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**USP1 deubiquitinase maintains phosphorylated CHK1 by limiting its DDB1-dependent degradation**

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**Objectives**

Checkpoint kinase 1 (CHK1) activity controls cell cycle checkpoints and DNA repair. It is a small part of an interconnected web of DNA repair mechanisms and cell cycle checkpoints. Phosphorylation of CHK1 can be caused by ataxia telangiectasia and RAD3 (ATR), and CLASPIN for optimal activation. The phosphorylated CHK1 will eventually control cell cycle arrest and DNA repair. CHK1 also regulates proliferating cell nuclear antigen (PCNA) and Fanconi anemia, complementation group D2 (FANCD2) monoubiquitinylation, as well as activation of a homologous recombination protein, RAD51.

Not only does phosphorylation activate CHK1, it also promotes its degradation. The phosphorylated form S345 may be marked by cullin-4a- or cullin-1-ubiquitinylation for proteasomal degradation. Another checkpoint termination possibility is the degradation of CLASPIN, which is needed for the initial activation of CHK1. Without CLASPIN, ATR-mediated phosphorylation may not take place.

Fanconi anemia (FA) patients have mutations in DNA damage response (DDR) genes, and must use the FANC pathway to resist interstrand DNA crosslinks (ICLs) produced from toxic lesions. There are 15 necessary FANC proteins. Eight are needed for the FANCcore complex to cause monoubiquitinylation of FANCD2 (mono-Ub-FANCD2) during S phase or in response to DNA damage. Also, CHK1 activity affects the accumulation of FA cells in late S/G2 phase. Researchers wanted to look at CHK1 activation in cells with a constitutively activated FANC pathway, and did so by knocking down deubiquitinase ubiquitin-specific peptidase 1 (USP1), which is responsible for stopping the monoubiquitinylation of FANCd2. This led to the main objective of the paper, which was to observe the USP1-regulated and damage-specific DNA-binding protein 1 (DDB1)-dependent ways to downregulate activated CHK1, with and without the FANC pathway.

**Experimental Approach and Results**

Since FANCD2 monoubiquitinylation can restrain checkpoint signaling of CHK1, a higher level of mono-Ub-FANCD2 should create a lower amount of phosphorylated CHK1. Depleting USP1 would increase the amount of FANCD2 monoubiquitinylation, allowing a closer look at the FANC pathway’s effect on CHK1.

Researchers did a series of experiments using siRNAs. First, USP1 deubiquitinase was depleted in U2OS cells and HeLa cells. This created larger amounts of mono-Ub-FANCD2 and mono-Ub-PCNA, without genomic stress. In the U2OS cells, this depletion showed a reduction in the amount of CHK1, but the results were more inconsistent with the HeLa cells. However, when seven different experiments were quantitatively analyzed, HeLa cells showed that a USP1-deficiency created significantly lower CHK1 levels than the HeLa cells that had USP1. These experiments revealed that the decrease in CHK1 steady-state levels was due to the reduction of USP1. When FANCcore complex proteins FANCC or FANCD2 were depleted along with USP1, the steady-state level of CHK1 was replenished. So, monoubiquitinylation of FANCD2 could be largely involved in USP1-dependent control of CHK1 steady-state levels.

Also, the experiments used mitomycin C and ultra violet C (MMC and UVC) to create genotoxic stress in order to observe “DNA damage-induced” p-CHK1 in USP1-depleted cells. When USP1 was depleted, there was a large reduction of the level of phosphorylated s345-CHK1 in both types of cells. Researchers used several controls to show that this reduction was specific. Also, the removal of the FANCcore complex protein FANCA, which is needed in the FANC pathway for FANCD2 monoubiquitinylation, showed a restoration of p-CHK1 in the stressed USP1-depleted cells. This shows that the FANC pathway, and especially monoubiquitinylation of FANCD2, may be needed to show the USP1 depletion effects.

A chicken form of monoubiquitinylated FANCD2 was expressed in HEK293T cells to observe the role of mono-Ub-FANCD2 in CHK1 regulation. This ectopic expression increased p-CHK1 and phosphorylated histone 2 AX, showing DNA stress. When the monoubiquitinylated FANCD2 was overexpressed along with genotoxic stress, however, phosphorylated and non-phosphorylated forms of CHK1 were lessened. The histone 2 AX served as a control and did not change to show the effects ofFANCD2 monoubiquitinylation were specific for CHK1.

To be certain that CHK1 level and phosphorylation of CHK1 are controlled by mono-Ub-FANCD2, a vector with CHK1-FLAG was expressed in MRC5 fibroblasts. This was done after depletion of USP1 or FANCD2. Of course, when USP1 was absent, there was an increase amount of FANCD2 and less CHK1. When FANCD2 was depleted, there was a higher amount of CHK1, as well as phosphorylation of the protein.

Next, FANCD2 knockout DT40 cells were observed under MMC exposure, which again represents genotoxic stress. The knockout cells were also ectopically corrected with WT FANCD2, or with FANCD2 cells that had a non-monoubiquitinylable lys 563 arg (KR). MMC treated DT40 cells without FANCD2 showed hyperphosphorylation of CHK1. Expression of WT FANCD2 brought CHK1 phosphorylation of CHK1 to normal, but the FANCD2-KR form did not correct the hyperphosphorylation. This shows the role of the FANC pathway, specifically the monoubiquitinylation of FANCD2, in CHK1 level and phosphorylation.

To see if factors besides mono-Ub-FANCD2 control CHK1 levels, USP1 was removed from Fanconi anemia patient cells FA-C, FA-G, and FA-D2. In these cells, when USP1 was removed, CHK1 levels were decreased as well as its phosphorylation. This meant that mono-Ub-FANCD2 is not the only CHK1 regulation factor that is affected by USP1. Also, the FANC pathway inactivation in these FA cells will cause them to find another way to survive without that particular pathway for checkpoints and DNA repair.

Next, due to the nature and role of CHK1, researchers wanted to make sure the decrease in CHK1 wasn’t just due to experimental changes of the cell cycle and/or changes made to the DNA repair mechanisms when USP1 had been depleted. They did so by observing the cell cycle distribution of the cells without USP1. The results showed that there was no major effect on the cell cycle without MMC genotoxic stress. Therefore, the lowered level of CHK1 seen in the experiments with depleted USP1 cells was not due to changes made in the cell cycle because of the lack of USP1. Also, researchers looked at cell cycle recovery of both U2OS cells and HeLa cells in the presence of MMC. The U2OS cells that were depleted of USP1 proved to reenter the cell cycle more rapidly from the G2 checkpoint than the control cells. This could mean the depletion of USP1 could speed up recovery from the checkpoint. However, the HeLa cells with the USP1 deficiency had an increase of genotoxic-induced G2/M content when compared to the control cells with USP1, which showed a longer G2 checkpoint. The main difference in HeLa cells and U2OS cells lies in their p53 status, so these experiments were performed again in the presence of a p53 inhibitor, but the results were the same. To look further for differences, a western blot of CHK1 and another checkpoint kinase CHK2 in each of the cells lines was done. HeLa cells proved to have higher overall phosphorylated amounts of each of the checkpoint kinases. This could explain the longer G2 checkpoint when USP1 is depleted and CHK1 is decreased. However, another study, using mouse embryonic fibroblasts, showed high levels of phosphorylated CHK2, but not CHK1, after genotoxic stress. So, in the end, researchers concluded the CHK1 decrease in USP1 depleted cells does not affect G2 checkpoints.

Researchers then wanted to find whether the lowered levels of CHK1 and p-CHK1 were due to increased DNA repair capabilities in the USP1-depleted cells. A dot blot was performed looking at the repair kinetics of 6-4 pyrimidine-pyrimidone lesions that were made by UVC exposure. These lesions were repaired equally well in cells with and without USP1. Therefore, the repair capabilities worked the same, with that particular lesion, whether or not USP1 is present.

 To show the carefully regulated ubiquitinylation/monoubiquitinylation cycle of FANCD2, immunofluorescence analysis of FANCD2 foci formation was observed. Since USP1 depletion leads to higher amounts of mono-Ub-FANCD2, it was possible that enhanced DNA repair by mono-Ub-FANCD2 was causing the lessened CHK1 activity. However, there was a defect in FANCD2 foci formation, and ϒH2AX, which is usually needed for FANCD2 subnuclear foci assembly, formed normal foci in the absence of USP1 and was therefore not the cause. This was another result that showed CHK1 degradation is due to USP1 depletion, and not by better DNA repair capabilities in USP1-depleted cells.

 Finally, the research focused on the DDB1-dependent degradation of CHK1. CLASPIN is a controller of phosphorylated CHK1, but researchers showed that when CLASPIN was depleted, only the UVC-induced phosphorylated form of CHK1 was lowered, and not the steady-state amounts. However, USP1 depletion they had earlier shown to be a regulator of steady-state and p-CHK1, but, significantly, did not affect CLASPIN amounts. Co-depletion of CLASPIN and USP1 combined created an even lower amount of CHK1 and p-CHK1, which shows that CLASPIN does not affect USP1 regulation of CHK1, but the two are on a convergent pathway to activate or restrain CHK1.

 DDB1’s role in the steadiness of CHK1 was then directly tested by DDB1 depletion. Researchers were surprised to find that when DDB1 was silenced, USP1 levels were reduced. However, the amount of USP1 in the DDB1-depleted cells was still enough to keep equal levels of mono-Ub-FANCD2 to FANCD2. When USP1 and DDB1 were both depleted, there were higher CHK1 levels than when USP1 was alone depleted. Also, mono-Ub-FANCD2 was not affected by depletion of DDB1 compared to depletion of USP1. This could show that in USP1-depleted cells, CHK1 down-regulation will have to happen downstream of FANCD2. A proteasome inhibitor MG-132 shows increased CHK1 in USP1-depleted cells only, which shows CHK1 downregulation to be an effect of proteasomal degradation. Finally, the interaction was seen between DDB1 and endogenous CHK1 in the cell line HEK293T, to associate the degradation of CHK1 with DDB1.

**Conclusion**

 Researchers showed CHK1 activity to be controlled by USP1 in order to rescue damaged cells. CHK1 holds a part in cell cycle regulation as well as DNA repair. CHK1 also causes monoubiquitinylation of PCNA and FANCD2, which help as well with replicative stress. DDB1 appears to be activated by mono-Ub-FANCD2 and degrade activated CHK1. In this paper, researchers showed USP1 to control the DDB1-dependent degradation of CHK1, in order to save DNA damaged cells.

**Future Research**

 Since a recent PCNA/FANCD2 interaction has been identified, future research in this area might look into whether this interaction is needed for mono-Ub-FANCD2. Also, as DDB1 needs CUL4-associated factors (DCAFS) for targeting, perhaps there is a specific DCAF for CHK1 that is regulated by mono-Ub-FANCD2? Finally, a FA patient, without typical symptoms, has been identified that contained bi-allelic mutations in both *FANCA* and *FANCM.* The *FANCM* mutation appears to perhaps compensate for FANCA deficiency. FANCA causes overactive CHK1, but FANCM is needed for CHK1 activation by ATR. Without the overactive CHK1, there are no FA symptoms. This case is very interesting and perhaps a further look could into the bi-allelic mutations.