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Role of the XIAP-Copper Axis in Prostate Cancer

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14. ABSTRACT During the last year we have been able to identify, confirm, and characterize a novel functional and physical interaction between the X-linked inhibitor of apoptosis (XIAP) and the copper chaperone for superoxide dismutase (CCS). We performed a targeted genetic screen in yeast to identify proteins involved in delivery of copper (Cu) to XIAP. This screen identified CCS as a primary mediator of Cu delivery to XIAP in yeast, and we subsequently determined that CCS delivers Cu to XIAP in mammalian cells as well. In addition, XIAP targets CCS for ubiquitination through its E3 ubiquitin ligase activity. This ubiquitination event seems to be proteasome-independent and leads to enhancement of CCS activity rather than CCS degradation. Taken together, our results over the first year of this project have shed substantial light on the interplay between XIAP and copper homeostasis. These studies have the potential to significantly improve our understanding of not only prostate cancer but also other disorders in which XIAP or Cu metabolism is deregulated.					
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INTRODUCTION

X-linked inhibitor of apoptosis (XIAP) is commonly deregulated in a number of solid tumor types, including prostate cancer. Similarly, copper (Cu) homeostasis is often altered in malignant tumors and has been implicated in angiogenesis and metastasis, among other processes. XIAP was recently shown to play a role in regulating cellular Cu metabolism and to be regulated by binding to Cu. This project aims to further delineate the role of XIAP in Cu metabolism and determine how deregulation of Cu handling and XIAP function might cooperate in the onset and progression of prostate cancer.

BODY

I have made substantial progress toward completing the tasks outlined in the original Statement of Work, as outlined below:

AIM 1. How does XIAP affect copper homeostasis in prostate tumors?

These experiments are in progress. Due to time constraints and technical issues with our in-house atomic absorption spectrometer, we have not completed measurements of copper content from prostate tissue samples as described in Aim 1. During year two of this project, we intend to obtain copper measurements from these samples using the more sensitive ICP-MS instrument available on campus in the University of Michigan Department of Geological Sciences.

AIM 2. How is copper delivered to XIAP?

Optimize protocol for monitoring delivery of copper to human XIAP in *S. cerevisiae*.

I was able to establish a reliable protocol for growing yeast transformed with a plasmid encoding human XIAP in Cu-free selective medium. Supplemental Cu was added to the medium 1-2 hours before cells were harvested for western blotting, which allowed me to accurately monitor delivery of Cu to XIAP. This objective is complete.

Introduce human XIAP into yeast deletion strains. We selected 16 deletion strains from the same background as our wild-type control (BY4741) for analysis. These 16 strains represented deletions all of the available Cu chaperones and transporters including the major Cu importer (Ctr1) and the Wilson/Menkes disease protein homolog (Ccc2). Also included in the 16 strains were several representing putative Cu-binding proteins (eg. Cox11, Cox19, Pet309) and enzymes required for synthesis of the Cu-binding small molecule glutathione (Gsh1, Gsh2). These 16 strains were obtained from Open Biosystems and transformed with the XIAP expression plasmid. This objective is complete.

Assess yeast deletion mutants for delivery of copper to XIAP. After transformation with the XIAP-expressing plasmid, the 16 yeast deletion strains were compared to wild-type yeast in Cu delivery to XIAP, as determined by western blotting. All mutants tested seemed to deliver Cu to XIAP with comparable efficiency to wild-type yeast except for the strain lacking the copper chaperone for superoxide dismutase (Ccs). In side-by-side comparisons with wild-type yeast and yeast lacking superoxide dismutase (Sod1), the Ccs

deletion strain showed a unique and highly reproducible defect in delivery of Cu to XIAP. This objective is complete.

Confirm findings from yeast in mammalian cells. Embryonic fibroblasts from mice deficient in Ccs, along with littermate controls, were generously provided by Dr. Jonathan Gitlin (Washington University, St. Louis). Fibroblasts lacking Ccs consistently demonstrated a defect in Cu delivery to endogenous Xiap compared to littermate controls, confirming our findings in yeast with ectopically expressed human XIAP. As additional confirmation, we reduced CCS expression in human embryonic kidney (HEK) 293T cells by transient transfection of small interfering RNA (siRNA) oligonucleotides. Reduction in CCS expression produced a corresponding decrease in Cu delivery to XIAP. Conversely, overexpression of human CCS in HEK 293 cells enhanced Cu delivery to endogenous XIAP. Taken together, our data from yeast and mammalian cells demonstrate a role for CCS in delivering Cu to XIAP. This objective is complete.

AIM 3. How does copper availability affect prostate tumor development in the presence and absence of XIAP?

The experiments proposed in Aim 3 have not been initiated in the first year as we originally planned. We hope it will be possible to complete the animal trial proposed in Aim 3 during the second year of the project.

In addition to the progress described above, I have made substantial progress in characterizing the physical and functional interaction between XIAP and CCS. As described in Aim 2, CCS seems to be the primary mediator of Cu delivery to XIAP in mammalian cells. The role of CCS in Cu delivery seems to be direct, as CCS does not have any effect on Cu export or uptake in mammalian cells (1). In addition, XIAP binds to CCS in HEK 293 cells in a manner that depends on the second domain of CCS. This is the same CCS domain that mediates its interaction with SOD1, suggesting that XIAP might dock with CCS to receive Cu in a similar manner to SOD1. Interestingly, a point mutant of XIAP that lacks E3 ubiquitin ligase activity (H467A) co-precipitates with CCS more efficiently than wild-type XIAP, suggesting that CCS might be a target for ubiquitination by XIAP. We have found that XIAP does in fact induce ubiquitination of CCS, but it seems to be a proteasome-independent event that does not lead to CCS degradation. Rather, XIAP-mediated ubiquitination of CCS seems to positively regulate CCS activity, as CCS-mediated metallation of SOD1 is subtly but consistently reduced in lysates from cells in which XIAP expression is reduced by siRNA transfection.

Regulation of CCS by ubiquitination has been previously described, whereby Cu-bound CCS is targeted for proteasome-mediated degradation (2). In order to determine whether XIAP-mediated ubiquitination of CCS and Cu-induced ubiquitination of CCS represented different pathways, we decided to map the ubiquitinated lysine (Lys) residues of CCS by mass spectrometry. By expressing untagged XIAP with affinity tagged CCS and ubiquitin in HEK 293 cells, we used a two-step purification scheme to obtain ubiquitinated CCS for analysis and identified four lysines on CCS that were conjugated to ubiquitin. Substitution of all four lysines with arginine does not alter binding of CCS to SOD1, suggesting that these lysines are not important to the overall three-dimensional structure of CCS. However, absence of the ubiquitinated lysines does seem to impair

activation of SOD1, which suggests a functional role for ubiquitination in CCS-mediated metallation of SOD1. Interestingly, XIAP seems to preferentially ubiquitinate CCS at Lys 241, while the Cu-induced ubiquitination of CCS does not exhibit a strong preference for any of the four lysines. The presence of Lys 241 seems to be critical for XIAP to affect activation of SOD1 by CCS, suggesting that ubiquitination of CCS at Lys 241 specifically enhances CCS activity. Studies are ongoing to determine the mechanism by which ubiquitination might enhance either Cu acquisition or Cu release to SOD1 by CCS.

KEY RESEARCH ACCOMPLISHMENTS

- Ccs was identified as the primary mediator of Cu delivery to exogenous human XIAP in yeast.
- Mammalian CCS was determined to mediate Cu delivery to endogenous XIAP in mouse and human cells.
- A physical interaction between XIAP and CCS was identified and found to depend on the second domain of CCS.
- XIAP was found to enhance activation of SOD1 by CCS, perhaps through CCS ubiquitination.
- The sites of ubiquitination on CCS were mapped by mass spectrometry to four lysine residues.
- XIAP-mediated ubiquitination of CCS was found to occur predominantly at Lys 241.
- Substitution of Lys 241 in CCS was found to alter its activation of SOD1 while leaving its ability to bind to SOD1 intact.
- The effect of XIAP on CCS-mediated SOD1 activation was found to depend on Lys 241, suggesting that ubiquitination by XIAP at this residue specifically enhances Cu acquisition or Cu transfer by CCS.

REPORTABLE OUTCOMES

This work was formally presented in the following settings:

- University of Michigan Medical Scientist Training Program Annual Retreat (August 2008)
- University of Michigan Graduate Program in Cellular and Molecular Biology Annual Symposium (September 2008)
- University of Michigan Department of Pathology Annual Symposium (October 2008)
- University of Michigan Department of Pathology Research Seminar Series (December 2008)

CONCLUSION

In the first year of this project, I have made substantial progress toward a better understanding of the role of XIAP in cellular Cu metabolism. The identification of CCS as a primary mediator of Cu delivery to XIAP is intriguing and presents a potential

therapeutic approach for targeting XIAP in prostate tumors. Cu-bound XIAP is unstable, yet XIAP levels remain high in many tumors despite elevated intracellular Cu levels. A drug that could mimic CCS or promote interaction of endogenous CCS with XIAP might prove useful by increasing the amount of Cu bound to XIAP and thus reducing XIAP levels.

Regulation of SOD1 activation by XIAP through ubiquitination of CCS is surprising and suggests a potential mechanism by which XIAP might enhance tumor cell survival independently of its well-described function in directly blocking apoptosis, by promoting activation of an enzyme that detoxifies reactive oxygen species. Together, the results of year one provide valuable insights into the junction between XIAP and Cu biology and how deregulation of XIAP might intersect with aberrant Cu homeostasis in prostate cancer development.

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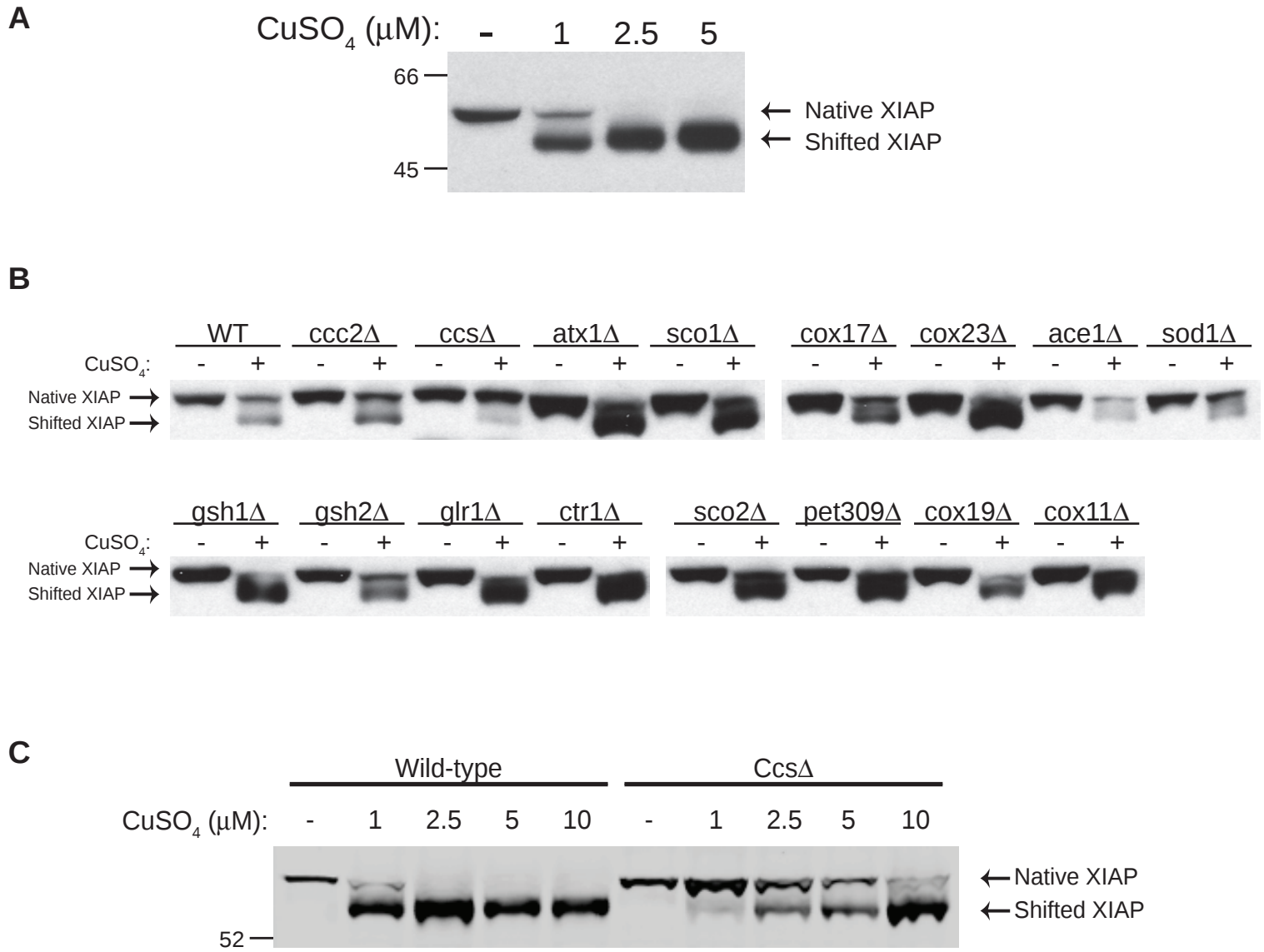


Figure 1. The copper chaperone for superoxide dismutase-1 (Ccs) mediates Cu delivery to human XIAP expressed in *Saccharomyces cerevisiae*. (A) Wild-type yeast were transformed with a plasmid containing the coding sequence of human XIAP, grown in selective medium, and treated with 0-5 μM CuSO₄ for 2 hours. Cu delivery to XIAP was determined by SDS-PAGE and western blotting with an antibody directed to human XIAP. Note that Cu-bound XIAP migrates faster than native XIAP under denaturing, reducing SDS-PAGE conditions. (B) Wild-type yeast and the indicated yeast deletion strains were transformed with human XIAP and treated with 0 or 5 μM CuSO₄ for 2 hours prior to analysis by western blot as in (A). (C) Wild-type yeast and yeast lacking Ccs were transformed with human XIAP and treated with 0-10 μM CuSO₄ for 1 hour.

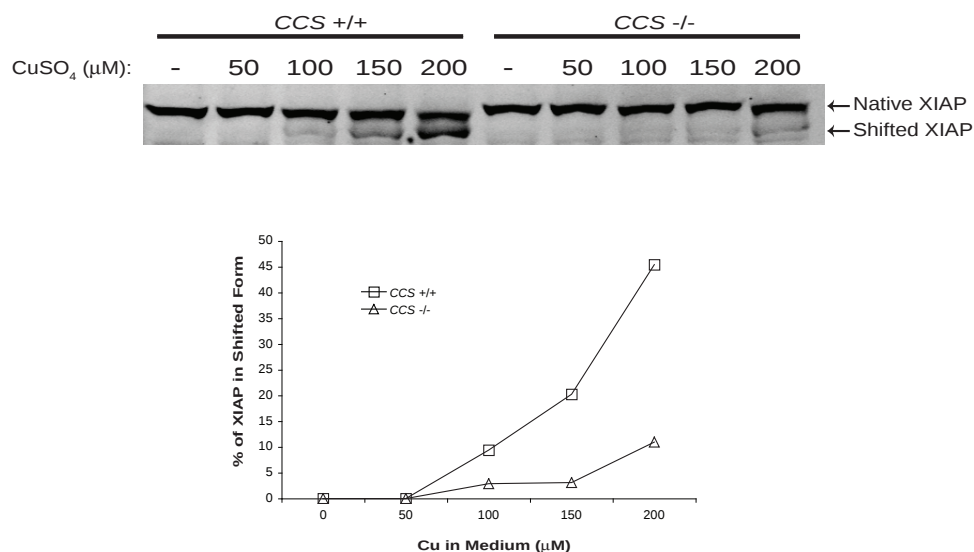
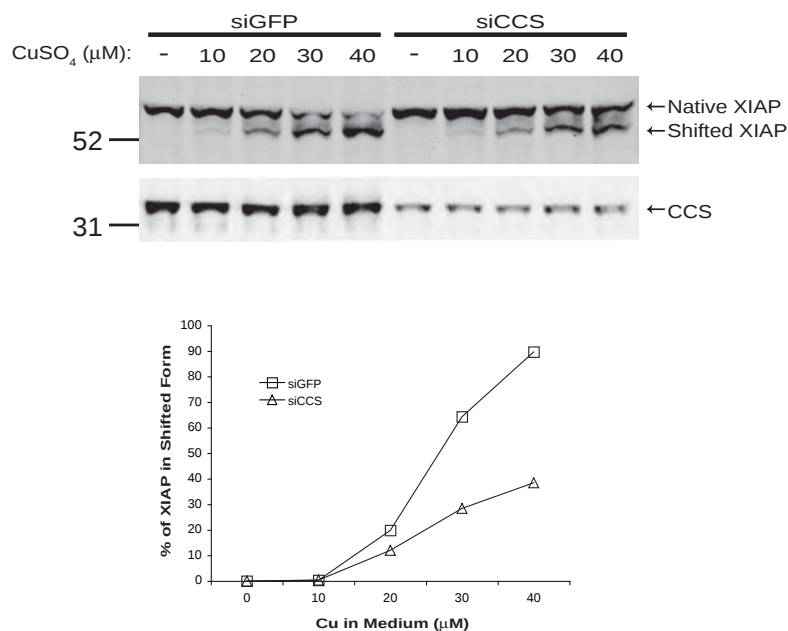
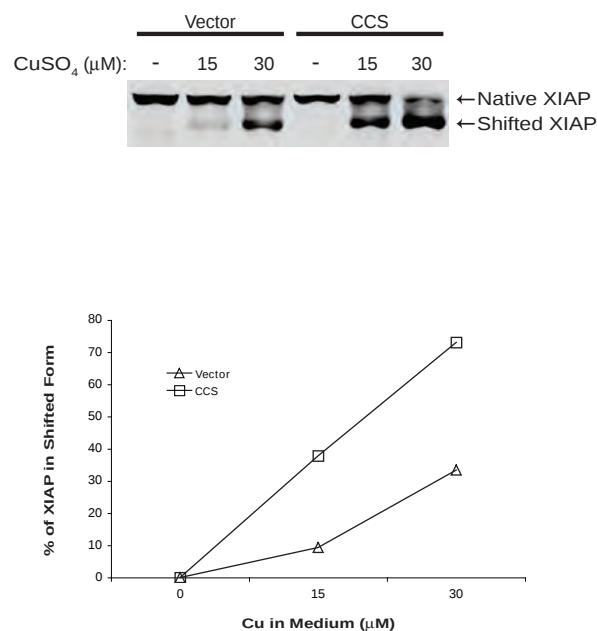
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Figure 2. CCS mediates Cu delivery to XIAP in mammalian cells. (A) Embryonic fibroblasts derived from either wild-type or *Ccs*-deficient mice were treated with 0-200 μM CuSO_4 for 48 hours. Cu delivery to XIAP was determined by SDS-PAGE and western blotting. **(B)** HEK 293T cells were transfected with siRNA oligos targeting either CCS or GFP, incubated for 48 hours, and treated with 0-40 μM CuSO_4 for 24 hours prior to lysis and analysis by western blotting. **(C)** HEK 293 cells were transfected a plasmid encoding human CCS or empty vector, then treated with 0-30 μM CuSO_4 for 48 hours and analyzed by western blot as in (A) and (B).

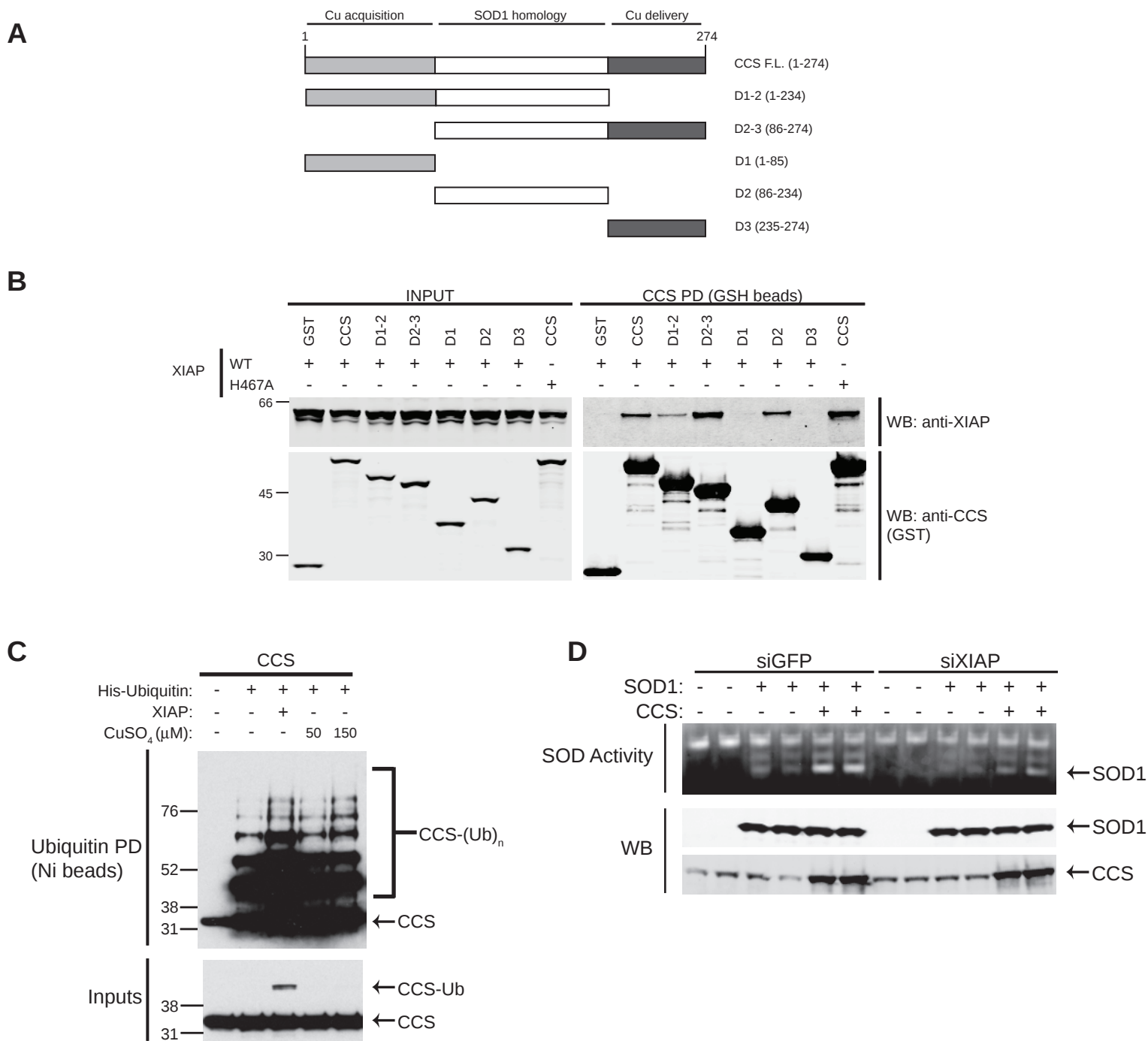


Figure 3. XIAP binds to and ubiquitinates CCS. (A) Schematic representation of CCS domains. (B) Full-length CCS and the indicated CCS deletion constructs with a C-terminal GST tag were co-expressed in HEK 293 cells with either wild-type XIAP or a point mutant of XIAP that lacks E3 ubiquitin ligase activity (H467A). CCS was precipitated from whole cell lysates with glutathione (GSH) beads, and pulldown samples were analyzed by western blotting. (C) CCS was co-expressed in HEK 293 cells with histidine-tagged ubiquitin, and cells were treated by either co-transfecting XIAP or adding 50-150 μM CuSO₄ to the culture medium for 24 hours. Ubiquitinated material was recovered from whole cell lysates by incubation with nickel-coated beads and analyzed by western blotting. (D) Human SOD1 was expressed in HEK 293T cells alone or with human CCS. One day prior to transfection of plasmid DNA, cells were transfected with siRNA oligos targeting either GFP or XIAP. Cell lysates were analyzed for SOD1 activity by in-gel nitroretetrazolium staining.

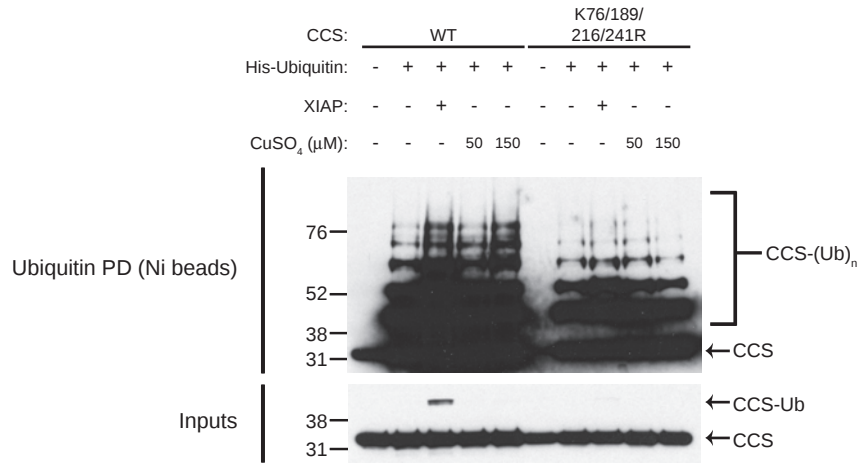
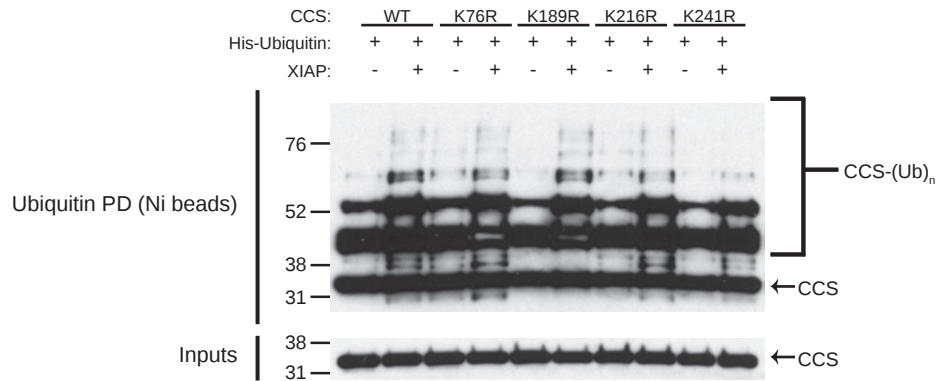
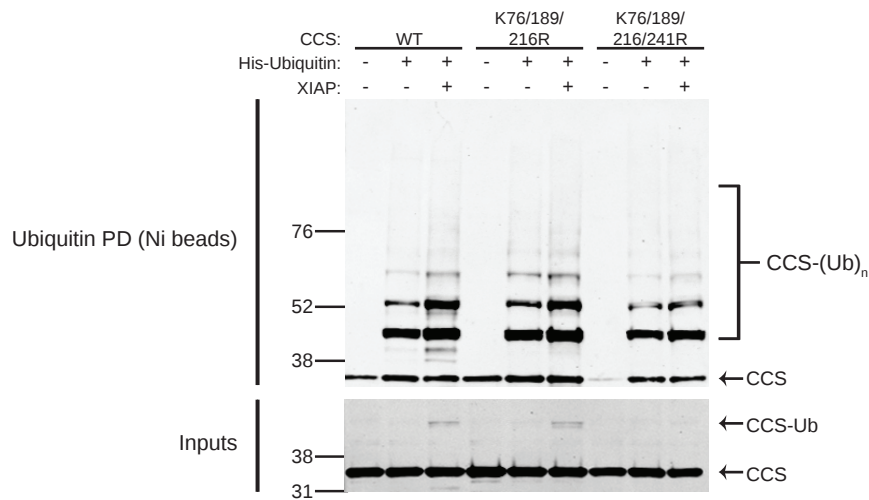
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Figure 5. Preferential ubiquitination of CCS at lysine 241 by XIAP. (A) Wild-type CCS or a mutant of CCS in which lysines 76, 189, 216, and 241 were replaced by arginines was expressed in HEK 293 cells with histidine-tagged ubiquitin. The effects of XIAP and Cu treatment on CCS ubiquitination were determined by western blotting of nickel pulldowns. (B) The effect of XIAP on wild-type CCS and individual lysine mutants was determined as in (A). (C) The effect of XIAP on the K76/189/216R mutant of CCS was compared directly to wild-type CCS and the quadruple lysine mutant of CCS as in (A) and (B).

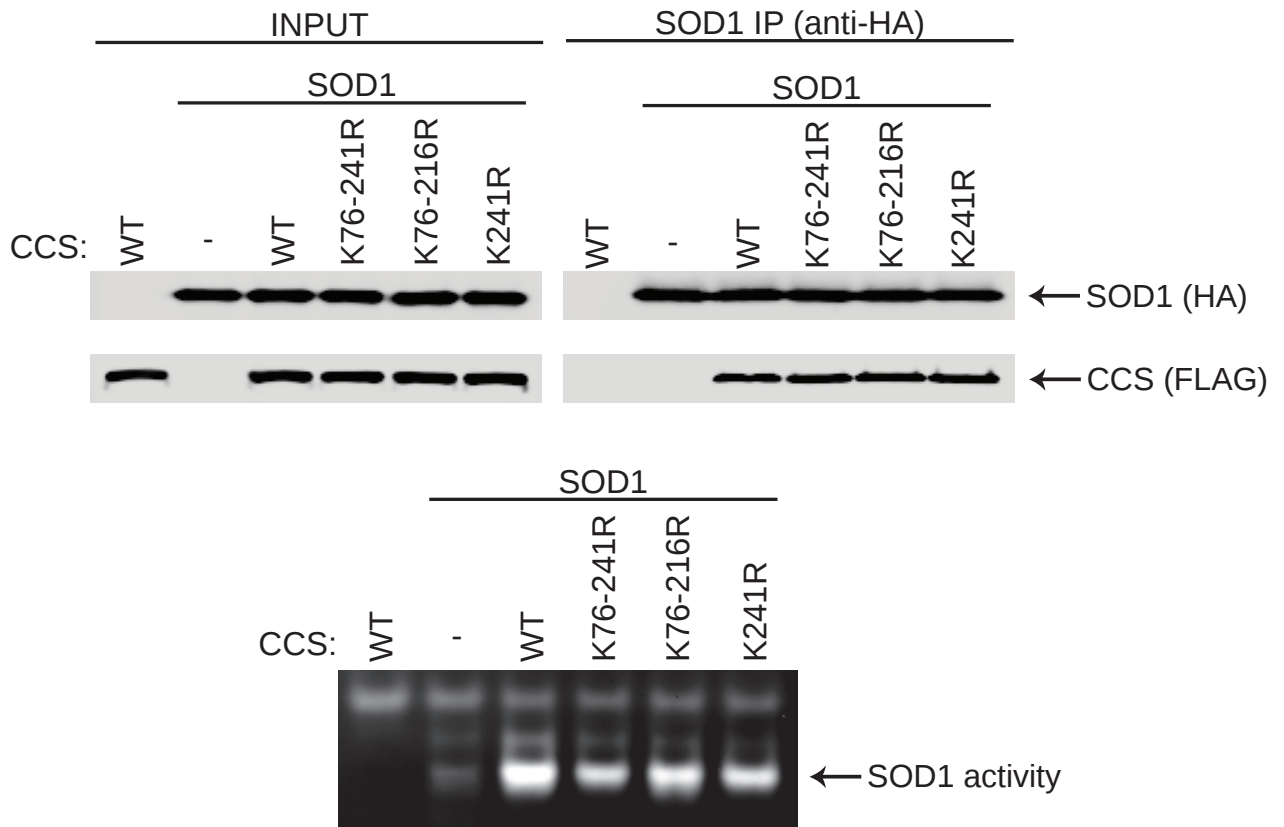
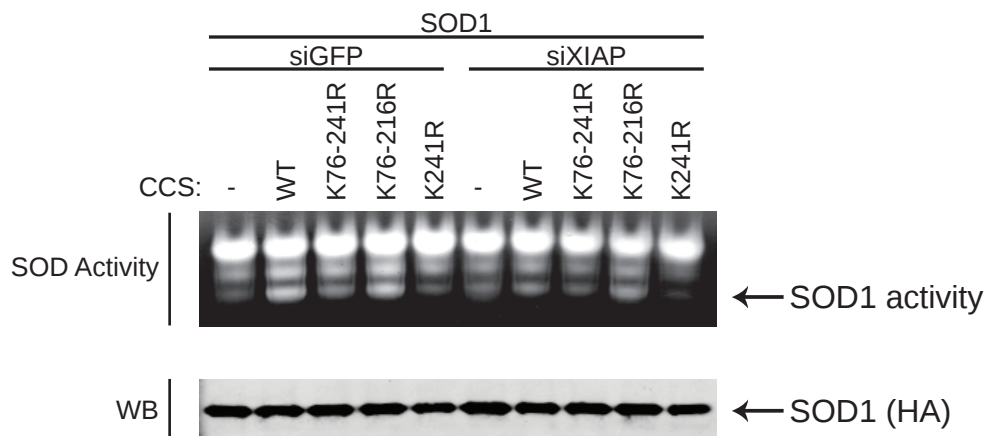
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Figure 6. XIAP-mediated ubiquitination of CCS positively regulates SOD1 activation in cells. (A) Human SOD1 was expressed in HEK 293T cells with wild-type CCS or the indicated lysine mutants of CCS. Two days after transfection, SOD1 was precipitated with an antibody directed to the HA tag, and CCS was detected in the immunoprecipitate with an anti-FLAG antibody (top panel). Whole lysates were analyzed for SOD1 activity by in-gel nitrotriazolium staining (lower panel). (B) The effect of XIAP on CCS activity requires the presence of Lys 241. The indicated CCS mutants were expressed together with SOD1 in cells that had been transfected one day prior with siRNA targeting either GFP or XIAP.