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SUMO stimulates the dephosphorylation of an inhibitory phosphoserine residue in Raf

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by

Min Woo Kim

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ABSTRACT OF THE THESIS

SUMO stimulates the dephosphorylation of an inhibitory phosphoserine residue in Raf

by

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Master of Science in Biochemistry, Molecular, and Structural Biology

University of California, Los Angeles, CA 2016

Professor Albert J. Courey, Chair

Small ubiquitin-related modifier (SUMO) becomes covalently conjugated to many proteins regulating a variety of cellular and development processes. A previous study demonstrated that multiple components of the Ras/MAPK signaling pathway are directly conjugated to SUMO in the early *Drosophila* embryo, and that the Ras/MAPK signaling cascade requires SUMO for activation in insulin or Spitz-stimulated *Drosophila* S2 cells. SUMO knockdown leads to reduced MAPK phosphorylation in response to either signal. Here, we elucidate a mechanism by which SUMO positively regulates the Ras/MAPK signaling pathway by showing that the dephosphorylation of Raf at serine 346 to activate Ras/MAPK signaling requires SUMO. Furthermore, site-directed mutagenesis of serine 346 to alanine, preventing its phosphorylation, bypasses the requirement for SUMO in the activation of the Ras/MAPK pathway. Earlier studies have shown that serine 346 dephosphorylation is required for membrane

localization of Raf, which is a pre-requisite for Raf activation. We show that fusing the Raf catalytic domain to the transmembrane and extracellular domains of Torso artificially tethering Raf to the plasma membrane restores Ras signaling in SUMO knockdown cells leading to SUMO independent Ras/MAPK signaling. We conclude that SUMO stimulates Ras/MAPK signaling by promoting the removal of an inhibitory phosphate group, which is a pre-requisite for Raf membrane localization and activation. While there is no evidence that Raf itself is sumoylated, several Raf interacting proteins, such as 14-3-3ζ and PP2A are members of the SUMO-ome suggesting an indirect role for SUMO in Raf localization.

The thesis of Min Woo Kim is approved.

Guillaume Chanfreau

James W. Gober

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INTRODUCTION

Cells communicate with each other through multiple signaling pathways responding effectively to changes in their surroundings by transducing environmental cues across the plasma membrane and into the nucleus to alter the transcriptional state of the cell. A prominent example of cellular signaling pathways is the Ras/mitogen-activated protein kinase (MAPK) pathway, which is widely used in the eukaryotic domain to coordinate the regulation of cellular differentiation, proliferation, and motility (Dhillon *et al*., 2007; McCubry *et al*., 2006; Torri *et al*., 2006). Moreover, due to its central role in modulating varying cellular processes, misregulation of the Ras/MAPK pathway has been shown to be involved in the pathogenicity of various diseases, including Alzheimer's disease, Parkinson's disease, and various types of cancers galvanizing interest in elucidating the mechanistic details by which the pathway functions (Dhillon *et al*., 2007; Hashimoto *et al*., 2003; Miller *et al*., 2009).

Here, I look more closely into the activation of one MAPK, the extracellular signal-regulated kinase (ERK). ERK activation begins with the binding of a variety of activating ligands including epidermal growth factor (Egf), insulin (In), and Trunk (Trk) to their cognate transmembrane receptor tyrosine kinases (RTKs). Ligand binding induces the dimerization of the RTKs promoting autophosphorylation at distinct tyrosine residues on their intracellular domains, which stimulates the recruitment of an adaptor protein, Downstream of receptor kinase (Drk). Drk then recruits Son of sevenless (Sos), a guanine nucleotide exchange factor that triggers the release of GDP allowing for binding of GTP positively regulating the small GTPase Ras. This is required for the direct interaction of Ras with Raf, a serine/threonine kinase, recruiting Raf to the plasma membrane. Raf then initiates the canonical protein phosphorylation cascade, whereby Raf phosphorylates MAPK/ERK Kinase (MEK), which in turn phosphorylates ERK triggering

its translocation to the nucleus where it interacts with various target substrates, primarily transcription factors, to alter the transcriptional program of the cell.

Previous research has defined a role for the ERK module in mitosis, identifying its interaction with mitogenic proteins in the nucleus that modulate cell proliferation. One example is the ERK-catalyzed phosphorylation and positive regulation of ETS domain-containing protein Elk-1, a sequence-specific transcription factor that coordinates the expression of immediate-early genes (Gille *et al*., 1995). ERK has also been shown to interact with products of immediate-early genes, which are transiently induced in response to an extracellular stimulus, stabilizing c-Fos proteins thereby allowing for its interaction with c-Jun to foster the formation of the AP-1 complex, which plays a prominent role in regulating cell cycle progression (Shaulian and Karin, 2001; Whitmarsh and Davis, 1996).

A variety of RTKs have previously been identified in *Drosophila melanogaster* to be essential during embryonic development, influencing cellular proliferation and growth as well as the proper patterning of the embryo. One such RTK, the *Drosophila* Torso receptor (Tor) modulates gene expression specifying cell fate and differentiation at both the anterior and posterior embryonic termini (Duffy and Perrimon, 1994). Thus, binding of the Trk ligand to the Tor receptor activates the Ras/MAPK pathway inactivating both the repressor Capicua (Cic) and the corepressor Groucho (Gro) thereby allowing for the terminal expression of *tailless* (*tll*) and *huckebein* (*hkb*) (Cinnamon *et al*., 2008; Jimenez *et al*., 2000). Both *tll* and *hkb* are transcription factors required for the proper antero-posterior patterning of the early *Drosophila* embryo (Gui *et al*., 2011; Reuter and Leptin, 1994). Whether through regulation during embryonic development or in the maintenance of cellular proliferation, the Ras/MAPK pathway plays a critical role in modulating varying cellular processes essential for viability.

Small ubiquitin-related modifier (SUMO) is directly conjugated to many protein targets, thereby modulating multiple cellular as well as developmental processes. Although, exhibiting minimal primary sequence similarity to ubiquitin, SUMO still adopts the characteristic ubiquitin tertiary fold (Bayer *et al*., 1998). Whereas, ubiquitylation is often, but not always, associated with proteasomal degradation, SUMOylation functions by conferring specificity to protein-protein interactions and by regulating protein subcellular localization.

The process by which SUMO becomes directly conjugated to target proteins parallels the ubiquitin conjugation pathway with the utilization of a different set of E1 activating, E2 conjugating, and E3 ligase enzymes (Kerscher *et al*., 2006). The SUMO conjugation pathway begins with an initial maturation step that involves removal of a C-terminal SUMO extension by one of two ubiquitin-like proteases (Ulp1 or Ulp2) revealing a C-terminal diglycine motif (Figure 1). The mature form of SUMO then undergoes ATP dependent thioester conjugation, in which the C-terminal glycine of SUMO is linked to a specific cysteine residue in the E1 activating enzyme, a heterodimer consisting of SAE1 and SAE2 subunits. SUMO is subsequently transferred from the E1 enzyme to a catalytic cysteine residue on the SUMO-conjugating enzyme, Ubc9 (Gareau and Lima, 2010). Ubc9 then, in an E3 ligase-independent manner, recognizes a consensus ΨKxE motif (where Ψ represents a hydrophobic residue and x represents any amino acid) in which the lysine residue acts as the acceptor site for SUMO conjugation, modifying the target protein and thereby modifying its function (Gareau and Lima, 2010). Unlike the ubiquitin pathway, which requires an E3 ligase to transfer ubiquitin from the E2 enzyme to a specific substrate, Ubc9 appears to be sufficient to catalyze the formation of an isopeptide linkage between the C-terminal glycine residue of SUMO and a lysine residue of the target protein. However, a number of proteins, including the PIAS family proteins, RanBP2, and

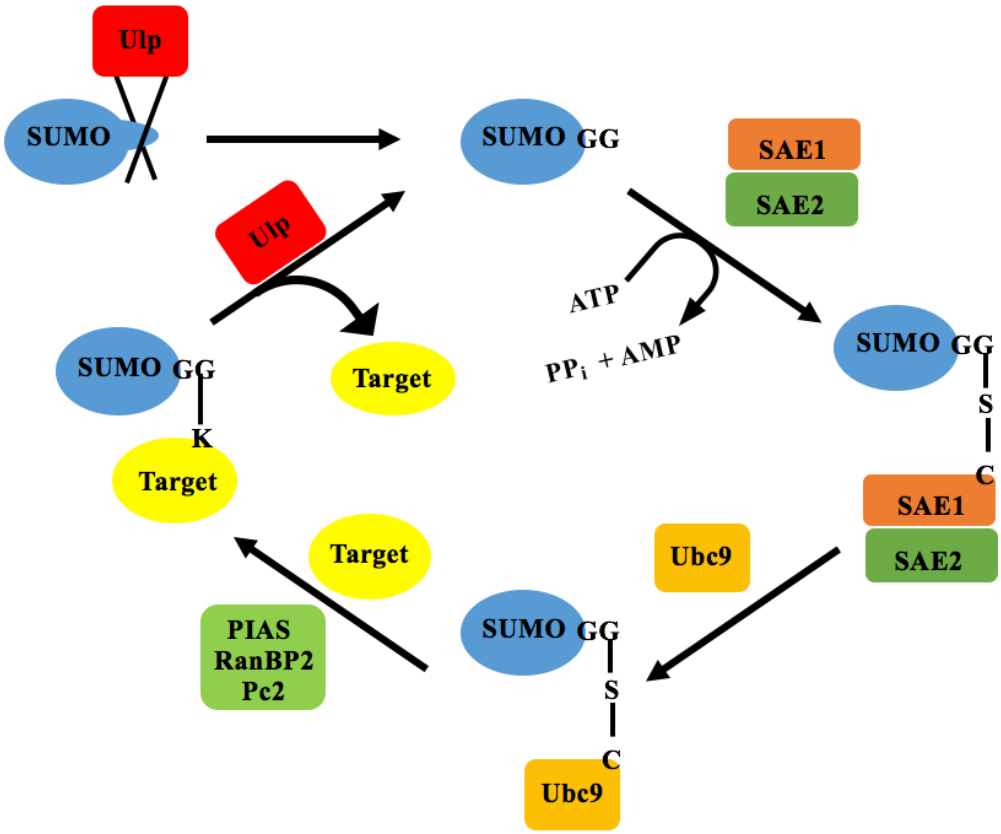

Figure 1: The SUMO Conjugation Pathway. SUMO begins as an immature protein and SUMO undergoes an initial maturation step with a cleavage occurring at its C-terminus by ubiquitin-like protease (Ulp) revealing a diglycine (GG) motif. Subsequently, it undergoes an ATP dependent thioester conjugation linking its C-terminal glycine to a cysteine residue on the E1 activating enzyme (SAE1 and SAE2 heterodimer). This gets transferred to a cysteine residue in Ubc9, an E2 conjugating enzyme. Oftentimes, one of several E3 ligases, such as the PIAS family proteins, RanBP2, and Polycomb group member proteins Pc2, assist Ubc9 in catalyzing the formation of an isopeptide linkage between SUMO and its target protein. SUMO conjugated proteins can be deconjugated in a reaction whereby Ulp cleaves the isopeptide linkage between SUMO and the target protein.

Polycomb group member Pc2 have been shown to possess SUMO ligase activity (Geiss-Friedlander and Melchior, 2007). E3 protein ligases coordinate the formation of an isopeptide bond in one of two ways: facilitating the interaction between Ubc9 and the respective target protein to promote substrate specificity or by stimulating the discharge of SUMO from the active

site cysteine residues of Ubc9, if the substrate already interacts directly with Ubc9 (Gareau and Lima, 2010).

In addition to the direct conjugation of SUMO to target proteins, SUMO facilitates noncovalent protein interactions. A previous study utilizing NMR spectroscopy and subsequently corroborated by structural studies characterized the SUMO interaction motif (SIM), which is comprised of two essential hydrophobic amino acid residues often flanked by acidic amino acids that bind to a hydrophobic pocket on the surface of SUMO (Song *et al*., 2004; Reverter and Lima, 2005; Song *et al*., 2005). Moreover, phosphorylated serine residues mimicked the functionality of the flanking acidic amino acid residues as evidenced by PML, EXO9, and the PIAS proteins, which utilize phospho-SIMs to facilitate interactions with lysine residues of SUMO (Hannich *et al*., 2005; Hecker *et al*., 2006; Stehmeier and Muller, 2009). Thus, the SUMO-SIM interaction presents a mechanism by which SUMO stabilizes protein-protein interactions, in which the usage of phosphorylated serine residues (phospho-SIMS) adds an extra layer of regulation and specificity for the proper selection of SUMO interaction targets.

SUMO modulates the function of a wide variety of target proteins, and thus, becomes involved in a myriad of cellular processes. Previous studies examined the impact of nutrient stress on the target of rapamycin complex 1 (TORC1) pathway and associated proteins downstream defining a role for SUMO in the regulation of genes involved in cellular proliferation and growth in response to alterations to the cellular environment. More specifically, varying components of RNA polymerase III (RNAP III), essential for the transcription of tRNA, lacked a conjugated SUMO during times of nutrient stress affecting their respective functionality (Chymkowitch *et al*., 2015). Although the role of SUMO in RNAP III regulation as well as the inhibitory mechanism impeding SUMOylation of RNAP III components remains elusive, the

proper functioning of RNAP III requires SUMO in the TORC1 pathway. Moreover, nutrient stress induced inhibition of RNAP III SUMOylation resulted in reduced tRNA expression levels further suggesting the significance of SUMO in modulating the translational capacity of the cells by gauging the availability of resources (Chymkowitch *et al*., 2015). These findings suggest that SUMOylation plays an essential role in maintaining cellular homeostasis helping to link the nutrient status of the environment to cellular growth.

Moreover, previous research demonstrated a connection between SUMOylation and mitosis, in which a number of proteins associated with the centromeres and the kinetochores as well as the Aurora B kinase, essential for proper attachment of the mitotic spindle to the centromere, and cohesion, which ensures the cohesion of sister chromatids, were identified as targets of SUMO conjugation (Almedawar *et al*., 2012; Wan *et al*., 2012). Uncontrolled mitosis fosters cancer cell proliferation, and thus, a previous genome-wide RNAi screen identified genes corresponding to different components of the SUMO conjugation pathway as essential for cellular proliferation suggesting a role for SUMO conjugation in tumorigenesis (Schlabach *et al*., 2008). Furthermore, varying cancers including breast cancer and melanoma revealed enhanced levels of the SUMO-conjugating enzyme Ubc9, and Myc-dependent breast cancer required the SAE2 subunit of the SUMO E1 activating enzyme (Mo *et al*., 2005; Moschos *et al*., 2007; Kessler *et al*., 2012). Utilization of shRNAs to knockdown either SAE2 or Ubc9 in human cancer cells demonstrated the ability to inhibit tumorigenesis by inhibiting SUMO conjugation (He *et al*., 2015). The potential role for SUMO in fostering tumorigenesis stimulates interest in further elucidating its mechanism of action with implications for different components to the SUMO conjugation pathway as targets for cancer therapy.

Courey and coworkers previously determined the *Drosophila* embryonic SUMO-ome, that is, they identified proteins that were directly conjugated to SUMO, by generating a transgenic *Drosophila* embryo expressing a dual $(His)_6$- and Flag-tagged SUMO and performing a tandem affinity purification followed subsequently by LC-MS/MS analysis (Nie *et al*., 2009). In doing so, multiple components of the Ras/MAPK pathway were found to be SUMO modified, including 14-3-3 family proteins as well as both the structural and catalytic subunits of the protein phosphatase 2A (PP2A) complex. Since the screen identified components of the Ras/MAPK pathway as SUMO modified proteins, Nie *et al.* examined the effect on MAPK phosphorylation of knocking down SUMO in *Drosophila* S2 cells, and discovered a SUMO requirement for activation of Ras/MAPK signaling (Nie *et al*., 2009)

Nie *et al*. generated SUMO mutant germline clone (GLC) embryos using a hypomorphic SUMO allele, successfully reducing levels of SUMO by more than five fold. Through both immunoblot and in situ antibody staining analysis, Courey and coworkers demonstrated that GLC embryos for SUMO exhibited reduced pMAPK at the embryonic termini (unpublished data). Knowing that activation of the Ras/MAPK signaling through Tor inactivates both a repressor Cic and a corepressor Gro, which serve to negatively regulate transcription of genes, such as *tll* and *hkb*, necessary for the proper anteroposterior patterning of the *Drosophila* embryo, the Courey lab quantified *tll* mRNA levels by using RT-qPCR and showed increased repression of *tll* in SUMO GLC mutant embryos (unpublished data). Since the Ras/MAPK pathway inhibits repression of *tailless* by Capicua, these observations are consistent with a positive role for SUMO in Ras/MAPK signaling, confirming the requirement for SUMO in the Ras/MAPK signaling pathway *in vivo*.

The proteomic study described above identified both 14-3-3 family proteins and two subunits of the PP2A complex as SUMO conjugation targets. Both 14-3-3 family proteins and the PP2A complex are involved in the regulation of Raf suggesting that SUMO may interact with the Ras/MAPK signaling pathway through Raf. Raf contains three conserved regions (CR): CR1 composed of the Ras-binding domain (RBD) and a cysteine-rich domain (CRD) facilitating the Ras-Raf interaction, CR2 consisting of a serine-threonine rich domain, which upon phosphorylation of specific residues allows for the binding of 14-3-3 regulatory proteins to Raf, and CR3 composed of the Raf kinase domain near the C-terminus (Rapp *et al*. 1986; Roskoski, 2010). While in humans, there are three different forms of Raf (A-Raf, B-Raf, C-Raf), the *Drosophila* genomes encodes one form of Raf (D-Raf), which is closely related to human C-Raf (Wellbrock *et al*., 2004).

The regulation of Raf is complex, as it requires not only membrane localization, but also alterations to its phosphorylation state that either promote or obstruct protein-protein as well as lipid-protein interactions required for its activation (Leevers *et al*., 1994; Mason *et al*., 1999). 14-3-3 family proteins negatively regulate C-Raf by binding to a nonphosphorylated region on the cysteine rich domain as well as to two specific phosphorylated serine residues (Ser259 and Ser621) sequestering Raf to the cytoplasm and thereby rendering it inactive (Muslin *et al*., 1996; Clark *et al*., 1997; Michaud *et al*., 1997). The PP2A complex, a serine-threonine phosphatase, positively regulates Raf by dephosphorylating serine 259, and this is sufficient to relieve the 14-3-3 mediated repression of C-Raf (Abraham *et al*., 2000; Jaumot *et al*., 2001). An amino acid sequence alignment between human C-Raf and *Drosophila* D-Raf reveals serine 346 on D-Raf to be homologous to serine 259 residue in C-Raf (Figure 2). While the phosphoserine residues at positions 259 and 621 inhibit C-Raf function by preventing membrane localization,

phosphoserines at positions 338 and 494, phosphothreonine 491, and phosphotyrosine 341 play stimulatory roles, promoting Raf kinase activity (Chong *et al*., 2001; Zhu *et al*., 2005). Thus, the activation of Raf not only requires the phosphorylation of specific residues to stimulate the functionality of the kinase domain thereby allowing for the phosphorylation of MEK to further transduce the signal, but also requires the dephosphorylation of a specific phosphoserine residue (S259) by the PP2A complex to relieve 14-3-3 binding and promote Raf recruitment to the membrane facilitating the Ras-Raf interaction.

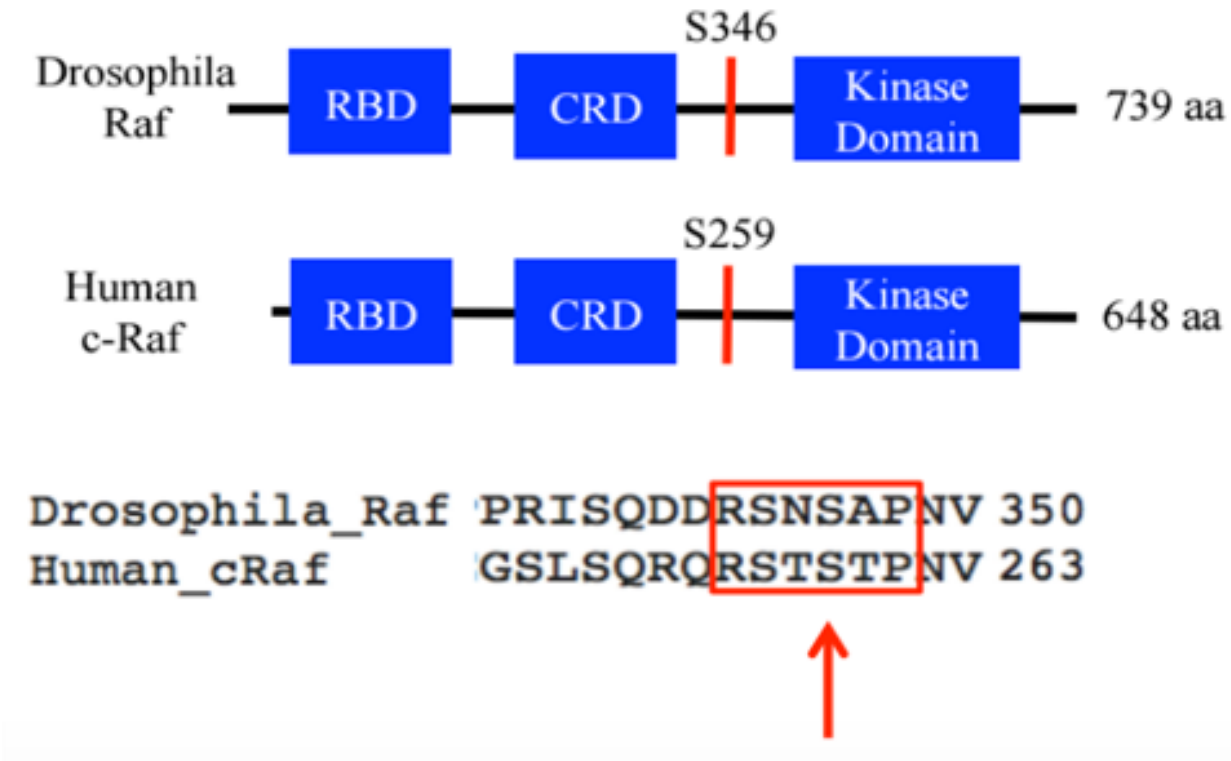

Figure 2: Human C-Raf and *Drosophila* D-Raf Protein Sequence Alignment. An amino acid sequence alignment between human C-Raf and *Drosophila* D-Raf reveals serine 346 of D-Raf to be homologous to serine 259 (indicated by the red arrow). The red box indicates the consensus sequence recognized by 14-3-3 family proteins, which bind Raf to negatively regulate its activity by sequestering it in the cytoplasm.

Here, we present a mechanism by which SUMO activates the Ras/MAPK signaling pathway by modulating the activation of Raf. Through phosphopeptide analysis utilizing tandem

mass spectrometry, we show that the dephosphorylation of serine 346 on D-Raf requires SUMO. Moreover, we show that mutagenesis of serine 346 to alanine, which prevents phosphorylation, leads to SUMO-independent Ras/MAPK signaling. Thus, SUMO may positively regulate the Ras/MAPK signaling pathway by stimulating the dephosphorylation of an inhibitory phosphoserine residue in Raf.

MATERIALS AND METHODS

Pyo-Raf Stable Cell Line

The pMET-Pyo-Raf vector was a gift from Dr. Marc Therrien's lab (Roy et al, 2002). To generate stable cell lines, the vector was co-transfected with pCo-Puro into Schneider 2 (S2) cells by calcium phosphate precipitation. One day after transfection with both the pMET-Pyo-Raf expression vector and the pCo-Puro selection vector, the media was replaced with fresh Schneider's medium supplemented with 10% fetal bovine serum (FBS). The cells were then allowed to grow for two days to allow for expression of the selection vector to confer resistance to puromycin. Three days post-transfection, successfully transfected S2 cells were selected with puromycin for 2 weeks establishing a stable cell line containing the pMET-Pyo-Raf expression vector.

Pyo-Raf Transient Transfection and $CuSO_4$-Induced Expression

Seeding at a density of $1x10^6$ cells/ml with a total volume of 3 ml ($3x10^6$ S2 cells in total), the S2 cells were incubated for a day at 23°C to allow for growth increasing the cell density to $2\sim4x10^6$ cells/ml. The pMET-Pyo-Raf expression vector was transfected into the S2 cells by calcium phosphate precipitation. One day after transfection with the pMET-Pyo-Raf expression vector, the medium was replaced with fresh Schneider's medium supplemented with 10% fetal bovine serum (FBS). Immediately afterwards, the S2 cells were provided with $CuSO_4$ to a final concentration of 0.5 mM to induce the metallothionein promoter for 48 hours to optimally express the pMET-Pyo-Raf expression vector.

SUMO knock down

In six-well plates, $3x10^6$ S2 cells were incubated in 1.5 ml of serum free Schneider media with 5 μg of SUMO dsRNA for 45 minutes. The SUMO dsRNA was generated by amplifying the full-length SUMO cDNA of which was used as template to make SUMO dsRNA by *in vitro* transcription. After 45 minutes, the S2 cells were provided with 1.5 ml of Schneider media supplemented with 20% FBS bringing the final volume to 3 ml. The cells were then incubated at 23°C for five days to optimally reduce the levels of the SUMO protein.

Pyo-Raf Pulldown

Using our Pyo-Raf stable cell line, cells were either left untransfected or transfected with SUMO dsRNA for five days to optimally knock down levels of the SUMO protein. Subsequently to activate the Ras/MAPK pathway, $300x10^6$ Pyo-Raf expressing S2 cells were resuspended in serum free medium ($4x10^6$ cells/ml) for 1 hour and treated with 10 μg/ml of insulin for 7 minutes. Cells were then lysed with RIPA buffer containing 50 mM NaF and 0.2 mM sodium orthovanadate, which act as serine/threonine and tyrosine phosphatase inhibitors respectively. Lysates were incubated with antibodies against the Pyo epitope (Millipore AB3788) overnight at 4°C. Subsequently the antibody-antigen complexes were pulled-down with Protein A magnetic beads (Life Technologies) and washed thoroughly with RIPA buffer supplemented with 500 mM NaCl. Pyo-Raf was eluted with 2x SDS loading buffer prior to loading onto a 10% SDS-PAGE gel.

Raf's Phosphorylation Profile Identification via LC-MS/MS

Pyo-Raf pulled down from S2 cells (wild type uninduced, wild type induced with insulin, and SUMO knock down cells induced with insulin) was excised from the SDS-PAGE gel, subjected to in-gel tryptic digestions, and submitted to Dr. James Wohlschlegel's lab at the University of California, Los Angeles for LC-MS/MS analysis.

Pyo-torso Raf Rescue Experiments

The pMET-pyoTor4021c expression vector was a gift from Dr. Marc Therrien's lab (Douziech *et al*., 2003). The pMET-pyoTor4021c vector was utilized as a template to amplify the pyoTor4021c insert which was cloned into the pMT vector (Life Technologies) using an EcoR1 restriction site. A $(His)_6$- and Flag- tag were also incorporated at the C-terminus of the pMET-pyoTor4021c expression vector and similarly cloned into the pMT vector (Life Technologies) using an EcoR1 restriction site. All constructs were transiently transfected by calcium phosphate precipitation and expression was induced with 0.5 mM $CuSO_4$ for 48 hours prior to harvesting. Cells were lysed with 2x SDS-PAGE loading buffer.

RESULTS

Previous work from our laboratory demonstrated a role for SUMO in the Ras/MAPK pathway, a pathway known to regulate cellular growth, differentiation, and mobility, in response to multiple signaling agents, including insulin and the Spitz ligand. SUMO knock down through the RNAi pathway led to reduced ERK/MAPK phosphorylation in response to either signal, demonstrating that SUMO positively regulates the Ras/MAPK pathway (Nie *et al*., 2009). Furthermore, the Courey laboratory previously identified 14-3-3 family proteins (14-3-3ε and 14-3-3ζ (Leonardo)) as well as both the structural and catalytic subunits of the PP2A complex as SUMO conjugation targets in *Drosophila* embryos (Nie *et al*., 2009). Previous studies defined a role for both protein families in modulating the activity of Raf, a critical component of the Ras/MAPK signaling pathway, by regulating its phosphorylation state and its recruitment to the plasma membrane, which is required for signal transduction (Leevers *et al*., 1994; Clark *et al*., 1997 Jaumot *et al*., 2001). We therefore attempted to define a mechanistic role for SUMO in activating the Ras/MAPK signaling pathway through its interaction with Raf.

Schneider 2 (S2) cells, a *Drosophila* macrophage-like cell line, expressing Raf tagged with a Pyo epitope, a polyoma virus-derived tag, were treated with SUMO dsRNA for five days to efficiently knock down SUMO in the cells (Figure 3A). Following SUMO dsRNA treatment, S2 cells were stimulated with insulin, which binds to the *Drosophila* insulin-like receptor (DInR) to activate the Ras/MAPK signaling cascade. Cells were subsequently harvested and lysed for a large scale immunoprecipitation utilizing a specific antibody against the Pyo epitope to pull down Pyo Raf from the cell lysate. Immunoprecipitations for the varying conditions (wild type S2 cells with no insulin stimulation, wild type S2 cells with insulin stimulation, and SUMO knockdown S2 cells with insulin stimulation) were resolved on an SDS-PAGE gel. We

confirmed the presence of Pyo-Raf by anti-Pyo immunoblot, detecting equal amounts of the Pyo-Raf protein being pulled down under the various conditions (Figure 3B). Bands corresponding to

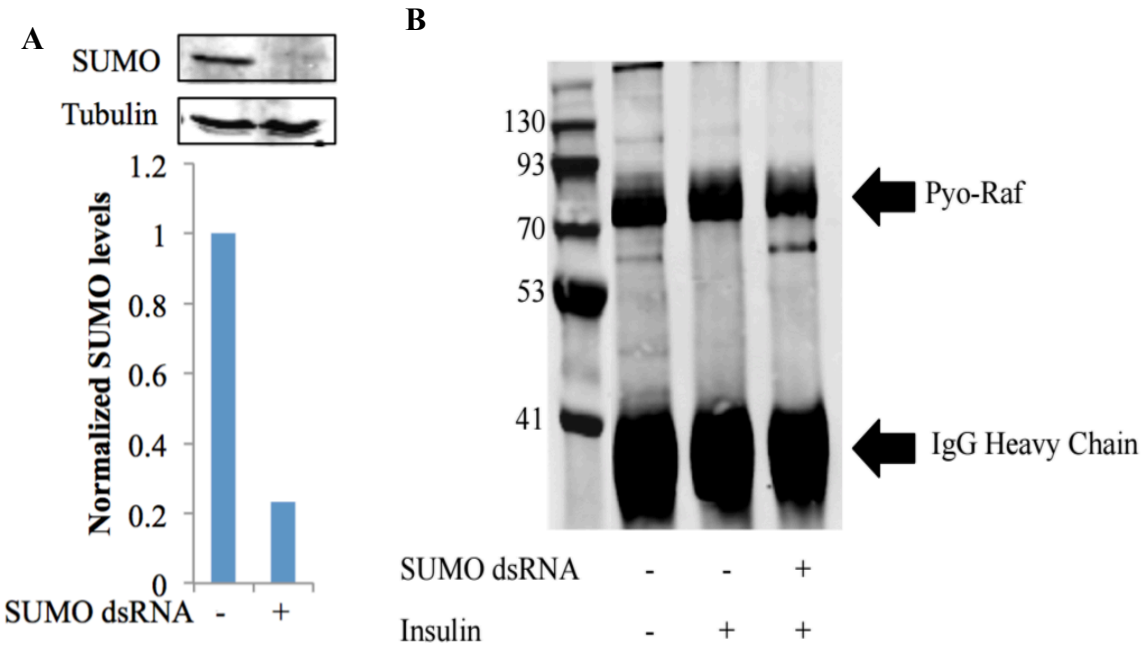

Figure 3: SUMO is Required for Dephosphorylation of Raf at Serine 346. Schneider 2 (S2) cells expressing Raf tagged with the Pyo epitope were treated with SUMO dsRNA for 5 days. (A) SUMO knock down through the RNAi pathway was effectively achieved reducing SUMO protein levels by approximately five-fold determined through a quantitative immunoblot analysis. Cells were then subsequently treated with insulin to stimulate the Ras/MAPK pathway through the *Drosophila* insulin-like receptor and lysed. (B) Cell lysates were then subjected to immunoprecipitation using an anti-Pyo antibody and run on an SDS-PAGE gel. The immunoblot for the immunoprecipitation showed that equal amounts of Pyo-Raf were being pulled down from cells grown under the various conditions (wild type S2 cells with insulin, wild type S2 cells without insulin, and SUMO knock down S2 cells with insulin). (C) Bands corresponding to Pyo-Raf were excised for LC-MS/MS analysis. Mass spectrometry identified two phosphorylated residues at serine 346 and 402, and their relative phosphorylation levels were quantified by normalizing peak areas to two separate unphosphorylated peptides. While phosphorylation of serine 402 of Pyo-Raf was not SUMO dependent, dephosphorylation of serine 346 of Pyo-Raf upon activation of cells with insulin required SUMO.

the Pyo-Raf were then excised from a duplicate gel and subjected to in-gel tryptic digestion prior to phosphopeptide analysis by tandem mass spectrometry. Through LC-MS/MS, we identified

two phosphorylated residues on Pyo-Raf at serine 402 and serine 346. Relative phosphorylation levels were determined by normalizing the peak areas of the phosphopeptides to two separate unphosphorylated Raf peptides.

Neither insulin stimulation nor SUMO knock down significantly affected the phosphorylation levels at serine 402. However, upon insulin stimulation, there was approximately a two-fold reduction in the level of phosphorylation at serine 346. However, dephosphorylation at serine 346 upon insulin stimulation was not observed in the absence of SUMO (Figure 3C). Utilizing a two-tailed t-test, we analyzed for statistical significance comparing the relative phosphorylation levels of the wild type insulin stimulated to the SUMO knockdown insulin stimulated S2 cells returning a p-value of 0.032. Thus, our initial findings through MS/MS phosphopeptide analysis suggested that SUMO stimulates the dephosphorylation of serine 346.

To further confirm SUMO's role in dephosphorylating serine 346 to positively regulate the Ras/MAPK pathway, we performed a site-directed mutagenesis whereby we mutated the serine residue at 346 to an alanine residue within the context of our Pyo-Raf expression construct. Since alanine cannot serve as a phosphoacceptor, we would expect alanine to mimick a desphosphorylated serine residue constitutively activating the Ras/MAPK pathway. We utilized *Drosophila* S2 cells and either left them untransfected or transfected them with either the Pyo-Raf expression construct or the S346A Pyo-Raf mutant construct. S2 cells were then treated with SUMO dsRNA for five days to knock down SUMO and were subsequently treated with insulin to activate the Ras/MAPK pathway. Cells were then lysed and subjected to immunoblot analysis using an antibody specific to phosphorylated MEK, indicative of an activated Ras/MAPK pathway.

As expected, upon insulin stimulation, wild type S2 cells and S2 cells overexpressing Pyo-Raf as well as the S346A Pyo-Raf mutant construct exhibited increased phosphorylation of MEK, indicative of an activated Ras/MAPK signaling pathway (lanes 1,3, and 5, compare with lane 6) (Figure 4). Irrespective of insulin stimulation, MEK was substantially phosphorylated in S2 cells overexpressing either the wild type or S346A Pyo-Raf construct suggesting that the

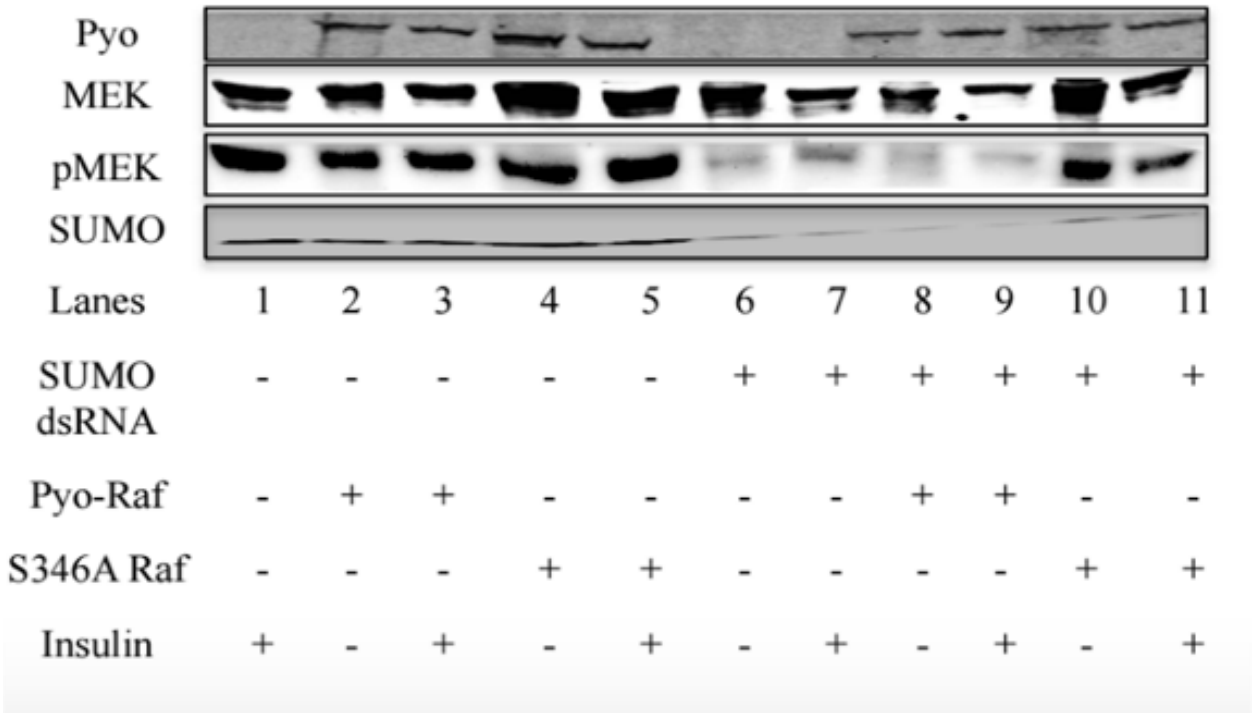

Figure 4: S346A Confirms SUMO Requirement for S346 Dephosphorylation. *Drosophila* wild type or SUMO knock down S2 cells were either left untransfected or transfected with either wild type or S346A mutant in the context of our Pyo-Raf construct. Cells were then stimulated by insulin to activate the Ras/MAPK pathway and lysed for immunoblot analysis using a specific antibody against phosphorylated MEK (pMEK), indicating an activated Ras/MAPK pathway. Under wild type conditions, insulin stimulated S2 cells as well as the overexpression of wild type or mutant Raf even without insulin stimulation sufficiently activates Ras/MAPK signaling. However, when SUMO is knocked down, even overexpression of wild type Raf cannot substantially activate Ras/MAPK signaling confirming the SUMO requirement. However, the S346A mutant form of Pyo-Raf bypasses the requirement for SUMO revealing bands for pMEK (compare lanes 10 and 11 to lanes 6 through 9). Thus, SUMO plays a role in the dephosphorylation of serine 346 on Raf to positively regulate the Ras/MAPK pathway.

overexpression of Raf activates the Ras/MAPK pathway even in the absence of a ligand to activate an RTK (lanes 2 and 4). However, SUMO knock down in either untransfected S2 cells or S2 cells transfected with the wild type Pyo-Raf construct, regardless of insulin treatment, inactivated the Ras/MAPK pathway, indicated by the loss of phosphorylation on MEK (Lanes 6-9). Thus, the overexpression of Pyo-Raf may have bypassed insulin stimulation, but could not bypass the requirement for SUMO further confirming SUMO's positive regulatory role in the Ras/MAPK pathway (Lane 9). In SUMO knock down S2 cells transfected with the S346A Pyo-Raf mutant construct, regardless of SUMO knock down, the phosphorylation of MEK was maintained at levels similar to wild type (compare lanes 4 and 10, 5 and 11). Thus, the constitutive activation of the Ras/MAPK signaling pathway through the S346A Pyo-Raf mutant eliminates the SUMO requirement for the dephosphorylation of serine 346 on Pyo-Raf further corroborating the findings from our MS/MS phosphopeptide analysis.

Previous studies have demonstrated that dephosphorylation of serine 259 on human C-Raf, which is homologous to serine 346 in D-Raf is essential for the efficient recruitment of Raf to the plasma membrane (Abraham *et al*., 2000; Jaumot *et al*., 2001). Furthermore, unpublished data from the Courey lab (J. Cao) confirms that SUMO, which we shown is required for serine 346 dephosphorylation (see above) stimulates membrane localization of D-Raf

To further study SUMO's role in modulating Raf activity via localization to the plasma membrane, we generated a chimeric protein fusing the extracellular and transmembrane domains of the Torso receptor to the Raf catalytic domain, thus artificially tethering Raf to the plasma membrane. A variety of fusion constructs were employed to control for nonspecific effects stemming from the epitope tag. S2 cells were transfected with the fusions were subjected to SUMO knock down for 5 days. We then lysed the cells and used an antibody against

phosphorylated MEK (pMEK), our readout for Ras/MAPK signaling. Regardless of SUMO knock down, without insulin stimulation, MEK phosphorylation was at basal levels (lanes 1 and 2) (Figure 5). Wild type S2 cells transfected with Torso-Raf exhibited Ras/MAPK pathway activation as indicated by the increased levels of pMEK (lanes 3, 5, and 7) and this rescue of MEK activation did not require SUMO (lanes 4, 6, and 8). Therefore, by bypassing the requirement for SUMO in localizing Raf to the plasma membrane, we eliminated the SUMO requirement for MEK activation.

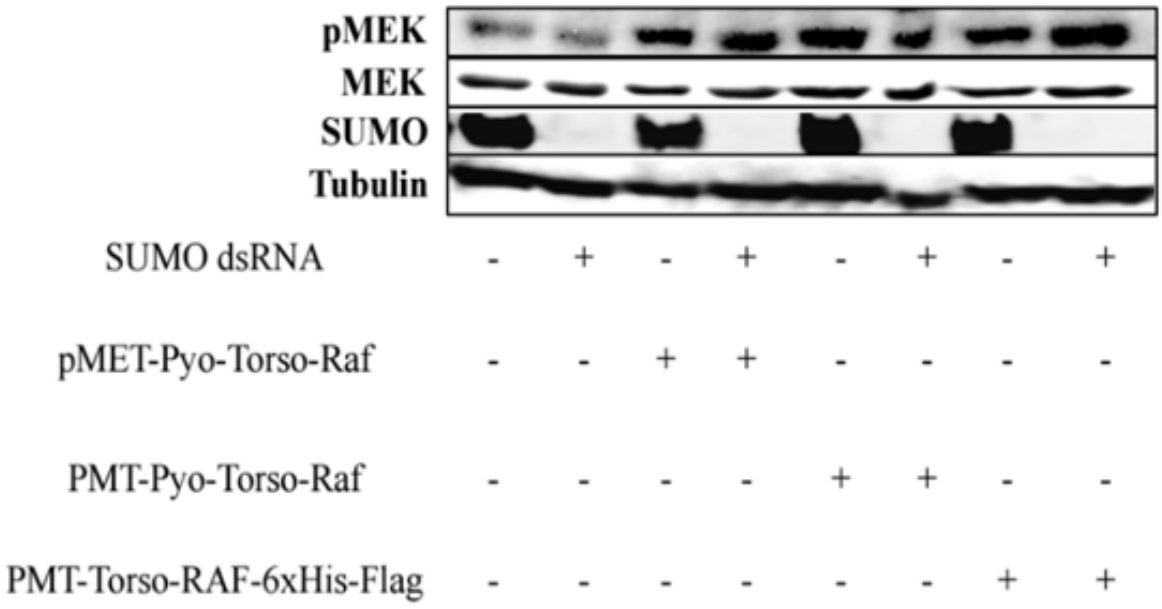

Figure 5: Constitutively Active Raf Rescues SUMO knock down S2 Cells. By fusing the extracellular and transmembrane domains of the Torso Receptor to the Raf kinase domain, we artificially tethered Raf to the plasma membrane (Pyo-Torso-Raf) resulting in constitutive activation of the Ras/MAPK pathway. S2 cells were transfected with different Pyo-Torso-Raf constructs to control for nonspecific effects due to the epitope tag and treated with or without SUMO dsRNA for five days. As expected without insulin stimulation, irrespective of SUMO knock down, there was no activation of Ras/MAPK signaling (lanes 1 and 2). However, regardless of SUMO knock down, the different Pyo-Torso-Raf constructs restore the Ras/MAPK signaling pathway.

DISCUSSION

The Ras/MAPK signaling pathway is induced by the binding of a wide variety of ligands, including Trunk (Trk), insulin (In), and the epidermal growth factor (EGF) to their receptors: the Torso Receptor (Tor), the insulin receptor (InR), and the epidermal growth factor receptor (EGFR), respectively. A previous study demonstrated that knocking down SUMO in S2 cells resulted in reduced Ras/MAPK signaling as indicated by reduced levels of MEK and MAPK phosphorylation. Furthermore, SUMO is required for signaling through Tor in the early Drosophila embryo (J. Cao and A. Courey, unpublished data). However, a mechanistic understanding of how SUMO interacts with the Ras/MAPK signal pathway was lacking.

The previous proteomic screen for SUMO-conjugated proteins (Nie *et al*. 2009) identified the 14-3-3 family proteins (14-3-3 ζ (Leonardo) and 14-3-3ε) as well as structural and catalytic subunits of the protein phosphatase 2A (PP2A complex). Previous studies have shown roles for the human 14-3-3 family proteins in binding specific phosphoserine residues (serine 259 and serine 621) in C-Raf to negatively regulate its activity by sequestering it to the plasma membrane (Muslin *et al*., 1996; Clark *et al*., 1997, Michaud *et al*., 1997). Moreover, both Abraham *et al*. and Jaumot *et al*. demonstrated the functionality of the PP2A complex in dephosphorylating serine 259 relieving the 14-3-3 mediated repression of Raf allowing for its localization to the membrane, a requirement for continued signal transduction through the Ras/MAPK pathway (Leevers *et al*., 1994). Considering that 14-3-3 family proteins and components of the PP2A complex become SUMO conjugated and that they both play roles in Raf regulation, specifically modulating its phosphorylation state as well as its membrane localization, we demonstrated here that SUMO stimulates the dephosphorylation of serine 346 in

D-Raf, which is homologous to serine 259 in human C-Raf, and that this, in turn, is probably required to target D-Raf to the membrane.

Utilizing S2 cells expressing Raf tagged with the Pyo epitope, we performed an immunoprecipitation experiment using an antibody specific to the Pyo epitope and subsequently performed phosphopeptide analysis by tandem mass spectrometry to analyze the differential phosphorylation levels of Pyo-Raf under various conditions: wild type S2 cells not stimulated with insulin, wild type S2 cells stimulated with insulin, and SUMO knockdown S2 cells stimulated with insulin. The phosphopeptide analysis by tandem mass spectrometry revealed that serine 346 phosphorylation was under SUMO control. Specifically, insulin treatment stimulated the dephosphorylation of serine 346 on Pyo-Raf in a SUMO-dependent manner. This provided initial evidence suggesting that SUMO may stimulate the dephosphorylation at serine 346 on Raf to positively regulate the Ras/MAPK pathway.

To further confirm this hypothesis, we performed a site-directed mutagenesis in which we mutated serine 346 to an alanine residue in the context of our Pyo-Raf expression construct. By thus blocking phosphorylation of residue 346, we expected constitutive activation of the Ras/MAPK pathway. Regardless of insulin stimulation, we found that overexpressing either the wild type or mutant S346A form of Pyo-Raf resulted in strong activation of the Ras/MAPK pathway. However, when SUMO was knocked down, the overexpression of wild type Pyo-Raf could not compensate for the loss of SUMO demonstrating a role for SUMO in activating the Ras/MAPK pathway. However, the S346A mutation abolished the requirement for SUMO.

Previous literature has demonstrated the importance of the dephosphorylation of human C-Raf at serine 259 (homologous to D-Raf serine 346) for the recruitment of Raf to the plasma membrane, to allow for signal transduction (Leevers *et al*., 1994; Abraham *et al*., 2000; Jaumot

et al. 2001). In accord with our finding that SUMO is required for serine 346 dephosphorylation, we show here that artificial tethering of Raf to the plasma membrane with a heterologous transmembrane domain overcomes the SUMO requirement for Ras/MAPK pathway activation. This finding that we can bypass the SUMO requirement by artificially tethering Raf to the membrane suggest that SUMO plays a role in the recruitment of Raf to the plasma membrane.

Thus, we propose that SUMO potentiates Ras/MAPK signaling by relieving the 14-3-3 mediated repression of Raf, perhaps by sterically hindering its binding interaction with Raf (Figure 6A). Moreover, as both the structural and catalytic subunits of the PP2A complex are conjugated to SUMO, we suggest that SUMO may regulate removal of the inhibitory phosphate from serine 346 by activating PP2A. Interestingly, Twins (Tws), the regulatory subunit of the PP2A complex contains a possible SUMO interaction motif (Figure 6B). Previous studies have identified a role for Tws not only in stimulating PP2A catalytic function, but also in regulating the subcellular localization as well as substrate specificity of the PP2A complex (Cegielska *et al*., 1994; Li and Virshup, 2002; McCright *et al*., 1996). Thus, SUMO could serve to stabilize the formation of the heterotrimeric enzyme to activate the phosphatase function of the PP2A complex. Furthermore, it may also provide a mechanism by which Tws confers specificity, recruiting the heterodimeric core enzyme to a particular subcellular compartment for selective activation of a subset of the many responses mediated by Ras/MAPK signaling.

The interaction of SUMO with the Ras/MAPK signaling pathway illuminates a mechanism by which SUMO facilitates cellular proliferation. Previous literature defined a role for the ERK module in mitosis activating the transcription factor, Elk-1 and also facilitating the formation of the AP-1 complex, both of which play pivotal roles in fostering cell cycle progression (Gille *et al*., 1995; Shaulian and Karin, 2001; Whitmarsh and Davis, 1996). Here, we

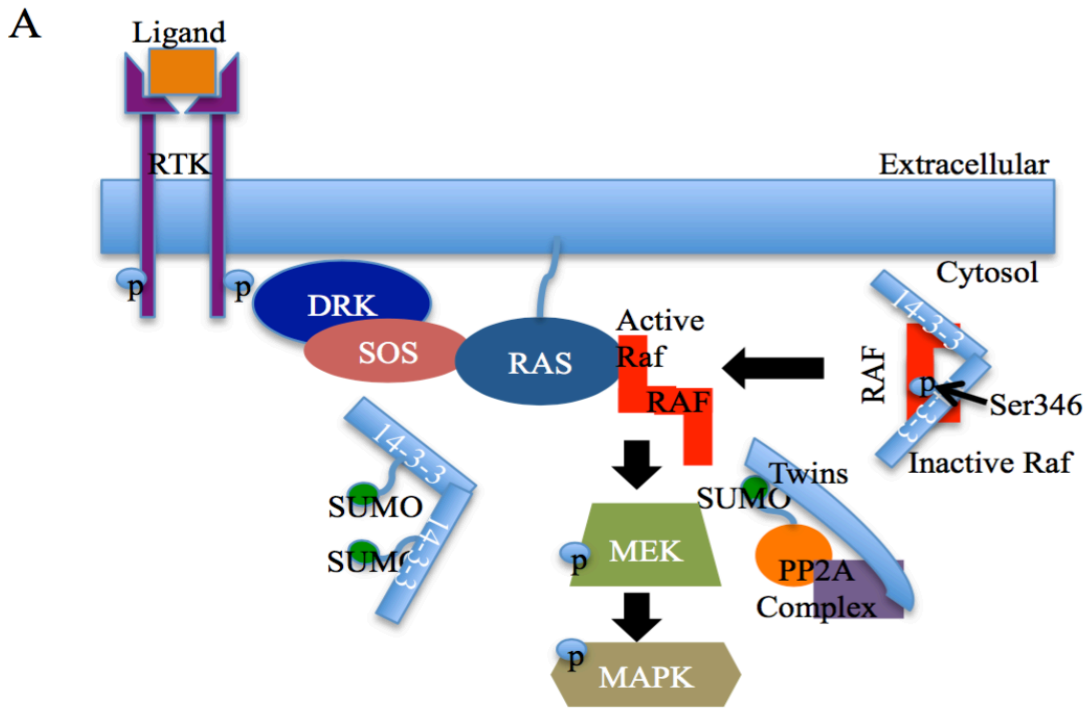

Figure 6: Model for SUMO Interaction with the Ras/MAPK Pathway. (A**)** The Ras/MAPK pathway initiates with binding of a ligand to a receptor tyrosine kinase (RTK) inducing dimerization followed by autophosphorylation at specific tyrosine residues. Downstream of receptor kinase (Drk) protein recognizes the phosphotyrosine residues on the RTK and recruits Son of sevenless (Sos), a guanine nucleotide exchange factor, that exchanges GDP for GTP to activate Ras. Ras then recruits Raf to the plasma membrane activating it to phosphorylate MEK, which then subsequently phosphorylates ERK/MAPK allowing for its translocation to the nucleus to alter the transcriptional program of the cell. SUMO is conjugated to the two *Drosophila* 14-3-3 family proteins as well as to the heterodimeric PP2A complex (consisting of structural and catalytic subunits) both of which function to regulate the membrane localization and activation of Raf. 14-3-3 family proteins bind to a phosphoserine residue at position 346 of D-Raf (equivalent to serine 259 in human C-Raf) sequestering Raf to the cytoplasm rendering it inactive. (B) A sequence analysis of Twins, a regulatory subunit for PP2A, identified a consensus SUMO interaction motif (SIM). Thus, upon stimulation by insulin, SUMO may aid PP2A activation by mediating Twins binding to the heterodimeric PP2A core. Once active, the PP2A complex may dephosphorylate serine 346 relieving the binding of 14-3-3 allowing for the recruitment of Raf to the plasma membrane. SUMO may also serve to potentiate signaling by directly relieving the 14-3-3 mediated inhibition of Raf.

demonstrate that SUMO modulates both the phosphorylation state and membrane localization of Raf to positively regulate the Ras/MAPK signaling pathway suggesting a mechanism by which SUMO promotes cell cycle progression. Previous studies defined a role for SUMO in responding to the availability of resources through the TORC1 pathway, whereby a lack of resources correlates with lower levels of SUMO conjugation in the cell (Chymkowitch *et al*., 2015). In a similar manner, SUMO may potentially serve as a mechanism for linking the Ras/MAPK signaling pathway to cellular stress. During times of nutrient stress, reduced SUMO conjugation could mimick our SUMO knock down conditions to inactivate the Ras/MAPK pathway. Biologically, this makes sense, as during times when nutrients are scarce, cells shift their transcriptional programs from that of proliferation to survival. Moreover, it is possible that SUMO confers specificity to complex formation by modulating the affinity of the PP2A catalytic core for its respective regulatory subunit, Tws. SUMO may serve as the initial scaffold to bring Tws into proximity of other binding interfaces in PP2A, fostering complex formation and thus activation. This in turn may allow for the selective activation of certain signaling pathways as previous studies have shown that SUMO conjugation levels vary depending on the extracellular environment (Chymkowitch *et al*., 2015).

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