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In charge: Targeting neddylation of UBE2M for anticancer therapy

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Abstract

- NEDD8 is a ubiquitin-like modifier protein whose pathway is organized similarly to the ubiquitin pathway with an E1-E2-E3 enzyme cascade.
- The NEDD8 pathway has garnered interest as a therapeutic target due to its upregulation in multiple cancer types.
- UBE2M, one of the two identified NEDD8 E2s, catalyzes two reactions: transthiolation of NEDD8 from a thioester on the E1 to a thiol on its active-site cysteine, and aminolysis transfer of NEDD8 to a target lysine. Both reactions require a 26-residue N-terminal docking peptide and a core catalytic domain.
- Described here are the effects of catalytic (C111A/C111S) and N-terminal peptide mutants on UBE2M NEDD8 charging, the effect on substrate neddylation and the DNA damage and re-replication phenotype induced by catalytic site UBE2M mutants.
- It was found that mutating the N-terminal peptide abrogates interaction with the E1. Interestingly, N-terminal mutants accumulate more neddylated UBE2M suggesting a defect in modifying downstream substrates.
- Results reveal the complexity of the neddylation cascade and raise the question if reducing discharge of NEDD8 from UBE2M creates a vulnerability in cancer cells that could be exploited by new therapeutics.

The neddylation pathway involves a NEDD8 transfer cascade

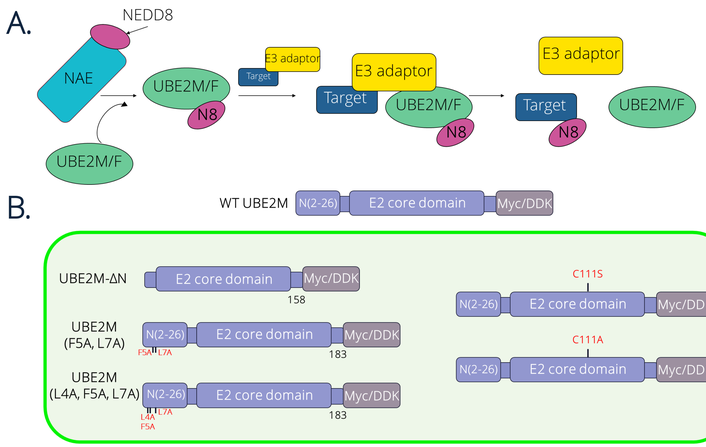

Figure 1: A) Illustration of the steps in the neddylation of target proteins. The pathway involves an activating enzyme (NAE), 2 E2s and multiple E3s. Cullins and their activity, critical E3s in the ubiquitin pathway, are major targets of the neddylation pathway. B) Domain structure of UBE2M and 5 mutants that display altered neddylation capabilities. C) Molecular dynamics (MD) of relaxed E1-E2 complex. D) Location of N-terminal deletion and previously annotated deleterious mutants. E) Probability distribution of fraction of short-range charged-E1/E2 complex binding potential energy (ΔU) attributed to the N-terminus of E2 (residues 3-26) across 10000 MD relaxation snapshots generated from five independent trajectories. Red: UBE2M, Orange/Gray: NEDD8, Blue/Yellow: NAE, Green beads: deleterious mutation sites. Model derived from RCSB PDB ID: 2NVU with remodeling performed to remove chimeric residues.

The UBE2M N-terminus and catalytic cysteine are required for NEDD8 discharge

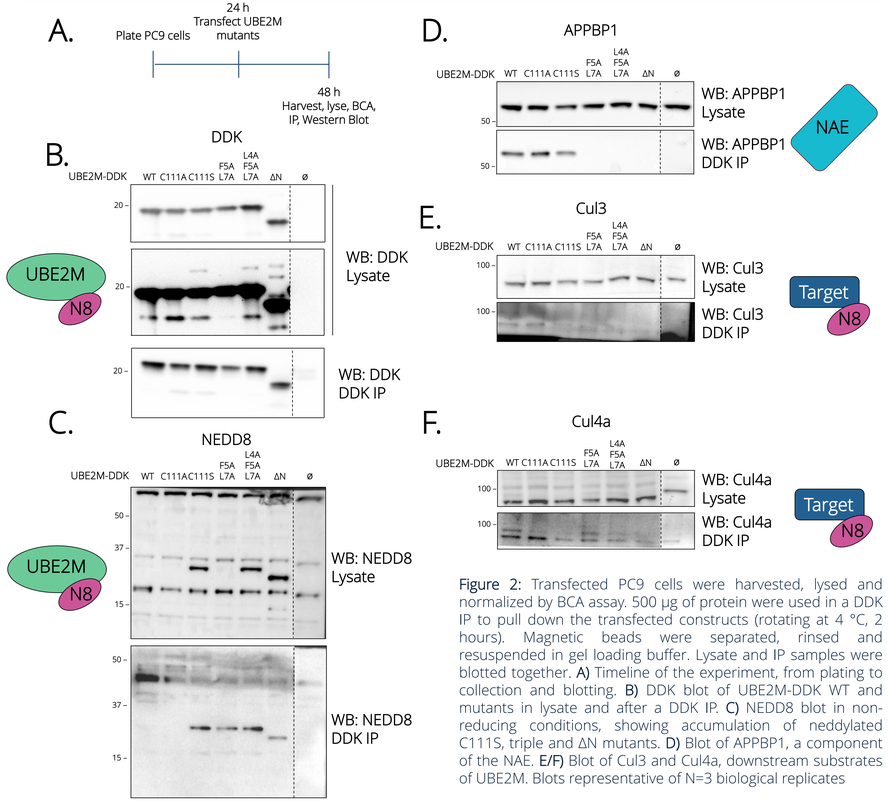

Figure 2: Transfected PC9 cells were harvested, lysed and normalized by BCA assay. 500 µg of protein were used in a DDK IP to pull down the transfected constructs (rotating at 4 °C, 2 hours). Magnetic beads were separated, rinsed and resuspended in gel loading buffer. Lysate and IP samples were blotted together. A) Timeline of the experiment, from plating to collection and blotting. B) DDK blot of UBE2M-DDK WT and mutants in lysate and after a DDK IP. C) NEDD8 blot in non-reducing conditions, showing accumulation of neddylated C111S, triple and ΔN mutants. D) Blot of APPBP1, a component of the NAE. E/F) Blot of Cul3 and Cul4a, downstream substrates of UBE2M. Blots representative of N=3 biological replicates

The C111 mutants of UBE2M are cytotoxic in the absence of UBE2F

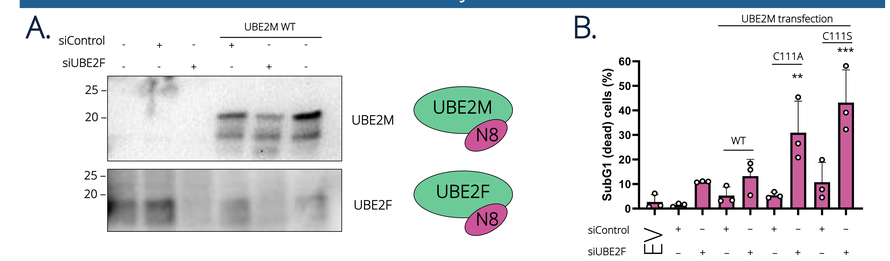

Figure 3: Knockdown of UBE2F was validated by Western Blot, with no significant effect on transfected UBE2M. The effect of UBE2M construct transfection on viability in a UBE2F WT/KD setting was assessed by flow cytometry. A) Western Blots from PC9 cells transfected with siUBE2F and the C111 mutants. PC9 cells have very low levels of endogenous UBE2M. B) The effect of the C111 mutants on cell death was assessed in a UBE2F WT/KD setting by flow cytometry. Significant cell death was observed in cells co-transfected with UBE2M mutants and siUBE2F, suggesting that UBE2F can compensate for deficient UBE2M. N=3, mean + SD. One-way ANOVA **: p<0.01, ***: p<0.001.

UBE2M C111 mutants induce re-replication of DNA in a Cdc7-dependent mechanism

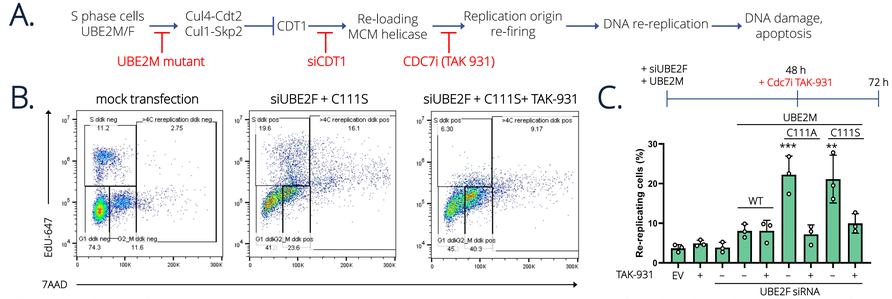

Figure 4: Cells transfected with WT or mutant UBE2M were stained with 7AAD and Edu-647 for cell cycle analysis. Cells to the far right have DNA content >4C and are undergoing re-replication. Inhibition of Cdc7 rescued the re-replication phenotype, demonstrating that cells are truly re-replicating and not aneuploid or undergoing another mitotic error. A) Pathway connecting UBE2M/F to re-replication through Cul1/Cul4, CDT1 and Cdc7. B) Gating strategy to identify cells in different cell cycle stages and undergoing re-replication during S-phase. C) Quantification of the effect of the C111 mutants on re-replication of DNA in PC9 cells. The C111S and C111A mutants induce re-replication in UBE2F KD cells, and this effect is rescued by pharmacologically inhibiting Cdc7, which controls replication fork firing. N=3, mean + SD **: p<0.01 ****: p<0.001 One-way ANOVA

UBE2M C111 mutants induce re-replication and DNA damage through CDT1

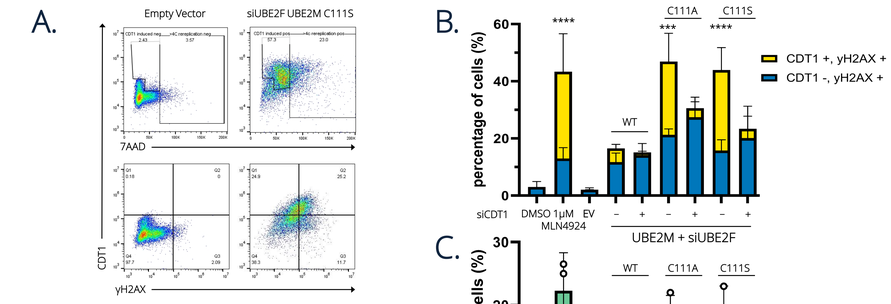

Figure 5: PC9 cells were labelled with a CDT1 antibody and either 7AAD or a γH2AX antibody to assess DNA damage and re-replication by flow cytometry A) Gating strategy to identify PC9 cells positive for re-replication (7AAD) and DNA damage (γH2AX). B) Bar graph of percentage of CDT1$^+$/γH2AX$^+$ and CDT1$^-$/γH2AX$^+$ cells expressing UBE2M mutants. C) Quantification of re-replication in cells expressing the UBE2M mutants. N=3 biological replicates ****: p<0.0001, ***: p<0.001, One-way ANOVA

Conclusions

- The UBE2M N-terminus and catalytic cysteine are required for proper E2 activity. Mutations in these critical regions inhibit the charging (C111A) or discharging (C111S, N-terminal mutants) of Nedd8 from UBE2M
- Dysfunctional discharge of Nedd8 from UBE2M results in DNA re-replication in a Cdc7-dependent mechanism. Cullin4-dependent CDT1 degradation, along with a CDT1-independent mechanism downstream of UBE2M neddylation are the primary mechanisms of DNA damage
- Inhibiting the discharge of Nedd8 from UBE2M creates a vulnerability in cancer cells that could be exploited by new therapeutics or combinations with current treatments