Wat1/pop3, a Conserved WD Repeat Containing Protein Acts Synergistically with Checkpoint Kinase Chk1 to Maintain Genome Ploidy in Fission Yeast *S. pombe*

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**Abstract**

Aberrant chromosome segregation defects can lead to aneuploidy, a common characteristic of human solid tumors. Aneuploidy is generated due to defects in the mitotic spindle or due to inefficient mitotic checkpoint response. We have isolated a novel mutant allele of *wat1*, a WD repeat containing protein that exhibits conditional synthetic lethality with *chk1* knock out. We observed only a marginal decrease in the level of α-tubulin protein level in *wat1*-17 mutants after prolonged exposure at semi permissive temperature. Interestingly the protein level of α-tubulin was reduced in the *chk1Δ wat1-17* double mutant at 18°C with defective microtubule structure. Consistent with loss of microtubule structure in the *wat1* deletion background, the double mutant of *wat1-17 chk1Δ* was hypersensitive to the microtubule destabilizing agent TBZ suggesting severe defects in microtubule integrity in *wat1*-17 mutant in the absence of Chk1. Combination of *wat1*-17 with the *chk1* deletion also aggravates the defects in the maintenance of genome ploidy. The mutation in *wat1*-17 was mapped to Cys 233 that was changed to tyrosine. Based on the molecular modeling studies, we hypothesize that the substitution of the bulky Tyr residue at Cys233 position in *wat1-17* mutant results in conformational changes. This in turn can affect its intercations with other interacting partners and perturb the overall functions of the Wat1 protein.

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**Introduction**

Accumulation of mutations and chromosomal aberrations is one of the hallmarks of cancer cells. These aberrations arise due to defects in the genome maintenance mechanisms that include DNA repair and cell cycle checkpoint pathways. Defects in these cell cycle checkpoints result in inappropriate proliferation. DNA damage checkpoints are responsible for maintaining the fidelity of genetic information by arresting cell cycle progression and facilitating DNA repair pathways. Several studies have identified a network of proteins that are involved during the DNA damage checkpoints response. Central to this network are protein kinases of the ATM/ATR family that work as sensors and transducers. These are also known as Tel1/Mec1 in budding yeast and Tel1/ Rad5 in fission yeast respectively [1]. Downstream of ATM and ATR are effector molecules Chk1 and Chk2 respectively. These are serine threonine kinases that sense DNA damage and phosphorylate a number of proteins that regulate cell cycle progression and DNA repair pathways [2]. ATR is the major upstream kinase that phosphorylates and activates Chk1 [3–7]. Chk1, an evolutionarily conserved protein kinase is an essential component of the DNA damage checkpoint [8–10]. In response to DNA damage, the protein kinase Chk1 is phosphorylated and inhibits mitotic entry by phosphorylating Wee1 and Cdc25 to prevent activation of Cdc2 [11].

The spindle assembly checkpoint blocks chromosome segregation until all the chromosomes are attached to the mitotic spindle. The anaphase-promoting complex (APC), a multi-subunit E3 ubiquitin ligase is required for the degradation of both cyclin B and cohesin to promote metaphase to anaphase transition. The activation of Mad2, a spindle assembly checkpoint protein prevents the association of APC with Slp1/Cdc20 and blocks the cells during metaphase until all the chromosomes are properly attached to the mitotic spindle [12]. Involvement of Chk1 pathway to delay metaphase to anaphase transition in response to DNA damage has also been shown in *S. pombe* and *Drosophila* [13,14].

The WD40-repeat motif was identified originally in the β-subunit of heterotrimeric G proteins [15] and subsequently has been found in a wide spectrum of regulatory proteins, where it functions in mediating protein-protein interactions. WD40-repeat proteins adopt a β-propeller structure, which can use one or two blades to interact with other proteins without affecting the other blades [16,17]. It is assumed that one (or more) WD repeat within a given protein specifically interacts with different partner proteins, thus creating multiple protein–protein interactions [18].

Fission yeast Wat1/pop3 is a homologue of Lst8 of budding yeast. Depletion of Lst8 in budding yeast cells results in a rapid arrest of cell growth [19,20]. The budding yeast LST8 functions in the delivery of Gap1 protein, and possibly other amino acid permeases, from the Golgi to the cell surface [20]. A mutant allele
of LST8 (lst8-1) exhibited synthetic lethality with the set13-1 mutation [20]. Fission yeast WAT1 has been shown to play an important role in the establishment of actin and microtubule cytoskeleton [21]. The role of Wat1 in mRNA maturation and its requirement for the maintenance of genome stability and microtubule integrity has been well studied [22]. Upon nutrient starvation, the \textit{wat1} mutant cells fail to arrest in the G1 phase and hence are sterile in fission yeast [21,23]. Mammalian LST8 is a functional component of mTOR signaling complex and interacts with the kinase domain of mTOR to stabilize its interaction with raptor. It also participates in regulating cell growth through the mTOR S6K1 signaling pathway. Recently the truncated mTOR (kinase domain) and LST8 protein have been co-crystallized. This has helped for the understanding of mTOR function and its inhibition by rapamycin and ATP competitive compounds [24].

In a genetic screen to identify conditional synthetic lethal mutants with \textit{chk1} null mutant, a novel temperature sensitive mutant allele of \textit{wat1} was isolated. Our study shows that the \textit{chk1} null mutant and \textit{wat1} mutant together pose a grave effect on the stability of leucine marker present on the plasmid. Plasmid distinguished from those with integrated plasmid by the mitotic segregation. Restriction digestion and sequencing analysis identify 11 kb region on chromosome II containing four open reading frames coding for \textit{inp2}, \textit{wat1}, \textit{pop3}, \textit{mfd} and SPBC21B10.03c genes. Further 3.3 kb \textit{HindIII} fragment containing full length \textit{wat1} gene was re-transformed in ts17/wat1-17 mutant strain for complementation analysis.

### Cloning of Mutant \textit{wat1} Gene by Gap Repair Method

Mutant \textit{wat1} gene (\textit{wat1-17}) was cloned by the gap repair method. Wild type \textit{wat1} gene cloned in pSP1 vector, was digested with \textit{NheI} and \textit{Nol} restriction enzyme that deleted 2524 bases from upstream to downstream of \textit{wat1} coding region. The linear fragment containing upstream and downstream sequences of \textit{wat1} gene with a leucine selectable marker was isolated by gel elution and transformed in \textit{wat1-17} mutant strain. Transformants containing plasmid with mutant allele of \textit{wat1} gene (\textit{wat1-17}) was distinguished from those with integrated plasmid by the mitotic stability of leucine marker present on the plasmid.

### Preparation of Whole Cell Lysate and Western Blot Analysis

Cells were grown up to mid log phase at 25°C then shifted at 18°C. Cells were harvested by centrifugation at indicated time interval and lysed using glass beads and a Fast Prep (Bio 101) vortex machine. Lysate in Phosphate Buffered Saline (PBS) was centrifuged at 10000 rpm in a microfuge for 5 min at 4°C. Supernatant was collected and protein estimation was performed using the Bradford assay method. For western blot analysis, 100 μg of total cell lysate was run on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with anti α-tubulin (Sigma, cat no. T6199) and anti-Cdc2 antibodies. A peroxidase-coupled secondary antibody and the enhanced chemiluminescence detection system (Millipore) were used to detect the immune complexes.

### Flow Cytometry

Aliquots of 10^6 cells were collected from mid log phase cultures, fixed in 1 ml of 70% ethanol before storing at 4°C. For flow cytometry, the cells were rehydrated by washing with 3 ml of 50 mM sodium citrate, resuspended in 0.5 ml of 50 mM sodium citrate containing 0.1 mg/ml RNase A, and incubated at 37°C for 2 h. Cells were stained by adding 0.5 ml of sodium citrate solution.
containing 10 µg/ml Propidium Iodide and stored at 4°C in dark for 1 hour and subjected to flow cytometry as described earlier [27]. Just prior to flow cytometry, samples were sonicated to avoid inaccurate readings resulting from the clumping of cells. Samples were analyzed with a Becton-Dickinson FACS Calibur.

Yeast Two-hybrid Analysis

For two-hybrid interaction studies pp2, wat1+ and wat1-17 mutant genes were amplified and cloned in pACT2 and pAS2 vector respectively using forward primer 5’-GATCCCATG-GATTT GTCTTCGAGATATC-3’, reverse primer 5’-GATCCGATCCATCACCCTAGTATGTT ATAG-3’ for pp2 and forward primer 5’-GATCCTATGTCGATACG-TATCGCACCA-3’, reverse primer 5’-GATCGGATCCACCT- TAAATTTGGATGCATTAG-3’ for wat1 gene. Plasmid containing Pp2 as prey fused with the GAL4 activation domain (pACT2) and the Wat1 protein fused to the DNA-binding domain of the GAL4 transcription factor were co-transformed in PJ69-4A strain (Clontech). Interaction studies were performed using LacZ and HIS3 as reporter gene on SD-leu-trp plates containing X-gal, (pACT2) and the Wat1 protein fused to the DNA-binding domain by complementation analysis as described in experimental knockout background as described earlier [31]. The ts17/wat1-17 mutant strain was isolated. The wat1-17 mutant strain was back-crossed with wild type strain and a pure mutant strain, isolated from the same genetic screen was back-crossed with mutant allele on expression of wat1-17 gene. Plasmid containing thiabendazole (Fig. 2A) indicating a conditional synthetic lethality of mutant cells were unable to form colonies (Figure 1).

Results

Isolation and Identification of Synthetic Lethal Mutants with chk1 Null Mutants

During a temperature sensitive screenings with chk1 null mutant background, five conditional mutants were isolated that were unable to form colonies at semi permissive temperature in chk1 knockout background as described earlier [31]. The ts17 mutant strain, isolated from the same genetic screen was back-crossed with wild type strain and a pure ts17+ mutant strain was isolated. The ts17+ mutant cells exhibited temperature sensitive phenotype and were unable to form colonies at 36°C. To further confirm the conditional lethality associated with the chk1 knockout, we checked the ability of ts17/wat1-17 and chk1Δ ts17/wat1-17 double-mutant strain to form colonies at 18°C. The ts17/wat1-17 single mutant were able to grow at 18°C but in chk1 deletion background the ts17/wat1-17 mutant cells were unable to form colonies (Figure 1) indicating a conditional synthetic lethality of ts17/wat1-17 with chk1 knockout.

The gene coding for the ts17/wat1-17 mutation, was identified by complementation analysis as described in experimental procedures. Sequencing and database analysis identified the 11 kb region on chromosome II containing four open reading frames coding for inp2, wat1+pop3, mlf1 and SPBC21B10.03c gene respectively. Further subcloning identified a 3.3 kb HindIII fragment containing full length wat1+pop3 gene was sufficient for the complementation of the temperature sensitive phenotype of ts17/wat1-17 mutation (data not shown).

Combination of wat1-17 Mutant with chk1 Knockout Renders the Cell Sensitive to Microtubule Destabilizing Agent

Earlier studies have shown ε-tubulin reduction and actin disorganization in wat1 mutants [21,22]. Wat1 protein has also been shown to be required for the maintenance of microtubule integrity. To further explore the role of wat1-17 mutant allele in microtubule stability, we tested the sensitivity of wat1-17 mutant with microtubule destabilizing drug. Contrary to earlier studies [22] we observed that the mutant allele of wat1-17 was not sensitive to microtubule destabilizing drug (Fig. 1B). Interestingly chk1Δ wat1-17 double mutant was hyper-sensitive to tubulin destabilizing agent and was unable to form colonies on plate containing thiabendazole (Fig. 2A) indicating a possible requirement of Chk1 for the recovery of wat1-17 mutant cells under defective microtubule condition. The previously [22] isolated wat1-5235 mutant is cold sensitive while the novel wat1-17 mutant is not, suggesting that the wat1-5235 mutation affects the function of Wat1 protein more severely than the wat1-17 mutation. We also monitored the cellular morphology of wat1-17 chk1Δ double mutant along with the wat1-17 single mutant at semi permissive temperature by staining the nuclei with DAPI. After 48 hr incubation at 18°C abnormal mitosis as defined by more than one DAPI -stained body was observed in about 8% of the wt1-17 chk1Δ cells while only <1% cells of the wat1-17 single mutant exhibited such abnormal nuclei (Fig. 2B) indicating severe defect in wat1-17 chk1Δ mutant.

Tubulin Level was Reduced in chk1Δ wat1-17 Double Mutant as Compare to wat1-17 Single Mutant

Previous work has identified Wat1 as a protein that is required for the maintenance of ε-tubulin level [22]. To explore the effect of wat1-17 mutant allele on expression of ε-tubulin, we examined the level of ε-tubulin after shifting the wat1-17 mutant cells to the non-permissive temperature. We did not observed reduction in ε-tubulin protein level at 36°C (data not shown) but there was reduction in the level of ε-tubulin protein after shifting the wat1-17 mutant cells to 18°C for 36 hr (Fig. 3A). Interestingly there was about 50% reduction in the protein level of ε-tubulin in chk1Δ wat1-17 double mutant as compare to wat1-17 single mutant just after 12 hr shift at 18°C (Fig. 3A). Consistent with the reduced ε-tubulin level in chk1 deletion background, the double mutant of wat1-17 chk1Δ were hypersensitive to microtubule destabilizing agent (Fig. 2A) suggesting severe defects in microtubule integrity in wat1-17 mutant in the absence of Chk1.

Microtubule Structure are Compromised in the wat1-17 Mutant at Semi Permissive Temperature

Reduction of ε-tubulin protein levels, in wat1-17 and wat1-17 chk1Δ delete cells prompt us to monitor the microtubule structure at permissive and semi permissive temperature. Immunofluorescence microscopy with anti ε-tubulin antibody showed normal microtubule structure in wild type cells at 25°C and 18°C (Fig. 3B left, upper and lower panel). Interestingly wat1-17 single mutant cells grown at 25°C have shorter microtubules (Fig. 3B, upper, middle panel) while at semi permissive temperature very few shorter microtubules were present (Fig. 3B, lower middle panel) indicating compromised microtubules at 18°C. In wat1-17 chk1Δ double mutant very few shorter microtubules were observed at permissive temperature (Fig. 3B, upper right panel) that were absent once the
cells were shifted at semi permissive temperature (Fig. 3B, upper lower panel) indicating a severe defect in microtubule structure at 18°C. Hypersensitivity of \textit{wat1-17 chk1}D double mutant with TBZ (Fig. 2A) is in agreement with the severe defects in microtubule structure in the double mutant.

Combination of \textit{wat1-17} with the \textit{chk1} Deletion Aggravates the Defects in Maintenance of Genome Ploidy

In earlier studies the \textit{wat1} mutant has been shown to required for the maintenance of genome ploidy and chromosome stability [22]. We used Phloxine B to observe the diploidising property of \textit{wat1-17} mutant cells. Phloxine B, a xanthene dye with a red colour often used to distinguish diploid strains of fission yeast from

Figure 1. The \textit{ts17/wat1-17} mutant allele exhibit conditional lethality with \textit{chk1} knockout. Indicated strains were grown at 25°C, serially diluted and spotted on YEA plates. Plates were incubated at indicated temperature for 3 days except 18°C plate that was incubated for 7 days before taking photographs.
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Figure 2. The \textit{wat1-17} \textit{chk1} delete cells are hypersensitive to microtubule destabilizing agent. A. Indicated strains were grown at 25°C, serially diluted and spotted on YEA plate or plate containing 10 ug/ml thiabendazole. Plates were incubated at 25°C for 3-4 days before taking photographs. B. Indicated strains were grown till mid log phase at 25°C and then shifted at 18°C for 36 hr, fixed with 70% ethanol and stained with DAPI. About 250 cells were counted for the presence of aberrant nuclei and percentage was calculated. Scale bar: 10 μm.
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haploid strains and has been used to detect the genome duplication [22,32]. The result shows that the colonies from wat1-17 and wat1-17 chk1Δ strains were slightly dark colored on plates containing Phloxine B as compared to wild type and chk1Δ cells, indicating the presence of diploid cells in these strains (Fig. 4A).

To observe the polyploidy in detail, DNA content of wat1-17, chk1Δ, wat1-17 chk1Δ mutants was measured by flow cytometry. The strains were grown at 25°C till mid log phase then shifted at semi permissive temperature (18°C), samples were collected and processed for FACS analysis. At 25°C the wild type, wat1-17 and chk1Δ cells exhibited the normal ploidy of diploid cells at each time point while most of the cells of the wat1-17 chk1Δ double mutant exhibited an increase in ploidy from 2N to 4N (Fig. 4B) indicating that the double mutant could be partially defective in mitosis even at the permissive temperature. More importantly the DNA peak in wat1-17 chk1Δ shifted towards polyploidy when these cells were shifted to 18°C (Fig. 4B) indicating severe defects in maintenance of genome ploidy in the double mutant.

Figure 3. The wat1-17 chk1Δ delete cells shows reduced α-tubulin levels and defects in microtubule structure. A. The wild type, wat1-17 and wat1-17 chk1Δ cells were grown at permissive temperature till mid log phase then shifted at 18°C for indicated time. Protein lysate was prepared as described in material and methods, samples were run on 10% SDS PAGE, transferred on nitrocellulose membrane and probed with anti α-tubulin antibody. Anti-cdc2 antibody was used as loading control. Signals were quantitated on Gel Doc system (Life Technologies) and protein ratio was calculated. The asterisk indicates a non specific band. B. Indicated strains were grown at 25°C and shifted at 18°C for 48 hr. Cells were processed for immunofluorescence microscopy using anti α- tubulin antibody. Scale bar: 10 μm.
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Mapping and Identification of wat1-17 Mutation by Gap Repair

To identify the mutation in wat1 gene we cloned the wat1-17 mutant gene by gap repair as described in material and methods. Sequencing and comparison with wild type sequence of wat1+ gene indicated a mutation from nucleotide G to A, that changes amino acid Cysteine to Tyrosine at position 233 in Wat1 protein (Fig. 5A).

Multiple sequence alignment studies show that Cysteine residue at 233 is conserved in yeast and human (Fig. 5B) indicating that this residue might be having important role in Wat1 function.

Mapping of wat1-17 Mutation based on Homology Modeling

To identify the structural basis for the function of the Wat1-17 mutant, homology modeling was performed as described in material and method. Sequence alignment revealed that Wat1 has significant sequence identity (~47%) with human Lst8 (Fig. 5B). Wat1 model depicted seven WD repeats consisting of only β-sheets (Fig. 6A, left). Overall structure appeared as β-propeller, where each repeat has four β-strands arranged in antiparallel fashion. Structural superimposition of Wat1 with Lst8 resulted in less than 0.5 Å root mean square deviation (rmsd), which confirms its relatedness at the structural level. In Wat1 model, we were more interested in exploring the position of C233Y mutation, which was found to be located in sixth repeat (Fig. 6A right). We hypothesize that the bulky nature of Tyrosine side chain at position 233 in wat1-17 mutant could alter the conformation of Wat1 protein (Fig. 6A, right, compare upper panel with lower panel) and hence affect the overall function of the protein.
The Mutant Wat1 Protein was Unable to Interact with Prp2

We further test the hypothesis that the substitution of Tyrosine residue at position 233 of Wat1-17 protein could affect its interaction pattern with their known interacting partners. Prp2 is the large subunit of U2AF and is required for pre-mRNA splicing [33,34]. Wat1 was isolated as interacting partner of Prp2 in a two hybrid screen [22]. Mutation in the prp2 (also known mis11) gene leads to the loss of mini-chromosomes indicating an important role of Prp2 in maintaining genomic stability [35]. We tested the interaction of Wat1-17 mutant protein with Prp2 by yeast two hybrid assays. As reported earlier [22] the strains expressing wild type copy of Wat1 and Prp2 protein produced blue color on plates containing X-gal and were able to form colonies on plate lacking histidine (Fig. 6B) suggesting a positive interaction between two proteins. More interestingly cells expressing Wat1-17 mutant protein and Prp2 protein were unable to produce blue color on...
plates containing X-gal and were unable to form colonies on plates lacking histidine (Fig. 6B) indicating the loss of interaction due to mutation in Wat1 protein.

Discussion

A complex haploinsufficient screening with the chkl knockout was carried out to identify the genes related to checkpoint function. This led to the identification of a ts17 mutant that code for the wat1 gene. Wat1 is a highly conserved protein that consists of seven WD repeats [18]. Budding yeast lst8, a homolog of wat1 is an essential gene for survival and acts as a positive regulator of the TOR complex [20,36]. Wat1 is also known to interact with Prp2, the large subunit of the essential splicing factor U2 auxiliary factor [23]. The conditional synthetic lethality of wat1-17 with chklΔ indicates the requirement of Chk1 for the recovery of wat1-17 mutant cells at semi permissive temperature (18°C). The reduction in the number of shorter microtubules in the wat1-17 mutant at semipermissive temperature could be due to the loss of cytoplasmic microtubules at low temperature as previously reported [37,38]. In the absence of Chk1, loss of microtubules might affect the survival of the cells due to the loss of spindle checkpoint pathway in yeast and human cells [13,39]. There is another possibility that the reduction of the α-tubulin protein level in wat1-17 chklΔ could result in shorter microtubules at 18°C. This could lead to chromosome segregation defects. In fact, the sensitivity of the chkl deletion wat1-17 double mutant towards the microtubule destabilizing drug, TBZ, suggests a possible requirement of Chk1 for the recovery of wat1-17 mutant cells under defective microtubule conditions. However only 8% chromosome segregation defect in double mutant does not coincide with the loss of survival at semi-permissive temperature, suggesting that the reduced viability at 18°C in wat1-17 chklΔ cells could be due to the defects in additional pathway such as stress response as Wat1 protein has been shown to interact with the components of TOR complex [40]. Target of Rapamycin (TOR), an evolutionally conserved phosphatidylinositol kinase –related protein controls cell growth in response to nutrients and growth factor.

At 18°C wat1-17 mutant exhibits genome diploidising defects as it fail in cell division after genome duplication. The broader DNA peak in wat1-17chklΔ cells at the semi permissive temperature indicates increase in ploidy. Increase in ploidy could be due to the chromosome segregation defect that has been visualized in the form of increased aberrant nuclei in the wat1-17chklΔ double mutant as compared to the single mutant. Two classes of genes have been implicated for the maintenance of ploidy. The first class of mutants is defective in regulating DNA replication and allows re-replication within one cell cycle [41,42]. The other class of mutants exhibit increase in ploidy and chromosome segregation defects due to the defects in spindle pole body duplication, kinetochore attachment and microtubule formation [43–46]. The wat1-17 chklΔ double mutant falls in the second class of mutants that posses significant defects as evidenced by the reduction in α-tubulin protein level, shorter microtubule structure, as well as a majority of the cells exhibiting increase in ploidy.

The protein kinase Chk1 is a well-established signal transducer in the DNA damage checkpoint. Recent studies have presented...
evidence to indicate that Chk1 also plays a critical role in the spindle checkpoint [13,39] and has also been implicated to delay metaphase to anaphase transition in *S. pombe* and *Drosophila* [31,13,14]. Chk1 has been shown to be required for the mitotic arrest in response to taxol treatment, a drug that stabilizes microtubules [47]. Genetic interaction studies have identified that Msc1, a multi-copy suppressor of Chk1, promotes cell survival in the absence of Chk1 and also that it requires an intact mitotic spindle checkpoint [48,49]. In the same series, the work presented here further emphasizes the requirement of Chk1 in response to defective microtubule and suggests a possible role for Chk1 in the mitotic spindle checkpoint pathway. However further work need to be done to strengthen our understanding of the spindle checkpoint involving Chk1 and Wat1.

The mutation in the *wat1*-17 mutant allele was found to be located at position 233 in the sixth repeat. This mutation changes the Cysteine residue to Tyrosine. Structural analysis suggests that the bulky nature of Tyrosine side chain in the *wat1*-17 mutant could alter the overall conformation of Wat1. This can then affect its interaction with other proteins and hence affect its function. Less likely alternate possibility is that the adjacent Cysteine residue at 265 position could be responsible for the formation of disulfide bond with Cys233. The presence of Tyrosine at this position in the *wat1*-17 mutant can result in the disruption of this disulfide bond, this in turn can affect the overall function of the Wat1 protein. In agreement with our hypothesis the Wat1-17 mutant protein was unable to interact with Prp2 suggesting that the bulky nature of Tyrosine residue indeed affects its interaction with the partner.

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**Author Contributions**

Conceived and designed the experiments: SV RR VK MS SA. Performed the experiments: SV RR VK. Analyzed the data: SV RR VK MS SA. Contributed reagents/materials/analysis tools: MS SA. Wrote the paper: MS SA.
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