UBC13 Deamidation by *Shigella flexneri* Disrupts its Native and Transient Interactions with TRAF6 to Impair Ubiquitination

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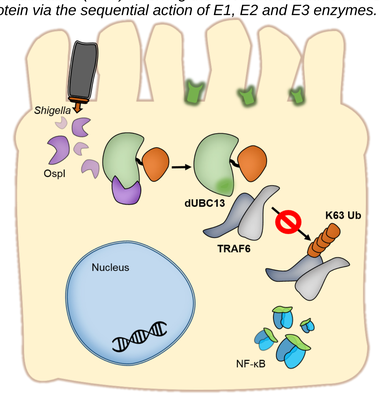
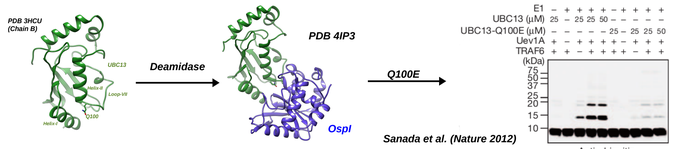
Introduction

Ubiquitination is a post-translational modification (PTM) involving the covalent attachment of Ubiquitin (Ub) onto the lysine residue of a target protein via the sequential action of E1, E2 and E3 enzymes.

In the TNF-α independent Nf-κB pathway, the UBC13(E2)/TRAF6(E3) complex is crucial for the downstream activation of TRAF6. This promotes the phosphorylation/ degradation of IκBα by the IKK complex which leads to entry of NF-kB into the nucleus



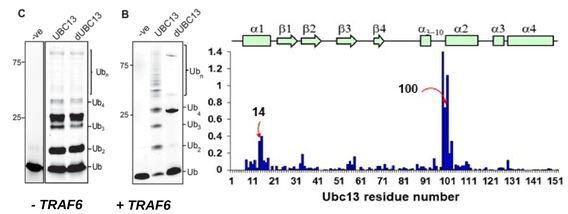
*The deamidase OspI secreted by Shigella flexneri weakens NF-κB activity by deamidating UBC13 at Q100 (**ref. 3**). In-vitro ubiquitination assays of TRAF6/UBC13 Q100E (dUBC13) revealed that ubiquitin chain formation was abolished.*



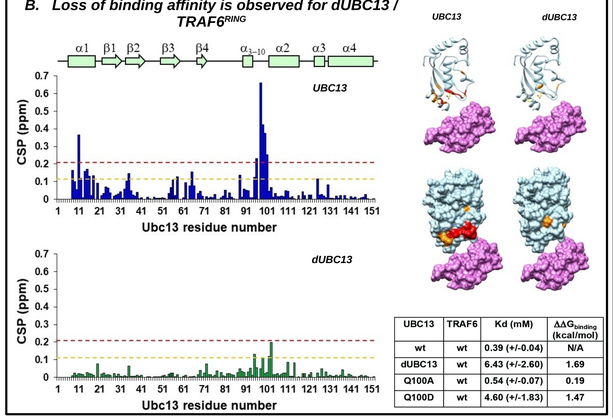
In this study, we employed NMR spectroscopy, MD simulations and in vitro activity assays to obtain mechanistic insights into the impairment of UBC13 activity with TRAF6^{RING} upon enzymatic deamidation.

Deamidation abolishes the interaction between UBC13 and TRAF6^{RING}

A. Structure and function of dUBC13 / dUBC13-Ub interaction remains unaffected



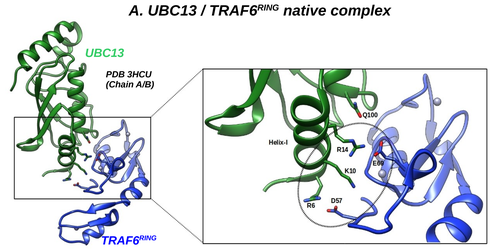
B. Loss of binding affinity is observed for dUBC13 / TRAF6^{RING}



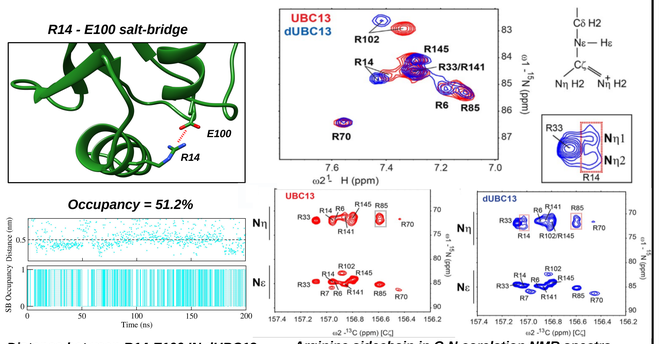
UBC13	TRAF6	Kd (mM)	$\Delta\Delta G_{binding}$ (kcal/mol)
wt	wt	0.39 (+/-0.04)	N/A
dUBC13	wt	6.43 (+/-2.60)	1.69
Q100A	wt	0.54 (+/-0.07)	0.19
Q100D	wt	4.60 (+/-1.83)	1.47

Deamidation triggers formation of an intra-molecular R14-E100 salt-bridge

A. UBC13 / TRAF6^{RING} native complex



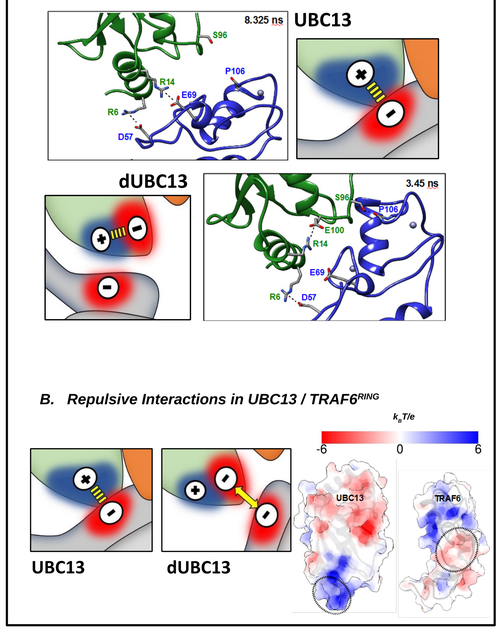
B. Intramolecular salt-bridge between R14-E100 was detected in dUBC13 in MD Simulation and NMR



Distance between R14-E100 IN dUBC13 — *Arginine sidechain in C-N corelation NMR spectra*

Salt-bridge competition destabilizes UBC13/TRAF6^{RING} in native complex

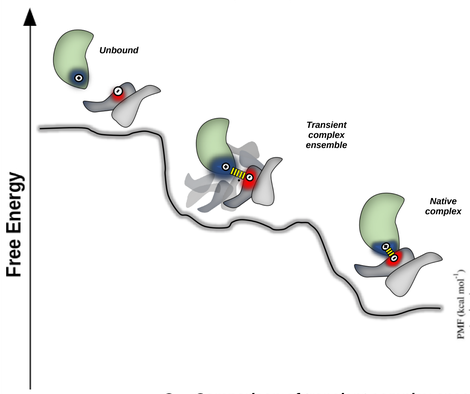
A. UBC13 / TRAF6^{RING} dissociation by Steered MD Simulation



B. Repulsive Interactions in UBC13 / TRAF6^{RING}

Deamidation enables dUBC13/TRAF6^{RING} dissociation in transient complexes by salt-bridge competition and repulsive interactions

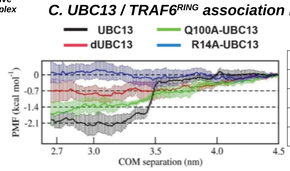
A. Mechanism of weak protein-protein association



B. Association Rate Calculation using Tran-comp webserver

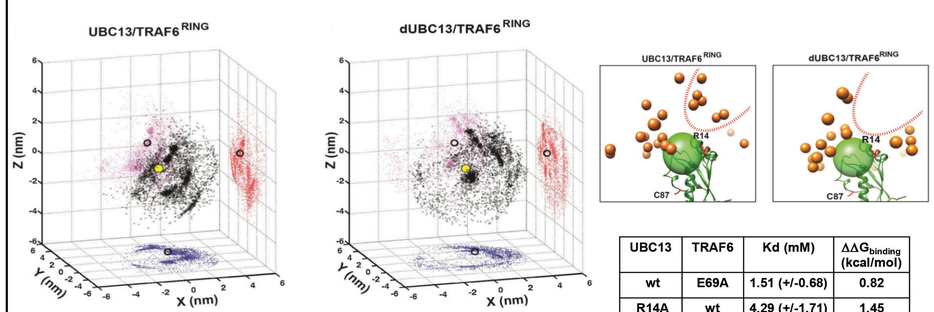
	10 mM NaCl		100 mM NaCl	
Transient complex Ensemble	ΔG_{el} (kcal mol^{-1})	k_{on} (10^6 .M^{-1}s^{-1})	ΔG_{el} (kcal mol^{-1})	k_{on} (10^6 .M^{-1}s^{-1})
UBC13/TRAF6	−2.32	28.7	−1.33	5.43
dUBC13/TRAF6	−1.59	9.36	−0.99	3.43
R14A-UBC13/TRAF6	−1.16	4.42	−0.62	1.78

C. UBC13 / TRAF6^{RING} association PMF by Umbrella Sampling



Complex	ΔG_{PMF} (kcal/mol)
UBC13/TRAF6	−2.03 (±0.27)
dUBC13/TRAF6	−0.67 (±0.50)
Q100A UBC13/TRAF6	−1.64 (±0.49)
R14A UBC13/TRAF6	+0.04 (±0.38)

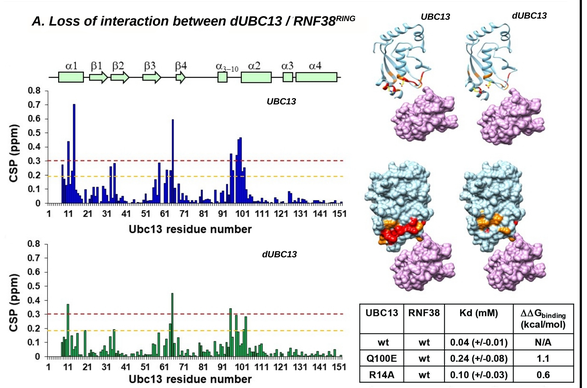
C. Comparison of transient complex ensembles of UBC13/dUBC13 with TRAF6^{RING}



UBC13	TRAF6	Kd (mM)	$\Delta\Delta G_{binding}$ (kcal/mol)
wt	E69A	1.51 (+/-0.68)	0.82
R14A	wt	4.29 (+/-1.71)	1.45

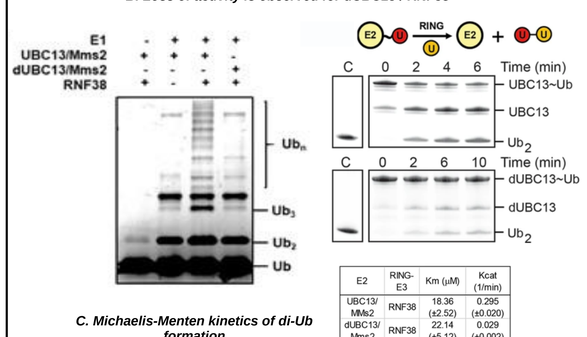
Deamidation selectively destabilises the transient complex ensemble of UBC13/RNF38^{RING}

A. Loss of interaction between dUBC13 / RNF38^{RING}



UBC13	RNF38	Kd (mM)	$\Delta\Delta G_{binding}$ (kcal/mol)
wt	wt	0.04 (+/-0.01)	N/A
Q100E	wt	0.24 (+/-0.08)	1.1
R14A	wt	0.10 (+/-0.03)	0.6

B. Loss of activity is observed for dUBC13 / RNF38^{RING}

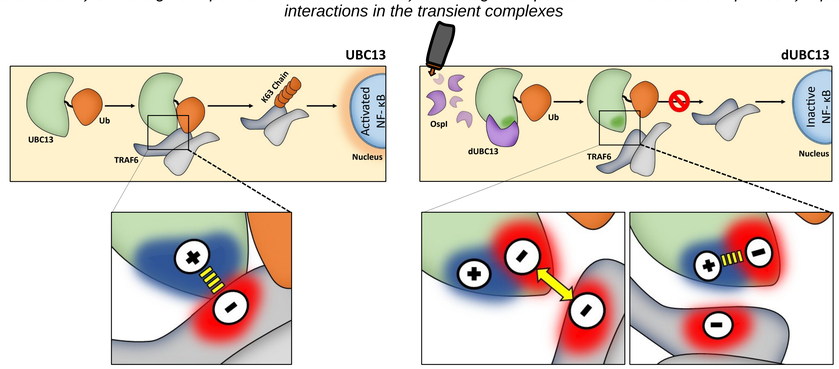


E2	RING-E3	Km (μM)	Kcat (1/min)
UBC13/ MMs2	RNF38	18.36 (±2.52)	0.295 (±0.020)
dUBC13/ Mms2	RNF38	22.14 (±5.12)	0.029 (±0.002)

C. Michaelis-Menten kinetics of di-Ub formation

Conclusion

UBC13 deamidation by Shigella effector disrupts its interaction with TRAF6 to hijack host immune response by three mechanisms: 1) Salt-bridge competition in the native state 2) Salt-bridge competition in the transient complexes 3) repulsive interactions in the transient complexes



Methods

- ***Initial structures and molecular modelling***. *Initial structures of UBC13 and TRAF6^{RING} for MD simulations and association rate constant (k_{on}) estimation were obtained from the PDB entry 3HCU (Chain A/B) which represents a model of the native complex. For MD simulations of TRAF6^{RING}, a C-terminal truncated model comprising of residues 50-148 was used which contains the RING domain within residues 70-109. For dUBC13 and other UBC13 variants, substitution mutations were introduced by replacing existing sidechains with the best aligning rotamer from the Dunbrack Rotamer library in UCSF Chimera. Electrostatic surface potentials of UBC13 and TRAF6^{RING} were calculated using the Adaptive Poisson-Boltzmann Solver (APBS) . In silico model of UBC13/RNF38^{RING} was generated by superposing RNF38^{RING} (PDB entry 4V3K, chain F) onto TRAF6^{RING} in its co-crystal complex with UBC13 using the structural alignment tool in UCSF Chimera.*
- ***MD simulations.*** *All simulation methodologies employed in the study were performed using the AMBER99SB*-ILDN force field in GROMACS version 4.6 and 5.1. The Zinc AMBER force field parameters were used to model the two zinc coordination sites within the TRAF6^{RING} domain. The initial structures were solvated in an appropriate cubic box using the TIP3P water model. The non-bonded ion parameters proposed by Joung and Cheatham were used to model Na^+, K^+ and Cl^- ions in TIP3P water. Additional non-bonded parameter corrections proposed by Yoo and Aksementiev for cation-chloride, amine-carboxylate and aliphatic carbon-carbon interactions were used for all simulations to eliminate an overestimation of the strength of these interactions. A suitable number of counter ions were added to neutralize the residual charge of the system, and additional ions were added to the box depending on the desired concentration. The electrically neutral, solvated system was then subjected to energy minimization using the steepest descent method for a maximum of 5000 steps until the maximum force on any atom was less than 1000 kJ mol^{-1} nm^{-1}. Production simulations were performed under periodic boundary conditions at a temperature of 300 K, and 1 bar pressure (NPT ensemble).*
- ***NMR spectroscopy.*** *All NMR titration experiments were recorded at 298K on 600 MHz Bruker Avance III HD spectrometer with a cryoprobe head. The samples were prepared in 25 mM Tris, 100 mM NaCl, pH 7.5 and 10% D_2O. Either ~2 mM TRAF6^{RING} or ~1 mM RNF38^{RING} was titrated into ~0.15 mM ^{15}N-UBC13, ^{15}N-dUBC13 and other mutants. The titration data was in 1:1 protein:ligand model using the equation $CSP_{obs} = CSP_{max}$ {{[P]$_t$+[L]$_t$+K_d) - [([P]$_t$+[L]$_t$+K_d)^2- 4[P]$_t$[L]$_t$]$^{1/2}$}/2[P]$_t$, where [P]$_t$ and [L]$_t$ are total concentrations of protein and ligand at any titration point.*
- ***Ubiquitination assay.*** *E1 (0.5 μM), UBC13 and MMS2 (5 μM) and Alexa Fluor Maleimide (Invitrogen) labelled UbS20C (20 μM) were incubated in 20 mM Tris, 5mM ATP, 5 mM $MgCl_2$ (pH 7.5) at 37°C for 30 minutes. The reaction mixtures were separated in 15% SDS gel, and the images were acquired in iBright FL1000.*

Acknowledgements

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