Understanding the Role of SUMOylation in the Function of Aac11/Api5

A thesis submitted in partial fulfillment of the requirements of IISER Pune's BS-MS dual degree programme.

Submitted by

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Certificate

This is to certify that this dissertation entitled "*Understanding the Role of SUMOylation in the Function of Aac11/Api5*" towards the partial fulfillment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research (IISER), Pune represents original research carried out by Darshini Ravishankar at IISER Pune under the supervision of Dr. Girish Ratnaparkhi, Associate Professor, Biology Division, IISER Pune during the academic year 2016-2017.

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Dr. Girish Ratnaparkhi

20/3/17

Date

Declaration

I hereby declare that the matter embodied in the report entitled "*Understanding the Role of SUMOylation in the Function of Aac11/Api5*" are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research (IISER), Pune, under the supervision of Dr. Girish Ratnaparkhi, IISER Pune, and the same has not been submitted elsewhere for any other degree.

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20/3/17

Date

Abstract

Api5 and Aac11 are orthologous anti-apoptotic proteins in humans and flies respectively. Api5 is gaining prominence as a target in therapy due to its ability to confer cancer cells with resistance to apoptosis. Api5 also confers stem-cell like properties to cancer cells through interactions with E2f1 and Fgf2. Post-translational modification of Api5 by way of acetylation has been demonstrated to be crucial to its function. In this study, I explore the regulation of Api5 and Aac11 function by SUMOylation. Bioinformatics tools predict that Api5 and Aac11 are SUMOylated at several putative target lysines, some of which are conserved between the two proteins. I demonstrate that both proteins are SUMOylated in vitro, and have generated reagents to test the same *in vivo*. I have also carried out extensive mutagenesis of the predicted lysine target sites for SUMO, but until date have not been able to abolish SUMOylation. This may indicate either that I have not targeted the correct SUMO site, or that Api5/Aac11 are promiscuous in terms of their SUMOylation. Additionally, I explore the possibility of rescuing Aac11 null flies with Api5. If the rescue is successful, known Api5 variants can be tested in *Drosophila* to uncover biological function.

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Acknowledgments

I am grateful to Dr. Girish Ratnaparkhi for the chance to work on this project, and for the encouragement, support, and guidance he has given me throughout this year. I am thankful to Dr. Mayurika Lahiri for her advice on different aspects of this study, and for generously providing *pGEX-Api5* and an Api5 antibody for our lab's use. I thank Prof. LS Shashidhara for his comments and advice as part of my TAC, and Dr. Richa Rikhy for input during our weekly lab meetings. Gratitude also goes towards the Fly Facility at NCBS under Dr. Deepti Trivedi.

I thank Amar Soory for generating the *pGEX-Aac11* construct and the first four *Aac11* mutants, but more importantly for the time and energy he invested this year in mentoring me, teaching me laboratory techniques, and taking time away from his own work to discuss mine. Everybody in the GR and RR labs has to be thanked for absorbing discussions and for providing motivation when I was in need of it. The support my parents, sister, and friends at IISER gave me cannot be understated, and I'm especially thankful to my labmate Neena Dhiman, with whom I shared my worries, many laughs, and many parathas.

Introduction

Post-translational modification of proteins is known to regulate, often crucially, their function, location, folding, activity, as well as interactions with other proteins or molecules (Gareau & Lima, 2010; Knorre et al., 2009). Numerous proteins and other moieties are known modifiers- ubiquitin and ubiquitin-like proteins are examples of the former, while phosphate and lipids are non-protein modifiers. SUMO, the Small Ubiquitin-related Modifier, is an ubiquitin-like protein of 12 kilo-daltons. Like the ubiquitin pathway, the SUMO pathway or cycle **(Figure 1)** involves enzymes that activate SUMO (E1 activating enzymes), covalently conjugate SUMO to a lysine on the substrate protein (E2 conjugating enzymes), and E3 ligases that aid conjugation (Flotho & Melchior, 2013; Gareau & Lima, 2010). Unlike ubiquitination, however, E3 ligases are not always necessary for SUMOylation, as seen in the case of RanGAP1, the first identified SUMO substrate (Bernier-Villamor et al., 2002).

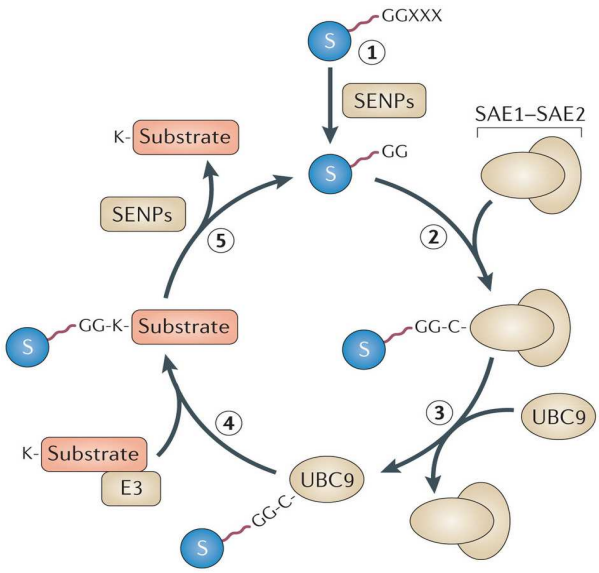


Figure 1: The SUMO cycle. 1) SUMO maturation (C-terminal cleavage) performed by the SENPs. 2) SUMO activation by the SUMO Activating Enzyme subunit 1 and subunit 2, SAE1-SAE2, which are E1 enzymes. 3) SUMO is transferred to the SUMO-conjugating enzyme, UBC9, which is an E2 enzyme. 4) SUMO conjugation to the substrate lysine by the E2 enzyme and E3 ligase. 5) SUMO deconjugation by the SENPs. The substrate may be SUMOylated again (Everett et al., 2013).

The lysine to which the SUMO is attached is often part of a sequence referred to as the SUMOylation consensus motif, ψ-K-X-D/E. These residues are known to interact with the E2 enzyme Ubc9, aiding SUMOylation (Sampson et al., 2001). The inverted consensus motif, D/E-X-K-ψ, has also been reported to be SUMOylated (Matic et al., 2010). A significant percentage of SUMO sites (25%, according to Beauclair et al., 2015, and 40%, according to Zhao et al., 2014) do not conform to such reported motifs, however, and nor is it necessary for a consensus/inverted consensus motif to be SUMOylated (Beauclair et al., 2015, Zhao et al., 2014). Humans have five isoforms of SUMO. Three of them are well-studied and named SUMO1, SUMO2, and SUMO3. SUMO4 and SUMO5 are recent discoveries (Gareau & Lima, 2010; Liang et al., 2016). The sequences of SUMO1 and SUMO2/3 are only 50% identical, whereas SUMO2 and SUMO3 are 97% identical. Yeast and *Drosophila melanogaster* have only one SUMO isoform, Smt3 (Flotho & Melchior, 2013; Gareau & Lima, 2010). Yeast cell cycles cannot progress without Smt3 (Li & Hochstrasser, 1999). Sequence alignment reveals that *Drosophila* Smt3 is 50% identical to SUMO1, and 66% identical to SUMO2/3 (McWilliam et al., 2013).

While ubiquitination was initially supposed to affect proteins mainly by directing them to the proteasome for degradation, SUMOylation has long been known to affect varied cellular processes in more diverse ways (Flotho & Melchior, 2013; Knorre et al., 2009). SUMO has been shown to modify proteins in most nuclear processes, such as splicing and DNA repair, and the activity of many transcription factors both positively and negatively, though repression is more common (Hendriks et al., 2014; Hilgarth et al., 2004). SUMO affects many signalling networks and cascades, and is therefore involved in processes such as development and differentiation, immunity, and mitosis (Wilson, 2009). SUMOylation has been observed to increase globally in response to stress conditions as well, such as heat, immune, or oxidative stress (Flotho & Melchior, 2013; Gareau & Lima, 2010; Handu et al., 2015). The variety of processes

SUMOylation is involved in, and the sheer number of SUMOylation substrates have made research on SUMO's involvement in disease a growing field (Flotho & Melchior, 2013; Wilson, 2009).

Api5 (*apoptosis inhibitor 5*) was discovered in a screen to uncover pro-survival genes by Tewari et al. in 1997, and was termed *AAC-11* (*antiapoptosis clone 11*). The protein product produced by this cDNA was observed to protect against apoptosis under the stress condition of growth factor withdrawal (Tewari et al., 1997). It is expressed ubiquitously in embryonic and adult tissue, and is conserved across phyla, from plants to invertebrates like *Drosophila melanogaster*, and mammals (Li et al., 2011; Rigou et al., 2009; Tewari et al., 1997). Also called FIF (fibroblast growth factor-2 interacting factor), Api5 was later shown to be upregulated in many human cancers, including cervical cancer (Koci et al., 2012; Song et al., 2015; Song et al., 2017; Van den Berghe et al., 2000). It was demonstrated to interact with the pRb (retinoblastoma)/E2F1 signalling pathway **(Figure 2)**, protecting cells from massive waves of cell death induced by E2F1 deregulation upon the inactivation of pRb. The loss of the *D. melanogaster* orthologue of Api5, Aac11, enhances E2F1-dependent phenotypes in the wing, bristle, and eye (Morris et al., 2006). Both Api5 and Aac11 have also been shown to prevent apoptosis caused by Acinus-mediated DNA fragmentation. The conserved leucine zipper domain is necessary for this function and allows oligomerisation. Api5/Aac11 then physically protects Acinus from cleavage by caspase-3, preventing the formation of an active fragment, p17, that effects DNA fragmentation (Rigou et al., 2009). More recently, Api5 has been confirmed to be sufficient to confer cancer stem-cell like properties to tumour cells, including the ability to form spheres and NANOG expression. Additionally, increased Api5 expression correlates with a poorer cervical cancer patient prognosis (Song et al., 2017). Another physical interactor of Api5 is the cell cycle checkpoint protein TopBP1 **(Figure 2)**, which plays a role in the ATR checkpoint signalling pathway following DNA damage (Kumagai et al., 2006; Parivesh & Lahiri, unpublished data).

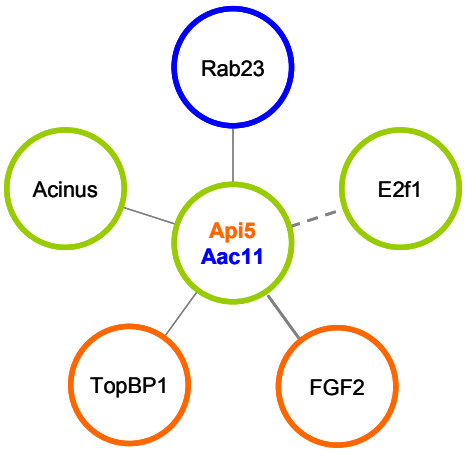
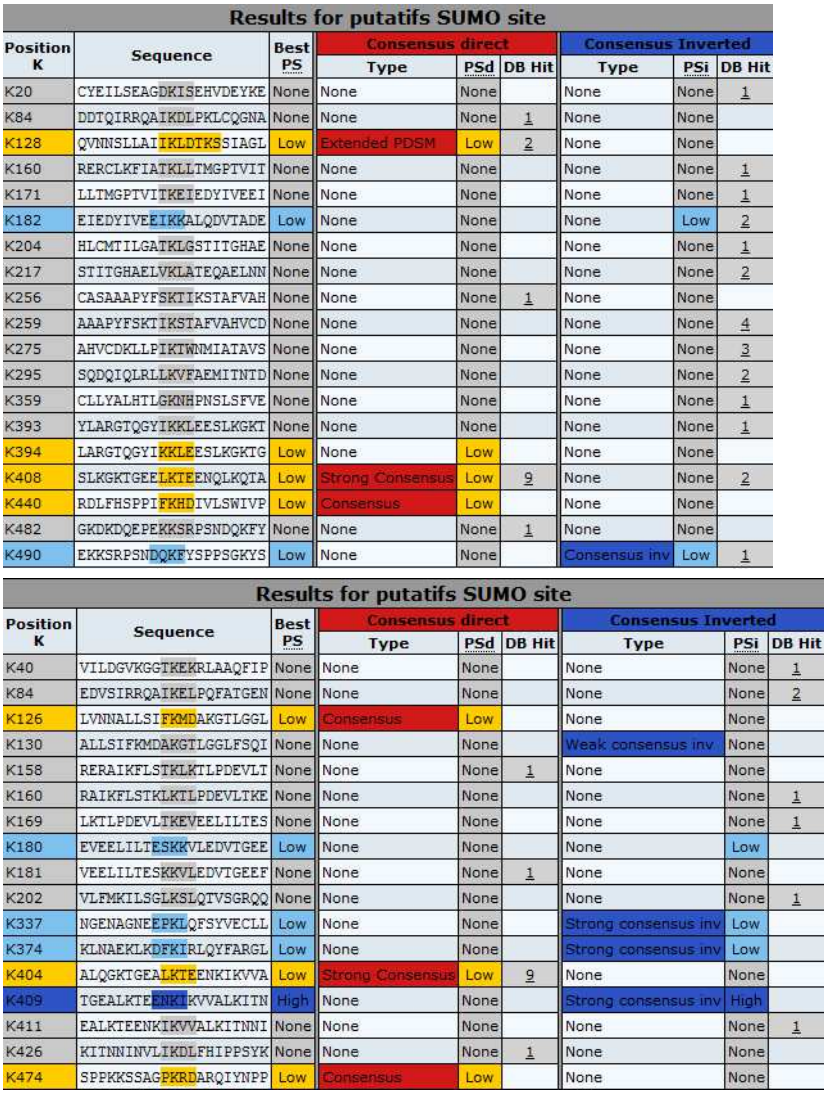


Figure 2: Summary of known *Api5/Aac11* interactions. The dashed lines indicate genetic interactions, the solid lines indicate physical interactions. The genes/proteins (Acinus, *E2F1)* in green bubbles represent those that interact with both *Api5* and *Aac11*. Orange bubbles signify those (TopBP1, FGF2*)* that are confirmed to interact with *Api5*, and blue (Rab23) with *Aac11* (Çiçek et al., 2016; Morris et al., 2006; Parivesh & Lahiri, unpublished data; Rigou et al., 2009; Van den Berghe et al., 2000).

These studies have brought to prominence the potential for developing therapies targeted at Api5 and the pathways that Api5 interacts with. To develop such therapies, we need to understand the regulation of Api5 better, including the effects of post-translational modification on protein structure, function, and activity. Han et al., who first determined the crystal structure of Api5, have shown that Api5 is acetylated at lysine 251, and that this modification is crucial to its stability and proper functioning as an anti-apoptotic protein. They proved that neither a constitutively acetylated variant of Api5 (lysine 251 mutated to glutamine) nor an acetylation deficient version (lysine 251 mutated to arginine) could regulate apoptosis effectively under the conditions of growth factor withdrawal (Han et al., 2012). Phosphorylation, on the other hand, has been suggested to enable Api5 to inhibit apoptosis through the NFκB pathway (Ren et al., 2010).

The goal for this project, in the context of Api5's emerging importance as a target in therapy and the lack of knowledge about how it is modified post-translation, is to explore the regulation of Api5/Aac11 by SUMOylation, as well as to determine whether *Drosophila melanogaster* can serve as a model system for studying Api5 function in development and disease. As a first step, we find that

algorithms that predict SUMOylation predict potential (strong) SUMO sites for both Aac11 and Api5. Joined Advanced SUMOylation Site and SIM Analyser (JASSA, Beauclair et al., 2015), for instance, was used as a bioinformatics analysis tool to predict whether Aac11 and Api5 contain lysine residues with the potential to be SUMOylated. JASSA's output **(Figure 3)** listed a number of putative SUMOylation sites for both proteins.



Results for putatifs SUMO site

Position K	Sequence	Best PS	Consensus direct			Consensus Inverted		
			Type	PSd	DB Hit	Type	PSi	DB Hit
K20	CYEILSEAGDKISEHVDEYKE	None	None	None		None	None	1
K84	DDTQIRRQAIKDLPKLCQGNA	None	None	None	1	None	None	
K128	QVNNSLLAIIKLDTKSSIAGL	Low	Extended PDSM	Low	2	None	None	
K160	RERCLKFIATKLLTMGPTVIT	None	None	None		None	None	1
K171	LLTMGPTVITKEIEDYIVEEI	None	None	None		None	None	1
K182	EIEDYIVEEIKKALQDVTADE	Low	None	None		None	Low	2
K204	HLCMTILGATKLGSTITGHAE	None	None	None		None	None	1
K217	STITGHAELVKLATEQAELNN	None	None	None		None	None	2
K256	CASAAAPYFSKTIKSTAFVAH	None	None	None	1	None	None	
K259	AAAPYFSKTIKSTAFVAHVCD	None	None	None		None	None	4
K275	AHVCDKLLPIKTWNMIATAVS	None	None	None		None	None	3
K295	SQDQIQLRLLKVFAEMITNTD	None	None	None		None	None	2
K359	CLLYALHTLGKNHPNSLSFVE	None	None	None		None	None	1
K393	YLARGTQGYIKKLEESLKGKT	None	None	None		None	None	1
K394	LARGTQGYIKKLEESLKGKTG	Low	None	Low		None	None	
K408	SLKGKTGEELKTEENQLKQIA	Low	Strong Consensus	Low	9	None	None	2
K440	RDLFHSPPIFKHDIVLSWIVP	Low	Consensus	Low		None	None	
K482	GKDKDQEPEKKSRPSNDQKFY	None	None	None	1	None	None	
K490	EKKSRPSNDQKFYSPPSGKYS	Low	None	None		Consensus inv	Low	1

Results for putatifs SUMO site

Position K	Sequence	Best PS	Consensus direct			Consensus Inverted		
			Type	PSd	DB Hit	Type	PSi	DB Hit
K40	VILDGVKGGTKEKRLAAQFIP	None	None	None		None	None	1
K84	EDVSIRRQAIKELPQFATGEN	None	None	None		None	None	2
K126	LVNNALLSIFKMDAKGTLGGL	Low	Consensus	Low		None	None	
K130	ALLSIFKMDAKGTLGGLFSQI	None	None	None		Weak consensus inv	None	
K158	RERAIKFLSTKLKTLPDEVLT	None	None	None	1	None	None	
K160	RAIKFLSTKLKTLPDEVLTKE	None	None	None		None	None	1
K169	LKTLPDEVLTKEVEELILTES	None	None	None		None	None	1
K180	EVEELILTESKKVLEDVTGEE	Low	None	None		None	Low	
K181	VEELILTESKKVLEDVTGEEF	None	None	None	1	None	None	
K202	VLFMKILSGLKSLQTVSGRQQ	None	None	None		None	None	1
K337	NGENAGNEEPKLQFSYVECLL	Low	None	None		Strong consensus inv	Low	
K374	KLNAEKLKDFKIRLQYFARGL	Low	None	None		Strong consensus inv	Low	
K404	ALQGKTGEALKTEENKIKVVA	Low	Strong Consensus	Low	9	None	None	
K409	TGEALKTEENKIKVVALKITN	High	None	None		Strong consensus inv	High	
K411	EALKTEENKIKVVALKITNNI	None	None	None		None	None	1
K426	KITNNINVLIKDLFHIPPSYK	None	None	None	1	None	None	
K474	SPPKKSSAGPKRDARQIYNPP	Low	Consensus	Low		None	None	

Figure 3: Prediction of *Drosophila* Aac11 and Human Api5 SUMOylation.
Joined Advanced SUMOylation Site and SIM Analyser (JASSA) (Beauclair et al , 2014 www jassa fr) output of polypeptide sequences of Aac11 (Uniprot #Q9V431) and Api5 (Uniprot #Q9BZZ5) indicate that they contain lysine residues that are potential targets of SUMOylation. In the table, Best PS refers to the best of two predictive scores, PSd (direct) and PSi (inverted). DB

Hit indicates whether the lysine and the sequence it is part of matches any in the JASSA training set.

Several of these are consensus motifs, and some, in particular lysine 128/126 and 408/404 on Aac11/Api5, are conserved. The locations of these lysines on the sequences of Aac11/Api5 are indicated in **Figure 4**. If Aac11 and Api5 are indeed SUMOylated, then SUMO-mediated regulation of Api5/Aac11 function would be of interest.

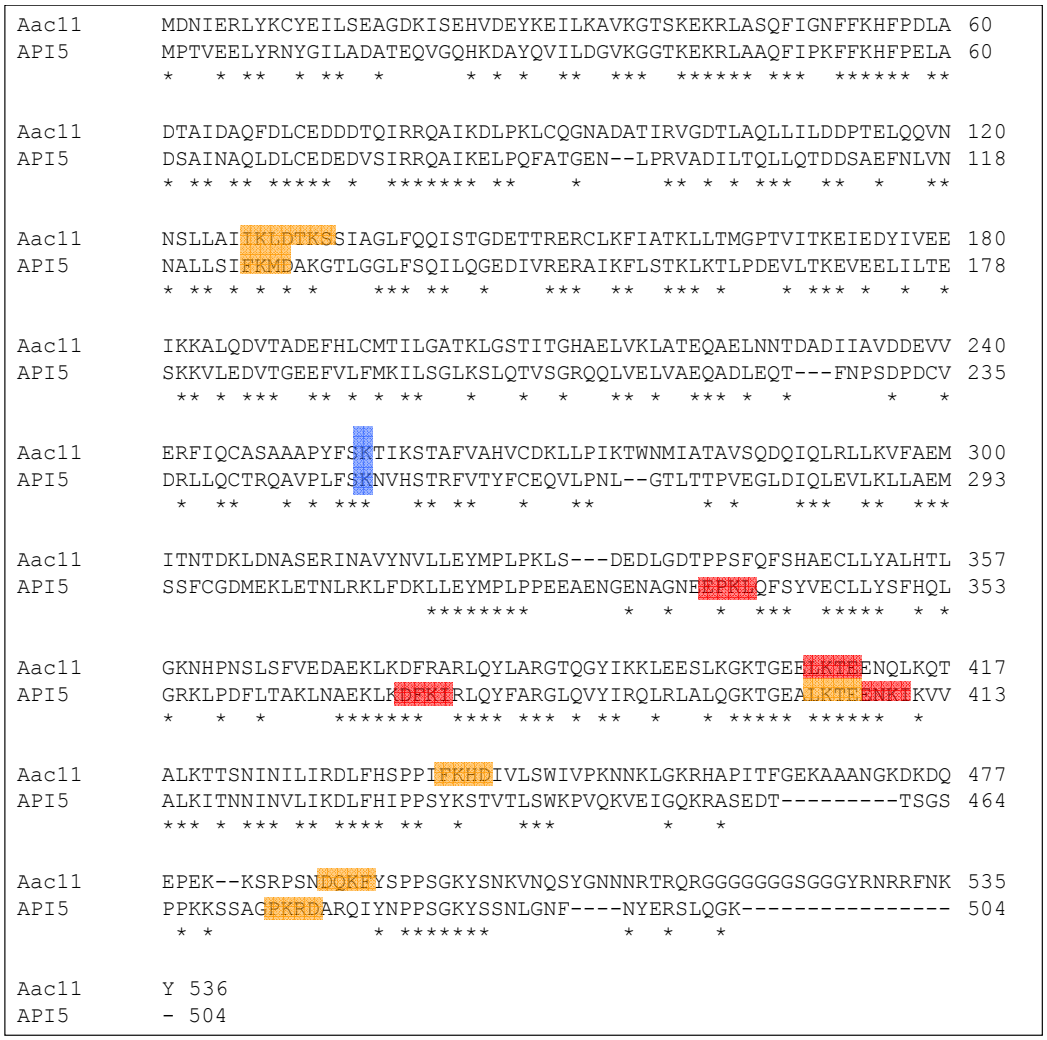


Figure 4: Putative SUMOylation sites are conserved on Aac11/Api5.
Alignment using Clustal Omega (McWilliam et al., 2013) of the sequences of Api5 and Aac11 indicates that the potential SUMOylation sites are conserved in evolution. The two proteins show 46% Sequence identity and 63% sequence similarity. The conserved sites are marked in the following colours: red for strong consensus or strong inverted consensus, orange for consensus. The acetylation site in Api5, K251 (Han et al., 2012), is marked in blue on both proteins to indicate the conservation of that lysine.

Hypothesis

SUMOylation modulates the biological function of Aac11/Api5.

Objectives

1. Demonstrate SUMOylation of Aac11/Api5.
2. Determine lysine targets of SUMO for Aac11/Api5 and generate protein variants that are resistant to SUMOylation.
3. Determine biological roles for Aac11/Api5 SUMOylation in flies.

Specific Aims

1. Are Api5 and Aac11 targets for the SUMO conjugation pathway? Test *in vitro*, in cells, and in flies.
2. Once Api5/Aac11 SUMOylation is demonstrated, mutate potential target lysines in both proteins and generate variants that cannot be SUMOylated.
3. In order to identify biological role(s) for Aac11/Api5 SUMOylation in the animal:

   (i) Generate transgenic flies that express Aac11 and Api5; both wild type and mutants.

   (ii) Demonstrate complete or partial rescue of null *Aac11* alleles in the fly by correct spatiotemporal expression of Aac11. If successful, test rescue with Api5 ('the humanised fly').

   (iii) Test the ability of Aac11 mutants that cannot be SUMOylated to rescue *Aac11-null* animals. Compare and contrast wild type vs mutant rescue to gain insight into roles for SUMOylation of Aac11.

Materials and Methods

Cloning and mutagenesis

The coding sequence of *Aac11* was obtained from the *Drosophila* Gold collection (DGRC). *Aac11* was subcloned into the *pGEX-4T-1* (Addgene catalogue number: 27458001, henceforth referred to as *pGEX*) expression vector. The vector incorporates an N-terminal GST tag. Four mutant variants of *Aac11* where a single lysine (K) had been mutated to an arginine (R) were also cloned. These were *pGEX-Aac11(K128R), pGEX-Aac11(K408R), pGEX-Aac11(K440R), and pGEX-Aac11(K490R)*. The aforementioned constructs were generated by Amar Soory (see Acknowledgements). *pGEX-Aac11* was used as the template for further cloning. The primers used to amplify the coding sequence, as well as the entire vector are tabled below.

Table 1: Primers used to subclone *Aac11* into *pGEX*-4T-1. Primers are listed 5'-3'. Blue indicates the portion of the sequence complementary to *Aac11*, while the 5' region is homologous to the vector sequence upstream and downstream of it.

	Forward primer	**Reverse primer**
pGEX-Aac11	CTGGTTCCGCGTGGATCCCCGGAATT CATGGACAACATAGAGCGACTG TAC	TCGTCAGTCAGTCACGATGCGGCCG CTTAGTACTTGTTGAATCGCCTGTT
pGEX	ATAGGGATCCATGTATCGCTGC	TATAGTCGACCTACATCAGGGCG

Overlap extension PCR was employed to generate five more single lysine mutants and a quadruple mutant, namely *pGEX-Aac11(K171R), pGEX-Aac11(K182R), pGEX-Aac11(K306R), pGEX-Aac11(K373R), pGEX-Aac11(K394R), and pGEX-Aac11(K128R, K408R, K440R, K490R)*. For the single lysine mutants, this was done using the following primers along with the *pGEX-Aac11* forward and reverse primers listed above. Two fragments of the coding sequence were generated with overhangs that were homologous to the 3' GST sequence on *pGEX* and the 5' region of *pGEX* immediately upstream and downstream of the desired point of insertion on the vector.

Table 2: Primers used to generate fragments for overlap extension PCR in the mutagenesis of *Aac11*. Each forward primer listed was used with *pGEX-Aac11* reverse, and each listed reverse primer was used with *pGEX-Aac11* forward.

Mutant	Forward primer	Reverse primer
Aac11 K170R	GTTATTACCAGGGAAATCGAGGAC	GTCCTCGATTTCCCTGGTAATAAC
Aac11 K182R	GAGGAGATCAGAAAGGCTTTGCA	TGCAAAGCCTTTCTGATCTCCTC
Aac11 K306R	CAACACAGACAGGCTGGACAAT	ATTGTCCAGCCTGTCTGTGTTG
Aac11 K373R	GAGGATGCAGAGAGACTAAAAGAC	GTCTTTTAGTCTCTCTGCATCCTC
Aac11 K394R	GGTTATATTAAGAGACTGGAGGAG	CTCCTCCAGTCTCTTAATATAACC

Platinum Pfx Polymerase (Invitrogen, catalogue number: 11708039) or PfuTurbo DNA Polymerase (Agilent, catalogue number: 600257) was used, with the associated buffers and according to the manufacturer's protocol. The following PCR programme was used.

Table 3: PCR programme employed during mutagenesis and overlap extension steps.

Step	Temperature	Duration	Cycles
First Denaturation	95 °C	5 minutes	
Denaturation	95 °C	30 seconds	30
Annealing	55 °C	30 seconds	
Extension	68 °C (Pfx)/72 °C (Pfu/Taq)	1 minute per kilobase	
Final Extension	68 °C (Pfx)/72 °C (Pfu/Taq)	10 minutes	
Hold	4 °C	-	

In the case of the quadruple mutant, two fragments, a 3' and middle fragment, were overlapped to create a third fragment. This third product was overlapped with the 5' fragment to generate the complete insert. The final insert generated was gel purified a second time instead of being used directly for transformation with linearised vector, as it was in the previous case.

Table 4: Primers and templates used to generate fragments for overlap extension PCR in the mutagenesis of four lysines of *Aac11* simultaneously. Blue in the *pGEX-Aac11* forward and reverse primers indicates portions complementary to *Aac11*.

Template	Primer Name	Primer sequence	Fragment generated
pGEX-Aac11 K128R	*pGEX-Aac11* forward	CTGGTTCCGCGTGGATCCCCGGAATT CATGGACAACATAGAGCGACTGTAC	Fragment One
	Aac11 K408R reverse	CTCCTCCGTCCTGAGCTCCTC	
pGEX-Aac11 K440R	*Aac11 K408R* forward	GAGGAGCTCAGGACGGAGGAG	Fragment Two
	Aac11 K440R reverse	ACAATGTCATGCCTGAATATGGG	
pGEX-Aac11 K490R	*Aac11 K440R* forward	CGCCCATATTCAGGCATGACATT	Fragment Three
	pGEX-Aac11 reverse	TCGTCAGTCAGTCACGATGCGGCCGC TTAGTACTTGTTGAATCGCCTGTT	
Fragment Two + Fragment Three	*Aac11 K408R* forward	(See above)	3' Fragment
	pGEX-Aac11 reverse	(See above)	
Fragment One + 3' Fragment	*pGEX-Aac11* forward	(See above)	Aac11 (K128R, K408R, K440R, K490R)
	pGEX-Aac11 reverse	(See above)	

The exact PCR programme detailed in Table 3 was used, with the exception of the annealing temperature, which was 60°C in this case.

The two fragments were then eluted from a 1% agarose gel, extracted using either the MinElute Gel Extraction Kit from Qiagen (Catalogue number: 28604) or the QIAEX II Gel Extraction Kit from Qiagen (Catalogue number: 20021) and both used as a template in an overlap extension PCR reaction using the *Aac11* forward and reverse primers listed above. The PCR product was purified using a QiaQuick PCR Purification Kit (Catalogue number: 28104) and checked for size and quality on an agarose gel. Simultaneously, *pGEX* was amplified using PCR with the *pGEX* primers listed in Table 1.

Linearised *pGEX* and the purified product of the overlap extension PCR were mixed in a molar ratio of 1:4. Where the concentration was not checked, a volume ratio of 1:4 was made. This mixture was used to transform 50 ul of competent PPY cells. Colonies were screened for the presence of insert-containing plasmid with a PCR reaction using an insert-specific (*Aac11 K374R* forward, listed in Table 2) and a vector-specific primer (*pGEX* reverse sequencing primer: 5'-CGGGAGCTGCATGTGTCAGAGG-3'). These colony PCRs were performed using Taq DNA Polymerase (Sigma-Aldrich, catalogue number: D4545).

Positive colonies were cultured and plasmid DNA was prepared from them using the GenElute^{TM} Plasmid Miniprep Kit from Sigma (Catalogue no: PLN70). The DNA concentration of these preps was checked using a nanodrop, and the quality was checked on an agarose gel. They were sequenced using 1st Base DNA Sequencing Services to confirm the presence of the desired point mutations and the absence of unwanted ones.

Cloning wild type *Api5* and mutants into *pGEX*-4T-1

The coding sequence of *Api5* was provided by Dr. Mayurika Lahiri as *pGEX-Api5*. Six *Api5* mutants were generated as they were for *Aac11*, detailed above. The primers used are listed below.

Table 5: Primers used to generate single lysine mutants of *Api5* through overlap extension PCR. The blue portions of the wild type forward and reverse primers represent the portion of the primer complementary to *Api5*.

Wild type	**Forward primer**	**Reverse primer**
pGEX-Api5	CTGGTTCCGCGTGGATCCCCGGAATTCATGCCGACAGTAGAGGAGCTT	TCGTCAGTCAGTCACGATGCGGCCGCTCAGTAGAGTCTTCCCCGACT
Mutant	Forward primer	Reverse primer
Api5 K126R	CTATTAAGTATATTTAGAATGGATGCAAAAGG	CCTTTTGCATCCATTCTAAATATACTTAATAG
Api5 K337R	GAAGAACCCAGGCTACAGTTCAGT	ACTGAACTGTAGCCTGGGTTCTTC

Api5 K374R	CTCAAAGATTTCAGAATCAGGCTGCAG	CTGCAGCCTGATTCTGAAATCTTTGAG
Api5 K404R	GGTGAGGCCTTAAGAACAGAAGAG	CTCTTCTGTTCTTAAGGCCTCACC
Api5 K409R	ACAGAAGAGAACAGGATTAAAGTCGTT	AACGACTTTAATCCTGTTCTCTTCTGT
Api5 K474R	GCAGGACCAAGAAGAGATGCC	GGCATCTCTTCTTGGTCCTGC

Cloning *Aac11* and *Api5* into *pRmHa-3*

The coding sequences of *Aac11* and *Api5* were cloned into the *pRmHa-3* expression vectors for use in *Drosophila* S2 cells. They were cloned with N-terminus 6xHis and HA tags. The primers used are listed below, and the cloning scheme made use of homologous recombination in the PPY strain as detailed previously. In this case, the homologous regions were the sequences for the His tag in the 5' region and the *pRmHa-3* vector in the 3' region.

Table 6: Primers used to clone the coding sequences of *Aac11* and *Api5* into *pRmHa-3* with N-terminus His tags. The portions in blue indicate the His-tagged portions. The blue and green portions were the sites of homologous recombination.

	Forward primer	**Reverse primer**
pRmHa-3 His-Aac11	ATGCACCACCATCACCACCATGGAGGCGGAATGGACAACATAGAGCGACTGTAC	GCAGGTCGACTCTAGAGGATCCTTAGTACTTGTTGAATCGCCTGTT
pRmHa-3 His-Api5	ATGCACCACCATCACCACCATGGAGGCGGAATGCCGACAGTAGAGGAGCTT	GCAGGTCGACTCTAGAGGATCCTCAGTAGAGTCTTCCCCGACT
pRmHa-3 forward	GGATCCTCTAGAGTCGACCTGC	-
pRmHa-3 His reverse	-	TCCGCCTCCATGGTGGTGATGGTGGTGCATGGTACCGAGCTCGAATTCCCTT

Transposable constructs for generating fly lines

The coding sequences of *Aac11* and *Api5* were also cloned into the *pUASp-AttB* vector with N-terminal HA tags for the generation of transgenic flies. The vector was digested at the BamHI site of the multiple cloning site using

BamHI-HF from NEB (Catalogue number: R3136S). The linearised vector was then gel purified and treated with Calf Intestinal Alkaline Phosphatase from NEB (Catalogue number: M0290L) to prevent self-ligation. The product was purified using the QiaQuick PCR purification kit from Qiagen listed previously. HA tags were first appended to the coding sequences of *Aac11* and *Api5* using *pRmHa-3* forward primers, and an *Aac11/Api5* primer with the *pUASp-AttB* reverse homology region. A second set of PCR reactions was then done with a forward primer specific to the HA tag and containing a 5' *pUASp-AttB* homology region, and the same reverse primer as the previous set. These homology regions are around the BamHI site. The primers are listed below.

Table 7: Primers used to clone the coding sequences of *Aac11* and *Api5* into *pUASp-AttB* with N-terminus HA tags. Blue indicates portions complementary to the sequence for the HA tag. Green indicates homology regions for *pUASp-AttB*. Amplification was first done with the *pRmHa-3* forward and *pUASp-AttB* reverse, and then with the *pUASp-AttB* forward and reverse.

	Forward primer	**Reverse primer**
pRmHa-3 HA Aac11 forward, *pUASp-AttB* reverse	TACGATGTTCCAGATTACGCTGGAGGCG GAATGGACAACATAGAGCGACTGTAC	ACGTTCGAGGTCGACTCTAGAGGAT CCTTAGTACTTGTTGAATCGCCTGTT
pRmHa-3 HA Api5 forward, *pUASp-AttB* reverse	TACGATGTTCCAGATTACGCTGGAGGCG GAATGCCGACAGTAGAGGAGCTT	ACGTTCGAGGTCGACTCTAGAGGAT CCTCAGTAGAGTCTTCCCCGACT
pUASp-AttB HA forward	GCATAGGCCACTAGTGGATCTGGATCCA TGTACCCATACGATGTTCCAGATTAC	-

Homologous recombination was used as previously detailed to generate constructs containing these HA-tagged sequences, termed *pUASp-AttB-HA-Aac11* and *pUASp-AttB-HA-Api5*, followed by confirmation of the presence of the insert using a colony PCR, and sequencing.

Cloning *Aac11* into pET45b+ with an N-terminal 6xHis tag

Aac11 was amplified with a forward primer containing a KpnI restriction site (ATGCGGTACCATGGACAACATAGAGCGACTGTAC), and a reverse primer to supply a BamHI site (GCTAGGATCCTTAGTACTTGTTGAATCGCCTGTT). This amplicon and the pET45b+ vector were then digested with KpnI and BamHI (NEB catalogue numbers: R3142S and R3136S) according to NEB's recommended protocols, and ligated overnight with Ligation Mighty Mix (Clontech, catalogue number: 6023) at 16 °C. The ligation mixture was transformed into DH5α cells. A colony PCR was done to screen for transformants using a T7 promoter primer and the *Aac11 K182R* reverse primer listed above.

In-bacto expression using the GST fusion system

Proteins encoded by the *pGEX* vectors were co-transformed into the BL21(DE3) strain of *E. coli* along with either the Q^{SUMO} or $Q^{\Delta GG}$ vectors as described by Nie et al., 2009. The Q vectors encode *Drosophila* SUMO (Smt3) and three enzymes involved in the SUMO pathway, SAE1, SAE2, and Ubc9. Q^{SUMO} encodes mature SUMO with a terminal –GG motif that can conjugate to SUMO targets. $Q^{\Delta GG}$ encodes SUMO without this –GG motif, therefore acting as a negative control. The SUMO encoded by both vectors is 6xHis-tagged. Single colonies were used to inoculate primary cultures in LB containing 0.1 mg/ml ampicillin (*pGEX* confers ampicillin resistance) and 0.05 mg/ml kanamycin (The Q vectors confer kanamycin resistance). These were allowed to grow overnight at 37 °C with shaking at 200 rpm. Secondary cultures were inoculated with 1-2% of their volumes of saturated primary culture and allowed to grow until an OD600 of 0.5-0.8, then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at 37 °C for 3-3.5 hours or 25 °C for 6 hours. 800 ul of culture was withdrawn from each sample, centrifuged to yield a pellet at 6000 rpm for 5 minutes, washed once with 1x PBS, centrifuged again, and boiled for 10 minutes at 95 °C in 80 ul 1x PBS and 20 ul 5x SDS sample buffer for analysis with SDS-PAGE. The remaining culture was spun down at 4°C and stored at -80°C until further processing was done.

Transfection and protein expression in *Drosophila* 529SU cells

Drosophila 529SU cells that overexpress Smt3 (*Drosophila* SUMO) under control of the metallothionein promoter were cultured in Gibco™ Schneider's *Drosophila* Medium from ThermoFisher Scientific (Catalogue number: 21720024) supplemented with 10% Gibco™ Heat Inactivated Fetal Bovine Serum (FBS) and passaged every 3-4 days. Cells were transiently transfected with *pRmHa-3-His-Aac11* and *pRmHa-3-His-Api5* using 2 ul of TransIt- Insect Transfection Reagent from Mirus (Catalogue number: MIR 6106) per microgram of plasmid used. The transfection mixture was prepared in Schneider's medium without added FBS. 100 ul of transfection mixture was prepared and used for each milliliter of cells. Protein expression in these cells was induced with $CuSO_4$ at a final concentration of 500 uM 4 -16 hours after transfection, and processed after 48 hours.

Fly handling

Flies were reared on corn meal agar at 25 °C unless stated otherwise. The following fly lines were used.

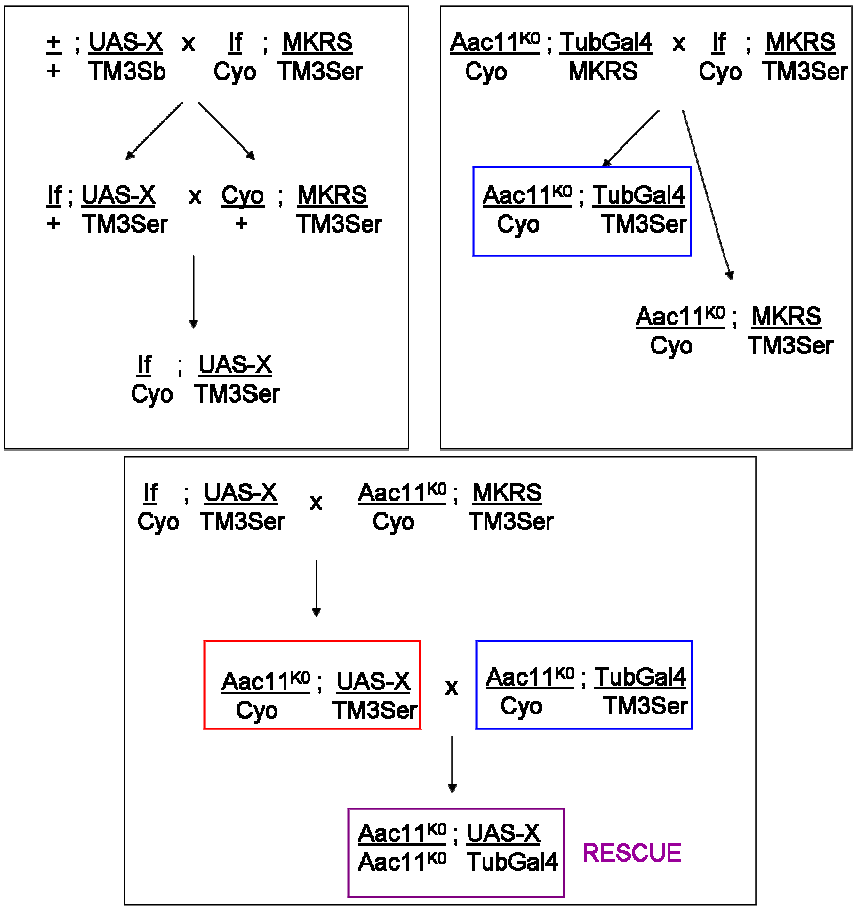
Table 8: Fly lines used for experiments.

S. no	Fly line	Description	Abbreviation	Bloomington stock number
1	y1 sc* v1; P{TRiP.HMC03881}attP40	siRNA for *Aac11* under UAS promoter	Aac11i	55187
2	y^1 w^{67c23}; P{lacW}$Aac11^{k06710}$/CyO	*Aac11* mutant, recessive lethal	$Aac11^{K0}$/Cyo	10645
3	TubGal4/MKRS	Tubulin Gal4, expressed ubiquitiously.	TubGal4	
4	ScaGal4	Scabrous Gal4, expressed in the mother cell ganglia in the thorax.	ScaGal4	
5	EnGal4/CyoActGFP	Engrailed Gal4, expressed in the posterior compartment of every segment.	enGal4	
6	MS1096Gal4/FM7A	MS1096 Gal4, expressed in the dorsal compartment of the wing.	MSGal4	
7	C155Gal4/FM7A	C155 Gal4, expressed in all CNS neurons.	-	
8	EyelessGal4/Cyo	Eyeless Gal4, expressed in the head and brain glia.	eyGal4	

Knockdown experiments were done by crossing virgin females of the Gal4 lines to males of the Aac11i line, or vice versa, at 18 °C, 25 °C, or 29°C. For wing images, the flies were first anesthetised and frozen at -80°C. The wings, including the hinge, were then pulled off with forceps, mounted in clove oil, and imaged with a stereomicroscope.

Rescue of the Aac11^{K0} mutant was done according to the following scheme.

Schematic 1: Crosses for the genetic rescue of the Aac11 null fly with Aac11/Api5. In the above scheme, UAS-X refers to either UAS-HA-Aac11 or UAS-HA-Api5. Aac11^{K0} refers to the Aac11 null allele.



Fly transgenics

pUASp-AttB-HA-Aac11 or *pUASp-AttB-HA-Api5* positive PPY strain colonies were used to inoculate 50 ml primary cultures containing 0.1 mg/ml ampicillin for positive selection. These were grown overnight at 37 °C. Plasmid was prepared from these cultures using the QIAGEN Plasmid Midi Kit (Catalogue

number: 12143) according to the protocol provided by the manufacturer. The resulting product was checked on a 1% agarose gel for quality. The concentration was measured using a nanodrop. 40 micrograms of plasmid were concentrated to dryness in a speed vac. The plasmids were submitted to the Fly Facility under Dr. Deepti Trivedi at NCBS, Bangalore to be injected into fly embryos expressing phiC31 integrase and containing 3rd chromosome attp2 sites for site-specific integration into these sites in the genome.

Affinity purification and western blotting

GST affinity pulldown using bacterial lysates

Bacterial cell pellets were lysed through vigorous pipetting in pre-chilled lysis buffer, which comprised 100 mM NaCl, 50 mM Tris, 1 mM EDTA, 2 mM DTT, 0.25 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1x protease inhibitor cocktail from Sigma (Catalogue number: S8820). 1 ml of lysis buffer was used for culture sizes of 10-30 ml, while 2 ml was used for 50 ml cultures. Once resuspended, the cells were placed on a nutator at 4°C for 30 minutes. Thereafter, they were sonicated for 30 seconds in pulses of 1 second ON, 3 seconds OFF at 60% amplitude. The sonicated lysates were centrifuged for 30 minutes at 4°C at 15000 rpm. The supernatant was added to glutathione agarose beads from ThermoFisher Scientific (Catalogue number: G2879) that had been equilibrated in extraction buffer (100 mM NaCl, 50 mM pH 8.0 Tris , 1mM EDTA) using three washes of 1 ml each, and left on a nutator overnight at 4°C. The beads were then washed thrice in 0.1% TBS-Triton, and boiled in 80 ul extraction buffer + 20 ul 5x SDS sample buffer for 10 minutes at 95 °C. 8 ul of sample was run on a 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue to check that the pulldown had worked, and to determine the amount of sample to load on an SDS-PAGE gel for immunoblotting. The sample run on the gel thereafter was transferred to a PVDF membrane at a constant current of either 275 mA for 3-4.5 hours or 130 mA overnight. The membrane was blocked for 1 hour with 5% milk powder in 0.1% TBST (Tween) as the blocking reagent, and incubated in primary antibody diluted in blocking reagent (mouse-anti-GST-

1:5000, mouse-anti-His- 1:1000 or 1:2000) for one hour at room temperature, (mouse-anti-GST), two hours at room temperature (mouse-anti-His), or overnight at 4°C (mouse-anti-GST or mouse-anti-His). The mouse-anti-GST and mouse-anti-His antibodies were purchased from Santa Cruz Biotechnology (Catalogue numbers: SC-8036 and SC-136 respectively). The membrane was washed for 3 x 15 minutes with TBST, and incubated in secondary antibody (horseradish peroxidase conjugated goat anti-mouse from Jackson ImmunoResearch, catalogue number: 115-035-003, 1:10000) for one hour. Final washes of 3 x 15 minutes with TBST were given, and the immunoblotted membrane was developed in a LAS4000 machine with developing reagent.

His affinity pulldown using 529SU cell lysates

529SU cells were grown, transfected, and induced as described under section 3. They were processed for Ni-NTA His affinity pulldown assays by first spinning them down for fifteen minutes at 4°C at 1500 rpm, washing twice with 1x PBS, and resuspending vigorously in lysis buffer consisting of 8M urea, 100 mM Tris (pH = 8.0), 100 mM NaH_2PO_4, and 10 mM imidazole. They were incubated in this lysis buffer for twenty minutes at room temperature before centrifugation for twenty minutes at room temperature at 15000 rpm. Ni-NTA Superflow beads from Qiagen (Catalogue number: 30430) were equilibrated in lysis buffer with 2 x 5 minute 1 ml washes. 50 ul of the supernatant from the centrifugation step was saved and boiled with 10 ul of 5x SDS sample buffer to analyse on an SDS-PAGE gel. The remaining supernatant was added to the beads and left on a nutator for one hour. The beads were then washed 3 x 5 minutes with 1 ml wash buffer comprising 8M urea, 100 mM Tris (pH = 6.3), 100 mM NaH_2PO_4, and 20 mM imidazole, and boiled in 80 ul wash buffer + 20 ul 5x SDS sample buffer for 10 minutes at 95 °C. They were then run on an SDS-PAGE gel, and transferred to a PVDF and immunoblotted as described earlier, using mouse-anti-His (1:1000) to visualise overexpressed *Aac11* and *Api5,* and mouse-anti-Flag from Sigma-Aldrich (1:2000, catalogue number: F1804) to visualise overexpressed SUMO.

HA/His affinity pulldown using fly lysates

Flies were heat-shocked for one hour at 37 °C prior to the pulldown assay to promote Gal4 expression. They were frozen at -80°C and then crushed in pre-chilled 1x RIPA buffer containing 1x protease inhibitor cocktail using a Dounce homogeniser on ice. 1 ml of buffer was used for 100 flies. The lysate was incubated on ice for 30 minutes and then centrifuged for 30 minutes at 4°C at 15000 rpm. If the supernatant contained floating debris, it was transferred to another microcentrifuge tube and spun down for 10-15 minutes. HA-agarose beads from Sigma-Aldrich (Catalogue number: A2095) were equilibrated by washing 3 x 5 minutes in 1 ml 1x RIPA buffer. The supernatant from the centrifugation step was then added to and incubated with the beads overnight on a nutator at 4°C. The beads were washed thrice in 1x RIPA and boiled in 40 ul 1x RIPA + 10 ul 5x SDS dye. The entirety of the sample was loaded onto an SDS-PAGE gel, transferred to a PVDF membrane and immunoblotted as described in section earlier. The primary antibody used was rabbit-anti-HA from Sigma-Aldrich (1:5000, catalogue number: 04-902), and the secondary antibody was horseradish peroxidase conjugated goat anti-rabbit from Jackson ImmunoResearch (1:10000, catalogue number: 111-035-003). For His affinity pulldowns, the flies were prepared using the Dounce homogeniser as outlined above, with 8M urea lysis buffer described in section 6b replacing 1x RIPA. Incubation with Ni-NTA beads, washing, and other steps were performed as in section described earlier, at room temperature.

Protein expression for antibody generation

pGEX-Aac11 was transformed into BL21(DE3) and grown overnight on an ampicillin-dosed LB agar plate (0.1 mg/ml ampicillin) at 37 °C. A single, isolated colony on such a plate was used to inoculate 1 ml of LB as a primary culture. This was allowed to grow overnight at 37 °C with shaking at 200 rpm. 50 ml secondary cultures were inoculated with 500 ul of primary culture and allowed to grow at 37 °C with agitation until OD600 0.5-0.8, and induced with IPTG at a final

concentration of 1 mM for 6 hours at 25 °C. Expression was checked on an SDS-PAGE gel as described in section 2, and a GST pulldown was done as in section 6a. The protein-bound glutathione beads were incubated in thrombin (HiMedia, catalogue number: RM5469) overnight at 4°C on a nutator. The amount of thrombin used was calculated according to the amount of protein estimated to be pulled down and the manufacturer's instructions. The eluate was collected, and the beads were washed twice with 0.1% TBST, with eluates being collected each time. Each eluate was boiled with 5x SDS sample buffer and analysed on an SDS-PAGE gel. A His-tagged version of *Aac11* was also cloned for this purpose, described under 'Cloning and mutagenesis'.

Results

Aac11 and Api5 are SUMOylated *in-bacto.*

The SUMOylation assay used was as described by Nie et al., in 2009 and was performed on GST-Aac11 and GST-Api5. The assay yielded evidence that the two proteins are indeed SUMOylated in the bacterial system **(Figure 5A)**. The assay was repeated with an additional thrombin cleavage step performed to exclude the possibility that the His bands represent GST SUMOylation **(Figure 5B)**.

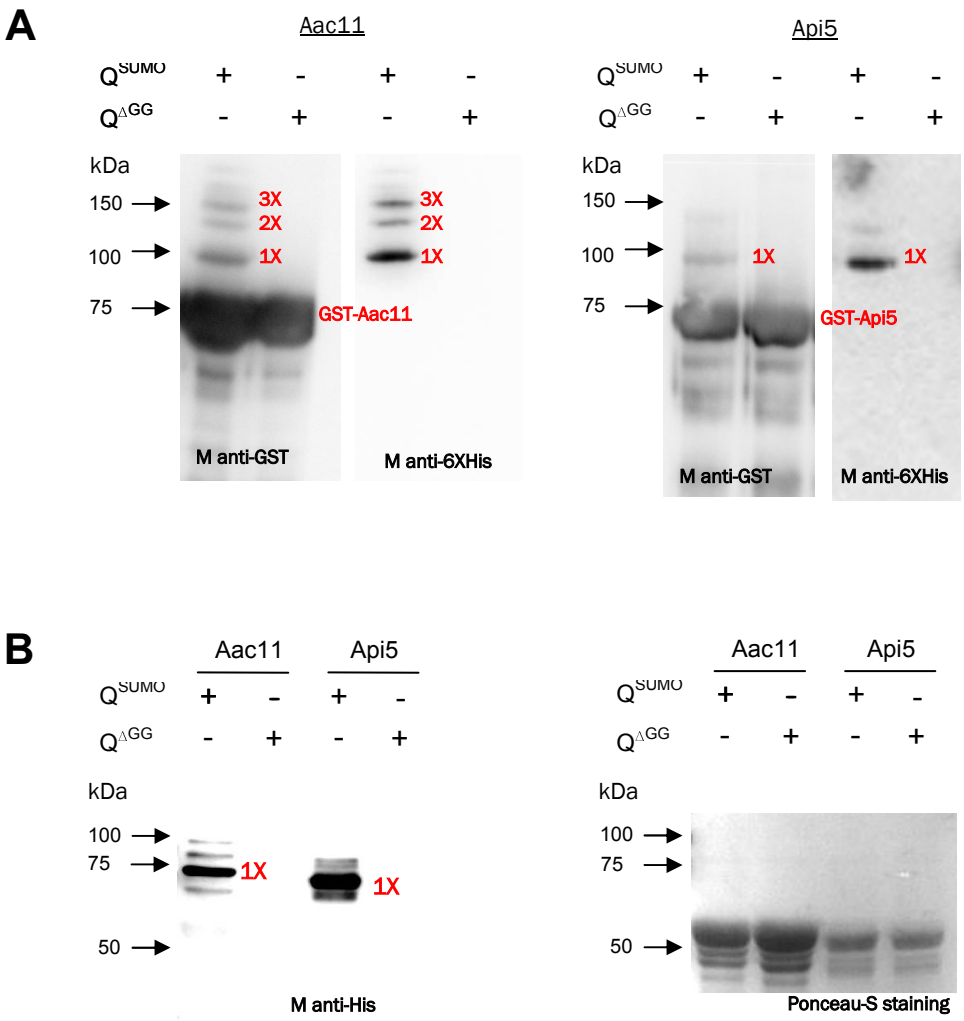


Figure 5: SUMOylation of *Drosophila* Aac11 and Human Api5.
A) *In-bacto* SUMOylation assay for both Aac11 and Api5 suggest that both proteins are SUMOylated. Both proteins are expressed in *E. coli* as GST fusions and SUMOylated in independent experiments. The protein products are affinity purified using glutathione agarose

beads and the enriched products loaded on two SDS-PAGE gels for western blotting. The duplicate blots are probed with Mouse Anti-GST (M anti-GST) and Mouse Anti-6X-His (M anti-His), Based on the banding patterns, there appear to be multiple SUMOylated species for SUMO (1X, 2X, 3X) and these have been marked/assigned based on their approximate molecular weights.
B) In order to rule out GST SUMOylation, the affinity purified proteins are cleaved with the protease thrombin. Thrombin cleavage separates the protein (Api5/Aac11) from bead-bound GST. The presence of SUMOylated species in the supernatant, confirmed by M anti-His staining confirms that both Api5 and Aac11 are SUMOylated. Ponceau-S staining (right panel) was done as a loading control. The molecular weight of Aac11 is 59.9 kDa, and Api5 59.0 kDa.

It was observed that Api5 is SUMOylated more readily than Aac11 *in bacto*, as the bands corresponding to the SUMOylated species are thicker in the case of Api5. Multiple SUMOylation bands were observed for both Aac11 and Api5, each shifted by about 20 kDa, which tallies with the size of SUMO when run on a gel. Their absence in the negative control provided confidence that these were true SUMO bands and not non-specific ones.

Nine individual single lysine -> arginine Aac11 mutants showed no loss of SUMOylation.

In order to identify the SUMO sites on Aac11, the four JASSA-predicted lysines with the strongest consensus motifs were chosen (128, 408, 440, and 490, marked in Figure 4) and individually mutated to arginines. These mutant Aac11 constructs were subjected to the SUMOylation assay **(Figure 6A)**. None of these lysine -> arginine mutants abolished SUMOylation or changed the pattern of His bands compared to the wild type, leading us to test five additional lysines (171, 182, 306, 373, 394, see Figure 4 for sequences) chosen on the basis of the presence of the consensus motif, and conservation across phyla. The latter arose from our hypothesis concerning the importance of SUMOylation to the function of Aac11- by extension, if SUMOylation is crucial, it is likely to be conserved. These five additional Aac11 mutants were put through the SUMOylation assay under the same conditions, but did not yield a difference in the number of bands seen upon immunoblotting against His **(Figure 6B)**.

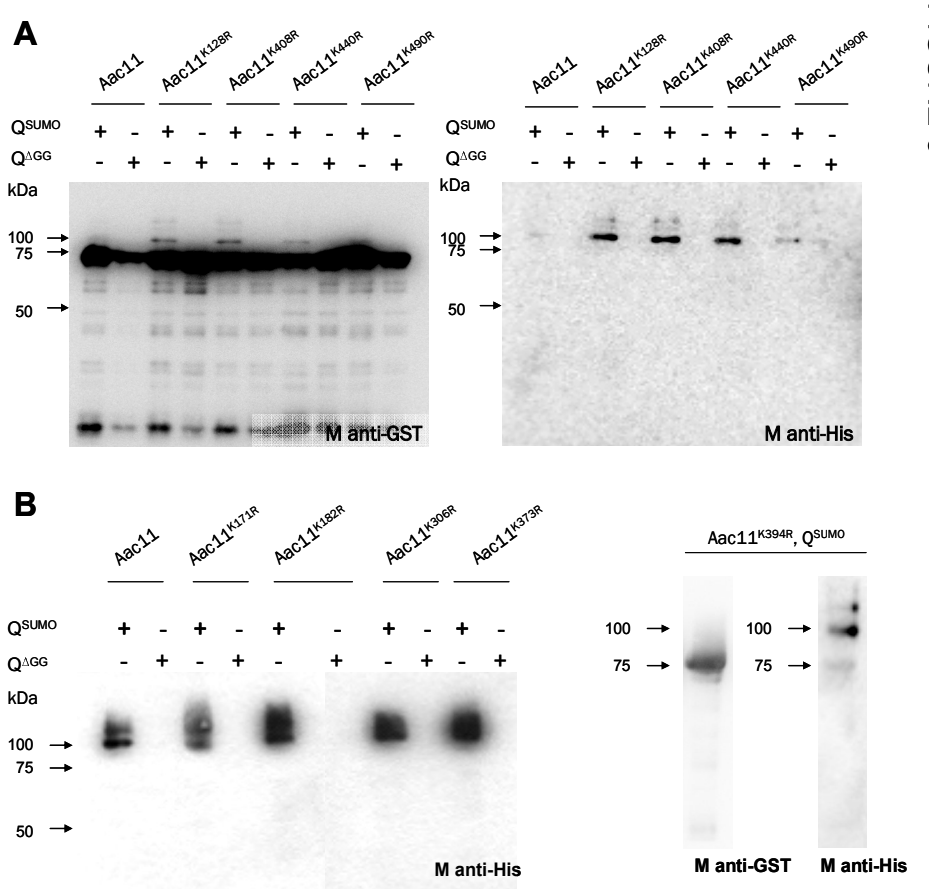


Figure 6: Discovering the lysine target for the SUMOylation machinery for Aac11.
Based on the JASSA output, a number of site directed Lys → Arg mutants were made in order to generate an Aac11 variant that did not get SUMOylated, as indicated in the panel.
A) Four mutants, namely K128R, K408R, K440R, and K490R were generated and tested using the *in-bacto* system. Induction was performed at 37 °C for 3 hours, using 1 mM IPTG.
B) Five additional mutants, K171R, K182R, K306R, K373R, and K394R were generated and tested using the *in-bacto* system. Induction was performed at 37 °C for 3 hours, using 1 mM IPTG.

Six individual single lysine -> arginine Api5 mutants showed no loss of SUMOylation.

Six lysines on Api5 were chosen based on the JASSA output (Figure 1) and mutated individually to arginines. The SUMOylation assay was performed on these Api5 mutants **(Figure 7)**. As with the experiment on the nine Aac11 mutants, no loss of SUMOylation or alteration of the pattern of His bands was observed for any of the mutants. This included the conserved lysines that were picked up as SUMO targets for Aac11 as well, 126 (128 on Aac11) and 404 (408 on Aac11).

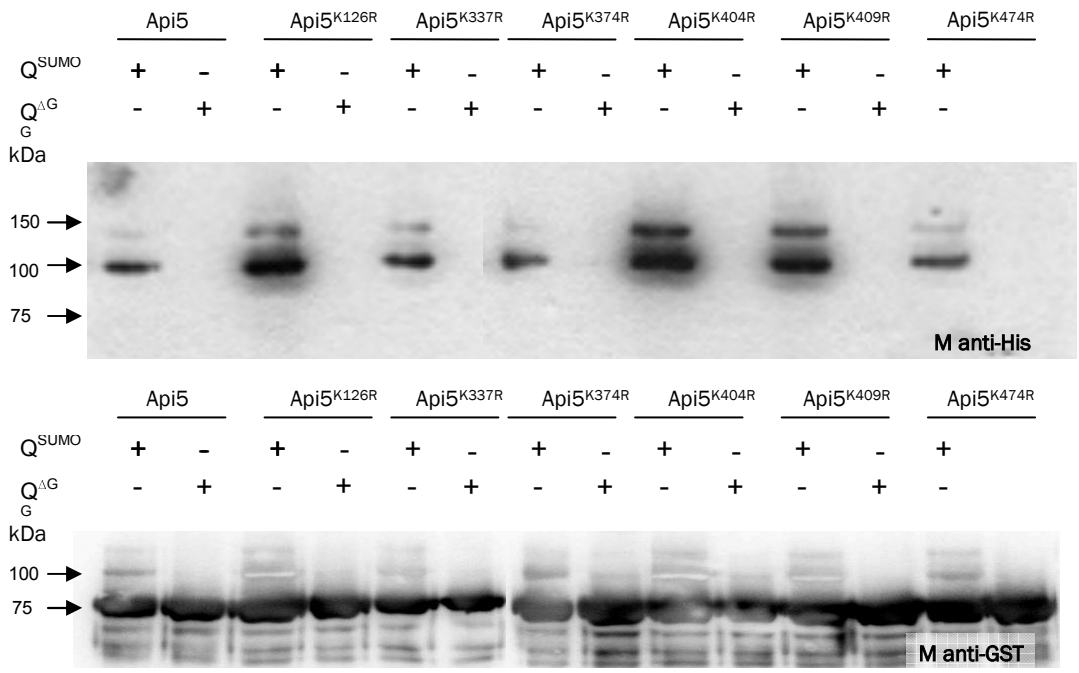


Figure 7: Discovering the lysine target for the SUMOylation machinery for Api5.
Based on the JASSA output, a number of site directed Lys → Arg mutants were made in order to generate an Api5 variant that did not get SUMOylated. As indicated, a total of six mutants, namely K126R, K337R, K374R, K404R, K409R, and K474R were generated and tested by the *in-bacto* system. Induction was performed at 25 °C for 6 hours, using 1 mM IPTG.

Simultaneous loss of four consensus motif lysines showed no loss of SUMOylation in Aac11.

Examples of proteins that are SUMOylated on multiple lysine residues are known, such as aPKC. It has been demonstrated that in some cases, multiple residues need to be mutated simultaneously in order to abolish SUMOylation completely. It has also been shown, as in the case of aPKC, that single mutations may not show a difference in banding pattern post-immunoblotting for SUMO, while multiple mutations of the same lysines do (Yadav et al., 2016). This led us to generate a quadruple-mutant version of Aac11, where lysines 128, 408, 440, and 490 were replaced with arginine. These were, as marked in Figure 1, the lysines predicted to be SUMO sites with the greatest confidence by JASSA. Lysines 128 and 408 are also conserved in evolution. This Aac11 variant was designated Aac11^{Quadruple}, and was put through the SUMOylation assay with an

additional thrombin cleavage step to separate the GST tag from Aac11. **(Figure 8)**. This mutant, too, did not show any change in banding pattern using the *in bacto* system in comparison with the wild type. Ponceau-S staining was done as a loading control, and shows comparable loading, with the wild type protein level being slightly higher.

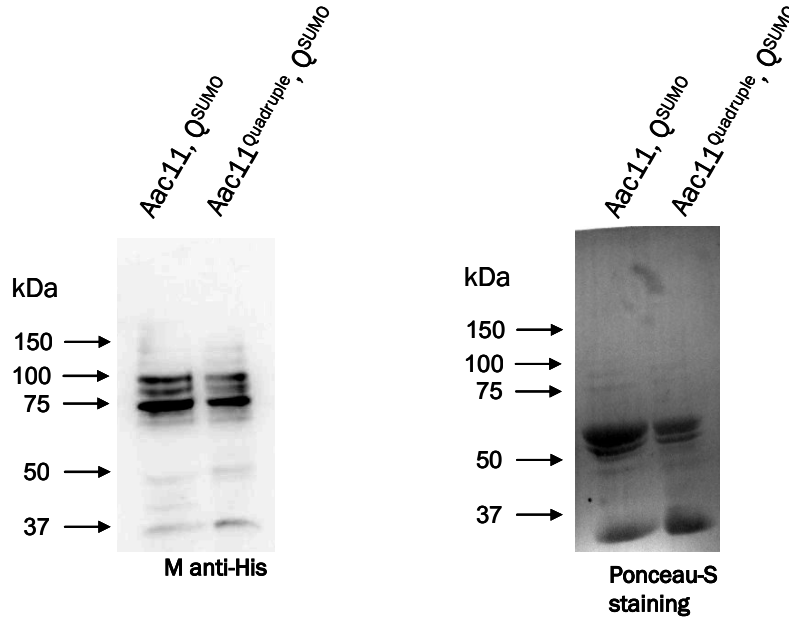


Figure 8: Four simultaneous lysine mutations for Aac11 were made and tested using the SUMOylation assay.
The SUMOylation assay was performed as with the other mutants, but with an additional thrombin cleavage step. Induction was performed at 37 °C for 3 hours, using 1 mM IPTG. The thrombin cleavage was done overnight at 4°C, and the supernatant containing the cleaved protein was processed for immunoblotting.

SUMOylation of Api5 and Aac11 in S2 cells in culture.

Since Api5 and Aac11 were SUMOylated in vitro, the next critical step was to demonstrate SUMOylation *in-vivo*. *Aac11* and *Api5* were cloned into *pRmHa-3* with N-terminus 6xHis tags **(Figure 9A)**. Transfection into 529SU cells and subsequent immunoblotting against 6X-His and Tubulin proved that these constructs express in the cell culture system **(Figure 9B)**. Experiments to demonstrate SUMOylation in S2 cells are ongoing using these vectors.

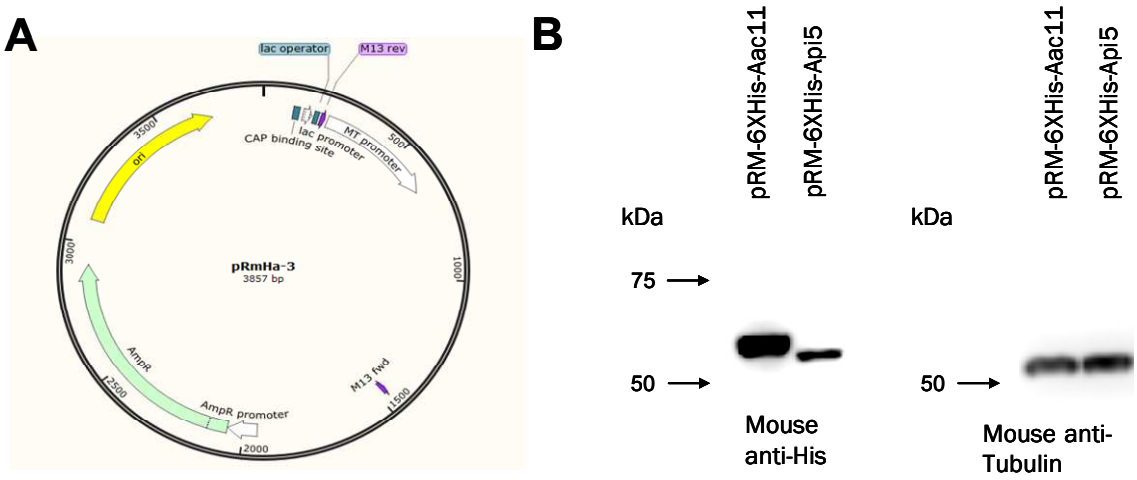


Figure 9: Demonstrating SUMOylation of Aac11 and Api5 in Schneider (S2) cells in culture.
A) *Aac11* and *Api5* were cloned into the *pRmHa-3* vector (pRM), with N-terminal 6xHis tags, under the control of a metallothionein promoter.
B) *pRM-6xHis-Aac11* and *pRM-6xHis-Api5* were transfected into *Drosophila* 529SU cells. Expression was induced using 500 uM $CuSO_4$ for 48 hours. The cells were processed for immunoblotting and probed with antibodies against 6xHis and Tubulin.

The experiments will involve affinity purification of FLAG-tagged SUMOylated species after co-expression of Aac11/Api5 with FLAG-SUMO.

SUMOylation of Api5 and Aac11 in flies.

UAS-HA-Aac11 and UAS-HA-Api5 lines were generated via site-directed integration into the fly genome using the *pUASp-AttB* vectors **(Figure 10)**. These constructs were cloned as described in the Materials and Methods section. The plasmids were injected in flies containing X-chomosome phiC31 integrase and 3rd chromosome attp2 sites by the Fly Facility under Dr. Deepti Trivedi at NCBS.

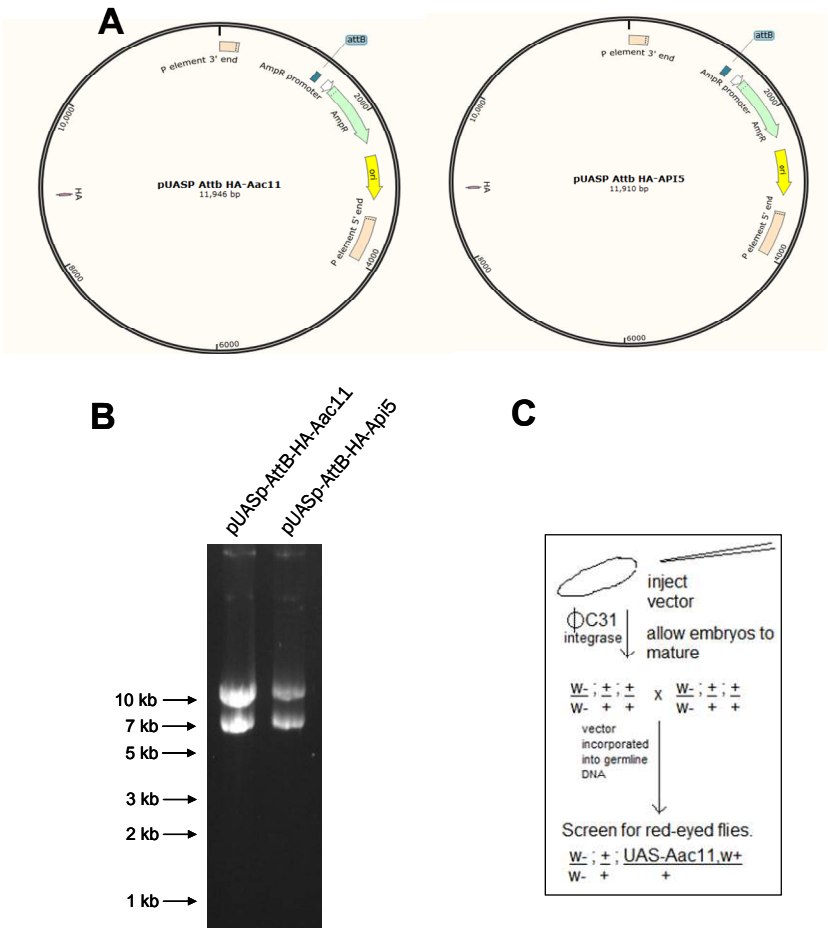


Figure 10: Generation of transgenic lines for expression of Api5 and Aac11 in flies.
A) *pUASp-AttB* was the vector used to subclone *Aac11* and *Api5* for expression in flies. Both were cloned with N-terminus HA tags.
B) Cloned *pUASp-AttB*-HA-Aac11 and *pUASp-AttB*-HA-Api5 on an agarose gel. These vectors were prepared for injection into fly embryos.
C) Injection and subsequent screening protocol for the *pUASp-AttB*-HA-Aac11/Api5 plasmids into fly embryos. The vector, containing a mini-white gene, is injected into the germ plasm embryos of a w- (white-eyed) attP2 line expressing phiC31 integrase for site specific integration into the attP2 site. The embryos are allowed to mature into adults, which are crossed to another line of white-eyed flies. The next generation is screened for red eyes.

Expression in the lines was induced at 29 °C using a double DaughterlessGal4 driver **(Figure 11)**. An hour-long heat shock at 37 °C was provided to increase global levels of SUMOylation (Gareau & Lima, 2010). Immunoblotting against HA was performed on the fly lysate. Bands are visible for each of the four lines, corresponding to the expected molecular weights. No other bands are seen, and no signal is seen in the negative control, for which

DaughterlessGal4 flies were used. The expression levels of Api5 seem low in comparison to Aac11.

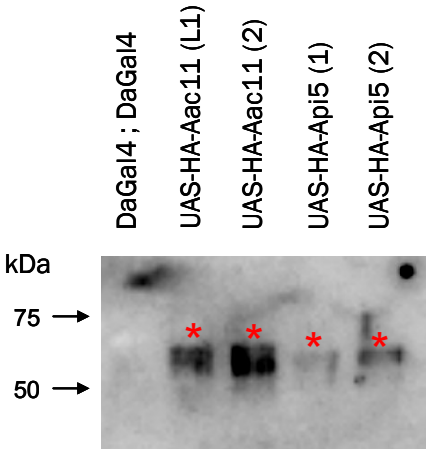


Figure 11: Expression of Aac11 and Api5 in transgenic flies.
Expression in each of the four transgenic lines (two UAS-HA-Aac11 lines and two UAS-HA-Api5 lines) was driven by a double DaughterlessGal4 (DaGal4) line. Flies were heat-shocked at 37 °C and lysed. HA-tagged Aac11/Api5 were purified from these lysates using HA-affinity beads and prepared for immunoblotting. DaGal4 ; DaGal4 flies were used as a negative control.

Rescue of Aac11 null flies with Aac11 and Api5.

The *Aac11*^{K06710} mutant is recessive lethal. Death occurs in the embryonic stages, pointing to the necessity of Aac11 in development. Crosses to rescue the Aac11 null fly through the overexpression of Aac11 and Api5 were carried out as indicated in Schematic 1. Overexpression was driven by the Tubulin promoter, but the rescue failed for both Aac11 and Api5. Hatching assays will be done to check if a partial rescue has taken place, and alternative drivers will be tested to see if the rescue works with a different spatiotemporal pattern of expression.

Rescue of wing defects in Aac11 RNAi flies with Api5 expression.

Knocking down Aac11 levels using an RNAi line against *Aac11* with certain tissue specific and ubiquitous Gal4 drivers leads to death at different stages prior to eclosion, corroborating the lethality seen in homozygous *Aac11* mutant flies. Knockdown with TubulinGal4, EngrailedGal4, and C155Gal4 leads

to lethality at 18 °C and 25 °C. Knockdown with ScabrousGal4 is 100% lethal at the pupal stage at 25 °C, but flies survive at 18 °C. Knockdown with MS20196Gal4 is predominantly lethal at 25 °C and 18 °C, but a few flies emerge, all with wing defects **(Figure 12)**. The majority of flies die as pupae, fully formed but unable to eclose. More flies with wing defects emerge at 18 °C than at 25 °C.

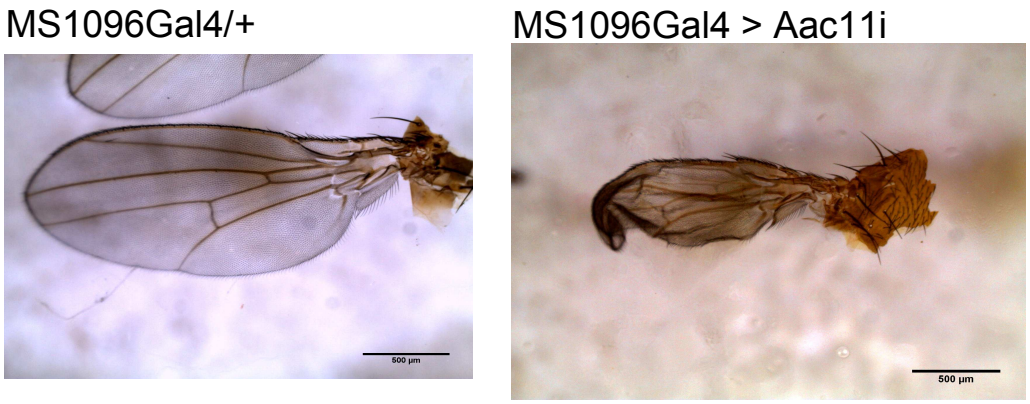
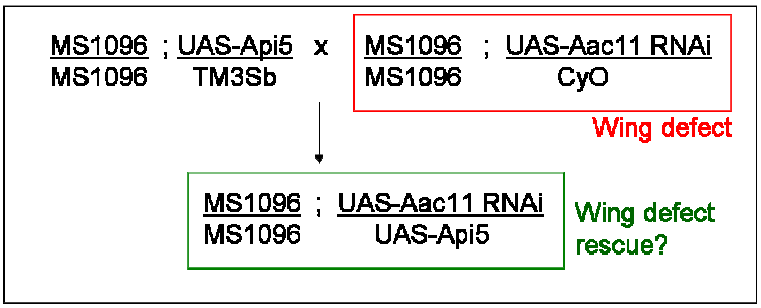


Figure 12: Aac11 knockdown driven by MS1096Gal4 yields pupal lethality and wing defects in the escapers.
The left panel depicts the control wing. The right panel depicts the wing defect in flies where Aac11 was knocked down under MS1096 control. The wing appears reduced in size and shrivelled. The scale bar is 500 microns.

Since the *Aac11* RNAi will affect only *Aac11* transcripts and not *Api5*, this provides another way to test if Api5 can rescue the loss of Aac11 function. Given below is a schematic **(Schematic 2)** that indicates a genetic cross to test Api5 rescue of Aac11 loss of function in the wing development.

Schematic 2: Cross to rescue wing defects caused by loss of Aac11 function by the expression o f Api5.



Discussion

Apoptosis is programmed and controlled cell death marked by DNA fragmentation, blebbing, and the formation of apoptotic bodies that are consumed or cleared by phagocytes. Apoptosis is initiated through three broad pathways- the intrinsic, extrinsic, and granzyme B pathways. The former is triggered through proteins internal to the cell that are normally inhibited by the pro-survival Bcl-2 family proteins. The extrinsic pathway is activated via death receptors such as Fas or the TNF receptors by their respective ligands (FasL and TNFα). The granzyme B pathway involves the entry of this enzyme into the cell through channels in the plasma membrane. The result of the activation of each of these pathways is the release of cytochrome c from the mitochondria through the BAX-BAK channels and the subsequent activation of the caspases, leading to the cleavage of cellular protein and the dismantling of the cytoskeleton. Several pathways feed into, control, and can trigger apoptosis, making it an intricate and tightly regulated process (Taylor et al., 2008).

SUMO conjugates several proteins involved in apoptotic processes (**Figure 13** (Hendriks & Vertegaal, 2016)). It is currently unclear whether a global increase in SUMOylation would enhance or reduce levels of cell death. It is known, however, that SUMO modification can act to support or enable the pro- or anti-apoptotic function of some proteins, and inhibit others. Rodriguez et al. showed that UV-induced apoptosis caused an accumulation of p53, the tumour suppressor gene, and, significantly, SUMO-modified p53. p53-dependent apoptosis is enhanced by SUMOylation, as SUMO modification of p53 at lysine 386 activates its transcriptional activity. The murine double minute 2 (Mdm2) protein, which acts as an ubiquitin E3 ligase, causes the degradation of p53, can also be SUMOylated (Buschmann et al., 2000). This causes self-ubiquitination, allowing p53 to persist and activate genes downstream of it, eventually bringing

about apoptosis (Buschmann et al., 2000; Rodriguez et al., 1999). In this case, SUMOylation promotes apoptosis.

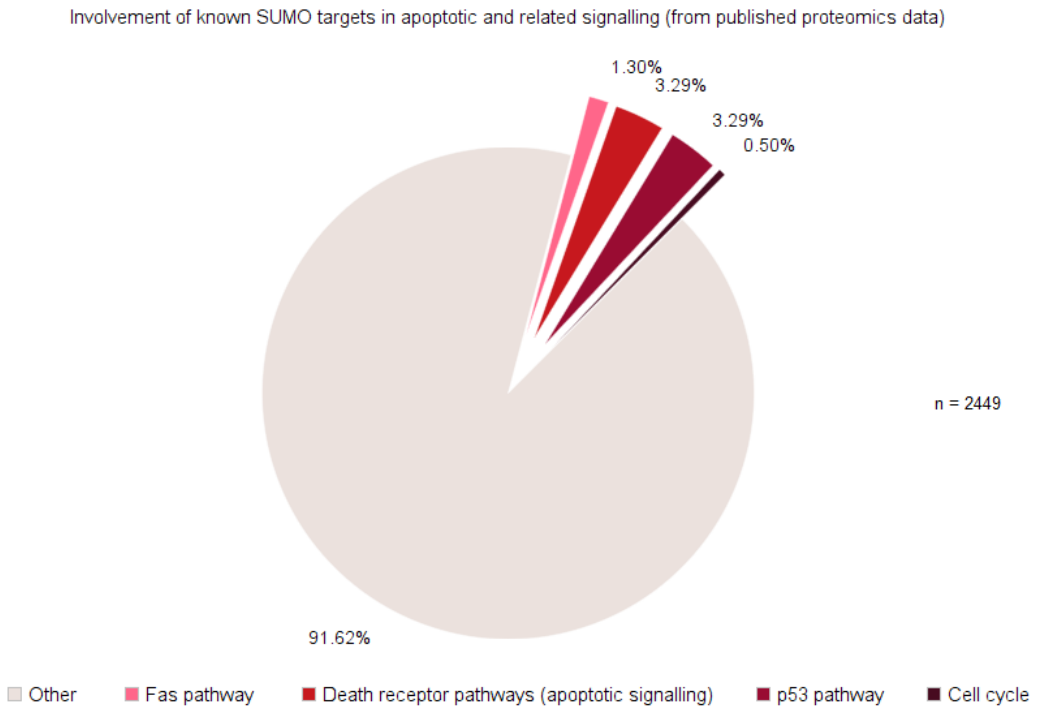


Figure 13: SUMOylation modifies proteins involved in apoptosis. Hendriks et al. compiled information from a number of proteomic studies to create a database of known SUMOylated proteins and the sites at which they are modified (Hendriks & Vertegaal, 2016). That database, when fed through the gene classification system PANTHER (Thomas et al., 2003), showed that a percentage of these proteins are involved in apoptosis, either in the death receptor pathways, the p53 pathway, the Fas pathway, or cell cycle signalling, which were chosen as representative pathways (number of genes, n = 2449). This representation excludes some SUMOylated proteins, such as caspase-8 (Besnault-Mascard et al., 2005), and substrates that were reported after the publication of this study, such as FADD (Choi et al., 2016).

PML, the promyelocytic leukemia protein, is also modified by SUMO-1. PML forms nuclear bodies that are the sites of recruitment of several other proteins, and SUMOylation has been demonstrated to be critical for the formation of these speckle-like structures (Zhong et al., 2000). Daxx, a protein in the TGFβ signalling pathway closely associated with PML bodies, is recruited to these nuclear bodies upon SUMOylation. This sequestration is pro-apoptotic. When Daxx is free in the cytoplasm, on the other hand, it aids the suppression of

apoptosis triggered by the TGFβ pathway by interacting with SUMOylated Smad4. SUMO regulation of the latter protein, in this case, is important for the inhibition of apoptosis (Hatake et al., 2009). Similarly, it has been reported that SUMO-1 overexpression aids the resistance of rheumatoid arthritis synovial fibroblasts to cell death (Meinecke et al., 2007).

Aac11 and Api5 are anti-apoptotic proteins that are orthologues of each other in fruit flies and humans respectively. Given the literature that exists on the SUMO regulation of apoptosis, we sought to explore the SUMO modification of these proteins. Both were predicted to be SUMOylated by the bioinformatic tool JASSA (Beauclair et al., 2014). In the course of this study, Aac11 and Api5 have been proven to be SUMOylated *in vitro* by *Drosophila* SUMO, Smt3. The banding pattern observed on the anti-His immunoblot (Figure 5) could indicate the SUMOylation of multiple residues, poly-SUMOylation, or both. To identify the SUMO site, lysines on Aac11/Api5 were mutated to arginines. Nine lysines on Aac11 and six Api5 lysines were tested as putative SUMOylation sites. The results of this screen for the SUMO acceptor site on Aac11/Api5 were inconclusive, despite the lysines mutated being part of strong SUMOylation consensus motifs, and conserved across evolution. A quadruple lysine -> arginine mutant version of Aac11 was then generated to test for loss of SUMOylation, based on examples of proteins on which multiple mutations were performed at a time to abrogate SUMOylation completely, such as aPKC and Cubitus interruptus (Lv et al., 2016; Yadav et al., 2016). This too, led to no change in the pattern of His-SUMO bands. This was surprising as it implied that the SUMO sites on Aac11 are not the conserved consensus motifs with the highest predictive score calculated by JASSA, but other lysines. It is also possible that there are several SUMO sites, *including* the ones we have mutated, and that the mutation of all of these lysines is necessary to see a complete loss of SUMOylation. This result indicates that a new approach to identifying the SUMO site(s) on Aac11/Api5 needs to be taken, such as the LC/MS method (Hendriks et al., 2014).

To demonstrate SUMOylation *in vivo*, we generated constructs for use with the *Drosophila* S2 cell system. These constructs have been shown to express under the control of the metallothionein promoter, and experiments are ongoing to show SUMO conjugation of Aac11 and Api5 in this context. Transgenic lines that express Aac11 and Api5 were also generated, to attempt a genetic rescue of the Aac11 null fly with Aac11 and Api5, which failed on our first attempt with the Tubulin driver. The fly cannot survive without Aac11 in early development, evidenced by the embryonic death of $Aac11^{K0}$ homozygous flies. This points towards an important role for Aac11 in fly development. Knocking down Aac11 using RNA interference in subsets of cells and ubiquitously were in agreement with this supposition. The rescue experiment would provide insight into whether Api5 can take over this important role that Aac11 plays in fly development. If Api5 can indeed rescue the *Aac11* null fly, this opens up the possibility of using the *Drosophila* system to perform experiments with Api5 that are more difficult in systems such as cell culture. On the other hand, if it does not, it could mean that sequence conservation in this case does not translate into functional conservation, despite the leucine zipper domain being conserved (Rigou et al., 2009).

The continuation of this study would involve producing an antibody against Aac11, for which a His-tagged version of Aac11 has been subcloned into pET45b+. It would also involve using the LC/MS approach to find out in a comprehensive manner which lysines are SUMOylated, and mutating them to create a variant that shows no SUMOylation. The genetic rescue experiment will then be done with this SUMOylation-resistant version of Aac11/Api5. Based on phenotypes seen thereafter, our hope is that we gain insight into the function that SUMOylation plays in the regulation of these proteins.

Summary

Aac11 and Api5 are SUMOylated *in vitro.* Nine lysines on Aac11 and six on Api5 were tested as possible SUMO sites but yielded inconclusive results. Reagents have been generated to test SUMOylation *in vivo,* in the fly and in S2 cells, including transgenic flies that express Aac11 and Api5. The rescue of the Aac11 null fly with Aac11 and Api5 failed with the Tubulin driver, but will be attempted with other drivers.

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