Processing of Sde2 from different eukaryotes by deubiquitinating enzymes of *Schizosaccharomyces pombe*

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A dissertation submitted for the partial fulfilment of
MS Degree in Science

Under the Supervision of
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CERTIFICATE OF EXAMINATION

This is to certify that the dissertation titled "Processing of Sde2 from different eukaryotes by deubiquitinating enzymes of *Schizosaccharomyces pombe*" submitted by Mr. Anuraag Ghosh (Registration number MP19005) for the partial fulfilment of the MS degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report is accepted.

Dr. Purnanada Guptasarma Dr. Rajesh Ramachandran Dr. Shashi Bhushan Pandit

Dr. Shravan Kumar Mishra
(Supervisor)

Dated:

DECLARATION

The work presented in the dissertation has been carried out by me under the guidance of Dr. Shravan Kumar Mishra at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or Institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me, and all sources listed within have been detailed in the bibliography.

Anuraag Ghosh

Dated:

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr Shravan Kumar Mishra
(Supervisor)

Acknowledgment

I want to take this opportunity to thank my M.S. thesis supervisor Dr. Shravan Kumar Mishra, for being such an incredible mentor and for nurturing and developing my scientific skills. I feel incredibly fortunate to have had the opportunity to acquire training under his supervision. It was an absolute pleasure to discuss biology with him at length during the course of my journey in the UBL lab, and I will definitely miss our extensive and fruitful scientific discussions once I am gone. Additionally, I would also like to express my most profound sense of gratitude to Dr. Shravan Kumar Mishra for being a constant source of emotional and mental support to all members of the lab during the testing times of the COVID-19 pandemic and for taking care of each one of us and for keeping us sane amidst such turbulent times.

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I would also like to thank Shristi and Pronoy (My two best friends) for constantly encouraging and motivating me to push my limits and to strive for better. You guys are fantastic. Thank you so much for being there.

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Last but not least, I would like to take this opportunity to acknowledge the efforts and contributions of my parents in helping me reach where I am today. Without their continuous backing and support, I would not have come so far. Thank you so much for everything you have done for me over the years. I love you both.

This thesis is dedicated to my late grandfather, Rabindra Nath Ghosh.

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Notations

Sde2-UBL	N-terminal domain of Sde2
Sde2-C	C-terminal domain of Sde2
DUBs	Deubiquitinating enzymes
Sp	*Schizosaccharomyces pombe*
Sj	*Schizosaccharomyces japonicus*
At	*Arabidopsis thaliana*
Ce	*Caenorhabditis elegans*
Dm	*Drosophila melanogaster*
Dr	*Danio rerio*
Hs	*Homo sapiens*
Sc	*Saccharomyces cerevisiae*

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Chapter 1: Profiling DUB activity against various Sde2 chimeras

1.1Introduction

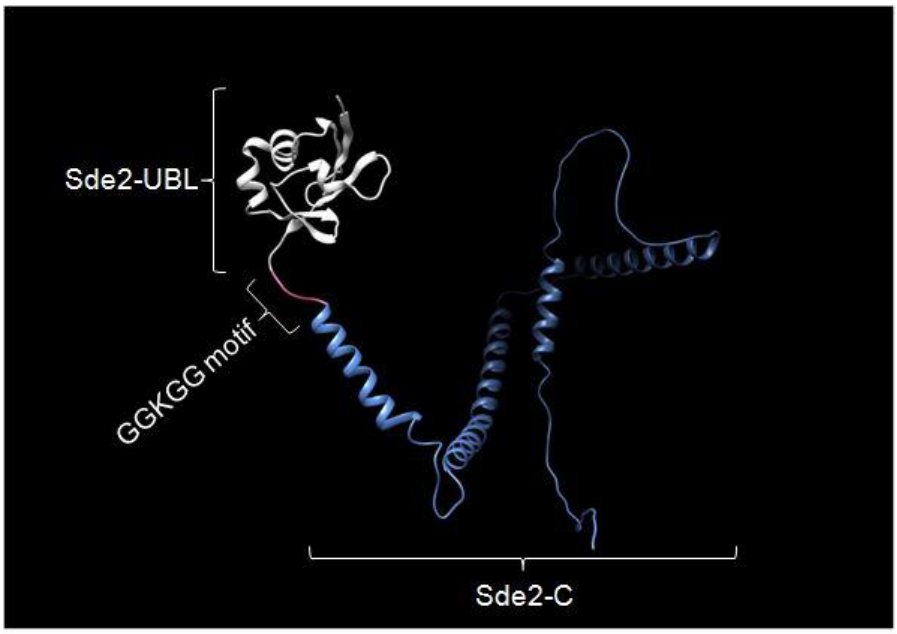

Figure 1.1 Predicted structure of *S. pombe* Sde2

The protein I have been working with is Silencing defective 2 (Sde2). It was first identified in fission yeast and was indicated to be involved in genome stability and telomere silencing(Sugioka-Sugiyama and Sugiyama, 2011). The inactive precursor form of the protein has an N-terminal ubiquitin-like fold domain that attains a β-grasp fold called the Sde2-UBL followed by an invariant GGKGG motif and a loosely folded Helical-carboxyl terminal domain called Sde2-C (Figure 1.1). The putative orthologs of Sde2 are present in all eukaryotes from fission yeast to humans; however, their orthologs are notably absent in eukaryotic organisms like *Saccharomyces cerevisiae, Candida albicans, and Pichia pistoris.* As the name suggests, the N-terminal Sde2-UBL or ubiquitin-like fold domain in Sde2 structurally resembles a small, 76 amino acid-long protein called ubiquitin. On comparing the sequence and structure of these two molecules, it was found that despite sharing very low sequence similarity, the two molecules share good structural similarity(Figure 1.2). Furthermore, upon performing a multiple sequence alignment with the Sde2-UBL sequences from various eukaryotic organisms, it was found that the sequences are very poorly conserved across eukaryotes, unlike ubiquitin which is highly conserved across the eukaryotic taxa.

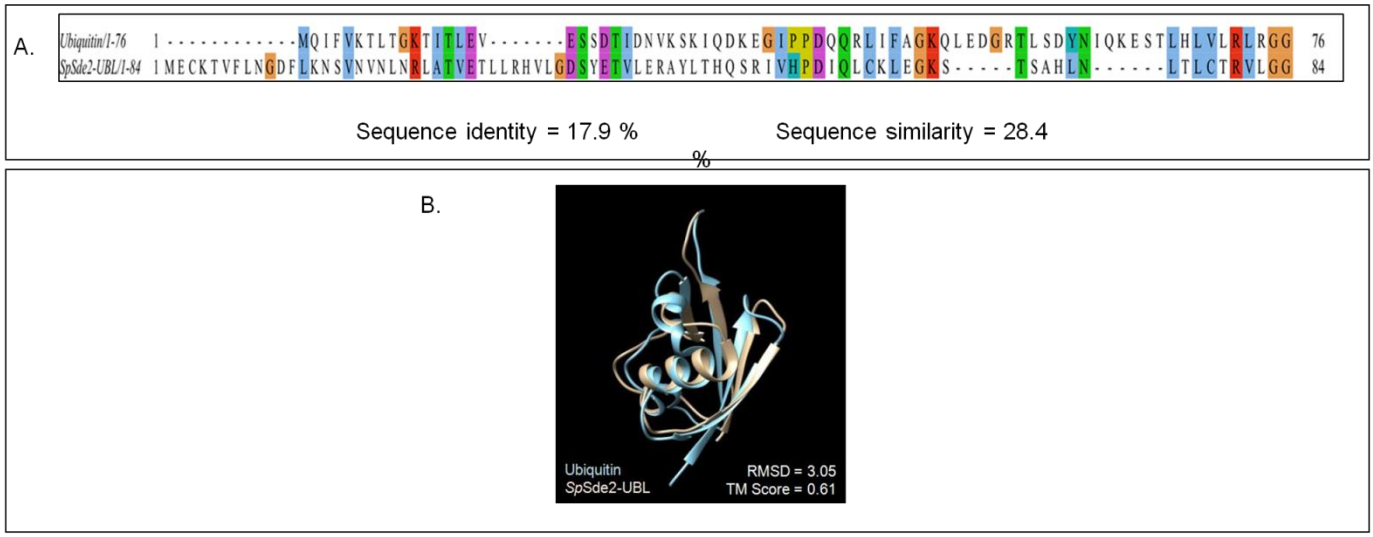

Figure 1.2: Sequence and structure comparison of Ubiquitin and *Sp*Sde2-UBL

A. Sequence alignment of Ubiquitin and *Sp*Sde2-UBL

B. Structural alignment of Ubiquitin and *Sp*Sde2-UBL

The precursor form of Sde2 in *S.pombe* has been reported to be cleaved after the first diglycine motif by two deubiquitinating enzymes or DUBs, namely Ubp5 and Ubp15, to generate the free Sde2-UBL and the functionally active Sde2-C form which can then associate with the spliceosome to carry out intron specific splicing of select genes(Thakran et al., 2017)(Figure 1.3).

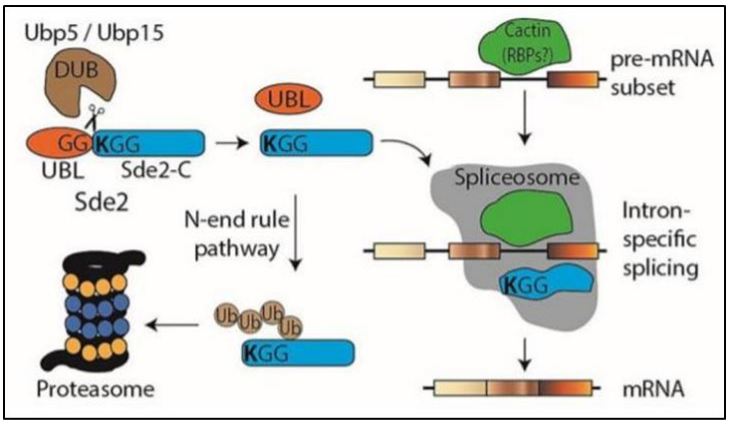

Figure 1.3: Schematics of Sde2 function and regulation.
{Figure source: (Thakran et al., 2017)}

Deubiquitinating enzymes are a critical group of proteases that regulate the process of ubiquitination by removing ubiquitin molecules from substrate proteins by hydrolyzing the isopeptide bond formed between ubiquitin and its targets (reversing the function of E3 ligases). In addition, DUBs are also intricately involved in fine-tuning the extent or nature of ubiquitination of substrate proteins and therefore play a crucial role in regulating the function of these

targeted proteins and determining their fates. In contrast to mammalian cells, which have nearly 100 DUBs(Nijman et al., 2005), fission yeast has been reported to have only 20 DUBs which belong to one of these four subgroups-USP, UCH, OTU, and JAMM(Kouranti et al., 2010).

Ubiquitin is synthesized either as a precursor fused with a ribosomal protein or as polyubiquitin. Like Sde2, processing of the ubiquitin precursor is also necessary for the activation of the fused ribosomal protein and ubiquitin. Free ubiquitin moieties and functionally active ribosomal proteins are generated through a process dependent on the DUB-mediated cleavage of these precursor molecules and is quite similar to the DUB-mediated processing that precursor Sde2 molecules go through.
The similarity between Sde2-UBLs and ubiquitin in terms of their structural architecture and their DUB-dependent processing to generate the functionally active forms of the protein intrigued us to study and decipher the evolutionary relationship between the two molecules. In this study, we aim to gain deeper insights into this evolutionary relationship between ubiquitin and Sde2-UBL as we try to determine the region in the ubiquitin fold that might be responsible for providing specificity to the DUBs towards ubiquitin or Sde2-UBL.

1.2 Materials and Methods:

Complementation assay

Fivefold serial dilutions of cells were spotted on SC-Leu and Emm-Leu plates. The plates were kept at 25°C, 30 °C and 37 °C and the cells were allowed to grow for 3-4 days. The constructs were expressed either under the nmt81 promoter (thiamine repressible promoter) or the pENO promoter (overexpression promoter) depending upon the aim of the experiment.

Protein expression in *S. pombe*

Freshly transformed cells were inoculated in SC-Leu media and allowed to grow for 24 hours at 30 °C. A secondary culture was inoculated with 2% primary culture and incubated at 30 °C. 1.5 OD_{600} of logarithmically growing cells were harvested and stored at -80 °C after flash freezing in liquid nitrogen.

Co-transformation in *E. coli*

The plasmids with Sde2 chimeras were transformed in chemical competent *E. coli* BL21 cells. New chemical competent cells were prepared using a colony from the transformed cells. These Sde2 chimera-containing competent cells were then transformed with the DUB containing plasmids.

Protein expression in *E. Coli*

Freshly co-transformed cells were inoculated in LB media with appropriate antibiotics and allowed to grow overnight at 37 °C. Thereafter, a secondary culture was inoculated with 2% primary culture and allowed to grow at 37 °C for

2.5 hours. IPTG was added to the culture at a final concentration of 100 µM and the cells were incubated at 18 °C for 16 hours. 0.5 OD_{600} cells were harvested and stored at -80 °C after flash freezing in liquid nitrogen.

Western Blot

S. pombe cells were lysed using TCA lysis method and *E. coli* BL21 cells were lysed using B-PER lysis method. The cell lysates were loaded on SDS-PAGE. Proteins were separated on the gel, and transferred to PVDF membrane using semi-dry transfer assembly. The PVDF membrane was then probed for Sde2-C using either anti-FLAG for yeast lysate, or anti-Sde2-C for bacterial lysate. The DUBs in the bacterial lysate were probed with anti-His antibody. Thereafter, appropriate secondary antibodies (anti-mouse/anti-rabbit) were used to probe specifically for the protein of our interest.

Bioinformatics

The structure of Sde2 was obtained from the AlphaFold database(Jumper et al., 2021). UCSF Chimera was used to perform structural alignment of ubiquitin and *Sp*Sde2-UBL. It was also used to visualize protein 3D structures(Pettersen et al., 2004). JalView was used for performing pair-wise and multiple sequence alignment (Waterhouse et al., 2009). JalView was also used to view the protein sequence alignment.

1.3 Hypothesis:

Given the facts that the same machinery of DUBs can process both ubiquitin as well as Sde2-UBLs, and the ubiquitin sequence is highly conserved in eukaryotes as opposed to Sde2-UBL which is poorly conserved, a former lab colleague (Bhargesh Patel) along with Dr. Shravan Kumar Mishra hypothesized that Sde2-UBL sequences emerged from, and were the same as ubiquitin at some point of time. The idea was that these proto-Sde2-UBLs in the past were processed by many DUBs, just like ubiquitin. But due to the sheer redundancy in the number of DUBs and ubiquitin genes, the proto-Sde2-UBLs diverged from ubiquitin and evolved freely as long as at least one of the DUBs was able to process it (Fig. 1.4)

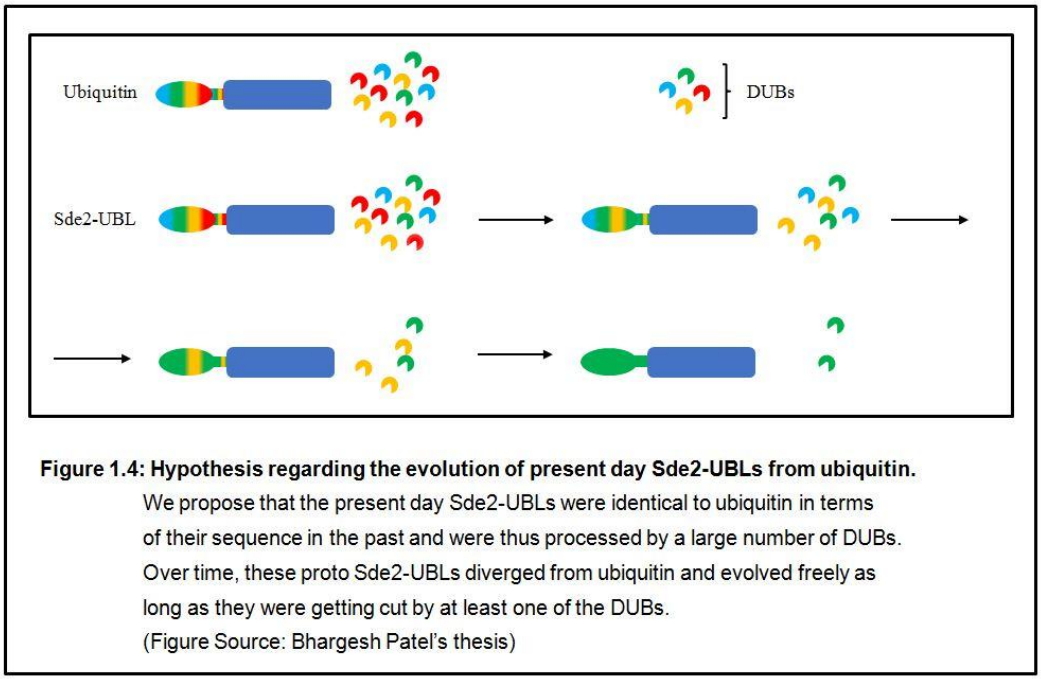

Figure 1.4: Hypothesis regarding the evolution of present day Sde2-UBLs from ubiquitin. We propose that the present day Sde2-UBLs were identical to ubiquitin in terms of their sequence in the past and were thus processed by a large number of DUBs. Over time, these proto Sde2-UBLs diverged from ubiquitin and evolved freely as long as they were getting cut by at least one of the DUBs. (Figure Source: Bhargesh Patel's thesis)

1.4 Results

1.4.1 Processing of distinct Sde2-UBLs from a variety of eukaryotes in *S.pombe.*

In order to obtain distinct Sde2-UBL sequences, we identified six different organisms – *Schizosaccharomyces japonicus (Sj), Arabidopsis thaliana (At), Caenorhabditis elegans (Ce), Drosophila melanogaster (Dm), Danio rerio (Dr), and Homo sapiens (Hs)* whose Sde2-UBL sequences showed low homology to ubiquitin as well as S. pombe Sde2-UBL (Fig.1.5). We fused these six Sde2-UBL sequences with Sde2-C from *S. pombe (Sp)* to negate any artefacts from variable C-termini.

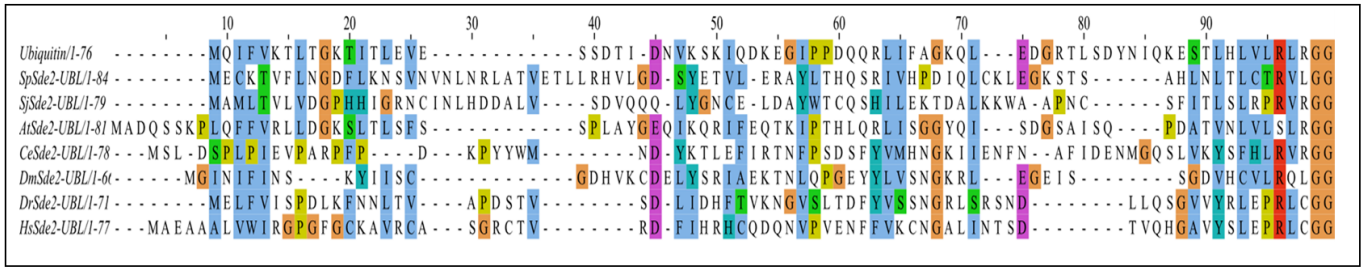

Figure 1.5: Multiple sequence alignment of Sde2-UBL's from a variety of eukaryotes.

Sp - Schizosaccharomyces pombe, Sj - Schizosaccharomyces japonicus, At- Arabidopsis thaliana, Ce - Caenorhabditis elegans, Dm - Drosophila melanogaster, Dr - Danio rerio, and Hs - Homo sapiens

Following this, a PhD student in the lab, Rakesh performed complementation of the *S. pombe* Δsde2 strain using these chimeric Sde2s. We found that only the *At*Sde2$_{UBL}$~Sde2-C chimera was able to fully complement the phenotype of Δsde2. Moreover, we also observed that the *Sj*Sde2$_{UBL}$~Sde2-C and *Ce*Sde2$_{UBL}$~Sde2-C chimeras complemented the phenotype only partially whereas the *Dr*Sde2$_{UBL}$~Sde2-C and *Hs*Sde2$_{UBL}$~Sde2-C chimeras almost completely did not complement the phenotype of Δsde2 (Fig. 1.6A). We tried to check the processing of the chimeras by western blotting. Since the constructs were tagged with a C-terminal FLAG-tag, a simple anti-FLAG blot revealed the processing. We found that there was a fair amount of correspondence in the processivity of the Sde2 chimeras to the Δsde2 complementation results. The Sde2-UBLs from *S. japonicus, A. thaliana*, and *C. elegans* got strongly processed, whereas the Sde2-UBLs from *D. melanogaster, D. rerio*, and *H. sapiens* got very weakly processed (such that the processed form was detectable only at high exposures) in *S. pombe* (Fig. 1.6B).

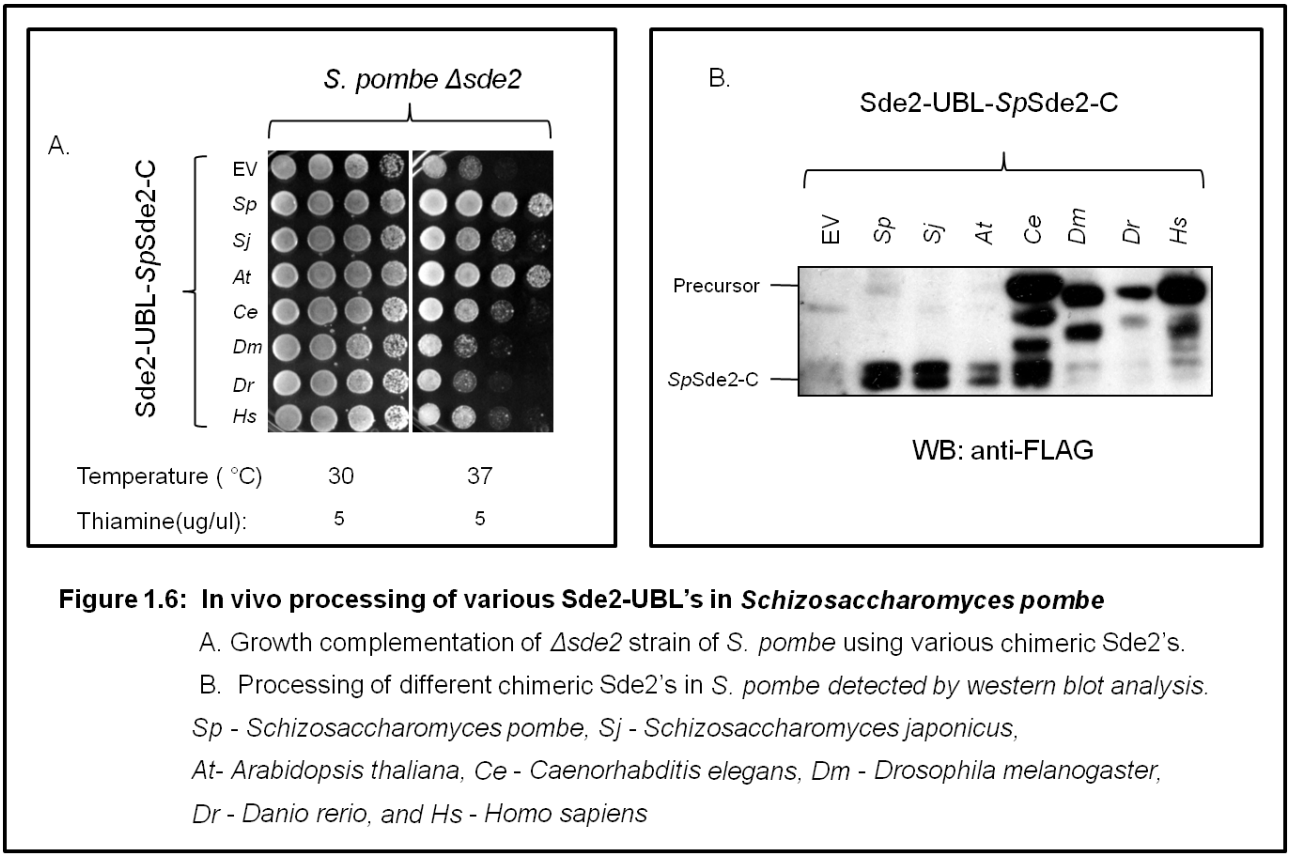

Figure 1.6: In vivo processing of various Sde2-UBL's in *Schizosaccharomyces pombe*

A. Growth complementation of *Δsde2* strain of *S. pombe* using various chimeric Sde2's.

B. Processing of different chimeric Sde2's in *S. pombe detected by western blot analysis.*

Sp - Schizosaccharomyces pombe, Sj - Schizosaccharomyces japonicus,

At- Arabidopsis thaliana, Ce - Caenorhabditis elegans, Dm - Drosophila melanogaster,

Dr - Danio rerio, and Hs - Homo sapiens

1.4.2 Identifying the *S. pombe* DUBs that process each of these Sde2 chimeras

The aim of the study was to find out the individual *S. pombe* DUBs that process each of the Sde2 chimeras. Since there are a large number of DUBs in *S. pombe* (20), it becomes challenging to use an in vivo system for this purpose. So, we used an in vitro expression system to overcome this obstacle. I added further clones to the *S. pombe* DUB library created by Bhargesh in a bacterial expression vector (pCDFDuet-1) to make the library more complete and I used the various Sde2 chimeras that he had cloned in a separate, compatible bacterial expression vector(pET28a) for my experiments. Following this, I first ran a control western blot each for the entire set of the 20 DUBs and the eight Sde2 chimeric constructs along with appropriate controls to check and confirm that each of these clones were correct and were expressing the respective proteins properly. Upon doing so, we found that all the eight Sde2 chimeras were fine and their unprocessed forms could be detected through western blot (Figure. 1.7).

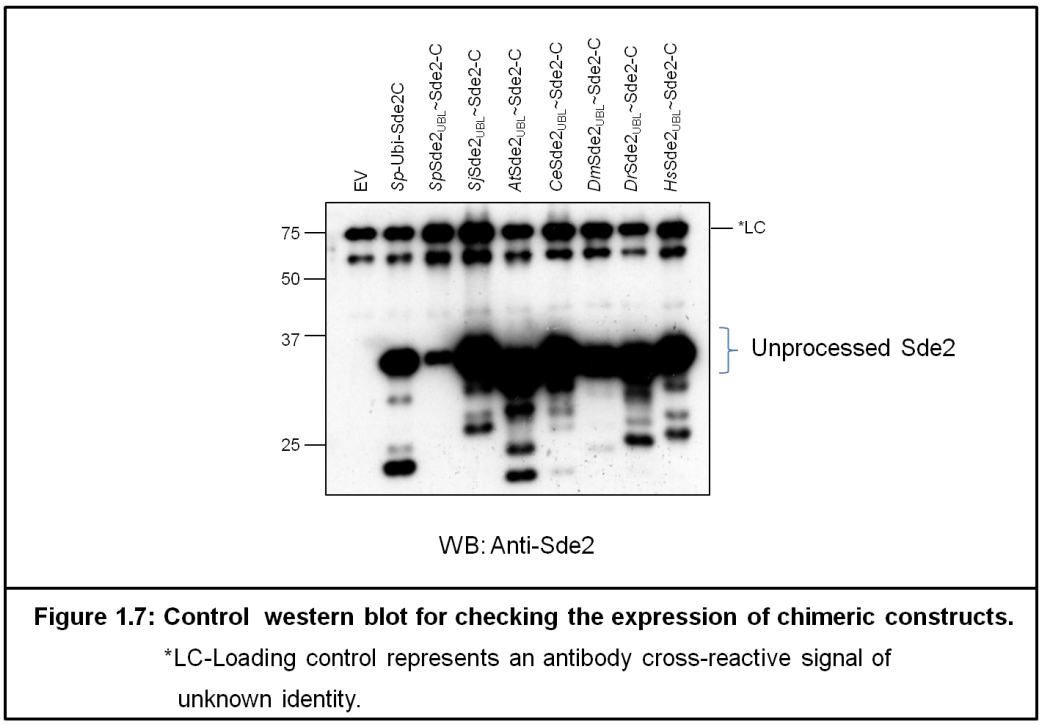

Figure 1.7: Control western blot for checking the expression of chimeric constructs. *LC-Loading control represents an antibody cross-reactive signal of unknown identity.

As far as the DUBs were concerned, expression for 17 out of the 20 DUBs could be detected through the control western blot but for three of the DUBs (ubp1, ubp7 and ubp9) no expression could be detected (Figure. 1.8).

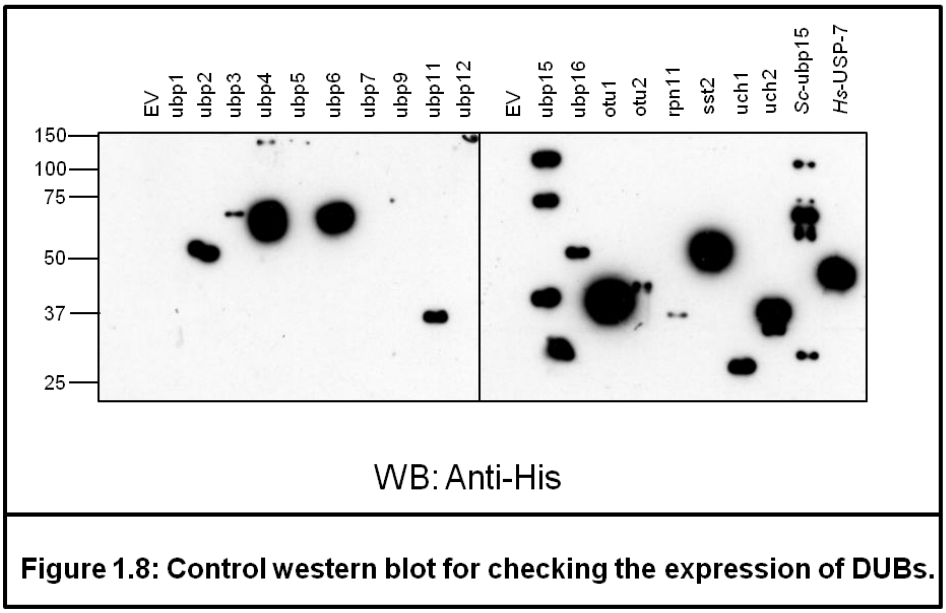

Figure 1.8: Control western blot for checking the expression of DUBs.

However, we later confirmed through sequencing that the ubp1, ubp7 and ubp9 clones were correct. Moreover, we also found that all three clones, namely, ubp1, ubp7 and ubp9 when co-expressed with *Sp*-Ubi-Sde2C chimeric construct were able to process the given chimera. All of these evidences hinted towards the fact that both the DUB

clones were correct and therefore, we included them in our analysis. The reason for not detecting the proteins on the control western blot could be that these proteins are extremely unstable in the heterologus *E.coli* system and therefore, their detection through western blot becomes difficult.

Once the correctness of the clones and their expression levels were checked, I co-expressed each of the chimeras with every DUB in a one-to-one fashion, in *E. coli*. Processing of the chimeras was checked by western blot (Fig. 1.9).

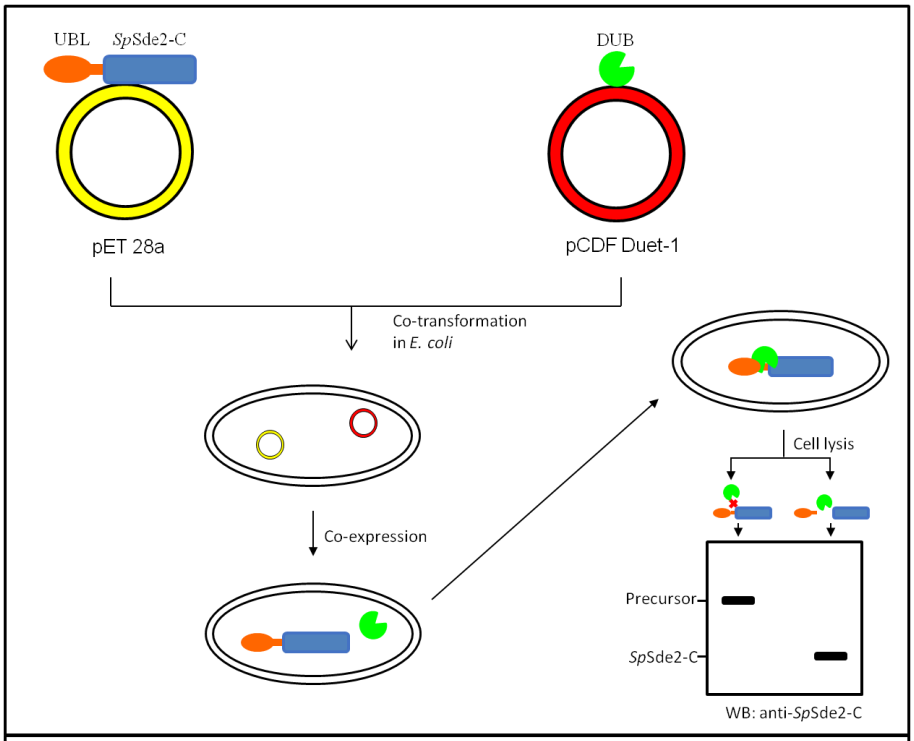

Figure 1.9: Workflow of the experiment.

Two *S. pombe* DUBs, namely, ubp14 and ubp8 were excluded from this screen since there were some difficulties in cloning it and the clone could not be obtained in time. Two additional DUBs - USP7 from *Homo sapiens* (referred hereafter as *Hs*USP7) and ubp15 from *Saccharomyces cerevisiae* (referred hereafter as *Sc*ubp15) were also included for our analysis since they are close paralogs of the *S. pombe* DUBs ubp5 and ubp15, which process *S. pombe* Sde2. We found that the chimeras containing Sde2-UBL's from *D. melanogaster* and *H. sapiens* did not get processed by any of the 20 DUBs in vitro. The Sde2-UBL from *C. elegans* got processed by the greatest number of DUBs (6) whereas Sde2-UBLs from *S. pombe* and *A. thaliana* got processed by the least number of DUBs (2 each). The chimeras containing Sde2-UBL's from *S. japonicus* and *D. rerio* got processed by four and three DUBs respectively. Three DUBs - Rpn11, Otu2, and Ubp9 did not show processivity towards any of the chimeras, whereas all the other DUBs process some or the other chimera (Fig. 1.10).

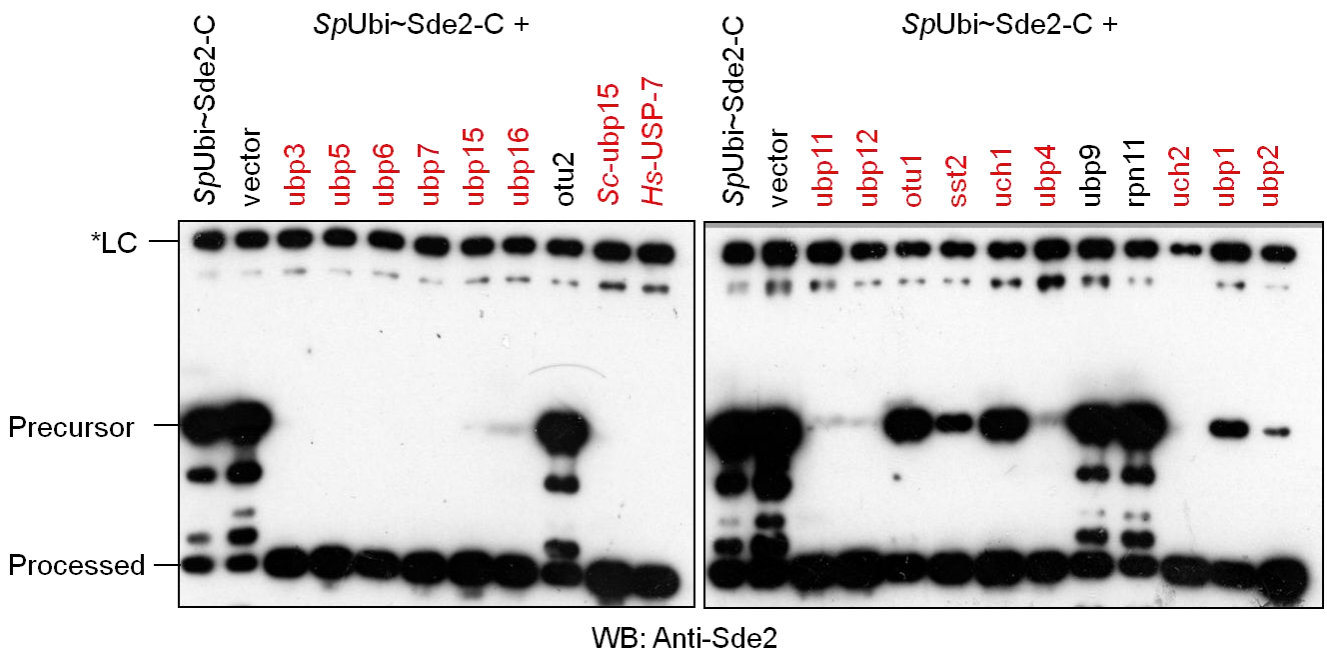

Figure 1.10.1: Processing of *Sp*Ubi~Sde2-C by *S.pombe* DUB'S.

The lanes showing a complete disappearance/reduction in the levels of the precursor form of the protein along with a concomitant increase in the amounts of the processed form of the protein were considered to be processed by the respective *S.pombe* DUB's and are indicated with red colour.

Abbreviations used here are same as indicated in Fig 1.6

*LC-Loading control represents an antibody cross-reactive signal of unknown identity.

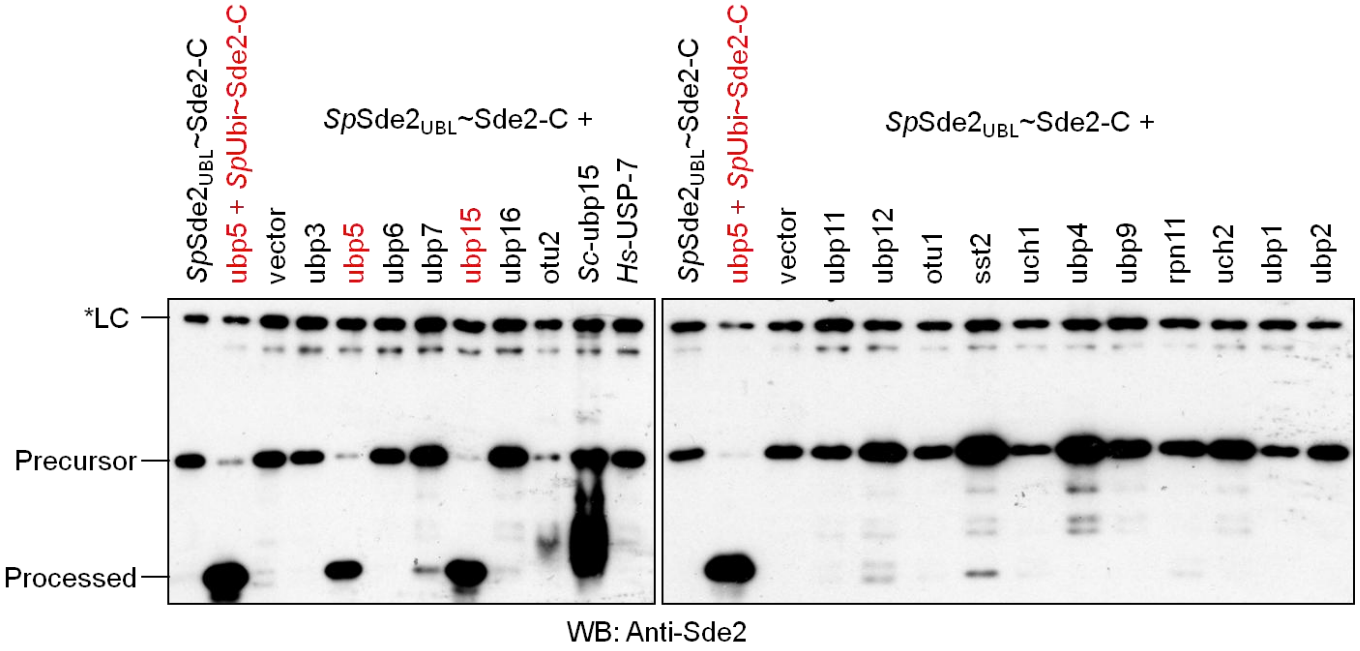

Figure 1.10.2: Processing of by *Sp*Sde2$_{UBL}$~Sde2-C *S.pombe* DUB'S.

Data analysis was carried out in a manner identical to figure 1.10.1.

ubp5 and ubp15 were found to process the *Sp*Sde2$_{UBL}$~Sde2-C chimera.

Abbreviations used here are same as indicated in Fig 1.6

*LC-Loading control represents an antibody cross-reactive signal of unknown identity.

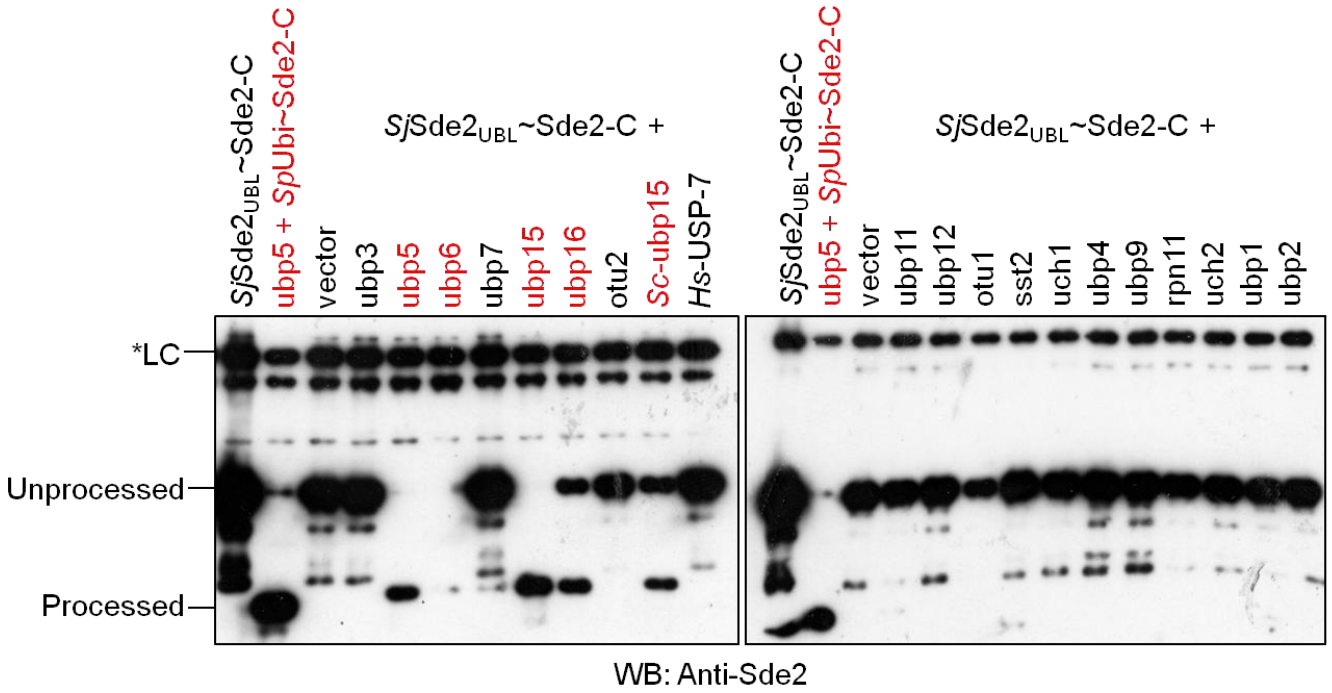

Figure 1.10.3: Processing of *Sj*Sde2$_{UBL}$~Sde2-C by *S.pombe* DUB'S.

Data analysis was carried out in a manner identical to figure 1.10.1.

ubp5, ubp6, ubp15, ubp16 and *Sc*-ubp15 were found to process the *Sj*Sde2$_{UBL}$~Sde2-C chimera.

Abbreviations used here are same as indicated in Fig 1.6

*LC-Loading control represents an antibody cross-reactive signal of unknown identity.

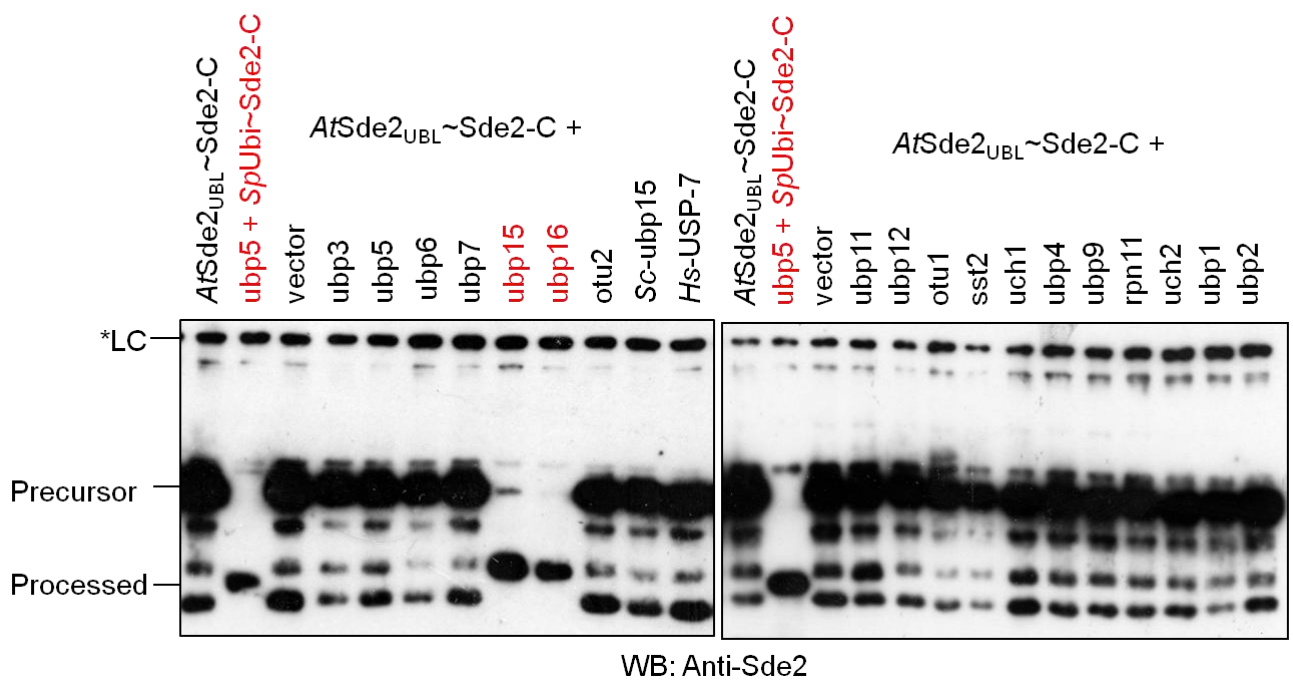

Figure 1.10.4: Processing of *At*Sde2$_{UBL}$~Sde2-C by *S.pombe* DUB'S.

Data analysis was carried out in a manner identical to figure 1.10.1.

ubp15 and ubp16 were found to process the *At*Sde2$_{UBL}$~Sde2-C chimera.

Abbreviations used here are same as indicated in Fig 1.6

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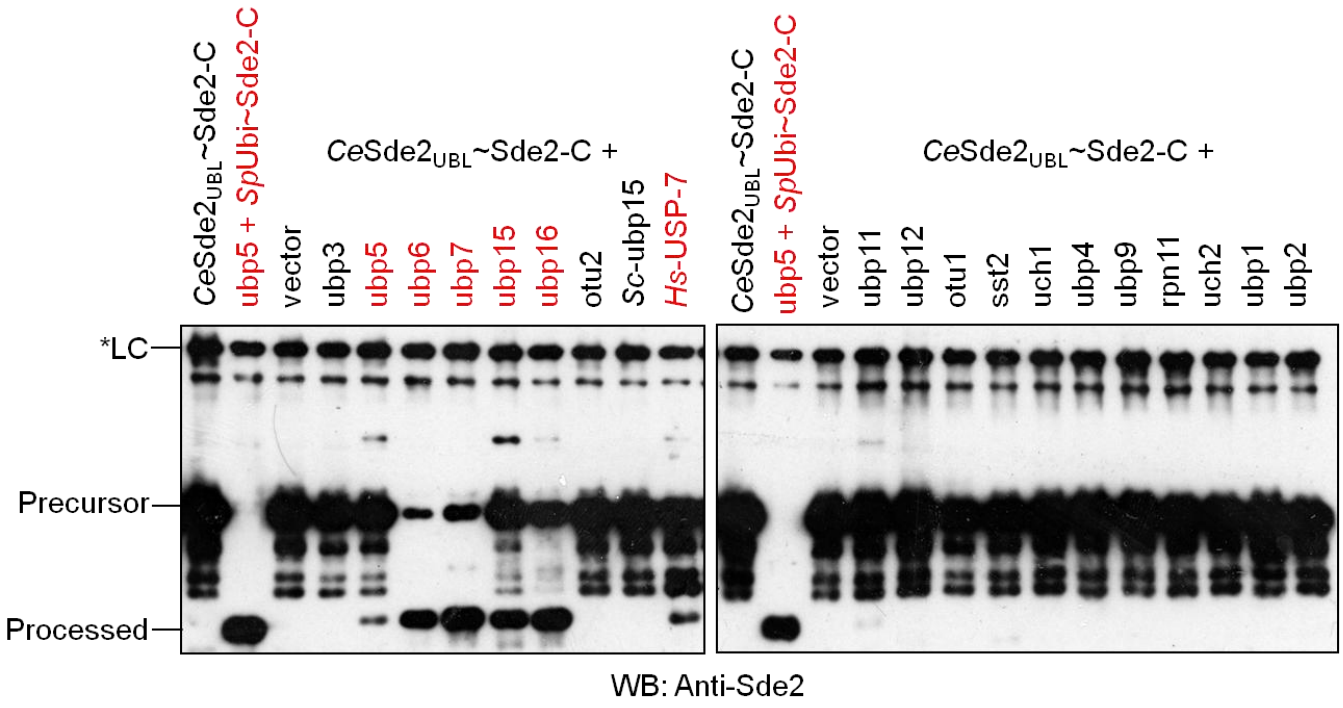

Figure 1.10.5: Processing of *Ce*Sde2$_{UBL}$~Sde2-C by *S.pombe* DUB'S.

Data analysis was carried out in a manner identical to figure 1.10.1.

ubp5, ubp6, ubp7, ubp15, ubp16 and *Hs*-USP-7 were found to process the *Ce*Sde2$_{UBL}$~Sde2-C chimera.

Abbreviations used here are same as indicated in Fig 1.6

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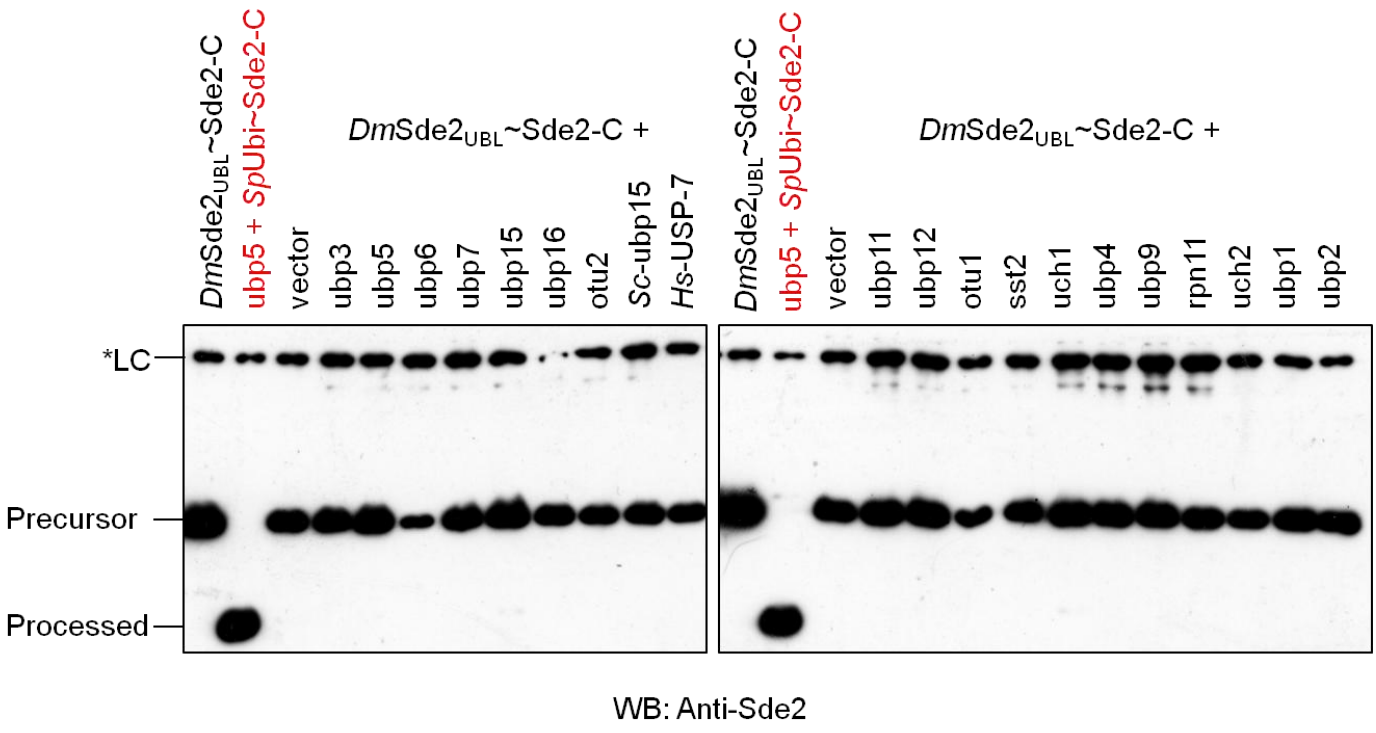

Figure 1.10.6: Processing of *Dm*Sde2$_{UBL}$~Sde2-C by *S.pombe* DUB'S.

Data analysis was carried out in a manner identical to figure 1.10.1.

None of the 20 *S.pombe* DUB's were found to process the *Dm*Sde2$_{UBL}$~Sde2-C chimera.

Abbreviations used here are same as indicated in Fig 1.6

*LC-Loading control represents an antibody cross-reactive signal of unknown identity.

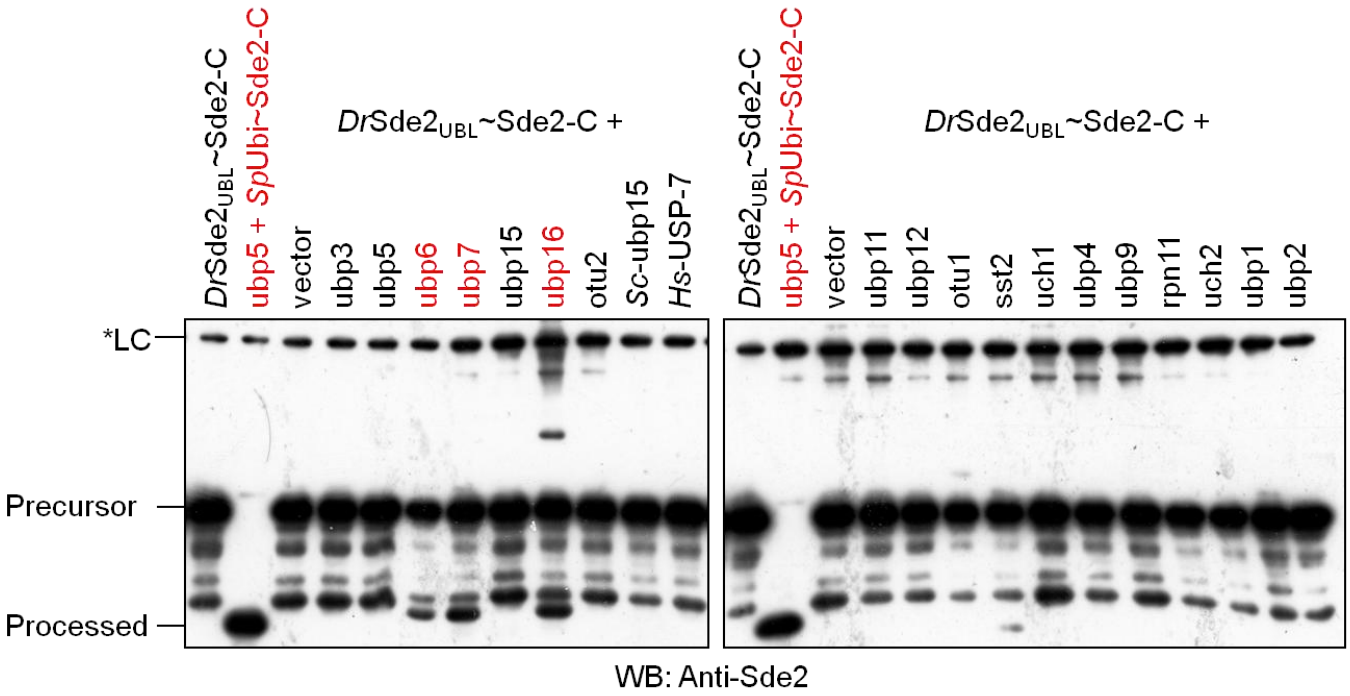

Figure 1.10.7: Processing of by *Dr*Sde2$_{UBL}$~Sde2-C *S.pombe* DUB'S.

Data analysis was carried out in a manner identical to figure 1.10.1.

ubp6, ubp7 and ubp16 were found to process the *Dr*Sde2$_{UBL}$~Sde2-C chimera.

Abbreviations used here are same as indicated in Fig 1.6

*LC-Loading control represents an antibody cross-reactive signal of unknown identity.

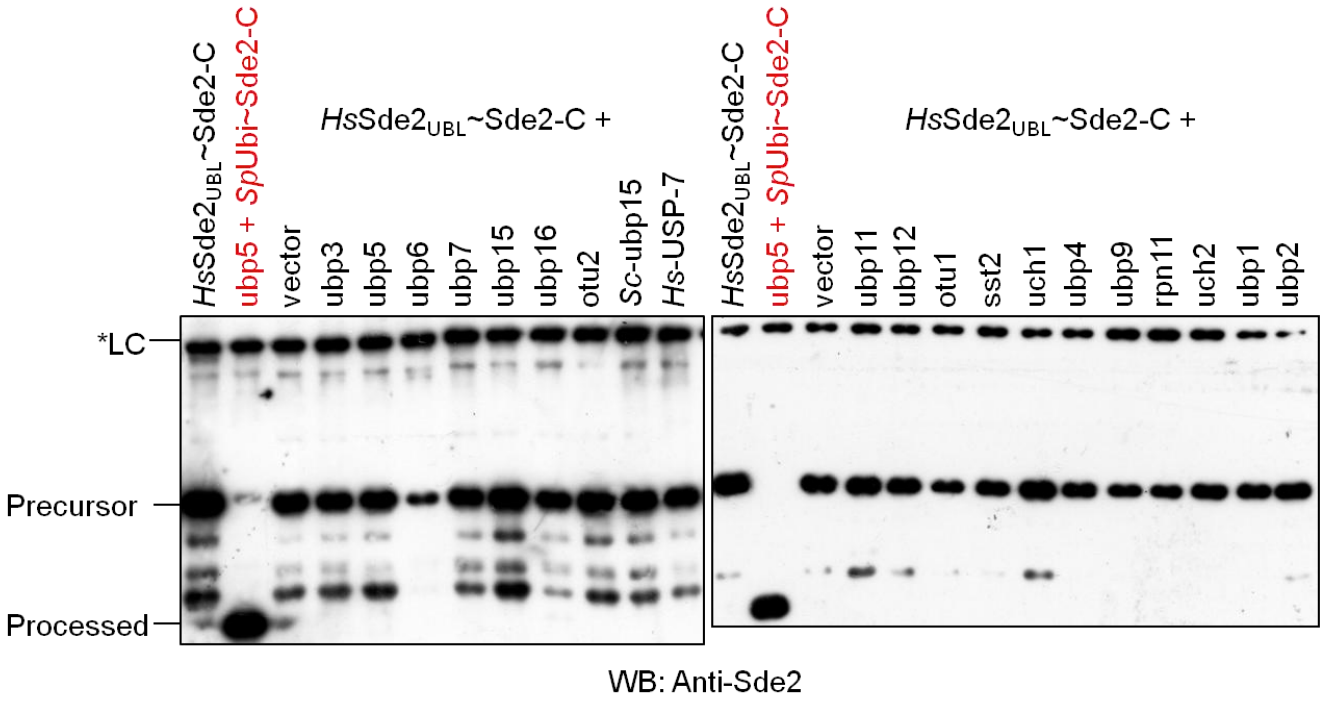

Figure 1.10.8: Processing of *Hs*Sde2$_{UBL}$~Sde2-C by *S.pombe* DUB'S.

Data analysis was carried out in a manner identical to figure 1.10.1.

None of the 20 *S.pombe* DUB's were found to process the *Hs*Sde2$_{UBL}$~Sde2-C chimera.

Abbreviations used here are same as indicated in Fig 1.6

*LC-Loading control represents an antibody cross-reactive signal of unknown identity.

The DUBs' processivity results have been summarized in Table 1.1. Most interestingly, we did not observe any correlation between the sequence similarity of the Sde2-UBLs with ubiquitin and the number of DUBs that are able to process them, as we were expecting from the predictions of our hypothesis. The complete table which contains data from all the controls in addition to the test samples is appended to this thesis.

	***Sp* Ubi-Sde2C**	***Sp* UBL-Sde2C**	***Sj* UBL-Sde2C**	***At* UBL-Sde2C**	***Ce* UBL-Sde2C**	***Dm* UBL-Sde2C**	***Dr* UBL-Sde2C**	***Hs* UBL-Sde2C**
SDE2-UBL's % similarity to ubiquitin	100%	28.40%	34.80%	54.10%	31.90%	45%	33.30%	14.20%
***Sp* Ubi-Sde2C+*Sp* ubp5**	1	1	1	1	1	1	1	1
***Sp* ubp3**	0.40	-0.01	-0.09	-0.10	-0.02	0.00	0.00	-0.03
***Sp* ubp5**	1	1.19	1.59	0.04	0.07	0.00	0.00	-0.05
***Sp* ubp6**	14.31	-0.01	0.08	-0.14	0.71	0.00	0.06	-0.05
***Sp* ubp7**	5.05	0.02	-0.04	0.00	0.72	0.00	0.09	-0.05
***Sp* ubp15**	2.35	1.17	2.06	3.90	0.39	0.00	0.00	-0.05
***Sp* ubp16**	0.08	0.00	0.48	6.52	0.43	0.00	0.08	-0.05
***Sp* otu2**	0.00	0.04	-0.06	0.03	-0.01	0.00	0.00	-0.05
***Sc* ubp15**	0.15	0.01	0.20	-0.12	-0.01	0.00	0.00	-0.05
***Hs* USP7**	17.55	0.00	-0.07	0.00	0.13	0.00	0.00	-0.05
***Sp* Ubi-Sde2C+*Sp* ubp5**	1	1	1	1	1	1	1	1
***Sp* ubp11**	0.65	0.00	0.00	0.04	0.00	0.00	0.00	0.00
***Sp* ubp12**	0.85	0.00	-0.01	-0.16	-0.01	0.00	0.00	0.00
***Sp* otu1**	0.02	0.00	-0.01	-0.22	-0.01	0.00	0.00	0.00
***Sp* sst2**	0.07	0.00	0.00	-0.22	-0.01	0.00	0.01	0.00
***Sp* uch1**	0.03	0.00	-0.01	-0.03	-0.01	0.00	0.00	0.00
***Sp* ubp4**	0.13	0.00	-0.01	-0.09	-0.01	0.00	0.00	0.00
***Sp* ubp9**	0.00	0.00	-0.01	-0.10	-0.01	0.00	0.00	0.00
***Sp* rpn11**	-0.02	0.00	-0.02	-0.10	-0.01	0.00	0.01	0.00
***Sp* uch2**	0.32	0.00	-0.01	-0.08	-0.01	0.00	0.00	0.00
***Sp* ubp1**	0.11	0.00	0.00	-0.13	-0.01	0.00	0.00	0.00
***Sp* ubp2**	0.94	0.00	-0.01	-0.06	-0.01	0.00	0.00	0.00
No. of *S. pombe* DUB's that process the chimera	17	2	4	2	6	0	3	0

Table 1.1: Summary of the in vitro processing of the Sde2-UBL's by DUBs.

The quality/extent of processivity of each of these chimeric constructs by different DUBs has been calculated using the ImageJ software by comparing the signal intensities of the processed forms of the protein to the precursor forms and by normalising the obtained values with respect to the positive control (*Sp* Ubi-Sde2C+*Sp* ubp5). The formula used for acquiring the values is mentioned below –

$$\frac{(\text{Processed/Precursor})_{\text{Test}} - (\text{Processed/Precursor})_{\text{Negative Control}}}{(\text{Processed/Precursor})_{\text{Positive Control}} - (\text{Processed/Precursor})_{\text{Negative Control}}}$$

where, Test = Sde2-UBL containing chimeric constructs co-expressed with various DUBs.
Negative Control = Sde2-UBL containing chimeric construct expressed without any DUB.
Positive Control = *Sp* Ubi-Sde2C co-expressed with *Sp* ubp5.

-The table provides both the absolute numerical values from these calculations and a graphical representation of the data in the form of Data Bars.
-The table also provides information regarding the percentage sequence similarity each of these Sde2-UBL's share with ubiquitin and the number of DUBs that were able to process them in vitro.
-Different Sde2-UBL containing chimeras have been represented with different colour shades.
-Abbreviations used here are same as indicated in Fig 1.6.

1.4.3 Possible explanation for the emergence of Sde2-UBL's from ubiquitin

The study aimed to figure out a possible explanation for the emergence of present-day Sde2-UBLs from ubiquitin. The initial hypothesis that we came up with was that Sde2-UBLs could have diverged away from ubiquitin as long as it could get cut by any one of the DUBs to generate the functionally active form of the protein (Sde2-C), and therefore, they evolved under very low/minimal selection pressure. In addition, the low conservation of Sde2-UBLs across different eukaryotic species could be explained by the fact that all of these organisms evolved under different environmental conditions, thus experiencing varying degrees of selection pressure. Moreover, the emergence of Sde2-UBLs from ubiquitin was not a case of directed evolution but was majorly a random process with minimal selection pressure. Hence, it makes perfect sense for the Sde2-UBL sequences across eukaryotes to lack conservation. However, the arguments presented above do not provide a convincing answer to the incumbent question of 'why Sde2-UBLs diverged out of ubiquitin or what purpose does the emergence of Sde2-UBLs out of ubiquitin serve?' The preliminary data presented in this section hints toward a possible and plausible answer to the question posed above. The complementation data indicates a minor growth defect at 37°C (Boxes marked with red in the figure) but a significant growth defect at 25°C (Boxes marked with blue in the figure) for *ΔSde2* cells transformed with the *Sp*-Ubi-Sde2C chimera as compared to those transformed with *Sp*Sde2$_{UBL}$~Sde2-C. Both the chimeric constructs were overexpressed using a strong promoter – pENO. On the other hand, the western blot data indicates the increased

formation of` ubiquitin conjugates of Sde2-C upon overexpression of *Sp*-Ubi-Sde2C at 37°C as compared to *Sp*Sde2$_{UBL}$~Sde2-C (Figure 1.11). These observations provide meaningful insights and propose 'ubiquitin toxicity in the nucleus' and 'increased ubiquitin conjugate formation of Sde2-C at elevated temperatures upon *Sp*-Ubi-Sde2C overexpression' as probable reasons for the emergence of Sde2-UBLs from ubiquitin through divergence.

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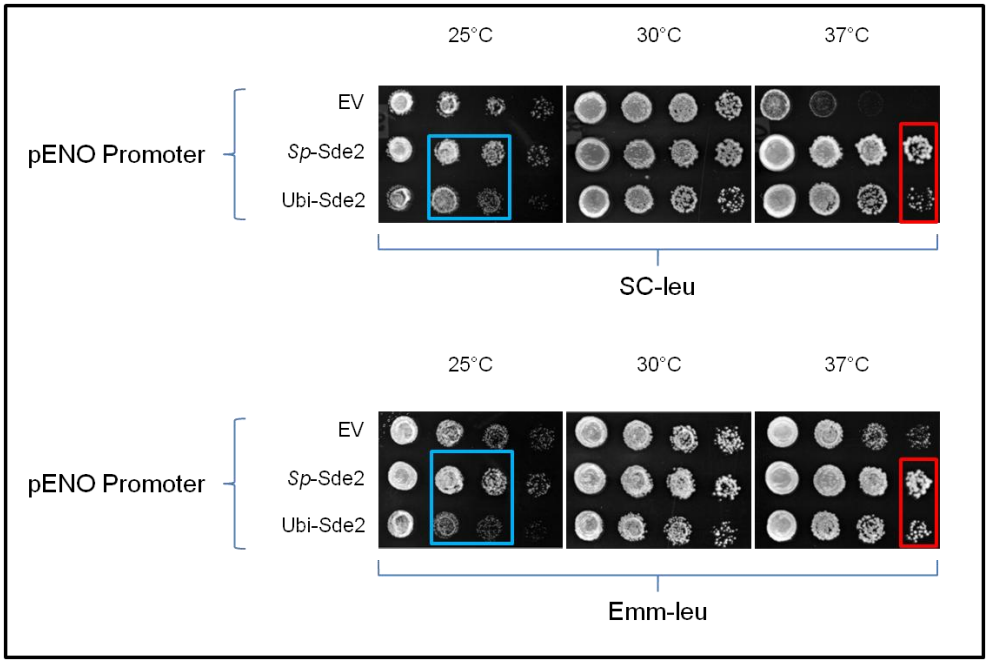

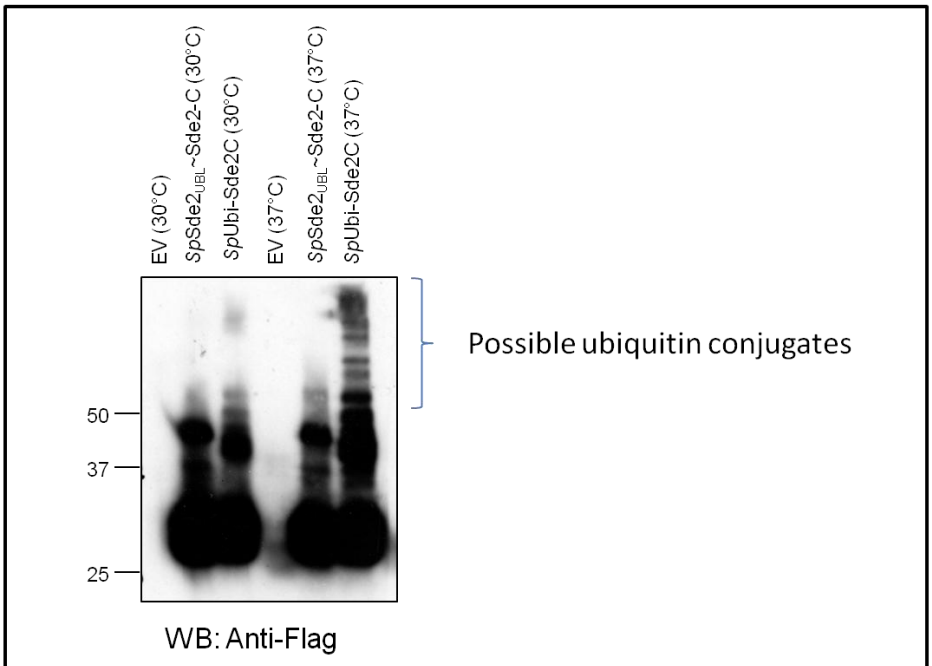

Figure 1.11: Complementation assay and western blot analysis to suggest ubiquitin toxicity in the nucleus and increased ubiquitin conjugate formation of Sde2-C.

A. Growth complementation of *Δsde2* strain of *S.pombe* with *Sp*Sde2$_{UBL}$~Sde2-C and *Sp*Ubi-Sde2C.

B. Processing of *Sp*Sde2$_{UBL}$~Sde2-C and *Sp*Ubi-Sde2C in *S.pombe* detected by western blot analysis.

Abbreviations used here are same as indicated in Fig 1.6

Discussion:

The data obtained from the extensive in-vitro experiment of co-expressing various Sde2 chimeras with each of the 20 DUBs in a one-to-one fashion to identify the subset of DUBs that process each of these chimeric constructs yielded very interesting results. To begin with, we found that only three DUBs – Rpn11, Otu2 and Ubp9 do not process the *Sp* ubiquitin–*Sp*Sde2-C fusion protein that we used as a positive control in our in vitro experiment for checking the processivity of the various Sde2-UBL chimeras by *S. pombe* DUBs. One reason for this could be that these DUBs might be involved in the cleavage of ubiquitin by recognizing the ubiquitin-ubiquitin isopeptide bond (which is formed during polyubiquitination) instead of the peptide bond present in ubiquitin–SpSde2-C. In addition, the lack of

any correlation between the amount of sequence conservation each of the Sde2-UBLs share with ubiquitin and the number of DUBs processing them indicate that overall sequence conservation is not is not an important factor when we talk about DUB-mediated recognition and processivity of these chimeras. Thereafter, upon examining the Sde2-UBL sequences from our experiment more carefully, we realized that the last eight amino acids (Sde2-UBL tails) showed relatively lesser variation as compared to the amino acids comprising the ubiquitin fold. Further, we noticed that among the non-hydrophobic residues present in the Sde2-UBL tails, arginine was the only amino acid that was conserved. The observation that the *C. elegans* and *S. japonicus* Sde2-UBLs gets processed by the greatest number of DUBs (six and four respectively) suggests that the Sde2-UBL tails from these organisms might be the most ideal for processing by DUBs. These ideal tails have either two or three arginines, each alternating with a hydrophobic residue, in the sequence. Finally, Sde2-UBLs from *S. pombe, A. thaliana, D. melanogaster, D. rerio*, and *H. sapiens* have only one arginine in their tails and none of these Sde2-UBLs gets processed strongly by more than 2 DUBs. (Such a trend suggests that presence of alternating arginines in the Sde2-UBL tails might be largely responsible for determining the specificity of the DUBs towards the Sde2-UBL. Presence of more arginines in the Sde2-UBL tails might increase the specificity of the DUBs towards the Sde2-UBL and presence of a smaller number of arginines in the Sde2-UBL tails might permit only the DUBs with a promiscuous catalytic cleft to be active over the Sde2-UBL.

In order to establish the importance of the Sde2-UBL tails in the DUB-mediated recognition and processing of these chimeras, one can make tail specific mutants of these Sde2-UBL chimeras to improve or deteriorate the tail sequence and then repeat the one to one co-expression study to check whether making such tail specific mutations, changes the efficiency of processing of these chimeras by the DUBs. One can also check whether there is an increase or decrease in the number of DUBs that are able to process the chimera post the introduction of the suggested mutations in the tails. These experiments will provide further insights into the mechanism through which the DUB recognizes and processes these Sde2-UBL chimeras and will also shed light on how important the Sde2-UBL tail sequences are in this regard.

Parallely, a colleague in the lab (Aiswarya) is also trying to decipher the contributions of the ubiquitin-like fold of the Sde2-UBL in this process of DUB mediated recognition and processing of the chimeras.

Finally, another interesting idea would be to look at the similarities and differences between the USP domains of the DUBs that have been identified through the in-vitro experiment to process more than two Sde2UBL-chimeras and to uncover critical features/residues that might be playing an important role in facilitating the recognition and processing of the multiple Sde2UBL-chimeras by these DUBs. This would allow us to better understand the features that provide such promiscuity to the specific DUBs.

Sde2-UBL from:	Similarity of the Sde2-UBL to Ubiquitin (in %)	Sde2-UBL tail sequence	No. of DUBs that strongly process the Sde2-UBL
S. japonicus	34.8	L R P R V R G G	4
C. elegans	31.9	F H L R V R G G	4
A. thaliana	54.1	L V L S L R G G	2
S. pombe	28.4	L C T R V L G G	1
D. melanogaster	45	C V L R Q L G G	0
D. rerio	33.3	L E P R L C G G	0
H. sapiens	14.2	L E P R L C G G	0
S. pombe Ubiquitin	-	L V L R L R G G	12

Table 1.2: Correlation between Sde2-UBL tail sequences and processivity by DUBs.

The data presented in section 1.4.3 of this thesis indicates a nuclear toxicity event that becomes prominent at 25°C and 37°C. The nuclear toxicity is most likely caused by the ubiquitin generated from the *Sp*-Ubi-Sde2C chimera since these constructs localize in the nucleus due to the presence of an inherent Nuclear Localization Signal (NLS) in the Sde2-C. The nuclear toxicity due to ubiquitin overexpression at elevated temperatures (37°C) can be explained by the fact that upon overexpression of ubiquitin in the nucleus, the levels of ubiquitin conjugated Sde2-C increases, thus handing such cells a growth disadvantage at such elevated temperatures. What remains to be seen is whether the increased ubiquitin conjugates observed for Sde2-C at 37°C occurs through a novel self-ubiquitinating process or does it adopt the regular mechanism of ubiquitination which is already reported in literature. The plasmids we have used in this study also have an N-terminal myc tag apart from the C-terminal FLAG tag which has been used to detect Sde2 in our experiments. To preliminarily understand whether the ubiquitin generated by the processing of *Sp*-Ubi-Sde2C itself initiates the ubiquitination of the activated Sde2-C or not, one can pull down Sde2-C using anti-FLAG antibodies and then probe for the myc-tagged ubiquitin (A part of the chimeric construct) through anti-myc antibodies. If the proposed mechanism of self-ubiquitination of the given chimeric construct at elevated temperatures is true and can be established through further experiments, then it will provide a very strong explanation for – 'Why ubiquitin fused to Sde2-C evolved/diverged to form the present day Sde2-UBL.

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Chapter 2: Potential Promiscuity in SUMO-interaction motifs (SIMs)

2.1 Introduction

It is known that ubiquitin molecules can bind to particular proteins non-covalently by attaching specifically to the ubiquitin binding domains (UBD) present in such proteins. The interaction between SUMO (Small Ubiquitin-like Modifier) proteins and proteins containing SIMs (SUMO-interaction motifs) is analogous to the interaction described above. However, the work presented in this chapter identifies through a yeast two- hybrid (Y2H) screen, potential interactions between the *Saccharomyces cerevisiae* Hub1 protein (A distant relative of the SUMO proteins) and other SIM containing proteins hinting towards broader roles/functions of the ubiquitin-like protein Hub1. The experiments conducted in this study were performed by me and Amjadudheen Varikkapulakkal (Lab colleague) under the expert supervision and able guidance of Dr. Shravan Kumar Mishra. Our study was reviewed and accepted by 'Micropublication Biology' and was published as a micropublication on 25th January, 2022. In order to maintain the integrity and originality of the work, a copy of the published manuscript has been attached below.

2.2 Copy of the Published Manuscript

Broader roles of the ubiquitin-like protein Hub1 indicated by its yeast two-hybrid interactors

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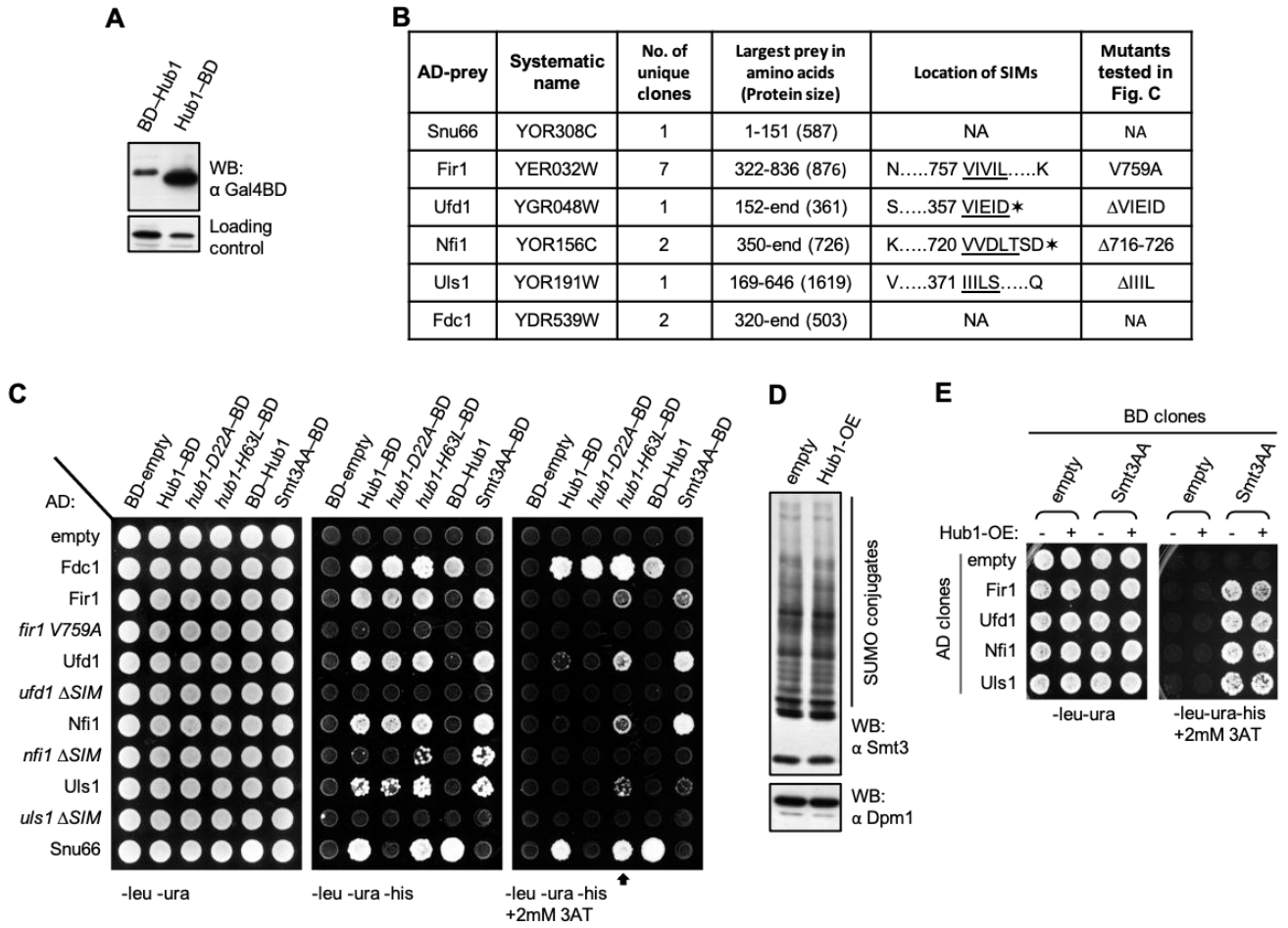
Abstract

The conserved ubiquitin-like protein Hub1/UBL5 functions in RNA splicing, DNA repair and mitochondrial unfolding responses. It binds proteins specific to these pathways and modifies their functional properties. However, the identities of other Hub1 substrates remain unknown. We have found unreported interactors of *Saccharomyces cerevisiae* Hub1 from a yeast two-hybrid (Y2H) screen. Proteins containing SIMs (small ubiquitin-like modifier SUMO-interaction motifs) and ferulic acid decarboxylase Fdc1 are identified as potential Hub1 interactors. Further experiments are required to establish these interactions and their physiological relevance, nevertheless, data presented here point towards larger and intriguing roles of Hub1.



AD-prey	Systematic name	No. of unique clones	Largest prey in amino acids (Protein size)	Location of SIMs	Mutants tested in Fig. C
Snu66	YOR308C	1	1-151 (587)	NA	NA
Fir1	YER032W	7	322-836 (876)	N.....757 VIVIL.....K	V759A
Ufd1	YGR048W	1	152-end (361)	S.....357 VIEID✶	ΔVIEID
Nfi1	YOR156C	2	350-end (726)	K.....720 VVDLTSD✶	Δ716-726
Uls1	YOR191W	1	169-646 (1619)	V.....371 IIILS.....Q	ΔIIIL
Fdc1	YDR539W	2	320-end (503)	NA	NA

Figure 1. Potential interactors of Hub1 identified from the Y2H screen: (A) Expression of Hub1 fusion clones with N- or C- terminal Gal4-BD. Proteins from total yeast lysate were detected by a monoclonal antibody specific to Gal4-BD. BD at Hub1's C-terminus is detectable in larger amounts possibly because of a chaperone-like activity of Hub1 on C-terminal extensions (chaperoning activities are known for ubiquitin and SUMO). Loading control represents an antibody cross-reactive signal of unknown identity detected in the lysates. **(B)** Clones obtained from Y2H screen. Hub1-Snu66 interaction was reported previously. Preys encoding Fir1, Ufd1, Nfi1, and Uls1, known to non-covalently bind yeast SUMO (Smt3) through their SIMs (underlined), were found as Hub1 interactors. SIM mutants used in Fig. C are indicated. In addition, two independent clones of Fdc1 were also obtained as Hub1 interactors (NA, not applicable, proteins lack SIM). Asterisks indicate stop codons. **(C)** Targeted Y2H assays monitoring interactions between indicated baits and the largest prey clones obtained in the screen. Each spot represents one of the 66 combinations of baits and preys co-transformed in the yeast. Full length (FL) Snu66 was used as prey control, which as expected interacted with Hub1 but not the D22A mutant. Yeast SUMO with its di-glycine motif changed to di-alanine (Smt3AA) was used as a control for monitoring SIM binding. Point mutant or deletions of SIMs in Fir1, Ufd1, Nfi1, Uls1 defective in Smt3AA binding did also not bind Hub1. SIM binding to *hub1-D22A* mutant was unaltered, while the *H63L* mutant showed stronger interactions (indicated by an open arrow). The N-terminal BD-Hub1 fusion did not bind SIM-containing clones either due to lower protein expression (Fig. A) or because these interactions require a free N-terminal surface of Hub1. By contrast, binding to Snu66 and Fdc1 was unaffected by BD's location. **(D)** Covalent SUMO conjugates (detected by anti-Smt3 antibody) were unaltered in yeast overexpressing (OE) Hub1. **(E)** Y2H assays between

indicated baits and preys upon Hub1 overexpression. SUMO-SIM interactions were not weakened upon Hub1 overexpression.

Description

Hub1/UBL5 has been reported to function in pre-mRNA splicing (Chanarat and Mishra, 2018; Wilkinson *et al.*, 2004), DNA repair (Oka *et al.*, 2015) and mitochondrial unfolding responses (Benedetti *et al.*, 2006). It associates with proteins only non-covalently thereby modifying their functional properties (Chanarat, 2021). Through its Asp-22 surface Hub1 binds HIND segments on the splicing factor Snu66/SART1 and through its His-63 surface Hub1 binds the RNA helicase Prp5 required for spliceosome formation (Karaduman *et al.*, 2017; Mishra *et al.*, 2011). An earlier study of Hub1 adducts in *S. cerevisiae* has indicated that this UBL may also have a larger number of substrates (Lüders *et al.*, 2003). However, identifying Hub1 substrates has been challenging, possibly due to its transient, weak or substoichiometric associations. We have performed a Y2H screen using *S. cerevisiae* Hub1 as bait fused to the DNA binding domain (BD) of the Gal4 transcription factor at the C-terminus. This fusion raises the possibility of identifying substrates binding through the N-terminal surface. Also, this fusion protein accumulated to higher levels in yeast (Fig. A).

In Y2H screens, Gal4BD fused to the protein of interest is used as bait for identifying its binding partners from a library of preys (DNA clones fused to the transcription activation domain (AD) of the same transcription factor). Once the bait binds a prey, functional Gal4 transcription factor gets reconstituted, whose activity is measured by various *GAL* promoters driven reporters, for example, *HIS3* (conferring histidine auxotrophy), *ADE2* (conferring adenine auxotrophy) (James *et al.*, 1996). Screens with partial and random clones become more useful when full-length baits and preys perform poorly due to improper expression, folding, or non-nuclear localisation of fusion proteins. Preys used in the screen were Gal4AD coding sequence fused to a random *S. cerevisiae* genomic DNA library in all three reading frames. ~5×10^5 bait-prey co-transformed yeast were screened for each reading frame. Besides the previously reported splicing factor Snu66, clones encoding Fir1, Nfi1, Uls1, and Ufd1 with their SIMs, and Fdc1 were identified as Hub1 interactors (Fig. B).

SIMs are found in proteins with diverse roles. Through a short stretch of hydrophobic amino acids with an acidic region, often unstructured SIMs bind SUMO non-covalently. Upon binding, SIMs adopt partial beta-strand conformations (Lascorz *et al.*, 2021). Among the clones containing SIMs identified here, Fir1 is involved in 3' mRNA processing, Nfi1/Siz2 is a SUMO E3 ligase, Uls1/Ris1 is a SUMO-targeted ubiquitin ligase, and Ufd1 is a polyubiquitin-binding substrate recruiting cofactor of Cdc48. SIMs in Fir1, Nfi1, Uls1, and Ufd1 clones (Hannich *et al.*, 2005) were verified by their positive interactions with yeast SUMO, whereas their SIM mutants were defective in SUMO binding. Smt3 in general interacted more strongly with SIM-containing clones than Hub1. Hub1-specific substrates Snu66 and Fdc1 lacked SIM and did not bind Smt3 (Fig. C). *hub1-D22A* mutant defective in Snu66 binding (Ammon *et al.*, 2014; Mishra *et al.*, 2011) interacted normally with SIM-containing clones, suggesting SIM associates to another surface of Hub1. On the contrary, the *hub1-H63L* mutant defective in binding to the splicing RNA helicase Prp5 (Karaduman *et al.*, 2017) interacted strongly with SIMs (Fig. C). The Leu63 variant's improved interactions suggest a hydrophobic mode of association with SIM and potential overlap with the Prp5-binding site. SIM point mutation, or deletions of the hydrophobic regions abrogating SUMO binding, did also not bind Hub1 (Fig. C), thereby indicating similar hydrophobic interactions between Hub1 and SIMs. The data also indicate potential promiscuity in SIMs. The hydrophobic associations of Hub1 possibly represent some of its adducts reported previously (Lüders *et al.*, 2003).

Notwithstanding the above evidence, binding sites for Smt3/SUMO and Hub1 on these SIMs need to be defined better by making point mutants of SIMs and comparing their interactions with the two UBLs. Similarly, SIM binding site on Hub1 needs to be defined better. Furthermore, SIMs even though are found in unstructured segments of proteins (Lascorz *et al.*, 2021), where point mutations and deletions are less likely to alter overall structures, protein folding and stability issues for the mutants need to be ruled out. The overlapping binding sites of Hub1 and SUMO suggested potential competition in the non- covalent associations of the two UBLs. However, owing to stronger SUMO-SIM associations, Hub1 overexpression neither altered covalent SUMO conjugates (Fig. D) nor the non-covalent SUMO-SIM binding (Fig. E). However, these assays are not highly quantitative or physiological for ruling out potential crosstalk between Hub1 and SUMO. Further biochemical assays are needed to confirm Hub1-SIM associations and follow up studies would reveal the function and mechanism of Hub1 with these molecules.

Fdc1-Hub1 interaction was not affected in the *hub1-D22A* mutant. Fdc1 lacks SIM and did not bind SUMO. Thus, Hub1 interaction with Fdc1 is distinct from its interaction with Snu66 or SIMs, for which another yet to be identified surface of Hub1 might be used (Fig. C). Diverse applications of ferulic acid (Kumar and Pruthi, 2014) make the Hub1-Fdc1 association an interesting research subject. Taken together, our data indicate that Hub1 employs additional surfaces for protein-protein interactions. Through hydrophobic associations, Hub1 might play broader roles that include possible crosstalk with other UBLs and potential decarboxylation of aromatic carboxylic acids.

Methods

Request a detailed protocol

Strains and plasmids used for yeast two-hybrid screens are as described previously (James *et al.*, 1996). The hub1 deletion strain was described earlier (Mishra *et al.*, 2011). Standard yeast protocols were followed for yeast transformation, growth assays and protein analysis (Knop *et al.*, 1999).

Western blots: A monoclonal antibody specific for Gal4-BD (Clonetech) was used for monitoring the expression of BD fusions in total yeast lysates. Smt3/SUMO conjugates were monitored in total yeast lysates using an anti-Smt3 polyclonal antibody. Cells were treated with 10mM NEM for 1 hour prior to harvesting. Preparation of sample for western blot analysis was described before (Knop *et al.*, 1999).

Plasmids construction: Plasmids were constructed through restriction enzyme cloning to ligate desired inserts into appropriate vector backbones following their amplification from *S. cerevisiae* genomic DNA using specific primer sets. The pGBDU-C1 plasmid was used to prepare bait clones with N-terminal BD. C-terminal BD clones were prepared in a uracil positive YEplac195 vector by inserting ADH promoter (between EcoRI and BamHI restriction sites), Hub1 or Smt3AA coding sequences (between BamHI and PstI restriction sites), BD coding sequence (at PstI restriction site), and ADH terminator (between PstI and SphI restriction sites), in that order. Random genomic DNA libraries were prepared in all three coding frames in pGAD-C1, C2 and C3 plasmids by inserting yeast genomic DNA fragments digested with ClaI restriction enzyme (James *et al.*, 1996). All prey clones and their mutants were made in the pGAD-C1 plasmid. The point mutants were made by quick-change site-directed mutagenesis and SIM deletions through splicing by overlap extension (SOE) PCR or by inserting a premature stop codon in the C-terminally located SIM of Ufd1. Hub1 was overexpressed either by using a centromeric vector with TEF2 promoter or an episomal vector with ADH promoter.

Yeast two-hybrid screen and assays: Y2H screen was performed essentially following published protocols (Gietz *et al.*, 1997; James *et al.*, 1996). In brief, the *S. cerevisiae* strain PJ69-7A was transformed with Hub1-BD bait and transformants were

selected on synthetic complete media lacking uracil. The bait transformed yeast was cultured, made competent and transformed with the leucine positive Gal4AD genomic DNA libraries at 60X scale in all three reading frames. An aliquot was plated on media lacking uracil and leucine to estimate the total number of transformants screened. The remaining transformation mixture (~5×10^5 transformants in each reading frame) were plated on media lacking uracil, leucine and histidine (–ura –leu –his) to select putative positive interactors. After 5 days of incubation at 30°C, putative positive colonies were replica plated on different reporter plates lacking histidine with/out 3AT and lacking adenine. For autoactivation test of prey fusions, first positive colonies were streaked on –leu + 5-FOA plates to shuffle out the bait marked with uracil, then autoactivation test of prey-containing colonies was done by streaking on –leu –his plates. AD plasmids from non- autoactivating colonies were isolated by yeast shuttle prep. Prey clones showing positive interaction after retransformation with the bait were sequenced. For targeted Y2H assays, the PJ69-7A strain was co-transformed with different combinations of AD and BD clones. The transformants were selected by plating on appropriate synthetic dropout plates and by growing them at 30°C for 2-3 days. Cells from each co-transformants were suspended in 500µl sterile water and their OD_{600} was measured.

Appropriate volumes of sterile water and the cell suspensions were mixed in a 96-well microtiter plate to get a final cell density of 1.0 OD_{600} / ml. 0.005 OD_{600} cells were spotted on various selection plates. The spotted cells were allowed to grow at 30°C for 3 days.

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Appendix

	Sp Ubi-Sde2C	*Sp* UBL-Sde2C	*Sj* UBL-Sde2C	*At* UBL-Sde2C	*Ce* UBL-Sde2C	*Dm* UBL-Sde2C	*Dr* UBL-Sde2C	*Hs* UBL-Sde2C
SDE2-UBL's % similarity to ubiquitin	100%	28.40%	34.80%	54.10%	31.90%	45%	33.30%	14.20%
Minus DUB	0	0	0	0	0	0	0	0
Ubi-Sde2C+ubp5	1	1	1	1	1	1	1	1
EV	0.00	0.00	-0.05	0.11	-0.01	0.00	0.01	0.02
***Sp* ubp3**	0.40	-0.01	-0.09	-0.10	-0.02	0.00	0.00	-0.03
Sp ubp5	1	1.19	1.59	0.04	0.07	0.00	0.00	-0.05
Sp ubp6	14.31	-0.01	0.08	-0.14	0.71	0.00	0.06	-0.05
Sp ubp7	5.05	0.02	-0.04	0.00	0.72	0.00	0.09	-0.05
Sp ubp15	2.35	1.17	2.06	3.90	0.39	0.00	0.00	-0.05
***Sp* ubp16**	0.08	0.00	0.48	6.52	0.43	0.00	0.08	-0.05
***Sp* otu2**	0.00	0.04	-0.06	0.03	-0.01	0.00	0.00	-0.05
***Sc* ubp15**	0.15	0.01	0.20	-0.12	-0.01	0.00	0.00	-0.05
***Hs* USP7**	17.55	0.00	-0.07	0.00	0.13	0.00	0.00	-0.05
Minus DUB	0	0	0	0	0	0	0	0
Ubi-Sde2C+ubp5	1	1	1	1	1	1	1	1
EV	0.01	0.00	0.01	0.04	-0.01	0.00	0.01	0.00
***Sp* ubp11**	0.65	0.00	0.00	0.04	0.00	0.00	0.00	0.00
***Sp* ubp12**	0.85	0.00	-0.01	-0.16	-0.01	0.00	0.00	0.00
***Sp* otu1**	0.02	0.00	-0.01	-0.22	-0.01	0.00	0.00	0.00
***Sp* sst2**	0.07	0.00	0.00	-0.22	-0.01	0.00	0.01	0.00
***Sp* uch1**	0.03	0.00	-0.01	-0.03	-0.01	0.00	0.00	0.00
***Sp* ubp4**	0.13	0.00	-0.01	-0.09	-0.01	0.00	0.00	0.00
***Sp* ubp9**	0.00	0.00	-0.01	-0.10	-0.01	0.00	0.00	0.00
***Sp* rpn11**	-0.02	0.00	-0.02	-0.10	-0.01	0.00	0.01	0.00
***Sp* uch2**	0.32	0.00	-0.01	-0.08	-0.01	0.00	0.00	0.00
***Sp* ubp1**	0.11	0.00	0.00	-0.13	-0.01	0.00	0.00	0.00
***Sp* ubp2**	0.94	0.00	-0.01	-0.06	-0.01	0.00	0.00	0.00
No. of *S. pombe* DUB's that process the chimera	17	2	4	2	6	0	3	0

Strains Used

SP20 - h+ JY741 Δsde2::Nat-NT2

SC2 - p J69-7a

Plasmid List

Stock ID	Plasmid
D001	pGADC1
D002	pGBDUC1
D006	pET28a
D007	pGBDUC2 HUB1
D052	pREP81x Sp 3MYC–SDE2–3FLAG
D067	pPROEX Sp 6HIS–SDE2
D076	pREP81x
D263	pENO-sp 3MYC-sde2-3flag
D436	pCDFduet empty
D437	pCDFduet 6HIS-Spubp5
D439	pCDFduet 6HIS-Spubp15
D441	pPROEX 6HIS-ubi1GG-KGGsde2-C
D442	pCDFduet 6HIS-Spubp16
D571	pENO-3MYC–SpUbi4–Sde2-C–3FLAG
D671	ubp1 in pCDFDuet-1
D672	ubp2 in pCDFDuet-1
D673	ubp3 in pCDFDuet-1
D674	ubp4 in pCDFDuet-1
D675	ubp6 in pCDFDuet-1
D676	ubp7 in pCDFDuet-1
D677	ubp9 in pCDFDuet-1
D678	ubp11 in pCDFDuet-1
D679	ubp12 in pCDFDuet-1
D680	otu1 in pCDFDuet-1
D681	rpn11 in pCDFDuet-1
D682	sst2 in pCDFDuet-1
D683	uch1 in pCDFDuet-1
D684	uch2 in pCDFDuet-1
D685	Scubp15 in pCDFDuet-1
D688	HsUSP7 USP domain in pCDFDuet-1
D690	SjSde2-UBL-SpSde2-C in pET28a(+)
D691	AtSde2-UBL-SpSde2-C in pET28a(+)

D692	CeSde2-UBL-SpSde2-C in pET28a(+)
D693	DmSde2-UBL-SpSde2-C in pET28a(+)
D694	DrSde2-UBL-SpSde2-C in pET28a(+)
D695	HsSde2-UBL-SpSde2-C in pET28a(+)
D939	pGADC1-Fir1 (322-836 a.a.)
D751	YC22 pTEF Sc Hub1
D940	pGADC1-Fir1-SIM- point mutant (V759A)
D941	pGADC1-Ufd1 (152-end a.a.)
D942	pGADC1-Ufd1-SIM mutant (ΔVIEID-deletion after E356)
D943	pGADC1-Ris1 (169-646 a.a.)
D944	pGADC1-Ris1-SIM mutant (Δ371-374)
D945	pGADC1-Siz2 (350-enda.a.)
D946	pGADC1-Siz2-SIM mutant (Stop codon inserted after Q715)
D947	pGADC1-Fdc1 (320-enda.a.)
D948	otu2 in pCDFDuet-1
D976	pYE195 pADH Sc Hub1-BD GAL 4 tADH
D977	pYE195 pADH Sc Hub1D22A-BD GAL 4 tADH
D978	pYE195 pADH Sc Hub1H63L-BDGAL 4 tADH
PSKM0097	pYE195 pADH Smt3 AA-BD-tADH