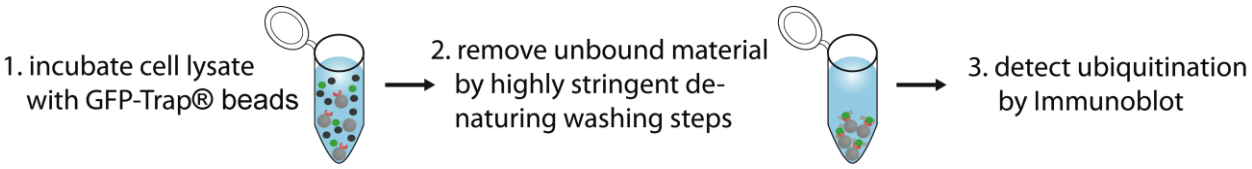
One step purification to test ubiquitination of GFP-tagged proteins



Background

Validation of candidate ubiquitinated proteins is often performed *in vitro* to avoid high-background from endogenous ubiquitinated proteins in the cell. However, *in vitro* systems do not reflect the *in vivo* situation. Critical questions whether those proteins would be modified in a more physiological setup or in different cell types remain to be answered. Here fluorescent fusion proteins provide an efficient alternative to conventional *in vitro* systems based on recombinant protein, since they can be expressed within different mammalian cell lines.

In our approach to analyze ubiquitination of individual candidate proteins in living cells we co-transfect the GFP-tagged protein with FLAG-tagged ubiquitin. Using the GFP-Trap®, GFP-tagged proteins are efficiently extracted from cellular extracts under non-denaturing conditions. Due to strong binding of the GFP-Trap® in combination with covalent ubiquitination, highly specific washing steps under denaturing conditions (8M Urea and 1% SDS) are possible to remove unspecifically bound proteins. Samples can be further analyzed by immunoblotting and mass spectrometry.

Materials and Methods

GFP-tagged candidate ubiquitin substrates were expressed in BG2 *Drosophila* neuronal cells in a 6-well plate, together with FLAG-tagged ubiquitin. Transfected cells were lysed in 300 µl of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% Triton, 1x Proteases Inhibitor cocktail (Roche Applied Science), 0.7% N-ethylmaleimide). 15 µL of pre-equilibrated GFP-Trap®A suspension was added to the cleared supernatant and incubated at RT for 150 min with gentle rolling. The beads were pelleted by centrifugation at 2700xg for 2 min. The supernatant was removed and the beads were washed once with dilution buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1x Proteases Inhibitor cocktail, 0.7% N-ethylmaleimide) followed by three times washing with stringent washing buffer (8 M Urea, 1% SDS in 1x PBS) and once with 1% SDS in 1x PBS. Bound proteins were eluted in SDS-sample buffer (250 mM Tris-HCl pH 7.5, 40% glycerol, 4% SDS, 0.2% bromphenol blue) boiled at 95ºC for 10 min and loaded onto 4-15% Tris-Glycine gels. Immunoblot was performed and transferred to PVDF membrane

using the iBlot system (Invitrogen). After antibody incubation, the membrane was developed using ECL kit (GE Healthcare) following the manufacturer's instructions. The following antibodies were used: monoclonal mouse/rat anti-GFP antibody; monoclonal mouse anti-Flag M2 HRP-conjugated antibody.

Results

Using GFP only expressing cells as a negative control, we tested for ubiquitination of 6 candidate proteins previously identified as being ubiquitinated in the developing brain of *Drosophila* (1). Immunoblot analysis reveals that all six proteins displayed FLAG immunoreactivity above their corresponding unmodified band (Figure 1), as expected by addition of one or several ubiquitin moieties (each Ub-FLAG chain should add ~8 kDa). No ubiquitin FLAG signal was detected on the free GFP control construct despite using a very long exposure. The GFP antibody does not detect the ubiquitin modified forms of the GFP-fusion proteins, due to their low abundance, but only the main unmodified fraction for each of the substrates.

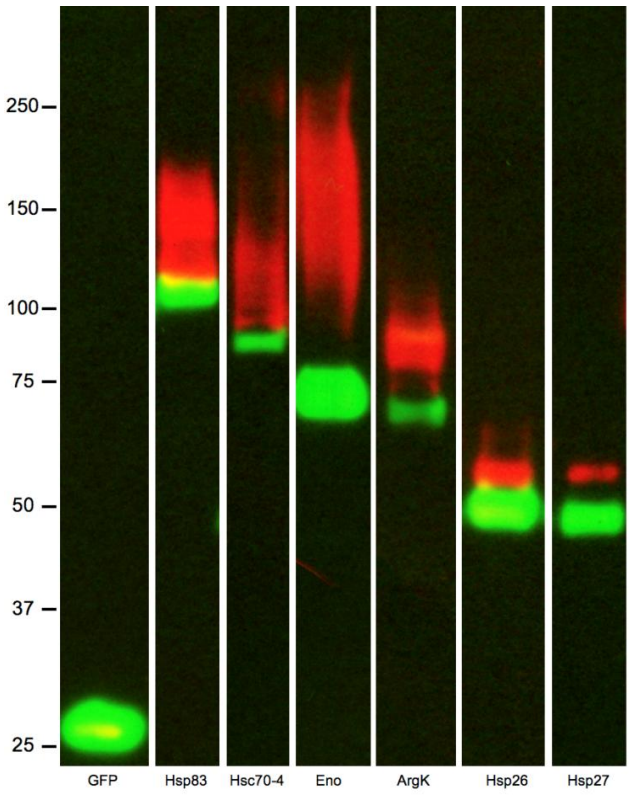


Figure 1.
Ubiquitination assay for six candidate ubiquitin substrate proteins in BG2 neuronal cell culture. Rat anti-GFP antibody was used for detecting the captured proteins (shown in green), and anti-FLAG HRP-conjugated antibody for monitoring their ubiquitinated fraction (shown in red). On the first lane, GFP on its own displays no ubiquitination. Other types of ubiquitin-tagging could be used for improved detection, for example biotin (2) as well as available ubiquitin antibodies to detect specific chain linkage.

Conclusion

We showed that the GFP-Trap® can withstand extremely stringent washes (8M Urea and 1% SDS) without losing its affinity to its antigen, a GFP-fusion protein. This facilitates the isolation and characterization of individual proteins which are subjected to posttranslational modifications, like ubiquitination. As this modification is covalently attached to its target it is not disrupted by the highly denaturing buffers used herein. As modified protein samples are eluted from the beads by boiling with sample loading buffer, this approach is compatible with both immunoblotting and Mass spectrometry after in-gel digestion. The strategy presented here based on the high affinity GFP-Trap® in combination with stringent washing procedures can be expanded for other covalent posttranslational modification like sumoylation.

References/relevant literature

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(2) M. Min, U. Mayor and C. Lindon. *Ubiquitination site preferences in APC/C substrates*.
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Cellular and Molecular Life Sciences (2013) doi:10.1007/s00018-013-1526-7

Author

Ugo Mayor
Catherine Lindon

Ordering Information

Product	Quantity	Code
GFP-Trap®_A	20 reactions	gta-20
GFP-Trap®_A	250 reactions	gta-250
GFP-Trap®_A kit	20 reactions	gtak-20
GFP antibody	100 µl	3h9
GFP antibody	150 µl	66002-1-Ig

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