

Next, a comparative analysis of canonical pathways with both up- and down-regulated proteins were performed on the QIAGEN Ingenuity Pathway Analysis (IPA). The top three significant pathways in the comparative analysis were Hypercytokinemia /heyperchemokinemia in Pathogenesis of Influenza, Interferon Signalling, and the Cell Cycle Control of Chromosomal Replication. Interestingly, in the comparative analysis also all the proteins present in the canonical Cell Cycle Control of Chromosomal Replication pathway were only upregulated. Thereafter, an activation *z-score* of biological function was calculated in order to understand the increase in activation of a biological function upon silencing of *TRIM8*. In this calculation a positive *z-score* signifies the increase in activation of biological function, whereas a negative *z-score* signifies the decrease in activation of biological function. Here, upon silencing of *TRIM8* in RPE cells, "Cell Cycle Control of Chromosomal

Replication" emerged as the top hit with a -log (B-H p-value) of 4.95 and a positive z-score of a positive z-score of 2.449 among the canonical pathways.

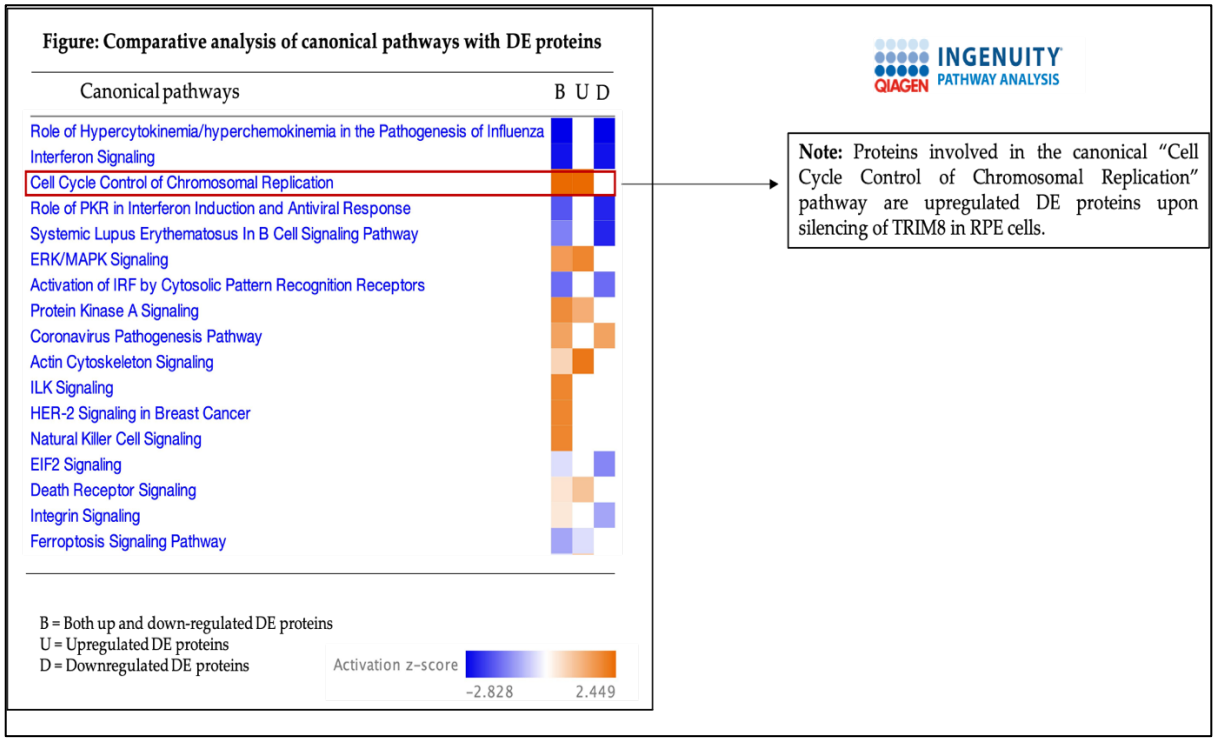


Figure 5. Comparative analysis of canonical pathways both differentially expressed up- and down-regulated proteins. All differentially expressed proteins in the "Cell Cycle Control of Chromosomal Replication" pathway are upregulated.

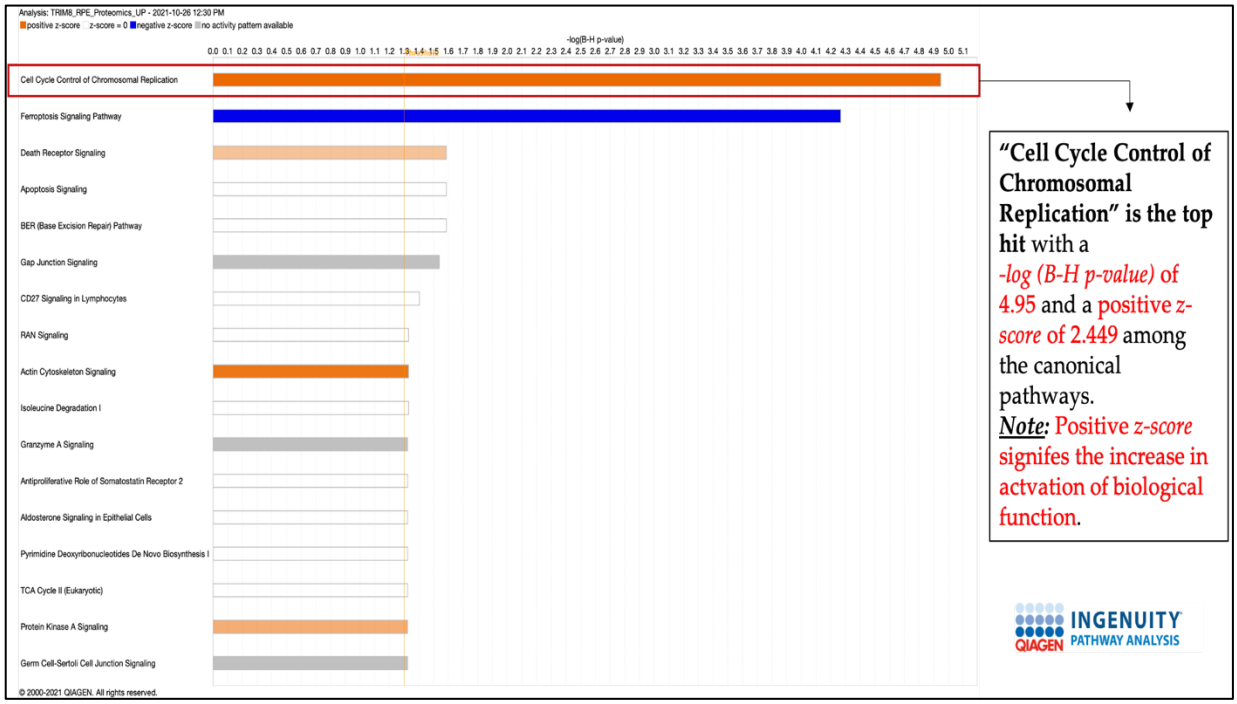


Figure 6. *z-score* calculation predicts silencing of *TRIM8* activates the canonical *"Cell Cycle Control of Chromosomal Replcation"* pathway. Positive *z-score* signifies the increase in activation of a biological function as quantified in IPA.

5.1.4. Silencing of TRIM8 significantly downregulates "Interferon Signalling" pathway

For the downregulated proteins in the differential proteomics study, an abundance in perturbation of immunity-related biological processes/pathways is observed. Particularly, Interferon Signalling" pathway was found to be significantly downregulated (Figure 1.C). This reflects a recent report where TRIM8 is shown to be required for virus-induced IFN response in human plasmacytoid dendritic cells.[17] The interferon-induced proteins with tetratricopeptide repeats (IFITs), IFIT3, IFIT2, IFIT1, known to host innate immune response to viruses, are most significantly downregulated (Figure 7**)**. Interestingly, which was not known before is that silencing of *TRIM8* can downregulate two other members of the TRIM family proteins, TRIM19 (also known as PML) and TRIM25 (Appendix. Supplementary Table 2).

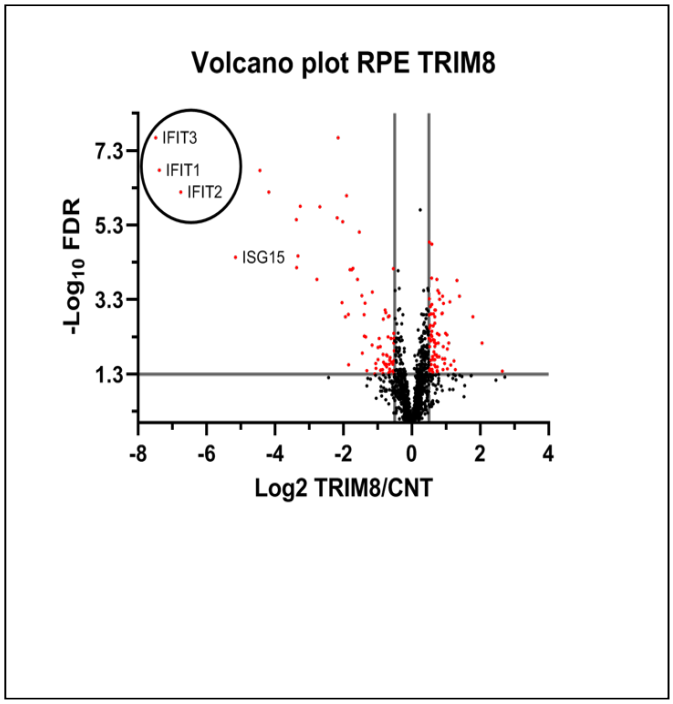


Figure 7. Volcano plot of downregulated proteins upon silencing of TRIM8. IFIT3, IFIT2, IFIT1, known to host innate immune response to viruses are most significantly downregulated

5.2. Silencing of *TRIM8* impacts key translational proteins and translational activity: Study of polysomes fractionation on sucrose gradients

Further analysis of 163 differentially expressed proteins revealed that *TRIM8* downregulation impacted ten translational proteins (Figure 8). Out of which seven were upregulated and three were downregulated. Seven upregulated translational proteins are: RPS16 (40S ribosomal protein S16), WARS (Tryptophan--tRNA ligase, cytoplasmic), RPS9 (40S ribosomal protein S9), CARS (Cysteine--tRNA ligase,

cytoplasmic), RPL18A (60S ribosomal protein L18a), ETF1 (Eukaryotic peptide chain release factor subunit 1), and RPL27A (60S ribosomal protein L27a). Three upregulated translational proteins are: PABPN1 (Polyadenylate-binding protein 2), LRRC47 (Leucine-rich repeat-containing protein 47), and RSL1D1 (Ribosomal L1 domain-containing protein 1). Considering the impact TRIM8 downregulation on important translational proteins an additional study of polysomes fractionations on sucrose gradients was performed to measure the actively translated mRNAs. Polysome profiling separates translated mRNAs on a sucrose gradient according to the number of bound ribosomes and fractions are measured on 254 OD after the sucrose-gradient centrifugation. Thereafter, polysome to monosome ratio has been calculated to observe the changes in translation. Very interestingly, the data shows that upon silencing of *TRIM8* in RPE cells the polysome to monosome ratio has significantly increased. This gives an indication of increase in translational activity upon silencing of TRIM8 (Figure 9).

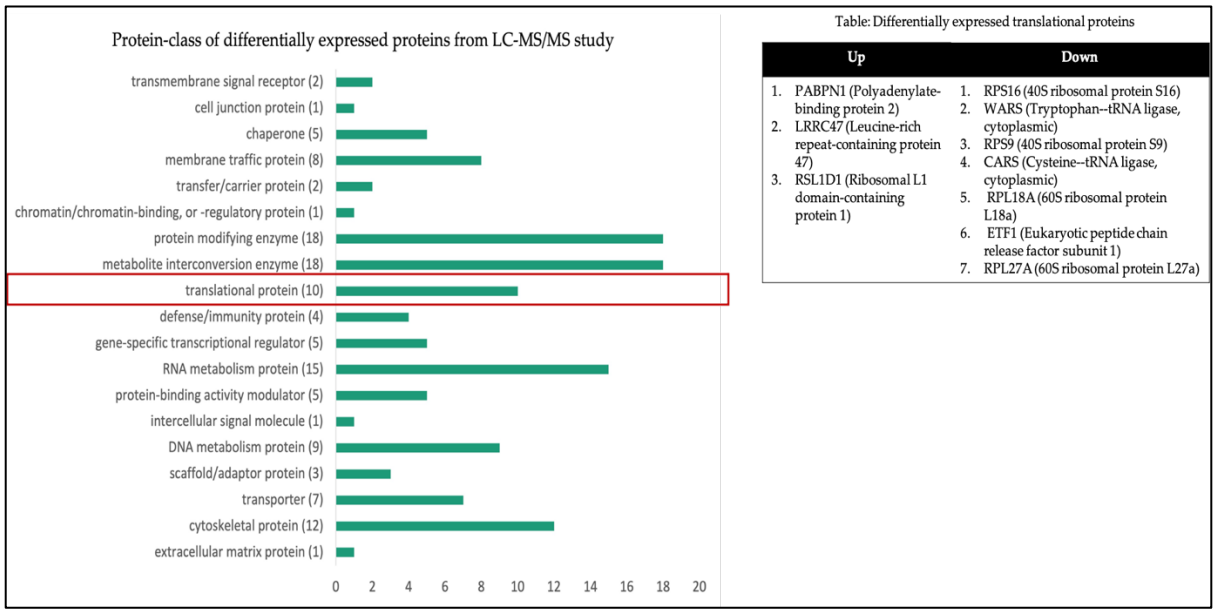


Table: Differentially expressed translational proteins

Up	Down
1. PABPN1 (Polyadenylate-binding protein 2)	1. RPS16 (40S ribosomal protein S16)
2. LRRC47 (Leucine-rich repeat-containing protein 47)	2. WARS (Tryptophan--tRNA ligase, cytoplasmic)
3. RSL1D1 (Ribosomal L1 domain-containing protein 1)	3. RPS9 (40S ribosomal protein S9)
	4. CARS (Cysteine--tRNA ligase, cytoplasmic)
	5. RPL18A (60S ribosomal protein L18a)
	6. ETF1 (Eukaryotic peptide chain release factor subunit 1)
	7. RPL27A (60S ribosomal protein L27a)

Figure 8. Identification of differentially expressed translational proteins/translation activity related proteins in the differential LC-MS/MS study upon silencing of *TRIM8.* Total ten translational proteins have been differentially expressed upon silencing of *TRIM8* in RPE cells.

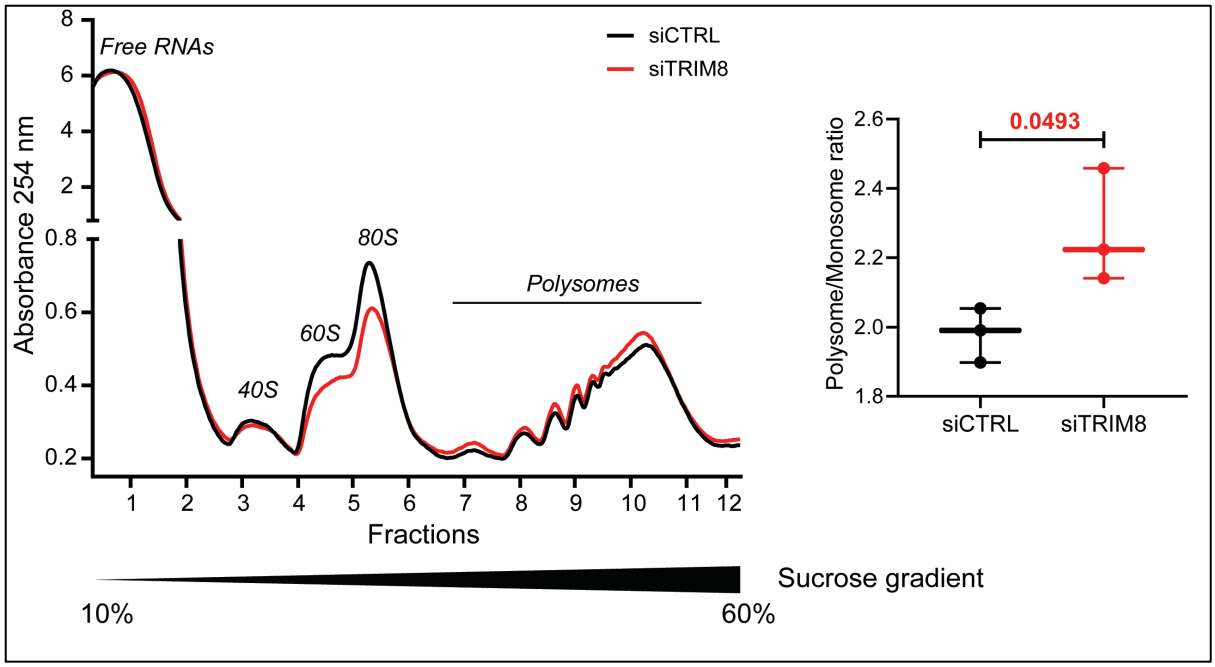


Figure 9. Study of polysomes fractionations on sucrose gradients upon silencing of *TRIM8* in RPE cells. The absorbance of fractions at 254 nm after sucrose-gradient centrifugation (left). Silencing of TRIM8 has increased the polysome to monosome ratio (right).

5.3. Rise of TRIM8-CEP170 axis

5.3.1. TRIM8 knockdown upregulates centriolar protein CEP170

As described in the introduction of the thesis, an earlier mass-spectrometry based interactome study showed that TRIM8 physically interacts with centrosomal protein, CEP170 along with key mitotic assembly proteins.[8] But it was not known whether silencing of *TRIM8* can impact the expression level of those proteins. Here in the differential proteomics study, among the earlier known TRIM8-interacting proteins, CEP170 has been found to be upregulated upon silencing of *TRIM8* (Appendix. Supplementary Table 1).

5.3.2. TRIM8 and CEP170 co-localise at the centrosomes during all phases of mitosis

CEP170 is constantly expressed throughout cell cycle and associates with centrosomes during interphase and with spindle microtubules during mitotis. It can be used to discrimnate bonafide centriole overduplication from centriole amplification that results from aborted cell division. CEP170 is also a physiological substrate of Plk1 kinase and can be phosphorylated by Plk1.[21] Venuto, Santina et al. (2019) showed earlier that TRIM8 can colocalise with Plk1 at the midbodies. As found in the differential proteomics study (5.2.1) that silencing of *TRIM8* upregulates CEP170, next an immunofluorescence study was performed to understand the co-localisation of

TRIM8 with CEP170. And, what is observed is that TRIM8 colocalises with CEP170 at the centrosomes during all phases of mitotic cell division in HeLa cells, from the beginning of interphase till the end of telophase. During the mid-interphase TRIM8 is bit scattered in the nucleus but its colocalization with CEP170 remains constant specifically at the centrosomal region (Figure 10).

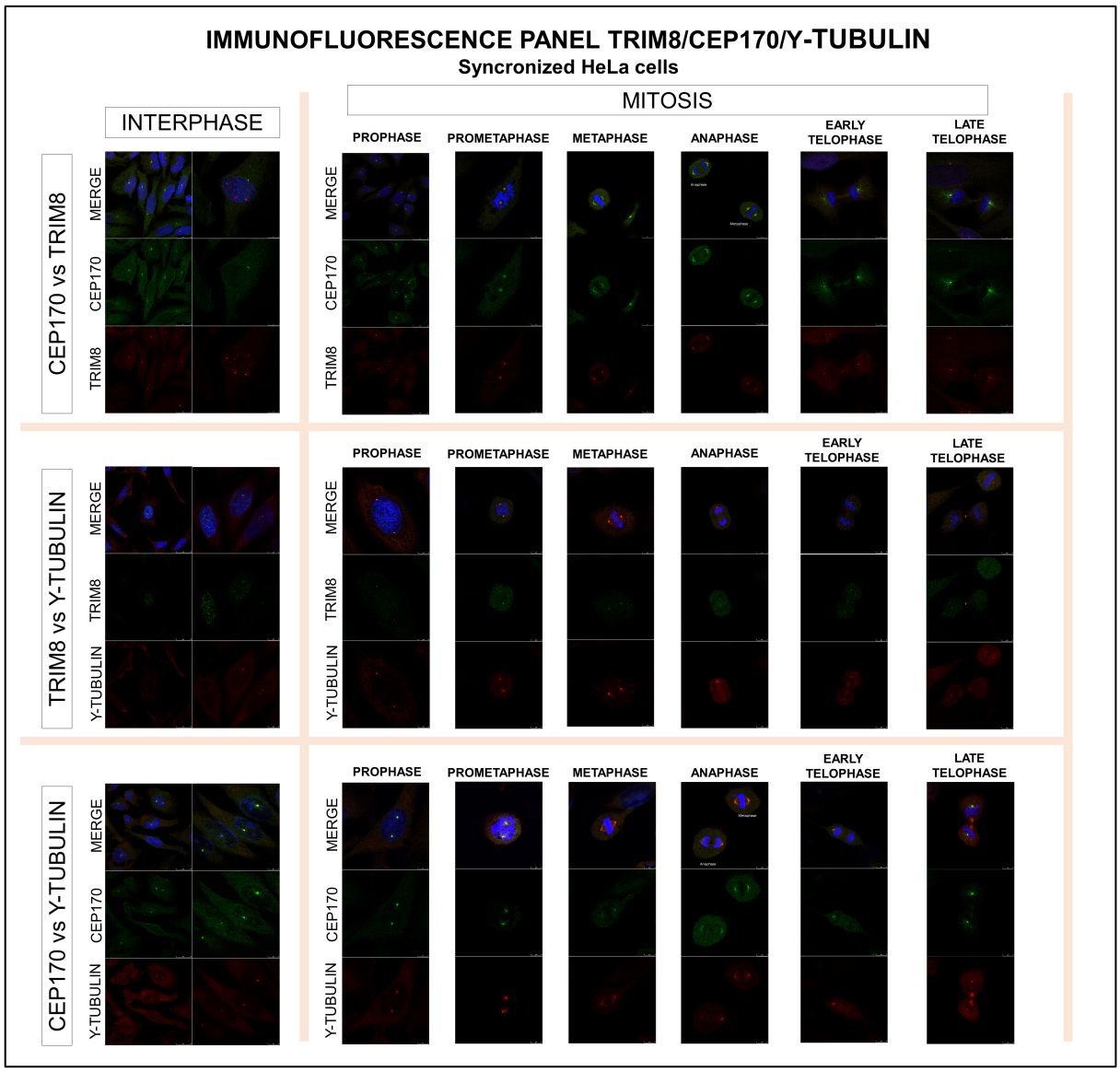


Figure 10. TRIM8 and CEP170 co-localise at the centrosomes during all phases of mitosis. During the interphase (possibly mid-interphase), TRIM8 has a tendency to be scattered in the nucleus, but its colocalization with CEP170 remains constant at the centrosomal region during all phases of mitotic cell cycle.

5.4. Differential transcriptomic study upon silencing of *TRIM8*

5.4.1. Principal component analysis (PCA): identification of the cluster of cells for control and silenced conditions

Differential proteomics study revealed that silencing of *TRIM8* has significantly activated the "Cell Cycle Control of Chromosomal" pathway (Chapter 5.1.3). To understand this impact of *TRIM8*-silecning at RNA-level we also performed the single-cell RNA sequencing. In the first step, we performed a POOL-level analysis (without discriminating the stages of cell cycle) of BD Rhapsody single-cell RNA-seq data. Based on the sample tagging during library preparation, we divided cells into control and silenced conditions. In this step of POOL level analaysis, I first attempted to understand whether Principal Component Analysis (PCA) can generate two clusters, i.e., one for control RPE cells and the other for TRIM8-silenced condition. Interestingly, PCA has revealed the POOL of cells can be differentiated into two distinct clusters, one for each sample tag. PCA on POOL of cells was done upon selction of the highly dispersed genes, as prescribed in the BD Rhapsody protocol. Sample Tag 05 (green) refers here the control RPE cells and the Sample Tag 06 (red) refers here the TRIM8-silenced RPE cells. This clear segregation shows the good quality cells, and the credibility for further analysis. The data shows a total of 2378 cells for the control cluster (green) and 2581 cells for the TRIM8-silenced cluster (red) as generated on the PCA plot (Figure 12). The sufficient number of cells in each condition also meets the criteria to proceed for the further analysis.

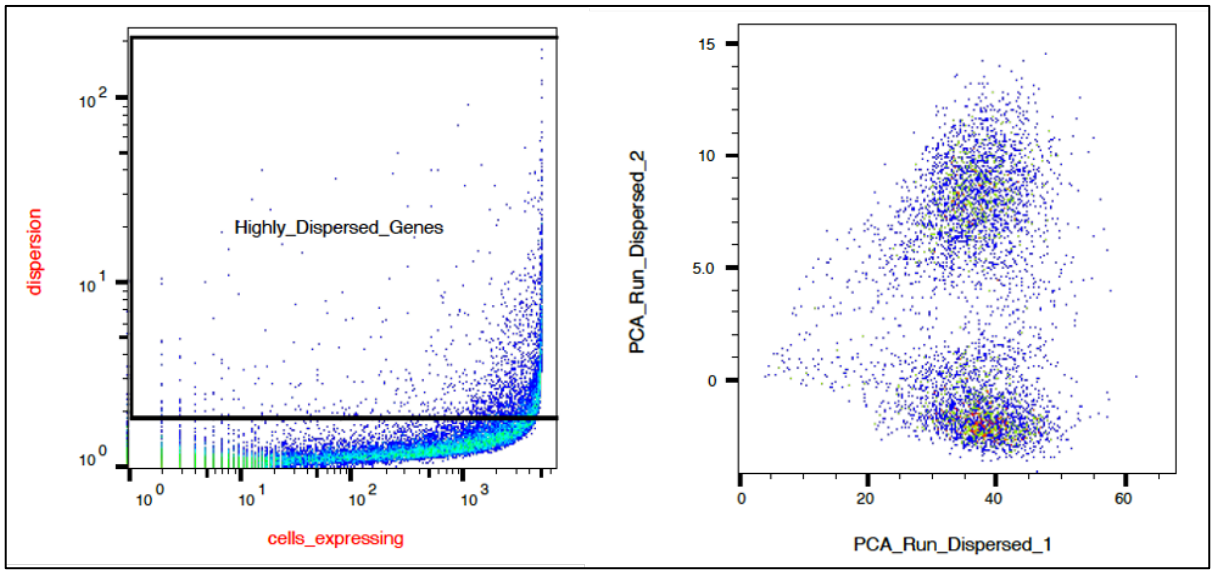


Figure 11. Principal Component Analysis (PCA) on the POOL of cells (both *TRIM8*-silenced and control RPE cells). First, highly dispersed genes were selected to perform PCA (left) and PCA was performed on POOL of cells that shows two distinct clusters (right).

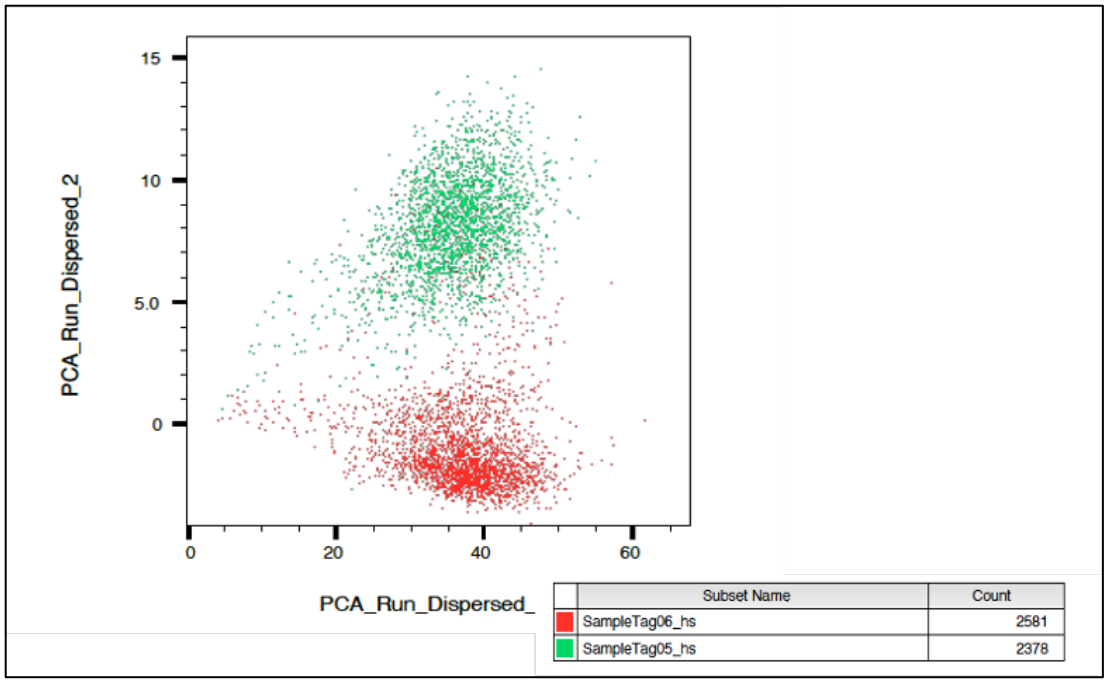


Figure 12. Colouring of the clusters in Principal Component Analysis (PCA). Sample Tag 05 (green) refers here the control or wild type RPE cells and the Sample Tag 06 (red) refers here the *TRIM8*-silenced RPE cells. A total of 2378 quality cells for the control cluster (green) and 2581 quality cells for the TRIM8-silenced cluster (red) has emerged on the PCA plot.

5.4.2. POOL-level analysis: silencing of TRIM8 impacts the canonical "Cell Cycle Control of Chromosomal Replication" pathway proteins at RNA level

In the POOL-level analysis, where differential expression of genes has been identified between control and silenced conditions (without dividing them into cell cycle stages), a total of 410 upregulated and 433 downregulated highly significant genes have been identified at the threshold of q-value with or less than 0.05 and a SeqGeq™ calculated fold change cut-off value of +/- 1.5 (+1.5 or above for upregulated genes and -1.5 or below for downregulated genes) after rounding off (Figure 13). All downstream POOL-level analysis has been down with this list of highly significant up and downregulated genes. QIAGEN IPA analysis of the upregulated DE genes revealed "Cell Cycle Control of Chromosomal Replication" pathway among the top five hits, along with Kinetochore Metaphase Signalling Pathway, Mitotic Roles of Polo-Like Kinases, ATM Signalling and Base Excision Repair Pathway (Figure 14**).** This shows that TRIM8 is controlling directly or indirectly cell cycle associated pathway proteins, particularly those belong to the cell cycle control of chromosomal replication from the gene level. Genes that are impacted upon silencing of TRIM8 at the RNA level from the POOL level analysis of single-cell analysis are TOP2A, CDK2, CDK4, CDK6, DBF4, MCM2, PCNA, RPA2 (Figure 16). Very interestingly, in the line of differential proteomics study (as described in Chapter 5.1.1), downregulated genes from the

single cell transcriptomic study also shows the tendency toward immunity-related pathways (Figure 15).

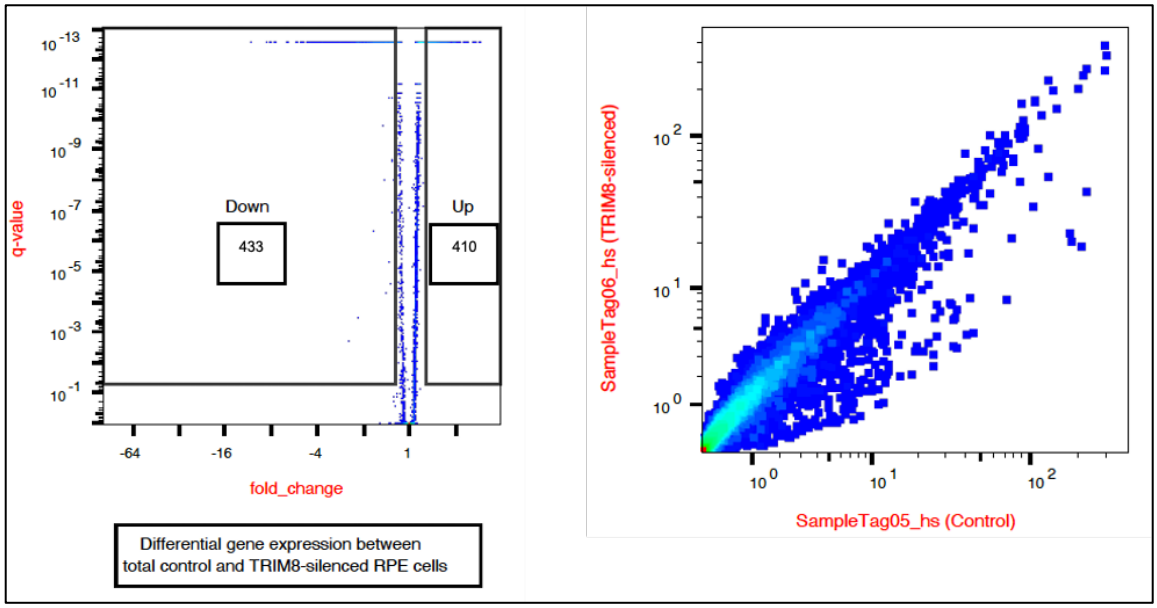


Figure 13. Identification of highly significant differentially expressed genes from the POOL level analysis of single-cell transcriptomic study. A total of 410 upregulated genes and 433 downregulated highly significant genes have been identified at the threshold of q-value with or less than 0.05 and a minimum SeqGeq™ calculated fold change of +/- 1.5 (rounded off). The actual calculation for fold change in SeqGeq™ per gene is: (Mean Expression of Test Population + 1) / (Mean Expression of Control Population + 1).

Top canonical pathways for upregulated DE genes (scRNA-seq)

Name	p-value	Overlap
Kinetochore Metaphase Signaling Pathway	7.34E-32	29.7 % 33/111
Mitotic Roles of Polo-Like Kinase	1.74E-07	15.2 % 10/66
Cell Cycle Control of Chromosomal Replication	4.30E-07	16.1 % 9/56
ATM Signaling	8.61E-07	11.3 % 11/97
BER (Base Excision Repair) Pathway	9.08E-06	15.9 % 7/44

Figure 14. QIAGEN Ingenuity Pathway Analysis (IPA) for upregulated genes from the single-cell transcriptomic study. Similar to differential proteomics study, "Cell Cycle Control of Chromosomal Replication" is also among the top five hits.

Top canonical pathways for downregulated DE genes (scRNA-seq)

Name	p-value	Overlap
Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	1.16E-23	30.2 % 26/86
Interferon Signaling	3.23E-18	44.4 % 16/36
Neuroinflammation Signaling Pathway	8.12E-14	10.2 % 32/315
Antigen Presentation Pathway	4.21E-13	33.3 % 13/39
Coronavirus Pathogenesis Pathway	4.38E-12	11.8 % 24/203

Figure 15. QIAGEN Ingenuity Pathway Analysis (IPA) for the downregulated genes from single-cell transcriptomic study (POOL level).

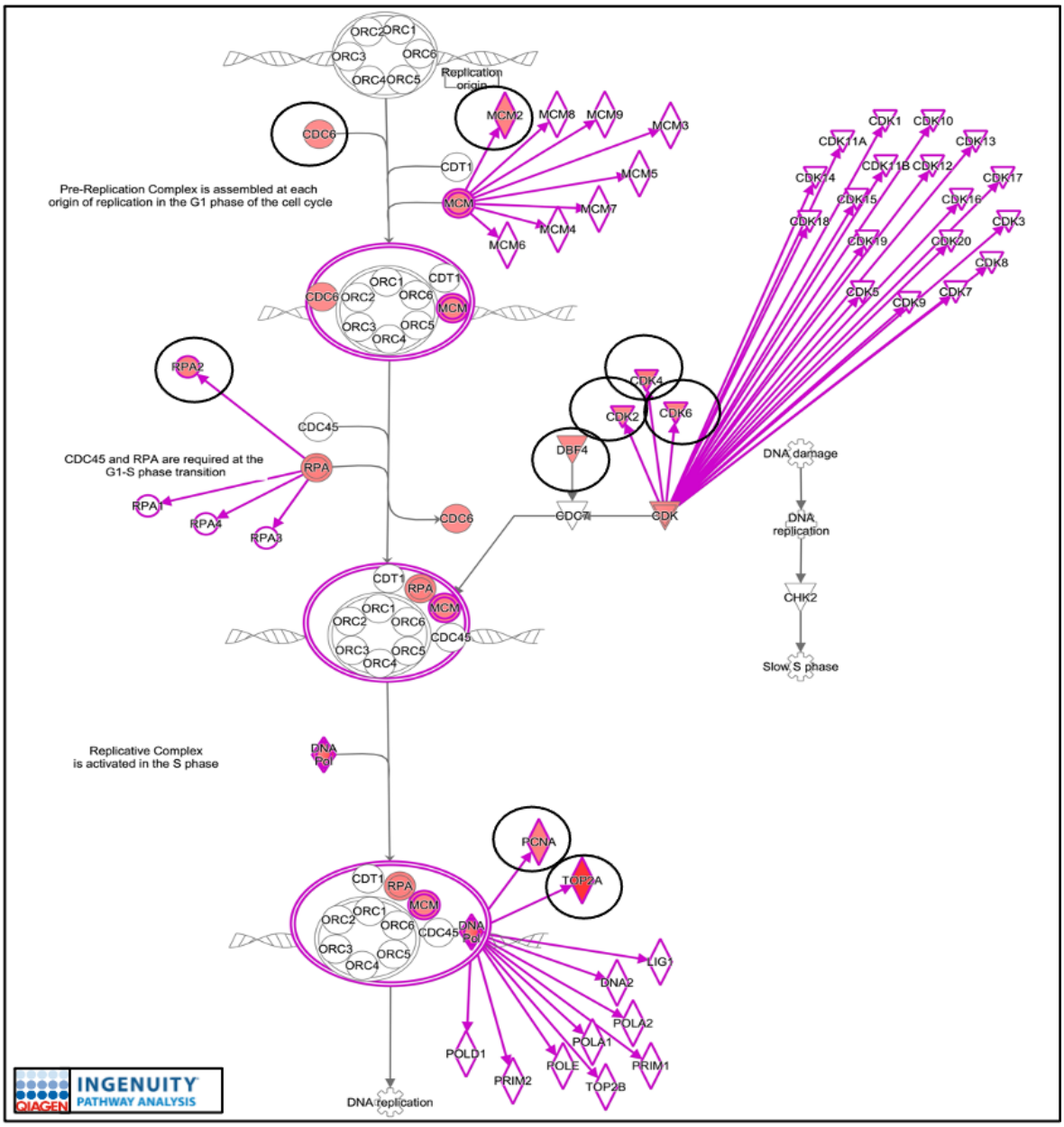


Figure 16: The canonical *"Cell Cycle Control of Chromosomal Replication"* pathway. Genes significantly impacted by *siTRIM8* in differential transcriptomic analysis are highlighted in red (e.g. *TOP2A, CDK2, CDK4, CDK6, DBF4, MCM2, PCNA, RPA2*). *TOP2A* is the highest upregulated gene in the pathway and also among the DE genes.

5.4.3. Silencing of TRIM8 significantly impacts the expression of TOP2A (DNA Topoisomerase II Alpha) at both RNA and protein level

As described in the analysis of differential proteomics study (Chapter 5.1.1) that silencing of *TRIM8* significantly upregulates the TOP2A, that is known to be essential for DNA replication, is also an upregulated candidate gene among the differentially

expressed genes from the scRNA-seq study. Indeed, among all upregulated genes in the single cell transcriptomic study, TOP2A is the most significant upregulated gene (Figure 17).

Table: Top 5 highly significant hits

UP (DE LC-MS/MS)	UP (DE scRNA-seq POOL)
TPM1	TOP2A
NUCKS1	HIST1H4C
MKI67	ODC1
SQSTM1	TMEM158
TOP2A	NT5DC2
Down (DE LC-MS/MS)	**Down (DE scRNA-seq POOL)**
IFIT3	IFIT2
IFIT1	IFIT1
IFIT2	IFIH1
ISG15	DDX58
DDX58	ISG15

Figure 17. List of top five differentially genes/proteins in both scRNA-seq and LC-MS/MS study. DNA Topoisomerase II Alpha (TOP2A), a key regulator of DNA replication is significantly (top five candidates) impacted upon silencing of *TRIM8* at both RNA and Protein level along with IFIT1, IFIT2, DDX58, ISG15.

5.4.4. TRIM8 and TOP2A correlation study in cancers: TCGA data analysis

As described in the previous section that silencing of *TRIM8* significantly impacts *TOP2A* expression at both RNA and protein level, so next I attempted to identify the significance of the inverse corelation between TOP2A and TRIM8 in the context of cancers by analysing the publicly available The Cancer Genome Atlas (TCGA) data. This analysis reveals that in many of the cancers where *TRIM8* is downregulated there *TOP2A* gets upregulated. For instance, in Glioblastoma Multiforme (GBM) where *TRIM8* has relatively low expression compared to its normal counterpart and *TOP2A* has significantly higher expression. This shows a causal link between the *TRIM8* and *TOP2A*, that are inversely co-related and this relation is also mimicable in normal cell line like RPE, at both RNA and protein level, as discussed before.

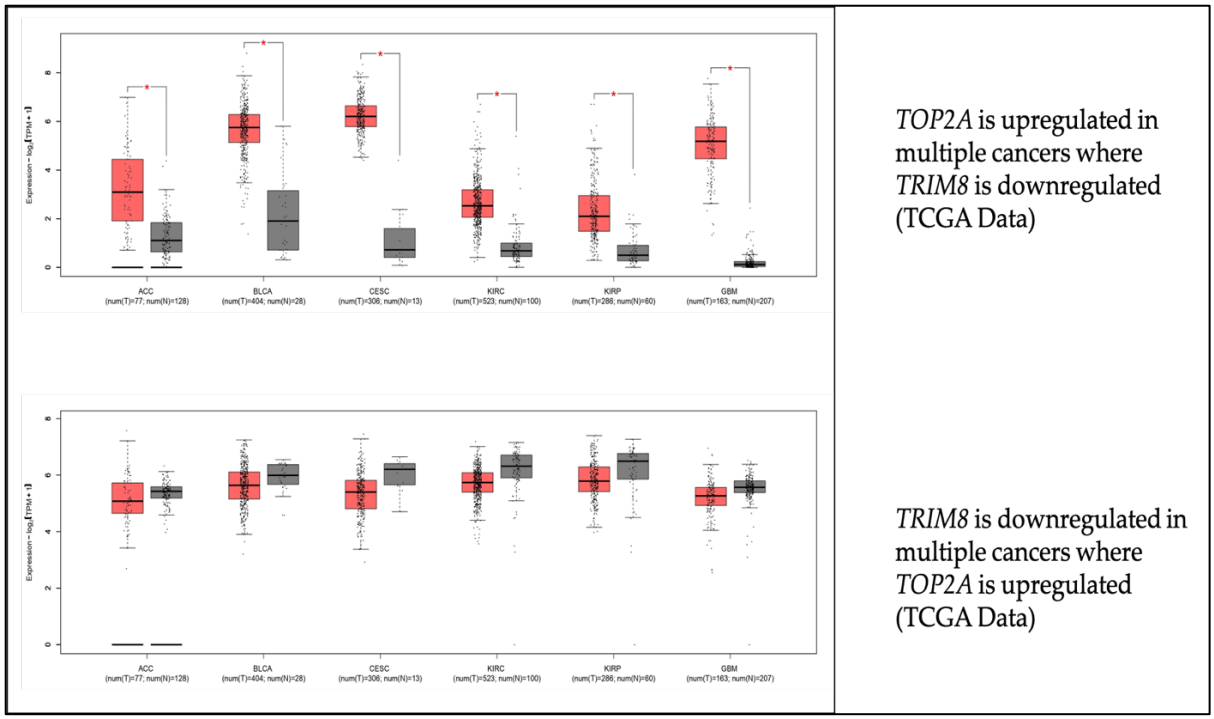


Figure 18. The inverse co-relation between *TOP2A* and *TRIM8* is also reflected in multiple cancers as observed in The Cancer Genome Atlas (TCGA) data. In the cancer datasets where *TOP2A* is upregulated (above), there *TRIM8* is downregulated(below).

5.4.5. Mitotic stage specific analysis of the impact of TRIM8-silencing

As many of the cell cycle related genes can vary in their expression among the cells that are at the different stages of cell cycle, so next I tried to understand whether the impact of TRIM8 silencing on cell cycle associated genes like TOP2A, MCM family proteins, CDKs are real or they are affected by the cell cycle heterogeneity of the single-cell RNA sequencing data. To address this, in the next of analysis, we divided the population of cells into different stages of cell cycle (using know candidate markers from Seurat website) and at every stage we compared control vs. *TRIM8*-silenced cells, so that the real impact of *TRIM8* downregulation can be visible on cell-cycle associated gene. Performing PhenoGraph machine learning algorithm on tSNE-plot has revealed three distinct clusters specific to G0/G1, S, and G2/M respectively. When compared among each other Cluster 6 has highest number of differentially expressed G2/M marker genes giving an indication of the cluster to being G2/M phase cells. Similarly, Cluster 2 tends to be S phase cells, and thus the remaining Cluster 1 tends to be G0/G1. The number of cells found in each cluster found are 1238, 789, and 497 for G0/G1, S, and G2/M respectively (Figure 19). Thereafter, in each cluster the total number of differentially expressed genes were identified. For instance, Cluster 6, the possible G2/M phase cluster of cells, has been identified from the POOL and genes that tend be upregulated in the G2/M cluster is also identified (Figure 20), followed by

the pathway enrichment analysis for differentially expressed genes in each cluster (Figure 23).

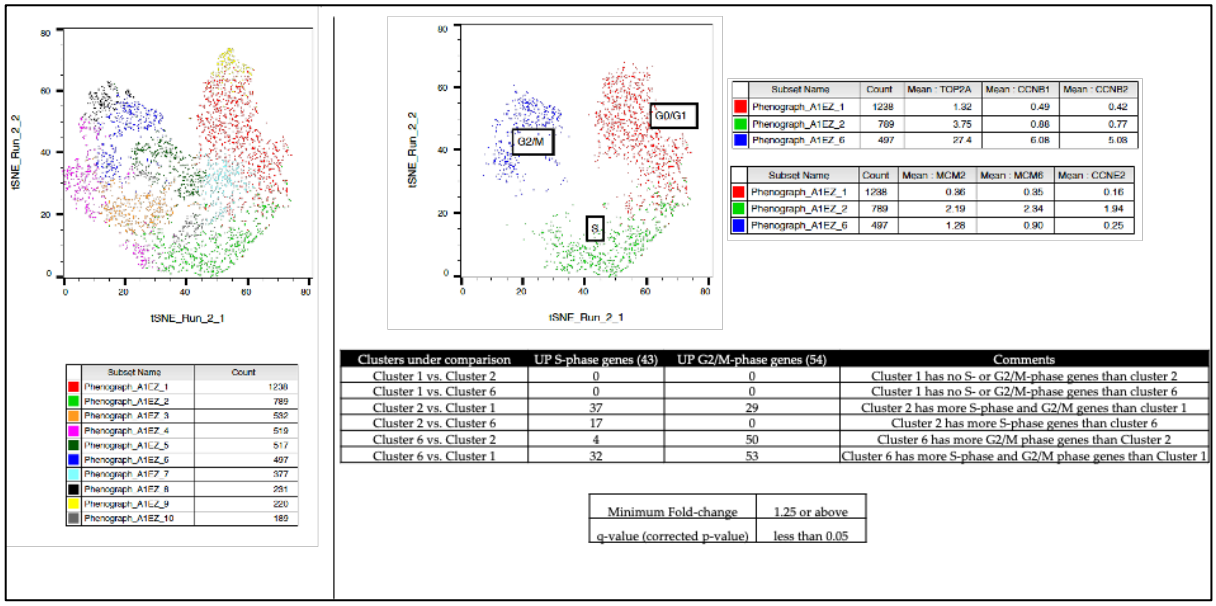


Figure 19. t-SNE plot on the total population of cells and generation of clusters using PhenoGraph machine learning algorithm (left). Generation of different clusters specific to different stages depending on 97 cell cycle marker genes in humans (43 S-phase marker genes, 54 G2/M marker genes). The number of cells found in each cluster found are 1238, 789, and 497 for G0/G1, S, and G2/M respectively. Cluster 6 has the highest number of differentially expressed G2/M marker genes whereas Cluster 2 has the highest number of S-phase marker genes (right).

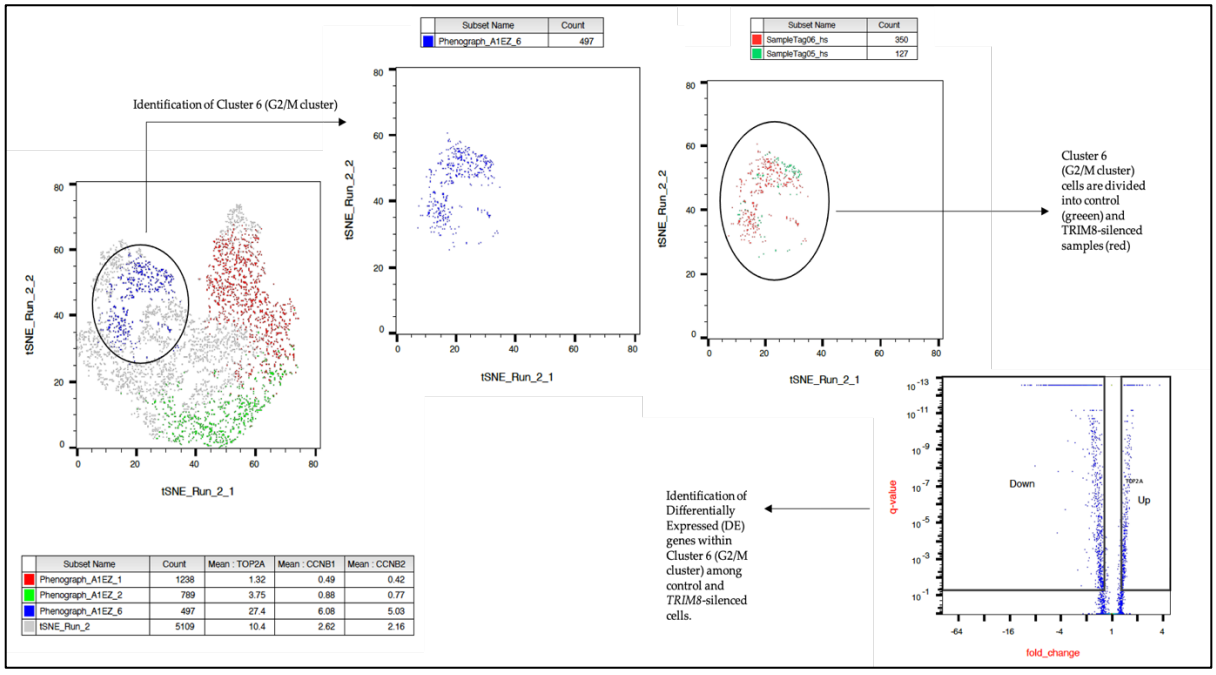


Figure 20. Scheme of identification of G2/M cluster, and identification of differentially expressed genes in the G2/M cluster.

5.4.6. RT-qPCR validation of the DE genes in the "Cell Cycle Control of Chromosomal Replication" pathway

Genes (*TOP2A, CDC6, CDK2, CDK4, CDK6, DBF4, MCM2, PCNA, RPA2*) that were differentially expressed in the POOL level analysis and were involved in the canonical "Cell Cycle Control of Chromosomal Replication" pathway (as described in Chapter 5.4.2) were computationally analysed at the different stages as mentioned in the previous section (Chapter 5.4.3). Except *DBF4*, all other genes were found to be differentially expressed in at least one stage of the cell cycle in the stage-specific cluster-level analysis (Figure 21). Candidate genes like *TOP2A CDK4, and CDK6* were found to be upregulated in all three stages of the cell cycle. Interestingly, genes like *TOP2A* and *CDK6*, that were found to be upregulated in all three clusters, showed better yield in *RT-qPCR* (Figure 22).

Genes	Cluster 1 (G0/G1 phase)	Cluster 2 (Mid S phase)	Cluster 6 (G2/M phase)
TOP2A	Yes	Yes	Yes
CDK4	Yes	Yes	Yes
CDK6	Yes	Yes	Yes
MCM2	No	Yes	Yes
PCNA	Yes	No	Yes
RPA2	No	Yes	Yes
CDC6	No	Yes	No
CDK2	No	No	Yes
DBF4	No	No	No

Figure 21. Cluster specific differential expression (yes/no) summary of genes found initially in the "Cell Cycle Control of Chromosomal Replication" pathway from the POOL level analysis of scRNA-seq study.

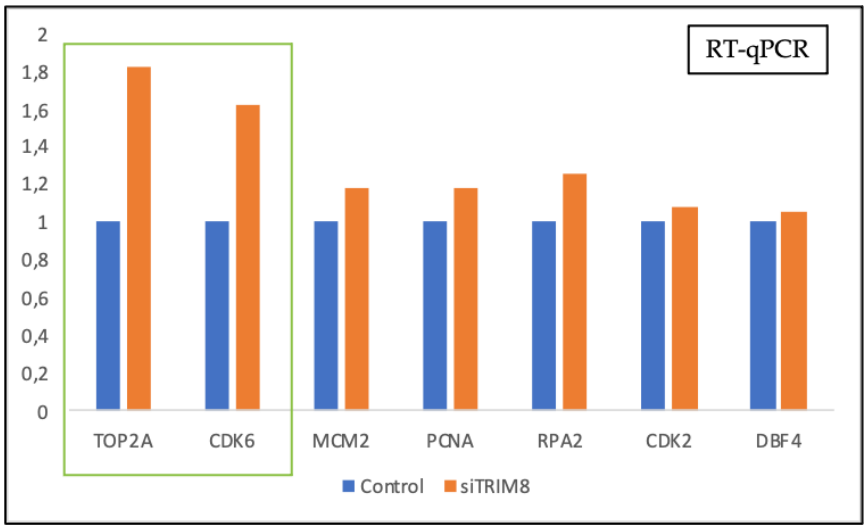


Figure 22. *RT-qPCR* validation of the DE genes in the "Cell Cycle Control of Chromosomal Replication" pathway from the POOL level analysis of scRNA-seq study. Genes like *TOP2A* and *CDK4*, that were found to be upregulated in all three clusters in computational analysis, showed better yield in *RT-qPCR* validation with cDNA isolated from "unsynchronized" control and TRIM8-silenced cells.

5.4.7. Mitotic stage-specific analysis reveals TRIM8 is involved in "Cell Cycle: G1/S Checkpoint Regulation" and "Kinetochore Metaphase Signaling" pathways

A further and in-depth pathway analysis of the DE genes in a stage specific manner also assures the involvement of TRIM8 in different cell cycle pathways alongside the previously described cell cycle control of chromosomal replication pathway. Particularly, analysis of the upregulated DE genes in Cluster 2 that tends to be the S-phase, shows the enrichment in "Cell Cycle: G1/S Checkpoint Regulation" pathway (Figure 23). Also, the same analysis in Cluster 6 that tends to be G2/M phase, shows the enrichment of Kinetochore Metaphase Signaling Pathway. This goes in the line of the earlier published work on TRIM8, that showed TRIM8 to physically interact with KIF11 and KIFC1, two kinetochore family proteins known to be the master regulators of mitotic spindle assembly.

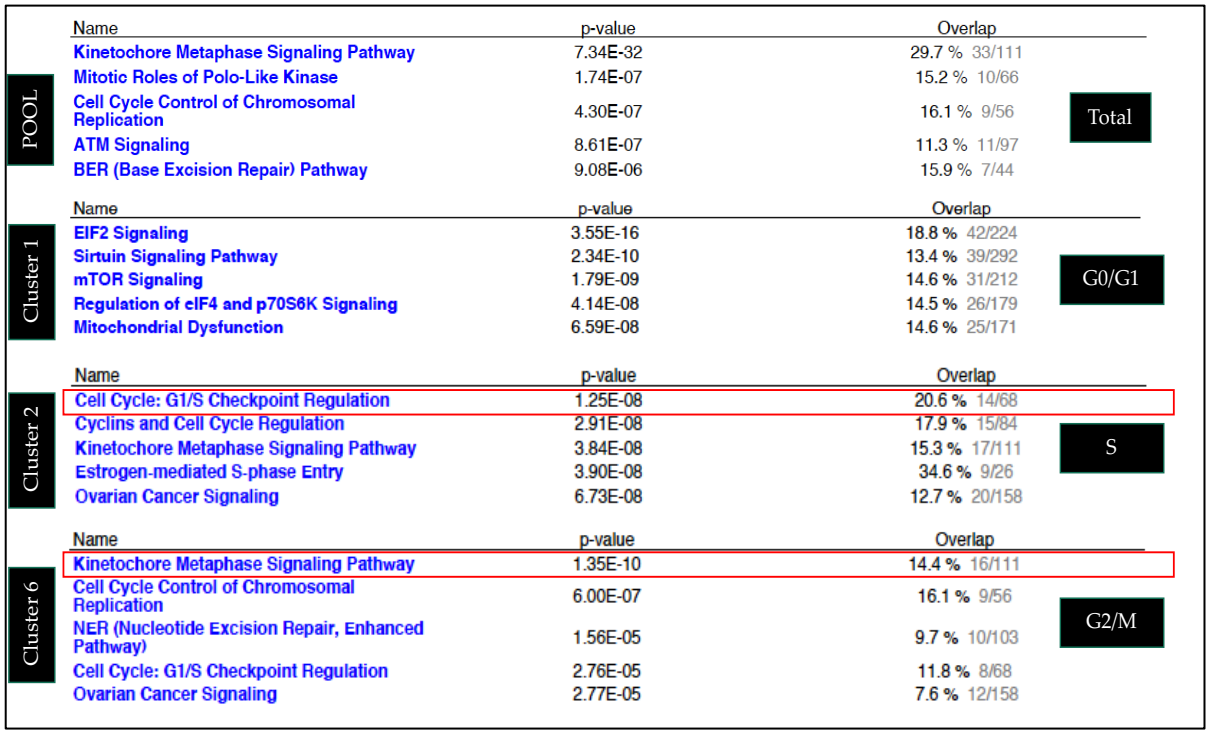


Figure 23. QIAGEN Ingenuity Pathway Analysis (IPA) of differentially expressed genes in three different clusters that resembles three mitotic stages (G0/G1, S, G2/M) along with the pathway analysis for POOL level DE genes.

5.5. New insights on TRIM8 at primary cilium

5.5.1. Silencing of TRIM8 impacts ciliary genes/proteins NEDD9 and NEK7 at RNA level

Digging into the single-cell RNA-sequencing data revealed silencing of TRIM8 impacts two important ciliary proteins NEDD9 and NEK7 at RNA level. NEDD9 and NEK7 both are associated with the process of ciliary disassembly, and NEK7 is required to promote G1 progression and procentriole formation.[23, 24] NEDD9 and NEK7 have been founded to be downregulated upon silencing of TRIM8 in the analysis of single cell transcriptomic data.

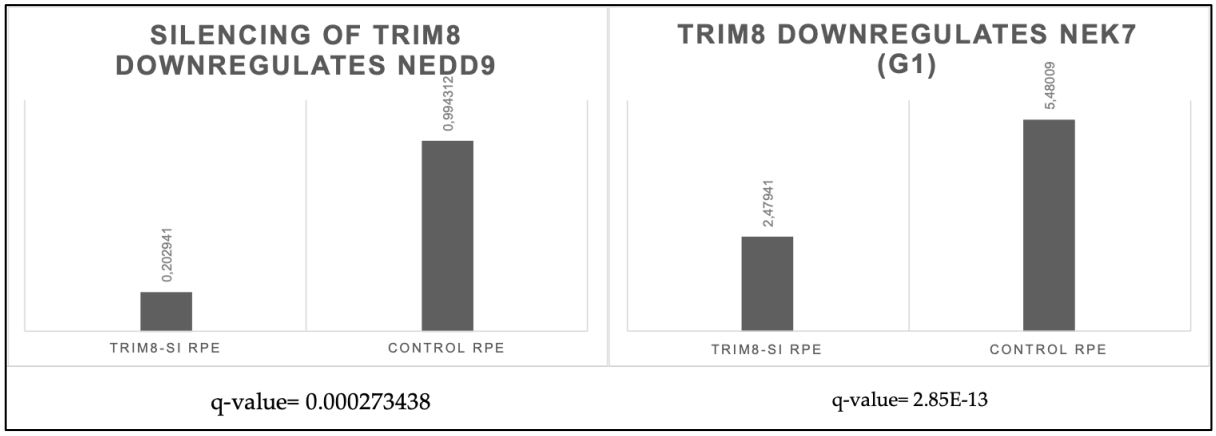


Figure 24. Silencing of *TRIM8* downregulates two ciliary proteins in RPE cells. Data presented here is shown from computational analysis of the transcriptomic data.

5.5.2. TRIM8 localises at the site of primary cilium formation during G0 phase

As shown before that TRIM8 colocalises with CEP170 at the centrosomal region during all phases of mitosis (Chapter 5.3.2) and CEP170 is known to associate with the the subdistal appendages, typical of the mature mother centriole, site of primary cilium formation.[21] CEP170 is also known to promote cilium disassembly in neural progenitors.[25] Therefore, an immunofluorescence study was carried out to show whether TRIM8 can localise at primary cilium that typically forms during G0. The study reveals that TRIM8 localises at the site of primary cilium formation in RPE cells (Figure 25).

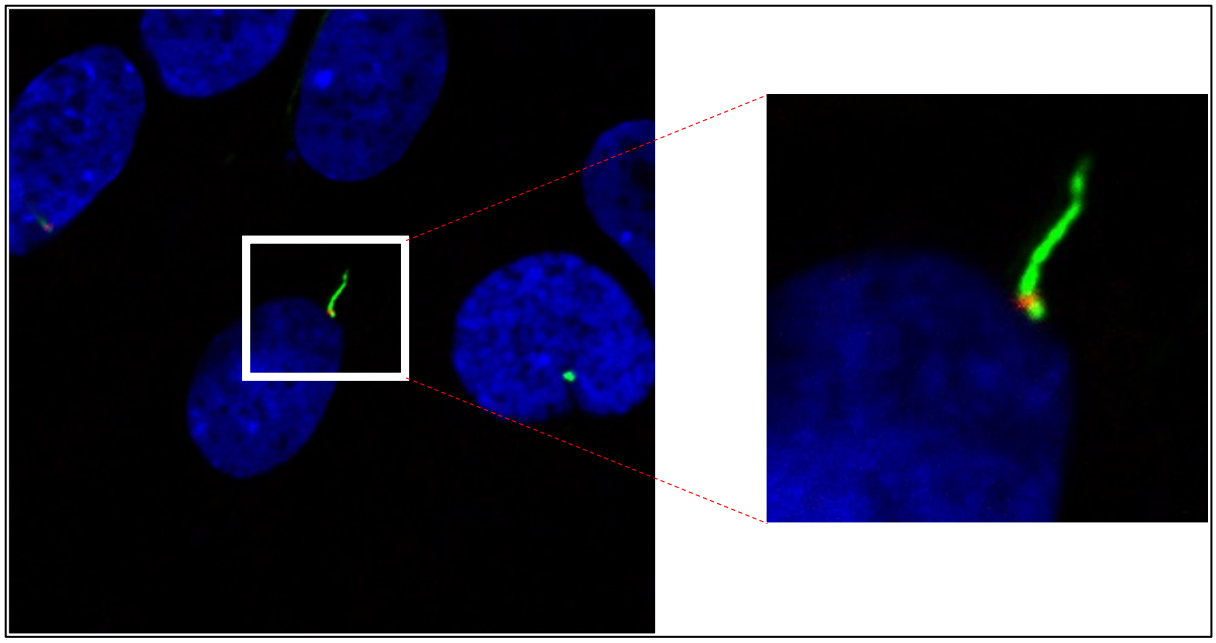


Figure 25: Localisation of endogenous TRIM8 at the site of primary cilium formation. Immunofluorescence study reveals that TRIM8 localises at the site of primary cilium formation in RPE cells.

Chapter 6. Discussion

In standard computational single-cell RNA-seq analysis from tissues, cell-cycle heterogeneity is removed and thus limits the scope of studying the impact on cell-cycle genes in a gain or loss of function study. Here, with aid of existing algorithms I standardised a pipeline that analyses single-cell transcriptomic data from a single-cell line, hTERT RPE-1 (hTERT-immortalized retinal pigment epithelial cell line), in a mitotic stage specific manner (G1, S, G2/M) without mitigating the effect of cell cycle heterogeneity so that we can assure a differentially expressed mitotic cell cycle gene hit is not due to cell cycle variability but due to the real impact of the gain or loss of function. For instance, high TOP2A expression, a marker of G2/M has been compared in a stage specific manner in both control and TRIM8-silenced condition in single-cell BD Rhapsody RNA-seq data from RPE cells. Analysis at each stage in control vs. treated condition assures that the differential expression is not due to cell cycle heterogeneity of a single cell transcriptomic data but due to the real impact of the silencing of TRIM8, in this case. As I showed alongside the transcriptomic study, proteomic study is also done upon the silencing of TRIM8 in the same cell line RPE to estimate the significant pathway enrichment upon the differential expression of genes/proteins. And, also, I showed the results from the immunofluorescence study of TRIM8 at the primary cilium and the colocalization study with a centrosomal protein, CEP170.

6.1. TRIM8-TOP2A-MCM axis under the lights of liquid–liquid phase separation (LLPS)

Overall, this descriptive thesis on TRIM8 brings out a picture a of this E3 ubiquitin ligase from different angles during the course of cell cycle. From, the transcriptomic and proteomic data it is very much clear that silencing of TRIM8 impacts the expression level of TOP2A at both RNA and protein level. And the inverse corelation between TOP2A and TRIM8 expression level can be found in many cancers, i.e., where TRIM8 is downregulated there TOP2A gets upregulated. Interestingly, this inverse corelation between TRIM8 and TOP2A is also mimicable in a normal cell line of epithelial lineage like RPE1 as I mentioned in the results. TOP2A or DNA Topoisomerase II Alpha is an enzyme that controls and alters the topologic states of DNA during transcription and TOP2A is also essential for the maintenance of mitotic chromosome structure.[26] Earlier studies have shown that silencing of TRIM8 leads to the aneuploidy, particularly an increase in cells with less than 46 chromosomes.[8] Interestingly, expression of *TOP2A* and aneuploidy are significantly co-related and associated with many cancers.[27] This thesis on TRIM8, gives an interesting route to understand the TRIM8 and TOP2A axis in the context of chromosomal replication and stability. Furthermore, the *Z-score* calculation on differential proteomics data predicts that the silencing of *TRIM8* activates the canonical "*Cell Cycle Control of Chromosomal Replcation"* pathway, where TOP2A is known for its function to introduce temporary double-stranded breaks into the DNA that are necessary for DNA replication during

the S-phase. In this line, not only TOP2A, but silencing *TRIM8* also significantly impacts the MCM complex proteins (MCM3-7) at the protein level, as discussed in the results. MCM complex proteins, that are crucial in the canonical "*Cell Cycle Control of Chromosomal Replcation"* pathway, are also important in the regulation of once per cell cycle DNA replication in eukaryotic cells.[22] The upregulation of both MCM complex proteins (MCM3-7) and TOP2A upon silencing of *TRIM8* thus gives a novel insight into the involvement of a TRIM E3 ligase, TRIM8, in chromosomal replication and stability. In recent past, Liquid–liquid phase separation (LLPS) has attracted significant attention. LLPS is a new concept that attempts to elucidate the accurate temporal and spatial regulation in living cells. LLPS compartmentalizes nucleic acids and proteins into micron-scale, liquid-like membrane less bodies that are now termed as biomolecular condensates with specific functions which were earlier called as membrane less organelles (MLO).[28] TOP2A is an experimentally identified "client" (member genes in an MLO that resides under certain conditions) of the newly identified Liquid–liquid phase separation (LLPS) hub genes that can be the part of four varieties of biomolecular condensates, nucleolus, centrosome, spindle pole body and P-body. And, as a Liquid–Liquid Phase Separation-Related gene TOP2A is associated with the distinct type of cancers like lower-grade glioma (LGG). Particularly, as a LLPS hub gene TOP2A expression goes high in LGG,[29] where TRIM8 expression is known to be low. Alongside, TOP2A, many of the MCM complex proteins, particularly MCM4-7 are the experimentally identified "regulators" (Clients of a condensate that are not part of an MLO often but can regulate the formation of the condensate). Like TOP2A, these MCM proteins also belong to the common biomolecular condensate, that is "centrosome/spindle pole body" in humans, as described in the data source of liquid-liquid phase separation (https://llps.biocuckoo.cn). Now, the localisation of TRIM8 at the centrosomal region during all phases of mitosis and the impact of the silencing of *TRIM8* on TOP2A and MCM complex proteins (as described in the results section) demands future experimental studies to establish the functional relationship of TRIM8-TOP2A-MCM axis. There is a huge possibility that the once per cell cycle mechanism that is associated with the chromosomal replication has also its precursors at the stage of centrosomal duplication and at the centrosome/spindle pole body condensate, virtually that remains as one of the most active centres of mitosis throughout the course of cell cycle. Very interestingly, the plausible functional study and future work on TRIM8 at the centrosomal region can draw some inspirations from a recent report where the role of another TRIM family protein has been established with a serine/threonine kinase, polo-like kinase 4 (Plk4) and a centrosomal protein, CEP192.[30]

6.2. A triangular TRIM-CEP-PLK axis for centriole and cilium biogenesis

Overexpression of TRIM37, a member of TRIM E3 ubiquitin ligase family that comes under the Class VIII with a MATH domain on the C-terminus, significantly degrades

the centrosomal protein CEP192 in RPE-1 cells, and also its overexpression of in RPE-1 cells inhibits acentrosomal spindle assembly, that leads to mitotic failure; whereas the loss of TRIM37 ameliorates proliferation and facilitates acentrosomal spindle assembly. Serine/threonine kinase, polo-like kinase 4 (Plk4) is an essential kinase that controls the centriole duplication.[30, 31] Indeed, to initiate the centriole duplication Plk4 has to be recruited to the centrosome. This recruitment of Plk4 to a distinct sub-centrosomal region takes place by two centrosomal proteins, CEP192 and CEP152.[32] In this juncture, a recent model showed that TRIM37 regulates CEP192 stability and TRIM37 also ubiquitinates Plk4.[30] The model shows that TRIM37 averts self-assembly of PLK4 to form biomolecular condensates that engage other centrosomal members and work as abnormal microtubule-nucleating centers, and more importantly TRIM37 acts to prevent PLK4 self-assembly rather than to control its expression because *TRIM37*-knocked out RPE cells did not notice any transcriptional changes of Plk4. Overall, the model depicts that TRIM37 can function in a bi-directional manner. When the level of TRIM37 is low, PLK4 makes molecular condensates that catalyze the formation of a robust and acentrosomal spindle assembly. In contrast, when the TRIM37 level is high, CEP192 level gets degraded and there are no PLK4 condensates that leads to the failure of acentrosomal spindle assembly.[30] Against the backdrop of TRIM37-Plk4-CEP192, if I attempt to sketch the data from this thesis along with the previously published results, I see three important points on the rise of TRIM8-Plk1-CEP170. 1) Silencing of *TRIM8*, upregulates CEP170 (as described in differential proteomics data), a physiological substrate of polo-like kinase1 (Plk1). This lies in the similar fashion of TRIM37, overexpression of which can lead to the degradation of a centrosomal protein CEP192. 2) Although TRIM8 and Plk1 colocalises at the midbodies (Venuto, Santina et al. 2020), silencing of *TRIM8* does not impact Plk1 at neither RNA nor only protein level. This is in the line of TRIM37 that acts to prevent self-assembly of Plk4 but not to change its expression. 3) TRIM8 and CEP170 co-localise at the centrosomal region during all phases of mitosis (as described in the immunofluorescence study). Now, as I described that TRIM8 can physically localise at the site of primary cilium formation during G0 phase, in order to broaden our knowledge about the regulation of primary cilium formation and centriole maturation and duplication, this new TRIM8-Plk1-CEP170 axis can be an important reference point of future study. Also, some of the recent studies have reported the involvement of Plk1 in primary cilium disassembly,[33] and it is known that CEP170 can be phosphorylated by Plk1.[21] Now, the important question in the frontier of centriolar and ciliary biology, is whether TRIM8 can ubiquitinate Plk1 or not, and how this axis can be involved in the process of ciliary assembly and disassembly. Nevertheless, during the complex process of primary cilium assembly/disassembly and during the centriolar duplication, the rise of a triangular interaction (one TRIM E3 ubiquitin ligase, Serine/threonine polo-like kinase, and one centrosomal protein) will be worth studying in near future.

6.3. TRIM8 in primary cilium assembly/disassembly

Furthermore, I showed in the transcriptomic study that silencing of *TRIM8* downregulates two important ciliary genes/proteins NEDD9 and NEK7, and Guarguaglini et al. (2005) showed with immuno-electron microscopy that CEP170, during G1 to early G2, resides only at the mother centriole, that is now known to be the site of primary cilium formation during G0/G1 stage.[21] As I discussed before that TRIM8 and CEP170 co-localise with each other at the centrosomal region during all phases of mitosis, and TRIM8 can also localise at the site of primary cilium formation during the resting phase G0, so it is highly obvious that TRIM8 is functionally linked to the mother centriole during G1 to early G2, and it may be involved in the regulation of primary cilium assembly or disassembly. Pugacheva, E.N., et al. (2007) showed earlier that the stimuli which instigate cell cycle can also induce the expression and the basal body localization of NEDD9, activator of AURKA. Activated AURKA triggers ciliary disassembly. And downregulation of NEDD9 can affect ciliary disassembly and can cause mitotic delay.[23] Also, Gupta, A., et al. (2017) showed that the depletion of NEK7 induces ciliogenesis in RPE.[24] Together with the immunofluorescence study on TRIM8-CEP170 and the expression data of NEDD9 and NEK7 upon silencing of *TRIM8* it can now be stated that TRIM8 may play some role in the primary cilium assembly/disassembly, and further mechanistic study will be required to bring out a clear picture of TRIM8 in the short span of primary cilium assembly/disassembly.

Chapter 7. Conclusion

From the above-mentioned results it can now be confirmed that TRIM8 is involved in the canonical "Cell Cycle Control of Chromosomal Replication" pathway, and that the involvement of TRIM8 in mitotic cell cycle now stands beyond doubt. On the other hand, the study also reveals for the first time that TRIM8, a member of the TRIM E3 ubiquitin ligase family, is localised at the site of primary cilium formation during the G0 phase, and that TRIM8 co-localises with CEP170, a physiological substrate of Plk1, during all phases of mitosis at the centriolar region. As CEP170 is already known to localise at the mother centriole during G1 to early G2 and as it is well-studied that mother centriole facilitates the formation of primary cilium, from future perspective, it would be interesting to argue that TRIM8-CEP170 axis can have a role in the formation of primary cilium. Nevertheless, we can now conclude three interesting facts from this study - 1) *TRIM8*-silencing upregulates DNA topoisomerase 2-alpha (TOP2A) at both RNA and protein level, and positively impacts the canonical "Cell Cycle Control of Chromosomal Replication" pathway, 2) TRIM8 colocalises with centriolar protein CEP170 in all phases of mitosis at the centrosomes and *TRIM8*-silecing upregulates CEP170 at protein level, and 3) TRIM8 localises with the site of primary cilium formation during G0 stage and *TRIM8*-silencing downregulates ciliary genes/proteins *NEDD9* and *NEK7*. Earlier work on NEDD9 and NEK7 showed their downregulation is associated with ciliary disassembly and mitotic delay, whereas TRIM8 colocalizes with Plk1 at midbodies during the end of cytokinesis. Moreover, it is published in literature that silencing of *TRIM8* delays mitotic cell cycle progression, and TRIM8 interacts with KIFC1 and KIF11, two master regulators of mitotic spindle assembly and cytoskeleton reorganization. Altogether, it can now be stated that TRIM8 keeps itself continuously present at the centrosome, one of the active centres of mitosis, throughout the course of cell cycle, and can impact different stages of cell cycle from centrosomal duplication to cell cycle control of chromosomal replication. Thus, this study brings out an impression of TRIM8 as a multifunctional workhorse during the course mitosis as the title of this thesis reads.

Chapter 8. Appendix

8.1. Supplementary Table 1

Codes	Protein names	Gene name	Peptides	Mol. weight [kDa]	FDR	Log2 Fold Change	FC TRIM8/CNT
P09493	Tropomyosin alpha-1 chain	TPM1	20	32,708	4,20 E-02	2,65	6,27
Q9H1E3	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1	NUCKS1	6	27,296	7,32 E-03	2,06	4,16
P46013	Proliferation marker protein Ki-67	MKI67	26	358,69	1,45 E-03	1,79	3,45
Q13501	Sequestosome-1	SQSTM1	6	47,687	4,05 E-04	1,39	2,63
P11388	DNA topoisomerase 2-alpha	TOP2A	14	174,38	1,52 E-04	1,32	2,50
P62328	Thymosin beta-4	TMSB4X	5	5,0526	3,79 E-02	1,28	2,43
Q3KQU3	MAP7 domain-containing protein 1	MAP7D1	4	92,819	2,19 E-02	1,23	2,35
P07741	Adenine phosphoribosyltransferase	APRT	5	19,608	2,82 E-02	1,14	2,21
Q96M27	Protein PRRC1	PRRC1	5	46,701	5,74 E-04	1,12	2,17
P21399	Cytoplasmic aconitate hydratase	ACO1	9	98,398	7,32 E-03	1,08	2,12
P30405	Peptidyl-prolyl cis-trans isomerase F, mitochondrial	PPIF	4	22,04	3,63 E-02	1,05	2,06
O00499	Myc box-dependent-interacting protein 1	BIN1	4	64,699	1,07 E-02	1,04	2,06
O95347	Structural maintenance of	SMC2	11	135,65	4,40 E-03	1,03	2,05

	chromosomes protein 2						
P98179	RNA-binding protein 3	RBM3	7	17,17	4,04 E-03	0,99	1,99
P52597	Heterogeneous nuclear ribonucleoprotein F	HNRNPF	12	45,671	1,93 E-02	0,97	1,96
P43246	DNA mismatch repair protein Msh2	MSH2	5	104,74	2,57 E-02	0,96	1,95
P49023	Paxillin	PXN	6	64,505	6,29 E-04	0,93	1,90
Q6P2E9	Enhancer of mRNA-decapping protein 4	EDC4	4	151,66	9,19 E-03	0,92	1,90
P31350	Ribonucleoside-diphosphate reductase subunit M2	RRM2	6	44,877	3,81 E-02	0,92	1,89
Q96EP5	DAZ-associated protein 1	DAZAP1	7	43,383	4,05 E-04	0,90	1,87
P27695	DNA-(apurinic or apyrimidinic site) lyase	APEX1	11	35,554	1,94 E-02	0,90	1,87
P53814	Smoothelin	SMTN	6	99,058	1,15 E-03	0,89	1,86
Q8N1F7	Nuclear pore complex protein Nup93	NUP93	9	93,487	1,19 E-03	0,88	1,84
Q12874	Splicing factor 3A subunit 3	SF3A3	7	58,848	4,92 E-04	0,82	1,76
Q27J81	Inverted formin-2	INF2	10	135,62	4,40 E-03	0,80	1,75
P35580	Myosin-10	MYH10	38	229	3,32 E-04	0,80	1,74
Q9BWD1	Acetyl-CoA acetyltransferase, cytosolic	ACAT2	6	41,35	1,71 E-02	0,80	1,74
Q02750	Dual specificity mitogen-activated protein kinase kinase 1	MAP2K1	7	43,439	7,32 E-03	0,77	1,71

P09104	Gamma-enolase	ENO2	15	47,268	2,83 E-04	0,77	1,70
O75326	Semaphorin-7A	SEMA7A	5	74,823	4,10 E-03	0,75	1,69
Q92896	Golgi apparatus protein 1	GLG1	9	134,55	3,18 E-02	0,75	1,68
Q8WXX5	DnaJ homolog subfamily C member 9	DNAJC9	6	29,909	4,29 E-02	0,75	1,68
Q8TAQ2	SWI/SNF complex subunit SMARCC2	SMARCC2	4	132,88	1,70 E-02	0,75	1,68
O75531	Barrier-to-autointegration factor	BANF1	5	10,058	4,07 E-02	0,75	1,68
P52292	Importin subunit alpha-1	KPNA2	14	57,861	1,43 E-04	0,74	1,67
P12270	Nucleoprotein TPR	TPR	24	267,29	1,53 E-02	0,72	1,65
P43487	Ran-specific GTPase-activating protein	RANBP1	6	23,31	7,63 E-03	0,71	1,63
Q96PK6	RNA-binding protein 14	RBM14	8	69,491	9,62 E-04	0,70	1,62
P25205	DNA replication licensing factor MCM3	MCM3	8	90,98	3,18 E-02	0,70	1,62
Q9H910	Jupiter microtubule associated homolog 2	JPT2	5	20,063	1,50 E-02	0,68	1,60
O95817	BAG family molecular chaperone regulator 3	BAG3	11	61,594	9,62 E-04	0,68	1,60
P09622	Dihydrolipoyl dehydrogenase, mitochondrial	DLD	9	54,177	9,09 E-03	0,68	1,60
P46087	Probable 28S rRNA (cytosine(4447)-C(5))-methyltransferase	NOP2	7	89,301	2,02 E-02	0,68	1,60
Q14008	Cytoskeleton-associated protein 5	CKAP5	7	225,49	2,15 E-03	0,68	1,60

Q9UQE7	Structural maintenance of chromosomes protein 3	SMC3	12	141,54	1,46E-03	0,67	1,60
Q9UKY7	Protein CDV3 homolog	CDV3	6	27,335	2,82E-02	0,67	1,59
Q96CT7	Coiled-coil domain-containing protein 124	CCDC124	7	25,835	6,17E-03	0,66	1,58
Q9UKV3	Apoptotic chromatin condensation inducer in the nucleus	ACIN1	6	151,86	6,98E-03	0,66	1,58
Q12849	G-rich sequence factor 1	GRSF1	7	53,126	2,05E-02	0,65	1,57
Q8N1G4	Leucine-rich repeat-containing protein 47	LRRC47	4	63,472	1,26E-02	0,65	1,57
P33316	Deoxyuridine 5-triphosphate nucleotidohydrolase, mitochondrial	DUT	7	26,563	1,22E-03	0,63	1,55
Q14566	DNA replication licensing factor MCM6	MCM6	8	92,888	5,99E-03	0,63	1,55
P10412	Histone H1.4	HIST1H1E	11	21,865	2,52E-03	0,63	1,54
Q96K17	Transcription factor BTF3 homolog 4	BTF3L4	5	17,27	4,42E-02	0,62	1,54
Q5SW79	Centrosomal protein of 170 kDa	CEP170	11	175,29	1,68E-02	0,61	1,52
O76021	Ribosomal L1 domain-containing protein 1	RSL1D1	10	54,972	2,05E-02	0,60	1,52
Q9UI30	Multifunctional methyltransferase subunit TRM112-like protein	TRMT112	5	14,199	3,71E-02	0,60	1,51
Q9P258	Protein RCC2	RCC2	10	56,084	4,85E-03	0,59	1,51

Q9UDY2	Tight junction protein ZO-2	TJP2	8	133,96	6,73 E-04	0,59	1,51
Q13283	Ras GTPase-activating protein-binding protein 1	G3BP1	12	52,164	1,62 E-05	0,59	1,50
Q9NYF8	Bcl-2-associated transcription factor 1	BCLAF1	7	106,12	3,54 E-03	0,59	1,50
P33993	DNA replication licensing factor MCM7	MCM7	9	81,307	1,33 E-04	0,58	1,50
Q14157	Ubiquitin-associated protein 2-like	UBAP2L	16	114,53	1,21 E-03	0,57	1,49
O14737	Programmed cell death protein 5	PDCD5	5	14,285	2,75 E-02	0,57	1,49
Q9UNS2	COP9 signalosome complex subunit 3	COPS3	5	47,873	4,40 E-03	0,57	1,48
P55957	BH3-interacting domain death agonist	BID	4	21,994	3,87 E-02	0,57	1,48
Q9UQ35	Serine/arginine repetitive matrix protein 2	SRRM2	11	299,61	6,46 E-03	0,55	1,47
O43765	Small glutamine-rich tetratricopeptide repeat-containing protein alpha	SGTA	5	34,063	2,48 E-03	0,55	1,46
Q86U42	Polyadenylate-binding protein 2	PABPN1	4	32,749	7,67 E-04	0,55	1,46
Q99729	Heterogeneous nuclear ribonucleoprotein A/B	HNRNPAB	8	36,224	2,14 E-02	0,55	1,46
P33992	DNA replication licensing factor MCM5	MCM5	6	82,285	2,48 E-03	0,54	1,46
P16949	Stathmin	STMN1	9	17,302	5,92 E-03	0,54	1,45
P04792	Heat shock protein beta-1	HSPB1	22	22,782	3,74 E-02	0,54	1,45

P36507	Dual specificity mitogen-activated protein kinase kinase 2	MAP2K2	6	44,424	4,03 E-02	0,53	1,45
P50402	Emerin	EMD	5	28,994	9,19 E-03	0,53	1,44
O75367	Core histone macro-H2A.1	H2AFY	7	39,617	9,23 E-03	0,52	1,44
Q460N5	Protein mono-ADP-ribosyltransferase PARP14	PARP14	4	202,8	3,53 E-02	0,52	1,43
Q04760	Lactoylglutathione lyase	GLO1	8	20,777	3,79 E-02	0,52	1,43
Q13242	Serine/arginine-rich splicing factor 9	SRSF9	7	25,542	2,94 E-03	0,52	1,43
P31943	Heterogeneous nuclear ribonucleoprotein H	HNRNPH1	14	49,229	1,42 E-05	0,52	1,43
Q14498	RNA-binding protein 39	RBM39	4	59,379	4,76 E-04	0,51	1,43
Q9BUF5	Tubulin beta-6 chain	TUBB6	25	49,857	1,29 E-03	0,51	1,43
P33991	DNA replication licensing factor MCM4	MCM4	10	96,557	5,50 E-03	0,50	1,42

8.2. Supplementary Table 2

Codes	Protein names	Gene name	Peptides	Mol. weight [kDa]	FDR	Log 2 Fold Change	FC TRIM8/CNT
P13674	Prolyl 4-hydroxylase subunit alpha-1	P4HA1	8	61,049	0,0060082 9	-0,5022643	0,70599783
P62249	40S ribosomal protein S16	RPS16	12	16,445	0,0118187	-0,5086657	0,70287219
P59998	Actin-related protein 2/3 complex subunit 4	ARPC4	4	19,667	0,0499773 4	-0,5098515	0,70229474
Q01469	Fatty acid-binding protein 5	FABP5	12	15,164	0,0493899 1	-0,5113713	0,70155529
Q9UJ70	N-acetyl-D-glucosamine kinase	NAGK	8	37,375	0,0054972 6	-0,51861	0,69804406
P05121	Plasminogen activator inhibitor 1	SERPINE1	15	45,059	0,0040436 7	-0,5219603	0,69642492
Q92734	Protein TFG	TFG	8	43,447	0,0073534	-0,522109	0,69635311
P32119	Peroxiredoxin-2	PRDX2	15	21,892	0,0219322 5	-0,5229184	0,69596257

Q86VP6	Cullin-associated NEDD8-dissociated protein 1	CAND1	26	136,37	0,0406895	-0,5353619	0,68998556
P21980	Protein-glutamine gamma-glutamyltransferase 2	TGM2	35	77,328	7,401E-05	-0,5373656	0,68902795
P51149	Ras-related protein Rab-7a	RAB7A	14	23,489	0,0272246	-0,5493151	0,68334444
P62495	Eukaryotic peptide chain release factor subunit 1	ETF1	12	49,03	0,0420306	-0,5580788	0,67920606
Q9Y3B3	Transmembrane emp24 domain-containing protein 7	TMED7	4	25,171	0,0237768	-0,5908298	0,66396088
Q13409	Cytoplasmic dynein 1 intermediate chain 2	DYNC1I2	15	71,456	0,0054972	-0,6010551	0,65927161
P46776	60S ribosomal protein L27a	RPL27A	8	16,561	0,0179020	-0,6302993	0,6460424
Q13724	Mannosyl-oligosaccharide glucosidase	MOGS	7	91,916	0,0364586	-0,641737	0,6409408
P62136	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit	PPP1CA	18	37,512	0,0431111	-0,6453416	0,63934141
Q15075	Early endosome antigen 1	EEA1	10	162,46	0,0209903	-0,6463903	0,63887683
Q12797	Aspartyl/asparaginyl beta-hydroxylase	ASPH	18	85,862	0,0270461	-0,65	0,63543227

						41897	
Q9UL46	Proteasome activator complex subunit 2	PSME2	12	27,401	0,0013732	-0,6593771	0,63315161
O95747	Serine/threonine-protein kinaseR1	OXSR1	9	58,022	0,0467492	-0,6716687	0,62778015
P78417	Glutathione S-transferase omega-1	GSTO1	17	27,566	0,0014575	-0,6864945	0,62136182
Q9BTV4	Transmembrane protein 43	TMEM43	6	44,875	0,0049885	-0,6880404	0,62069636
Q15008	26S proteasome non-ATPase regulatory subunit 6	PSMD6	13	45,531	0,0160839	-0,7190135	0,6075127
O15144	Actin-related protein 2/3 complex subunit 2	ARPC2	17	34,333	0,0315791	-0,7322706	0,60195579
P09486	SPARC	SPARC	4	34,632	0,0152831	-0,7358017	0,60048425
P46781	40S ribosomal protein S9	RPS9	10	22,591	0,0271827	-0,7447491	0,59677166
Q9Y6N5	Sulfide:quinone oxidoreductase, mitochondrial	SQOR	8	49,96	0,0011307	-0,7534857	0,59316868
Q02543	60S ribosomal protein L18a	RPL18A	6	20,762	0,0282378	-0,7638286	0,58893136

P61086	Ubiquitin-conjugating enzyme E2 K	UBE2K	6	22,406	0,0275477	-0,7686828	0,58695313
Q9NUQ6	SPATS2-like protein	SPATS2L	4	61,728	0,0009636	-0,7759345	0,5840102
P19105	Myosin regulatory light chain 12A	MYL12A	11	19,794	0,0266689	-0,7883622	0,57900103
O00629	Importin subunit alpha-3	KPNA4	7	57,886	0,0237768	-0,8205004	0,56624552
P49589	Cysteine--tRNA ligase, cytoplasmic	CARS	5	85,472	0,0016777	-0,8307041	0,56225479
P61923	Coatomer subunit zeta-1	COPZ1	5	20,198	0,0482223	-0,8361324	0,56014321
P49257	Protein ERGIC-53	LMAN1	9	57,548	0,0149001	-0,8384256	0,55925353
P17096	High mobility group protein HMG-I/HMG-Y	HMGA1	8	11,676	0,0380115	-0,8531946	0,55355764
Q08AF3	Schlafen family member 5	SLFN5	4	101,05	0,0383159	-0,8995889	0,53603945
P27824	Calnexin	CANX	30	67,567	0,0397316	-0,910305	0,53207259
Q06323	Proteasome activator complex subunit 1	PSME1	15	28,723	0,0092331	-0,91	0,52912178

						83283	
P54709	Sodium/potassium-transporting ATPase subunit beta-3	ATP1B3	10	31,512	0,00552016	-0,9485769	0,51814331
Q8TDB6	E3 ubiquitin-protein ligase DTX3L	DTX3L	5	83,553	0,0204986 8	-0,9832484	0,5058395
P80217	Interferon-induced 35 kDa protein	IFI35	4	31,546	0,0096914 8	-0,9860493	0,50485838
O94855	Protein transport protein Sec24D	SEC24D	8	113,01	0,0349198 9	-1,043985	0,48498598
P49721	Proteasome subunit beta type-2	PSMB2	5	22,836	0,0256648 4	-1,047356 9	0,48385379
Q53GQ0	Very-long-chain 3-oxoacyl-CoA reductase	HSD17B12	6	34,324	0,0496803 8	-1,077365 2	0,47389349
Q14258	E3 ubiquitin/ISG15 ligase TRIM25	TRIM25	11	70,973	0,0003141 1	-1,148715	0,45102677
P46977	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A	STT3A	4	80,529	0,0084116 3	-1,154435 8	0,44924184
P13726	Tissue factor	F3	5	33,067	0,0406891 5	-1,307878 5	0,40391441
P42574	Caspase-3	CASP3	4	31,608	0,0049888 5	-1,344728 2	0,39372817

Q7Z2W4	Zinc finger CCCH-type antiviral protein 1	ZC3HAV1	11	101,43	0,0006197	-1,361661	0,38913386
P09543	2,3-cyclic-nucleotide 3-phosphodiesterase	CNP	11	47,578	0,0047955	-1,381371	0,38385371
P23381	Tryptophan--tRNA ligase, cytoplasmic	WARS	30	53,165	0,0012709	-1,391369	0,38120268
P49720	Proteasome subunit beta type-3	PSMB3	4	22,949	0,0135709	-1,445841	0,36707799
Q9NZT2	Opioid growth factor receptor	OGFR	5	73,324	0,0003932	-1,455000	0,36475486
Q9H223	EH domain-containing protein 4	EHD4	13	61,174	7,6211E-06	-1,526693	0,34707187
P23497	Nuclear autoantigen Sp-100	SP100	8	100,42	0,0001432	-1,584461	0,33344918
P28838	Cytosol aminopeptidase	LAP3	22	56,166	7,1945E-05	-1,715010	0,30460039
Q8TCS8	Polyribonucleotide nucleotidyltransferase 1, mitochondrial	PNPT1	10	85,95	7,8085E-05	-1,749145	0,29747785
P29590	Protein PML	PML	9	97,55	7,8085E-05	-1,801376	0,28690075
P84077	ADP-ribosylation factor 1	ARF1	13	20,697	0,027889	-1,84	0,27909406

						11767	
O75436	Vacuolar protein sorting-associated protein 26A	VPS26A	4	38,169	0,0012585	-1,8493452	0,2775183
P10321	HLA class I histocompatibility antigen, Cw-7 alpha chain	HLA-C	13	40,648	8,0985E-07	-1,9046825	0,26707513
P32455	Guanylate-binding protein 1	GBP1	16	67,93	0,0014575	-1,935183 2	0,26148803
O14933	Ubiquitin/ISG15-conjugating enzyme E2 L6	UBE2L6	5	17,769	4,0557E-06	-2,016067 5	0,24723116
Q9BYX4	Interferon-induced helicase C domain-containing protein 1	IFIH1	11	116,69	0,0006042	-2,036756 8	0,24371098
P19525	Interferon-induced, double-stranded RNA-activated protein kinase	EIF2AK2	14	62,094	2,2484E-08	-2,148302 4	0,22557789
Q9Y6K5	2-5-oligoadenylate synthase 3	OAS3	8	121,17	3,1885E-06	-2,176850 3	0,22115805
Q9BQE5	Apolipoprotein L2	APOL2	9	37,092	1,6185E-06	-2,684470 5	0,15555854
Q03518	Antigen peptide transporter 1	TAP1	9	87,217	0,0001432	-2,773300 5	0,14626936
P42224	Signal transducer and activator of transcription 1-alpha/beta	STAT1	33	87,334	1,5727E-06	-3,253982 5	0,10482229

P52630	Signal transducer and activator of transcription 2	STAT2	13	97,915	3,3673E-05	-3,322539	0,09995766
Q16666	Gamma-interferon-inducible protein 16	IFI16	25	88,255	6,8814E-05	-3,361304 3	0,09730756
O15162	Phospholipid scramblase 1	PLSCR1	6	35,049	3,5925E-06	-3,368633 6	0,09681446
Q9Y3Z3	Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	SAMHD1	17	72,2	6,4553E-07	-4,173378 9	0,05542271
O95786	Probable ATP-dependent RNA helicase DDX58	DDX58	22	106,6	1,7064E-07	-4,438605	0,04611548
P05161	Ubiquitin-like protein ISG15	ISG15	6	17,887	3,6268E-05	-5,151630 4	0,02813227
P09913	Interferon-induced protein with tetratricopeptide repeats 2	IFIT2	29	54,632	6,4553E-07	-6,749729 2	0,00929242
P09914	Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	30	55,36	1,6896E-07	-7,376676 9	0,00601726
O14879	Interferon-induced protein with tetratricopeptide repeats 3	IFIT3	36	55,984	2,2484E-08	-7,484107	0,00558546

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