UbiQ
targeting the ubiquitin system

Triple E probes
the first cascading activity-based probes for ubiquitin and ubiquitin-like protein E1-E2-E3 enzyme cascades

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Here we present our *Triple E* probe design (Ub-Dha, Figure 1), which to our knowledge represents the first type of cascading activity-based probe for E1-E2-E3 enzymes.[1]

The post-translational modification of proteins with ubiquitin (Ub) and ubiquitin-like proteins (Ubls) is orchestrated by a cascade of E1, E2 and E3 enzymes. To study these enzymes, several activity-based probes (ABPs) have been developed.[2] However, these ABPs are based on the Ub-AMP intermediate (Figure 1) and thus only target the initial E1 activity directly.

Akin to native Ub/Ubls, the ATP-dependent activation of the ***Triple E*** probe by E1 (Fig. 2) and sequential native trans-thioesterifications allow the probe to travel downstream to the E2 and E3. However, unlike the native Ub/Ubl, at each step along the cascade, the Dha probe can react irreversibly with an active site cysteine residue. Thus our probe 'hops' and 'traps' catalytically active E1, E2 and (HECT/RBR) E3 enzymes (Fig. 1). Our founder methodology can be used for activity-based protein profiling (ABPP) using SDS-PAGE (Fig. 3), structural studies (Fig. 4), ABPP in living cells (Fig. 6) and proteomics (Fig. 5). Overall, our *Triple E* probes represent the first tools of their kind that allow the monitoring of full E1-E2-E3 cascade activity.

Figure 1 – mechanism-based inactivation of E1-E2-E3 cascade by *Triple E* probes.

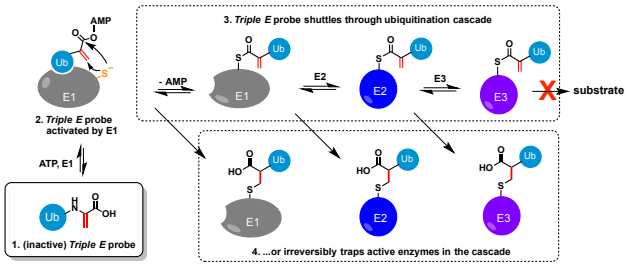


Fig. 2 – E1 labeling by Ub-Dha is ATP dependent

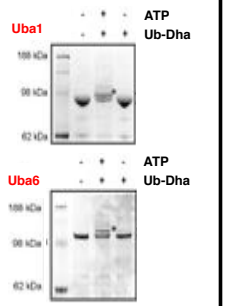


Fig. 3 – activity-based protein profiling with Cy5-Ub-Dha (in-gel fluorescence imaging).

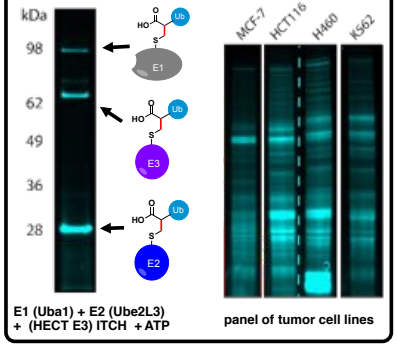
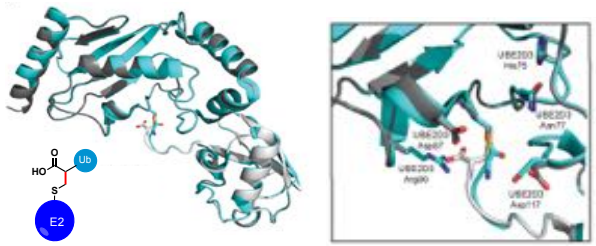
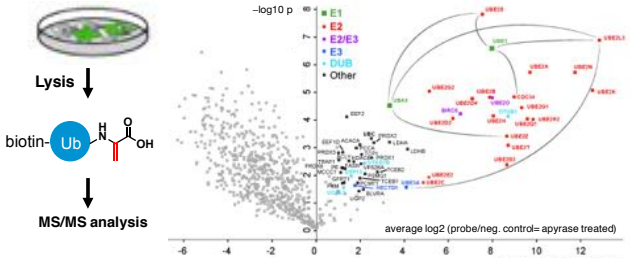


Fig. 4 – structural analysis [Ub-Dha-UBE2D3] conjugate (formed via Ub E1)



Superposition of thioether linked UBE2D3-Ub-Dha conjugate (gray) over the earlier reported oxyester-linked UBE2D3-Ub conjugate (cyan, PDB 3UGB).[3]

Fig. 5 – proteome-wide activity profiling of Ub ligation in HeLa cells



Volcano plot: pairwise comparison of proteins bound to biotin-Ub-Dha relative to the apyrase-treated negative control (y-axis) as a function of average log2 fold enrichment (x-axis). Known cascade connections are highlighted with gray lines. Confidently identified Ub machinery components (average log2 ratio >1 and with p< 0.05) are marked; proteins unrelated to Ub cycle are marked black, those below the threshold are shown in gray.

Fig. 6A - distribution of Cy5-Ub-Dha (magenta) in HeLa cells that ectopically express GFP-UBE1 (green) relative to untransfected cells.

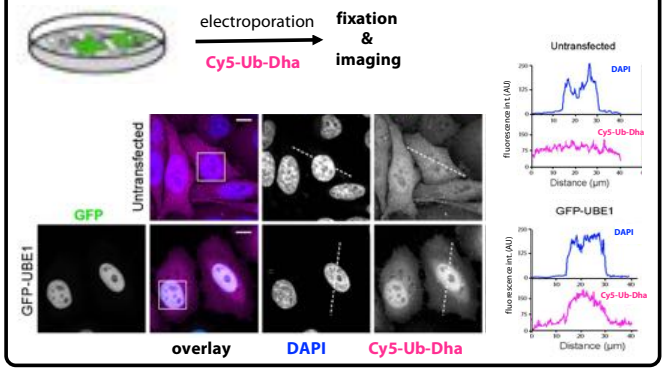
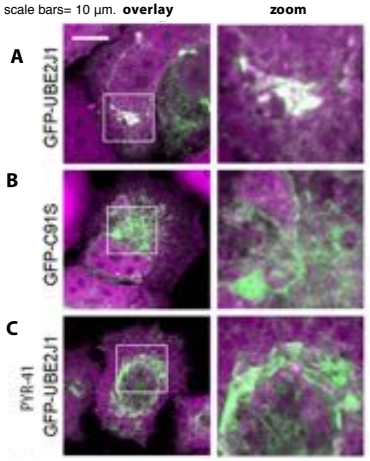
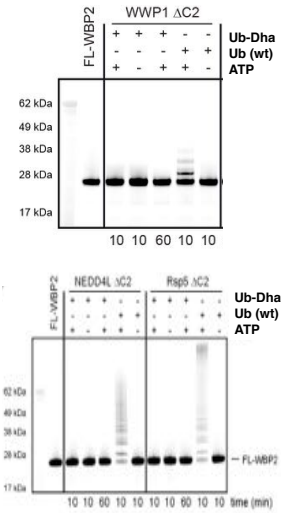


Fig. 6B - visualizing E2 activity in HeLa cells with (electroporated) Cy5-Ub-Dha



A) GFP-UBE2J1 + Cy5-Ub-Dha (magenta).
B) GFP-UBE2J1 C91S mutant + Cy5-Ub-Dha.
C) GFP-UBE2J1 + Cy5-Ub-Dha + PYR-41 (Ub E1 inhibitor, 50 µM, 30 min pre-incubation).

Fig. 7 – *Triple E* probes are not transfered to substrates



take home messages

- ***Triple E* probe: the first cascading activity-based probe for E1-E2-E3 pathways**
- **probe reactivity is ATP-dependent (Fig. 2)**
- **probe is <u>not</u> transfered to substrates (Fig. 7)**
- **probe has relatively low reactivity with Ub/Ubl proteases (data not shown)**
- **thioether linked Ub(l)-Dha-E2 conjugates act as stable (competitive) inhibitors of RING type E3 ligases (data not shown)**

applications *Triple E* probe

- **gel based activity-based protein profiling (Fig. 3)**
- **structural biology (Fig. 4)**
- **proteomics (Fig. 5)**
- **activity-based protein profiling in living cells (Fig. 6)**

drug discovery

- **we are currently using the mode of action of our (proprietary) *Triple E* probe[2b] for the development of mechanism-based small molecule inhibitors of E1-E2-E3 cascades.**

References. [1] (a) Mulder et al., *Nat. Chem. Biol.* **2016**, *In press.* (b) MPC Mulder, F. El Oualid and H. Ovaa. *Adenylation enzyme inhibitors.* Patent pending (filed 26 Aug 2014).
[2] (a) Lu et al. *J. Am. Chem. Soc.* **2010**, *132*, 1748. (b) Olsen et al. *Nature*, **2010**, *463*, 906. (c) H. An and A.V. Statsyuk, *J. Am. Chem. Soc.* **2013**, *135*, 16948. (d) H. An and A.V. Statsyuk *Chem. Comm.* **2016**, *52*, 2477.
[3] Page et al. *Biochemistry* **2012**. *51*, 4175.