Sections were viewed in a Philips CM10 electron microscope at 60 kV. Scale bars 500 nm in B and E, and apply to C and F, respectively. Scale bar 200 nm in G.

Conclusion

Through two simple changes in the composition of the fixative for freeze-substitution of cryo-immobilized specimens, we are able to ameliorate the reproducible visibility of the plasma membrane in *D. melanogaster* embryos and larvae. The ultrastructural analysis of membrane-defective phenotypes of embryos fixed by our modified method should allow us a refined view on the plasma membrane and give us new insight in the function of the respective factors in particular and in membrane biology in general.

Acknowledgments: We are grateful to the technical assistance of Frauke Meyer, Ursula Müller, and Brigitte Sailer. This work was supported by the German Research Foundation (DFG, MO1714/2-1).

References: Buser, C., and P. Walther 2008, J. Microsc. 230: 268-277; McDonald, K., and M.K. Morphew 1993, Microsc. Res. Tech. 24: 465-473; McDonald, K., and T. Muller-Reichert 2008, J. Microsc. 230: 252; McDonald, K.L., 2009, J. Microsc. 235: 273-281; McDonald, K.L., M. Morphew, P. Verkade, and T. Muller-Reichert 2007, Methods Mol. Biol. 369: 143-173; Moussian, B., 2010, Insect Biochem. Mol. Biol. 40: 363-375; Moussian, B., H. Schwarz, S. Bartoszewski, and C. Nusslein-Volhard 2005a, J. Morphol. 264: 117-130; Moussian, B., C. Seifarth, U. Muller, J. Berger, and H. Schwarz 2006a, Arthropod Struct. Dev. 35: 137-152; Moussian, B., J. Soding, H. Schwarz, and C. Nusslein-Volhard 2005b, Dev. Dyn. 233: 1056-1063; Moussian, B., E. Tang, A. Tonning, S. Helms, H. Schwarz, C. Nusslein-Volhard, and A.E. Uv 2006b, Development 133: 163-171; Moussian, B., J. Veerkamp, U. Muller, and H. Schwarz 2007, Matrix Biol. 26: 337-347; Steinbrecht, R.A., 1980, Tissue Cell, 12: 73-100; Studer, D., M. Michel, and M. Muller 1989, Scanning Microsc. Suppl. 3: 253-268, discussion 268-259; Tonning, A., S. Helms, H. Schwarz, A.E. Uv, and B. Moussian 2006, Development 133: 331-341; Uv, A., and B. Moussian 2010, Eur. J. Cell Biol. 89: 208-211; Walther, P., and A. Ziegler 2002, J. Microsc. 208: 3-10; Zhang, S., and E.H. Chen 2008, Methods Mol. Biol. 475: 275-297.

Affinity purification of FLAG-tagged protein complexes: A cautionary tale.

Pronovost, S.M.[1], E. Kokai[2], P. Friedrich[3], and J.L. Kadrmas[1,4]. [1]Huntsman Cancer Institute at the University of Utah, Salt Lake City, UT, USA; [2]Department of Medical Chemistry, University of Debrecen, Debrecen, Hungary; [3]Institute of Enzymology, Biological Research Center, Hungarian Academy of Science, Budapest, Hungary; [4]Department of Oncological Sciences, University of Utah, Salt Lake City, UT, USA; julie.kadrmas@hci.utah.edu.

Introduction

Tagging proteins with a FLAG peptide epitope (DYKDDDDK) or a variant thereof has become a standard laboratory technique both to detect proteins for which there are no available

antibody reagents, and for facile protein purification via α-FLAG resins (Brizzard *et al.*, 1994). The strength of the FLAG tag system lies in the antigenicity of the FLAG sequence and in the absence of naturally occurring FLAG-like sequences across many species. It is presumed that in most protein contexts, the small size of the FLAG tag makes it unlikely to affect significantly the expression, stability, folding, or function of the protein to which it is fused. We show here that this is not universally true.

We describe the purification of artifactual RSU1-FLAG complexes from adult *Drosophila* lysates. By SDS-PAGE and subsequent silver staining and western analysis, these complexes contain RSU1-FLAG and established RSU1 partners, which serve as a positive control for the purification procedure. In addition, novel bands were observed and identified by mass spectrometry as Transglutaminase (Tg) and Calpain A (CalpA). We were unable to confirm the specificity of the RSU1 interaction with CalpA by co-immunoprecipitation with native, untagged RSU1. Our data indicate that the co-purification of RSU1-FLAG and CalpA, and presumably Tg as well, is not dependent upon RSU1, but rather is a FLAG-specific artifact. This underscores the importance of independently confirming the specificity of any new protein-protein interaction discovered by tagged-protein technologies.

Methods

• *Generating RSU1-FLAG flies*

A genomic fragment containing the RSU1 coding sequence plus 3 kb upstream of the start ATG was cloned into pCaSpeR. Sequence encoding a 3xFLAG tag (DYKDHDGDYK DHDIDYKDDDDK) was inserted at the C-terminus of the RSU1 coding sequence immediately upstream of the stop codon. This plasmid was injected into w^{1118} *Drosophila* embryos, and transgenic animals were identified and mapped by standard techniques. The RSU1-FLAG transgene was crossed into an RSU1 (*ics*) null background.

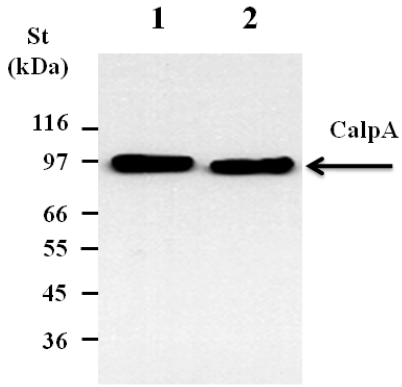

Figure 1. The α-CalpA antibody specifically recognizes the 94 kDa CalpA protein. Lane 1: 50 ng purified recombinant CalpA. Lane 2: 25 μg protein from adult Oregon R crude lysate.

• *Generating an α-Calpain A antibody*

After taking blood samples for the preparation of control sera, 250-250 μg purified recombinant CalpA (Jekely and Friedrich, 1999) (expressed by Attila Farkas, Institute of Enzymology, Biological Research Center, Hungarian Academy of Science, Budapest) was injected into two male rabbits (Charles River Laboratories) with 50% (v/v) Complete Freund Adjuvant (Calbiochem). Boosting injections were repeated with the same amount of the recombinant protein and Incomplete Freund Adjuvant (Sigma) after the 2nd, 4th, and 6th weeks. Antisera were prepared 12 days after the last injection and were tested with bacterially expressed CalpA protein in dot blot (not shown) and western blot (Figure 1). All animal experiments were completed at the Department of Pharmacology and Pharmacotherapy of the Medical Faculty and were approved by the Animal Care and Protection Committee at the University of Debrecen in

accordance with the European Community Council Directions. For western blotting, CalpA antiserum from rabbit #1 was used at a 2500-fold dilution.

• *FLAG purification*

For each sample, 300 adult flies were dounce homogenized in 10 mL TLB (50 mM Tris pH 7.9, 150 mM NaCl, 0.1% Triton X-100) plus protease inhibitor cocktail. Lysates were centrifuged 10 min at 16,000 ×g. The resulting supernatant was filtered (0.45 μm) before pull-down with 220 μL α-FLAG M2 slurry (Sigma) per manufacturer's instructions. Protein was eluted from the resin with 100 mM glycine pH 3.5. Eluates were neutralized by addition of Tris pH 8.8 to 100 mM prior to SDS-PAGE analysis. Gels were either silver stained, stained with Colloidal Coomassie (Dyballa and Metzger, 2009), or transferred for western blotting.

• *Mass spec analysis*

Bands of interest were excised from a Colloidal Coomassie visualized gel and subjected to an in-gel tryptic digest prior to nano-LC/MS/MS. Data was analyzed using the Mascot database for protein identification.

• *Co-Immunoprecipitations*

Lysates were prepared as for FLAG purification with 30 flies per sample in 2 mL TLB plus protease inhibitors. Each lysate was precipitated with 20 μL of Protein A Sepharose slurry mixed with 5μL α-RSU1-N or α-CalpA antibody.

• *Western blotting*

Proteins were immunoblotted using standard techniques. Primary antibodies used for western blotting were as follows: rabbit α-RSU1-N (Kadrmas *et al.,* 2004), rabbit α-PINCH-C (Clark *et al.,* 2003), and rabbit α-CalpA (above). Secondary antibodies were either goat (Sigma) or donkey (G.E. Healthcare) anti-rabbit IgG conjugated to horseradish peroxidase.

Results and Discussion

The Leucine-rich repeat (LRR) protein RSU1, encoded by *ics* in *Drosophila*, has been demonstrated to bind the LIM protein PINCH and participate in integrin dependent processes (Kadrmas *et al.,* 2004). Because the LRR motif, a protein interaction interface (Kobe and Kajava, 2001), is the only identified domain structure within RSU1, we were interested in whether RSU1 binds additional proteins. To identify novel partners, we undertook an affinity purification scheme using a FLAG-tagged RSU1 transgene introduced into an *ics* null *Drosophila* background to exclude endogenous RSU1 from competing for binding partners during the FLAG purification procedure.

As expected, in the negative control FLAG pull-downs from w^{1118} lysate (which does not express any FLAG-containing protein), only non-specific background bands were observed on silver stained gels (Figure 2A, lane 1), and there was no western signal for RSU1, PINCH, or CalpA (Figure 2C, lane 1).

A western blot of purified RSU1-FLAG complexes shows a band for the established RSU1 partner, PINCH (Figure 2C, lane 2). PINCH empirically stains poorly and cannot be readily discerned on silver stained gels (Figure 2A, lane 2). A silver-stained gel does show the tertiary and quaternary partners ILK and Parvin, which associate with RSU1 via PINCH (Figure 2A, lane 2). Of particular interest were three novel bands at approximately 87, 94, and 125 kDa (Figure 2A, lane 2).

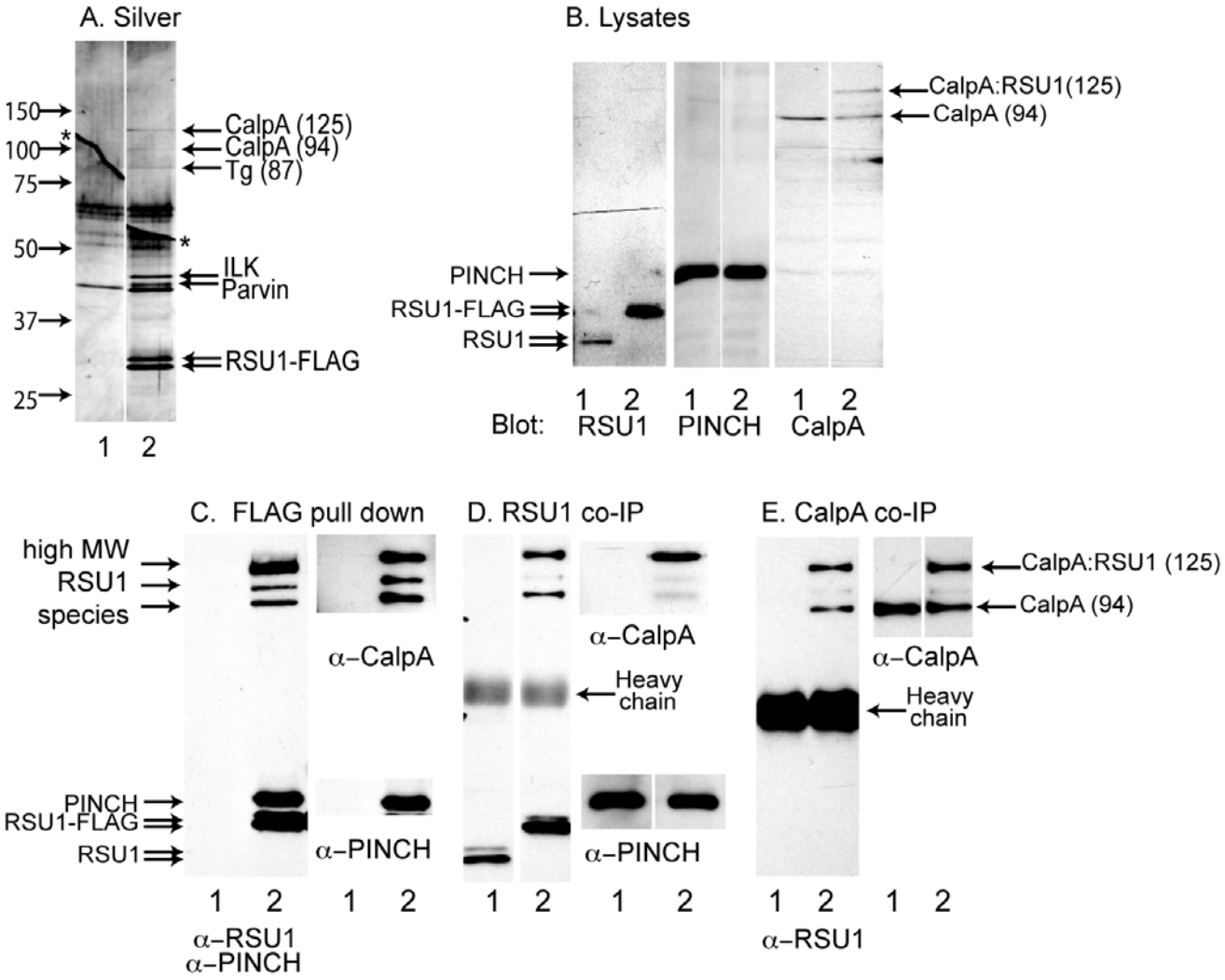

Figure 2. The FLAG-tag on RSU1 produces and artifactual interaction with Tg and CalpA. For all panels, lane 1 contains w^{1118} sample. Lane 2 contains *w; ics P[w+ RSU1-FLAG]*. A.) Silver stained gel of FLAG pull-downs. * indicates a crack through the gel. B.) Representative adult lysates for each sample show expression levels of RSU1/RSU1-FLAG (which can run either as a doublet or a single band), PINCH, and CalpA. C.) FLAG pull-down in the RSU1-FLAG sample co-purifies RSU1-FLAG, PINCH, and CalpA. The w^{1118} sample does not express FLAG and is, therefore, devoid of signal. D.) An RSU1 co-IP shows that both RSU1 and RSU1-FLAG co-purify PINCH. Only RSU1-FLAG co-purifies CalpA signal. E.) A CalpA co-IP robustly purifies CalpA from both samples. Only RSU1-FLAG shows the 125 kDa CalpA species, and RSU1 signal is only present in the high molecular weight species of the RSU1-FLAG sample.

The proteins in these bands were identified by mass spectrometry. The 87 kDa band contains the Ca^{2+}-dependent protein cross-linking enzyme Transglutaminase (Tg) (predicted M_r = 87 kDa). Both the 94 and 125 kDa bands contain the Ca^{2+}-dependent protease CalpA (predicted M_r = 94 kDa). The presence of CalpA in both of these bands was confirmed by western analysis (Figure 2B and 2E).

Notably, the 125 kDa band corresponds to the predicted molecular weight of CalpA plus RSU1, and this band (and to a lesser extent the 87 and 94 kDa bands) contained both Calpain and RSU1 peptides in the mass spec analysis. A western blot for RSU1 of purified FLAG-RSU1 complexes demonstrates that RSU1 is present in three high molecular weight bands (Figure 2C). Because Tg is a protein cross-linking enzyme (Iismaa *et al.*, 2009), we hypothesized that RSU1-FLAG in these high molecular weight species may result from a Tg catalyzed cross-linking reaction between RSU1-FLAG and CalpA.

In order to confirm independently the RSU1-CalpA interaction, we performed co-immunoprecipitations using α-RSU1 antibody (Figure 2D). Both native RSU1 (in the w^{1118} sample) and RSU1-FLAG co-purify PINCH, which serves as a positive control for the IP. In contrast to RSU1-FLAG (Figure 2D, lane 2), however, native RSU1 was unable to co-precipitate any CalpA-containing bands (Figure 2D, lane 1). Altered expression levels do not explain this difference, because equal amounts of CalpA and comparable amounts of RSU1 and RSU1-FLAG are present in the starting material (Figure 2B). Our data indicate that the FLAG-tag is producing an artifactual interaction. We also demonstrate this via an α-CalpA co-IP. CalpA co-purifies high molecular weight species of RSU1-FLAG, but not free RSU1-FLAG (Figure 2E, lane 2). The RSU1-FLAG:CalpA interaction was, however, completely absent in the w^{1118} sample, which expresses only untagged native RSU1 (Figure 2E, lane 1). We also observed a lack of CalpA interaction with RSU1-GFP and with a mutant RSU1-FLAG defective in PINCH binding and presumed to be mislocalized (data not shown).

These data indicate that RSU1, CalpA, and Tg are likely to be in close physical proximity in at least some *Drosophila* tissues. This is not surprising, given that mammalian RSU1 and Calpain can both reside in integrin adhesive complexes (Zaidel-Bar *et al.*, 2007) and that Tg can be bound, cleaved, and activated by α-Calpain (Zhang, *et al.*, 1998). One plausible explanation for our data is that the FLAG tag may confer upon RSU1 the spurious ability to act as a substrate in a proximal, nascent Tg reaction with CalpA. The 3xFLAG contains four lysine residues, any one of which is theoretically competent to act as the second substrate in the Tg catalyzed cross-linking reaction. By FLAG-tagging RSU1, we have created conditions that yield a very specific, but false, interaction.

Our results demonstrate the necessity for independently confirming the specificity of any new protein-protein interaction uncovered by tagged-protein technologies. In the absence of antibody reagents for a confirmatory co-IP with the native proteins, repeating the co-purification with different tags fused to different locations within the protein sequences is critically important.

Acknowledgements: We thank Attila Farkas, Mary Beckerle, and the University of Utah Mass Spectrometry Shared Resource (supported by NIH P30CA042014). This work was supported by NIH R01 GM084103 to JLK, a János Bolyai Research Scholarship to EK, and by the Hungarian Scientific Research Fund OTKA 60723 to PF.

References: Brizzard, B.L., R.G. Chubet, and D.L. Vizard 1994, Biotechniques 16: 730-735; Clark, K.A., M. McGrail, and M.C. Beckerle 2003, Development 130: 2611-2621; Dyballa, N., and S. Metzger 2009, J. Vis. Exp. 30: http://www.jove.com/index/details.stp?id=1431; Iismaa, S.E., B.M. Mearns, L. Lorand, and R.M. Graham 2009, Physiol. Rev. 89: 991-1023; Jekely, G., and P. Friedrich 1999, J. Biol. Chem. 274, 23893–23900; Kadrmas, J.L., M.A. Smith, K.A. Clark, S.M. Pronovost, N. Muster, J.R. Yates III, and M.C. Beckerle 2004, J. Cell Biol. 167: 1019-1024; Kobe, B., and A.V. Kajava 2001, Curr. Opin. Struct. Biol. 11: 725-732; Zaidel-Bar, R., S. Itzkovitz, A. Ma'ayan, R. Iyengar, and B. Geiger 2007, Nature Cell Biol 9: 858-867; Zhang, J., R.P. Guttmann, and G.V. Johnson 1998, J. Neurochem. 71: 240-247.