Role of Jab1 in regulating Ubiquitin carboxy-terminal hydrolase-1 (UCHL1) activity

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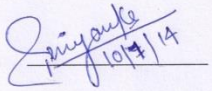

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Approval Sheet

This thesis entitled "Role of Jab1 in regulating Ubiquitin carboxy-terminal hydrolase-1 (UCHL1) activity" by Priyanka Mishra is approved for the degree of Master of Technology/ Doctor of Philosophy from IIT Hyderabad.

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Abstract

UCH-L1 is a member of the ubiquitin C-terminal hydrolase (UCH) family of proteins that is expressed predominantly in neuronal tissues. Previous studies have shown that UCH-L1 is highly expressed in different types of cancers, suggesting a role in tumorigenesis. Currently, it is not clear how UCH-L1 activity is regulated in vivo. We hypothesized that enzymatic catalysis of UCH-L1 may allosterically be regulated by interacting proteins. A screen to identify UCH-L1 interacting protein revealed that JAB1/CSN-5, a COP-9 signalosome (CSN) subunit, to be the UCH-L1 interacting protein. The present study is aimed at understanding the role of Jab1 interaction on UCH-L1 activity. Jab1 is known to alter the activity of the other interacting protein either by phosphorylation or promote degradation by ubiquitin proteasome pathway. We performed UCHL1 activity assay on Ubiquitin substrate having C-terminal peptide extension and found that the presence of JAB1 in the assay is completely inhibiting UCHL1 deubiquitinase activity, without phosphorylation or ubiquitylation. Our results indicate a novel mode of regulation of UCHL1 activity by physical interaction with Jab1. The results from the present study will be helpful for further understanding of regulation of UCHL1 activity. It would also be of interest to see if similar mechanism exists for other UCH family and also will explore substrate specificity, stability and posttranslational modification of UCH-L1 in presence of Jab1. The outcome of this study may facilitate the design of drugs to treat cancers where UCH-L1 is overexpressed.

Nomenclature:

1.	aa	amino acid.
2.	APS	Ammonium per sulfate
3.	Amp	Ampicillin
4.	AP-1	Activator protein1
5.	β-ME	Beta merceptoethanol
6.	bp	base pair
7.	°C	Degree Celsius.
8.	Cdna	Complementary dna
9.	COP	Constitutive photo morphogenesis
10.	CSN	COP9 signallosome
11.	DNA	Deoxyribonucleic acid
12.	DTT	Dithiothretol
13.	DUB	Deubiquitinase
14.	E.Coli	Escherichia coli
15.	et.al	and other
16.	EDTA	Ethylene Di amino tetra acetic acid
17.	g	gram or gravity depending on context
18.	HF	High fidelity
19.	His	Histidine
20.	IPTG	Isopropyl thio galactopyranosidase
21.	JAB1	Jun activation domain binding protein1
22.	JAMM	JAB1/MPN/Mov34 metalloenzyme
23.	JNK	c-jun N-terminal kinase
24.	kb	kilo base pair
25.	kD	kilo dalton
26.	LB	Luria Bertani.

27.	M	Molar
28.	mg	milligram
29.	min	minute
30.	ml	milliliter
31.	Mw	molecular wt
32.	n	nano
33.	NCBI	National centre for Biotechnology
34.	Ni-NTA	Nickel Nitrilo tri acetic acid
35.	PAGE	Polyacrylamide gel electrophoresis
36.	PCR	Polymerase chain Reaction
37.	pH	-log10(H+)
38.	RNAase	Ribonuclease
39.	RT	Room temperature
40.	sec	Second
41.	SDS	Sodium do decyl sulphate
42.	SOC	Super optimal broth with catabolic repression
43.	TE	Tris EDTA
44.	TEMED	Tetra methyl ethylene di amine
45.	TAE	Tris base, Acetic Acid, EDTA
46.	Tris / THAM	Tris hydroxyl methyl amino methane
47.	TB	Tariffic Broth
48.	Ub	Ubiquitin
49.	UCH	Ubiquitin C-terminal Hydrolase.
50.	UCH-L1	Ubiquitin C-terminal hydrolase- 1
51.	UCHL-3	Ubiquitin C-terminal hydrolase- 3
52.	UPS	Ubiquitin proteasome system
53.	USP	Ubiquitin specific proteases
54.	USP15	Ubiquitin specific protease 15
55.	U.V	Ultra violet
56.	v/v	volume in volume
57.	w/v	weight in volume
58.	µ	micro

59.	µg	micro gram
60.	µl	micro liter

Contents

Introduction *Chapter 1*

1.1. Ubiquitin

Ubiquitin (Ub) is a highly conserved small (76 amino acid) eukaryotic protein that is covalently attached to proteins via its carboxy terminal glycine residue by the consecutive actions of three distinct enzymes. Ub is first activated by the Ub-activating enzyme (E1), transferred to a Ub-conjugating enzyme (E2), and then attached to a target protein under the control of a Ub ligase (E3). There are only a few E1 enzymes, ~ 30 E2s, and over 600 E3s in humans, which together generate a variety of different ubiquitinated forms of many thousands of proteins by monoubiquitination as well as by polyubiquitinations [51].Importantly, ubiquitin itself has seven lysine residues, all of which can act as acceptors for further ubiquitination generating polyubiquitin chains. Lys 48-linked (K48) polyubiquitin targets substrate proteins for proteasomal degradation whereas most non-proteolytic functions of ubiquitin chains are currently associated with Lys 63-linked (K63) ubiquitin polymers. A variety of ubiquitinated products are then specifically recognized either by Ub receptors containing Ub-binding domains (UBD), leading to downstream effects, or by deubiquitinating enzymes (DUBs), which catalyze the reverse reaction. Thus, the Ub system is extremely versatile and can play multiple essential roles in various cellular processes by regulating not only protein stability but also protein interactions, trafficking, and activation.

1.2. UCH-L1, a deubiquitinating enzyme: Protein ubiquitination, like protein phosphorylation, is a reversible process. Deubiquitinating enzymes (DUBs),

which belong to the superfamily of cysteine proteases, remove Ub from protein substrates. DUBs are grouped into two subfamilies: the ubiquitin C-terminal hydrolases (UCHs) and the ubiquitin-specific proteases (USPs/UBPs). In general, UCHs catalyze hydrolysis of C-terminal peptide bond and USPs/UBPs hydrolyse isopeptide bond between the K48 and K63 linked polyubiquitin chains [20]. To date, four members have been known in UCH family in human: UCH-L1, UCH-L2 (also known as BRCA1-associated protein-1 or BAP1), UCH-L3, UCH-L5 (UCH37). All the UCH family members have a conserved catalytic domain consisting of about 230 amino acids. The crystal structures of UCH-L1, UCH-L3 and UCH-L5 have been solved [11, 16, 32], showing that the UCH-domain contains three conserved residues, cysteine, histidine and aspartate. The UCH enzymes prefer to cleave relatively small protein substrates from the C-terminus of Ub, and the size limit is imposed by a loop that partially occludes the active site of these enzymes [20].

Ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1, aka PGP9.5) is an abundant neuronal protein consisting of 223 amino acids [53]. UCH-L1 is an abundant neuronal protein, comprising approximately 2% of total brain protein [54]. Although low levels of UCH-L1 protein was reported to be present in kidneys, breast epithelium, and reproductive tissues, UCH-L1 was absent in most other tissues [5]. Mice lacking functional UCH-L1 developed gracile axonal dystrophy, a recessive neurodegenerative disease [35, 36]. At the subcellular level, UCH-L1 was primarily found in the cytoplasm, but recent reports indicate that a subpopulation of UCH-L1 can be transiently localized to the nucleus[4, 24]. *In vitro* biochemical studies revealed that UCH-L1 hydrolyzes Ub after its C-

terminal glycine residue to generate monomeric Ub and that this activity was dependent upon the catalytic residues C90 and H161 [19]. Analysis of UCH-L1 crystal structure revealed that the catalytic residues were not accessible to large polymers of Ub and suggested that UCH-L1 and could accommodate small adducts of Ub [27]. It is possible that substrate binding and/or the presence of interacting proteins may induce a conformational change, allowing UCH-L1 to process larger Ub chains. However, this has not yet been demonstrated *in vitro* or *in vivo*.

1.3. UCH-L1 function: Although the exact cellular function of UCH-L1 is not fully understood, several studies suggest that UCH-L1 regulates the cellular pool of free Ub. In most organisms, Ub is expressed as a linear polymer, pro-ubiquitin, consisting of multiple copies of Ub and one or more amino acids appended to the C-terminus of the final Ub. For example, in humans polyubiquitin-C (UbC) is expressed as 9 Ub monomers followed by a Val and polyubiquitin-B (UbB) as 3 monomers followed by a Cys [52]. UCH-L1 was reported to cleave the Ub gene products UbB and UbC and the ribosomal ubiquitin fusion protein UbA80 to generate monomeric Ub when co-expressed in *E. coli* [20]. In contrast to other DUBs, *in vitro* studies revealed that UCH-L1 could not directly catalyze the deubiquitination of ubiquitinated protein substrates [20]. Moreover, no *in vivo* UCH-L1 substrates have been identified thus far.

UCH-L1 and UCH-L3 are composed entirely of the UCH domain and are capable of cleaving amino acids linked by peptide bonds to the C-terminus of Ub, yet they are inactive towards di-Ub. In contrast, BAP1 and UCH37 are capable of acting on di-Ub and poly-Ub chains. The basis of this specificity stems from a

loop that crosses over the UCH catalytic site, forming a pore through which the C-terminus of Ub must be threaded. The most striking structural feature relating to substrate specificity is presence of this loop in UCH-L1 and UCH-L3. There are two models explaining function of this loop. In one scenario substrates would enter the active site cleft by passing underneath the arch created by the active site crossover loop. This model would readily explain the ability of UCHs to hydrolyze small Ub C-terminal extensions, as the loop itself would likely impose significant size restrictions on potential substrates. However, the recent reports of larger substrates hydrolyzed by UCH enzymes conflict with the model of substrate threading through the crossover loop. The maximum theoretical internal diameter of the active site crossover loop is ~12–15 Å. Polyubiquitinated substrates would be impossible to accommodate underneath this loop. The alternate model for UCH hydrolysis was thus required to account fully for the observed action of these enzymes. According to this model, to accommodate larger substrates in the active site of UCHs one must relieve steric constraints by peeling away the active site crossover loop from the active site cleft. Besides proteolysis, UCH-L1 was also reported to bind free Ub monomers and prevented its lysosomal degradation [33]. Association of UCH-L1 with monomeric Ub occured independently of the catalytic C90 residue, indicating that mono-Ub binding did not depend upon UCH-L1 hydrolase activity [33]. The role of UCH-L1 in the regulation of the free Ub pool was also supported by the observation that levels of monomeric Ub were decreased in *g*racile *a*xonal *d*ystrophy (*gad*) mice, which lacked functional UCH-L1 [33]. Although the best understood function of UCH-L1 is maintenance of cellular pool of free Ub, UCH-L1 was

reported to possess putative, dimerization-dependent E3 ligase activity in addition to its hydrolase function. *In vitro* experiments showed that dimeric UCH-L1 promoted K63-linked polyubiquitination of α-synculein [23]. Unlike other E3 ligases, UCH-L1 E3 ligase activity was observed in the absence of ATP, which was different from the mechanism of conventional ubiquitination. It is currently not known whether UCH-L1 exhibits E3 ligase activity *in vivo*. Further investigation into UCH-L1 enzymatic function is needed to understand its role in health and disease. Recently, a post-translational modification of UCH-L1 that controls the function of UCH-L1 has been identified [26]. It was observed that UCH-L1 was monoubiquitylated on a lysine residue near the active site (K157). This modification restricted ubiquitin binding. However, UCH-L1 deubiquitination activity removed this Ub and prevented permanent mono-ubiquitination.

1.4. UCH-L1 and cancer: Although the expression of UCH-L1 is limited to neuronal tissue [53], UCH-L1 levels were found to be increased in various types of malignancies including acute lymphoblastic leukemia, breast cancer, leukemia, medullary thyroid carcinoma, non-small cell lung cancer; neuroblastoma, prostate, esophageal, colorectal and pancreatic cancer indicating the involvement of UCHL1 as oncogene in the pathogenesis of these tumors [14, 21, 28, 29, 34, 38, 44, 45, 56, 57]. Overexpression of UCH-L3 and UCH-L5 were reported in breast cancer and cervical carcinoma [28, 40] and UCH-L2/BAP1 overexpression was found to be associated with lung and breast cancer [9, 15].Contrary to previous reports proposing cancer-promoting role of UCH-L1, recent studies revealed that UCH-L1 attenuated tumor growth and maturation in prostate cancer

and breast cancer [48, 49]16, 52]. In contrast to previous studies demonstrating that UCH-L1 is upregulated in breast tumors, UCH-L1 mRNA expression was reported to be decreased in several breast carcinoma cell lines [55]. Therefore it is clear that although there has been growing evidence on the relation between UCH enzymes and oncogenesis, its role in carcinogenesis is still unknown. Elevated Ub level is essential for the survival and proliferation of the cancer cells; but not for the viability of normal cells. Thus, the down regulation of Ub levels would be preferentially detrimental to cancer cells. Cancer cells acquire their malignant capabilities through variuos mutations and extensively reprogrammed pathways, which inevitably generate a variety of stresses, including proteotoxic stress [13]. Cancer cells must tolerate these increased stresses through the activity of Ub-proteasomal system by degrading unnatural proteins and thus considered attractive cancer therapeutic targets [25, 41]. Therefore, the suppression of the Ub level, for example by UCH-L1 inactivation, may lead to stress sensitization by weakening the stress-supporting system.

1.5. UCH-L1 interacting protein-Jab1: Using UCH-L1 as bait in a yeast 2-hybrid assay with an expression cDNA library derived from fetal brain, it was shown to interact with JAB1 (Jun activation domain-binding protein 1) [6]. The study also confirmed that the two proteins interact *in vivo* via co-immunoprecipitation. Other proteins that are known to interact with UCH-L1 are UBC9 and Ran binding protein (RanBP).

1.6. Jab1/CSN5 function: The COP9 signalosome (CSN) complex is composed of 8 subunits (CSN1-8) and well conserved in all eukaryotes from yeast to humans and involved in various biological responses. CSN function was orginally

discovered in *Arabidopsis*. In the absence of light signals, the CSN represses photomorphogenesis through the degradation of the HY5 transcription factor. Mutations in the CSN lead to *constitutive photomorphogenesis* (*cop*) in the dark. Among the 8 components of the CSN, the fifth subunit of the CSN (CSN5, also known as Jab1) is unique in many ways. Mammalian CSN5 was originally identified as a protein binding to the transcription factors c-Jun, a member of AP-1 transcription factors family, and so termed as Jun-activation-domain-binding protein-1 (Jab1). AP-1 (activating protein-I) is a collective term referring to dimeric transcription factors composed of Jun, Fos or ATF (activating transcription factor) subunits that bind to a common DNA site, the AP-l-binding site. Jab1 determines the specificity of AP-1 transcription factors. The AP-1 complex can respond immediately to many different extracellular stimuli, epidermal growth factor (EGF), and serum. Although c-Jun and c-Fos have very similar DNA binding and dimerization domains, they seem to activate distinct sets of target genes [33-35]. Jab1/CSN5 specifically stabilized the protein-DNA complexes of c-Jun and Jun-D with its cognate AP-1 DNA binding sites. Jab1/CSN5 involvement as a specificity factor was demonstrated for a number of proteins in addition to c-Jun. Jab1/CSN5 is a transcriptional coactivator and potential specificity factor for E2F-1, IκB, p105, NFκB and p53 [2, 8, 12, 39], yet the mechanism by which it acts is poorly defined. Phosphorylation is one mechanism of regulation that could be attributed to Jab1/CSN5-associated kinase. In case of p53, CSN-specific phosphorylation of Thr155 was an important prerequisite for degradation of the tumor suppressor by the Ub–26S proteasome system [2]. Inhibition of the CSN-associated kinase by curcumin or a competitor

peptide derived from p53 resulted in the accumulation of endogenous p53. CSN phosphorylated c-Jun at the N-terminal transactivation domain including Ser63 and Ser73, which prevented ubiquitylation and degradation of the transcription factor [30]. Jab1 also controls the nuclear export and 26S proteasome-dependent degradation of several nuclear proteins, such as cyclin-dependent kinase inhibitor $p27^{Kip1}$, LHR, Smad, estrogen receptor, topoisomerase II and p53 [2, 3, 7, 17, 22, 46, 50, 58]. It was shown that CSN-unassociated free form of Jab1/CSN5 interacted with and promoted the export of $p27^{Kip1}$ from the nucleus to the cytoplasm and enhances its degradation by a proteasome [46]. However, the relationship between Jab1 and the 26S proteasome complex during protein degradation is not clearly understood. It was recently reported that in cooperation with CSN, Jab1 mediates the deneddylation of the cullin component of SCF ubiquitin-protein isopeptide ligase, which may promote cullin-dependent proteolysis (24).

1.7 Jab1 interaction domain: Small domain containing conserved Asp and Leu residues was discovered within the Jab1/CSN5 interacting region of $p27^{kip}$. Sequence analysis revealed that this domain was present in other Jab1 interacting proteins like c-Jun, LFA1β, suggesting an essential role in interaction with Jab1/CSN5 [47].

Objective *Chapter* 2

Significance of Jab1-UCH-L1 interaction is not known. Jab1 is known to alter the protein function by phosphorylation, promoting degradation or introducing conformational change [8, 10, 31]. UCH-L1 crystal structure revealed that the catalytic site was not accessible to large polymers of Ub. However, in an apparent contradiction to these results, UCH-L1 was also reported to be involved in deubiquitination of number of cellular proteins (Introduction section, page 4). Apart from the exact *in vivo* function and substrate of UCH-L1 almost nothing is known about the regulation of UCH-L1 inside the cell. Therefore studying Jab1-UCH-L1 interaction might help us in understanding cellular function or regulation of UCH-L1.

Objective: Effect of Jab1 interaction on UCH-L1 activity.

Specific aim-I: Cloning, expression, purification of recombinant UCH-L.

Specific aim-II: Site-directed mutagenesis of UCH-L1 to generate C90A active site mutant and purification of recombinant C90A UCH-L1.

Specific aim-III: Cloning, expression, purification of recombinant Ubiquitin.

Specific aim-IV: *In vitro* assay of UCH-L1 with C90A UCH-L1 as control.

Specific aim-V: Cloning, expression, purification of recombinant Jab1.

Specific aim -VI: *In vitro* assay of UCH-L1 in presence of recombinant Jab1.

3. Molecular biology methods:

3.1. Primers used for PCR: Primers were designed based on cDNA sequences of genes (taken from NCBI http://www.ncbi.nlm.nih.gov/nucleotide). Suitable restriction enzymes sites were added to 5'ends of each primer to promote directional cloning. A NEB tool (http://tools.neb.com/NEBcutter2/index.php) was used to select appropriate restriction enzymes. Stuffer sequences of 4-5 nucleotides were also inserted in primers flanking the restriction sites at the ends, which facilitate binding of restriction enzymes to their respective sites. Chemically synthesized oligonucleotides (Imperial Life Sciences) were dissolved in molecular grade water by vigorous vortexing to a final concentration of 100µM and stored at -20°C.

Table 1: Oligonucleotide primers used in study.

Gene	Name of the Primer	Sequence (5'-3')
UCHL1	UCHL1-BamHI-Sen	CCTGGGATCCATGCAGCTCAAGCCGATGGAGAT CAA CCCC
	UCHL1-SalI-HindIII-Anti	AAAAGTCGACAAGCTTTTAGGCTGCCTTGCAGAGAG CCACGG C
Ubiquitin	Ub-NdeI-Sen	GATATACATATGCAGATCTTCGTGAAGACTCTGACTGG
	Ub-HindIII-Anti	GCCTAGAAGCTTCTAGCAATGATGATGATGATGCTT CCCACCTCTGAGACGGAGTACC.
JAB1	JAB1-BamHI-Sen	AGTCGGATCCATGGCGGCGTCCGGGAGCG
	JAB1-SalI-Anti	GCGCGTCGACTTAAGAGATGTTAATTTGATTAAAC

3.2 .Polymerase chain reaction: To amplify specific DNA sequence PCR was done using primers. Phusion DNA polymerase enzyme (New England Biolabs, Cat.no M0530S) was used in all the PCR reactions. Composition and reagents used for standard PCR reaction mixture of 50µl is given below in Table 2. Templates used in the PCR reaction were of high quality, supercoiled plasmid or

cDNA. The volume of template DNA was diffrerent for different target amplification; for example, for amplifying UCHL1, 1µl of pGEFPC3-hUCHL1 was used as template; for amplifying Jab1, 1µl of human cDNA was used as template; for amplifying Ubiquitin, 0.5µl of pET-21b was used as template. All components were thawed and mixed properly prior to use, in the following order,10x buffer was first diluted with nuclease free water and the dNTPs were added. Template and then primers were added sequentially. Finally, just before starting the reaction Phusion DNA polymerase was added. Reactions were set up on ice in 0.2ml PCR tubes (Axygen, Cat. No: PCR-02-C). All components were mixed together by finger tapping followed by short centrifugation (5000rpm, 2min, RT). Thermal cycling condition for routine PCR performed is given in Table 3. PCRs were performed in Veriti 96 well thermal cycler from Applied Biosystems (model no.9902). After completion of the PCR, amplified DNA was analyzed by electrophoresis on agarose gel as described in section 3.3.

Table 2.Composition of PCR reaction mixture.

Components	Volume used (in µl)	Final conc.
HF Phusion buffer (5x)	10µl	1X
dNTP	10mM	0.5µl
Template DNA	As mentioned above	1µg
Forward and Reverse primers	0.25µl	200nm
Phusion DNA Polymerase	1.0µl	2U

Table 3. Thermal cycling condition for PCR

For UCHL1 amplification:

Steps	Temperature	Time
Initial denaturation	98°C	30 sec
32 cycles	98°C	10 sec
	57°C	30 sec
	72°C	1 min
Final Extension	72°C	10min

Ubiquitin amplification:

Steps	Temperature	Time
Initial denaturation	98°C	30 sec
32 cycles	98°C	10 sec
	57°C	30 sec
	72°C	1:30 min
Final Extension	72°C	10min

For JAB1 amplification:

Steps	Temprature	Time
Initial denaturation	98°C	30 sec
32 cycles	98°C	10 sec
	57°C	30 sec
	72°C	1 min
Final Extension	72°C	10min

3.3. Agarose gel electrophoresis: Horizontal agarose gel (0.8-1.0%) electrophoresis were routinely performed to separate DNA frangments of various lengths (ranging from 100bp-10kb). Appropriate amount of agarose (SeaKem®LE, LONZA, Cat. no. 50005) was dissolved in 1X TAE buffer (40mM Tris-acetate; 1mM EDTA pH 8.0) by heating in microwave oven. After cooling, Ethidium bromide was added of concentration 1µg/ml into the gel solution and then poured into gel a mould and a comb was inserted to generate wells. After 30-45 minutes, when the gel got solidified completely, comb was removed gently and gel mounted into electrophoresis chamber containing 1X TAE (40mM Tris-acetate; 1mM EDTA pH 8.0). DNA samples and size marker were mixed with appropriate volume of 1X DNA loading dye (6x stock: 0.25% bromophenol blue; 0.25 xylene cyanol FF; 30% v/v glycerol,(Cat.no:R0611, Thermo scientific). Electrophoresis were generally carried out at 80 volt till the xylene cynol dye migrated to distance of half of the gel.DNA bands were visualized in gene documentation system from SynGene (Model no: Chemi XR5, S.No DR4V2/2355). DNA ladder of either 100kb (catalog no: SM0241, Thermo scientific) for ubiquitin and 1kb ladeer (Cat no: SM0311 Thermo scientific) for JAB1and UCHL1, linearized plasmid DNA were used, respectively, for fragment size determination.

3.4. PCR Clean up: Before using in further experiments, PCR amplified DNA fragments were cleaned to remove the traces of dNTPs, enzyme, residual primers etc. Gene jet PCR clean Up kit (Thermo scientific, Cat.no K0691) was used for

this purpose to get pure amplified DNA free from contaminants. The process was carried out according to manufacturer's instructions.

3.5. Restriction Digestion and gel extraction of DNA: Restriction endonucleases were used to promote directional cloning. All the restriction enzymes were purchased from New England Biolabs. For cloning Ubiquitin, double digestion of the pRSETA vector and the PCR DNA were performed using NEB buffer 4, which is compatible for both enzymes. Cloning was successful when 34µl (71.2ng/µl) of PCR DNA was digested with BamH1 and NdeI for 6 hours and pRSETA vector was digested with BamH1 and NdeI for 16 hours. Digested products were first checked on 0.8% agarose gel for completion of digestion. For cloning UCHL1, double digestion of the pRSETA vector and the PCR DNA were performed using NEB buffer 4, which is compatible for both enzymes. Cloning was successful when 34µl of (46.6)ng/µl of PCR DNA was digested with BamH1 and HINDIII for 6h at 37°C incubation and pRSETA vector was digested with BamH1 and HINDIII for overnight digestion at 37°C. For cloning JAB1, double digestion of the pGEX6P1 vector and the JAB1 PCR DNA were performed using NEB buffer 4, which is compatible for both enzymes. Cloning was successful when 34µl of (187.7ng/µl) of PCR DNA was digested with BamH1 and SalI for 6 hours and pGEX6P vector was digested with BamHI and SalI for 16 hours. Digested products were first checked on 0.8% agarose gel for completion of digestion. In order to perform gel elution of the desired band, agarose gel electrophoresis were carried out for restriction digested PCR DNA and vector DNA on 0.8% agarose gel as described earlier. Gels were visualized under U.V light (345nm) in a trans-illuminator. DNA bands were carefully

excised with a sterilized scalpel and placed into a pre-weighed of 2ml micro centrifuge tube and record the gel weight. DNA was extracted by using Gene Jet Gel Extraction kit (Thermo scientific, Cat.no K0691), following manufacturers protocol.

3.6. Ligation Reaction: Ligation reactions were performed using T4-DNA ligase (New England Biolabs, Cat. no: M0202S).. For cloning UCH-L1, in total reaction volume of 10µl contained 1µl of restriction digested and gel extracted vector (28.5ng), 7µl of restriction digested and gel extracted PCR DNA (81.5ng) ,1µl (400U) of T4-DNA ligase and 1µl of ligation buffer containing ATP. Ligation was performed at 16°C for 10 hours and the whole ligation reactions were used for transformation into E. coli (DH5α). For cloning Ubiquitin, in total reaction volume of 10µl contained 1µl of restriction digested and gel extracted vector (23.95ng), 7µl of restriction digested and gel extracted PCR DNA (58.1ng) ,1µl (400U) of T4-DNA ligase and 1µl of ligation buffer containing ATP. Ligation was performed at 16°C for 10 hours and the whole ligation reactions were used for transformation into E. coli (DH5α).For cloning JAB1, in total reaction volume of 10µl contained 1µl of restriction digested and gel extracted vector (18ng), 7µl of restriction digested and gel extracted PCR DNA (297.5ng) ,1µl (400U) of T4-DNA ligase and 1µl of ligation buffer containing ATP. Ligation was performed at 16°C for 10 hours and the whole ligation reactions were used for transformation into E. coli (DH5α).

3.7. Preparation of competent *E.Coli* cells: Competent *E. coli* cells of DH5α and BL21 (DE3) pLysS, were prepared described by Alexander [1] by using $MnCl_2$ and $CaCl_2$. A sterilized inoculating loop was used to streak *E.coli* strains

(DH5α and BL21 (DE3) pLysS) directly from frozen glycerol stock onto an LB agar plate containing no antibiotic. Plate was incubated for 16 hours at 37°C.A single colony was picked and inoculated in 5 ml of LB medium for overnight in a shaking incubator at 200 rpm. 1ml of this overnight grown culture was inoculated in a pre -warmed 100 ml of LB medium (1% tryptone, 0.5% yeast extract) prepared in 250ml Erlenmeyer flask. Inoculated culture was grown for 4hrs at 30°C, 100 rpm in shaking incubator under monitoring culture growth by measuring OD_{600} spectrophotometer every 20 minutes. When culture had reached OD_{600} of 0.35 it was taken out and incubated on ice for 1hr. Cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 12 ml of acid salt buffer-A (ASB-A, sodium acetate-40 mM, $CaCl_2$-100 mM, $MnCl_2$-70 mM, pH 5.5) and incubated on ice for 1 hour. The ASB treated cells were then pelleted by centrifugation carried out at 3500 rpm for 15 min at 4°C and resuspended in 4ml of ASB-B (Sodium acetate-40 mM, $CaCl_2$-100 mM, $MnCl_2$-70 mM, 15% glycerol, pH 5.5) and were stored in Eppendorf tubes (aliquots of 50µl) at -86 °C for future use.

3.8 Transformation of competent E. coli cells:

(I) *Transformation of competent DH5α cells*: DH5α competent E.coli cells were prepared as described earlier. 10µl of a ligation mixture were added to 50µl aliquots of competent DH5 α cells and incubated on ice for 30 minutes. After that heat shock was given at 42°C for 30 seconds, cells were again briefly incubated on ice for 5 minutes and transformed competent cells were plated on LB- agar plates containing 100µg/ml ampicillin. Plates were incubated at 37°C overnight.

(II) *Transformation of BL21 (DE3) pLysS competent cells:* BL21 (DE3) pLysS competent E.coli cells were prepared as described earlier. 4µl of a plasmid DNA was added to 50 µl aliquots of cells and the mixture was incubated on ice for 10 minutes. After a 42°C heat shock for 30 seconds, the cells were briefly incubated on ice for 5 minutes. After this incubation, entire transformation mixture was plated on LB-agar plates containing 100µg/ml ampicillin. Plates were incubated at 37°C overnight for growth of cells.

3.9. Plasmid DNA Isolation from mini bacterial culture: A single colony was picked out from the plate containing colonies of transformed cells and inoculated in 5ml of LB medium containing ampicillin. The culture was incubated in a shaking incubator at 37°C, 200 rpm for overnight. 2ml of this culture was used for isolation of plasmid DNA using Plasmid Mini-Prep kits (Fermentas Life Sciences, cat. no K0502) following the instructions recommended by the manufacturer.

3.10. Site directed Mutagenesis: To study the enzymatic role of UCH-L1, the key residue (Cys90) from catalytic domain were mutated by PCR mediated site directed mutagenesis. Cys codon 'TGT' was mutated to Ala codon GCT. Primers were designed to create substitutions by incorporating the desired nucleotide changes in the center of forward primer including 19 complementary nucleotides on 5' and 3' end of the mutation. The New England Biolabs guidelines were followed to design the oligonucleotides, PCR protocols and transformation. The entire plasmid containing wild type UCH-L1 was amplified by oligonucleotides that carried the specific mutation. PCR specifications are as follows; 25 PCR cycles (with 56°C annealing temperature) and 50µl total volume. PCR enzyme

used was Phusion High Fidelity DNA polymerase (New England Biolabs, Cat: no M0530S). To remove the non-mutated template plasmid, the entire PCR mixture was treated with 1µl of *Dpn*I digestion at 37^{o}C for 2h, which degrades specifically methylated DNA that is template plasmid purified from bacterial host. *Dpn*I digested PCR mixture was then transformed to 200µl DH5α competent cells and grown on LB agar plates containing 100µg/ml ampicillin. After overnight incubation, several colonies were observed. Single colony was inoculated to 2ml of LB-ampicillin and plasmid was purified from grown culture as described in section 3.9.

Table 4. Primers used for site directed mutagenesis PCR.

Gene	Name of the Primer	Sequence (5'-3')
UCHL1 mutagenesis	UCHL1-C90A-Sen	GCAGACCATTGGGAATTCCgctGGCACAATCGGACTTATT C
	UCHL1-SalI-HindIII-Anti	5'AAAAGTCGACAAGCTTTTAGGCTGCCTTGCAGAGAGCCA CGGC-3'

Protein techniques

3.11. SDS-PAGE: Recombinant proteins were analyzed through SDS-PAGE. SDS-PAGE was carried using discontinuous buffer system as described by Laemmli (18) using Biorad gel electrophoresis apparatus (Cat. no: 165-8001). Throughout this study 1.5 mm thick 12% polyacrylamide (30:0.8, acrylamide to bisacrylamide ratio) gel containing 0.1% SDS was used for electrophoretic separation of proteins. For some application Biorad precast gel were also used (Cat. no: 456-1086). The protein samples were mixed with an equal volume of 3X sample buffer (2.4 ml 1M Tris-HCl pH 6.5, 3 ml 20% SDS, 3 ml 100% Glycerol, 1.6 ml β-mercaptoethanol, 0.006 g Bromophenol blue) and heated at 100 °C for

10 min and were loaded into the wells of precast polyacrylamide gel. Electrophoresis was performed at a constant voltage of 100 or 120V by diluting 10x buffer (Tris base 30.3g, Glycine 144g, SDS 10g in 1000ml). After the electrophoresis, the gel was stained with coommasie brilliant blue (0.4% w/v Coomassie blue R250, 30% v/v Methanol, 10 % Acetic acid). Spectra multicolor broad range protein ladder was used to confirm the size of protein. Composition of 12.5% SDS-PAGE gel is given below:

Table7. SDS-PAGE gel composition

Resolving Gel (12.5 %)		**Stacking Gel (4 %)**	
30% Acrylamide	2.00 ml	30% Acrylamide	0.65 ml
Tris-HCl, pH 8.8, 1.5M	1.25 ml	Tris-HCl,pH 6.8, 0.5M	1.25 ml
H_2O	1.70 ml	H_2O	3.05 ml
SDS (10%)	50µl	SDS (10%)	50µl
TEMED	20µl	TEMED	10µl
APS	45µl	APS	25 µl

3.12. Expression Analysis of the recombinant clones: *E.coli* BL21 (DE3) pLysS cells transformed with recombinant plasmids were grown and induced for protein expression as described by Studier *et al* [42, 43]. A single colony was inoculated in 6 ml of Luria-Bertani medium containing 100µg/ml ampicillin and grown for 4 hrs at 37^{o}C in a screw-cap inoculation vial. From this culture 2ml culture was recovered as a reference for uninduced *E.coli* extract and the remaining 4ml culture was induced with 1mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG). Following induction the culture was further grown for 4 h at 37^{o}C and 1.5ml culture was recovered from the induced sample as a reference for induced *E.coli* extract and 750 µl was recovered from uninduced culture. The IPTG-induced and uninduced cells were harvested and

resuspended in 100µl protein extraction buffer (50 mM Tris pH 8.8, 250 mM NaCl, 0.05% Triton-X) and lysed by sonication. From the sonicated samples, 60µl were added to 30µl of 3x sample loading buffer [18], boiled for 10 min and were used for SDS-PAGE analysis in 12% gel. The electrophoresis was carried out as mentioned in section 3.11.

3.13. Purification of His tag UCHL1 protein through Ni^{+}-NTA Affinity purification: Before protein purification, His-tag proteins were first checked by SDS-PAGE for overexpression as described in section 3.12. Purification of N-terminal His-tag UCHL1 was carried out in one step by affinity purification using Ni-NTA chromatography. For this, few colonies of BL21, transformed with specific recombinant plasmid DNA, were inoculated into 200 ml LB-broth with 100µg/ml ampicillin and grown overnight on incubatory shaker at 37^{o}C at 200 rpm as "pre-inoculum". The grown culture was 10-fold diluted into fresh 2L of TB-broth with 100 µg/ml ampicillin. Further incubation was carried out at 37^{o}C at 200 rpm for 3 hrs, when $O.D_{600}$ of culture reached to 0.5, the culture was induced with 1mM IPTG for 5 hrs at 30^{o}C at 200 rpm. Cells were harvested after centrifugation at 8000 rpm for 15 minutes at 4^{o}C. The cell pellet was suspended in 30ml of 50mM Tris-HCl buffer (pH 8.0) containing 300mM sodium chloride, 0.1% Triton-X, 1mM Imidazole and sonicated on ice for 30 minutes using sonicator (Vibra cell, Model No: VCX 130). Sonication program for 2L pellet is following; 15sec on, 25% amplitude, and 10 sec off for 25 minutes. The sample was then centrifuged at 14,000 rpm for 20 minutes at 4^{o}C to remove insoluble debris. The soluble fraction was mixed with 2ml of Ni-NTA resin pre-equilibrated with the same buffer and allowed to rotate in rotary shaker for 4h at 4^{o}C to allow

the protein to bind to the resin. The sample was then centrifuged at 1000 g for 5 minutes at 4^{o}C and the washing is carried out in 3 steps with 25ml wash buffer in each step (50 mM Tris, pH8.0, 350 mM NaCl, 0.1% Triton-X and 5 mM Imidazole). Centrifugation was carried out at 1000g for 4 minutes at 4^{o}C. Ni-NTA beads bound protein was transferred to poly prep columns (BIORAD). Protein was eluted by passing elution buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 400 mM Imidazole) to the same column. First elute was collected in 1.5 ml of elution buffer. Likewise, 4 more elutes were collected. All elutes were checked in 12.5% SDS-PAGE. Purification of His tag UCHL1 protein through Ni+-NTA Affinity purification:

3.14. Purification of His tag Ubiquitin protein through Ni^{+}-NTA Affinity purification: Before protein purification, His-tag proteins were first checked by SDS-PAGE for overexpression as described in section 3.12. Purification of C-terminal His-tag Ubiquitin was carried out in one step by affinity purification using Ni-NTA chromatography. For this, few colonies of BL21, transformed with specific recombinant plasmid DNA, were inoculated into 200 ml LB-broth with 100μg/ml ampicillin and grown overnight on incubatory shaker at 37^{o}C at 200 rpm as "pre-inoculum". The grown culture was 10-fold diluted into fresh 2L TB-broth with 100 μg/ml ampicillin. Further incubation was carried out at 37^{o}C at 200 rpm for 3 hrs, when O.D$_{600}$ of culture reached to 0.5, the culture was induced with 1mM IPTG for 5 hours at 30^{o}C at 200 rpm. Cells were harvested after centrifugation at 8000 rpm for 15 minutes at 4^{o}C. The cell pellet was suspended in 30ml of 50mM Tris-HCl buffer (pH 8.0) containing 300mM sodium chloride, 0.1% Triton-X, 1mM Imidazole and sonicated on ice for 30 minutes using

sonicator (Vibra cell, Model No: VCX 130). Sonication program from 2L culture pellet is following; 15sec on, 30% amplitude, and 10 sec off for 35 minutes. Lysate was then centrifuged at 14,000 rpm for 20 minutes at 4^{o}C to remove insoluble debris. Supernatant (soluble fraction) was collected and heated at 70°C for 10mins. Since, ubiquitin is heat stable protein. This step caused denaturation of heat unstable soluble proteins of bacteria which on centrifugation at high speed of 14000rpm form insoluble aggregate while heat stable soluble proteins remain in the scoop and thus reducing the chances of nonspecific contamination of purified Ubiquitin. After heating, solution was kept for centrifugation at 14,000 rpm for 20min. The soluble fraction was mixed with 2ml of Ni-NTA resin pre-equilibrated with the same buffer and allowed to rotate in rotary shaker for 4h in a cold chamber at 4^{o}C to allow the protein to bind to the resin. The sample was then centrifuged at 1000 g for 5 minutes at 4^{o}C and the washing is carried out in 3 steps with 25ml wash buffer in each step (50 mM Tris, pH8.0, 350 mM NaCl, 0.1% Triton-X and 5 mM Imidazole). Centrifugation was carried out at 1000g for 4 minutes at 4^{o}C. Ni-NTA beads bound protein was transferred to poly prep columns (BIORAD). Protein was eluted by passing elution buffer (10 mM Tris, pH 8.0, 100 mM NaCl, 400 mM Imidazole) to the same column. First elute was collected in 1.5 ml of elution buffer. Likewise, 4 more elutes were collected. All elutes were checked in 12.5% SDS-PAGE.

3.15. Purification of GST-tag Jab1 Protein: GST tag Jab1 was checked for overexpression as mentioned in section 3.11 and purified by GST-glutathione affinity purification method. Recombinant plasmid DNA showing over expression of recombinant GST fusion protein in expression analysis were

transformed into BL21 (DE3) pLysS cells as mentioned in section 3.8(II). 270ml LB broth with amp100µg/ml was grown for preinoculum, as described in section 3.13 and inoculated to 2.7L of TB containing amp of concentration100µg/ml. Following induction, cells were pelleted at 8000 rpm, 15min, 4°C. Pellet was stored at-86°C. Pellet was resuspended in 10ml of Extraction buffer containing the protease inhibitor tablet "cOmplete" (Roche , REF 11836170001). Dissolved pellet was sonicated as described in section 3.13. A 20µl aliquot of cell lysate was store at -86°C Cell lysate was centrifuged at 14000 rpm, 20 min at 4°C to separate soluble and insoluble fraction. Supernatant was collected in 50ml falcon tube. A 20µl aliquot of supernatant and insoluble fraction was stored for sds-page analysis to identify the presence of fusion protein in the fractions. 800µl of glutathione sepharose 4B slurry was added to the supernatant and kept it for binding to at 4°C cold chamber on rotary shaker for 2hrs. The the tube was centrifuged at 500g for 5min at 4°C to sediment the protein bound glutathione matrix. Supernatant was collected as flow through (unbound fraction) in a 50 ml falcon tube. A 20µl aliquot of flow through was also collected for sds-page analysis. Beads settled on the bottom of tube were washed with 10ml wash buffer for 10 min on rotary shaker at 4°C and then centrifuge at 500g/4 min/4°C. The supernatant (the unbound fraction) was collected by gentle pipetting. Washing step was repeated three times. Glutathione beads bound to protein were transferred to 2ml tube. 1.0 ml of elution buffer (50mM Tris (pH 8.0), 150mM NaCl, 0.05% TritonX, 10mM reduced glutathione, 10% glycerol) was added to beads and kept on rotary shaker for 25 min. To elute the protein, beads were centrifuged at 500g/5min 4°C and supernatant was collected. This process was repeated two more times and total of

3 elutions of 1ml each were collected. Aliquot 5µl of each elution was analysed by SDS-PAGE, as described in section 3.11. Eluted proteins were stored at -20°C.

3.16. Establishment of *in vitro* assay for UCHL1 Activity: Deubiquitinase activity of UCHL1 was examined *in vitro* by using an Ubquitin substrate. Ubiquitin with eight amino acids C-terminal extension peptide (Lys-His-His-His-His-His-His-Cys) was designed as UCH-L1 was reported to cleave the bond present between C-terminal glycine of Ubuiqitin and a short peptide, preferably starting with lysine. Therefore, the peptide substrate was designed with lysine after the last amino acid of Ub (Glycine) followed by six His and a Cys. This unique Ub C-terminal extension peptide would help in Ni-NTA purification of ubiquitin (Figure 14). The C-terminal eight amino acids (containing the Lys^{77}, 6x His and Cys^{83}) was added to ubiquitin sequence during cloning using a specific PCR primer (Table1: Ub-HindIII-Antisense primer sequence). The *in vitro* assay of UCHL1 deubiquitinating activity on Ubiquitin C-terminal peptide substrate was performed by using 15.0µM (48µg) of UCHL1 and 100µM (50µg) of Ubiquitin in a 50µl reaction in 5µl assay buffer. Reaction mix was incubated overnight at 30°C. Identical reaction was also carried out with catalytic mutant of C90A UCH-L1 as control. Activity of wild type UCH-L1 and the mutant UCH-L1 on the Ub C-terminal extension peptide was analyzed by gradient SDS PAGE gel (4-15%) from BIORAD.

3.17 *In vitro* Assay for analysis of Jab1 interaction effect on deubiquitinating activity of UCH-L1:

To examine the effect of Jab1 interaction with UCH-L1 on its deubiquitinating activity an *in vitro* time course and concentration dependent assay were

performed. For time course assay, four reactions of final volume 50µl reactions were set up. Each reaction contained 20µl (6µg) of Jab1, 5µl of UCHL1 (14.3µg), 5µl (12.5µg) of Ubiquitin substrate and 5µl of 1X activity assay buffer and 15µl of water and incubated at 30°C temperature. Control reactions without Jab1 were also set up of final volume 50µl. At each mentioned time point, one of the reactions and one of control were taken out and reaction were stopped by adding 3X –SDS page loading dye and boiled for 10 mins. To examine the JAB1 effect on UCH-L1 deubiquitinase activity in concentration dependent manner, we gradually increased JAB1 amount. Four reactions were set each having 5µl of U8CHL1, 5µl of Ubiquitin and 5µl of 1x activity assay buffer but different concentration of JAB1 starting from 0µl,5µl,10µl and 20µl. Final volume of each reaction was maintained by water to 50µl. Reactions were incubated for 2hrs at 30°C.After 2hrs reaction were stopped by adding 3X SDS page loading dye and boiled for 10 mins on dry bath. Results were analyzed by SDS page gel electrophoresis using pre-casted gradient gels (4-15%).

3.18. *In vitro* assay of UCHL-1 with Ubiquitin-AMC:

UbAMC was dissolved water to make stock solution of final concentration of 100 µM. UCH-L1 (10nM) and UbAMC (5µM) were mixed in reaction buffer (10 mM Tris•HCl, pH 8.0) was added to initiate the enzymatic reaction (100 µL final volume). For Jab1 analysis XXµg of Jab1 was added. The rate of AMC cleavage was monitored at 25 °C with a Molecular Devices microplate spectrofluorometer with excitation at 340 nm and emission at 440 nm.

3.19 Media and Solutions: De-ionized water was used for all buffers solutions and media.

Media	Composition
LB media	1%Tryptone w/v, 0.5% w/v Yeast Extract
TB media	12g Tryptone , 24 g Yeast Extract 4ml glycerol in 1L
SOC media	2.0% Tryptone w/v, 0.5 % w/v Yeast extract.

3.20 Other solutions:

Isopropyl β-D-1-thiogalactopyranoside (1M):IPTG 4.7g, H_2O 20ml.

20% SDS: SDS (20g), H_2O (80ml). Make final volume 100 ml with stirring.

Store at room temperature.

20% Ammonium per Sulphate: APS (0.2g), H_2O (0.8 ml).

General buffers used in the study:

Buffers	Composition
TE (Tris-EDTA) 50X	1mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0. A 10X stock solution was routinely used to prepare 1X TE.
SDS-PAGE Resolving gel buffer	1.5 M Tris-HCl, (for Resolving Gel) ,pH 8.8
SDS -PAGE Stacking gel buffer	0.5 M Tris-HCl (for stacking gel), pH 6.5
10X SDS-PAGE gel running buffer	Tris base 30.3g, Glycine 144g, SDS 10g (add last) , makefinal volume to 1000ml.
1X Gradient gel running buffer	25mM Tris Base**,** 192mM Glycine**,** 0.1 % (w/v) SDS, methanol 20%.
3X SDS-PAGE loading dye (10ml)	1M Tris-Cl, pH 6.5 (2.4 ml), 20% SDS (3 ml), Glycerol (100%) (3 ml), β-mercaptoethanol (1.6 ml), Bromophenol blue (0.006g).
SDS-PAGE staining solution.	Coomassie blue R250- 0.4 % w/v, Methanol- 30 % v/v, Acetic acid 10 %v/v
SDS-PAGE destaining solution	Methanol- 30 % v/v, Acetic acid 10 %v/v.
UCHL1 10X assay Buffer	200mM Tris-Cl (pH-8.0), 5mM EDTA, 50mM DTT, 40% glycerol.

Protein Purification Buffers

For Ni^+-NTA purification:	
Extraction Buffer	50 mM Tris (pH 8.0), 300mM NaCl**,** 0.1% TritonX**,** 1mM imidazole.
Wash buffer	50 mM Tris (pH 8.0), 350mM NaCl**,** 0.1% TritonX**,** 5mM imidazole.
Elution Buffer	50 mM Tris (pH 8.0), 100mM NaCl**,** 0.1% TritonX**,** 250mM imidazole.
For GST Purification:	
Extraction Buffer	50mM Tris (pH 8.0), 550mM NaCl.
Wash Buffer	50 mM Tris (pH 7.4), 1M NaCl.
Elution Buffer	50mM Tris (pH 8.0), 150mM NaCl**,** 0.05% TritonX**,** 10mM reduced glutathione, 10% glycerol.

Results and Discussion *Chapter* 4

4.1. Cloning of human UCHL1: Full length UCH-L1 was PCR amplified using pEGFP-C3-hUCHL1 (a generous gift from Kerstin Brinkmann, Kashkar lab CECAD, Institute for Medical Microbiology ,Immunoloogy and hygiene ,Germany) as template and specific primers (UCHL1-BamHI-Sen and UCHL1-SalI-HindIII-Anti; Table 1). PCR amplification was examined by agarose gel electrophoresis and ethidium bromide staining. The expected PCR amplification of size 672 bp was observed. The positive clone was identified due to slower mobility of the plasmid carrying UCH-L1 DNA to plasmid vector pRSETA.

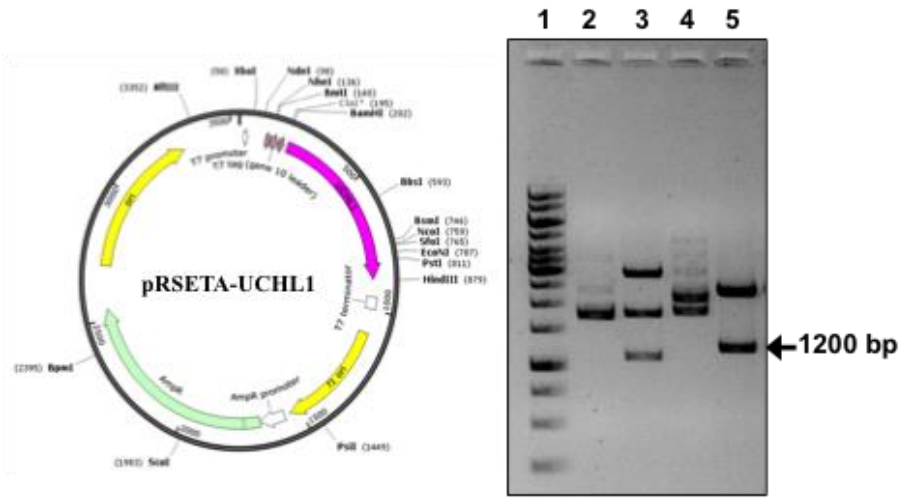

Figure 1. Restriction analysis of pRSETA-UCHL1 plasmid for conformation of positive clones. Lane 1, 1kb Dna Marker; lane 2, pRSETA vector; lane 3, NcoI and ScaI digested pRSETA vector; lane 4, pRSETA-UCHL1; lane 5:NcoI and ScaI digested prsetA-UCHL1. NcoI and ScaI site are present within UCH-L1 gene and pRSETA-vector, respectively.Thus, digestion with these enzymes release a fragment of 1200 bp.

To confirm the presence of UCH-L1 in right orientation, restriction analysis of the slow moving clones was performed using enzymes NcoI and ScaI. Insert of size 1200 bp confirmed the presence of UCH-L1 gene in correct orientation (Fig. 1A). A complete plasmid map of pRSET-UCHL1 is shown in Fig 1B.

4.2. Expression of recombinant human UCHL1 in bacteria. For expression analysis pRSET-UCH-L1 plasmid were transformed into *E. coli* BL21-DE3 (plysS) strain and checked for over-expression (Fig. 2). Over expression of protein was confirmed by visualizing appropriate protein in the total cell extract (Fig. 2)**.**

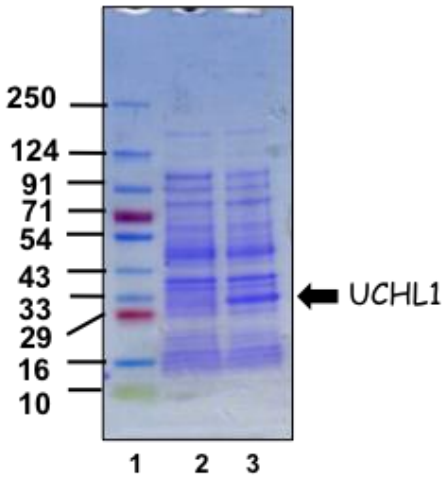

Figure 2. Expression of Recombinant UCHL1 in *E. coli*. Lane 1:Protein molecular wt marker; lane 2, total cell extract; lane 3, IPTG-Induced total cell extract showing overexpression of UCHL1

4.3. Purification of UCHL1: UCHL1 was expressed as N-terminal His-tag fusion protein and purified by Ni^{+}-NTA affinity purification. The samples were analyzed on 12.5 % SDS-PAGE (**Fig. 3**). During purification most of the protein was eluted in the first fraction and the yield was 2.8571mg/ml of UCH-L1 as analyzed by standard BSA quantification gels (**Fig. 7**).

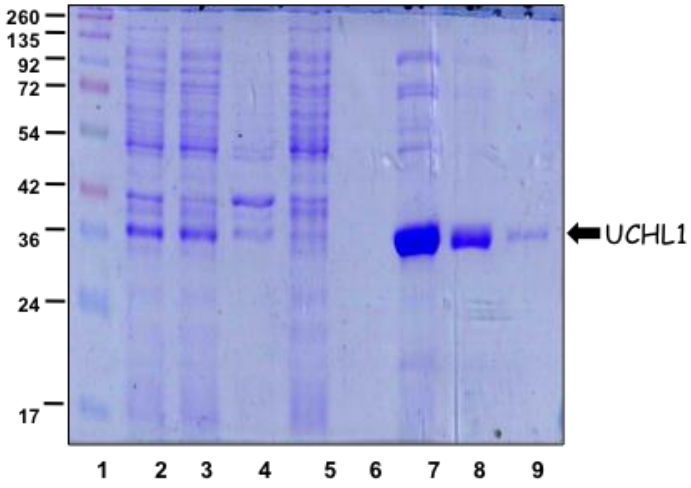

Figure 3. Affinity purification of His-tag UCHL1. Lane 1, Molecular wt marker; lane 2, total cell extract; lane 3, soluble fraction; lane 4, insoluble fraction; lane 5, unbound flow-through fraction; lane 6, wash fraction; Lane 7-9, fraction eluted with 250mM imidazole.

4.4. Site directed mutagenesis: To study the enzymatic role of UCH-L1, the key residue (Cys90) from catalytic domain were mutated by PCR mediated site directed mutagenesis. Cys codon 'TGT' was mutated to Ala codon GCT. The designed oligonucleotides were complementary to the site of mutation with 19 nucleotides flanking on both side non-complementary sequences (Table 4). The mutation was confirmed by sequence determination (**Fig. 5**).

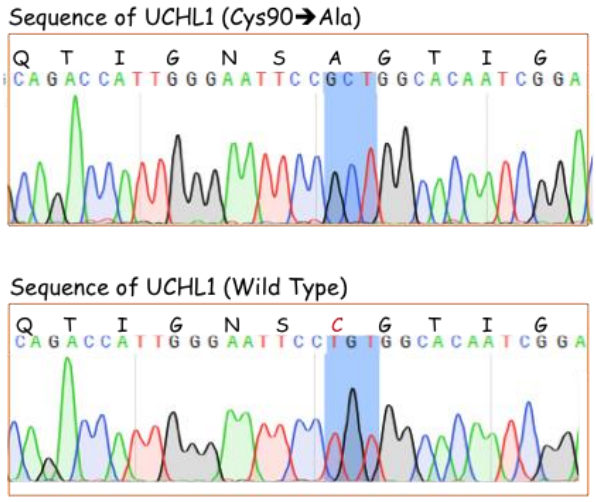

Figure 5. Confirmation of C90A mutation in pRSET-A UCH-L1 by sequencing.

4.5. Purification of mutant UCHL1C90A: UCHL1C90A was expressed as N-terminal His-tag fusion protein and purified by Ni^{+}-NTA affinity purification. The samples were analyzed on 12.5 % SDS-PAGE (**Fig. 6**). During purification most of the protein was eluted in the first fraction and the yield was 2.8571mg/ml of UCH-L1 as analyzed by standard BSA quantification gels

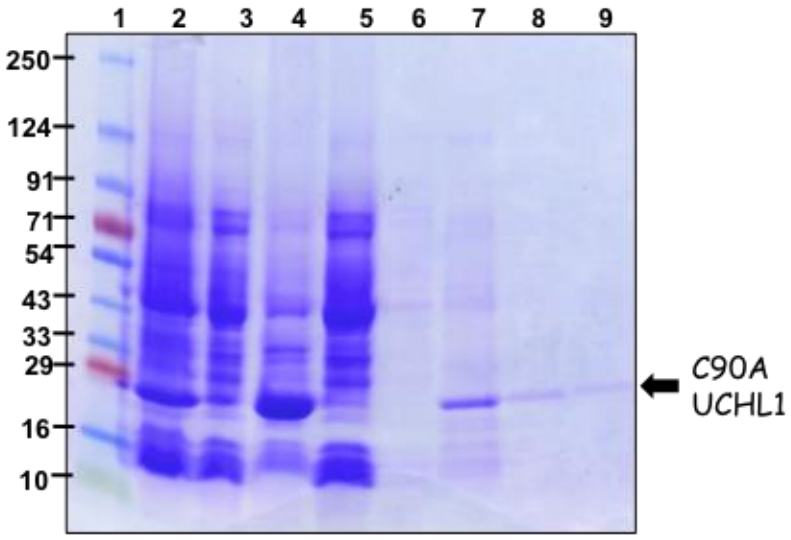

Figure 6. Affinity purification of His-tag C90A UCHL1. Lane 1, Molecular wt marker; lane 2, total cell extract; lane 3, soluble fraction; lane 4, insoluble fraction; lane 5, unbound flow-through fraction; lane 6, wash fraction; Lane 7-9, fraction eluted with 250mM imidazole.

4.6. Cloning of Ubiquitin: Full length human ubiquitin DNA was PCR amplified using pET21b-Ubq (produced in own lab) as template and result of amplification was examined by 1.0% agarose gel electrophoresis using 1µg/ml ethidium bromide to allow visualization of the DNA under UV light. The expected PCR amplification of size 204bp was observed. The full length UCHL1 PCR product and plasmid pRSETA were sequentially digested by NdeI and BamHI. Plasmid carrying ubiquitin was identified as this moving slower compared to negative control (pRSETA) when analyzed on agarose gels. To confirm that the ubiquitin

is present in right orientation, restriction digestion was carried out with enzymes NdeI and BamHI. The insert release of 204 bp confirmed the presence of UCH-L1 gene in correct orientation.

4.7. Expression of Ubiquitin: For expression analysis, pRSET-Ubiquitn plasmid were transformed into *E. coli* BL21-DE3 (plysS) strain and checked for their over-expression. When total cell extracts were analyzed by SDS-PAGE, over expression of ubiquitin was confirmed (**Fig. 8A**).

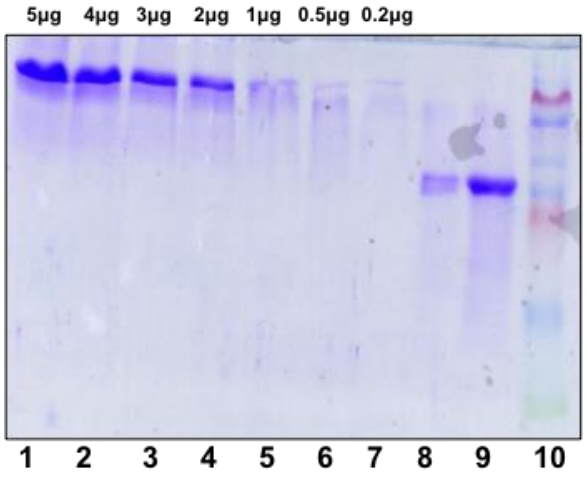

Figure 7: BSA Gel for quantification of proteins.

4.8. Purification of Ubiquitin: Since the ubiquitin constructs has the C-terminal His tag, they were purified by Ni-NTA affinity purification. Due to heat-stable nature of ubiquitin, total extract was heated and most of the *E.coli* protein was denature except ubiquitin. When the eluted fractions were analyzed on 4-15 % gradient gel by SDS-PAGE (**Fig 8B**), we observed purified ubiquitin protein.

4.9. *In Vitro* Assay for UCHL1 Activity: Deubiquitinating activity of UCHL1 was examined *in vitro* by using an Ubiquitin substrate. Ubiquitin with eight amino acids C-terminal extension peptide (Lys-His-His-His-His-His-His-Cys)

was designed as UCH-L1 substrate. UCHL1 mediated peptide cleavage was analyzed by gradient SDS PAGE gel (4-15%) from BIORAD. The reaction mixture with wild type UCHL1 resulted two bands of the substrate: Ub C-terminal extension peptide (9.6kDa) and a band of cleaved ubiquitin substrate (devoid of peptide extension) of (8.5kDa). As expected, the mutant UCH-L1 could not cleave Ub C-terminal extension peptide and thus resulted to a single band (**Fig. 9**).

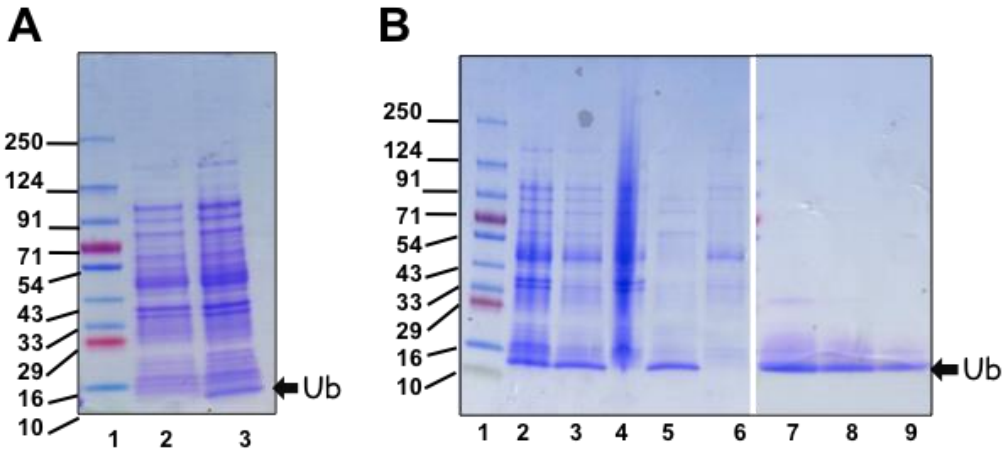

Figure 8. Expression of Recombinant Ub in *E. coli*. and affinity purification of His-tag Ub A. Expression of Ub. Lane 1:Protein molecular wt marker; lane 2, total cell extract; lane 3, IPTG-Induced total cell extract showing overexpression of Ub**. B.** Purification of Ub. Lane 1, Molecular wt marker; lane 2, total cell extract; lane 3, soluble fraction; lane 4, insoluble fraction; lane 5, unbound flow-through fraction; lane 6, wash fraction; Lane 7-9 fraction eluted with 250mM imidazole

4.10. *In vitro* Assay for UCHL1 Activity with Ub-AMC: UCH-L1 catalyze the hydrolysis of ubiquitin-AMC (Ub-AMC, 7-amido-4-methylcoumarin C terminus derivative of ubiquitin). Upon formation of the acyl-enzyme intermediate, AMC is released from the ubiquitin C-terminus. The free AMC exhibits enhanced fluorescence, and the rate of AMC release was monitored by fluorescence spectroscopy (λ_{em} 440nm, λ_{ex} 340nm). In each reaction, 10nM of UCH- L1 was

reacting with 5µM of Ub-AMC substrate for 1h.

4.11. Cloning of *JAB1*: Full length Jab1 was PCR amplified and the amplification was examined by 0.8% agarose gel electrophoresis. The expected PCR amplification of size 1005 bp was observed. Plasmid carrying Jab1 DNA was moving slower compared to negative control (pGex6p1). To confirm the presence of JAB1 in right orientation, restriction analysis of the slow moving clones was performed using enzymes BamHI and SalI. The insert release of 1005 bp confirmed the presence of Jab1 gene in correct orientation.

4.12. Purification of Jab1: For expression analysis Pgex6p-JAB1 plasmid were transformed into *E. coli* BL21-DE3 (plysS) strain and checked for their over-expression by SDS-PAGE and coomassie blue staining (**Fig. 11**). Jab1 was expressed as N-terminal GST fusion protein and purified using Glutathione-sepharose affinity matrix (**Fig. 12**). Purification yielded .85mg/ml of UCH-L1 in Elute1 as analyzed by standard BSA quantification.

4.13. *In Vitro* assay to study effect of Jab1 on deubiquitinating activity of UCH-L1: As reported in literatures that Jab1 interacts with UCHL1 but the effect of this interaction is still unknown. To examine the effect of Jab1 interaction with UCH-L1 on its deubiquitinating activity, *in vitro* assay were performed. We added 14.3 µg (5µl) of UCHL1, 12.5µg of Ubiquitin with 6µg (20µl) of Jab1 and 5µl of 1X assay buffer. Total reaction volume was made 50µl and incubated the reaction at 30°C in the presence of for 8h. Control reactions without Jab1 were also set up simultaneously. At 2, 4, and 8h, 50µl aliquots from this reaction and control were taken out and stopped by adding SDS page loading dye and boiled for 10 minutes and analyzed by SDS-PAGE gel electrophoresis. As shown in **Fig.**

13, at 0h there was no substrate cleavage, as no band of cleaved peptide was observed on the gel. Interestingly, at all the time points, especially at 2h time point, it was observed that reaction without Jab1 had more cleavage compared to the reaction with Jab1 (**Fig. 13**). These results suggest that presence of Jab1 prevents UCH-L1 protease activity.

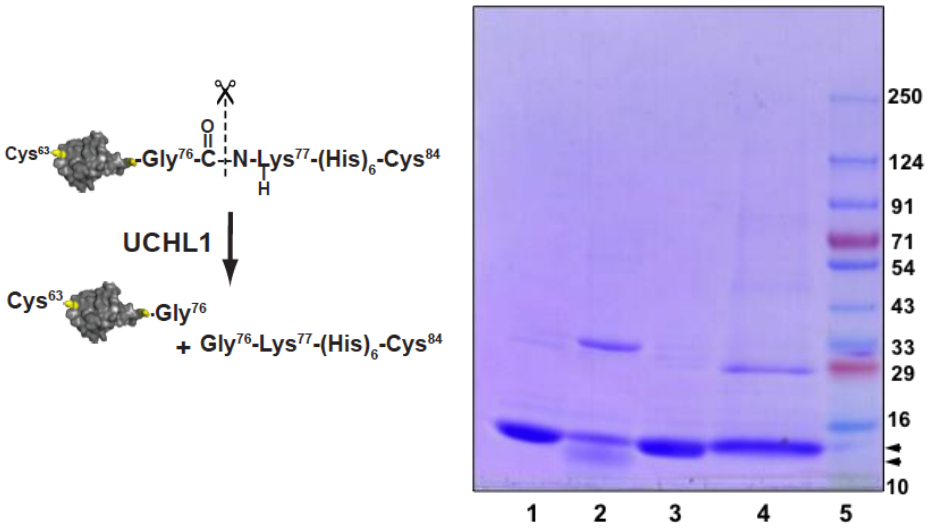

Figure 9. UCHL1 assay using Ub-C-terminal peptide as substrate. Ub-C-terminal substrate was incubated with UCH-L1 and analyzed by SDS-PAGE. Lane 1 and 3, Ub--C-terminal substrate without UCH-L1; lane 2, UCH-L1 with Ub substrate; lane 4, C90A UCHL1 with Ub-C-terminal substrate; lane 5, protein Mw marker. Arrows indicate the position of the cleaved substrate.

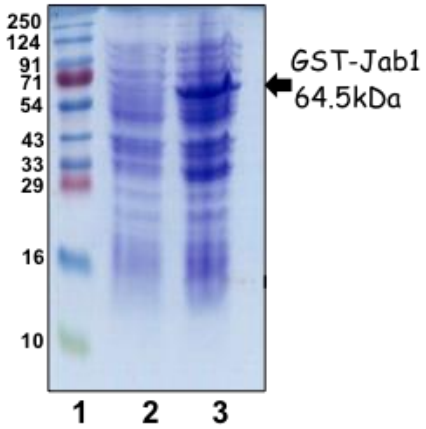

Figure 10. Expression of Recombinant Jab1 in *E. coli*. Lane 1:Protein molecular wt marker; lane 2, total cell extract; lane 3, IPTG-Induced total cell extract showing overexpression of UCHL1

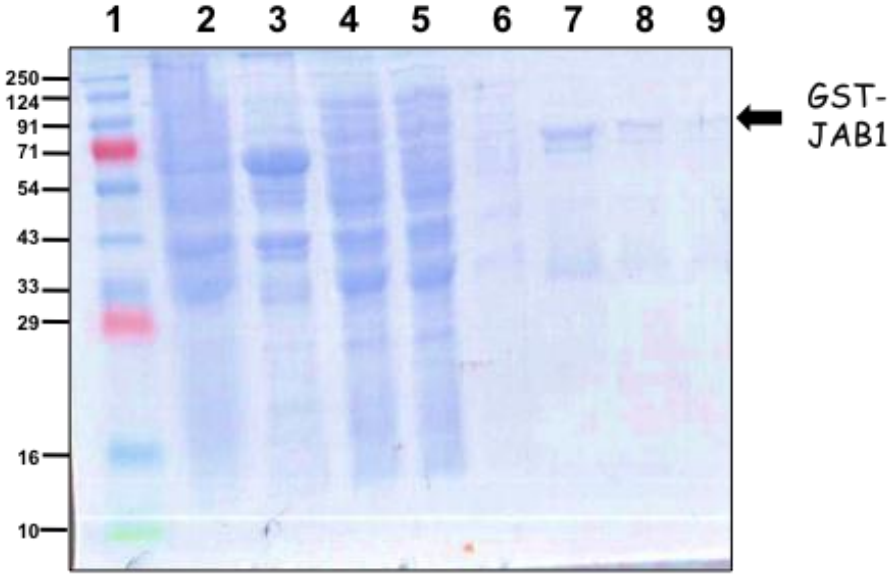

Figure 11. Affinity purification of GST-tag Jab1. Lane 1:Protein molecular wt marker; lane 2, total cell extract; lane 3; soluble fraction; lane 4, Insoluble fraction Lane 5, unbound flowthrough fraction; lane 6 wash fraction; lane 7-9, elution fractions

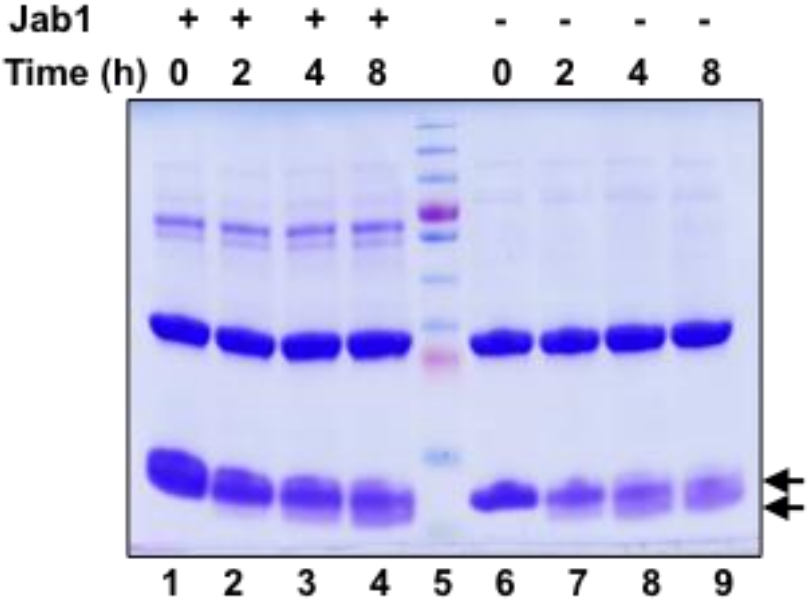

Figure 13. Cleavage activity of recombinant UCHL-1 protein is regulated by Jab1. Purified GST-UCHL1 was incubated with Ub substrate in presence (lanes 1-4) and absence (lanes 6-9) of Jab1. Samples were analyzed after 2, 4 and 8 hours to detect cleavage of Ub substrate; lane 5, molecular weight markers. The position of cleaved proteins are shown by arrows.

4.14. *In Vitro* Ub-AMC assay to study effect of Jab1 on deubiquitinating activity of UCH-L1: When Jab1 (18µg; 2.7nM) was incubated with UCHL1 (10nM) in presence of 5µM of Ub-AMC, it clearly inhibited AMC release, suggesting inhibitory role of Jab1 (Fig14). Together, these results suggest that UCHL1 deubiquitinating activity on our designed ubiquitin C-terminal extension peptide substrate and also on Ub-AMC substrate is transiently inhibited by the presence of JAB1. The plausible explanation of such transient inhibition effect can be the location of Jab1 binding region on UCHL1. The Jab1 interaction motif is present on UCHL1 at a loop3 which is also covers residues present in Ubiquitin binding domain (P-site) of

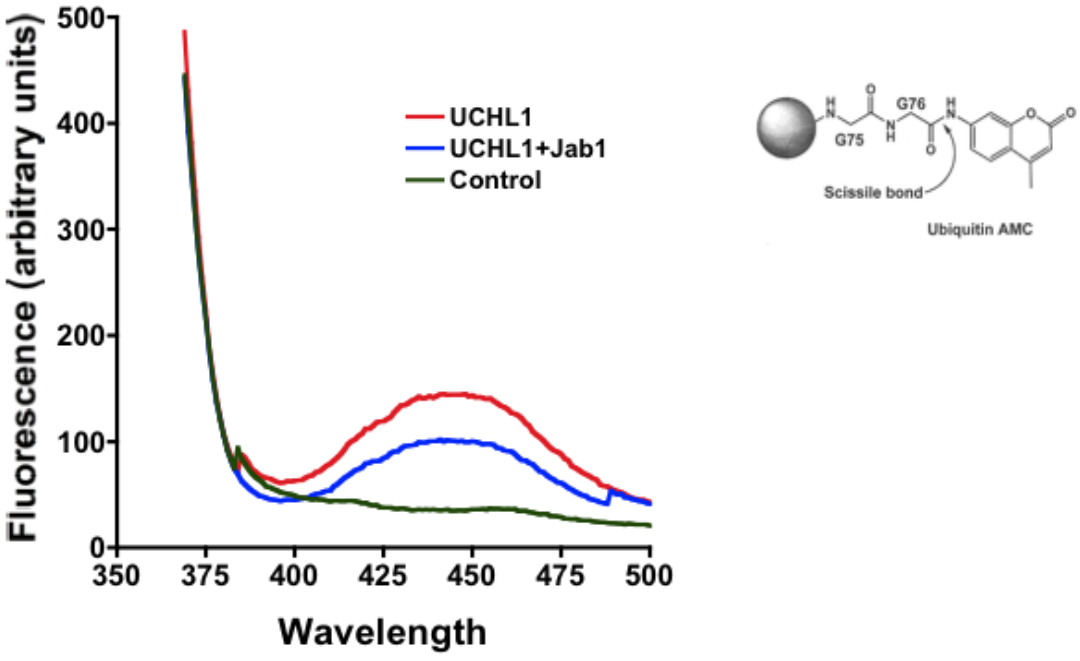

Figure 14. Effect of Jab1 on ubiquitin-AMC cleavage by UCHL1. Ubiquitin-AMC (5µM) was incubated with wild-type UCHL1 or UCHL1 and Jab1. Cleavage of ubiquitin-AMC was measured after 1h by fluorescence (340 nm excitation/440 nm emission).

UCHL1. Since structure of UCHL1 shows that it undergoes a conformational change upon binding of substrate and then become catalytically active it is possible that slight coverage of ubiquitin binding site by Jab1 binding is resisting UCHL1 to undergo the required conformational change to make it catalytically efficient. It might be also possible that such transient interaction of JAB1 to UCHL1 is promoting Jab1 mediated phosphorylation or any other post transcriptional modification in UCHL1 since Jab1 is known to have kinase activity associated with it. Or, it is also possible that binding of Jab1 inducing some conformational change in UCHL1 and thus Jab1 cannot interact further with UCHL1. Indeed further studies are needed to solve the enigma of transient inhibitory effect of JAB1 interaction on UCHL1 which will allow to better understanding of UCHL1 role in signaling pathways and will also be very helpful to understand the regulation of UCHL1 activity inside the cell.

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