Chapter-5

DISCUSSION
A genetic interaction is defined by the emergence of a surprising phenotype when two genes are disrupted together. A new type of reverse genetic screen to discover gene interaction is known as unlinked non-complementation (Baetz et al 2004). In such type of interaction a haploid mutant strain carrying a query mutation crossed with many haploid strains each carrying a single engineered mutation at a second (unlinked) locus. The diploid strains produced by mating carrying two heterozygous mutations. If the double-mutant strain shows reduced fitness relative to each single-mutant strain, this is known as unlinked non-complementation. Unlinked noncomplementation was further elaborated by Haarer et al (2007) by putting forward a new sub-type of unlinked non-complementation interaction. In which at least one of the mutant loci carries a null mutation, and coined the term complex haploinsufficient interaction. The complex haploinsufficient interaction was used in this study. We used a *chk1* null mutant as a query mutation, screening this against nonessential genes for studying complex haploinsufficient interactions and the role of these nonessential genes.

During the study we have explored the role of a temperature sensitive mutant obtained by random mutation of *chk1Δ* strain using EMS (Ethyl Methane Sulfonate) and screening of the mutants having temperature sensitive phenotype. This screening identified *ts17* mutant that cannot form colonies at a temperature above than 31°C. Complementation analysis of *ts17* mutant identified 11 kb fragment on chromosome II. Further sub-cloning and sequence analysis of a 3.3 kb fragment identifies *ts17* coding for *wat1*+ gene. Wat1 is known to interacting with the Prp2 (U2AF59), the large subunit of essential splicing factor (U2AF) U2 auxiliary factor (Kominami and Toda 1997). Prp2 is involved in the binding to the Py-tract prior to the first trans-esterification reaction (Merendino et al 1999, Wu et al 1999). As *lst8* a homologue of *wat1* is essential for budding yeast survival; shows the importance of Wat1 and its homologs among organisms (Roberg et al 1997). Wat1/Pop3 protein is the part of both TOR complexes in fission yeast and Lst8 is a constituent as well as positive regulator of TOR complex in budding yeast while among mammals mLst8 is the component of mTOR (Chen and Kaiser 2003, Kim et al 2003, Weisman 2010).

Cloning and sequence analysis of *wat1-17* mutant gene results in identification of a point mutation in ORF, changing the amino acid, Cys233 to Tyr. Earlier studies by Kemp et al (1997) and Ochoterena et al (2001) has identified two mutants allele of *wat1* gene Ser278Phe and *wat1*-5235 mutant respectively.
Wat1 is a highly conserved protein made up of seven WD repeats (Neer et al 1994). The mutation in *wat1*-17 mutant allele was located in sixth WD repeat of forty amino acids. This temperature sensitive *wat1* mutant allele (*wat1*-17) exhibits genome diploidiising defect as it fails in cell division after genome duplication. Wat1 protein becomes unphosphorylated at restrictive temperature in *wat1*-17 mutant. The *wat1*-17 mutant defects become severe in absence of checkpoint function. The double mutants of *wat1*-17 and *chk1* deletion lose their viability faster than the single *wat1*-17 mutant at non permissive temperature as compared to single mutant suggesting that abolishing *wat1*-17 function might be activating a checkpoint response involving Chk1 kinase. To characterize its functional aspects we studied the effects of various damaging agents that affect the cell cycle progression. Use of a spindle destabilizing agent the benomyl and microtubule poison thiabendazole; results in *wat1*-17 mutant sensitivity at 25°C in *chk1* deletion background. The effect was severe than the *chk1Δ* mutant alone predicting involvement in spindle assembly checkpoint. Involvement of *chk1* in spindle checkpoint was shown by Zachos et al (2007). It has been shown the recombinant Chk1 could phosphorylate and activate aurora B kinase *in vitro* (Zachos et al 2007). It suggests the direct regulation of aurora B kinase by Chk1. Whereas DNA damaging agents like, Hydroxyurea and Ultra Violet rays had no effect on *wat1*-17 mutant but *wat1*-17*chk1Δ* double mutant cells were highly sensitive to UV suggesting that these protein might be involve in two different pathway. Genetic interaction of *wat1*-17 mutation with kinetochore proteins suggests an indirect role of *wat1* in chromosome segregation in cell cycle. FACS analysis shows *wat1*-17 mutant cells in absence of Chk1 exhibit high percentage of ploidy at non permissive temperature. At C-terminal of *wat1* gene three tandem repeats of FLAG octapeptide was inserted by using a fabricated pFA6a 3HA-kanMX6 module of Bähler et al (1998) as described by Ranjan et al (2010). The *wat1*-FLAG strain was used to study localization of Wat1 protein. Confocal microscopy shows presence of Wat1 throughout the cytoplasm prominently around nucleus. Chen and Kaiser (2003) found that Lst8 protein in budding yeast is associated with membranes and appears to localize to the endosomal/Golgi compartments. In *Chlamydomonas reinhardtii* Lst8 protein was concentrated in the proximity of basal bodies and was more abundant around the nucleus (Diaz-Troya et al 2008).

The most obvious phenotype caused by loss of Wat1 function in both the temperature sensitive and the null mutant was the change in cell shape. The *wat1* gene was named
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by watermelon shaped of wat1 deletion mutant previously described by Kemp et al (1997). This abnormal cell shape was not found by Ochoterena et al (2001) in either wat1/pop3-5235 mutant or wat1/pop3 deletion strain. We also did not found any abnormal cell shape in wat1 null mutant that we had constructed during this study. A possible explanation for this difference in phenotype could be due to the fact that the previously isolated deletion allele of wat1 (Kemp et al 1997) only removed part of the protein, while the entire ORF was deleted in wat1Δ in our study and by Ochoterena et al (2001). On the other hand the wat1-17 mutant cells become rounded in shape and very small in size as compare to wild type at restrictive temperature but we were unable to find any reduction in tubulin level in this mutant as reported earlier (Ochoterena et al 2001). It may be possible that this phenotype is allele specific because we are dealing with the mutation different from previous mutations studied earlier. Another possible explanation could be that the localization of F-actin might be disturbed in response to mutated or truncated form of Wat1 protein, which renders the rounded cells rather than cylindrical.

Since the wat1Δ mutant cells were resistant to temperature change, suggests that the wat1 gene is necessary for fission yeast but not essential for survival. The wat1Δ mutant was sterile and the cells of wat1Δ become larger than wild type cells and take almost double time in cell division. It was highly sensitive for osmotic stress and involved in genome diploidising phenomenon also shown by Ochoterena et al (2001). There was no effect on expression of tubulin protein in wat1-17 mutant but surprisingly the expression level of Ded1 was reduced significantly in wat1-17 mutant and wat1-17chk1Δ double mutant as compare to wild type. The Ded1 interacts with Chk1 and present in two forms in response to heat shock and depletion of carbon source (Liu et al 2002). We also observed two form of Ded1 at high temperature (36°C) in wild type cells but in chk1Δ cells these two forms were absent. This could be due to the fact that we use 36°C rather than 42°C for this experiment. These results indicate a possible role of Wat1 and Chk1 in stress response. Wat1 protein has already been shown to interact with TOR complex in fission yeast S. pombe (Ikai et al 2011). Chk1 has not been implicated in stress response but physical interaction of Chk1 with Ded1 has been established (Liu et al 2002).

The nup120 and dlc2 have been isolated as multicopy suppressors during a suppressor screen that rescue the cold sensitive phenomenon of wat1-17Chk1Δ mutant. The Nup120 (Nuclear pore protein 120) is an important member of Nup84 complex
The *nup120* is an essential gene in budding yeast. Deletion of *nup120* gene caused clustering of NPCs at one side of the nuclear envelope, moderate nucleolar fragmentation and slower cell growth (Aitchison et al. 1995). The Nup120 structure reveals a 7-bladed β propeller domain and α-helical domain that represents a novel fold (Fernandez-Martinez et al. 2012). The Wat1 is a WD repeat protein and WD repeat proteins exhibit β propeller structure (ter Haar et al. 1998). The Dlc2 (dynein light chain 2) was found as component of cytoplasmic dynein connect the complexes to their cargoes (Dick et al. 1996, Vallee et al. 2004). It is also found in Nup82 complex which situated at cytoplasmic side of nuclear pore complex and facilitate nuclear mRNA export. The Dlc2 works as molecular glue and responsible for dimerization of Nup159 (Stelter et al. 2007). How these suppressors are associated with *wat1*-17chk1Δ mutant is elusive. The Nup120 a component of Nup84 complex, which is involved in nucleocytoplasmic transport and Dlc2, is a component of Nup82 complex, well known for mRNA export (Hodge et al. 2011, Lutzmann et al. 2002). Both these nucleoporins may increase the export rate of mutant *wat1* mRNA, which increases the amount of mutated protein in cytoplasm, which in turn rescues the cold sensitivity of *wat1*-17chk1Δ double mutant.

Wat1 was emerged as interacting partner of Prp2 in a yeast two hybrid screen (Kominami and Toda 1997). We examined the interaction between mutant Wat1 protein with Prp2 and find the interaction was disrupted. Perhaps the mutation alters the binding site in mutant Wat1 protein, which may be essential for physical interaction of both proteins. In order to elucidate the role of *wat1* in cell cycle and interaction with *chk1* more experimental work is needed.