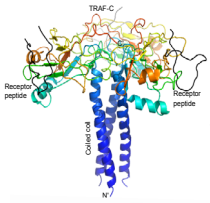
Understanding the extent of TRAF RING heterodimerization

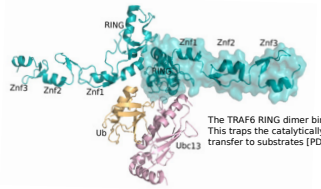
Anubrita Das, Adam J. Middleton, Peter D. Mace & Catherine L. Day
Department of Biochemistry, School of Biomedical Sciences
University of Otago, Dunedin, New Zealand

TRAFs as signalling proteins

- Tumour necrosis factor Receptor Associated Factors (TRAFs) are a family of seven adaptor proteins that regulate cell signalling in immune response pathways [1].
- TRAFs induce both cell survival and apoptosis via ubiquitylation of downstream substrates.
- Mutations in TRAFs have been linked to development of inflammatory diseases as well as certain types of cancer [1, 2, 3].



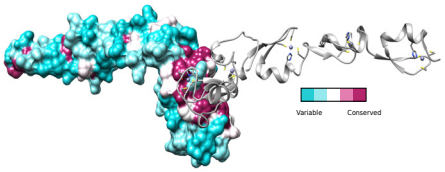
The TRAF-C and Coiled Coil (CC) domain form trimers [PDB: 1RF3]. Trimeric TRAFs interact with the cytoplasmic domains of various receptors.

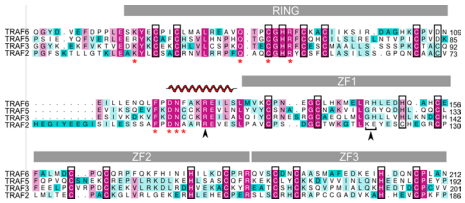


The TRAF6 RING dimer binds to the Ubc13~Ub conjugate. This traps the catalytically active form, allowing ubiquitin transfer to substrates [PDB: 5VO0].

- In TRAF6, the dimeric RING domain and Znf1 confer E3 ligase activity by binding to the E2 complex, Ubc13~Uev1a to promote formation of K63-linked polyubiquitin chains [4].
- The RING domain of one monomer interacts with Ubc13, whereas Znf1 of the other monomer stabilizes ubiquitin in the closed conformation. This explains why RING dimerization is required for E3 ligase activity.

Conserved RING dimer interface





Conservation surface mapping of the RING and Zf_1 of TRAFs 2, 3, 5 & 6 mapped on the structure of TRAF6 (PDB: 3HC5). Sequence alignment has same colouring as on the surface. Arrows indicate residues that stabilize ubiquitin.

The RING dimer interface of co-localized TRAFs is conserved, raising the possibility of TRAF RING heterodimer formation.

Acknowledgements

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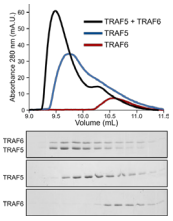
Aim

To determine whether TRAF RING domains form heterodimers that can promote ubiquitin transfer

TRAF RINGs form heterodimers

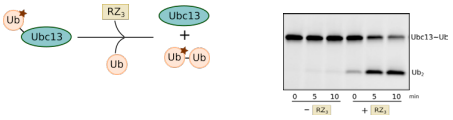


TRAF5 and TRAF6 RING & Znf_{1-3} (RZ_3) co-elute during analytical size exclusion chromatography, indicating RING heterodimer formation.

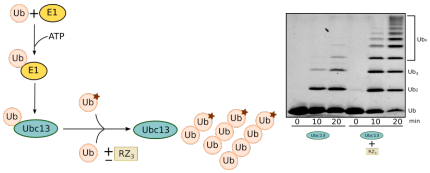
Activity assays

We have used two different assays to assess TRAF E3 ligase activity.

1. Discharge assay:



2. Chain-building assay:

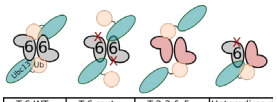


Ub Ubiquitin with a Cy3 fluorescent tag at its N terminal allows visualization using *Licor*.

TRAF RING heterodimers are active

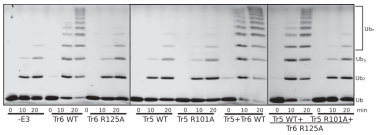
We have shown that homodimeric TRAF2, 3 and 5 are inactive with Ubc13. To establish if these TRAFs have an intact ubiquitin binding site we used an inactive TRAF6 Znf mutant that cannot bind Ub. If TRAF RINGs form heterodimers, and the Znfs of TRAF2,3 and 5 bind ubiquitin, we would expect recovery of activity.

Predicted Activity

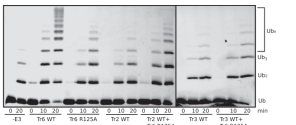


Tr6 WT	Tr6 mut	Tr2,3 & 5	Heterodimer
Active	Inactive	Inactive	Active

Assays



Activity was recovered with TRAF5/6 RZ_3 suggesting the Znf1 of TRAF5 can bind ubiquitin, implying the TRAF5/6 heterodimer is stable and active.

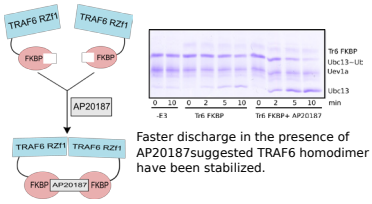


TRAF2 and TRAF3 do not recover activity. This suggests that ZnF1 of TRAF2 and TRAF3 do not bind ubiquitin, or that they do not form stable heterodimers with TRAF6.

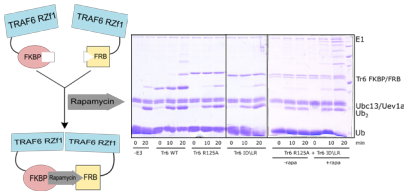
Controlling RING dimerization

To determine if RING dimer stability limited activity we established a system where RING dimerization can be controlled. We chose the Chemically Induced Dimerization (CID) technique that relies on small molecules to trap homo and heterodimers.

This technique has been established using TRAF6, which does not form a stable RING dimer.



Faster discharge in the presence of AP20187suggested TRAF6 homodimer have been stabilized.



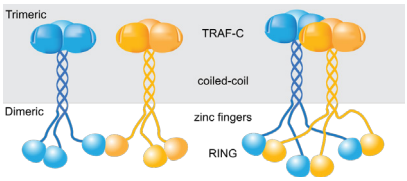
Rescue of ubiquitylation by TRAF6 mutants indicates stable heterodimer formation upon addition of rapamycin.

This technique can now be used to make stable homo- and heterodimers, and assess their activity.

TRAF Oligomerization

Structural studies show that the TRAF-C and coiled coil domains of TRAFs exist as homotrimers [1]. However, the RING domains are active as dimers. There is a symmetry mismatch between the trimers and the dimers.

Further investigations are required to dissect how the TRAF trimers and RING dimers organize to form signalling complexes at the membrane.



Summary

- TRAF6 RING dimerization is essential to lock Ubc13~Ub in the closed conformation.
- The dimer interface among TRAF RINGs is conserved.
- TRAF5 and TRAF6 form stable heterodimers.
- Like TRAF6, the Znf of TRAF5 can bind ubiquitin and the TRAF5/6 heterodimer is an active E3 ligase.
- CID technique can be used to make stable dimers. This will allow us to systematically analyse active TRAF pairs and E2s using a combinatorial approach.

A bidentate Polycomb Repressive-Deubiquitinase complex is required for efficient activity on nucleosomes

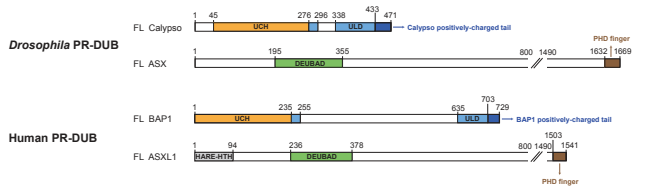
Martina Foglizzo[a], Adam J. Middleton[a], Abigail E. Burgess[a], Jennifer M. Crowther[b], Renwick C.J. Dobson[b,c], James M. Murphy[d], Catherine L. Day[a] & Peter D. Mace[a]

[a] Biochemistry Department, School of Biomedical Sciences, University of Otago, Dunedin, New Zealand.
[b] Biomolecular Interaction Centre, School of Biological Sciences, University of Canterbury, Christchurch, New Zealand.
[c] Bio21 Molecular Science and Biotechnology Institute, Department of Biochemistry and Molecular Biology, University of Melbourne, Parkville, Victoria, Australia.
[d] The Walter and Eliza Hall Institute of Medical Research & Department of Medical Biology, University of Melbourne, Parkville, Victoria, Australia.

UNIVERSITY of OTAGO
Te Whare Wānanga o Otāgo
NEW ZEALAND

Introduction

Attachment of ubiquitin to Histone 2A (H2A) on lysine residue 119 (H2AK119Ub) in humans is an epigenetic mark that plays a key role in the regulation of gene transcription. Conjugation of ubiquitin to H2AK119 requires the E3 ligase activity of the Polycomb-Repressive Complex 1 (PRC1), and is removed by the Polycomb Repressive-Deubiquitinase (PR-DUB) complex[1].



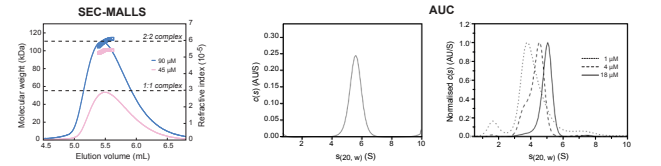
Schematic representing domain structure of *Drosophila* and human PR-DUB components.

BAP1 has recently emerged as an important tumor suppressor protein, which is frequently mutated in melanomas, mesotheliomas, carcinomas, myelodysplastic syndromes and other neoplasms[2-7].

This study aims to understand the mechanisms by which the PR-DUB complexes are regulated, and how their misregulation leads to tumorigenesis.

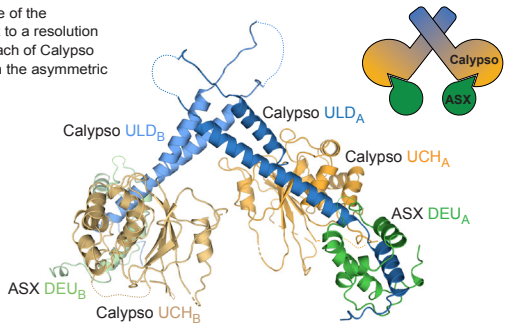
The *Drosophila* PR-DUB forms a bidentate complex

To determine the stoichiometry of Calypso–ASX we performed SEC-MALLS and AUC experiments. Results demonstrated that the *Drosophila* PR-DUB forms a 2:2 complex in solution, with a dissociation constant ~1-18 μM.



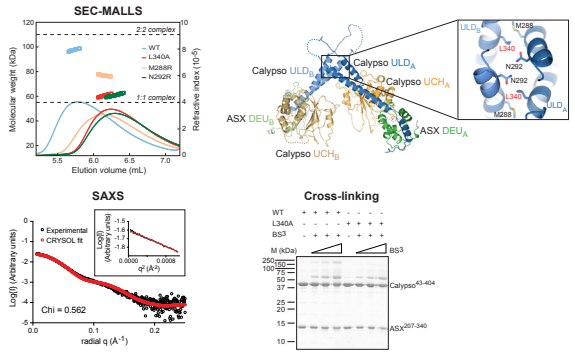
Structure of the *Drosophila* PR-DUB

We solved the structure of the Calypso–ASX complex to a resolution of 3.5 Å. Two copies each of Calypso and ASX are present in the asymmetric unit.



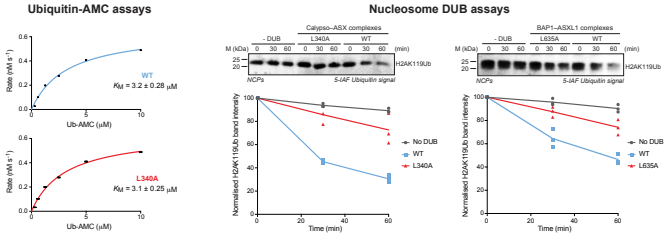
PR-DUB oligomerisation is mediated via the coiled-coil hairpin

We analysed various interaction surfaces within crystal contacts of the Calypso–ASX complex. Using SEC-MALLS, SAXS and chemical cross-linking, we showed that the interface formed by the Calypso coiled-coil hairpin plays a key role in mediating higher-order PR-DUB oligomerisation.



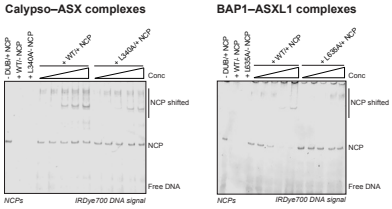
Bidentate complex assembly promotes activity on NCP

We tested wild-type and L340A Calypso–ASX proteins in activity assays against Ubiquitin-AMC and H2AK119Ub nucleosomes. Oligomerisation of Calypso–ASX via the coiled-coil region in Calypso is crucial for cleavage of H2AK119Ub. Mutation of the corresponding interface in BAP1–ASXL1 is also required for efficient activity of the human PR-DUB on nucleosomes.



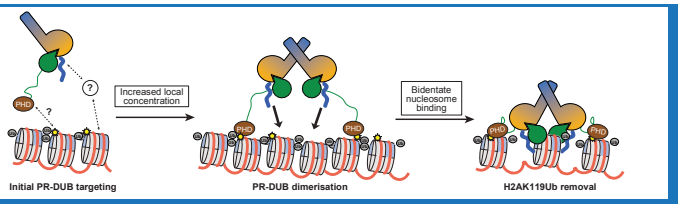
PR-DUB oligomerisation enables efficient nucleosome binding

Electrophoretic mobility shift assays demonstrated that the *Drosophila* and human bidentate PR-DUBs have a greater ability to bind to nucleosomes compared to the respective 1:1 complexes.



Model for PR-DUB regulation

We propose a model whereby enrichment of the 1:1 PR-DUB complex in specific regions of the genome would enhance the local concentration such that DUB oligomerisation and bidentate complex formation becomes favoured. This would allow the C-terminal tails of Calypso/BAP1 to be brought into an optimal orientation for efficient nucleosome recruitment and removal of H2AK119Ub.



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6. Napolitano et al. (2016) *Oncogene* **35**, 1986-2002.
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