FUNCTION AND RECRUITMENT OF CENTROMERIC HETEROCHROMATIN PROTEIN 1

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DEDICATION

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by

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FUNCTION AND RECRUITMENT OF CENTROMERIC HETEROCHROMATIN PROTEIN 1

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During early mitosis, the sister chromatids are held together by Cohesin, a protein complex composed of Smc3, Smc1, Scc1/Rad21 and Scc3. Cohesin is first released from the arms of chromosomes, leaving it intact at the centromere. At the metaphase – anaphase transition, centromeric cohesin is cleaved, allowing the chromatids to segregate to two daughter cells. Shugoshin (Sgo-1) is a known protector of cohesin at the centromere. It prevents phosphorylation of cohesin complex by Plk1 before the

metaphase – anaphase transition, which would otherwise lead to cohesin release, causing the two chromatids to separate untimely.

In this study we show that Sgo1 localizes on centromeres through HP1 during interphase in human cells. Also, Sgo1 binds all three forms of HP1 (i.e. alpha, beta, gamma) through its chromoshadow domain. We have determined the dissociation constant of this interaction to be in the sub-micromolar range. We have shown conclusively that Sgo1 binds to HP1 chromoshadow domain via one PxVxL motif.

We have further shown that, in mitosis, HP1 is recruited to centromeres by Incenp, a subunit of the chromosomal passenger complex via the chromoshadow domain of HP1. This interaction is most likely at the HP1 CSD dimer interface, where PxVxL motifs bind. Hence, it seems that Incenp may provide competition to Sgo1 for HP1 binding.

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LIST OF DEFINITIONS

^{0}C	Degree Celcius
μM	micromolar
Å	Angstrom
ATP	Adenosine triphosphate
bp	base pair
CDK	Cyclin dependent kinase
CENP-A	Centromeric protein A
CPC	Chromosome passenger complex
CSD	Chromoshadow domain
DTT	Dithiothreitol
E.coli	*Escheria coli*
G1 phase	Gap 1 phase
G2 phase	Gap 2 phase
G_0 phase	Gap 0 phase
GST	Glutathione S-transferase
Kd	Dissociation constant
H3	Histone 3
HP1	Heterochromatin protein 1
INCENP	Inner centromeric protein
ITC	Isothermal titration calorimetry
LB	Luria Bertani

M phase	Mitotic phase
min	minute
mL	milliliter
NaCl	Sodium Chloride
nm	nanometer
NMR	Nuclear Magnetic Resonance
PDB	Protein databank
PEG	Polyethylene glycol
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sgo1	Shugoshin 1
S phase	Synthetic phase
SCC1	Sister chromatid cohesion 1
SMC1	Structural maintenance of chromosome 1
SMC3	Structural maintenance of chromosome 3
TBS-T	Tris Buffered Saline-Tween20

CHAPTER ONE
Introduction

Division of a mother cell into two daughter cells is one of the hallmarks of life. This process is at the core of growth, development and reproduction of an organism and ultimately helps in the survival and propagation of a species. Cell division is an extremely well-coordinated and complex sequence of events that result in the handing down of genetic material from one generation to the next. Proper segregation of genetic material between two daughter cells is essential to maintain the genetic integrity of an organism. Therefore, the cell division cycle is a highly regulated event. Any defect in any of these regulatory mechanisms causes dire consequences for the cell and the organism such as the occurrence of several life threatening diseases including cancer.

The cell division cycle proceeds through several checkpoints which delay the cycle in the advent of unfavorable conditions. Several regulatory protein complexes at each checkpoint ensure that the conditions both inside and outside the cell are favorable for cell division to occur. Many of these regulatory mechanisms have been well studied and their mechanisms of action are known. However, there are several of these regulatory mechanisms whose functions are still largely unknown.

In this study, we focused on one of the regulatory mechanisms of cell cycle which protects the cohesion of sister chromatids at the centromere during interphase and early phases of mitosis until they are ready to be segregated to individual daughter cells. In this study, we characterize Heterochromatin protein 1 (HP1), which is the hallmark of hetrochromatin in eukaryotes and its interaction with Shugoshin1 (Sgo1), a genome protection protein and Incenp, a member of the chromosomal passenger complex (CPC). HP1 has been shown to interact with Shugoshin1 and recruit it to the kinetochores (Yamagishi et al., 2008). We have determined the roles of all potential binding motifs present on Sgo1 for HP1 and have also solved the structure of the complex which helps to further characterize the interaction. Also, we have characterized the interaction between a component of CPC and HP1 and postulated a possible role of this interaction in recruitment of HP1 at centromere during mitosis.

CHAPTER TWO
Review of the Literature

All cells divide to create new cells, which is essential for the growth, reproduction and ultimately survival of an organism and the species. Cell division is characterized by an orderly sequence of events that culminates in the duplication of the contents of the cell and passing them on to two daughter cells. This is an extremely precise event, controlled and monitored by several regulatory mechanisms. Failure at any of the regulatory steps can result in several life threatening diseases including cancer.

Cell Division cycle

The eukaryotic cell cycle is divided into four phases: the S phase, where the cells duplicate their chromosomes, the M phase which is characterized by equal separation of the genetic material and creation of two daughter cells and the gap phases (G1, between M phase and S phase and G2, between S phase and M phase), where cells grow. The period of time between two M phases is called interphase and is marked by cellular activities that help in preparing a cell for S phase and cell division. Only under favorable environmental conditions do cells enter M phase. If conditions are not favorable, the cells arrest in G1 or may enter

a resting state known as G_0. To commit to duplication and division, a cell must pass through a checkpoint known as the restriction point or start in mammalian cells and yeast respectively. Once a cell crosses the restriction point, it will divide even if the stimulus for cell division is removed. After the cell crosses S phase and enters G2 phase, it must cross a similar checkpoint known as the G2-M checkpoint. A third checkpoint is present in the M phase known as the metaphase-anaphase transition or spindle checkpoint. Together, these checkpoints are part of the regulatory mechanisms in place to ensure correct division of the genetic material. In the advent of any unfavorable conditions inside or outside the cells, the cell cycle is arrested at these checkpoints till favorable conditions return or the problems are fixed. The progress of the different stages of the cell cycle is controlled in part by the cyclin-dependent kinases (CDKs). The activity of these kinases cyclically rise and fall throughout the cell cycle and are controlled by several regulators. Most well known among these regulators are the cyclin proteins which undergo a cycle of synthesis and degradation during the cell cycle. There are different cyclins which form complexes with different CDKs. Assembly of these different cyclin-CDK complexes leads to progression through the cell cycle.

The M phase or mitotic phase is divided into six stages. Prophase is characterized by condensation of the replicated chromosomes which consist of closely associated sister chromatids held together at the kinetochore. The nuclear

envelope dissociates and marks the beginning of the prometaphase, when the chromosomes attach to opposite poles of the cells via their kinetochores and spindle microtubules. At metaphase, the chromosomes are arranged at the equatorial plane of the cell, halfway between the spindle poles. The spindle microtubules contract and pull each chromatid away from the equatorial plane toward a spindle pole to create segregation of the chromosomes at anaphase. Telophase and cytokinesis mark the last two stages of mitosis where the cell finally divides into two daughter cells, the nuclear envelope reforms and organelles are distributed evenly between the daughter cells.

Proper segregation of chromosomes is essential to maintain the genetic integrity of eukaryotic cells. Separation of sister chromatids, held together at the kinetochore by the cohesin complex of proteins, at anaphase is an important process that ensures that each daughter cell receives an equal complement of genetic material. Several proteins and protein complexes regulate this event. Some of the key players are described as follows.

Kinetochore

Kinetochores are distinct structures on chromosomes. They connect the microtubules to the chromosomes during mitosis so that they can be pulled

towards their individual poles (Gorbsky, 1995). The simplest of the kinetochores in yeast contain 7 protein complexes made up of around 40 proteins (McAinsh et al., 2003; Westermann et al., 2007). The loci of these structures on chromosomes are called centromeres. The simplest forms are called point centromeres consisting of about 125bp while in higher organisms they can be much larger stretches of DNA. Electron micrographs of mammalian kinetochores show an arrangement of 3 layers of different electron densities – inner and outer being dense with a lighter middle layer (McEwen et al., 1998). At the centromere – kinotochore interface, Cse4 (in budding yeast, CENP-A in higher organisms), a H3-histone variant recruits other protein complexes that make up its structure(Meluh et al., 1998).

The orientation of attachment of microtubules to the kinetochore is critical for cell division. The kinetochore has to be attached to microtubules emerging from both poles i.e. bi-orientation or amphitelic orientation, of the dividing cell such that it creates a tension on the kinetochore. This tension is essential to pass the spindle checkpoint at the metaphase-anaphase transition (Cheeseman et al., 2004; Pinsky and Biggins, 2005; Zhou et al., 2002). Failure of this tension to activate the checkpoint causes segregation of unequal number of chromosomes to both daughter cells i.e. aneploidy or polyploidy. Proper microtubule attachment to the kinetochore also ensures progression through the cell cycle by loss of cohesion between sister chromatids and degradation of cyclinB. Collectively,

these two feedback mechanisms are called spindle assembly checkpoint. Thus, the kinetochore plays an essential role in ensuring fidelity of separation of genetic material.

Cohesin

The cohesin protein complex binds the two sister chromatids together at the kinetochore. It is composed of 4 subunits – SMC1, SMC3, SCC1 (also known as Mcd1 and Rad21) and SCC3 (SA1 and SA2 in mammals). The SMC family of proteins is conserved in eukaryotes both sequentially and structurally. They are 1000-1300 amino acid proteins and have ATPase activity. The nucleotide binding walker A and walker B motifs are separated by a long 50nm long coiled coil region. SMC1 and SMC3 form a heterodimer and they collectively bind to SCC1 which together form a closed ring of about 50nm (Nasmyth and Haering, 2009; Peters et al., 2008). This ring is believed to embrace two chromatids keeping them together (Feeney et al., 2010; Gruber et al., 2003; Guacci, 2007; Nasmyth, 2005; Nasmyth and Haering, 2009). The fourth subunit of cohesin, SCC3, binds to SCC1 to complete the complex. (Fig 2-1)

The cohesin complex binds to sister chromatids at prophase in an inactive form and is removed during the metaphase-anaphase transition. However, a small pool of cohesin still remains on the chromatids at the kinetochore and helps to keep sister chromatids together. The cleavage of cohesin on kinetochores marks the point in anaphase where the sister chromatids are pulled towards their respective poles by microtubules originating from the two spindles(Diaz-Martinez and Yu, 2009; Feeney et al., 2010).

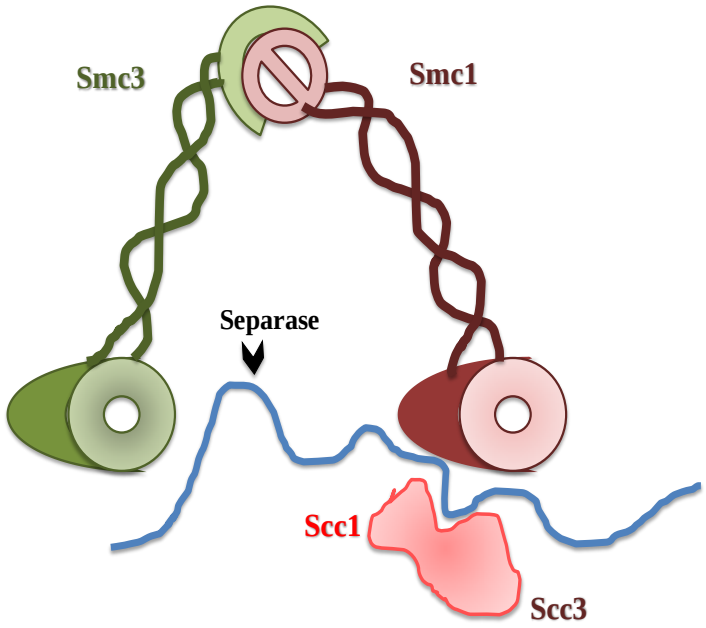

Figure 2- 1 Structure of Cohesin

Cohesin molecule made up of Smc1, Smc3, Scc1 and Scc3. They together form a 50nm circle that hold the two sister chromatids together. Adapted from Nasmyth et. al.

Centromeric region on the chromosomes is made of heterochromatin where H3 histone is tri-methylated at lysine-9 which binds to the N-terminal chromo domain of HP1 (Bannister, 2001; Fischle, 2005; Jacobs and Khorasanizadeh, 2002; Lachner et al., 2001a). The C-terminal chromoshadow domain of HP1 is responsible for recruitment of Sgo1 which protects cohesin from cleavage by separase (a protease) at Scc3 by a mechanism not well understood.

Heterochromatin Protein -1

The eukaryotic chromosome is divided into areas of active trasncription known euchromatin and transcriptionally silent areas known as heterochromatin. Methylation of histone H3 at the ninth lysine residue is an indicator of heterochromatin areas(Lomberk et al., 2006). Heterochromatin Protein-1 is a heterochromatin associated protein that binds to H3 histone via methylated-Lys-9(Jacobs and Khorasanizadeh, 2002; Lachner et al., 2001b). It controls expression of several hundred genes at both heterochromatin and euchromatin

Figure 2- 2 Structure of Heterochromatin Protein 1

regions(Lomberk et al., 2006). HP1 has 3 isoforms in mammals – HP1α, HP1β and HP1γ. HP1α is mainly localized on centromeres, HP1β is spread out all over the chromosomes and HP1γ is found mostly on the arms at euchromatin areas(Lomberk et al., 2006). They are highly similar in terms of sequence though each has slightly different functions. Their sequence comparison shows a N-terminal domain known as chromodomain and a C-terminal chromo-shadow domain (CSD) connected by a variable length and unstructured hinge (Fig 2-2) (Aasland and Stewart, 1995; Lomberk et al., 2006; Platero et al., 1995). Binding of HP1 to chromosomes has been shown to be dependent on both these domains. A number of binding partners of HP1 have been characterized and implicated in transcription regulation (TIF1α, TIF1β, TAFII130)(Cammas et al., 2002; Vassallo and Tanese, 2002), chromosome segregation (IDN3) and nucleosome assembly during DNA replication and repair(CAF1)(Thiru et al., 2004; Vassallo and Tanese, 2002). Sequence alignment of various binding partners show presence of a common motif - PxVxL/I involved in binding to HP1. The importance of this motif has been established by various mutation studies. Structures of the complex of HP1 chromoshadow domain and peptides with PxVxL/I motif have been solved (Huang et al., 2006; Thiru et al., 2004) and have revealed two modes of binding. The canonical and most prominent mode of binding is shown by a NMR structure of HP1βCSD – CAF1 complex (PDB ID:1S4Z). The structure shows two symmetrical monomers of HP1βCSD forming a dimer where each monomer

contributes a surface formed by its beta sheets forming a groove in which PxVxL/I motif bind (Brasher et al., 2000; Thiru et al., 2004). The non canonical mode of interaction seen in the structure of HP1βCSD – EMSY (PDB ID:2FMM) protein complex is similar except that instead of a PxVxL/I motif, LVPLM and FTVTA are the binding motifs in the β-sheet groove(Huang et al., 2006). This mode has been observed just in case of EMSY.

By virtue of this PxVxL/I motif, Sgo1 as well as Incenp have the potential to bind to HP1.

Shugoshin

Shugoshin (Japanese for "Guardian Angel") is a protein that has been described to protect cohesin at centromere from cleavage by separase in meiosis I (Kitajima et al., 2006; Macy et al., 2009; Watanabe, 2005). Sgo1 prevents separase from accessing cohesin by recruiting the phosphatase PP2A (Brar et al., 2006; Riedel et al., 2006). However, there is evidence that Sgo1 prevents cohesin cleavage by a mechanism other than by preventing cohesin phosphorylation(Brar et al., 2006). Sgo1 has been observed to be expressed both in meiosis II and during mitosis. During mitosis, it has been shown to promote accurate chromosome segregation by allowing sister chromatid biorientation(Indjeian et al., 2005; Kawashima et al., 2007; Vanoosthuyse et al., 2007). Its function of

centromeric cohesin protection is conserved from yeast to mammals(Lee et al., 2008).

Chromosome Passenger Complex

CPC is composed of four proteins – Aurora B Kinase, Inner Centromere Protein (INCENP), Survivin and Borealin. The complex localizes on the chromosome arms and then to centromere during early mitosis and finally to the midzone of cytokinesis telophase. It functions in chromosome condensation, chromosome alignment, microtubule – kinetochore interaction, spindle checkpoint function and in cytokinesis according to its localization (Ruchaud et al., 2007) which in turn depends on stage of mitosis the cell is in.

Aurora B Kinase is a Ser/Thr kinase and phosphorylates several proteins during mitosis including H3 histone at Serine-10. Phosphorylation of H3 S-10 will abolish the interaction of HP1 to Lysine9 of the histone protein (Hirota et al., 2005). It is therefore believed that HP1 should not be able to localize to the centromere when the CPC is localized during mitosis. Paradoxically, it is known that HP1 does localize to the centromere during mitosis. A previous report states that Incenp, a scaffolding component of CPC interacts with HP1 (Ainsztein et al., 1998). This interaction may be responsible for localizing HP1 to centromere during mitosis but this hypothesis has never been tested.

A key study in the field has been conducted by Yamagishi et al. where they showed that Shugoshin is recruited to the kinetochore via HP1 and that this interaction ensures proper chromosome segregation and hence cohesin protection. They studied this interaction further and have mapped the binding site of HP1 on Sgo1to a 5 residue conserved region PxVxL/I which is the known HP1 binding motif.

Studies in our laboratory have been focused to biophysically and biochemically study the role of HP1 in cohesin protection. In an unpublished study, along with this study in our laboratory, we have shown that HP1 – Incenp interaction is important for localizing Sgo1 at interphase. This interaction might have an effect of inhibiting Sgo1 during mitosis.

CHAPTER THREE
Materials and Methods

Heterochromatin Protein 1 cloning, expression and purification.

HP1α, HP1β, HP1γ constructs in pGEX6p-FA plasmid were obtained from the laboratory stocks. The chromoshadow domains (CSD) of HP1 were amplified using PCR and cloned into pGEX6p-FA. The plasmid pGEX6p adds Glutathione-S-Transferase protein tag upon expression of the cloned fragment.

The HP1α, HP1β, HP1γ, HP1αCSD, HP1βCSD, HP1γCSD plasmids were transformed into BL21DE3 E.coli competent cells, plated on LB Amp plates and incubated overnight. The following day colonies were picked from each of the plates and inoculated in 10 mL of LB with 100µg/ml Ampicillin as primary culture and grown overnight at 37°C on shaker. The primary culture was then inoculated into wide bottomed flasks with 1L LB and 100µg/ml Ampicillin and grown at 37°C on shaker until the OD reached 0.6. The culture was then cooled down to 16°C. IPTG was added to a concentration of 400µMolar to induce expression of proteins and the culture was maintained at 16°C for overnight. The following morning, the cells were pelleted at 4000g for 15 min.

The pellet was then resuspended in 25mM Tris-Cl pH 7.6, 200mM NaCl, 1mM DTT and 2.5%Glycerol buffer with protease inhibitors. Lysozyme was

added to these resuspended cells at 1mg/ml concentration. Each soup was then snap frozen in liquid nitrogen and then thawed to room temperature. The cells in each sample were lyzed in a pressurized cell-disrupter and then ultracentrifuge at 96000g at 4°C for 1hr 30min. The clear supernatant were then decanted and added to 50mL tubes with GST-sepharose beads and incubated for 1 hr with gentle shaking at 4°C. After 1 hour, the lysate with beads was added to columns and then flowthrough was collected. The column was then washed with 10 column volumes of 25mM Tris-Cl pH 7.6, 200mM NaCl, 1mM DTT and 2.5%Glycerol buffer. The protein was then eluted with 50mM Tris-Cl pH 8, 200mM NaCl, 1mM DTT and 2.5% Glycerol buffer with 10mM glutathione. 5 fractions of 2 column volumes were collected. The flow through, wash and elution fractions were then analyzed on SDS-PAGE Gel.

***In vitro* binding assay**

Fragments of Shugoshin protein were cloned into pCS2+-FA vector with a SP6 promoter site. The fragments were translated *in vitro* with ^{35}S labeled Methionine in Rabbit Reticulocyte Lysate System(Promega).

5 µg of GST and 5 µg of GST labeled HP1α, HP1β, HP1γ, HP1αCSD, HP1βCSD were immobilized on 10µL GST-sepharose beads(GE Lifesciences). The *in vitro* translated Sgo1products were incubated with the immobilized proteins for 1hour with vigourous shaking at room temperature. Unbound proteins were removed by washing with TBST+5% dry milk. The beads were then run on a tricine-SDS-PAGE gel and stained using commasie blue. The gels were dried on whatman filter paper and exposed to phosphorimager. The SDS-PAGE profile and the phosphorimage profile were compared to determine presence of binding between GST tagged protein and *in vitro* translated protein.

Similarly, *in vitro* binding assays for *in vitro* translated INCENP, INCENP (141-282) and INCENP (Δ141-282) with GST tagged HP1β were done.

Purification of HP1βCSD.

HP1βCSD expressed overnight at 16°C in 12 L of LB media. HP1βCSD was then purified in large quantities as described in the GST purification above. Before eluting the protein with 10mM Glutathione, the GST tag was removed by treating the beads with Precission Protease. The wash flow through was collected and run on SDS PAGE gel to determine purity. This sample was further run

through a ResourceQ column (GE) for anion exchange purification eluting with increasing NaCl concentration from 10mM to 500mM. The collected fractions were run on tricine-SDS-PAGE to identify fractions with pure HP1βCSD. The fractions with pure HP1βCSD were pooled and concentrated.

Isothermal Titration Calorimetry

The binding affinities of various fragment peptides of sgo-1 with the first and second PxVxL/I motif to HP1 was measured using MicroCal Omega VP-ITC calorimeter(GE Lifesciences). Three peptides of Sgo1 were synthesized at the Protein Chemistry Technology Center, UTSW. These peptides were SgP12 (Sgo1 423-457), SgP1 (Sgo1 425-443) and SgoP2 (Sgo1 443-458). Each of these peptides was solubilized in Phosphate buffer. The HP1βCSD protein was also

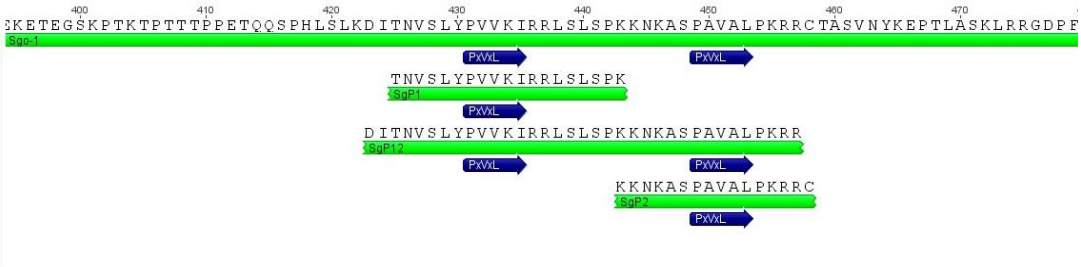

Figure 4- 1 Sequences of synthesized peptides for Isothermal Titration calorimetry

SgP1 contains proximal PxVxL/I motif. SgP12 contains both proximal and distal motifs. SgP2 contains the distal motif.

dialysed in phosphate buffer from the same stock. ITC measurements were done at 20°C. 400μM peptide was titrated into 50μM HP1βCSD in 28 steps of 10μL each. Resulting data ware analyzed as a single binding site model on MicroCal Origin 7.0 software.

Crystallization, data collection and structure determination of HP1βCSD-SgP1 complex

HP1βCSD was concentrated to 14mg/mL and then SgP1 was added in the ratio 1:1.2 with SgP1 being in excess. Using this sample, trial conditions for crystallization were examined using Phoenix RE protein dispensing robot (Art Robbins Instruments) and intelli-plate sitting drop system. Approximately 1200 conditions were screened after which promising condition candidates were setup on larger scale using 48 well hanging drop plates with different protein and mother liquor ratios. Best crystals were screened in an x-ray beam for diffraction at the Structural Biology Core of UTSW and then final data collected at Argonne National Laboratory's Advanced Photon Source in 19BM beam. The data were processed using HKL2000(Otwinowski and Minor, 1997) and CCP4 suite using the method of molecular replacement (Rossmann, 1990) for calculating phases.

Phenix(Adams et al., 2010) and Coot (Emsley and Cowtan, 2004; Emsley et al., 2010) were used for refinement. The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC was used to generate figures and graphics for manuscripts.

CHAPTER FOUR
Results and Discussion

The role of the two PxVxL/I binding sites for HP1 on Sgo1 was determined by *in vitro* binding assay. In this experiment, two fragments of Sgo1 gene corresponding to residues 443-477 and 451-527 were cloned into a mammalian *in vitro* translation vector. These fragments were then *in vitro* translated with ^{35}S methionine incorporated. GST tagged HP1α, HP1β, HP1γ, HP1αCSD and HP1βCSD were immobilized on glutathione sepharose beads and used as baits. GST was used as a negative control to rule out the effect of GST tag on the target fragment. These immobilized proteins were then used to pull down the *in vitro* translated Sgo1fragments. It was observed that disruption of the proximal binding site abolished the interaction of HP1 with Sgo1, while disruption of distal binding site did not (Fig 4-1). This in part confirms the observations made by Yamagishi et al where they show that mutation of the first PxVxL/I site in Sgo1abolishes its interaction with HP1.

We also synthesized three peptides from Sgo1 sequence (Fig 4-2). SgP1 has the first PxVxL/I motif, SgP2 has the second while SgP12 has both. Isothermal titration calorimetry (ITC) was used to determine the dissociation constants of SgP1, SgP2 and SgP12 against HP1βCSD. The K_d values observed for SgP1 was 0.18 µMolar and for SgP12 was 0.56 µMolar (Fig 4-3). For SgP2 the dissociation constant was not obtained because of small amount of heat

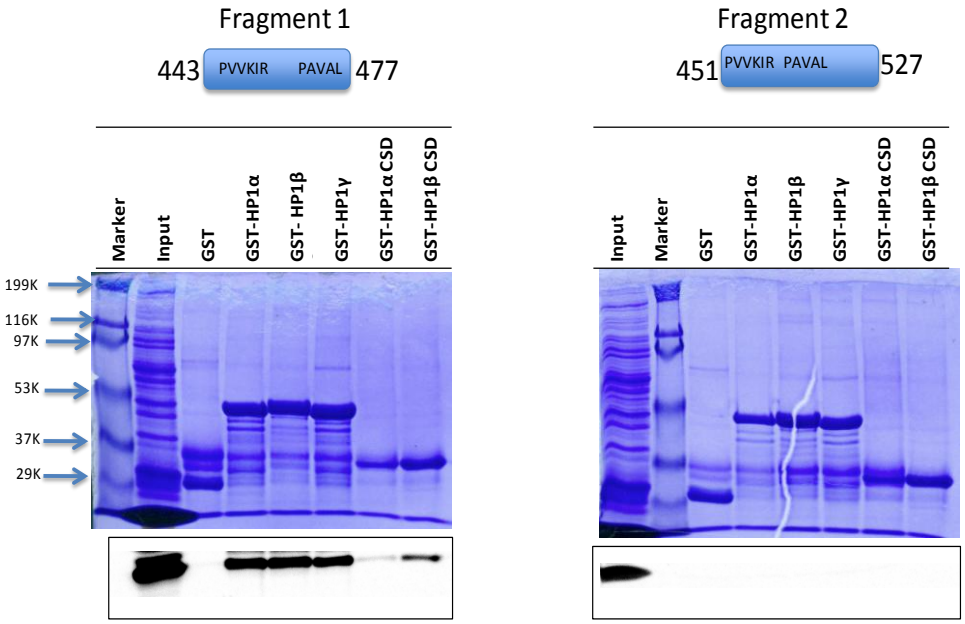

Figure 4- 2 HP1 binds to the proximal PxVxL/I motif on Sgo1

Constructs containing either the proximal PxVxL/I motif (Fragment 1) or the distal motif (Fragment 2) of Sgo1 were synthesized using in vitro translation in ^{35}S labeled rabbit reticulocyte lysate and their binding to HP1 was monitored using in vitro binding assay. 5µg Full length GST-tagged HP1 isoforms or chromo-shadow domains of HP1α or HP1β were immobilized on glutathione beads and 5µl of rabbit reticulocyte lysate containing either ^{35}S labeled fragment 1 in **A** or fragment 2 in **B** was incubated with them for one hour at room temperature. The beads were then washed and proteins eluted by boiling the beads in 5X Laemmli buffer. The proteins were resolved on 15% SDS-PAGE gel, stained with Commassie blue (upper panels) and binding visualized using autoradiography (lower panels). GST was used as a negative control.

observed indicative of weak binding. The dissociation constants suggest that the proximal PxVxL/I binding motif is the strongest while the distal PxVxL/I binding site has much lower affinity. Hence, these data suggest that only the proximal PxVxL/I site is the active binding site for HP1.

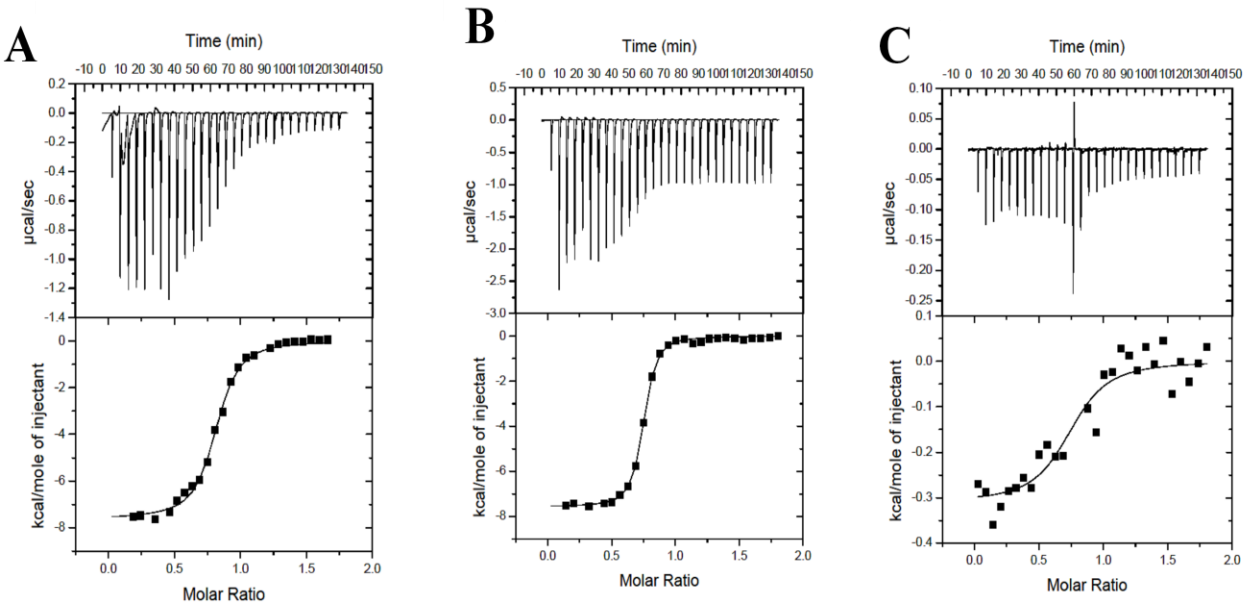

Figure 4- 3 HP1 binds to the proximal PxVxL/I motif on Sgo1.

Binding of peptides of Sgo1 containing both the proximal and distal motifs (**A**), the proximal motif only (**B**) or the distal motif only (**C**) to chromo-shadow domain of HP1β was monitored using isothermal titration calorimetry (ITC). ITC measurements were done at 20°C where 400µM peptide was titrated into 50µM HP1βCSD in 28 steps of 10µL each. Resulting data was analyzed as single binding site model on MicroCal Origin 7.0 software.

To confirm the interaction site of HP1βCSD and Sgo1, we crystallized and determined the structure of HP1βCSD and SgP1 complex. HP1βCSD was cloned and expressed in *E.coli* cells and purified using affinity column and size exclusion column (Fig 4-4). After primary screening of crystallization conditions, crystals (Fig 4-5) were found in two conditions after about 3 weeks - 0.1 M BisTris pH 6.6, 0.2M NaCl, PEG 3350 25% w/v and 0.1 M Tris pH 8.6, PEG 3350 25% w/v. The conditions were further optimized and crystals were found to be growing best in 0.1 M BisTris pH 6.6, 0.2M NaCl, PEG 3350 21% w/v. No single crystals were obtained hence a small piece was broken off from a cluster which could be put in the X-ray beam. This small crystal diffracted to about 3.5Å in the in-house diffraction collector and to about 2.0 Å in the synchrotron. The phase was calculated using molecular replacement method utilizing a HP1βCSD subunit from the crystal structure of HP1βCSD – EMSY complex(PDB ID:2FMM)(Huang et al., 2006). The refinement was done using Phenix and Coot and final R-factor was 21.8%. Other refinement statistics are listed in Table 4-1.

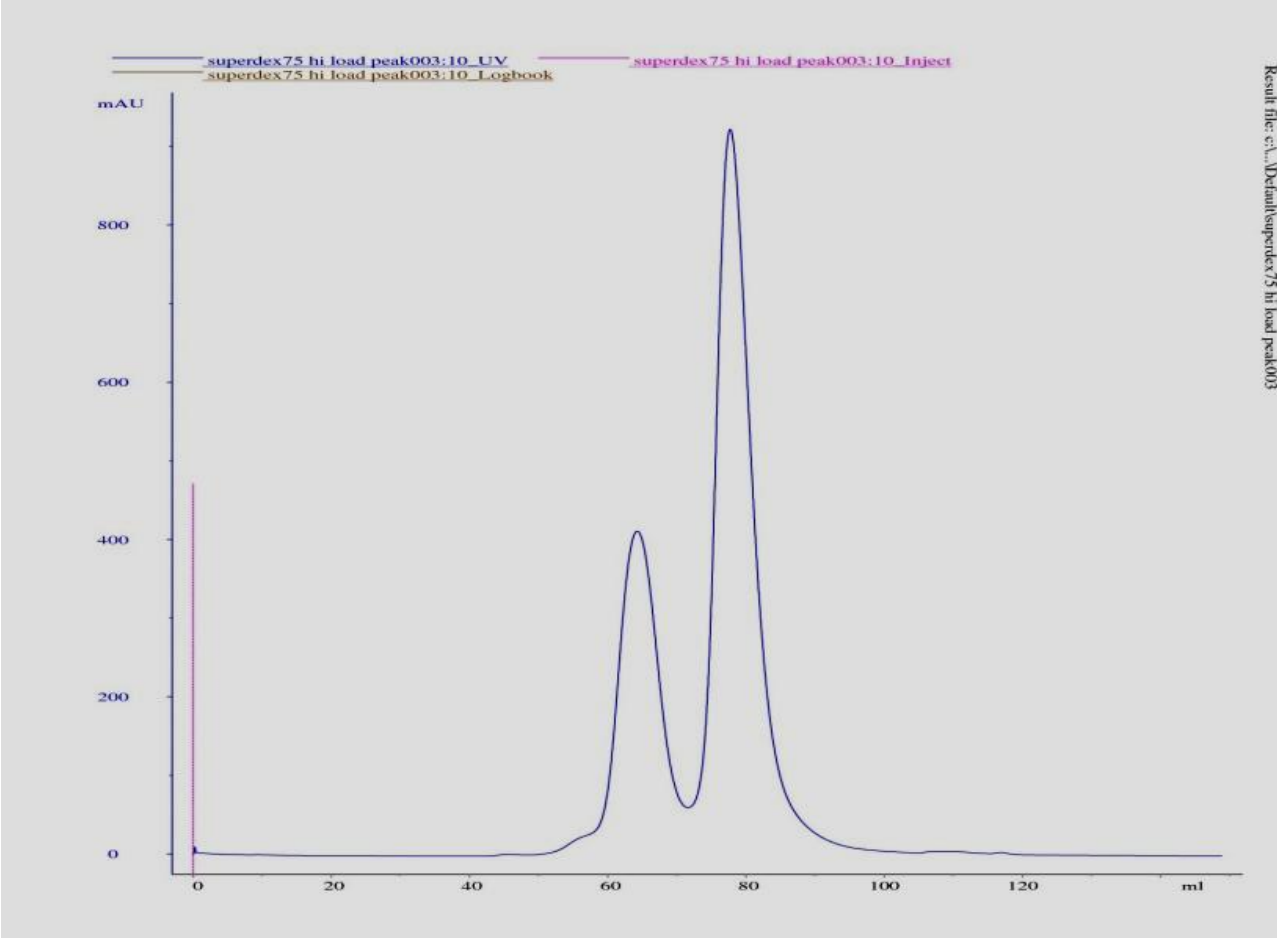

Figure 4- 4 Gel Filteration profile of HP1βCSD.

. The HP1βCSD elutes as the second peak in the profile and corresponds to approximately 14KDa molecular weight which is the size of a dimer.

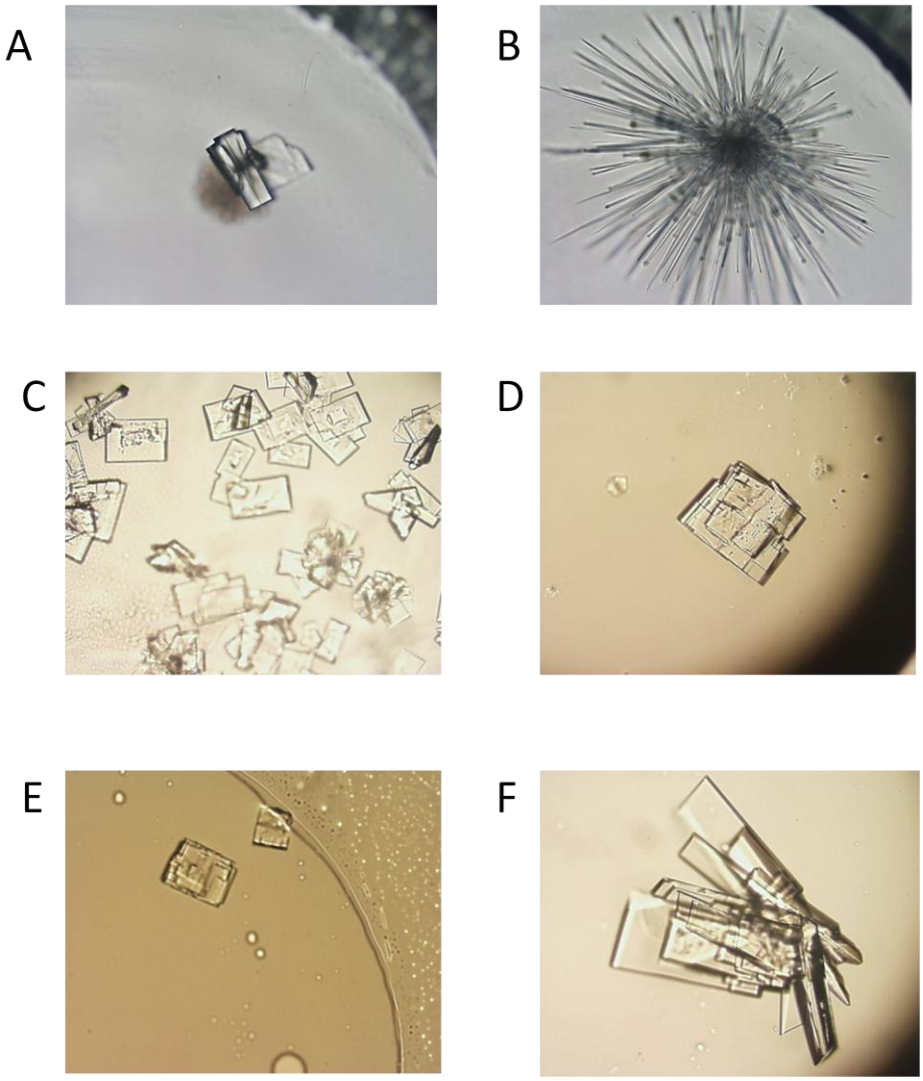

Figure 4- 5 Crystals of HP1βCSD – Shugoshin complex

A and B show crystals obtained in trials in the following conditions:

A) 0.1 M BisTris pH 6.6, 0.2M NaCl, PEG 3350 25% w/v

B) 0.1 M Tris pH 8.6, PEG 3350 25% w/v

C-F) 0.1 M BisTris pH 6.6, 0.3M NaCl, PEG 3350 24-32%

Table 4- 1 Data collection and refinement statistics for molecular replacement

Data collection		
Space group	P21 21 2	
Cell dimensions		
a, *b*, *c* (Å)	76.81, 108.89, 41.93	
a, b, g (°)	90, 90, 90	
Resolution (Å)	(2.12-2.05)	
*R*sym or *R*merge	51%	
$I / \sigma I$	5.1	
Completeness (%)	100%	
Redundancy	8.6	
Refinement		
Resolution (Å)		(50-2.05)
No. reflections		21603
*R*work / *R*free		21.81%/24.70%
No. atoms		
Protein		2288
Ligand/ion	0	
Water	186	
B-factors		
Protein		34.11
Ligand/ion		
Water	39.34	
R.m.s. deviations		
Bond lengths (Å)	0.007	
Bond angles (°)	0.956	

One crystal was used to collect the data.

The final structure revealed that the proximal PxVxL/I motif of Sgo1 binds to HP1 in the canonical HP1-PxVxL/I binding mode (Fig 4-6). It confirms that the dimer of HP1 CSD was required for PxVxL/I interaction. Also, the hydrophobic surface formed at the dimer interface where proline, valine and leucine insert into the hydrophobic cavity formed by the two hydrophobic surfaces between the dimer provided the most essential mode of interaction between HP1 and PxVxL/I peptide.

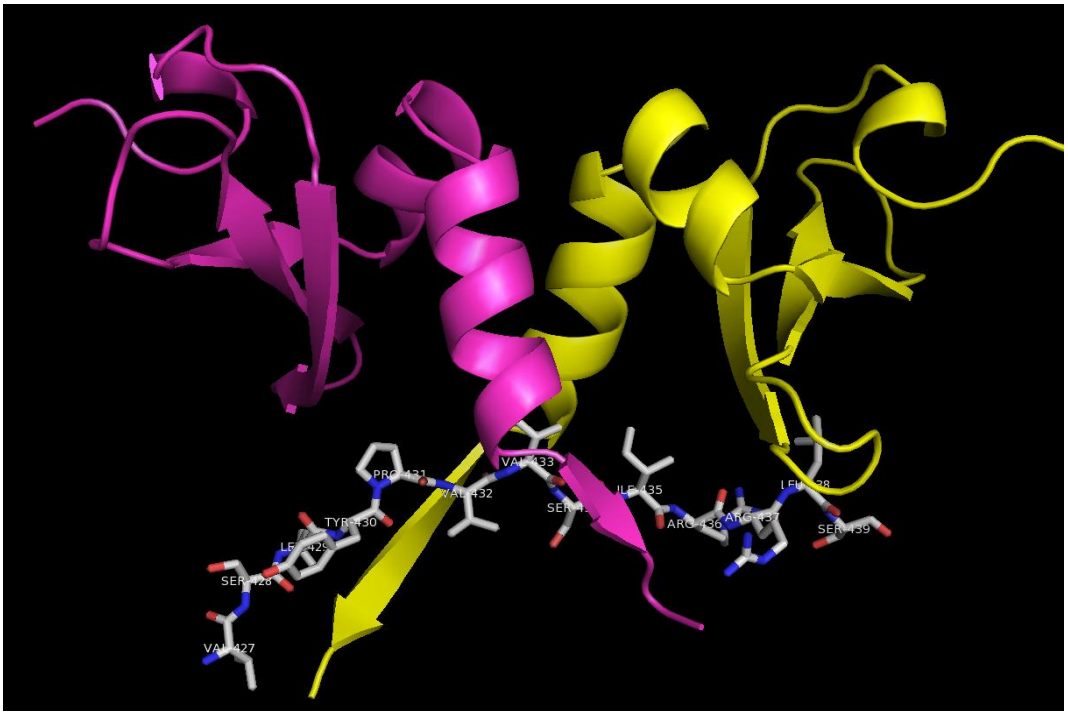

Figure 4- 6 Crystal structure of HP1βCSD – Sgo1 peptide complex

HP1βCSD and Sgo1 peptide complex was crystallized using the hanging drop method. Best crystals were obtained with 0.1 M BisTris pH 6.6, 0.2M NaCl, PEG 3350 21% w/v. Diffraction data was collected from the crystal at 2.0 Å at the synchrotron and the structure was solved using molecular replacement using a HP1βCSD subunit from the crystal structure of HP1βCSD – EMSY complex(PDB ID:2FMM)

The functional role of the HP1-Sgo1 interaction is not yet clear. It has been shown that both the wild type Sgo1and mutant Sgo1 lacking HP1 binding ability, rescue mitotic arrest caused by depletion of endogenous Sgo1(personal communication from Jungseog Kang, Hongtao Yu Laboratory). Also, the HP1 binding mutant of Sgo1 rescues premature separation of sister chromatids to the same extent as wild type Sgo1in endogenous Sgo1 depleted cells (Fig 4-7). This data suggests that HP1-Sgo1 interaction may not be essential for sister chromatid cohesion during mitosis. However, other experiments show that HP1-Sgo1 binding is important for Sgo1 localization during interphase (Fig 4-8(personal communication from Jungseog Kang, Hongtao Yu Laboratory). Hence, it is possible that in interphase, cohesin protection is facilitated by HP1-Sgo1 interaction. In mitosis however, this interaction plays no role in Sgo1 localization because HP1-binding mutant Sgo1 is properly localized at the centromere. This localization is contrary to the reports that phosphorylation of Serine-10 on H3 histone by Aurora B kinase, a component of CPC disrupts the interaction between adjacent tri-methylated Lysine-9 and HP1 chromo domain. This causes mislocalization of HP1 rendering it incapable of recruiting Sgo1in mitosis. But since we observe that HP1 is still localized at the centromere during mitosis, it suggests to us that HP1 must be recruited via some other mode other than by binding to histone H3.

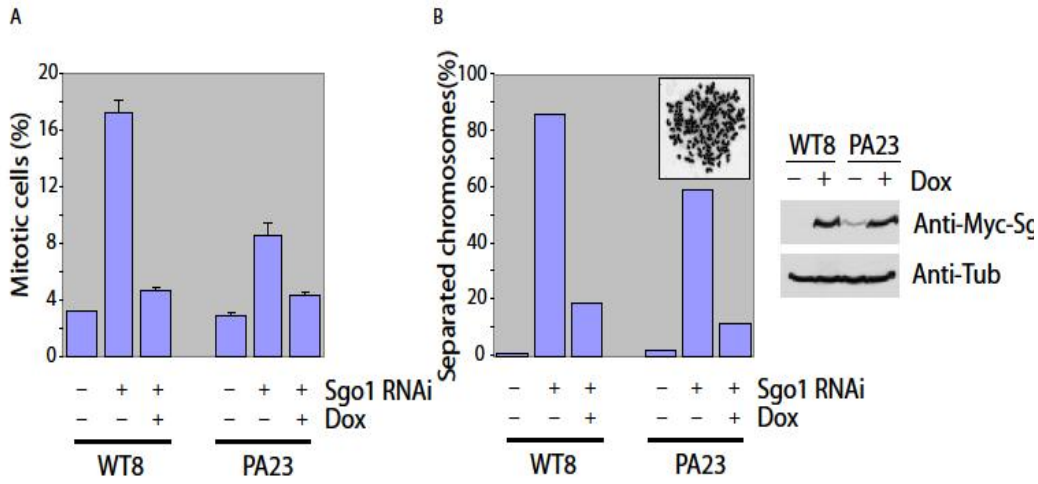

Figure 4- 7: Sgo1 interaction with HP1 is dispensable for Sgo1 localization and sister chromatid cohesion

(A) HeLa tet-on cells stable expression of Myc-Sgo1 WT or HP1-binding motif mutant (PA23) under doxycyclinepromoter were Sgo1 was knocked out by siRNA for 24 hr and analyzed with FACS for mitotic cells.
(B) Stable cell lines (PA23) transfected with Sgo1 siRNA treated with nocodazole and its mitotic chromosomes spread and stained with Giemsa. Cells exhibiting totally separated chromosomes phenotype and unseparated chromosome were counted. The western blot shows expression level of Myc-Sgo1 proteins in these cell lines.
Courtesy: Jungseog Kang, Yu Laboratory

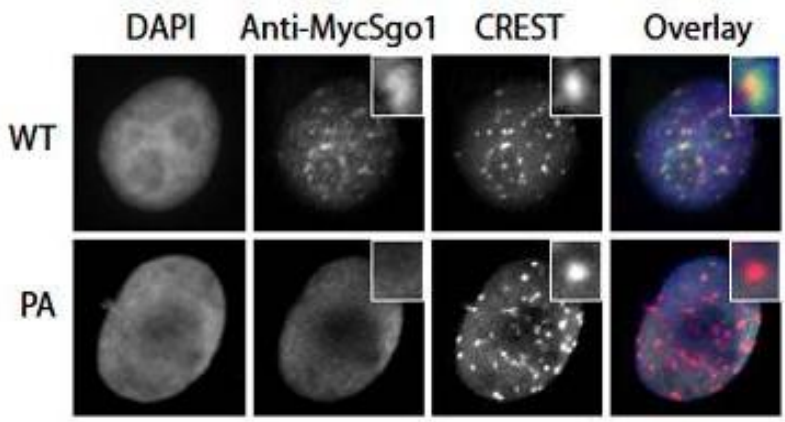

Figure 4- 8 Sgo1 interaction with HP1 is important for its interphase localization

HeLa tet-on cells stably expressing Myc-Sgo1 WT or HP1-binding motif mutant were Sgo1 was knocked down siRNA and their nucleus spread and processed for immunofluorescence using Myc or CREST antibody. Myc-Sgo1 is shown as green and CREST as red.

Courtesy: Jungseog Kang, Yu Laboratory

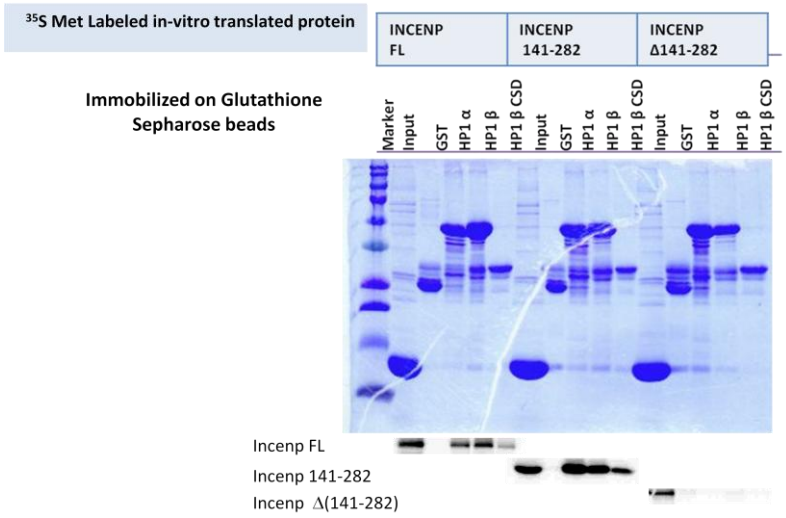

Figure 4- 9 HP1 interacts with INCENP in vitro.

Binding of INCENP to HP1 monitored using *in vitro* pulldown assay. 5µg of HP1α, HP1β or the chromo-shadow domain of HP1β was immobilized on glutathione beads. Full length INCENP protein, a fragment containing residues 141-282 or a deletion mutant of INCENP lacking these residues was synthesized in vitro using ^{35}S labeled rabbit reticulocyte lysate. 1ul of this lysate was incubated with the immobilized proteins for 1 hour at room temperature. The beads were then washed and proteins eluted by boiling the beads in 5X Laemmli buffer. The proteins were resolved on 15% SDS-PAGE gel, stained with Commassie blue (upper panel) and binding visualized using autoradiography (lower panel). GST was used as a negative control.

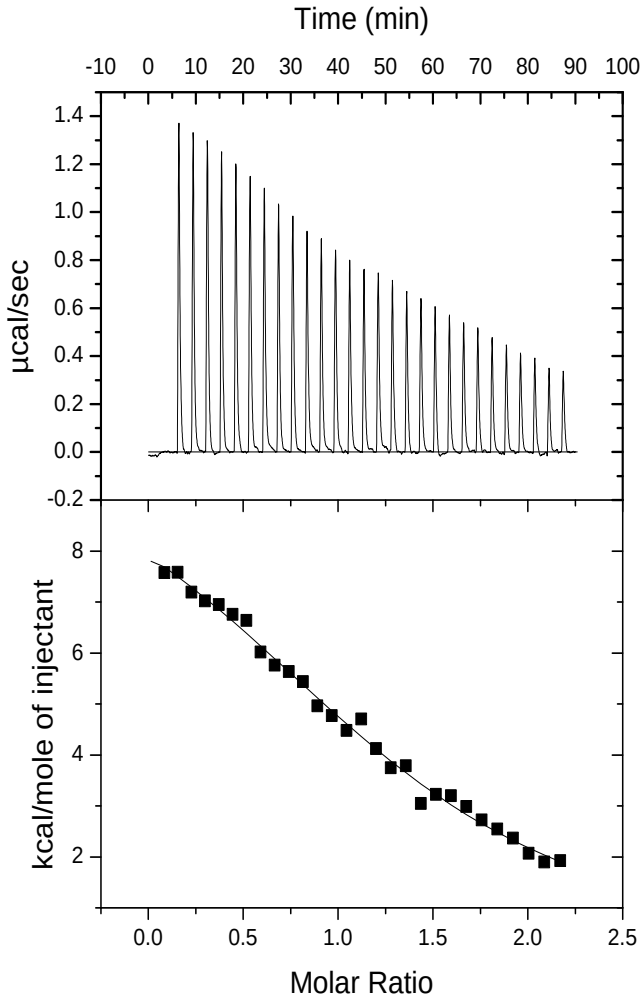

Figure 4- 10 : HP1 interacts with INCENP PxVxL motif weakly

Binding of INCENP peptide containing the PxVxL/I motif to the chromo-shadow domain of HP1 was monitored using ITC. ITC measurements were done at 20°C where 500µM peptide was titrated into 50µM HP1βCSD in 28 steps of 10µL each. Resulting data was analyzed as single binding site model on MicroCal Origin 7.0 software.

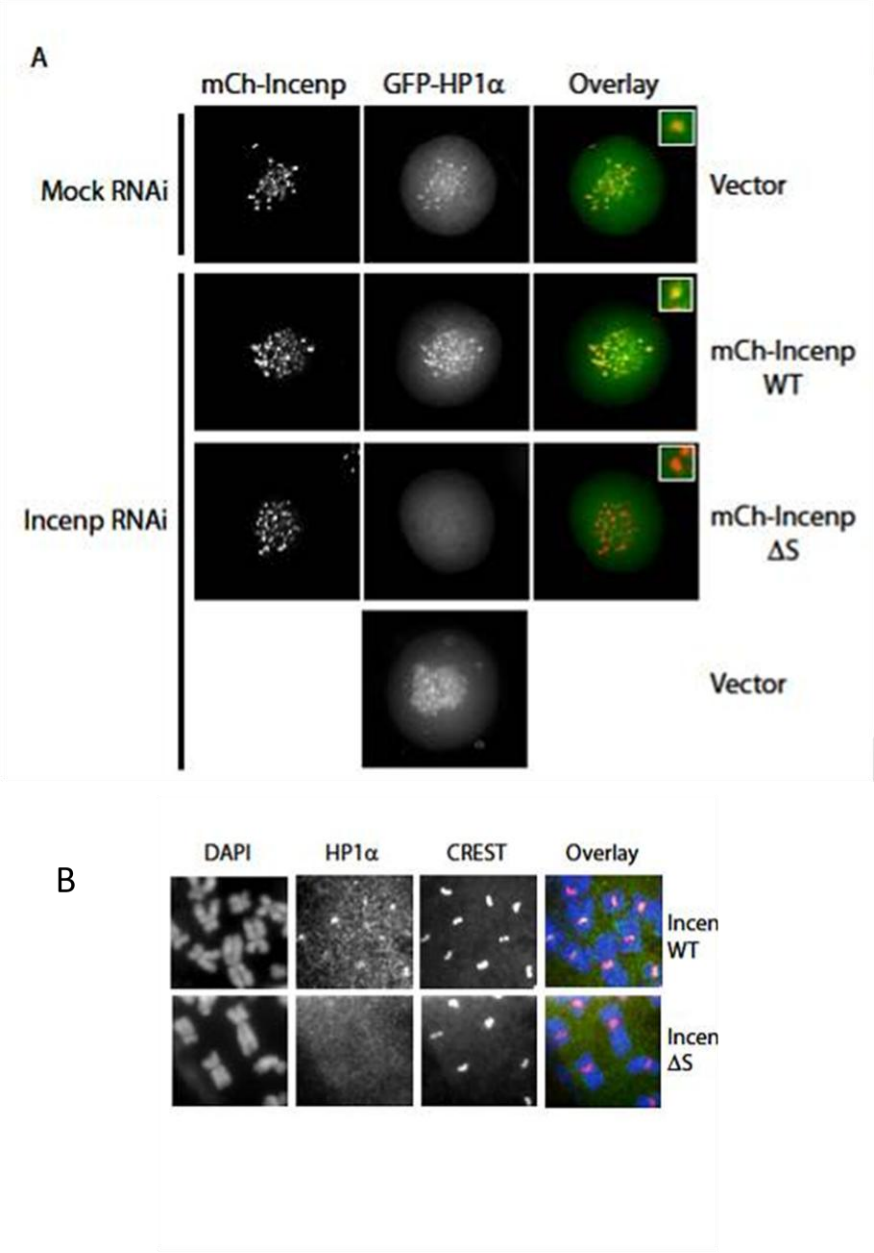

Figure 4- 11 INCENP recruit HP1α to the centromere

(A) HeLa tet-on cells cotransfected with mCh-INCENP and GFP-HP1α and then mock or INCENP siRNA. Live cells show mCh-INCENP in red and GFP-HP1α in green.

(B) HeLa tet-on cells stably expressing mCh-INCENP WT or non Hp1 binding deletion mutant transfected with INCENP siRNA and mitotic chromosomes spread. HP1α is shown as green and CREST as red.

Courtesy: Jungseog Kang, Yu Laboratory

An earlier report shows HP1 localized in the same vicinity as the chromosome passenger complex during mitosis. It has been known that HP1 interacts with Incenp, which is a component of chromosomal passenger complex(Ainsztein et al., 1998). It is possible that the CPC might be mediating HP1 localization on the centromere via Incenp during mitosis. An *in vitro* binding assay of HP1α, HP1β and HP1βCSD with *in vitro* translated Incenp shows that full length Incenp binds to these three forms of HP1. Another study mapped the binding site of HP1 on Incenp and determined that a region containing 124-248 residues of Incenp is sufficient for this interaction and that deletion of this region abolished HP1-incenp binding (personal communication from Jungseog Kang, Hongtao Yu Laboratory). In an *in vitro* binding experiment this same region is also shown to be essential to bind to HP1α, HP1β and HP1βCSD, where deletion of this region abolished the interaction (Fig 4-9). It has been reported that both the hinge region and the chromoshadow domain of HP1 is essential for this binding but experiments in our laboratory have also shown that the chromoshadow domain of HP1α is essential to localize it to centromere during mitosis, since the hinge mutant of HP1 properly localizes at the centromere during mitosis(data not shown). There is a PxVxL/I motif present in the 124-248 region of Incenp. Therefore it is very likely that HP1 is interacts with Incenp via this PxVxL/I motif. To test this, we synthesized another 25 residue peptide containing the sequence of Incenp around and including the PxVxL/I motif. An ITC experiment

was performed with HP1βCSD in the cell and Incenp peptide in the syringe. The results indicate that they interact extremely weakly and the protein in the cell could not be saturated because the dissociation constant could not be estimated with high confidence by ITC (Fig 4-10). However, it has been shown that mutation of the PxVxL/I motif on Incenp does not abolish the interaction between Incenp and HP1 (Fig 4-11) (personal communication from Jungseog Kang, Hongtao Yu Laboratory). Therefore it is possible that a region other that the PxVxL/I motif is significantly contributing to this interaction. The disruption of HP1-Incenp binding does interfere with localization of HP1 on centromere but not that of Incenp. This suggests that HP1 is recruited by Incenp onto the kinetochore during mitosis (Fig 4-12). However, the role of HP1 at the centromere during mitosis is not known and will require further investigation.

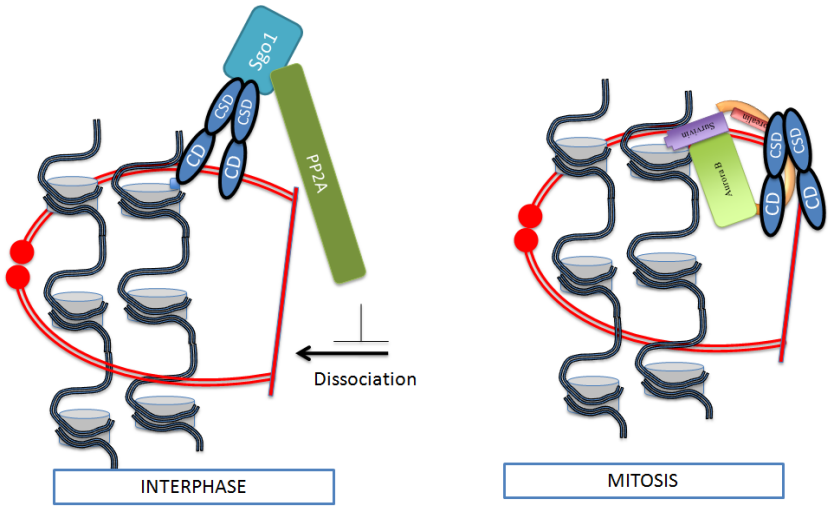

Figure 4- 12 Model for Heterochromatin protein 1 recruitment at centromere during interphase and mitosis

During interphase, HP1 is being recruited by Histone H3 via chromodomain. The Chromoshadow domain recruits Shugoshin which recruits PP2A inhibiting cohesin cleavage.
During mitosis, HP1 is being recruited by chromosomal passenger complex which in turn is recruited by Histone H3 Ser10.

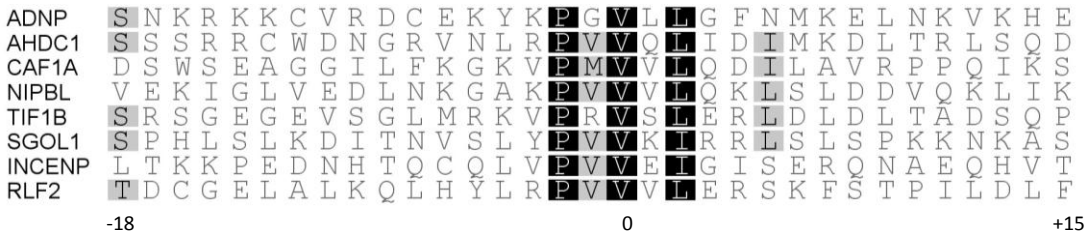

Figure 4- 13 Sequence alignment of HP1 Chromoshadow domain interacting protein.

V in the consensus PxVxL motif is marked as 0. ADNP - Activity dependent neuroprotector homeobox protein. ADHC – AT hook DNA binding motif containing protein 1. CAF1A- Chromatin assembly factor 1A. NIPBL – Nipped B like protein. TIF1B – Transcription intermediary factor. SGO – Shugoshin. Incenp – Inner Centomeric protein. RLF2 - Rearranged L-myc fusion gene protein.

CHAPTER FIVE
Summary

HP1 is localized on the centromere throughout the cell cycle. At interphase, HP1 is recruited to centromeric heterochromatin via lysine9 of Histone H3. This enables HP1 to recruit Sgo1 to the centromere. Sgo1 inhibits premature sister chromatid separation by protecting cohesin at the centromere. In this study, we have biochemically and biophysically characterized the interaction of Sgo1 with HP1. We show that Sgo1 binds through one of the two binding motifs to the chromoshadow domain of HP1 (Fig 4-12).

During mitosis, however, phosphorylation of Serine10 of Histone H3 inhibits recruitment of HP1 via Lysine9. We show that in this case, Incenp, a member of the CPC, recruits HP1 to the centromere via the chromoshadow domain of HP1 (Fig 4-12). Further work needs to be done to elucidate the physiological importance of this interaction. We propose that the function of Incenp-HP1 interaction may either be redundant or that in mitosis, HP1 inhibits Incenp or Sgo1.

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