**Title**
Investigating the role of 14-3-3ζ SUMOylation



**Author**
Nguyen, Hoang Anh Phuc

**Publication Date**
2013

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA
Los Angeles

# Investigating the role of 14-3-3ζ SUMOylation

A thesis submitted in partial satisfaction of the

requirements for the degree of Master of Science in

Biochemistry and Molecular Biology

By

Hoang Anh Phuc Nguyen

2013

**ABSTRACT OF THE THESIS**

Investigating the role of 14-3-3ζ SUMOylation

By

Hoang Anh Phuc Nguyen

Master of Science in Biochemistry and Molecular Biology

University of California, Los Angeles, 2013

Professor Albert J. Courey, Chair

SUMO modification is involved in several cellular processes such as signal transduction, cell division, and regulation of protein subcellular localization, specifically including Ras signaling and protein nuclear import/export. Previous studies have demonstrated that 14-3-3ζ, which is thought to have functions in both the nucleus and cytoplasm, is a SUMOylated protein and is required for Ras signaling. I have therefore sought to determine whether or not the SUMOylation of 14-3-3ζ promotes or inhibits its localization to the nucleus. Subcellular fractionation experiments fail to reveal a detectable change in the overall ratio of nuclear to cytoplasmic 14-3-3ζ upon knockdown of SUMO by RNAi in cultured Drosophila cells. However, immunofluorescence studies suggest that nuclear localization of this protein may increase in a subset of the SUMO knockdown cells.

The thesis of Hoang Anh Nguyen is approved.

Catherine F. Clarke

James W. Gober

Albert J. Courey, Committee Chair

University of California, Los Angeles

2013

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ATP | Adenosine-5'-triphosphate |
| CRM1 | Chromosome Region Maintenance 1 |
| DAPI | 4',6-diamidino-2-phenylindole |
| dpErk | Doubly phosphorylated Extracellularly Regulated Kinase |
| Erk | Extracellularly Regulated Kinase |
| Grb2 | Growth factor receptor-bound protein 2 |
| GTPase | Guanosine triphosphate (GTP) hydrolase enzyme |
| HDAC1 | Histone Deacetylase 1 |
| Hipk | Homeodomain-interacting Protein Kinase |
| MAPK | Mitogen-activated Protein Kinase |
| MEK | Mitogen-activated or Extracellular signal-regulated kinase kinase |
| Pc2 | Proprotein convertase 2 |
| PIAS | Protein inhibitor of activated STAT (signal transducer and activator of transcription) |
| PP2A | Phospho-protein phosphatase 2A |
| Raf | Rapidly Accelerated Fibrosarcoma protein, proto-oncogene serine/threonine-protein kinase |
| RanBP2 | Ran-binding protein 2 |
| Ran-GAP | Ran GTPase-activating protein |
| Ras | Rat sarcoma protein, small GTPase |
| Rpb3 | RNA polymerase II core subunit 3 |
| RTK | Receptor tyrosine Kinase |
| SAE1/2 | SUMO-activating enzyme 1/2 subunit |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| Shc | src (proto-oncogene tyrosine-protein kinase) homology and collagen protein |
| Siz | SAP and Miz-finger domain-containing protein, a SUMO E3 Ligase |
| SOS | Son of sevenless guanine nucleotide exchange factor |
| SUMO | Small ubiquitin-like modifier |
| TEL | Translocation-Ets-Leukemia |
| Ubc9 | Ubiquitin-conjugating enzyme 9 |
| Ulp | Ubiquitin-like protein specific protease |

## ACKNOWLEDGEMENTS

Thank you, Dr. Albert Courey for giving me the opportunity to work in your lab to explore SUMO's affect on protein function, for giving me guidance, and especially for being a role model as a scientist. Next, I would like to thank Joseph Cao, the graduate student researcher with whom I have been working directly, for introducing me to research, teaching me from the very start of my research project providing me insightful thoughts, support, and helpful directions. Last of all I thank the past and current members of the Courey lab, Mathew Smith, Wiam Turki-Judeh, Pak Wong, Michael Chambers, and Alberto Ponce, for providing me protocols, reagents, and thoughtful feedbacks when I needed it. I am also grateful for not only their support in lab but for their kindness and encouragement outside of the lab.

Secondly, I would like to thank my committee members, Dr. Catherine Clarke and Dr. James Gober, for their kind support and critique of my thesis.

Finally, I would like to thank my family for their unconditional love, support, and sacrifices they have made for me. They have always stood by me during the difficult times and given me the motivations to pursue my goals in life. With their strong value in diligence and education, they have guided me well on the route searching for knowledge. Without them, I would not be where I am now.

# INTRODUCTION

Post-translational modifications of proteins play critical roles in regulating protein function. Proteins can undergo phosphorylation, methylation, acetylation, SUMOylation, and ubiquitination to alter their functions by affecting their subcellular localization, degradation, activity, or interactions with other proteins. Since post-translational modifications are reversible, proteins can be rapidly turned on or off allowing cells to respond to external cues without the delays of transcription and translation. For example, in the Ras signaling pathway, Raf, MEK, and ERK are activated by phosphorylation (McCubrey *et al.* 2007). In addition, protein modifications of histones regulate transcription to prevent the wasteful production of unnecessary gene products.

Although Small Ubiquitin-related Modifier (SUMO) shares only modest sequence identity with ubiquitin, the two proteins possess highly similar three-dimensional structures. Like ubiquitin, SUMO attaches to target proteins through an ATP-dependent mechanism, in which it is first covalently attached to an E1 family activating enzyme (SAE1/2), then transferred to an E2 family conjugating enzyme (Ubc9), and finally transferred to a target protein, sometimes with the help an E3 family ligase. The final linkage is an amide bond (also termed an isopeptide bond) between the C-terminal carboxyl group of SUMO and an ε-amino group in a target protein lysine residue. The SUMO-acceptor lysine is often found in the consensus site ΨKXE (Ψ denotes a hydrophobic amino acid, K is lysine, X can be any amino acid, and E is glutamic acid) (Figure 1). SUMO is one of the several ubiquitin-like proteins that have diverse functions in cell and developmental biology because it regulates the target protein's degradation, sub-cellular localization, or activity (Gill, 2005; Seeler and Dejean, 2003; Verger *et al*., 2003).

Previously, Minghua Nie, from Dr. Albert Courey's laboratory, used liquid chromatography tandem mass spectrometry (LC-MS/MS) to identify both SUMO-modified and SUMO-interacting proteins in the developing embryo (Nie *et al.*, 2009). 6x-His-FLAG-tagged SUMO was expressed in early embryos followed by two different purification schemes using the His and/or FLAG affinity tags. In the first scheme, proteins were isolated under native conditions by anti-FLAG co-precipitation, an approach that is expected to yield SUMO-conjugated proteins as well as proteins that interact non-covalently with SUMO-conjugated proteins. In the second scheme, SUMOylated proteins were first purified under denaturing conditions using the His tag and then repurified under native conditions using the FLAG-tag. This latter two-step approach is expected to yield only proteins that are directly conjugated to SUMO. In each case, the purified proteins were then size-fractionated by SDS-PAGE. After in-gel trypsinolysis, the proteins were identified by liquid chromatography-multidimensional mass spectroscopy. The native single-step purification identified 247 proteins while the denaturing two-step purification yielded 144 proteins.

Among the proteins identified as described above were multiple components of the Ras signaling pathway including Ras, Erk, subunits of the PP2A complex, 14-3-3ε and 14-3-3 ζ (Nie *et al.* 2009). In confirmation of a role for SUMO in Ras signaling it was found that SUMO knock down in S2 cells impaired Erk activation. The canonical Ras pathway involves the activation of a receptor tyrosine kinase (RTK) via autophosphorylation on tyrosine residues. The phosphotyrosines then serve as binding sites for Shc and Grb2 adaptor proteins (Figure 2.). The adaptor proteins recruit Son of Sevenless (SOS), a guanine nucleotide exchange factor, which activates the small GTPase Ras. The Ras protein activates the protein kinase Raf leading to the sequential activation of the kinases MEK and Erk via a phosphorylation cascade (Campbell *et*

*al.*, 1998). PP2A is also important for the activation of Raf by dephosphorylating Raf at serine 259, a site of Raf inhibition when phosphorylated (Jaumot and Hancock, 2001). The 14-3-3 family proteins also bind to Raf for activation and recruitment of an unknown activating factor (Avruch *et al*., 2001; Dhillon *et al., 2009*).

The 14-3-3ζ protein was identified in both the native and denaturing purification schemes indicating that it is covalently conjugated to SUMO. A SUMOylation assay in *E. coli* was performed confirming that 14-3-3ζ is a SUMO target protein (Nie *et al.*, 2009). A search of the 14-3-3ζ sequence for matches for the SUMO acceptor-site consensus sequence identified lysine 125 as the highest probability SUMO acceptor-site, followed by two other lower probability sites at lysine 71 and lysine 6 (Figure 3.).

Previous studies have revealed that 14-3-3ζ is involved in cell signaling, cell division, and transcription regulation (Stokoe *et al.*, 1994; Leevers *et al*., 1994; Su *et al.,* 2001; and Winter *et al.,* 2008). Genetic and biochemical experiments have shown 14-3-3ζ can stimulate transcription of genes such as HDAC1 by interacting with phosphorylated histone H3 serine 10 (Winter *et al.*, 2008), while other studies have shown 14-3-3ζ stimulates Raf activity leading, via MEK, to the activation of Erk MAP kinase (Tzivion *et al.*, 1998; Dhillon *et al.*, 2009). *In vitro* and *in vivo* assays showed that 14-3-3ζ needs to bind the C-terminus of Raf for Erk activation. Li and coworkers revealed that 14-3-3ζ plays a role in Torso RTK signaling, which uses the Ras pathway during the development of the early embryo to activate the transcription of *tailless,* a gap gene important for *Drosophila* anteroposterior development (Li *et al*., 1997). Overexpression of 14-3-3ζ enhances Torso RTK signaling by expanding *tailless* expression beyond its normal domains at the anterior and posterior ends of the embryo. The overexpression suppresses *torso*

and *draf* loss of function phenotypes. However, the overexpression 14-3-3ζ did not affect *ras* mutant phenotype indicating that Ras is necessary for activation of the Ras pathway by 14-3-3ζ. In addition, Torso signaling is impaired in 14-3-3ζ maternally depleted embryos. Work by Joseph Cao in the Courey lab has also revealed that levels of activated Erk (dpErk) are dramatically reduced in SUMO depleted embryos. Therefore, I am interested in investigating the function of the SUMO modification of 14-3-3ζ.

One prominent role of SUMO modification is to influence protein subcellular localization (Seeler and Dejean, 2003). For example, SUMOylation of Bicoid, Dorsal, Hipk, and Medea are necessary for nuclear uptake (Epps and Tanda, 1998; Bhaskar *et al.*, 2002; Huang *et al.*, 2011; and Miles *et al.*, 2008). Therefore, SUMO could possibly affect 14-3-3ζ nuclear trafficking and thereby play a role in transcription regulation (Winter *et al.*, 2008). In order to understand how SUMOylation affects the function of 14-3-3ζ, I have performed experiments to determine if localization of 14-3-3ζ is dependent on SUMO. Subcellular fractionation was performed followed by immunoblotting to determine whether 14-3-3ζ's localization is affected by knocking down SUMO. Immunofluorescence was also performed on Drosophila Schneider 2 (S2) cells to determine if knocking down SUMO alters the localization of 14-3-3ζ.

# MATERIALS AND METHODS

1. **Subcellular Fractionation assay**

Centrifugation through a sucrose cushion was performed to separate nuclear from cytoplasmic components. The protocol was adapted from Khodor, Y., Rodriguez, J., Abruzzi, K., *et al*. (2011). About $80x10^6$ S2 cells treated or untreated with SUMO dsRNA as described below were lysed with 1 mL Buffer AT (15 mM HEPES pH 7.6, 10 mM KCl, 5 mM MgOAc, 3 mM $CaCl_2$, 300 mM Sucrose, 0.1% Triton X-100, 1 mM Dithiothreitol (DTT), 10 mM *N*-Ethylmaleimide (NEM), 1X Halt Protease inhibitor from Thermo Scientific). The 1 mL lysates from the previous step were transfered to a 7 mL Kimble Kontes Dounce Tissue Grinder in which cells were homogenized by douncing with pestle B for 30 strokes. Lysates were overlaid onto 1 mL of Buffer B (15 mM HEPES pH 7.6, 10 mM KCl, 5 mM MgOAc, 3 mM $CaCl_2$, 1 M Sucrose, 0.1% Triton X-100, 1 mM DTT, 10 mM NEM, 1X Halt Protease inhibitor). To separate the nuclear and cytoplasmic fractions, the samples were spun at 8000 rpm in microcentrifuge for 20 minutes at $4^0$ C. After the centrifugation, the nuclear pellets were resuspended in Buffer AT and overlaid onto 1 mL of Buffer B for another round of centrifugation. The cytoplasmic fraction, which remained at the top of the supertant, was overlaid onto 1 mL of Buffer B for a second spin identical to the first. Multiple spins were performed to enhance the separation and minimize cross contamination between the cytoplasmic and nuclear fractions. The entire procedure was performed in a cold room. The subcellular fractions were stored at $-20^0$ C and analyzed by Western blot.

2. **Immunofluorescence staining:**

**A) Formaldehyde fixation**

S2 cells ($3x10^6$) cells either treated or untreated with SUMO dsRNA as described below were pipetted onto cover slips coated with poly-lysine in 6-well plates for 30 minutes at room

temperature (RT). To fix cells, 1 mL of 4% formaldehyde in phosphate-buffered saline (PBS) (pH 7.4) was added on top of the cover slip and incubated at RT for 20 minutes before being permeabilized by the addition of 1 mL PBST (0.3% Triton X-100 in PBS). Cells were then blocked for one hour in PBST with 10% Bovine Calf Serum (BCS). Cells were incubated with primary antibody overnight at 4°C then incubated with secondary antibody for an additional hour at RT. Cells were mounted onto slides with ProLong® Gold Antifade Reagent with DAPI from Invitrogen. The primary antibody used was rabbit anti-Drosophila 14-3-3ζ (1:3000) (Skoulakis and Davis, 1996). The secondary antibody used was goat anti-rabbit Alexa Fluor 488 (1:1000) from Invitrogen.

**B) Methanol fixation:**

After treating $3x10^6$ S2 cells with either control or SUMO dsRNA, cells were fixed in 100% methanol at $-20^0$C for 10 minutes. The cell samples were not treated with PBST for permeabilization. Cells were stained in the same manner as those fixed by the formaldehyde method.

**C) Fluorescent Microscopy:**

The S2 cell samples were imaged using a Leica TCSSPE Confocal Microscope with a 100x objective. Alexa Fluor 488 attached to the secondary antibody was used to image 14-3-3ζ and was excited at 488nm. DAPI was excited at 405nm.

**3. Western Blotting**

Protein samples were run on 12% SDS-PAGE gels and then transferred to nitrocellulose membranes. To reduce the nonspecific background, the membrane was blocked in blocking solution (1X phosphate-buffered saline (PBS), 10% Bovine Calf Serum (BCS), 0.03% Tween-20) for one hour at room temperature. After the blocking, the membrane was incubated in

primary antibody overnight at $4^0$C. After extensive washing with PBST, the membrane was incubated for one-hour with IR-dye-labeled secondary antibodies, and, after additional PBST washes, imaged on a Licor Odyssey IR imager. The primary antibodies used were rabbit anti-14-3-3ζ (1: 30000) from Skoulakis (Skoulakis and Davis, 1996), rabbit anti-SUMO (1:5000) from the Courey lab (Smith *et al.,* 2004), rabbit anti-Rpb3 (1:5000) from John Lis (Ni *et al*., 2008), and mouse anti-tubulin (1:10000) from Sigma. The secondary antibodies (purchased from Licor) were goat anti-rabbit coupled to a fluororphore that emits at 800 nm, and goat anti-mouse coupled to a fluorophore that emits at 680 nm.

**4. RNA interference (RNAi)**

**A) SUMO dsRNA Synthesis**

The sequences of the primer used to generate the T7 SUMO template for *in vitro* transcription of SUMO double stranded RNA are TAATACGACTCACTATAGGGAGATTTGACCACTTAGCAGCTTCAACAAGC and TAATACGACTCACTATAGGGAGAACCATTTTCTTGTCTGCAAATGTTTTTG. The underlined sequence corresponds to the T7 promoter (Nie *et al.*, 2009). HiScribe T7 *In Vitro* Transcription Kit from NEB was used to generate the SUMO dsRNA.

**B) Conditions for RNAi in Drosophila cultured cells (S2 cells)**

For the subcellular fractionation experiment, $8x10^7$ S2 cells were harvested and resuspended in 40 mL of Schneider's serum free medium before transferring the cells into two T150 cell culture flasks (i.e. $4x10^7$ cells in 20 mL serum free medium for each flask). S2 cells at a density of $2x10^6$ cells per mL were then incubated with 66.7 μg of dsRNA in serum free medium in each flask for 45 minutes at room temperature. After 45 minutes, the cells were supplemented with an equal volume of Schneider's insect medium containing 20% fetal bovine

serum (FBS), which resulted in a final concentration of $1x10^6$ cells/mL in 10% FBS. The cells were incubated at $25^0$C for an additional 4-5 days.

For the immunofluorescence experiment, $3x10^6$ S2 cells in 1.5 mL Schneider's serum free medium were transferred to a 6-well cell culture plate. The cells were then incubated with 5 μg of dsRNA in the serum free medium for 45 minutes at room temperature. After this step, the cells were treated in the same manner as described for the RNAi treatment of S2 cells used for the subcellular fractionation.

**5. Site-Directed Mutagenesis**

14-3-3ζ was mutagenized at lysine 125, the highest probability SUMO acceptor site as predicted by SUMOplot™ (http://www.abgent.com/sumoplot) (Figure 2). The lysine was changed to arginine to generate the mutant K125R. Plasmid pUC19 containing the 14-3-3ζ cDNA was used as DNA template. The mutagenic primer was: 5′ AGCAAGGTGTTTTACCTGAAGATGA**G**GGGTGATTACTACAGGTATTTAGCC 3′. The mutagenized codon is underlined while the mutagenized base is bold. The 14-3-3ζ K125R mutant was generated using the QuikChange Multi Site-Directed Mutagenesis Kit (purchased from Stratagene). Mutants were confirmed by DNA sequencing.

# RESULTS

**Subcellular Fractionation does not reveal a detectable change in the overall ratio of nuclear to cytoplasmic 14-3-3 ζ upon SUMO knockdown.**

To determine if SUMO affects the localization of 14-3-3ζ in S2 cells, I centrifuged cell lysates through sucrose cushions to separate nuclear from cytoplasmic proteins starting with control cells or cells in which SUMO had been knocked down by RNAi (Figure 4). This was followed by western blotting to detect proteins in the total cell lysate, and in the nuclear and cytoplasmic fractions (Figure 5). Anti-SUMO blots indicate that levels of free SUMO and SUMO-conjugated proteins are reduced in the SUMO knockdown cells relative to the control cells (Figure 5B). Tubulin was used as a cytoplasmic marker demonstrating that cytoplasmic proteins did not significantly contaminate the nuclear fractions (Figure 5A, compare lanes 3 and 4 with lanes 5 and 6 in the tubulin blot). In addition, I used the RNA polymerase II subunit, Rpb3, to determine the extent to which nuclear proteins contaminated the cytoplasmic fraction. I found that, while the nuclear fractions were enriched for Rpb3 (Figure 5A, lanes 3 and 4 in the Rpb3 blot), there was also some Rpb3 in the cytoplasmic fraction (Figure 5A, lanes 5 and 6 in the Rpb3 blot). SUMO knockdown did not result in any detectable change in the overall ratio of nuclear to cytoplasmic 14-3-3ζ (Figure 5A, compare lane 3 with lane 4 and lane 5 with lane 6 in the 14-3-3ζ blot).

**Immunofluorescence**

To determine if SUMO knockdown resulted in a change in the localization of 14-3-3ζ in a small subset of the cells, I performed immunofluorescence studies on control and SUMO knockdown S2 cells. Western blots with antibodies against SUMO and tubulin demonstrate the effectiveness of the SUMO knockdown. For immunofluorescence, the fixation and

permeabilization steps are critical to the quality of the results. Two common methods for cell fixation are the use of cross-linking reagents and the use of organic solvents. Cross-linking reagents such as formaldehyde result in the formation intermolecular bridges and require a permeabilization step for antibodies to penetrate the cells and obtain access to the network of linked antigens; whereas organic solvents such as methanol remove lipids, dehydrate cells and precipitate proteins onto the cellular architecture. The formaldehyde fixation method is better than the methanol method in retaining the cell structures, but does not detect all antigens with the high sensitivity that can be obtained using the methanol fixation procedure. The formaldehyde procedure also requires a permeabilization step to allow the access of the antibody to the antigen, which may cause some subcellular leakage, whereas the methanol fixation method avoids that problem. Since every protein has its own optimal fixation conditions, two different fixation methods, formaldehyde and methanol fixation, were compared, in case the observed localization of 14-3-3ζ is sensitive to the method of fixation.

Both fixation methods yielded similar results regarding the effect of SUMO knockdown on the localization of 14-3-3ζ. To ensure objectivity, I scored the samples blindly (without being aware of which samples were the control samples and which were the SUMO knockdown samples). For each sample, I determined the percentage of cells that exhibited reduced levels of 14-3-3ζ in the nucleus as compared to the cytoplasm (Figure 6A, B, C, D). 71.5% of formaldehyde fixed and 72.5% of methanol fixed cells exhibited this phenotype (Table 1, Figure 7). However, in SUMO knockdown cells only 36.7% of the formaldehyde fixed and 30.5% of the methanol fixed cells showed lower levels 14-3-3ζ in the nucleus than in the cytoplasm (Table 1, Figure 7). Rather, the majority of SUMO knockdown cells exhibited equal levels 14-3-3ζ, in the nucleus and cytoplasm.

# DISCUSSION

**A possible role for SUMO in 14-3-3 ζ nuclear export.**

One of the processes with which SUMO has been closely associated is nuclear import and export and for targeting proteins to the nuclear pores (Seeler and Dejean, 2003). For example, the cytoplasmic nuclear import factor Ran-GAP requires SUMO modification for its translocation to the nuclear pore complex (NPC) (Matunis *et al.,* 1996). In addition, mutagenesis of the gene encoding the SUMO-conjugating enzyme Ubc9 appears to result in reduced nuclear import of the transcription factor Bicoid (Epps and Tanda, 1998). This explains the *hunchback*-like phenotype of *ubc9* mutants because zygotic expression of *hunchback is* dependent on Bicoid. Furthermore, SUMO can modulate export from the nucleus. The TEL protein, which is a Ras-regulated repressor of transcription, is exported from the nucleus when SUMOylated in NIH-3T3 cells (Wood *et al.,* 2002). As a result, the TEL SUMO-acceptor site mutant (TEL K99R) was found to be a better repressor of Ras target genes than was wild-type TEL.

Since previous studies have demonstrated that 14-3-3ζ is localized in both the nucleus and cytoplasm, I decided to determine whether SUMOylation of 14-3-3ζ regulates its localization to the nucleus. To assess this possibility, I compared control and SUMO knockdown cells by two methods: subcellular fractionation followed by anti-14-3-3ζ western blotting and anti-14-3-3ζ immunofluorescence studies. The two methods appear to give discordant results. While the subcellular fractionation studies do not reveal a change in the ratio of nuclear to cytoplasmic 14-3-3ζ following SUMO knockdown, immunofluorescence studies suggest that SUMO knockdown may lead to increased levels of nuclear 14-3-3ζ in a subset of cells. Thus, although these results are preliminary, they suggest that SUMO may interfere with 14-3-3ζ nucleocytoplasmic transport either by inhibiting nuclear import or stimulating nuclear export. Given that the SUMO knockdown cells never show 14-3-3ζ enrichment in the nucleus relative to

the cytoplasm, but only a loss of the cytoplasmic enrichment observed in the control cells, it is more likely that SUMO promotes nuclear export of 14-3-3ζ.

**Future Directions**

My findings can be followed up in a number of ways. For example, to determine whether there is an increase in nuclear localization of 14-3-3ζ in SUMO knockdown cells, I can perform chromatin precipitation - polymerase chain reaction (ChIP-PCR) experiments on known 14-3-3ζ target genes. This will allow me to reassess the possibility that SUMO affects the subcellular localization 14-3-3ζ and to determine whether 14-3-3ζ becomes enriched at chromatin perhaps in a target gene-specific manner in the absence of SUMO.

To further investigate the role of SUMO in 14-3-3ζ nuclear export, I could perturb protein nuclear export by various methods. For example, previous work has shown that Leptomycin B modifies and inhibits CRM1 (exportin 1), a factor required for the nuclear export of many proteins (Kudo *et al.*, 1999). In such an experiment, I would treat S2 cells with this inhibitor and then determine whether knocking down SUMO still affects 14-3-3ζ nuclear localization in the absence of functional CRM1. If I find that the change in nuclear localization is dependent upon CRM1, this would provide additional support for the idea that SUMO modulates 14-3-3ζ nuclear export.

In addition to the subcellular localization studies, additional in vivo work to determine how SUMOylation of 14-3-3ζ affects *Drosophila* development needs to be carried out. Previous experiments have demonstrated that the function of SUMO targets is sensitive to SUMO gene dosage. For example, Schnorr *et al*. showed defects in eggshell dorsoventral patterning (a Ras-

dependent process) in females heterozygous for mutant alleles of the genes encoding SUMO and Ras (Schnorr *et al*., 2001). Therefore, we will generate double heterozygotes for the genes encoding SUMO and 14-3-3ζ and look for defects in Ras-dependent development processes or other processes that could require 14-3-3ζ.

The most likely SUMO-acceptor residue in 14-3-3ζ is K125. I have therefore recently generated a 14-3-3ζ mutant allele in which this lysine has been converted to arginine (see Materials and Methods). To confirm that this residue is required for SUMOylation, the mutant protein will be examined by well-established in vitro and in vivo SUMOylation assays (Nie *et al*., 2009). Once this is confirmed, it will be possible to introduce this mutant allele into the Drosophila germ line and then carry out genetic studies to elucidate the function of the SUMO-modification of 14-3-3ζ. There are several possible phenotypes that these genetic experiments may reveal. For example, since 14-3-3ζ enhances Ras signaling (Freed *et al*., 1994), we can look for phenotypes known to result from Ras signaling defects, including eggshell dorsoventral patterning defects or embryonic anteroposterior patterning defects (Schüpbach *et al*., 1987; reviewed by Duffy and Perrimon, 1994). Additionally, since both SUMO and 14-3-3ζ have roles in mitosis, we can use approaches that have been previously employed to examine the effect of the 14-3-3ζ SUMO acceptor site mutation on mitosis (Su *et al*., 2001).

## TABLES AND FIGURES

**Table 1. Percentage of cells with low levels of 14-3-3ζ detected in the nucleus compared to cytosol**. Formaldehyde and methanol fixing methods were used for the immunofluorescence staining of WT and SUMO KD S2 cells. Percentage (%) corresponds to the ratio of number of cells with low levels of 14-3-3ζ in nucleus compared to cytosol and total cells.

## Table 1.

| Cell Fixing Method | Formaldehyde fixing | | Methanol Fixing | |
|---|---|---|---|---|
| | WT | SUMO KD | WT | SUMO KD |
| Number of cells with low levels of 14-3-3ζ in nucleus compared to cytosol | 98 | 11 | 37 | 18 |
| Total cells | 137 | 30 | 51 | 59 |
| Percentage (%) | 71.5% ± 3.9% | 36.7% ± 8.8% | 72.5% ± 6.2% | 30.5% ± 6.0% |

**Figure 1**. **The SUMO-modification pathway.** The SUMO peptides in immature form are first cleaved by Ulp to expose a di-glycine motif at the C-terminus. The SUMO with the exposed di-glycine motif attaches to SAE1/SAE2, the E1 activating enzyme in an ATP-dependent manner by the formation of a thioester bond before being transferred to Ubc9, the E2 conjugating enzyme. SUMO is then transferred from Ubc9 to a target protein, sometimes with the help of an E3 ligase. The consensus sequence found at the SUMO acceptor sites in target proteins is ψKXE, where ψ is a hydrophobic residue, K is the target lysine, X represents any residue, and E is glutamic acid. The SUMO-modified proteins also undergo deconjugation by the SUMO protease Ulp to release SUMO from the target proteins.

**Figure 1.**

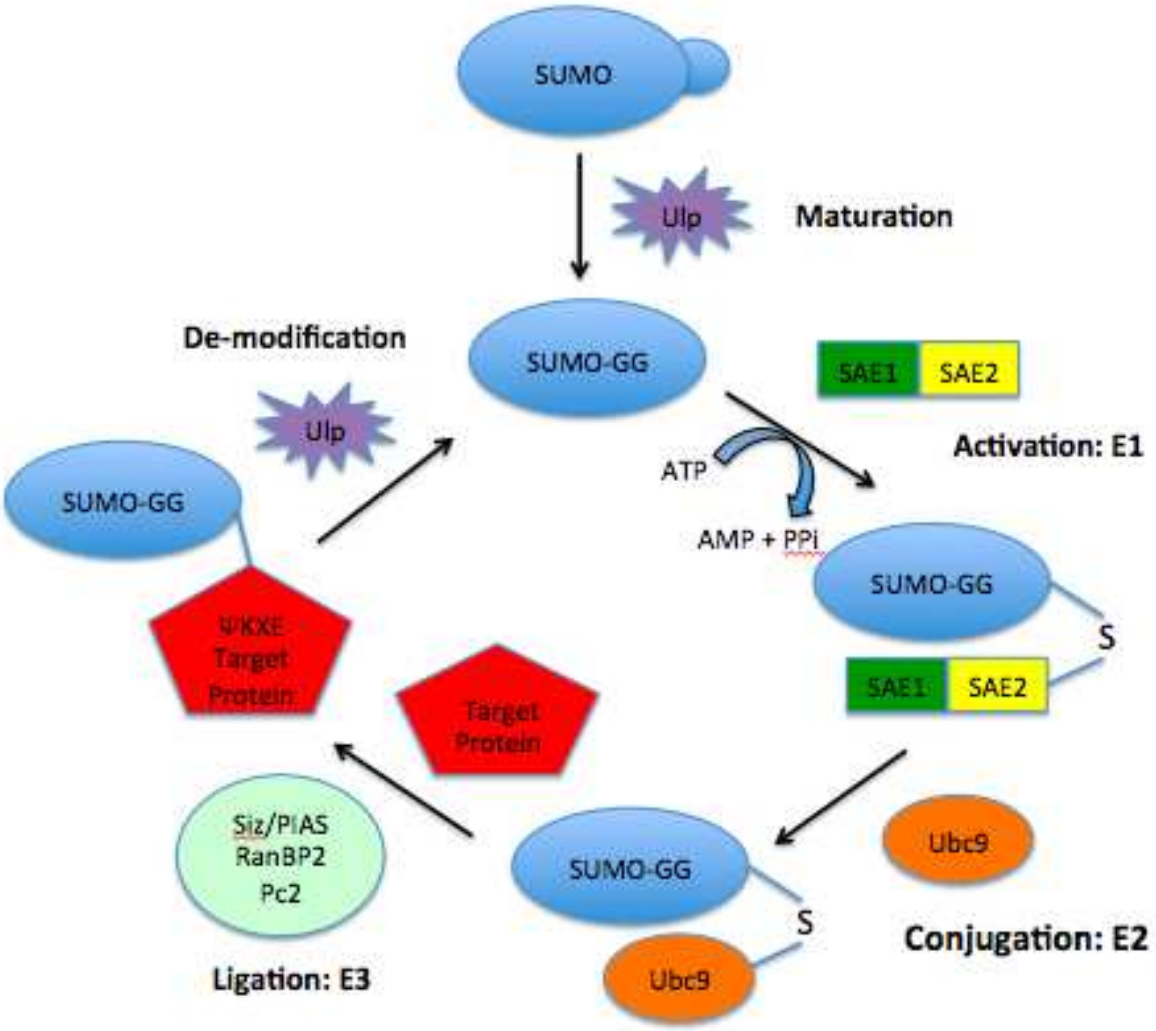

SUMO
Ulp
Maturation
De-modification
SUMO-GG
SAE1
SAE2
Activation: E1
Ulp
ATP
AMP + PPi
SUMO-GG
SUMO-GG
ΨKXE
Target
Protein
S
SAE1
SAE2
Target
Protein
Siz/PIAS
RanBP2
Pc2
SUMO-GG
Ubc9
S
Conjugation: E2
Ligation: E3
Ubc9

**Figure 2. The Ras signaling pathway.** The RTK first dimerizes leading to the autophosphorylation of particular tyrosines and providing a binding site for the adapter proteins Shc and Grb2. SOS is then recruited and activates Ras, followed by the sequential activation of Raf, MEK, and ERK by phosphorylation. Once ERK is activated, it is able to phosphorylate its target proteins. PP2A and 14-3-3ζ are also required for Raf activation.

**Figure 2.**

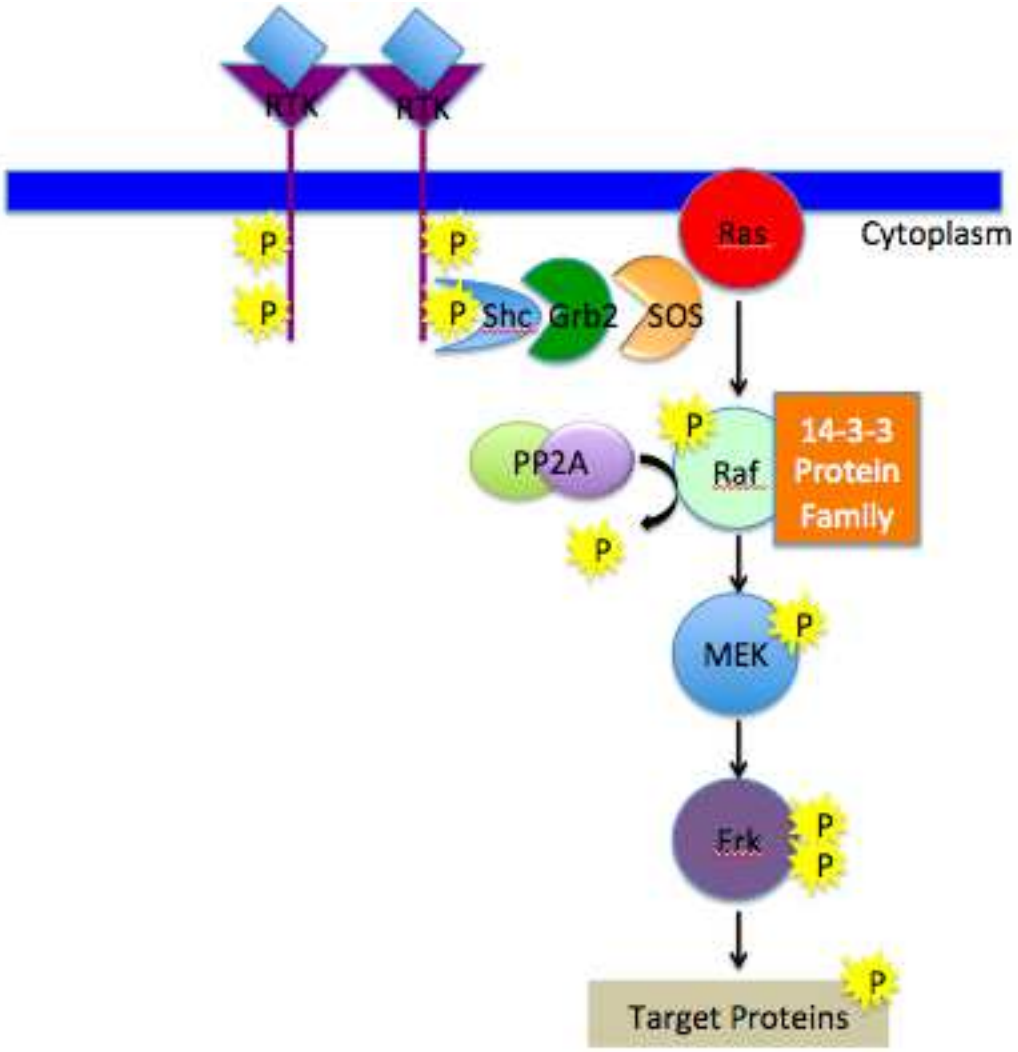

RTK
RTK
P
P
P
P
P
Shc
Grb2
SOS
Ras
Cytoplasm
P
PP2A
Raf
14-3-3
Protein
Family
P
MEK
P
Erk
P
P
Target Proteins
P

**Figure 3. Potential SUMO-acceptor sites of 14-3-3ζ are predicted by SUMOplot™.** The program predicts SUMOylation sites using information about the sequence context of SUMOylation sites in known SUMO targets. The score ranges from 0 to 1. K125 is the highest probability SUMO acceptor site with a score of 0.8, followed by the two lower probability sites at K6 and K71, both with scores of 0.5.

**Figure 3.**


| No. | Pos. | Group | Score |
|---|---|---|---|
| 1 | K125 | VFYLK **MKGD** YYRYL | 0.80 |
| 2 | K71 | ISSIE **QKTE** ASARK | 0.50 |

| No. | Pos. | Group | Score |
|---|---|---|---|
| 3 | K6 | MSTV **DKEE** LVQKA | 0.50 |

**Figure 4. Flowchart of the subcellular fractionation method used to study the localization of 14-3-3ζ in *Drosophila* cultured cells.** S2 cells are first lysed and dounce homogenized with the tight pestle to break open cells. The cell lysate is then centrifuged through a sucrose cushions to separate the nuclear fraction (pellet) from the cytosolic fraction (top layer of the supernatant). For a better separation, a second spin is performed on both the nuclear and cytosolic fractions. After the separation, the cellular fractions are used to perform western blots with antibodies against 14-3-3ζ, tubulin (a cytoplasmic marker), and Rpb3 (a nuclear marker).

**Figure 4.**

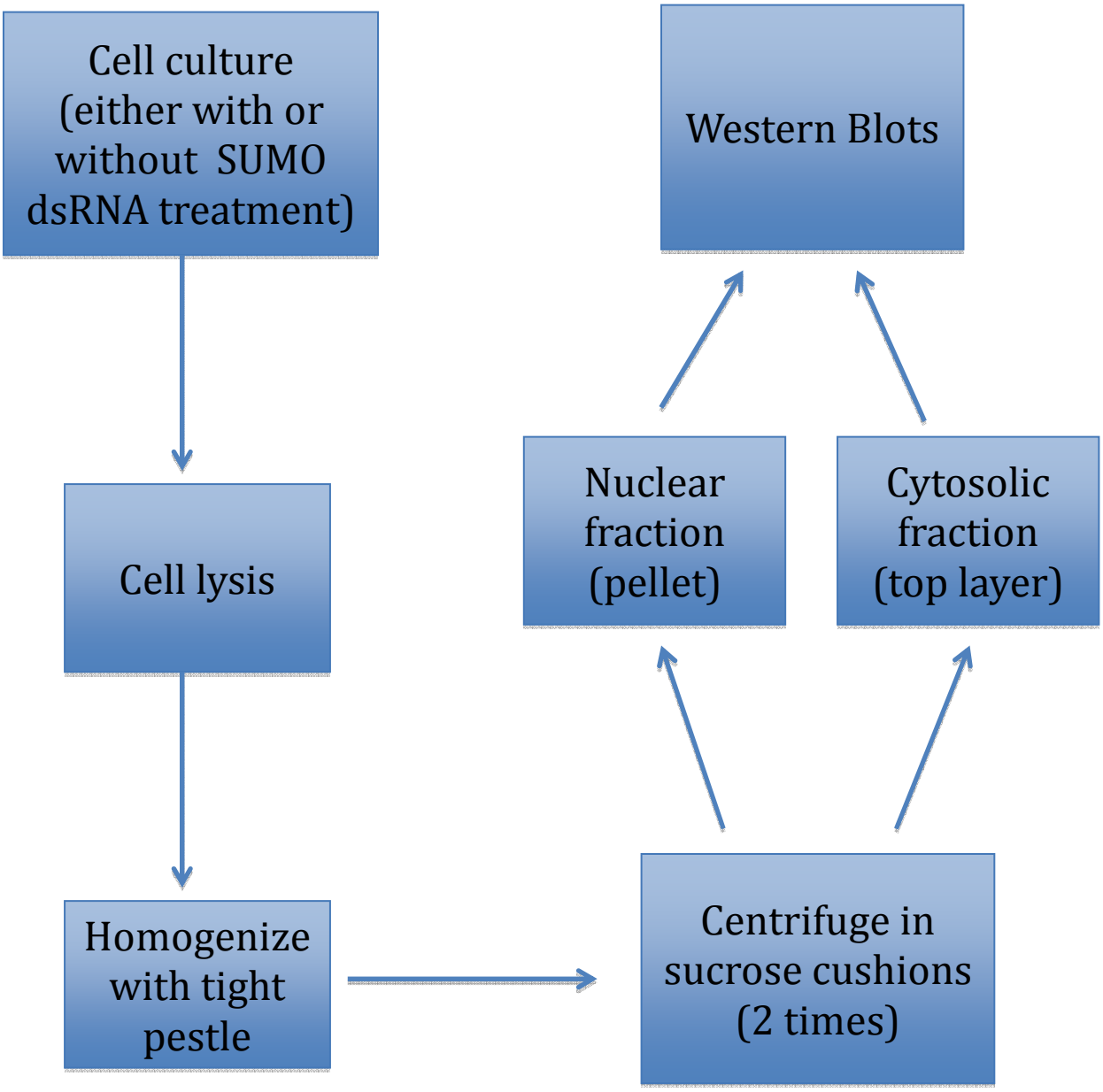
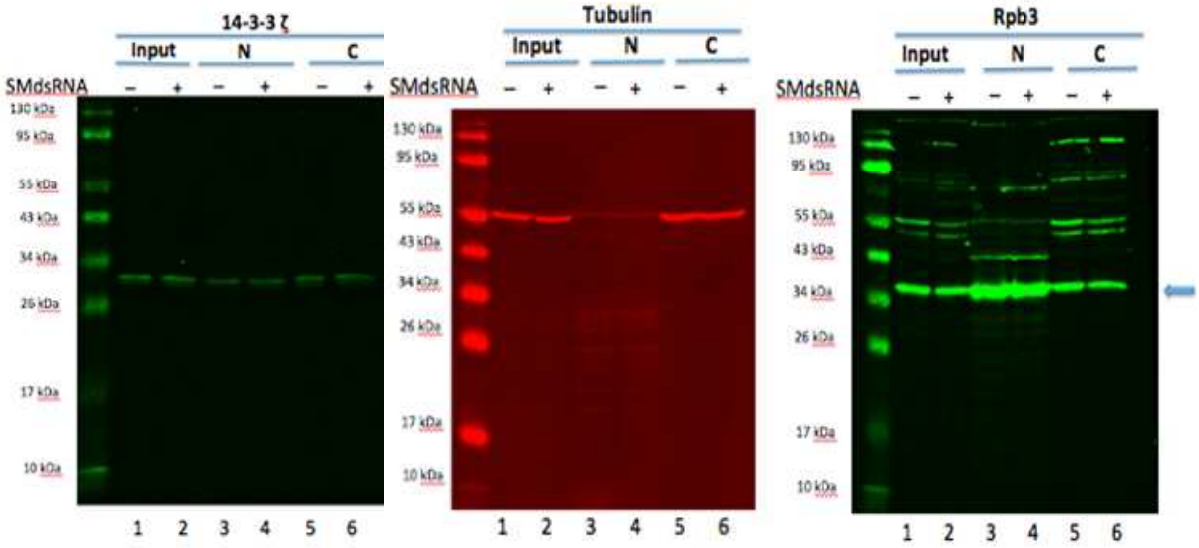

Cell culture (either with or without SUMO dsRNA treatment)
Cell lysis
Homogenize with tight pestle
Centrifuge in sucrose cushions (2 times)
Nuclear fraction (pellet)
Cytosolic fraction (top layer)
Western Blots

**Figure 5. Localization of 14-3-3ζ in *Drosophila* cultured cells (S2 cells) is determined by subcellular fractionation**. **A)** Western blot with 14-3-3ζ antibodies was performed to detect the level expression of 14-3-3ζ in the cell lysates (Input), nuclear fraction (N), and cytosolic fraction (C). The Rpb3 and tubulin serve as nuclear and cytosolic markers respectively. The cell samples used in the subcellular fractionation experiment included the control S2 cells (without treatment of SUMO dsRNA) denoted with "−" sign, and the SUMO dsRNA treated S2 cells denoted with "+" sign. The arrow in the western blot with Rpb3 antibodies points to the band representing Rpb3. **B)** Western Blot with SUMO antibodies shows the reduced levels of SUMO expression in cells treated with SUMO dsRNA (denoted with "+" sign) compared to the control sample without any treatment (denoted with "−" sign). Tubulin served as a control loading.

**Figure 5.**

**A.**


14-3-3 ζ
Input
N
C
SMdsRNA
– + – + – +
130 kDa
95 kDa
55 kDa
43 kDa
34 kDa
26 kDa
17 kDa
10 kDa
1 2 3 4 5 6
Tubulin
Input
N
C
SMdsRNA
– + – + – +
130 kDa
95 kDa
55 kDa
43 kDa
34 kDa
26 kDa
17 kDa
10 kDa
1 2 3 4 5 6
Rpb3
Input
N
C
SMdsRNA
– + – + – +
130 kDa
95 kDa
55 kDa
43 kDa
34 kDa
26 kDa
17 kDa
10 kDa
1 2 3 4 5 6

**B.**

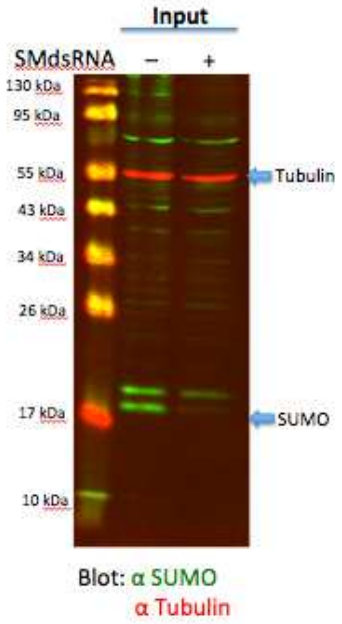
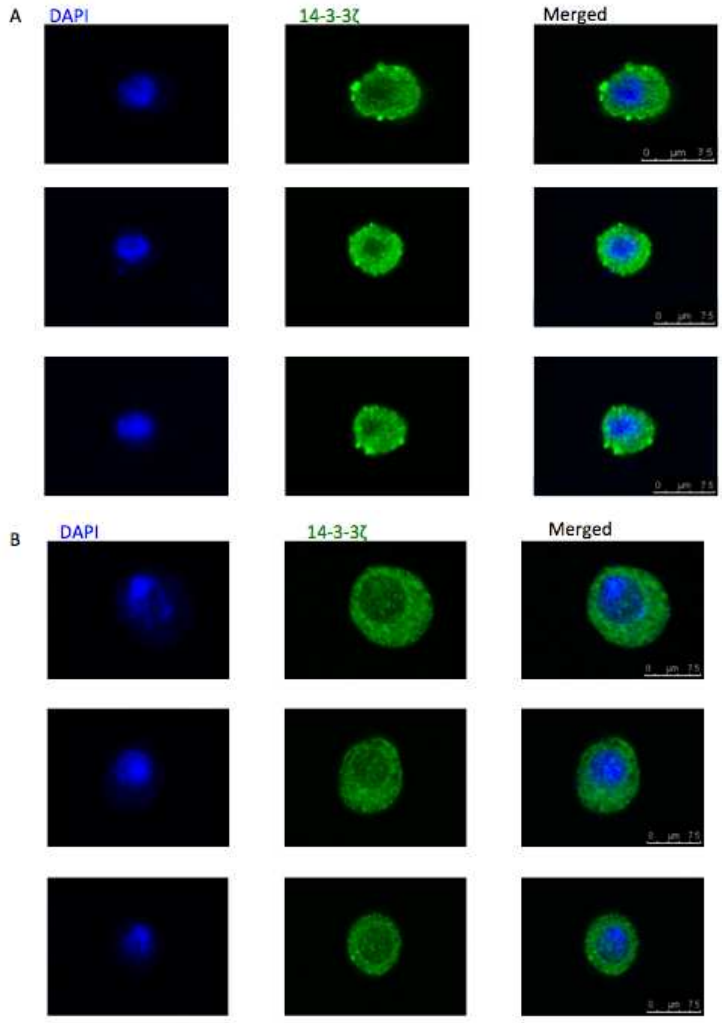
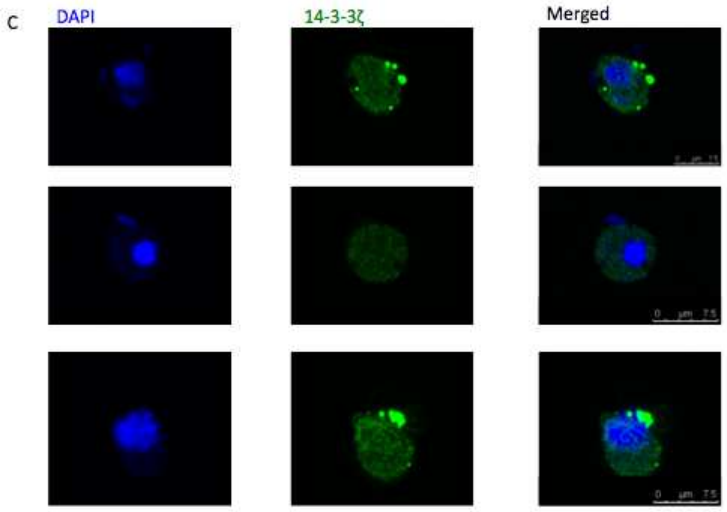
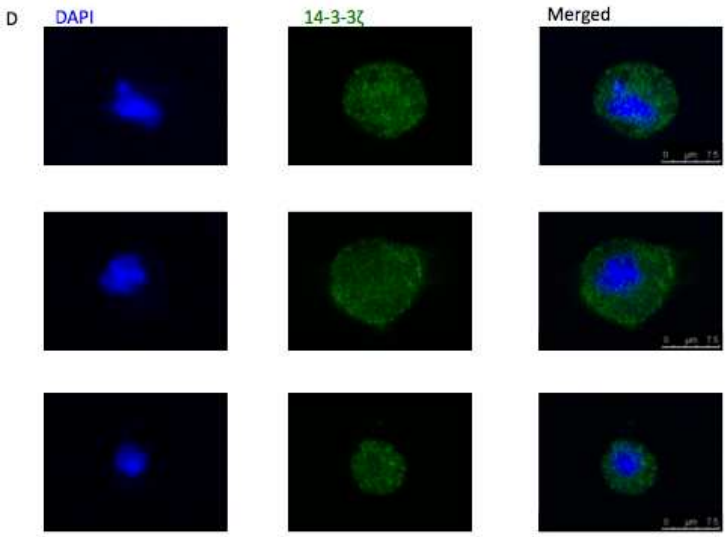

Input
SMdsRNA – +
130 kDa
95 kDa
55 kDa
43 kDa
34 kDa
26 kDa
17 kDa
10 kDa
Tubulin
SUMO
Blot: α SUMO
α Tubulin

**Figure 6. Localization of 14-3-3ζ in *Drosophila* cultured cells (S2 cells) imaged by immunofluorescence staining**. **A.** Three examples of control S2 cells fixed with formaldehyde. **B.** Three examples of control S2 cells fixed with methanol. **C.** Three examples of SUMO knockdown S2 cells fixed with formaldehyde. **D.** Three examples of SUMO knockdown S2 cell fixed with methanol. Cells were stained with DAPI (blue) and 14-3-3ζ antibodies (green). Bar scale for cell samples is 7.5 μm. **E.** Western Blot with SUMO antibody shows that levels of SUMO were reduced when S2 cells were treated with SUMO dsRNA. Tubulin served as a loading control.

**Figure 2-3.**




C
DAPI
14-3-3ζ
Merged


D
DAPI
14-3-3ζ
Merged

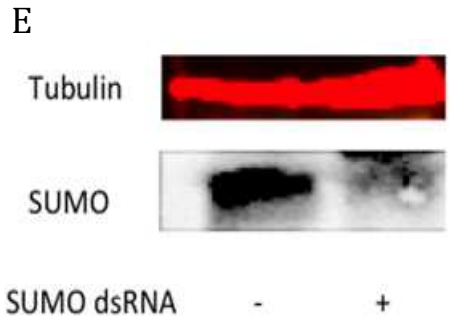
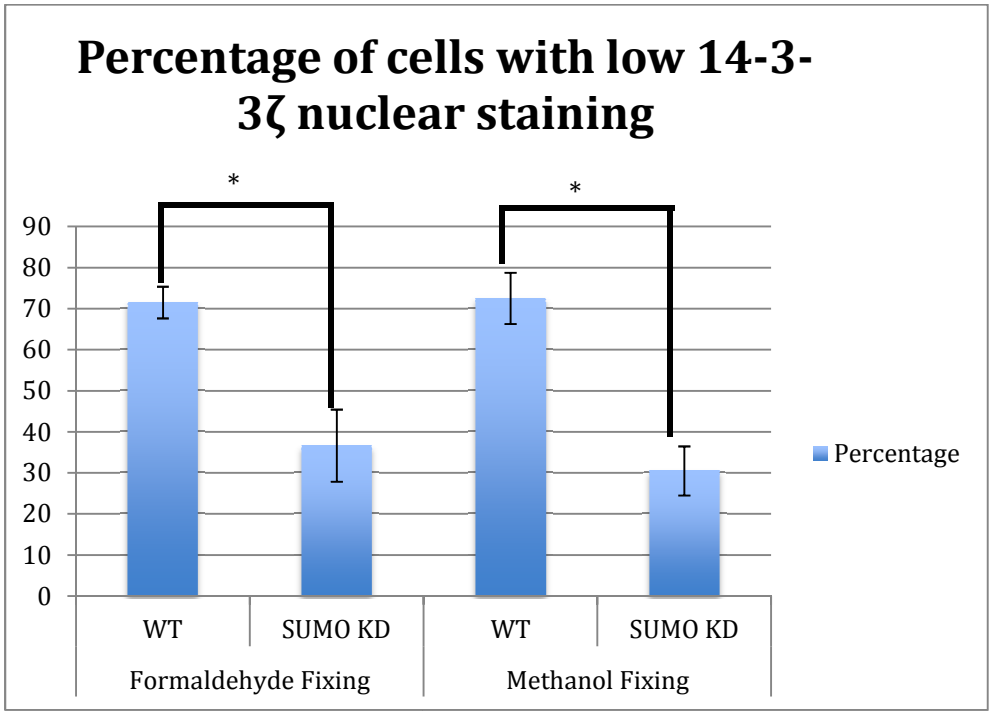

E
Tubulin
SUMO
SUMO dsRNA
-
+

**Figure 7. Quantitation of the immunofluorescence results.** The bar graph shows the percentage of S2 cells with lower levels of 14-3-3ζ in the nucleus than in the cytoplasm (see Figure 2-3, A and B for examples). The data are from Table 1. Error bars represent for the standard deviation for cell samples. * p-value < 0.001.

**Figure 7.**


Percentage of cells with low 14-3-3ζ nuclear staining
*
*
90
80
70
60
50
40
30
20
10
0
WT
SUMO KD
WT
SUMO KD
Formaldehyde Fixing
Methanol Fixing
Percentage

## REFERENCES

1. Avruch, J., *et al.* (2001) Ras activation of the Raf kinase: tyrosine recruitment of the MAP kinase cascade. *Recent Prog. Horm. Res.* 56, 127-155.
2. Bhaskar, V., *et al.* (2002) Conjugation of Smt3 to Dorsal May Potentiate the *Drosophila* Immune Response. *Mol Cell Biol.* 2, 492-504.
3. Bridges, D. and Moorhead, G. (2005) 14-3-3 Proteins: A Number of Functions for a Numbered Protein. *Science*.
4. Campbell, S. *et al*. (1998) Increasing complexity of Ras signaling. *Oncogene*. 17, 1395-1413.
5. Chang, H. and Rubin, G. (1997) 14-3-3ϵ positively regulates Ras-mediated signaling in Drosophila. *Genes Dev.* 11, 1132-1139.
6. Dhillon A. *et al*. (*2009*) The C-terminus of Raf-1 acts as a *14-3-3*-dependent activation switch. *Cell Signal* 21, 1645-51.
7. Duffy J. and Perrimon N. (1994) The torso pathway in Drosophila: lessons on receptor tyrosine kinase signaling and pattern formation. *Dev. Biol*. 166, 380–395.
8. Epps, J. and Tanda, S. (1998) The *Drosophila* semushi mutation blocks nuclear import of Bicoid during embryogenesis. *Curr Biol.* 8, 1277-1280
9. Freed, E., *et al.* (1994) Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science.* 265, 1713-1716.
10. Gill, G. (2005) Something about SUMO inhibits transcription. *Current Opinion in Genetics & Development.* 15, 536-541.
11. Hershko A**,** Ciechanover A, Heller H, Haas AL, and Rose IA**.** (1980) Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. Proc Natl Acad Sci USA. 77, 1783–1786.

12. Huang, H., *et al.* (2011) *Drosophila* Smt3 negatively regulates JNK signaling through sequestering Hipk in the nucleus. *Development.* 138, 2477-2485.
13. Jaumot, M. and Hancock, J. (2001) Protein Phosphatases 1 and 2a promote Raf-1 activation by regulating 14-3-3 interactions. *Oncogene.* 20, 3949-3958.
14. Khodor, Y., Rodriguez, J., Abruzzi, K., *et al*. (2011) Nascent-seq indicates widespread cotranscriptional pre-mRNA splicing in Drosophila. *Genes Dev*. 25, 2502-2512.
15. Kudo, N., *et al.* (1999) Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *PNAS* 16, 9112-9117.
16. Leevers, S., *et al.* (1994). Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature.* 369, 411-414.
17. Li, W., *et al.* (1997) The Drosophila 14-3-3 protein Leonardo enhances Torso signaling through D-Raf in a Ras 1-dependent manner. *Development.* 124, 4163-4171.
18. Matunis MJ, Wu J, Blobel G (1998). SUMO-1 modification and its role in targeting the Ran GTPase-activating protein, RanGAP1, to the nuclear pore complex. *The Journal of Cell Biology* 140, 499–509.
19. McCubrey, J., *et al.* (2007) Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim Biophys Acta.* 1773:1263-84
20. Miles, W., *et al.* (2008) Medea SUMOylation restricts the signaling range of the Dpp morphogen in the *Drosophila* embryo. *Genes Dev.* 22, 2578-2590.
21. Ni, Z., *et al*. (2008) P-TEFb is critical for the maturation of RNA polymerase II into productive elongation in vivo. *Mol. Cell. Biol. 28*, 1161--1170.
22. Nie, M., *et al.* (2009) Genetic and proteomic evidence for roles of Drosophila SUMO in cell cycle control, Ras signaling, and early pattern formation. *Plos 1.*

23. Schnorr, J. *et al.* (2001) Ras1 interacts with multiple new signaling and cytoskeletal loci in Drosophila eggshell patterning and morphogenesis. *Genetics.* 159, 609-622.

*24.* Schüpbach T. *1987* Germ line and soma cooperate during oogenesis to establish the dorsoventral pattern of egg shell and embryo in *Drosophila melanogaster. Cell* 49*,* 699–707*.*

25. Seeler, JS, and Dejean, A. (2003) Nuclear and unclear function of SUMO. *Nat Rev Mol Cell Biol*. 9, 690-699.

26. Skoulakis, E.M., Davis, R.L. (1996). Olfactory learning deficits in mutants for leonardo, a Drosophila gene encoding a 14-3-3 protein. *Neuron* 17, 931--944.

27. Smith *et al.* (2004) *Drosophila* Ulp1, a nuclear pore-associated SUMO protease, prevents accumulation of cytoplasmic SUMO conjugates. *JBC* 279, 43805-43814.

28. Stokoe, D., *et al*. (1994). Activation of Raf as a result of recruitment to the plasma membrane. Science 264, 1463-1467.

29. Su, T., *et al.* (2001) Cell cycle roles for two 14-3-3 proteins during *Drosophila* development. *Journal of Cell Science* 114, 3445-3454.

30. Tzivion *et al.* (1998) A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature* 394, 88-92.

31. Verger, A., Perdomo, J., and Crossley, M. (2003) Modification with SUMO. *EMBO.* 4, 137-142.

32. Winter, S., *et al.* (2008) 14-3-3 Proteins recognize a histone code at histone H3 and are required for transcriptional activation. *Embo.* 27, 88-99.

33. Wood, L., *et al.* (2003) Small ubiquitin-like modifier conjugation regulates nuclear export of TEL, a putative tumor suppressor. *PNAS* 6, 3257-3262.