"Exploring the Mitotic Function of a Subunit of the Molecular Motor Cytoplasmic Dynein"

A Major Project dissertation submitted

In partial fulfillment of the requirement for the degree of

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In

Biomedical Engineering

Submitted by

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2K13/BME/14

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CERTIFICATE

This is to certify that the M. Tech. dissertation entitled **"Exploring the Mitotic Function of a Subunit of the Molecular Motor Cytoplasmic Dynein"**, submitted by **AKANKSHA SMRITI SINGH (2K13/BME/14)** in partial fulfillment of the requirement for the award of the degree of Master of Engineering, Delhi Technological University (Formerly Delhi College of Engineering, University of Delhi), is an authentic record of the candidate's own work carried out by her under my guidance.

The information and data enclosed in this dissertation is original and has not been submitted elsewhere for honoring of any other degree.

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I hereby declare that the work entitled "**Exploring the Mitotic Function of a Subunit of the Molecular Motor Cytoplasmic Dynein**" submitted in partial fulfillment of of the requirement for the award of the degree of **Master of Technology** in Biomedical Engineering from Delhi Technological University (formerly DCE), is an authentic record of my work carried out under the guidance of **Professor Dr. Bansi D Malhotra.**

The information and data enclosed in this dissertation is original and has not been submitted anywhere for honoring of any other degree.

Date: Signature:

Place:

ACKNOWLEDGEMENT

The completion of any project requires a lot of guidance and support from many people and I am extremely fortunate to have got this all along during the course of my project work.

I take this opportunity to express my profound gratitude and deep regards to my guide Mr. Sayan Chatterjee for his exemplary guidance, monitoring and constant encouragement throughout the course of this work. The blessing, help and guidance given by him time to time shall carry me a long way in the journey of life on which I am about to embark.

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Last but not the least I wish to avail myself of this opportunity, express a sense of gratitude and respect to all my seniors for their moral support, strength and help.

Akanksha Smriti Singh

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Exploring the Mitotic Function of a Subunit of the Molecular Motor Cytoplasmic Dynein

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ABSTRACT

The key players in maintaining the gene function and inheritance are eukaryotic chromosomes. The mechanisms and architecture involved in controlling chromosome distribution at mitosis and meiosis are crucial to maintain the stability and activity of the genes and genomes. Much of the energy of a cell is expended in preserving their chromosomes probity. The chromosome imbalances during development causes embryonic casualities, and chromosome instability which prompts the onset of tumours. One serious cause of cancer development is chromosome instability and is associated with poor prediction and diagnosis. The misregulation and mutation of genes alters functioning at the kinetochores, spindle checkpoints, and sister chromatid cohesion are main cause of development of various cancer types.

To minimize the production of aneuploid progeny during mitosis, cells have developed a checkpoint competent of delaying sister chromatid separation in the presence of non-attached or improperly attached kinetochores. The function of SAC is to monitor the interaction between spindle microtubules and kinetochores and if chromosomes are not properly bioriented it will prevent the separation of sister chromatids and exit from mitosis. It is now becoming abundantly clear that misregulation of cell cycle checkpoints underlays not just the birth defects, but very much to the tumorigenesis. Thus, for better development of therapeutic approaches that could transform most cancers into chronic diseases allowing a more rational and adequate management of the disease, it has become all important to unravel the basic biology of cell.

CHAPTER 1: INTRODUCTION

An elaborate series of events that leads to duplication of chromosomes and other cytoplasmic components equally into two cells followed by growth phases constitutes the cell cycle. This process is highly directional and irreversible that causes change in a cell. An appropriate condition keeps a cell dividing and growing. Growth and development of every organism depends on multiplication and enlargement of its cells. In order to maintain the continuity the cells follow a particular order of division which is referred as cell cycle. This duplication of mammalian cells is a highly dynamic and event that is regulated at multiple levels in a defined order and with an exact timing, requiring precise control mechanisms. Mis-regulation in any of these critical pathways via these regulatory mechanisms leads to chromosome mis-segregation, aneuploidy and aberrant mitosis, which are precursors to major diseases like cancer. It is important to study the molecular mechanisms of mitotic regulation in order to understand the basis for cell division leading to differentiation, as well as for potential therapies against major diseases.

The major monitoring mechanism in the metaphase to anaphase transition is the spindle assembly checkpoint (SAC). The SAC ensures accurate segregation of chromosomes at the anaphase stage between daughter cells by arresting cells in metaphase until bipolar attachment of all sister chromatids and subsequent inter kinetochore tension are well satisfied. Mitotic spindle formation and functioning is not only dependent upon the assembly of microtubules but also upon the action of motor enzymes. One such motor complex is cytoplasmic dynein, which is made up of six different subunit families. The Light intermediate Chain1 (LIC1) subunit of the dynein motor plays a key role in spindle assembly checkpoint silencing. LIC1 is believed to silence the checkpoint by removing the checkpoint proteins from kinetochores in metaphase cells. Once the checkpoint proteins are stripped off from kinetochores, the cell cycle machinery allows the onset of anaphase and chromosomes are equally segregated into the two daughter cells. A Cdk1-cyclin B phosphorylated form of LIC1 at S207 is a supervisory modification required for SAC protein removal. Other sites reported to undergo phophorylation at the entry into mitosis are S398, S405 and T408 on rLIC1.

CHAPTER 2: HYPOTHESES

The effect of phopshorylation of LIC1 at various sites can bring about the idea of how these S207, S398, S405 and T408 sites are regulating the SAC function. It is been shown that phosphorylated form of LIC1 is important for silencing the SAC by removing the checkpoint proteins and transports them from kinetochores along spindle microtubules in metaphase. Phosphorylated form of LIC1 works with SAC proteins and transports them away from kinetochores along spindle microtubules.

When dynein is unable to bind SAC cargo this would result in the observed accumulation of SAC proteins at kinetochores. Upon entry into the mitosis cdk1 (a mitotic kinase) phosphorylates LIC1 at S207, S398, S405, and T408. However, preventing phosphorylation at just one of these sites, (S207 – by generating a non-phosphorylatable mutant A207), seems to make LIC1 incompetent for SAC protein removal from kinetochores. (Mylavarapu VS Sivaram, Thomas L Wadzinski, Sambra D Redick, Tapas Manna and Stephen J Doxsey (2009) Dynein light intermediate chain 1 is required for progress through the spindle assembly checkpoint. *EMBO*, 1–13)

This suggests that the S207 site needs to be in the phosphorylated condition for SAC inactivation by LIC1. It is unknown however, whether phosphorylation of the other three sites has any functional importance in SAC inactivation by dynein. It is possible that the remaining sites which are S398, S405 and T408 play important roles in controlling the SAC function and inactivating the checkpoint. There is no published information on the relevance of these phospho sites. This project is aimed to determine why and how mitotic phosphorylation of LIC1 at some of the other three phospho-sites is essential for mitotic progression mediated by dynein.

This proposal has very high relevance to the field of SAC regulation; there is no published data at present that answers the questions being addressed. The biochemical reactions that drive SAC silencing are poorly understood. Either depletion of LIC1, or suppression of S207 phosphorylation impairs dynein's ability to remove SAC proteins from kinetochores. The molecular mechanism of this process is of fundamental importance to understand. This project will determine why phosphorylated LIC1 is essential for mitotic progression mediated by dynein, and functionally characterize the contributions of these interaction partners in SAC inactivation. Answers to these questions will provide a major advance in understanding the intricate molecular mechanisms governing cell cycle regulation.

CHAPTER 3: REVIEW OF LITERATURE

Strict cell cycle regulation is vital to ensure faithful segregation of genetic material and thereby allow normal development and maintenance of multicellular organisms. Failure to coordinate such processes leads to genome instability, often associated with birth defects and cancer. Accordingly, cells have checkpoint controls ensuring that the correct sequence of events is firmly maintained. Checkpoints consist of at least three components: a sensor, that detects the error, a signal, generated by the sensor via a signal transduction pathway and, finally, a response element in the cell cycle machinery to block cell cycle progression. Three checkpoints have been amply documented: the DNA damage checkpoint, which arrests cells in G1, S phase, G2 or even mitosis in case of DNA lesions; the DNA replication checkpoint, which ensures that mitosis is not initiated until DNA replication is complete and also that no DNA is replicated twice; and, the Spindle Assembly Checkpoint (SAC), which delays anaphase onset until all chromosomes are properly attached to the mitotic spindle.

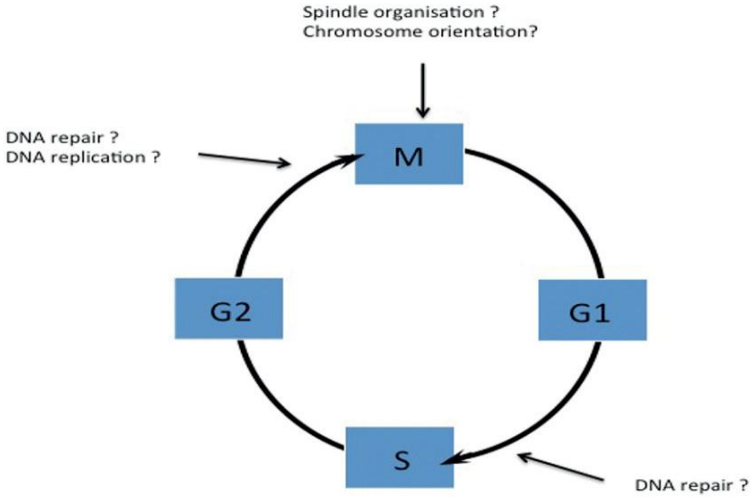

Figure 1: Cell cycle checkpoints

3.1- Metaphase (Spindle) Checkpoint

Separation of sister chromatids during anaphase should take place only when all chromosomes are attached to the bipolar mitotic spindle via their kinetochores. The spindle checkpoint guarantees this dependence. The checkpoints are activated when cells are treated with microtubule-depolymerizing drugs. If free kinetochores are present, cells do not exit mitosis phase or progress to anaphase, suggesting that the spindle checkpoint pathway blocks both the metaphase to anaphase and anaphase to telophase transitions.

Microtubules functions like tracks within the cells, on which cargoes of material like vesicles or organelles can be transported. Two families of motor protein called the kinesins and dynein that move along the microtubule, act like tow trucks, attaching to the cargo and pulling it along the tracks to the destination. There are many types of kinesins and dynein each of which has function in delivering a different cargo.

3.2- Dynein

Dynein is a motor protein (also called molecular motor or motor molecule) in cells. The mechanical energy of movement is obtained from the chemical energy contained in ATP with the help of dynein. Dynein aids the transportation of Various cellular cargos are by walking along cytoskeletal microtubules heading towards the the center of cell. Thus, they are usually referred to as "minus-end directed motors". This is the retrograde form of transport.

Dyneins are divided into two groups: cytoplasmic dyneins and axonemal dyneins. The movement of cytoplasmic dynein is processive along the microtubule which means one or the other of its stalks is connected to the microtubule always so that the dynein can "walk" a considerably significant distance along a microtubule without getting detached.

There is a probability that Cytoplasmic dynein helps to position and orient the Golgi complex and other important organelles in the cell (Gerald Karp, Kurt Beginnen, Sebastian Vogel, Susanne Kuhlmann-Krieg (2005). *Molekulare Zellbiologie* (in French). Springer. ISBN 978-3-540-23857-7). It also helps in transportation of cargos required for vital cell function such as vesicles made by the lysosomes, endoplasmic reticulum and endosomes (Karp, 2005). Dynein is associated with the movement of chromosomes and proper positioning the mitotic spindles for cell division. Dynein aide in a process which is called as retrograde axoplasmic transport because dynein support the movement of organelles, vesicles and possibly microtubule fragments along the axons of neurons toward the cell body.

3.2.1- Cytoplasmic dynein

The molecular mass of cytoplasmic dynein is about1.5 megadaltons (MDa) and is composed of approximately twelve polypeptide subunits: two identical "heavy chains" which are 520 kDa in mass, who compasses the ATPase activity and are responsible for generating movement along the microtubule, two intermediate chains of 74 kDa which are supposed to anchor the dynein to its cargo, four intermediate chains of 53–59 kDa, and several light chains whose functions are less apprehended.

The doughnut-shaped "head", of dynein heavy chain is the location of force-generating ATPase activity which is related to other AAA proteins, while two projections from the head helps in connecting it to other cytoplasmic structures. The "walking" along the surface of the microtubule by a repeated cycle of detachment and reattachment is done with one of the projection, the coiled-coil stalk and the other projection which is the extended tail "stem" binds to the other intermediate and light chain subunits which attach the dynein to its cargo.

The ability of a single dynein molecule to transport its cargo by "walking" a considerable significant distance along a microtubule without getting completely detached comes from the alternating activity of the paired heavy chains. The cytoplasmic dynein is activated by binding of dynactin in eukaryotes. Dynactin is another multisubunit protein that is vital for mitosis. Dynactin may modulate the activity of dynein, and possibly facilitates the attachment of dynein to its cargo.

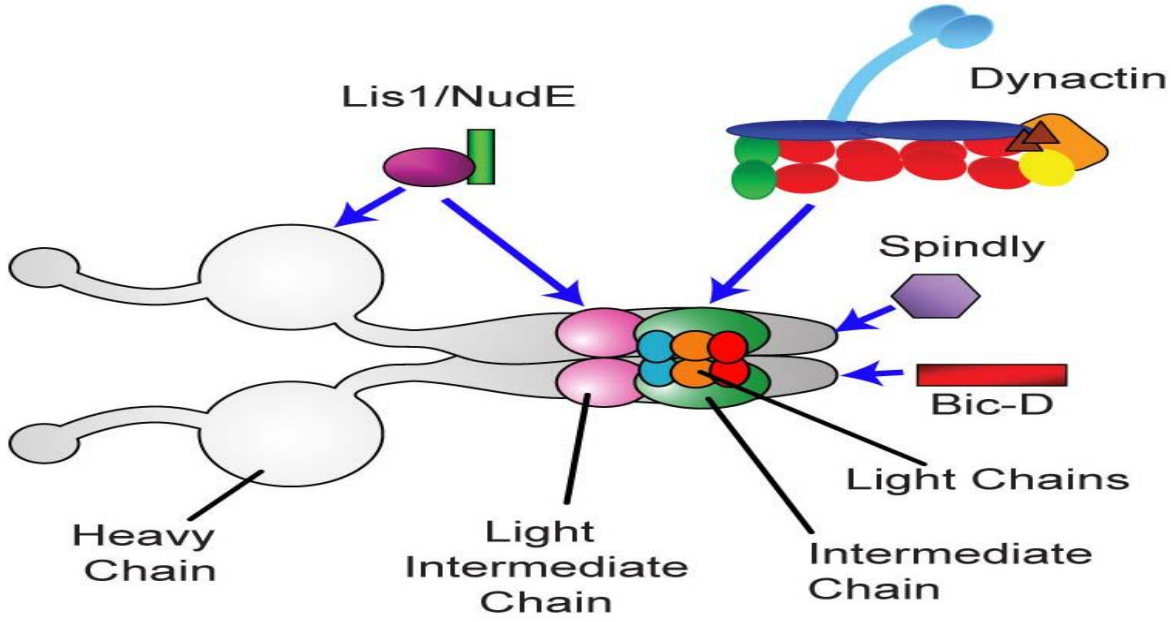

Fig 2: Structure of Dynein

Image courtesy:
http://www2.mrclmb.cam.ac.uk/groups/cartera/research/images/DyneinComplex_front2.jpg

3.3-Light intermediate chains

Two different light intermediate chain genes : LIC1 and LIC2 are bind to cytoplasmic dynein. In Caenorhabditis elegans, there is only one LIC for cytoplasmic dynein which is LIC1, and it has been found to be involved in playing key role in mitosis. When Several mutant alleles of this gene are checked they have resulted in failed divisions and when using RNAi LIC1 was depleted there were observed prominent problems with cleavage furrow formation. The other problems as observed upon LIC depletion or in LIC mutants were pronuclear migration and with centrosome separation resulting in monopolar mitotic spindles (Yoder and Han, 2001). LIC1 has been found to interact with several proteins. LIC1 interacts with Rab4A. Rab4A is a GTPase muddled in the regulation of membrane receptor recycling (Bielli et al., 2001). LIC1 also interacts with pericentrin, which is a centrosomal protein that is assembled to the centrosome by dynein.

This phosphorylation of the intermediate chain is in the middle of the binding domain for the dynein light chain LC8 family and results in the loss of this subunit from the dynein complex (Whyte et al., 2005). This is suggested to allow phosphorylated dynein to localize to the cortex of cells in late mitosis and then to the congressing furrows of cells undergoing anaphase and cytokinesis. A third example is phosphorylation of intermediate chain 1 at S84 (Vaughan 2001). This phosphorylation site is localized within the binding site for dynactin and appears to disrupt the interaction of dynactin with dynein. As dynactin is involved in targeting dynein to membranous cargoes the removal of dynactin would result in the loss of this cargo capacity.

These examples show the gain or loss of dynein subunits with phosphorylation. The third type of phosphorylation event directly affects the binding site on a dynein subunit for a particular cargo. The one example of this phenomenon is that during the transition from interphase to mitosis dynein is observed to lose its affinity for vesicle cargoes. Cyclin dependent kinase 1 (cdk1 or cdc2-cyclin B1) is a major mitotic kinase and it has been shown to phosphorylate light intermediate chain 1 in more than one position in mitotic extracts. These phosphorylations of light intermediate chain 1 have been implicated in this transition from interphase dynein binding to many membranous cargoes to mitotic dynein which binds far fewer membranous cargoes (Addinall et al., 2001). The mechanism of this change is not clear as light intermediate chain 1 has not been shown to be a pivotal subunit in binding membranous cargoes to dynein.

The Mad and Bub protein families which are the main molecular components required for the checkpoint, were originally shown to be required for the wait anaphase signal in yeast and later in humans. (Hoyt et al, 1991; Meraldi et al, 2004).
Mad2 and BubR1, the members of these families functions directly in the SAC by keeping a check on degradation of cyclin B and securins and inception of anaphase (Shah and Cleveland, 2000; Hoyt, 2001). Both ladle out as an indicators of ‘wait anaphase’ signal by localizing at the kinetochores (Waters et al, 1998; Howell et al, 2000; Hoffman et al, 2001) and both move off kinetochores before anaphase progression (Howell et al, 2004; Mayer et al, 2006; Zhang et al, 2007)

Another possibility is that the cargo, in this case the vesicles, have been altered in the transition from interphase to mitosis and then no longer interact with dynein. While this example is not the strongest example it demonstrates the idea of how a phosphorylation event could affect the binding of cargoes but not the overall dynein functionality or subunit make-up. one well demonstrated phosphorylation site on LIC1 is right in the middle of the AA201-225 region of low homology and cargo targeting. LIC1 has been shown to be phosphorylated by cdk1 at S207 by mutational analysis and mass spectrometry. Looking at the other potential cdc2 phosphorylation sites, they also fall within a region of fairly low homology from AA376 to AA425 in rLIC1, suggesting that there may be other proteins that bind in this area that are regulated with these phosphorylations.

Role of Dynein in maintaining spindle bipolarity: On depleting the LIC1 of Dynein with small interfering RNAs in human cell lines and *Xenopus laevis* early embryos it was shown that the microtubule gliding rate was normal, but for the formation of a bipolar spindle LICs are quintessential. In cells lacking LICs there was multipolar spindles with poles that contain single centrioles indicating that they are required for maintaining centrosome integrity. (Laura A. Jones, Cécile Villemant, Toby Starborg, Anna Salter, Georgina Goddard, Peter Ruane, Philip G. Woodman, Nancy Papalopulu, Sarah Woolner, and Victoria J. Allan Dynein light intermediate chains maintain spindle bipolarity by functioning in centriole cohesion)
Spindle morphology was distorted upon depletion of LICs in *Xenopus* embryos and 44% of mitotic cells found to have multipolar spindles found in by developmental stage.
PCM fragmentation or premature disengagement of daughter and mother centriole can be a cause of spindle pole fragmentation (Maiato and Logarinho, 2014) and this causes multipolar spindle formation. The possible explanation for the effect of LIC knoeck down on spindle morphology is that LICs depleted dyneins fails to enroll cargoes required for spindle pole organization.

Comparison between Homo sapiens dynein, cytoplasmic 1, light intermediate chain 1 (DYNC1LI1), mRNA and Rattus norvegicus dynein cytoplasmic 1 light intermediate chain 1 (Dync1li1), mRNA

- Showing 1.57kb region from base 105 to 1676.

Homo sapiens dynein, cytoplasmic 1, light intermediate chain 1 (DYNC1LI1), mRNA

NCBI Reference Sequence: NM_016141.3

GenBank Graphics

>gi|302129656:105-1676 Homo sapiens dynein, cytoplasmic 1, light intermediate chain 1 (DYNC1LI1), mRNA

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Protein:

>VIRT27707

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Rattus norvegicus dynein cytoplasmic 1 light intermediate chain 1 (Dync1li1), mRNA

NCBI Reference Sequence: NM_145772.1

GenBank Graphics

>gi|21955133:24-1595 Rattus norvegicus dynein cytoplasmic 1 light intermediate chain 1 (Dync1li1), mRNA

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Wild rLIC1 Protein seq:

cytoplasmic dynein 1 light intermediate chain 1 [Rattus norvegicus]

NCBI Reference Sequence: NP_665715.1

GenPept Graphics

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Comparision:

Nucletide:

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 Strand=Plus/Plus

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Query  901   GTTCAGAAACTATATGGATTTCCCTATAAGATTCCTGCTGTTGTTGTGGAAAAGGATGCA  960
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Query  961   GTATTTATTCCAGCAGGGTGGGATAATGATAAGAAAATAGGAATATTACATGAAAATTTT  1020
              || || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  1021  CAAACATTAAAAGCAGAAGATAATTTTGAAGACATCATAACTAAACCACCTGTTCGAAAG  1080
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Query  1558  GGAGAAGCTTCTTGA  1572
              |||||||| || |||
Sbjct  1558  GGAGAAGCCTCCTGA  1572
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Protein:
CLUSTAL 2.1 multiple sequence alignment

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VIRT27707     MAAVGRVGSFGSSPPGLSSTYTGGPLGNEIASGNGGAAAGDDEDGQNLWSCILSEVSTRS 60
VIRT28691     MAAVGRVGSFGSSPPGLASTYASGPLANELASGSGGPAAGDDEDGQNLWSRILREVSTRS 60
              ******************.****.:***.***.****.**.************** ** *******

VIRT27707     RSKLPAGKNVLLLGEDGAGKTSLIRKIQGIEEYKKGRGLEYLYLNVHDEDRDDQTRCNVW 120
VIRT28691     RSKLPTGKNVLLLGEDGAGKTSLIRRIQGIEEYKKGRGLEYLYLNVHDEDRDDQTRCNVW 120
              ******.********************.****************************************

VIRT27707     ILDGDLYHKGLLKFSLDAVSLKDTLVMLVVDMSKPWTALDSLQKWASVVREHVDKLKIPP 180
VIRT28691     ILDGDLYHKGLLKFSLDALSLRDTLVMLVVDMSKPWTALDSLQKWASVVREHVDKLKIPP 180
              ********************.**.****************************************

VIRT27707     EEMKQMEQKLIRDFQEYVEPGEDFPASPQRRNTASQEDKDDSVVLPLGADTLTHNLGIPV 240
VIRT28691     EEMKEMEQKLIRDFQEYVEPGEDFPASPQRRATAAQEDRDDSVVLPLGADTLTHNLGLPV 240
              *****.***************************** **.***.********************.**

VIRT27707     LVVCTKCDAISVLEKEHDYRDEHFDFIQSHIRKFCLQYGAALIYTSVKENKNIDLVYKYI 300
VIRT28691     LVVCTKCDAISVLEKEHDYRDEHFDFIQSHIRKFCLQYGAALIYTSVKENKNIDLVYKYI 300
              ************************************************************

VIRT27707     VQKLYGFPYKIPAVVVEKDAVFIPAGWDNDKKIGILHENFQTLKAEDNFEDIITKPPVRK 360
VIRT28691     VQKLYGFPYKIPAVVVEKDAVFIPAGWDNDKKIGILHENFQTLKIEDNFEDIITKPPVRK 360
              ********************************************** ***************

VIRT27707     FVHEKEIMAEDDQVFLMKLQSLLAKQPPTAAGRPVDASPRVPGGSPRTPNRSVSSNVASV 420
VIRT28691     FVHEKEIMAEDDQVFLMKLQSLLAKQPPTAAGRPVDASPRVPGGSPRTPNRSVSSNVASV 420
              ************************************************************

VIRT27707     SPIPAGSKKIDPNMKAGATSEGVLANFFNSLLSKKTGSPGGPGVSGGSPAGGAGGGSSGL 480
VIRT28691     SPIPAGSKKIDPNMKAGATSEGVLANFFNSLLSKKTGSPGGPGV-GGSPGGGAAGASTSL 479
              ********************************************** *****.***.*.*:.:*

VIRT27707     PPSTKKSGQKPVL-DVHAELDRITRKPVTVSPTTPTSPTEGEAS 523
VIRT28691     PPSAKKSGQKPVLSDVHAELDRITRKPASVSPTTPPSPTEGEAS 523
              ***:********** **************.:*******.*********
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CHAPTER 4: OBJECTIVES

LIC1 gets hyper-phosphorylated upon entry of the cell into mitosis in eukaryotes. The phosphorylation site S207 in mammals is homologous to the corresponding S197 in *Xenopus* LIC1, a residue that also gets phosphorylated upon mitotic entry. In addition to S207 the other three residues (S398, S405, T408) are phosphorylated by the master mitotic kinase cdk1-cyclinB. Also, the secondary structure prediction analysis reveals that S207 is situated in a predicted loop/ unstructured region, while the remaining three phosphorylation sites lie clustered together in another predicted loop distal to S207 . Given that all four phosphorylation sites are highly conserved across species, they all are likely to have their own important and conserved functions.

Main objective is to assess whether any or all of the three conserved phosphorylation events other than S207 contributes to SAC inactivation. Various single and double site directed alanine mutants (in several permutations) of these residues have been attempted to be generated, while keeping S207 undismayed. Replacement with alanine will render the particular sites phosphorylation- incompetent. The mutant LIC1 proteins are cloned into mammalian expression vectors and then are tested for their ability to functionally rescue LIC1 depletion phenotypes in cells upon exogenous transfection. The phenotypes that have been studied are arrested in metaphase. The functional readout will be to check which protein(s) phenocopy LIC1 upon depletion, i.e., shows prolonged arrest in metaphase.

Objective 1:

To generate site-specific phosphomutants at different sites of LIC1.

MAAVGRVGSFGSSPPGLASTYASGPLANELASGSGGPAAGD
DEDGQNLWSRILREVSTRSRSKLPTGKNVLLLGEDGAGKTSLI
RRIQGIEEYKKGRGLEYLYLNVHDEDRDDQTRCNVWILDGDL
YHKGLLKFSLDALSLRDTLVMLVVDMSKPWTALDSLQKWAS
VVREHVDKLKIPPEEMKEMEQKLIRDFQEYVEPGEDFPASPQ
RRATAAQEDRDDSVVLPLGADTLTHNLGLPVLVVCTKCDAIS
VLEKEHDYRDEHFDFIQSHIRKFCLQYGAALIYTSVKENKNIDL
VYKYIVQKLYGFPYKIPAVVVEKDAVFIPAGWDNDKKIGILHE
NFQTLKIEDNFEDIITKPPVRKFVHEKEIMAEDDQVFLMKLQS
LLAKQPPTAAGRPVDASPRVPGGSPRTPNRSVSSNVASVSPI
PAGSKKIDPNMKAGATSEGVLANFFNSLLSKKTGSPGGPGV
GGSPGGGAAGASTSLPPSAKKSGQKPVLSDVHAELDRITRKP
ASVSPTTPPSPTEGEAS.

Fig 3: Sequence of rLIC1

The sites in blue represent the amino acids where the mutation is required. The sites are required to be converted into alanine.

Fig 4: mutation sites of rLIC1

Objective 2:

To understand the functional role of phospho-deficient mutants of S398, S405 and T408 in mitotic progression.

The involvement of LIC1 in the spindle assembly checkpoint requires the phosphorylation of LIC1 at a putative Cdk1 phosphorylation site. This site is located in a domain of LIC1 that binds various proteins suggesting that this phosphorylation could also regulate these interactions.

For understanding the role of S398, S405, T408 in mitotic progression I had used the three recombinant single mutants made in several permutations of rat LIC1 on these residues keeping S207 unperturbed EAST, ESAT and ESSA.

The mutant LIC1 proteins had been tested for their ability to functionally rescue LIC1 depletion phenotypes (multipolar foci of spindle poles).

CHAPTER 5: METHODOLOGY

5.1- Strategy involved in mutant generation: The flow chart below depicts the experimental strategy that was employed to attempt generation of site-specific alanine mutations at the three residues indicated in the diagram.

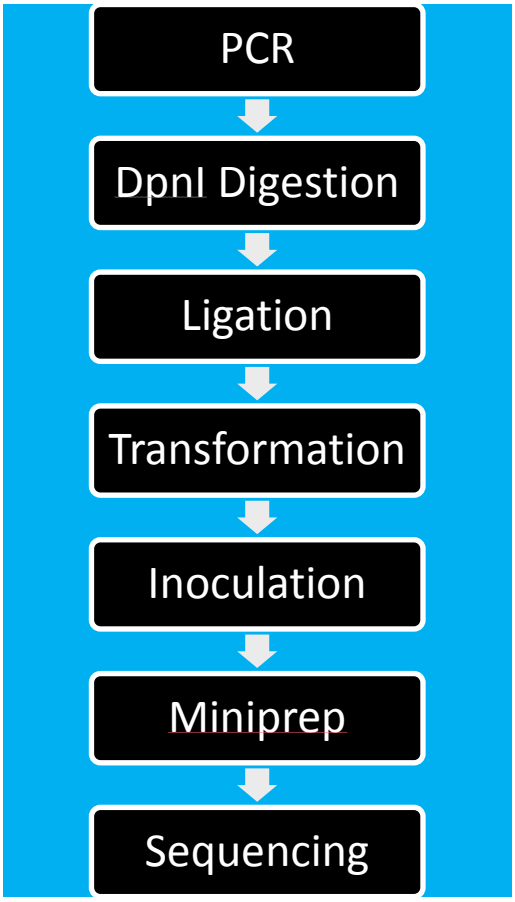


Figure 5: Strategic methodology followed for mutant generation

5.1.1- Site Directed Mutagenesis by POLYMERASE CHAIN REACTION (PCR):

The POLYMERASE CHAIN REACTION (PCR) is a molecular biology technique that is used to produce several copies of a DNA up to several orders of magnitude. PCR essentially consists of three basic steps - denaturation, annealing and elongation. PCR includes denaturation of DNA substrate, primer annealing and extension of the annealed primers by

the polymerase enzyme result in the hybridization of specific DNA fragment. Oligonucleotide DNA fragments (primers) complementary to the target DNA hybridize to the respective sequences. After primer binding/annealing, the elongation of the strand is catalyzed by a heat-stable DNA polymerase in the presence of suitable buffer, magnesium ions and dNTPs.

For the purpose of introducing site-directed mutations (SDM) at specific bases, the oligonucleotide primer was designed such that it had specific base(s) mutated at the position complementary to which the target mutation was desired (SDM primers). The SDM PCR was then performed using the SDM primers, but in a manner otherwise similar to the normal PCR reaction.

Materials Required: Double distilled water (dH_2O), dNTPs, Phusion DNA polymerase, HF buffer, DMSO, Template, Primers.

Reaction Mixture: Various concentrations were used in all different tries.

dH_2O- variable

dNTPs- 1 µl, 2 µl, 2.5 µl

Phusion DNA polymerase: 0.5 µl, 1 µl,

HF Buffer: 5 µl, 10 µl

DMSO- 1 µl, 1.5 µl

Template- EAST, ESAT, ESSA

Primer- 398, 405, 408 (1:10, 1:100)

The following table enlists the existing LIC1 template available in the lab and the desired mutations targeted:

	Template		**Targeted mutations**		
Wild type	S		S	S	T
Already in lab	E(always mimics P)		S	S	T
			We have to check whether these phosphorylation sites are important for LIC1 function		
We have made	E		**A**	S	T
	E		S	A	T
	E		S	S	A
New double mutants attempted in the project	**Template**		**Targeted mutations**		
	ESSA	E E	A S	S A	A A
	EAST	E E	A A	A S	T A
	ESAT	E E	A S	A A	T A

Table 1: Schematic of the various single and combinatorial mutants attempted

5.1.2- DpnI Digestion

The Process is then followed by digestion with Dpn1 digestion Methylated sites are cleaved by the action of DpnI thus it chews up the template plasmid and PCR product remains unaffected.

1µl DpnI Enzyme is added to the PCR product and kept in water bath for 2 hours at 37°C.

5.1.3- Transformation

The final reaction is transformed into the competent cells. Transformation is the process of incorporating an external DNA into a host cell.

Here I have used DH5α Ecoli Cells for the purpose.

1) 50µl of competent cells were added to 5µl of reaction.
2) Competent cells along with ligated reaction were kept on ice for 30 min.
3) Heat shock was given at 42^0 C for 90 second in a water bath.
4) Cells were immediately transferred on to ice and kept in ice for 10 min.
5) 1000 µl of LB broth was added and kept for 1 hour at 37℃.
6) Cells were plated on an LB agar plate containing kanamycin.
7) Plates were incubated overnight at 37◦C.
8) The next morning, Some colonies were picked and inoculated in 5ml media separately in each tube and incubated overnight at 37◦C with rotary shaking to generate the starter culture.

5.1.4- Miniprep:

1) 2 ml from each of the tube were taken (under laminar), the tubes were centrifugated at 132000 rpm for 4 minutes at room temperature.
2) Supernatant was decanted and to the pellet 250µl P1 buffer was added, thoroughly mixed by resuspending through pipette.
3) 250 µl P2 buffer was then added and gently mixed.
4) Then 350 µl of N3M buffer was added and mixed well by vortexing.
5) The tubes were then centrifugated at 132000 rpm at 4◦C for 20 minutes.
6) The supernatant (600 µl) was carefully collected and the pellet was discarded. To the supernatant 420 µl of isopropanol is added and again centrifuged at 132000 rpm at 4◦C for 40 minutes.
7) The faint white pellet was observed (DNA), this was resuspended in 50 µl of sterile water.

5.2- The array of different combination of mutants generated has been checked for their ability to rescue the phenotype of non mutated LIC1.

5.2.1- Transfection

Transfection is the process of introducing exogenous DNA (usually complementary DNA) into a mammalian cell host by encapsulating the DNA in a suitable transfection reagent. The DNA gets introduced into the target cells in varying copy numbers and is inherited to daughter cells through cytoplasmic transfer during cell division. The DNA is transcribed and translated by the host cell machinery and the protein encoded by the gene expressed in the cells. Transfection is used as an extremely useful tool to study the expression, localization and function of a gene.

- Plated HeLa cells into a 6 well plate containing cover slips inside the wells approximately 24 hours before transfection to ensure that cells are at optimal concentrations at the time of transfection.
- The concentrations of the generated phosphomutants was checked through the use of nanodrop, and it was observed as:

Phoshomutant	Concentration	260/280
ESAT	450.1 ng/µl	1.88
ESSA	245.9 ng/µl	1.89
EAST	396.5 ng/µl	1.88

Table 2: Concentration of DNA of phosphomutants generated checked using nanodrop.

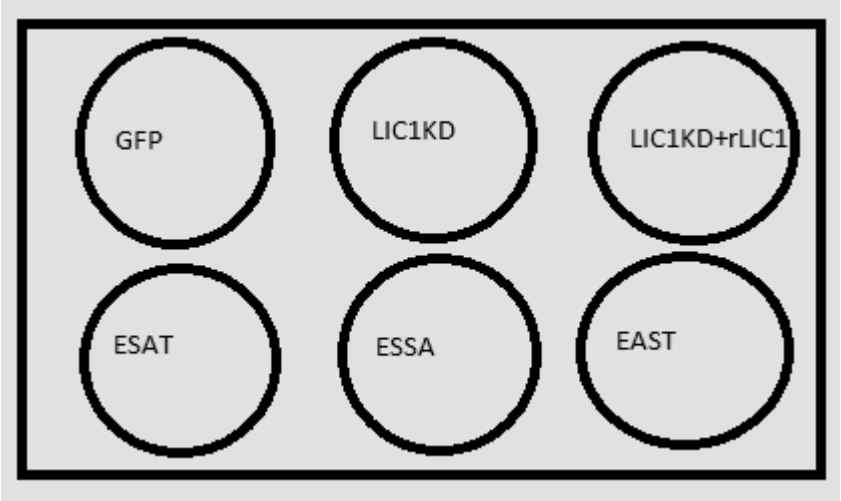


Figure 6: Representation of 6 well plate containing variously treated HeLa cells

- 6 well plate was taken out of the incubator, observed under microscope for confluency. Media was removed using aspirator and washed with PBS.
- First two wells were treated with 100µl trypsin and then 100µl DMEM media is added. A calculated amount of cells were removed from those to wells to get the 40% confluency by next day.
- In rest of the 4 wells added 700µl optiMEM media.
- In rest of the 4 wells following treatment was given, the tubes with following concentration were prepared and incubated for 15 minutes

Phosphomutant	DNA concentration	OptiMEM concentration
ESAT	4.5 µl	94.5 µl
ESSA	8.3µl	92.7µl
EAST	5.12 µl	94.88µl
Myc+Lic1	1.7µl	98.3µl
Lipofectamin	36.8µl	363.2µl

Table 3: Transfection protocol

- After 15 minutes 100µl from Lipofectamin+optimum tube is taken and added to rest of the 4 tubes equally and left for another 15 minutes for incubation.

- After the 15 minutes the contents were transferred to each of the respective wells and left for 4 hours of incubation.
- The optimum media was removed from the wells after the 4 hours and 2 ml fresh DMEM media is added to each well and kept in incubator for incubation overnight.

5.2.2- SiRNA Transfection

- For GFP control two tubes were prepared as follows: the tubes were kept for 15 minutes incubation.
- The contents of both the tubes were mixed and kept of another 15 minutes, and then added (400 µl) to the first well of GFP control.
- For rest of the 5 wells i.e. for phosphomutants and Lic1 KD another reagent tubes were prepared as follows and kept for 15 minutes incubation.
- The reagents of two tubes are mixed and again incubated for 15 minutes, after 15 minutes 400µl was taken and added to each of the well and old media is replaced with fresh 2ml DMEM media.
- The plate was kept in incubator for 48 hours.
- Cells were observed under microscope after 2 days and images were taken using camera.

5.3- Cell Fixation

- 6 well plate is taken, and the media is removed from the wells, appropriate amount of PBS is added to wash the cells.
- In another labeled 6 well plate, chilled methanol was added.
- Cover slips from the first well plate were picked using forcep and transferred to second plate containing methanol and stored under -20◦C till further use.

5.4- Lysate Preparation

- In a tube, 50µl β-mercaptoethanol and 950 µl 2X SDS loading buffer were mixed. (loading buffer consists of: SDS, Glycerol, Tris HCl and Bromophenol blue).
- From this preparation, 100 µl was taken and added to each of the well.

- A cell scrapper was used to scrape out the cells, and then collected into a eppendorf tube using a fine needle.
- After the cells of all the 6 wells were collected in tubes, tubes were then kept on preheated dry bath at 95◦C for 5 minutes.
- Tubes were removed from dry bath and stored at -20◦C/-80◦C

E) Immunostaining

- A well cleaned humidifying chamber was taken and a water soaked kimwipe was placed inside the chamber, this was done in order to main the humidity inside the chamber.
- Now the drops of PBS were put equal in number to the cover slips inside the chamber.

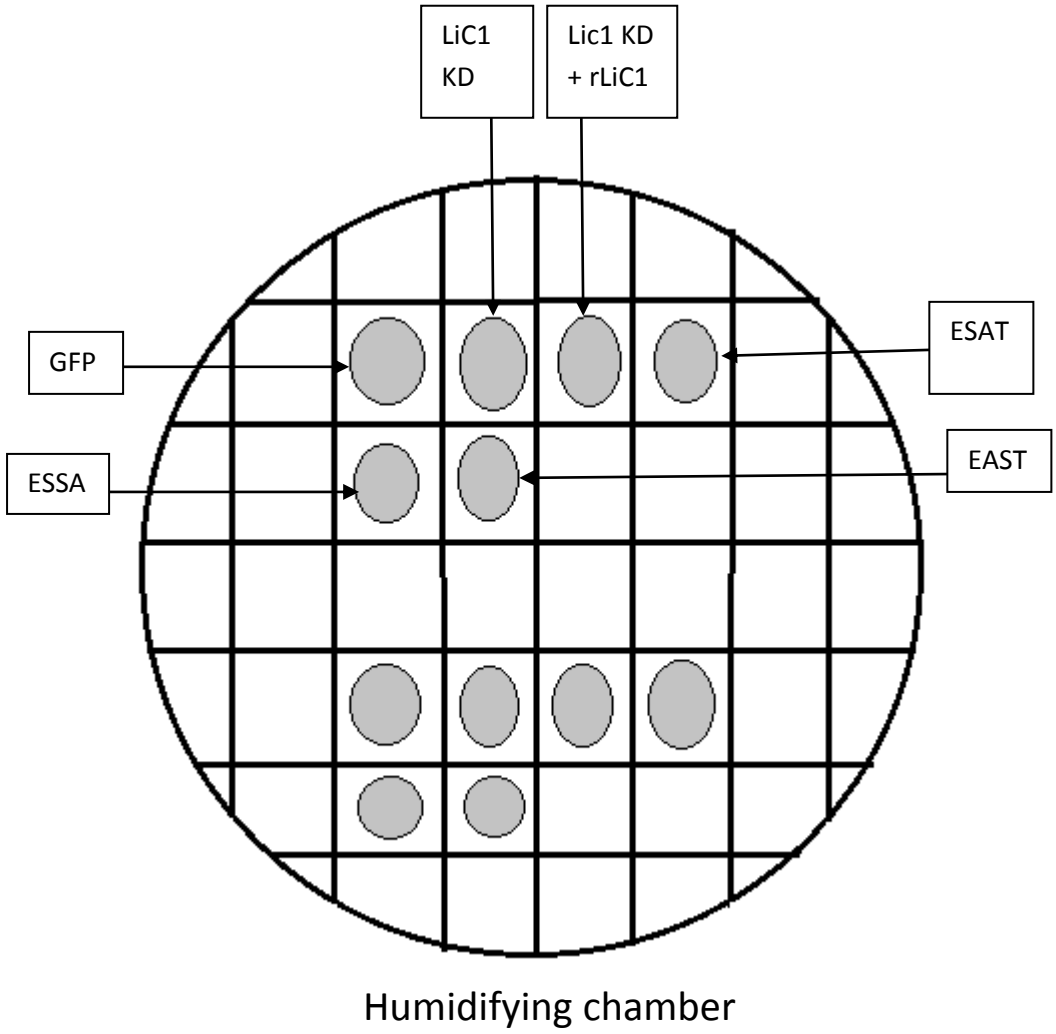


Figure 7: Humidifying chamber for performing Immunostaining

- Cover slips were taken out from the methanol and put over the PBS drops.
- PBS was removed using aspirator and then water drops added over the cover slips, after a few minutes aspirated the water.
- Before letting the cover slips dry drops of PBSAT were added on them, chamber was then closed and kept for 1 hour undisturbed.
- Primary antibody preparation:
  α-tubulin is used in 1:1000 concentration
  γ-tubulin is used in 1:250 concentration
  crest is used in 1:50 concentration
  Therefore amount used of the respective antibodies was:
  α-tubulin- 1µl, γ-tubulin-3µl, crest-16µl and mixed in 800 µl of PBSAT.
- 30µl of 1◦ antibody was tip over every coverslip and left for 1 hour undisturbed.
- Secondary antibody preparation:
  Now the amount of dyes respective to the primary antibody were calculated and Mixed in 800 µl of PBSAT.

α-tubulin	DL488	Green	1:800	1 µl
γ-tubulin	Cy-3	Red	1:800	1 µl
Crest	Cy-5	Far red	1:800	1 µl
DAPI		Blue		

Table 4: Primary antibody production

- After 1 hour primary antibody was removed from cover slips and washed with PBSAT 2-3 times thoroughly.
- 30 µl of 2◦ antibody was tip over every coverslip and left for 1 hour. And washed thoroughly with PBSAT after the 1 hour was over.
- 25 µl of DAPI was added over coverslips for 1 minute and washed with PBSAT again.
- The cover slips were then stacked over the microscopic slides upside down, before putting the cover slips, drops of 4 µl Prolong gold (antifading agent) were spilled over the slide. Left overnight the slides to let them dry and after that store them under -20◦C.

CHAPTER 6: RESULTS

Out of various combinations tried I have made three single phosphomutants which are EAST, ESAT and ESSA and the double mutants has not formed

1) **EAST**

   Got DAAPRVPGGSPRTP

   The 398 site has been mutated and converted into alanine.

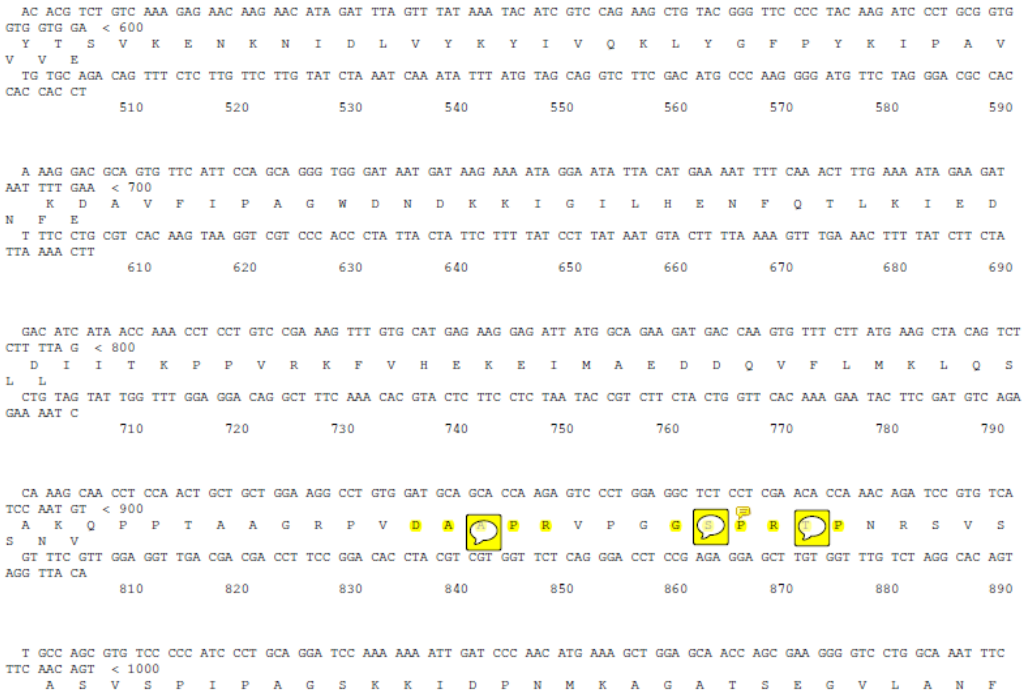


Figure 8: LIC1sequence mutated at 398 phosphorylation site

2) ESAT

Got DASPRVPGGAPRTP

The 405 site has been mutated and converted into alanine.

```
TAA GGT CGT
              510         520         530         540         550         560         570         580         590


  GGG TGG GAT AAT GAT AAG AAA ATA GGA ATA TTA CAT GAA AAT TTT CAA ACT TTG AAA ATA GAA GAT AAT TTT GAA GAC ATC ATA ACC AAA CCT
CCT GTC C  < 700
   G   W   D   N   D   K   K   I   G   I   L   H   E   N   F   Q   T   L   K   I   E   D   N   F   E   D   I   I   T   K   P
P   V
  CCC ACC CTA TTA CTA TTC TTT TAT CCT TAT AAT GTA CTT TTA AAA GTT TGA AAC TTT TAT CTT CTA TTA AAA CTT CTG TAG TAT TGG TTT GGA
GGA CAG G
              610         620         630         640         650         660         670         680         690


  GA AAG TTT GTG CAT GAG AAG GAG ATT ATG GCA GAA GAT GAC CAA GTG TTT CTT ATG AAG CTA CAG TCT CTT TTA GCA AAG CAA CCT CCA ACT
GCT GCT GG  < 800
  R   K   F   V   H   E   K   E   I   M   A   E   D   D   Q   V   F   L   M   K   L   Q   S   L   L   A   K   Q   P   P   T
A   A   G
  CT TTC AAA CAC GTA CTC TTC CTC TAA TAC CGT CTT CTA CTG GTT CAC AAA GAA TAC TTC GAT GTC AGA GAA AAT CGT TTC GTT GGA GGT TGA
CGA CGA CC
              710         720         730         740         750         760         770         780         790


  A AGG CCT GTG GAT GCA TCA CCA AGA GTC CCT GGA GGC GCC CCT CGA ACA CCA AAC AGA TCC GTG TCA TCC AAT GTT GCC AGC GTG TCC CCC
ATC CCT GCA  < 900
    R   P   V   D   A   S   P   R   V   P   G   G   A   P   R   T   P   N   R   S   V   S   S   N   V   A   S   V   S   P
I   P   A
  T TCC GGA CAC CTA CGT AGT GGT TCT CAG GGA CCT CCG CGG GGA GCT TGT GGT TTG TCT AGG CAC AGT AGG TTA CAA CGG TCG CAC AGG GGG
TAG GGA CGT
              810         820         830         840         850         860         870         880         890


  GGA TCC AAA AAA ATT GAT CCC AAC ATG AAA GCT GGA GCA ACC AGC GAA GGG GTC CTG GCA AAT TTC TTC AAC AGT CTG TTG AGT AAG AAG ACT
GGC TCT C  < 1000
   G   S   K   K   I   D   P   N   M   K   A   G   A   T   S   E   G   V   L   A   N   F   F   N   S   L   L   S   K   K   T
```

Figure 9: LIC1sequence mutated at 405 phosphorylation site

3) ESSA

Got DASPRVPGGSPRAP

The 408 site has been mutated and converted into alanine.

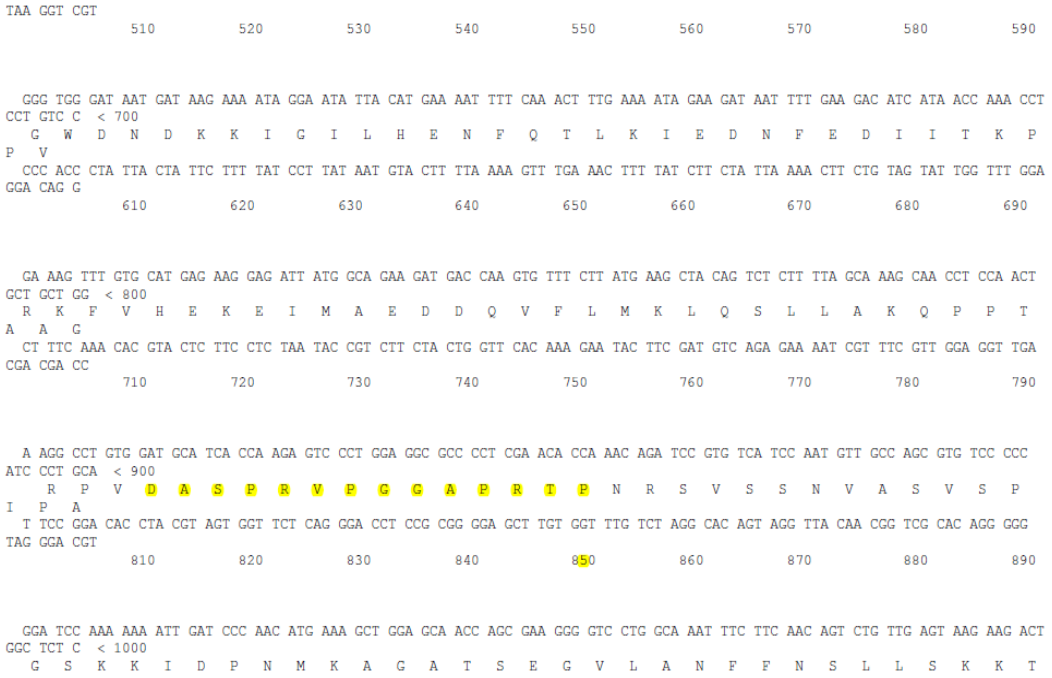


Figure 10: LIC1sequence mutated at 408 phosphorylation site

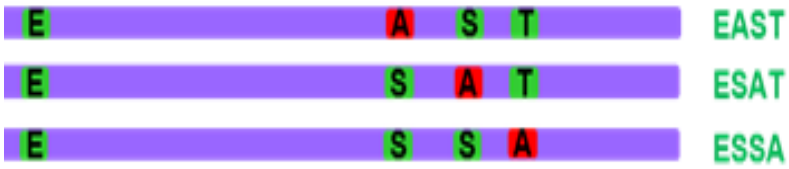
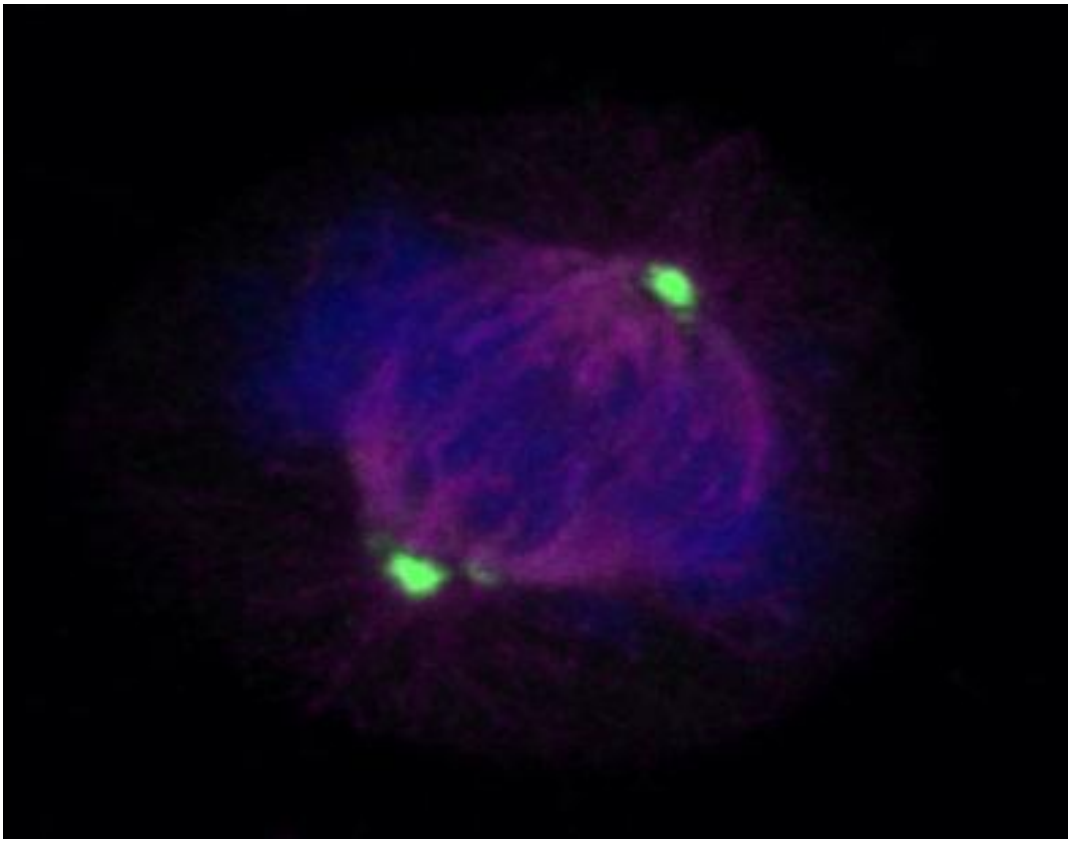


Figure 11: A series of phosphomutant constructs of rat LIC1(changing S207 to E207 and permutation at the other four sites with alanine (A) combinations. Green letters indicate mutants generated)

2) **Result of Confocal imaging**:

The images have been acquired by confocal microscopy after having performed the functional assays. The normal LIC1 phenotype shows spindle poles attached normally to the two equators. But LIC1 depleted cells will show multipolar spindles, which is being shown in the following images.

1) **GFP Control**



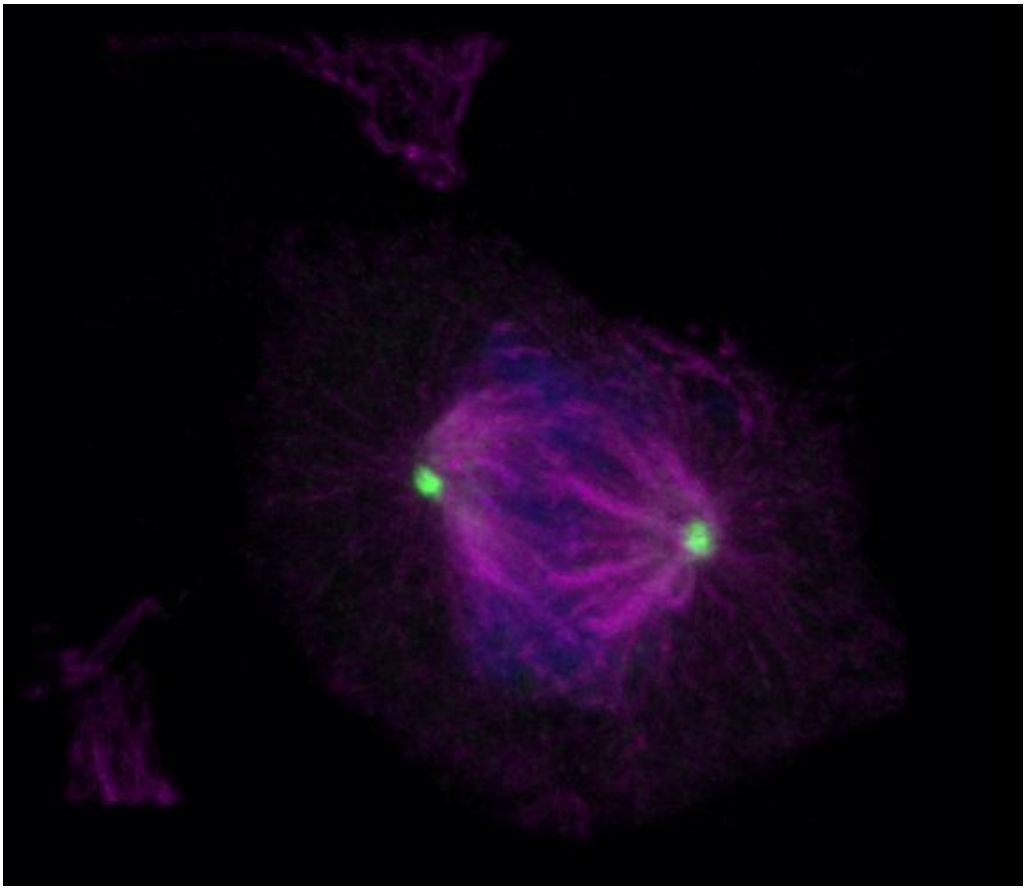


Figure 12: Confocal images of spindles in HeLa cells injected with (GFP control) or LIC 1 labeled with anti-α-tubulin and DAPI.

2) LIC1 KD (Knock Down):

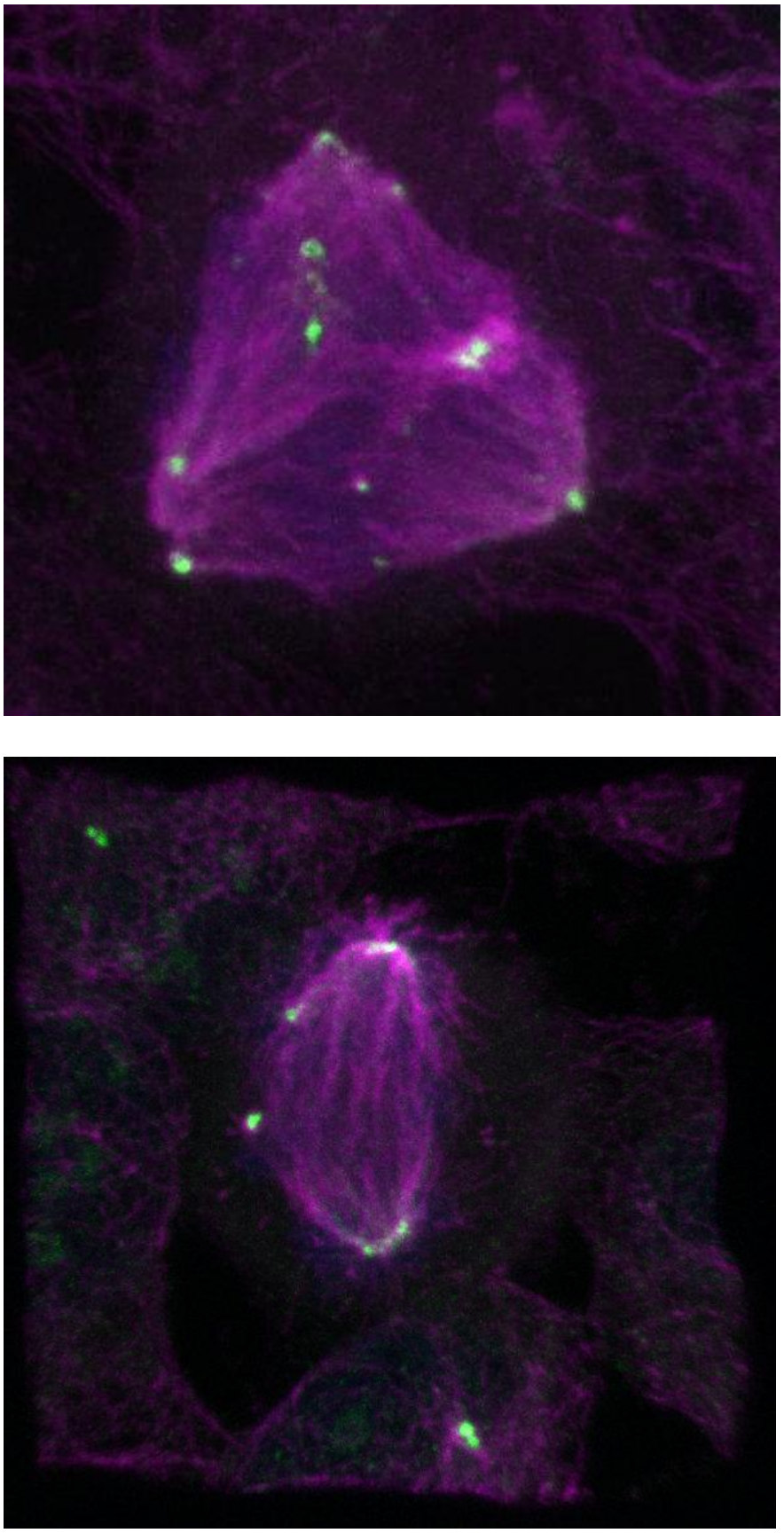


Figure 13: Confocal images of spindles in HeLa cells after depletion of LIC1.

When dynein activity is compromised, spindles become multipolar and disorganized, chromosomes fail to attach properly to the spindle, and cell cycle progression is slowed. (Robinson et al., 1999; Wojcik et al., 2001; Maiato et al., 2004; Mische et al., 2008; Tanenbaum et al., 2008; Firestone et al., 2012; Iwakiri et al., 2013; Raaijmakers et al., 2013)

3) LIC1 KD + rLIC1 (Rescue):

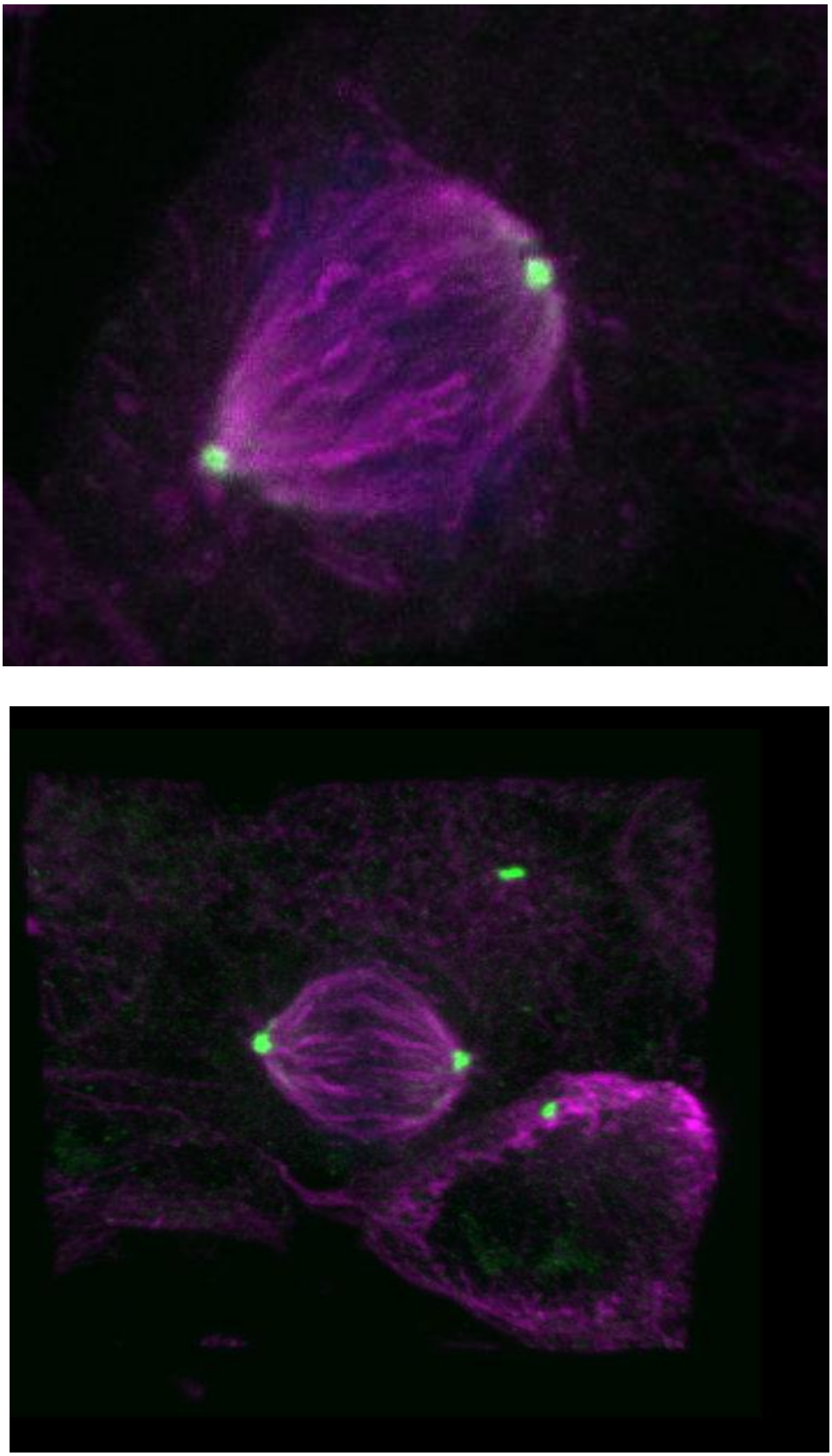


Figure 14: Confocal images of spindles in HeLa cells after depletion of LIC1 and rescued with rLIC1.

4) LIC1 + ESSA

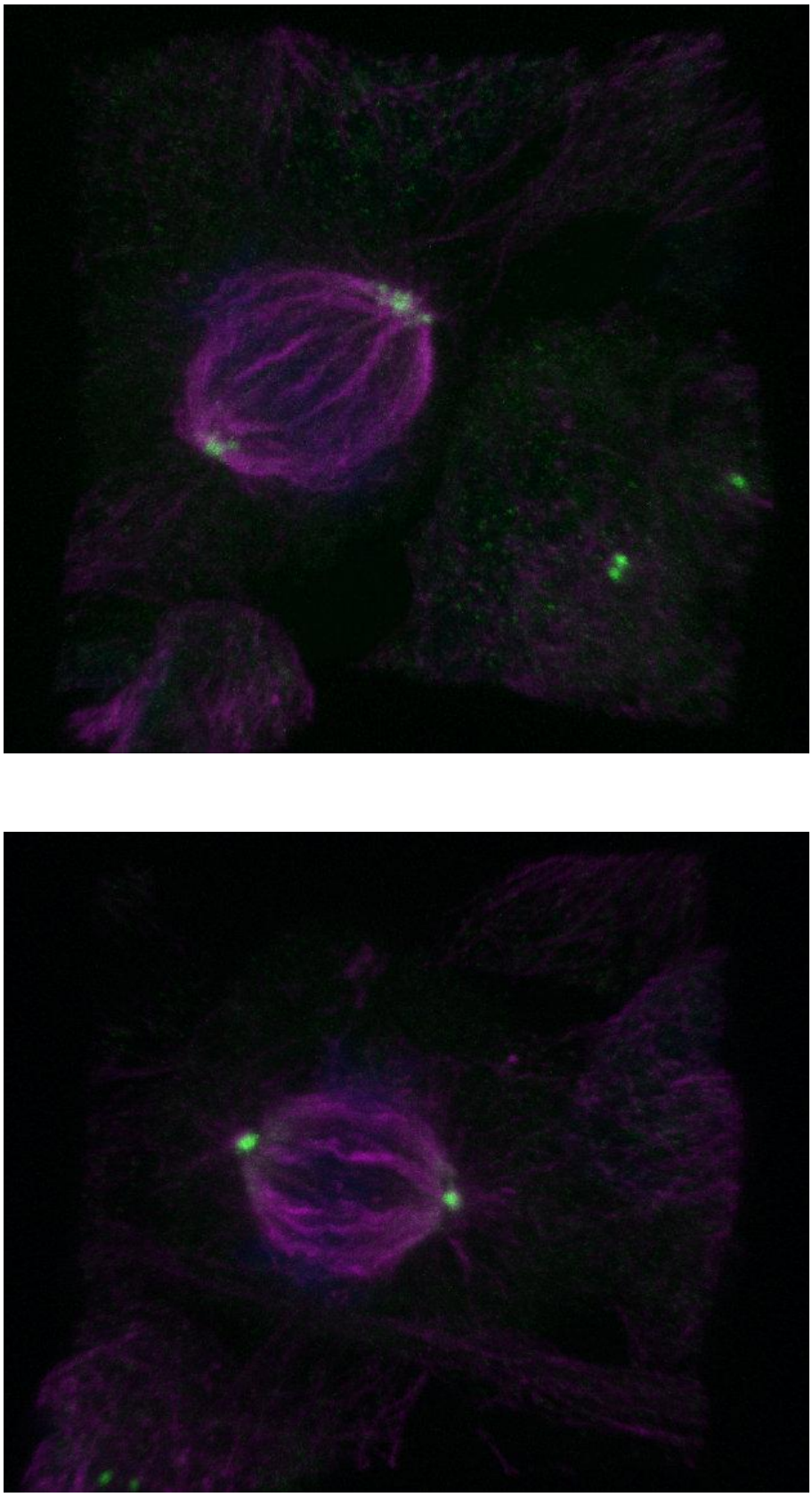


Figure 15: Confocal images of spindles in HeLa cells after depletion of LIC1 and rescued with mutant ESSA.

5) LIC1 + EAST

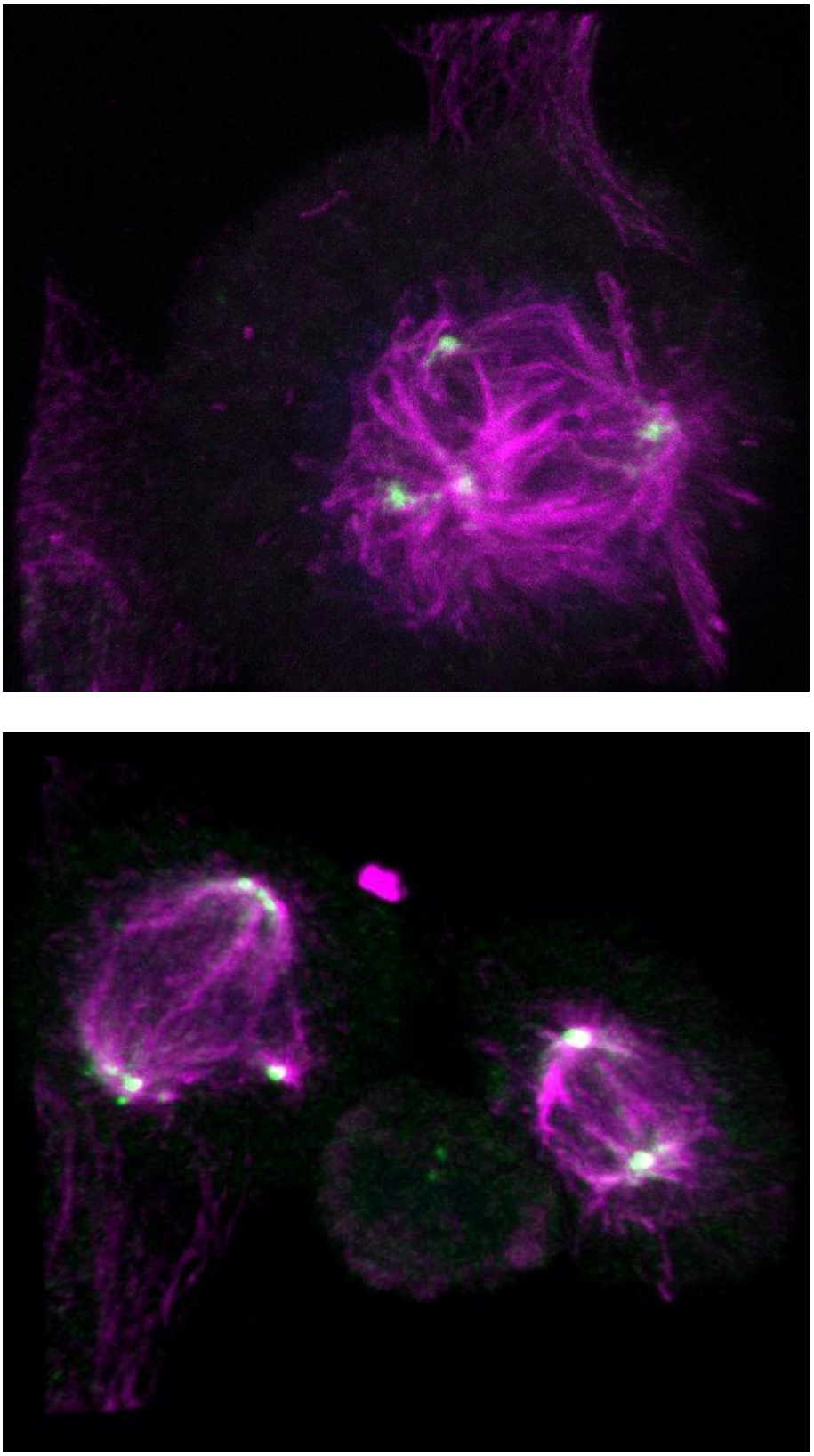


Figure 16: Confocal images of spindles in HeLa cells after depletion of LIC1 and rescued mutant EAST

6) LIC1 + ESAT:

We couldn't found any transfected cells in the plate, probably they got died and washed off in several washing steps.

No. of cells in mitotic phase and metaphase as visualized under phase contrast microscope:

1) GFP Control:

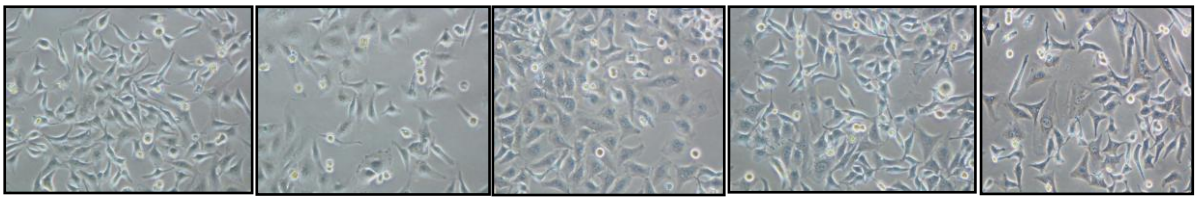


Figure 17: Hela cells (GFP Control) visualized under phase contrast microscope

GFP CNRTL					
field no	TOTAL CELLS	TOTAL MITOTIC	METAPHASE	Metaphase Index	Mitotic Index
1	70	7	2	2.85	10
2	47	3	1	2.12	6.382978723
3	57	2	2	3.5	3.50877193
4	51	9	3	5.88	17.64705882
5	49	3	1	2.04	6.12244898
				3.278	**8.732251691**

Table 5: Cells in GFP control

2) LIC1 KD (Knock Down)

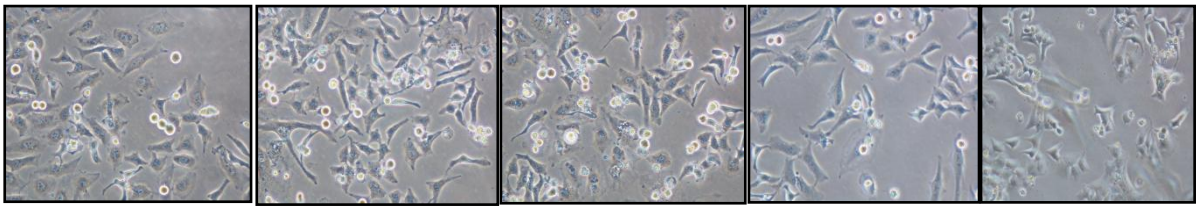


Figure 18: Hela cells (LIC1 Knock down) visualized under phase contrast microscope

LIC1 KD					
field no	TOTAL CELLS	TOTAL MITOTIC	METAPHASE	metaphase index	mitotic index
1	57	9	3	17.5	15.7
2	70	17	5	7.14	24.28
3	49	15	4	8.16	30.61
4	89	23	10	11.23	25.84
5	69	20	9	13.04	28.98
6	116	24	11	9.48	20.68
				11.0916666	**24.34833333**

Table 6: Cells in LIC1 KD

3) LIC1 KD + rLIC1

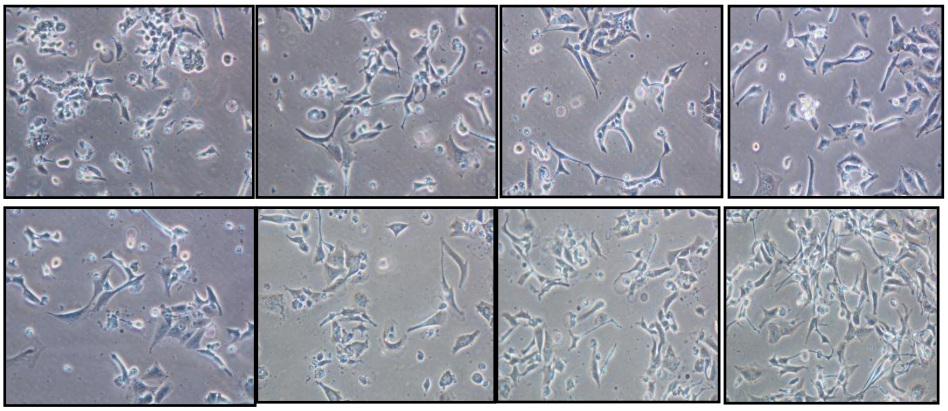


Figure 19: Hela cells (LIC 1 KD rescued with rLIC1) visualized under phase contrast microscope

LIC1KD+RLIC1 RESCUE					
field no	TOTAL CELLS	TOTAL MITOTIC	METAPHASE	metaphase index	mitotic index
1	50	5	1	2	10
2	25	5	1	4	20
3	25	2	0	0	8
4	20	4	2	10	20
5	24	2	1	4.16666	8.33333333
6	15	2	0	0	13.3333333
7	12	2	0	0	16.666666
8	16	2	1	6.25	12.5
				3.3020825	**13.604078**

Table 7: Cells in LIC1 KD + rLIC1

4) LIC1 KD + ESSA

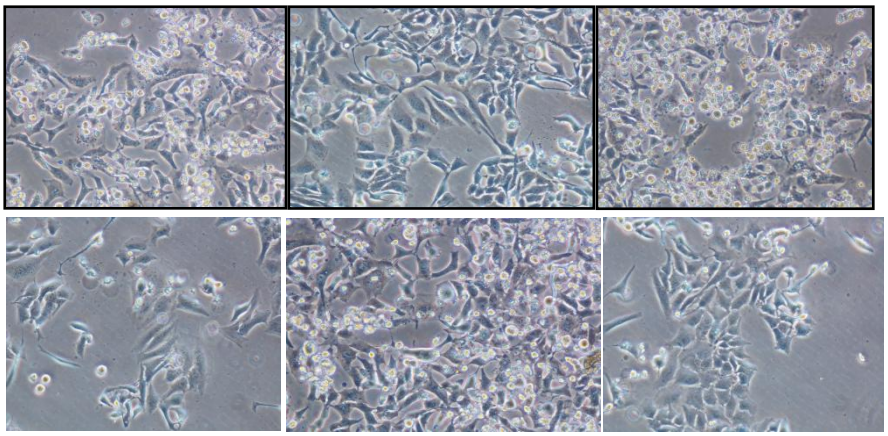


Figure 21: Hela cells (LIC1 KD rescued with mutant ESSA) visualized under phase contrast microscope

LIC1KD+ESSA					
field no	TOTAL CELLS	TOTAL MITOTIC	METAPHASE	metaphase index	mitotic index
1	86	10	4	4.65	11.6279
2	37	9	5	13.51	24.32432
3	65	26	12	18.46	40
4	48	15	7	14.58	31.25
5	40	8	5	12.5	20
6	49	9	4	8.16	18.3673
				11.97666667	**24.26158667**

Table 8: Cells in LIC1 KD + ESSA

5) LIC1 KD + ESAT

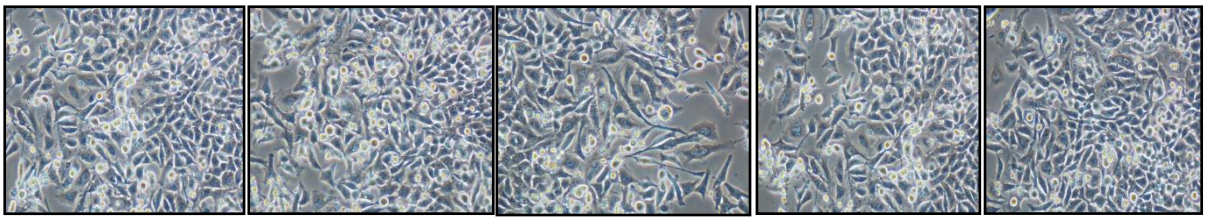


Figure 22: Hela cells (LIC1 KD rescued with mutant ESAT) visualized under phase contrast microscope

LIC1KD+ESAT					
field no	TOTAL CELLS	TOTAL MITOTIC	METAPHASE	metaphase index	mitotic index
1	140	36	19	13.5	25.7142
2	114	31	20	17.5	27.1929
3	102	40	19	18.6	39.2156
4	91	25	17	18.8	27.4725
5	111	32	27	24.3	28.8288
				18.54	**29.6848**

Table 9: Cells in LIC1 KD + ESAT

6) LIC1 KD + EAST

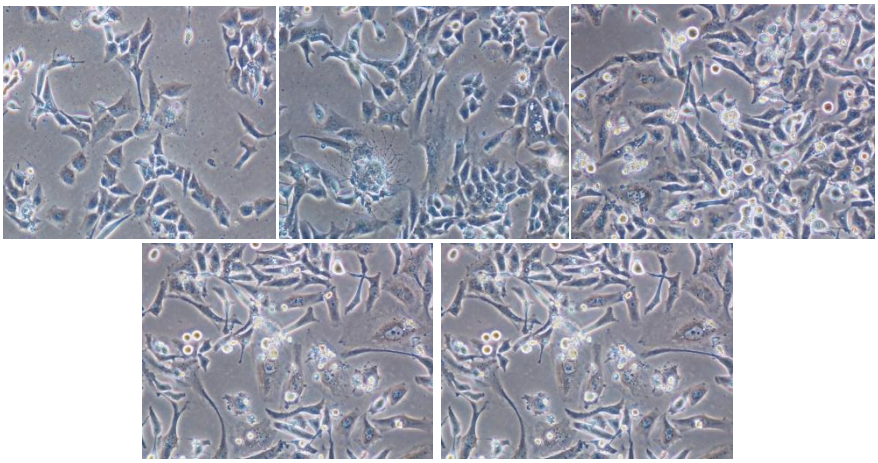


Figure 23: Hela cells (LIC1 KD rescued with mutant EAST) visualized under phase contrast microscope

LIC1KD+EAST					
field no	TOTAL CELLS	TOTAL MITOTIC	METAPHASE	metaphase index	mitotic index
1	68	5	2	2.94	7.3529
2	46	1	0	0	2.1739
3	44	6	4	9.09	13.6363
4	41	6	3	7.31	14.6341
5	62	13	6	9.67	20.9677
6	73	10	2	2.73	13.6986
				5.29	**12.07725**

Table 10: Cells in LIC1 KD + EAST

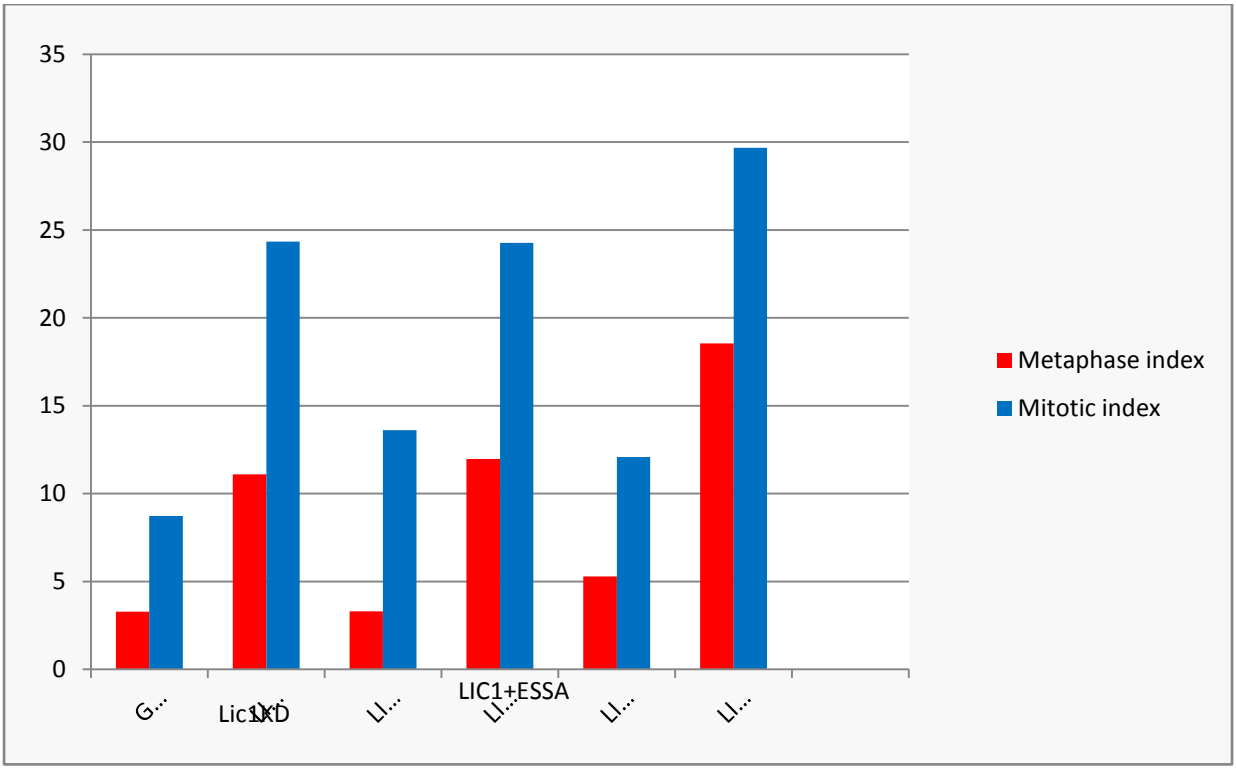


Fig 24: Cell count based on the state of cells (Mitotic or Metaphasic)

	GFP CNRTL	LIC1 KD	LIC1KD+Rlic1	LIC1KD+ESSA	LIC1KD+EAST	LIC1KD+ESAT
Metaphase index	3.278	11.0916666	3.3020825	11.97666667	5.29	18.54
Mitotic index	8.732251691	24.34833333	13.604078	24.26158667	12.07725	29.6848

Table 11: Average values of mitotic index and metaphase index calculated and plotted against graph

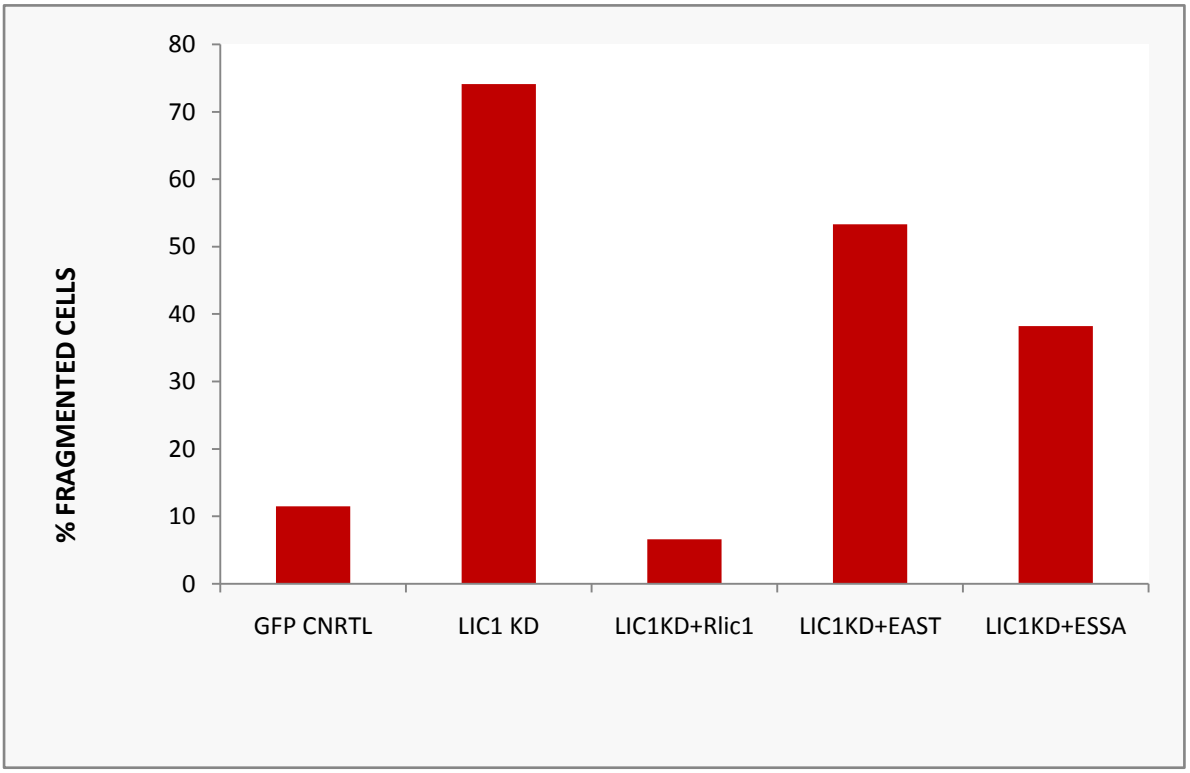


Fig 25: graphical representation of % fragmented cells (counted under phase contrast microscope)

GFP CNRTL	LIC1 KD	LIC1KD+Rlic1	LIC1KD+EAST	LIC1KD+ESSA
11.5	74.1	6.6	53.3	38.2

Table 12: fragmented cells (counted under phase contrast microscope)

Here the fragmented cells make reference to the cells which shows the multipolar spilndle formation activity which is directly related to the slowing of cell cycle progression.
1) The cells in GFP control are more normal type and have two distinct spindle poles at the two ends and thus have a normal rate of cell cycle progression.

2) The cells in which LIC1 is knocked down shows that it led to almost 75% of mitotic cells having multipolar spindles (Figure:23) and these fall into two categories: cells with three or more poles, and those with a primarily bipolar spindle that had auxiliary poles.

3) The cells in which knocked down LIC have been rescued by rat LIC1 shows the normal behavior of having two intact spindle poles, which means that rat LIC1 have rescued the function up to 96% .

4) The cells knocked down of LIC and rescued by mutant EAST shows that there is around 53% of cells which have the multipolar spindle formation which confers that mutant EAST (site S398 converted into A398) is only partially able to rescue the LIC function. Half of the cells shows the intact bipolar spindle nature whereas other half shows the multipolar spindle formation.

5) The cells knocked down of LIC and rescued by mutant ESSA shows that there is only 38% of cells which have been found to have mutipolar spindle formation and rest of the 62% have normal bipolar spindle formation which confers that mutant ESSA (site T408 converted into A408) is able to rescue the LIC function up to a prominent extent.

CHAPTER 7: CONCLUSION

From the results obtained it is clearly shown that one mutant which is ESSA (mutated at T408 site) is able to rescue the SAC inactivation defect and the second mutant which is EAST (mutated at S398 site) is only partially able to rescue the SAC inactivation defect. However nothing can be said about the mutant ESAT. However further experiments will uncover regulation, if any, of the critical S207 phosphorylation by the other three conserved phophorylation events and may also shed light on the temporal order of these phosphorylation events on LIC1. Overall, these results will illuminate the role of mitotic LIC1 hyper-phosphorylation in SAC inactivation.

CHAPTER 8:

DISCUSSION AND FUTURE PERSPECTIVE

Understanding of cell cycle and its control not only helps us in maintaining the correct cell structure and orientation but the information can be harbored for understanding how its failure can lead to cancer and various other diseases such as pituitary development disease, vaculoproliferative disease, alzheimer's disease, failure of signaling networks, genome instability and etc.

Cancer at the most basic level can be defined as a disease where the cellular proliferation has lost the control. This uncontrolled proliferative nature of cell can alter with the normal functioning of body. This uncontrolled growth is the heart of the disease, so in order to know more about this disease we need to understand what is the basic mechanism of cell division and in what various ways it is controlled.

The discoveries made so far in understanding of the cell cycle and its control have done through using various organisms by genetical and biochemical studies. But now we have human genome sequence available with us so its time for using it for making better understanding of the disease and its cure as well.

Pituitary gland is among one of the important organs of the body which controls metabolism, stress response, reproduction ageing and other physiological functions such as growth. Early genetic research and experiments explains us about us that the proper pituitary functioning is in lieu of genetic alteration of specific cell cycle regulators such as the retinoblastoma protein (pRB) or the cell cycle inhibitor $p27^{Kip1}$. The neoplasias of human pituitary which when molecularly analyzed has verified that cell cycle deregulation is significantly a cause in pituitary tumorigenesis. The proteins involved in cyclin-dependent kinase regulation are altered in nearly all human pituitary tumors. Recent experimental data suggest that these cell cycle regulators may have significant implications in the biology of putative progenitor cells and pituitary homeostasis. Insight and interpretation of how cell cycle regulation controls pituitary functioning & biology may bestow us with new therapeutic approaches against pituitary diseases.

The major drudgery work in front of clinical cardiologists is atherosclerosis and restenosis of epicardial vessels and the major components of the vasculoproliferative response are phenotypic modulation and proliferation of smooth muscle cells. The regulation of the proliferation is regulated by the network of regulatory proteins working at checkpoints in the cell cycle that alters the cellular growth. In this process the activation of the cell cycle and the

genetic control of its regulation and progression are final common pathways. In vasculoproliferative disorders the vascular injury is common and the responsive factors are such as mechanical interventions, hypertension, and hyperlipidemia. The vasculoproliferative response is mainly due to failure of antimitogenic events mediated by CDKIs. For proliferative responses the regulation of the cell-cycle machinery is a important pathway, so it is very evident that correlations exist between markers of cell-cycle progression and others factors such as cyclin dependent kinase and etc associated with the vasculoproliferative response. The first applications of antiproliferative therapies for vascular diseases were designed to inhibit specific signaling pathways.

Gene therapy can be another means to adjust the cell cycle in major vasculoproliferative diseases. The Gene therapy approach can involve two methods the first can be a method to modulate a gene's expression or activity and the second can be the gene to be modulated. Using antisense techniques the endogenous gene expression can be downregulated, which depends on the ability of antisense RNA and DNA molecules to bind to an mRNA molecule in a sequence-specific manner to increase degradation of the RNA. The negative regulation of gene activity can be done by using transcription factor limiting techniques, which utilize oligonucleotides bearing consensus binding sequences for transcription factors in order to competitively prevent their function.

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