In Vivo and *in Vitro A*ssays to Determine UBR7 in Association with sNASP and Histones H3/H4

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Research Question

Histone chaperones are essential for the reincorporation of evicted and newly synthesized histones back into the nucleosome. Genomic stability and health is highly dependent on the smooth running of this process and is therefore an area of great interest. Here we focus on the interaction between novel protein UBR7 with nuclear molecules. We also consider the possibility of ubiquitin, a highly abundant and conserved protein, being used a molecular tag to aid this reincorporation.

UBR7, a putative E3 ligase enzyme, has been observed in close association with histone chaperone sNASP and histones H3 and H4. However, detail on its binding mechanisms and binding sites are unknown. Truncations were performed on UBR7 and mutations on H3 to determine crucial amino acids for binding.

Here we show that L378, I401, F405 amino acids are important for binding of UBR7 to histones H3/H4 when present as a monomer through fluorescent two-hybrid analysis of UBR7/H3 truncations/mutations.

Methods

1. Western Blot analyses were carried out to demonstrate the putative E3 ligase activity of UBR7. Reaction buffer components were adapted from *Zhang et al. 2017* [1], SDS -Page gels were transferred to nitrocellulose membranes using an iBlot 2 Dry Blotting System (ThermoFisher), blocked with 2.5% BSA, and incubated with Streptavidin -HRP antibodies to detect bio-ubiquitin, anti -H3 to detect H3 histones at a 1:2000 dilution each for 2 hours, before being analysed using ECL.

2. Induced knockout of H3 monomer and dimer binding sites on sNASP in transfected HEK293 cells enabled subsequent analyses post-Co-IP using 4-20% gradient SDS-PAGE gels and western blots. Aimed to determine if NASP could pull down H3 in a non-ubiquitylation fashion.

3. Co-immunoprecipitations utilized GFP-trap Sepharose beads to isolate sNASP from transfected HEK293. Cells were lysed, followed by protein purification, and bead equilibration before use.

4. FIJI ImageJ and R programming were utilised to quantify the *in vivo* microscopy data, measuring and comparing light intensity of the region of interest spot.

Results

In Vitro Ubiquitination Assay

An ubiquitination assay was performed to demonstrate the ubiquitin ligase activity of UBR7 towards the potential substrate histone H3, which could increase its binding affinity for sNASP, based on findings of Pardal and Bowman (2022). However, this assay failed to demonstrate mono ubiquitination of H3 in vitro within the given assay conditions.

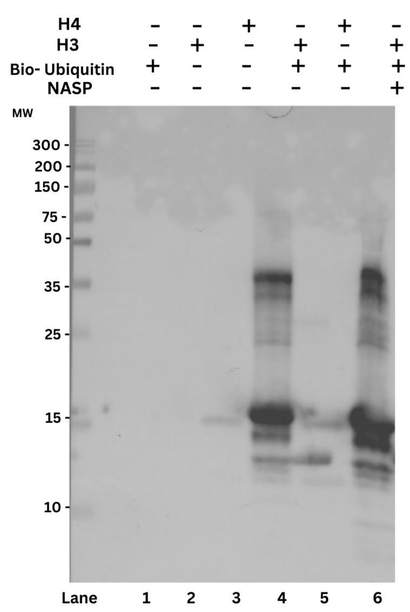

Figure 1: **Putative UBR7 ubiquitination activity of H3 histones.** Western Blot analysis of ubiquitination assay, probed with anti –H3 antibodies on 15% SDS-Page gel. All lanes contained UBA1 and UBC12 as the specific E1 and E2 enzymes. H4 was used as a negative control. Bands at around 38 kDa may indicate polyubiquitinated H3. No bands are seen higher than 48 kDA indicating that H3 has not bound to UBR7.

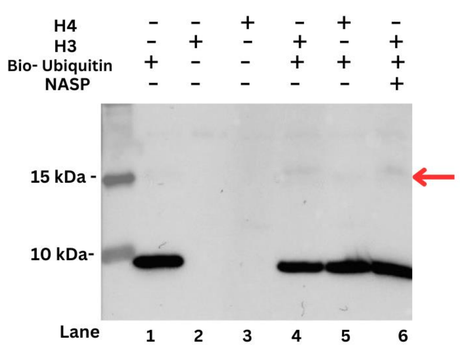

Figure 2. Probing interactions of biotinylated ubiquitin with streptavidin conjugated HRP. Clear reservoirs of ubiquitin were detected at 7 kDa, but none at the expected levels of 55kDa. Faint bands were detected at 15 kDa and higher, however, this was regarded as polyubiquitin chains formed in the reaction mixture.

H3 and H4 Mutations

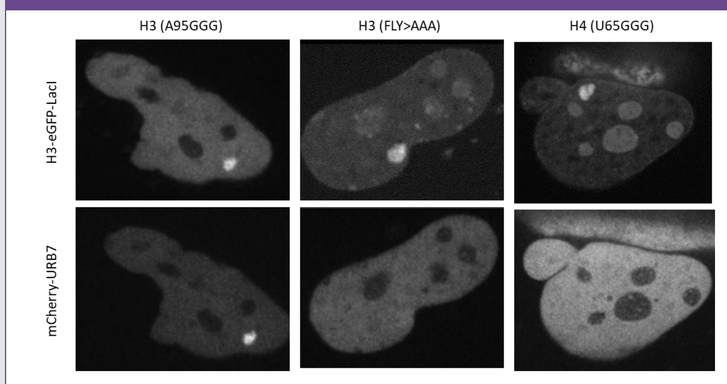

Figure 3. H3 and H4 mutations can affect H3 interaction with UBR7. *In vivo* confocal microscopy clearly show the specific residues required for UBR7 interaction with H3 and H4. Both H3 and H4 can recruit UBR7 despite difference in binding site.

UBR7 Truncations

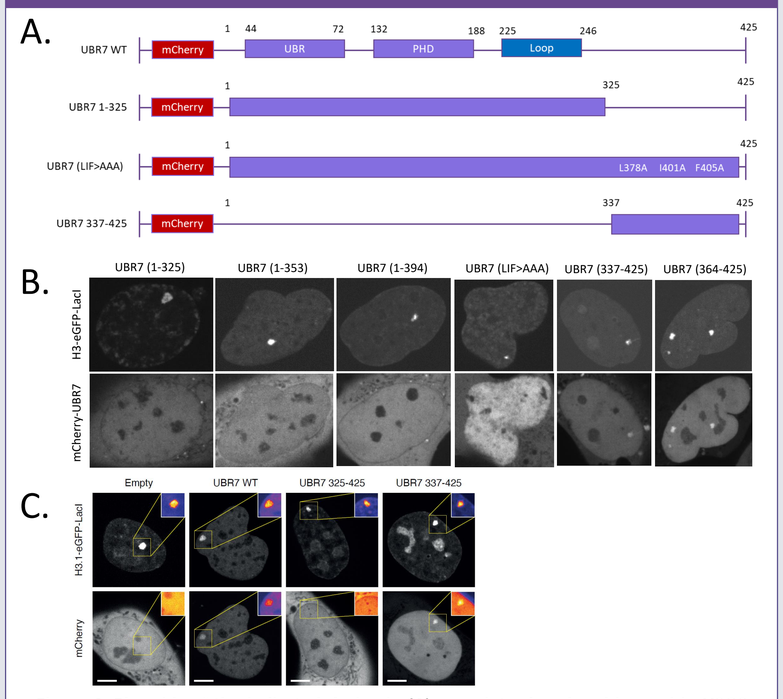

Figure 4. Plasmids of the indicated design in (A) were introduced and imaged in U2OS 2b2 cells. B. *In vivo* confocal microscopy showed clear association with UBR7 terminal C domain containing alpha helices involved in H3 binding. In their absence, no interaction can be seen. **C.** Colour and contrast added to highlight region of interest.

Co-Immunoprecipitations

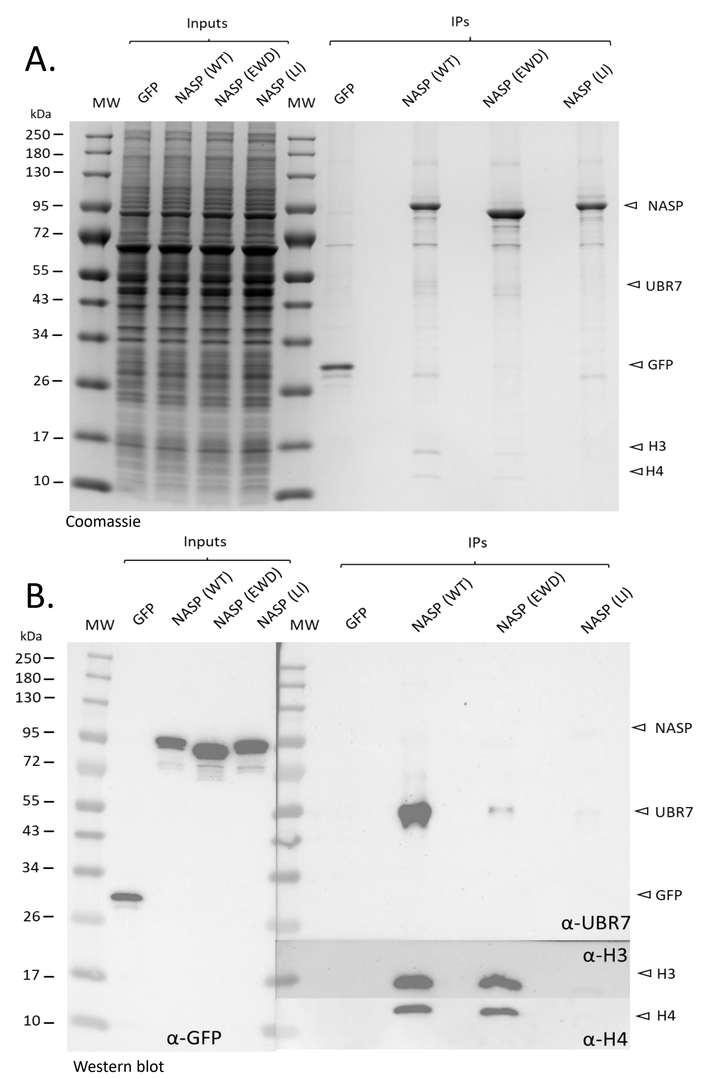

Figure 5. Both of NASP's H3/H4 binding sites appear essential for UBR7 recruitment. **A.** NASP Coomassie stained Co-IPs on 4-12% gradient SDS-PAGE. Co-IPs for GFP, eGFP-NASP wildtype (WT), monomeric NASP mutant (EWD), and dimeric NASP mutant (LI) from stable HEK293 cell lines. **B.** Western blot analysis of NASP-UBR7-H3/H4 interaction. Monomeric mutation results in decreased UBR7 recruitment, similarly for H3 and H4. Dimeric mutation greatly decreases NASP-UBR7 interaction, similarly for H3 and H4. Note UBR7, H3, and H4 are still discernible here.

Discussion

- Due to the highly specific nature of E2 enzymes, there is a possibility that UBC12 is not compatible with UBR7/sNASP, leading to a halt in ubiquitin conjugation to the protein. This theory could be further tested by incorporating other E2 enzymes into the assay such as UBE2K and UBCH5C.
- UBR7 C-terminal domain is necessary for H3 recruitment due to binding site alpha helices involved in the interaction. Issue with nuclear import, more experiments involving the signal peptide will need to be done to investigate the reason for poor localization.
- H3 monomer binding site KO on NASP expectedly affects UBR7 recruitment however the dimer binding site also affects this. Suggests H3/H4 may play a role in recruiting UBR7 to NASP as a mediator.
- Overall, this clearly demonstrates essential amino acids for binding sites for UBR7 recruitment. Further mass spectrometry could solidify findings.

References

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