$E3^{Histone}$/LASU1, a 500 kDa Novel Multi-functional Ubiquitin Protein Ligase

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Abstract

During spermatogenesis histones must be degraded in late round and early elongating spermatids to permit chromatin condensation. Ubiquitin conjugation is activated and histones are ubiquitinated at this stage, suggesting that histone degradation may be mediated by ubiquitination. The activation of ubiquitin conjugation during spermatogenesis is dependent on the ubiquitin conjugating enzyme (E2) UBC4. We therefore studied whether histones are ubiquitinated by a UBC4 dependent ubiquitin protein ligase (E3) during spermatogenesis. $E3^{Histone}$ was identified by a biochemical screen and purified to near homogeneity. Mass spectrometry identified $E3^{Histone}$ as LASU1, a 482 kDa HECT domain protein and $E3^{Histone}$ conjugates ubiquitin to all core histones *in vitro*. UBC4-1 and UBC4-testis were the preferred E2s for $E3^{Histone}$-dependent ubiquitination of histones. $E3^{Histone}$ was the major UBC4-1 dependent histone ubiquitinating E3 in testis. Anti-LASU1 antibody immunodepleted $E3^{Histone}$ activity. Immunohistochemistry showed that $E3^{Histone}$/LASU1 was predominantly expressed in nuclei from spermatogonia to mid-pachytene cells, but not detectable in spermatids. Histones are also ubiquitinated in spermatocytes. $E3^{Histone}$/LASU1 was widely expressed in different mouse tissues. It was mainly expressed in the cytoplasm in most tissues, except in neurons of the brain and in early germ cells of the testis where it was expressed in the nucleus. In most tissues, $E3^{Histone}$/LASU1 was expressed in epithelia. The wide expression of $E3^{Histone}$/LASU1 suggests the existence of substrates of this E3 other

than histones. Indeed, our assays showed that *in vitro* purified $E3^{Histone}$ stimulates polyubiquitination of Mcl-1, a BH3 region containing antiapoptotic protein. $E3^{Histone}$ may therefore regulate cell apoptosis by mediating degradation of Mcl-1. Since $E3^{Histone}$/LASU1 was found previously to affect gene transcription and histone monoubiquitination is known to regulate gene transcription, we also evaluated the role of $E3^{Histone}$/LASU1 in histone ubiquitination in somatic cells. Depletion of $E3^{Histone}$/LASU1 protein by siRNA did not affect the levels of free or ubiquitinated histones. In summary, $E3^{Histone}$/LASU1 is a novel multi-functional protein that may mediate histone ubiquitination during meiosis and may be involved in apoptosis by triggering Mcl-1 degradation. Its wide expression and large non-catalytic region indicate that there are likely many other substrates of $E3^{Histone}$/LASU1.

Résumé

Durant la spermatogenèse, les histones doivent être dégradées aux derniers stades de différentiation des spermatides ronds et les premiers stades des spermatides en allongement afin de permettre la condensation de la chromatine. L'activation de la conjugaison de l'ubiquitine et l'ubiquitination des histones se produisant au cours de cette période, suggèrent que la dégradation des histones pourait être entraînée par l'ubiquitination. La conjugaison de l'ubiquitine durant la spermatogénèse est dépendante de UBC4, une enzyme de conjugaison de l'ubiquitine (E2). Nous avons donc tenté de déterminer si les histones sont ubiquitinées par une ligase d'ubiquitine (E3) dépendante d'UBC4, durant la spermatogénèse. Une $E3^{Histone}$ a été identifiée par un criblage biochimique et purifiée presque à homogénéité. La spectrométrie de masse a permis d'identifier cette $E3^{Histone}$ comme étant la protéine LASU1, une protéine de 42kDa comportant un domaine HECT. De plus, la protéine $E3^{Histone}$ conjugue l'ubiquitine à toutes les histones, *in vitro*. UBC4-1 et UBC4-testis sont les E2 de préférence pour l'activité de liaison de l'ubiquitine aux histones dépendante de la protéine $E3^{Histone}$ tandis que $E3^{Histone}$ est la principale E3 impliquée dans l'ubiquitination des histones dépendante de UBC4-1 dans les testicules. L'anticorps contre LASU1 permet l'immuno-suppression de l'activité $E3^{Histone}$. Les études d'immunohistochimie ont démontré que $E3^{Histone}$ /LASU1 est exprimée de façon prédominante dans le noyau des cellules du stade de spermatogonies jusqu'au stade mi-pachytène mais n'est pas

détectée dans les spermatides. Les histones sont aussi ubiquitinées dans les spermatocytes. $E3^{Histone}$ /LASU1 est largement exprimée dans différent tissus murins. Elle est principalement exprimée dans le cytoplasme de presque tous les tissus à l'exception des neurones, du cerveau et des cellules germinales précoces des testicules où elle est exprimée dans le noyau. Dans la majorité des tissus, $E3^{Histone}$ /LASU1 est exprimée dans l'épithélium. L'expression généralisée de $E3^{Histone}$ /LASU1 suggère l'existence d'autres substrats que les histones pour cette E3. En effet, nos essais *in vitro* ont démontré que la protéine E3 purifiée peut stimuler la poly-ubiquitination de Mcl-1, une protéine anti-apoptotique contenant un domaine BH3. $E3^{Histone}$ peut donc réguler l'apoptose des cellules en provoquant la dégradation de Mcl-1. Puisqu'il a déjà été démontré que $E3^{Histone}$ /LASU1 affecte la transcription génique et que la mono-ubiquitination des histones est connue pour réguler la transcription génique, nous avons aussi évalué le rôle de $E3^{Histone}$ /LASU1 dans l'ubiquitination des histones dans des cellules somatiques. La suppression de la protéine $E3^{Histone}$ /LASU1 par ARN interférant n'a pas affecté le niveau d'histones ubiquitinées ou non-ubiquitinées. En résumé, $E3^{Histone}$ /LASU1 est une nouvelle protéine multi-fonctionnelle qui peut entraîner l'ubiquitination des histones durant la méïose et qui peut être impliquée dans le processus d'apoptose en déclenchant la dégradation de Mcl-1. Son expression généralisé et la présence d'un grand domaine non-catalytique indiquent qu'il y a probablement plusieurs autres substrats pour $E3^{Histone}$ /LASU1.

To my husband and parents

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Contribution of authors

Chapter 2. Text and figures in this chapter are reproduced from the paper "Characterization of $E3^{Histone}$, a Novel Testis Ubiquitin Protein Ligase Which Ubiquitinates Histones" by Zhiqian Liu, Rose Oughtred and Simon S. Wing in *Mol Cell Biol.* 2005 April; 25(7): 2819-2831. Dr. Rose Oughtred determined the substrate specificity and cognate E2s of $E3^{Histone}$. I did all the rest of the work in the paper, including preparation of the manuscript.

Chapter 3. This work is in preparation for publication. The authors are Zhiqian Liu, Dengsheng Miao, Qingwen Xia, Louis Hermo, Simon S. Wing. I performed all the studies in this paper (including preparation of the manuscript) except the EM immunohistochemistry study which was done by Dr. Louis Hermo's laboratory. I did the LM immunohistochemistry studies under the supervision of Dr. Dengsheng Miao, who helped me interpret the results. Qingwen Xia, a technician of Dr David Goltzman, isolated some mouse tissues, helped me prepare the tissue sections and taught me techniques for immunohistochemistry staining. Dr. Louis Hermo supervised my LM immunohistochemistry studies on the testis and helped me interpret the results.

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Abbreviation

AMSH (*A*ssociated *M*olecule with the *SH*3 domain of STAM)
APC/C (*A*naphase *P*romoting *C*omplex or cyclosome)
ARF-BP1 (*ARF* tumor suppressor *b*inding *p*rotein*1*)
EGFR (*E*pidermal *G*rowth *F*actor *R*eceptor)
DUB (*D*e*u*biquitinating *E*nzymes)
DUF 908 (*D*omain with *U*nknown *F*unction)
E1 (ubiquitin activating enzyme)
E2 (ubiquitin conjugating enzyme)
E3 (ubiquitin protein ligases)
E6AP (*E6 A*ssociated *P*rotein)
HECT domain (*H*omologous to *E*6AP *C*-*T*erminus)
hPRC1L (*H*uman *P*olycomb *R*epressive *C*omplex *1 L*ike)
HR6B (*H*uman homolog of *R*ad*6*)
JAMM (*JA*B1/*M*PN/*M*ov34 metalloenzyme)
LASU1 (*La*rge *S*tructure of *U*REb1)
LZ (*L*eucine *Z*ipper)
MHC I molecules (*M*ajor *H*istocompatibility *C*omplex)
MIR1 (the *M*odulators of *I*mmune *R*ecognition)
Mule (*M*cl-1 *u*biquitin protein *l*igase *E*3)
MJD (*M*achado-*J*oseph *D*isease protease)
NEM (*N*-*e*thyl*m*aleimide)
OTU (*O*varian *tu*mor)
PRE (*P*olycomb *r*esponse *e*lement)
SAGA (*S*pt-*A*da-*G*cn5-*A*cetyltransferase)
RING (*R*eally *I*nteresting *N*ew *G*ene)
SCF (*S*kp1–*C*ullin–*F* box) complex
Tom1 (*T*rigger *o*f *m*itosis)
TP (*T*ransition *P*roteins)
Ub (*Ub*iquitin)
UBA (*Ub*iquitin *A*ssociated domain)
UBL (*Ub*iquitin *L*ike Protein)
UCH (*U*biquitin *C*-terminal *H*ydrolase)
UreB1 (*U*pstream *r*egulatory *e*lement *B*inding protein)
USP (*U*biquitin *S*pecific *P*rotease)
VHL ('*v*on *H*ippel-*L*indau')

Chapter 1. Introduction

(Literature review)

1.1. Spermatogenesis

Spermatogenesis is a vital process leading to the formation of mature male gametes, the spermatozoa (Fig. 1). In this complex, but precisely regulated developmental process, stem cell spermatogonia are transformed into highly differentiated mature spermatids. This process can be divided into three phases: a proliferative or mitotic phase, a meiotic phase and the spermiogenic phase (Roosen-Runge 1977; Ewing, Davis et al. 1980; de Kretser, Loveland et al. 1998).

In the first phase, the stem cell spermatogonia divide mitotically to produce proliferative spermatogonia, which further divide into differentiating spermatogonia (Clermont and Bustos-Obregon 1968; Huckins 1971; Ewing, Davis et al. 1980). Spermatogonia can be recognized on the bases of the spherical or oval nuclei, and their localization on the basement membrane of the seminiferous tubule. At the end of the proliferative phase, the most mature spermatogonia divide to form primary spermatocytes.

The second phase is the meiotic phase in which the diploid number of chromosomes present in the primary spermatocytes are reduced to the haploid number present in spermatids. There are two sequential cell divisions in this phase. Cells in prophase of the first meiotic division are located in the middle of the seminiferous epithelium and are characterized by highly condensed chromosomes (Russell 1977; Russell and Frank 1978; Russell 1978). Prophase of the first meiotic division is exceptionally long, lasting about three weeks. The size of the primary spermatocytes and their nuclei progressively increase during prophase (Russell and Frank 1978). Nuclear changes are the morphologic basis for subclassifying primary spermatocytes. In the transition from preleptotene to leptotene, nuclei gradually lose their peripheral chromatin and form condensed chromatin threads. Meiotic prophase begins in the leptotene cells. In zygotene cells, the chromatin

threads have progressed to chromosomes, which begin to pair in the synaptonemal complex. The chromosomes are fully paired in pachytene cells. Crossing over, the genetic recombination, occurs during this period, allowing germ cell chromosomes to possess a unique combination of genetic material distinct from that of the somatic cells of that animal. Pachytene cells become highly synthetic in the middle of their development (Monesi 1965) and grow rapidly in size (Russell and Frank 1978). The following diplotene phase is brief. In this phase, the synaptonemal complex dismisses, allowing chromosomal pairs to separate except at regions known as chiasmata. Diplotene cells are the largest germ cells. Diplotene is the last phase in the prophase of the first meiotic division. Subsequent metaphase, anaphase and telophase of the first meiotic division are completed rapidly. The first meiotic division results in secondary spermatocytes, which are short lived. The second meiotic division follows rapidly, generating haploid spermatids.

The third phase of spermatogenesis is spermiogenesis, the process of remodeling haploid spermatids to form mature spermatozoa. In this process, round spermatids are transformed into elongating spermatids, during which the acrosome gradually forms and the flagellar apparatus develops. The volume of the spermatids is reduced to approximately 25% of its original size before sperm release (Sprando and Russell 1987). Mature spermatid loses almost all of its cytoplasm. The cytoplasmic fragments are phagocytosed by the adjacent Sertoli cells and transported within the cell to the base of the seminiferous tubule where they are digested (Kerr and de Kretser 1974). The nucleus also undergoes dramatic reduction in size. The volume of the mature spermatid is only about 5% of that of somatic cells (Sawada, Ochi et al. 1975). In mature spermatids, chromosomes are extremely compact. This is permitted by the substitution of histones by

Fig. 1

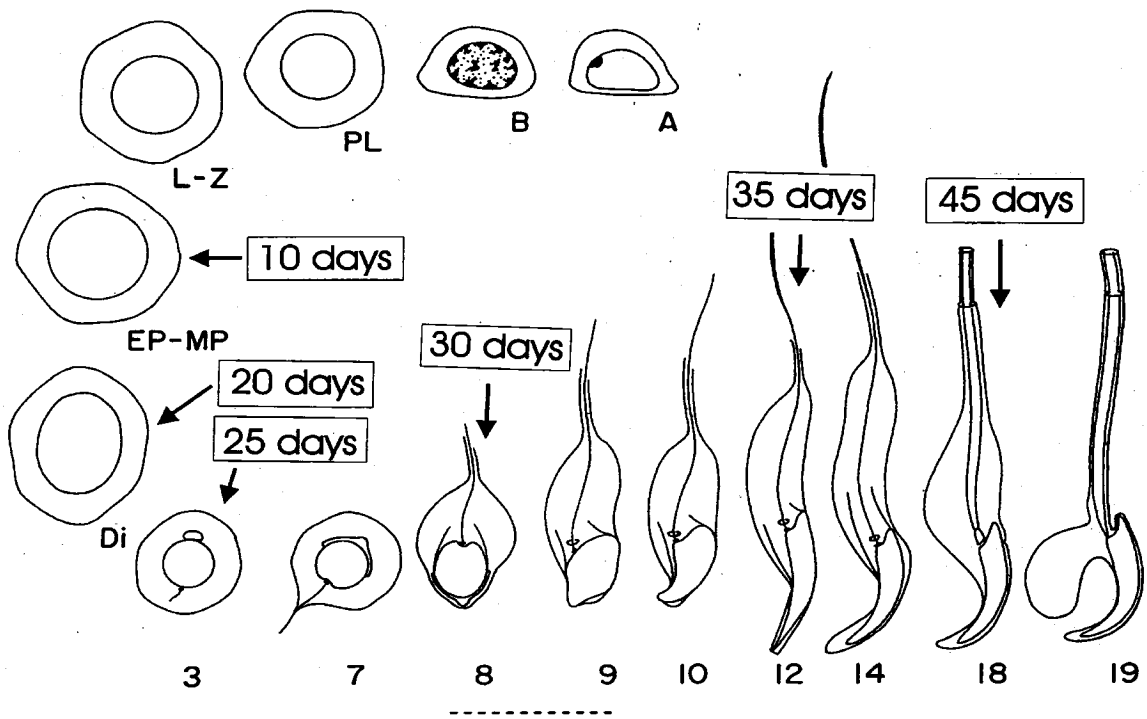

Fig. 1. Schematic representation of the various steps of spermatogenesis in the rat from type A spermatogonia (A) through type B spermatogonia (B) to spermatocytes (PL, preleptotene; L-Z, leptotene to zygotene; EP-MP, early to mid-pachytene; Di, diplotene) to spermatids (steps 3 to 19). The time (days after birth) of the first appearance of the germ cells is indicated. Discontinuous line shows the germ cells in which histones are degraded.

transition proteins and in turn by protamines (Mills, Van et al. 1977; Meistrich 1989). The protamine of E. cirrhosa condenses spermiogenic chromatin in a pattern which comprises fibres with a progressively larger diameter and lamellae that finally undergo definitive coalescence (Gimenez-Bonafe, Ribes et al. 2002). Protamine is not just Arg and Lys rich protein. It is also Cys rich. The effective cross-linking of protamines by disulphide bonds is critical for chromatin condensation. The spermatozoa are released into the lumen of the seminiferous tubule. All these processes take place in the highly organized organ, testis.

1.1.1. Organization of the testis

The testis is covered by a tough fibrous capsule. Each testis is composed of two major compartments: an exocrine part (seminiferous tubules) and an endocrine part (interstitial compartment (interstitium)) (Russell 1990). The testis is divided into lobules by septa consisting of loose areolar connective tissue. Several seminiferous tubules are found in each lobule, and interstitial cells are found in the connective tissue septa surrounding the seminiferous tubules. The interstitial compartment contains the blood and the lymphatic glands. The most abundant cell type in the interstitium is the Leydig cell (Christensen 1975; Mori and Christensen 1980). The Leydig cell is the major source of the androgen, testosterone and various other steroids. This cell possesses abundant smooth endoplasmic reticulum and mitochondria with tubular cristae, both of which contain the enzymes associated with steroid synthesis. The macrophage is another cell type in the interstitium and accounts for about 25% of the cells in the interstitium (Christensen 1975).

The seminiferous tubules are the exocrine portion of the testis producing and excreting spermatozoa (Russell, Saxena et al. 1989). The seminiferous tubules are

convoluted loops that have both ends connected into the beginning of the excurrent duct system (the rete testis) by the straight tubules (tubuli recti) (Fig. 2). The seminiferous tubules are lined by a stratified epithelium that consists of the developing germ cells and supporting cells (Sertoli cells).

Sertoli cells are the somatic cells present amongst and in close contact with the germ cells within the seminiferous tubule (Vilar, Perez Del Cerro et al. 1962; Schulze 1974; Fawcett 1975). Sertoli cells are thought to provide structural and metabolic support to the developing germ cells. A single Sertoli cell extends from the basement membrane to the lumen of the seminiferous tubule. However, its cytoplasm at the distal end is difficult to distinguish at the light microscopic level. Sertoli cells are characterized by the presence of a vesicular, oval, basally positioned nucleus which contains a prominent nucleolus. They are endocrine cells which secrete the polypeptide hormone, inhibin. Inhibin acts at the level of the pituitary to reduce the secretion of follicle stimulating hormone, a glycoprotein gonadotropin that is critical for spermatogenesis. Sertoli cells are also hypothesized to deliver nutrients to germ cells such as lactate, which inhibits apoptosis of germ cells (Vilar, Perez Del Cerro et al. 1962; Mita and Hall 1982). In addition, Sertoli cells phagocytose germ cells that degenerate in the normal course of spermatogenesis or as the result of damage from some deleterious agent or condition. Furthermore, during spermiogenesis, Sertoli cells phagocytose tubulobulbar complexes, a testis specific actin-based adherens junction type (Russell 1980; Russell 1993; Lin, Harman et al. 1997), resulting in elimination of cytoplasm (Russell and Clermont 1976) from the spermatid as well as elimination of the contacts between germ cells and Sertoli cells (Russell, Goh et al. 1988). Finally, the Sertoli cell also phagocytoses the large mass of discarded

Fig. 2

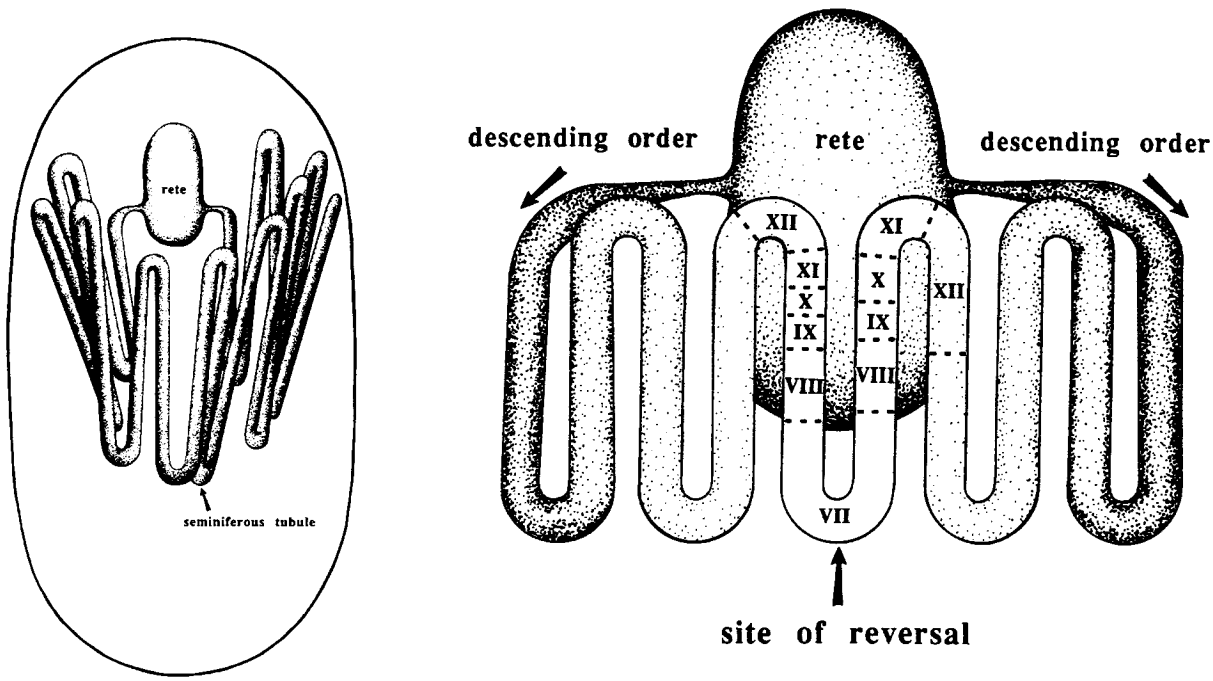

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Fig. 2. The seminiferous tubule. Left panel, the course of one convoluted rat seminiferous tubule and its connections to the rete testis are shown. The tubule joins the rete at both ends. The tubule is convoluted; however, its straightened portions course primarily in the long axis of the testis. The cranial end of this tubule is nearer the surface of the testis than the caudal ends (Russell 1990). Right panel, stages of the seminiferous tubule. Roman numerals indicate the stages. Segments descend in order from the rete. When the end of the series is reached (stage I), the series begins to descend from stage XIV again. The site of reversal marks the meeting place of two patterns of descent.

cytoplasm and organelles (residual bodies) extruded from the spermatid (Kerr and de Kretser 1974).

1.1.2. Stages of spermatogenesis (cycles of seminiferous epithelium)

The stratified epithelium of the seminiferous tubules is composed of different stages of developing germ cells. A cell association or stage is a defined grouping of germ cell types at particular phases of development in cross sectioned tubules. Each cell type of the cell association is morphologically integrated with others in its developmental processes. Each cell association or stage has a constant germ cell composition. In rats, there are fourteen designated cell association or stages (Fig. 3). A complete series of changes in cell associations (stages) arranged in the logical sequence of developmental progression is called the cycle of the seminiferous epithelium. Many studies have been done on staging in the rat (Roosen-Runge and Giesel 1950; Leblond and Clermont 1952; Leblond and Clermont 1952; Clermont and Perey 1957; Clermont and Harvey 1965; Lalli 1972). The important criteria used to determine the stage of the cycle of the seminiferous epithelium in the rat are listed in Table 1. Spermatids are the cell type commonly used to classify stages of spermatogenesis because the less mature spermatids have the most easily recognizable morphological features. Changes in the acrosomal system of the spermatids and their nuclear shape are used to distinguish different spermatids, and thereby to determine the stages of spermatogenesis.

1.1.3. Structure and function of acrosome

The acrosome is a sac of secreted enzymes required for penetration of egg vestments. Acrosomal formation is a slow but continuous process that is not complete until late spermatogenesis (Leblond and Clermont 1952; Lalli and Clermont 1981). Most immature rat spermatids contain no acrosome, but only a perinuclear Golgi apparatus. Shortly after

Table 1

Important Criteria Used to Determine the Stage of of the Cycle of the Seminiferous Epithelium in the Rat

STAGE	CRITERIA
I	No acrosomal system is seen in spermatids by light microscopy; step 1 spermatids are smaller than 2° spermatocytes.
II-III	The stage begins as the proacrosomal granules and/or acrosomal granule are first seen. The acrosomal vesicle has not yet flattened sufficiently to form a hemisphere on the nuclear surface.
IV	The stage begins as the acrosomal vesicle forms a hemisphere on the nuclear surface and ends before the angle subtended by the spreading acrosome (on the nuclear surface) becomes 40°.
V	The angle subtended by the acrosome extends from 40° to a maximum of 95°.
VI	The angle subtended by the acrosome extends from greater than 95° to a maximum of 120°; elongate spermatids remain within deep crypts of the Sertoli cell.
VII	Elongate spermatids move to the luminal aspect of the seminiferous epithelium and line the lumen; the angle subtended by the acrosome is greater than 120°; nuclei of round spermatids (acrosome intervening) have not yet made contact with the cell surface.
VIII	Nuclei of step 8 spermatids make contact with the plasma membrane, but their shape is not yet distorted from round or slightly oval.
IX	The spermatid nucleus first becomes deformed from its round or ovoid shape, but a ventral angle has not formed.
X	The spermatid shows a ventral angle, but no dorsal angle is yet seen.
XI	The spermatid elongates and shows a dorsal angle; the spermatid head displays a gently curved dorsal surface and has not yet taken on a "bent rod" appearance.
XII	The dorsal surface of the spermatid head has a bent rod appearance; dense staining of the chromatin is not yet complete, indicating chromatin condensation has not reached the caudal head.
XIII	Dense staining extends throughout the spermatid nucleus indicating dense chromatin is seen throughout the head; the stage ends before meiotic metaphase of diplotene spermatocytes begins.
XIV	The presence of meiotic anaphase or telophase of meiosis I, secondary spermatocytes, or any of the phases of meiosis II defines this stage.

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Fig. 3

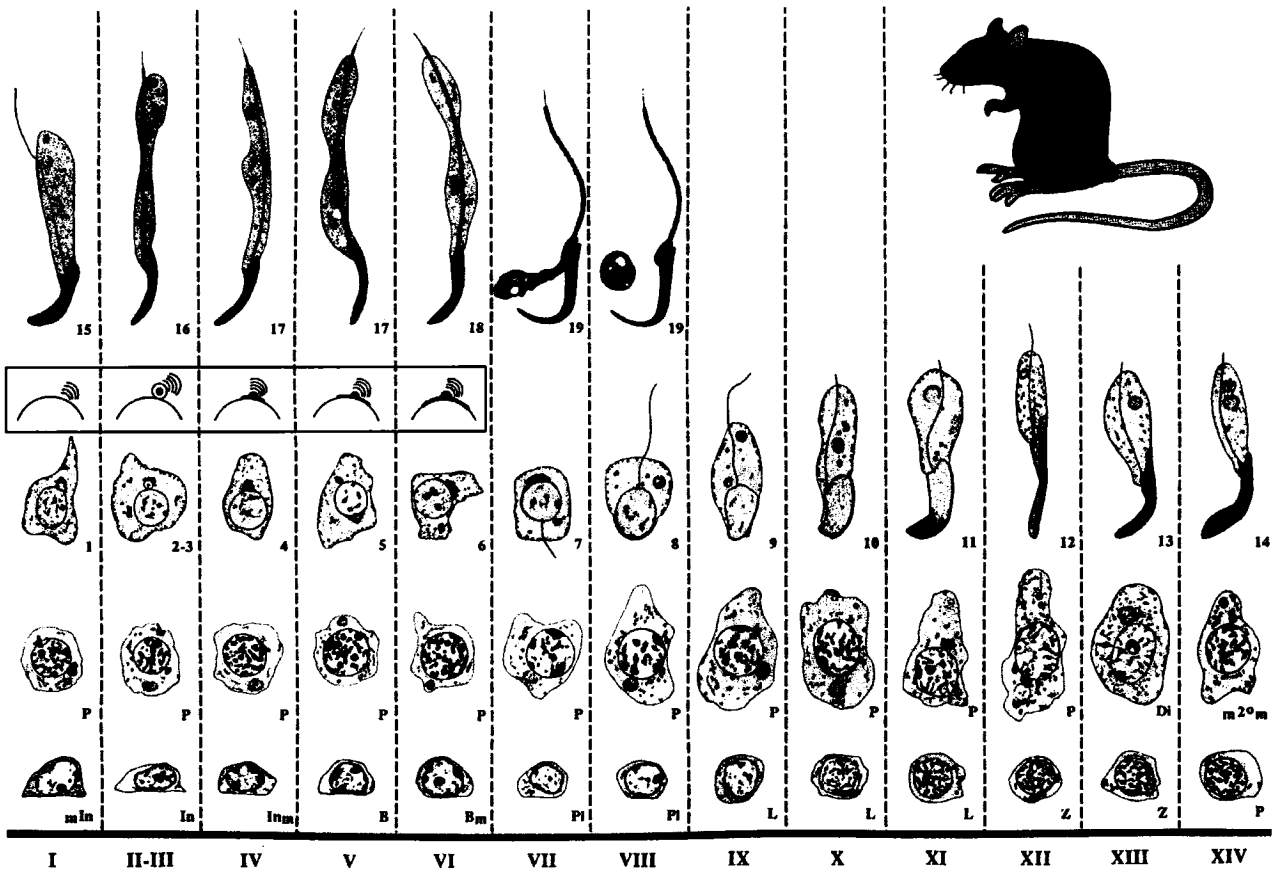

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Fig. 3. Cycle map of spermatogenesis for the rat. The vertical columns, designated by Roman numerals, depict cell associations (stages). In the scheme provided, stages II and III are combined into a single stage called II-III. A cycle is a complete series of cell associations which are placed in logical order. The developmental progression of a cell is followed horizontally until the right hand border of the cycle map is reached. The cell progression continues at the left of the cycle map one row up. The inset describes acrosome development, which is used to designate specific phases of cell development.

the formation of spermatids, the Golgi apparatus is involved in producing small condensing proacrosomal vesicles that contain dense material referred to as proacrosomal granules. The four very small proacrosomal granules eventually coalesce within one large membrane bounded vesicle containing a single granule known as the acrosomal vesicle. The acrosomal vesicle is rounded until it contacts with the nucleus, where after it becomes flattened on the side that makes contact with the surface of the nucleus and spreads out with the acrosomal granule intact and bulging. The spermatid nucleus moves to the cell surface through an unknown mechanism (Russell, Lee et al. 1983). The acrosomal region of the spermatid becomes apposed to the plasma membrane tightly. As time passes, the nucleus progressively elongates. The Golgi apparatus moves away from the acrosome and migrates to the caudal aspect of the cell. The progression of changes on the acrosome is the primary basis for classifying spermiogenesis into steps and for using these steps to classify cell associations into stages. The acrosome contains various hydrolytic enzymes, including typical lysosomal enzymes, which are released to digest the egg membrane to permit the fusion of egg and sperm during fertilization. It has been shown that the acrosome also contains functional molecules required for the complex steps of sperm-egg interaction, including binding to the zona pellucida, induction of the acrosome reaction and penetration through the zona (Saxena, Tanii et al. 1999; Saxena, Tanii et al. 2000; Kim, Foster et al. 2001).

1.1.4. Protein degradation in spermatogenesis

In the third phase of spermatogenesis, before sperm release, the spermatid volume is reduced to about 25% of its original size upon remodeling. There are at least three steps in the process of making spermatids smaller and more streamlined: water elimination, cytoplasm elimination by tubulobulbar complex and separation of cytoplasmic package

as a residual body (Huckins 1978). The cytoplasmic fragments are phagocytosed by the Sertoli cell (Elftman 1950). Exclusion of residual body is responsible for one fourth of the volume reduction (Huckins 1978). After cytoplasmic elimination, a small amount of cytoplasm, the cytoplasmic droplet, remains around the neck of the spermatid and is shed later in the epididymis.

In addition to elimination of the cytoplasm, nuclear volume decreases to only ~5% that of a somatic cell nucleus. The haploid genome is extremely compacted within the nucleus in the sperm head. This remarkable reorganization of the chromosomes is permitted by replacing histones with protamines, arginine and cysteine rich proteins that are even more basic than histones (Mills, Van et al. 1977; Meistrich 1989). Protamines organize the haploid male genome into a highly specialized chromatin structure that is much more compact than the classical nucleosomal architecture (Mills, Van et al. 1977; Meistrich 1989). The substitution of histones by protamines is probably related to the high compaction potential of nucleoprotamines and the requirement for a unique chromatin architecture that would enable a specific transcription schedule after fertilization (Sassone-Corsi 2002). In mammals, histones are not replaced directly by protamines. Transition proteins (TP1 and TP2) are small, basic nuclear proteins that replace histones in late round and early elongating spermatids. They are further replaced by protamines at around step 14 spermatids. Deletion of *TP1* or *TP2* genes, individually, does not yield a phenotype, suggesting a redundant role for these two transition proteins (Yu, Zhang et al. 2000; Zhao, Shirley et al. 2001). Mice lacking both *TP*s are sterile (Zhao, Shirley et al. 2004). In these double knockout mice, chromatin condensation is irregular in all spermatids, with many late spermatids showing DNA breaks (Zhao,

Shirley et al. 2004). Thus, mammalian TPs are required for normal chromatin condensation, fertility, and for preventing the loss of genomic integrity.

The molecular mechanism underlying this bulk reduction of cytoplasm and the degradation of specific proteins such as histones remains unclear. The phagocytic uptake of proteins by Sertoli cells may lead to degradation within lysosomes. However, a large body of accumulating evidence indicates that the ubiquitin proteasome pathway of degradation also appears to be involved.

1.2. Ubiquitin system

The ubiquitin system is a system involving multiple enzymes that regulate conjugation of ubiquitin to substrate proteins. Ubiquitination subsequently mediates protein degradation or modulates a number of other cellular processes. The ubiquitin system is increasingly recognized as playing a vital role in multiple cell functions (details are introduced later in this section).

Ubiquitin was originally isolated from the thymus and thought to be a thymic peptide hormone (Goldstein, Scheid et al. 1975). It is a ubiquitous and highly conserved 8 kDa protein of 76 amino acids. Not long later, an ATP dependent proteolytic system was identified in reticulocyte lysates (Etlinger and Goldberg 1977; Ciehanover, Hod et al. 1978). Later studies established that this ATP dependent degradation required a heat stable factor in the lysate. The factor was subsequently identified as the peptide ubiquitin. The ATP was shown to be required at two steps: the conjugation of ubiquitin to substrate proteins and the degradation of the ubiquitinated substrates by a large protease, proteasome (Ciechanover, Heller et al. 1980; Hershko, Ciechanover et al. 1980). In the following 30 years and especially in the last decade, much has been learned about the ubiquitin system. Although originally thought to be required only for degradation of

misfolded proteins, the ubiquitin system is now recognized to degrade the majority of cell proteins (Rock, Gramm et al. 1994). Furthermore, it can serve as a multifunctional signaling mechanism, and has also many nonproteolytic functions such as regulation of membrane protein trafficking (Hicke 1999; Hicke and Dunn 2003), ribosomal function (Spence, Gali et al. 2000), postreplication DNA repair (Spence, Sadis et al. 1995; Hofmann and Pickart 1999), the initiation of the inflammatory response (Deng, Wang et al. 2000), and the function of certain transcription factors (Kaiser, Flick et al. 2000).

Ubiquitin was the first protein shown to be covalently conjugated to other intracellular proteins (Hochstrasser 1996; Hershko and Ciechanover 1998). Ubiquitination is a highly precise, temporally controlled, and tightly regulated process that modulates, in a specific manner, numerous cellular proteins (Fig. 4) (Varshavsky 1997; Hershko, Ciechanover et al. 2000; Pickart 2001). It is carried out by the sequential action of three enzymes: ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2 and ubiquitin protein ligase E3 (Hershko, Heller et al. 1983) (Fig. 4). El catalyses the first step which is the formation of a high energy thioester bond between Gly76 of ubiquitin and a specific Cys residue of E1. This step requires ATP hydrolysis. The El linked ubiquitin is then moved from El to a Cys residue of an E2, and from there to a Lys residue of an ultimate protein substrate, resulting in a ubiquitin protein conjugate. This last step requires E3, which is the principal substrate recognition factor that determines the specificity of ubiquitination. In some cases there are additional protein components involved such as E4 proteins, (polyubiquitin chain elongation factors) (Koegl, Hoppe et al. 1999; Hoppe, Cassata et al. 2004), which serve to lengthen the polyubiquitin chains (Kuhlbrodt, Mouysset et al. 2005).

In many cases, proteins are modified with multiple moieties of ubiquitin that generate a branched polyubiquitin chain. Most frequently the C-terminus of each ubiquitin unit is linked to Lys48 of the previous ubiquitin. Lys48 linked polyubiquitination results in degradation of the ubiquitinated protein by the 26S proteasome, a reaction that also requires ATP hydrolysis (Fig. 4A) (Chau, Tobias et al. 1989; Hochstrasser 1996; Hershko and Ciechanover 1998). The polyubiquitin chain is generally not degraded but removed from the substrate, and then depolymerized into free ubiquitin.

Although the first and best known property of ubiquitination is to target proteins for degradation via attachment of Lys 48 linked chains to substrates, more recent findings clearly reveal other functions of ubiquitination which include modification of the activity, interactions with other proteins, and subcellular localization of the target proteins. These nonproteolytic functions appear to be modulated by monoubiquitination or by polyubiquitination via Lys residues of ubiquitin other than Lys 48. Monoubiquitination, which does not target proteins for degradation, has important functions in endocytosis, gene transcription, DNA repair, and DNA replication (Hicke 2001; Sigismund, Polo et al. 2004). Polyubiquitination does not always generate Lys 48 linked polyubiquitin chains. Ubiquitin has seven lysine residues, and so there could be other types and mixed types of polyubiquitination. Indeed, in yeast ubiquitin itself was found to be conjugated to other ubiquitin molecules at all seven lysine residues, with a relative abundance order of Lys48 > Lys63 and Lys11 >> Lys33, Lys27 and Lys6 (Peng, Schwartz et al. 2003). A chain linked through the Lys63 residues of its ubiquitin moieties appears to have roles in DNA repair (Spence, Sadis et al. 1995; Ulrich and Jentsch 2000; Andersen, Zhou et al. 2005), transcription (Yu, Zhang et al. 2000; Van Landuyt, Lissens et al. 2001; Andersen, Zhou

Fig. 4

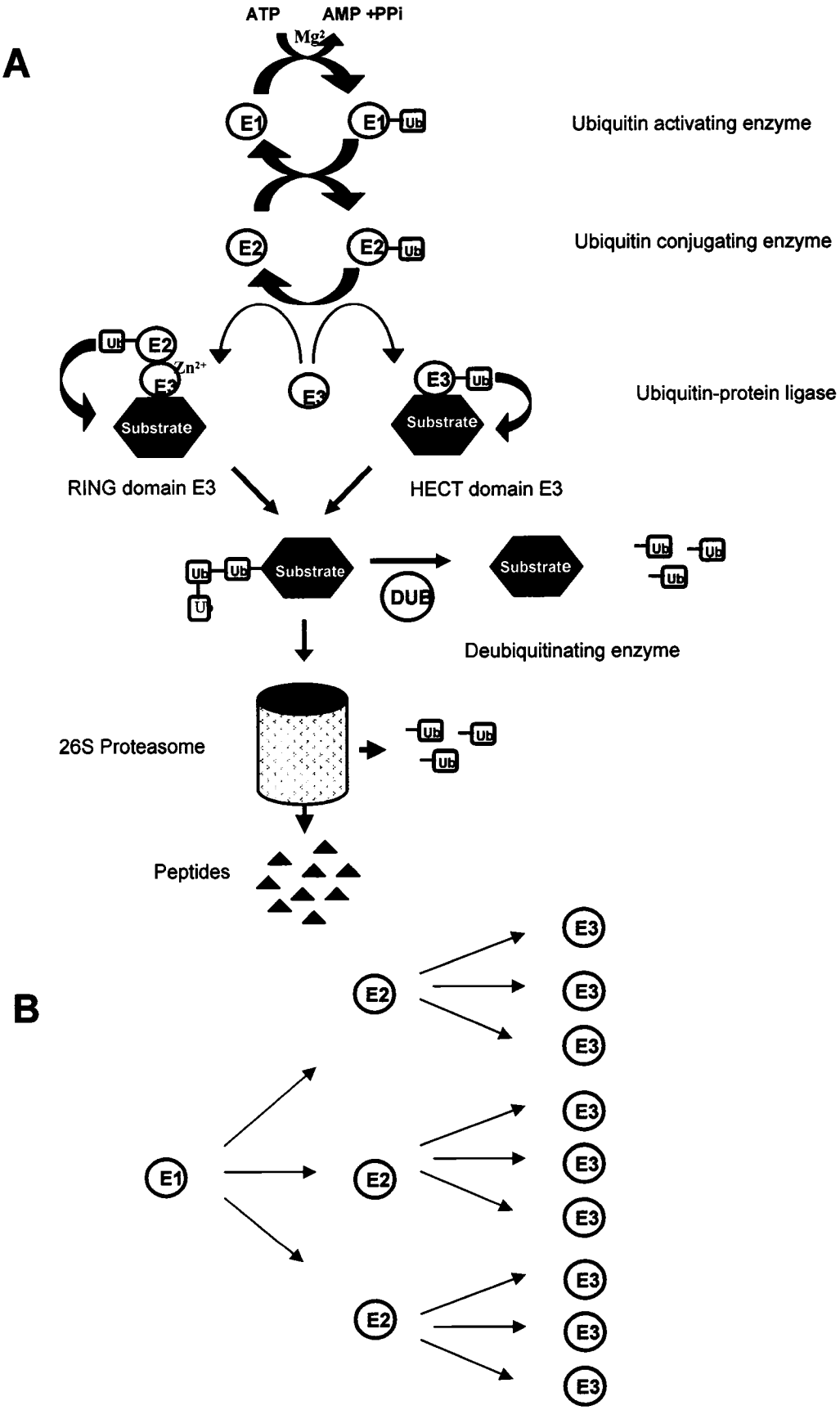


Fig. 4. Ubiquitin proteasome pathway. Details are in the text.

et al. 2005), endocytosis (Hein, Springael et al. 1995; Galan and Haguenauer-Tsapis 1997; Huang, Kirkpatrick et al. 2006), mitochondrial inheritance (Fisk and Yaffe 1999), ribosomal function (Spence, Gali et al. 2000) and cell cycle (Bothos, Summers et al. 2003). Not surprisingly, in view of these many functions, deregulated ubiquitin system has been implicated in the pathogenesis of many diseases, including certain malignancies, neurodegenerative disorders, and pathologies of the inflammatory and immune response (Scheffner, Werness et al. 1990; Kishino, Lalande et al. 1997; Loda, Cukor et al. 1997; Matsuura, Sutcliffe et al. 1997; Staub, Gautschi et al. 1997; van Leeuwen, de Kleijn et al. 1998; Joazeiro, Wing et al. 1999; Maxwell, Wiesener et al. 1999; Saigoh, Wang et al. 1999; Waterman, Levkowitz et al. 1999; Bignell, Warren et al. 2000; Lam, Pickart et al. 2000; Shimura, Hattori et al. 2000).

While a series of enzymes conjugate ubiquitin to substrates, there are also at least 19 deubiquitinating enzymes (DUBs) in yeast and 95 DUBs in human that remove ubiquitin from polyubiquitin chains or from the substrate (Wilkinson 1997; D'Andrea and Pellman 1998; Wilkinson 2000; Wing 2003; Soboleva and Baker 2004). DUBs can negatively regulate ubiquitination to protect proteins from degradation or to terminate the signal or property changes generated by ubiquitination (Fig. 5). Deubiquitination of the ubiquitin chains released by the proteasome from proteolytic substrates, after the substrates have been targeted there, is necessary to maintain a sufficient pool of free ubiquitin within the cell for normal ubiquitination. In this way, DUBs keeps the 26S proteasome free of unanchored ("free") ubiquitin chains that can compete with ubiquitinated substrates for ubiquitin binding sites (Amerik and Hochstrasser 2004; Nijman, Luna-Vargas et al. 2005). Moreover, DUBs are also responsible for processing inactive ubiquitin precursors, generated by polyubiquitin genes which contain tandemly repeated copies of the

Fig. 5

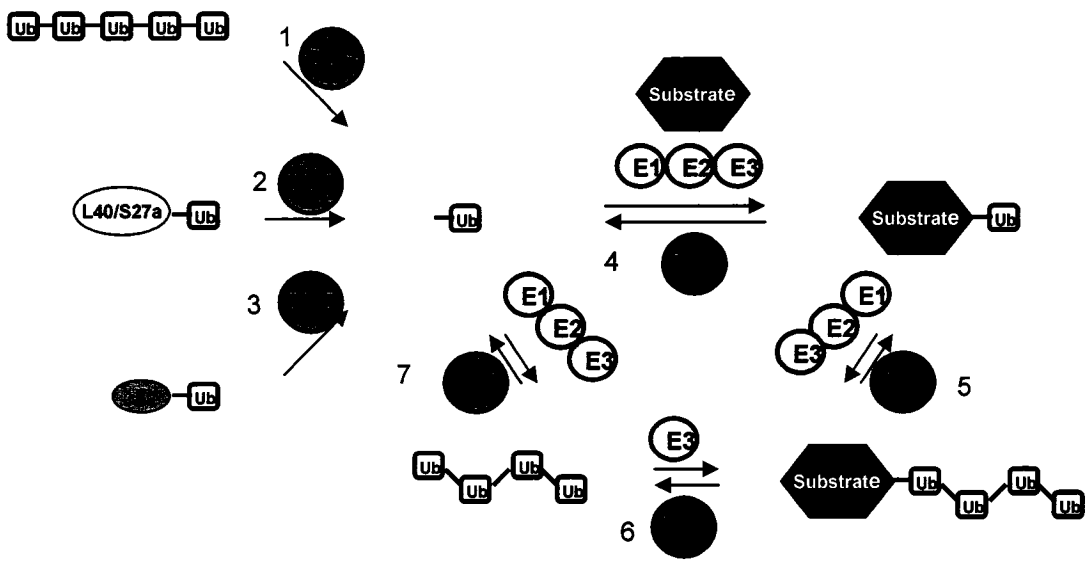


Fig. 5. Functions of DUBs in the ubiquitin pathway. Ubiquitin is encoded in the genome as polyubiquitin genes (1) and as fusion genes to either the L40 or S27a subunits of the ribosome (2). Deubiquitinating enzymes process the gene products to the monomeric proteins (1) and (2). DUBs can also serve to salvage ubiquitin from adducts ligated to abundant small nucleophiles such as glutathione or polyamines (X) (3). DUBs can recycle ubiquitin (4) or ubiquitin chains (6) from ubiquitinated proteins. The process can also serve to rescue inappropriate ubiquitinated proteins. Finally, DUB can disassemble anchored (5) or unanchored ubiquitin chains (7).

ubiquitin coding sequence (Dworkin-Rastl, Shrutkowski et al. 1984; Ozkaynak, Finley et al. 1984).

1.2.1. Ubiquitin conjugation system

As described above, it has now been well established that the E1-E2-E3 cascade is the enzymatic machinery responsible for attaching ubiquitin to various protein substrates (Hershko, Heller et al. 1983). Although ubiquitination of all proteins uses the same E1, there are about 40 different E2s and 500 different E3s or E3 multiprotein complexes for ubiquitination of different proteins in mammalian cells (Pickart 2001; Shcherbik and Haines 2004). E1 works with all E2s, while each E2 has specificity for several E3s (Fig. 4B). Conversely, an E3 can also be supported by several specific E2s.

1.2.1.1. Ubiquitin activating enzyme-E1

E1 is a ~100 kDa protein that contains the nucleotide binding motif Gly-X-Gly-X-X-Gly. E1 activates ubiquitin and thereby starts ubiquitination. The end result of this activation is the C-terminal Gly of ubiquitin is bound to the active site Cys residue via a high energy thioester bond. The initial step of this reaction is the nucleophilic attack of ATP with ubiquitin, which results in the formation of ubiquitin adenylate and the release of PP_i. The active site Cys then reacts with the ubiquitin adenylate, resulting in the binding of ubiquitin to the Cys residue of E1 in a thioester linkage, with the release of AMP (Huang, Miller et al. 2004). The E1 can then adenylate another molecule of ubiquitin. Each fully loaded E1 molecule therefore carries two molecules of activated ubiquitin: one bound as a thioester, the other as an adenylate. The ubiquitin loaded E1 can interact with the full spectrum of ubiquitin E2 enzymes, and in a transthiolation reaction, ubiquitin is transferred from the active site cysteine of the E1 to the active site cysteine of the E2. The catalysis by E1 is very efficient. The process of ATP-AMP

exchange, which requires all steps from ATP binding to thioester formation, occurs at a maximum turnover number of 1–2 s^{-1} (Haas and Rose 1982), while the catalytic rate (k_{cat}) of substrate ubiquitination reported later is 10- to 100- fold slower (Mastrandrea, You et al. 1999). This relatively high efficiency of E1 allows production of sufficient activated ubiquitin for the entirety of downstream conjugation reactions, even though the E1 concentration is usually less than the total E2 concentration (Hershko, Heller et al. 1983).

While structural determination of the E1 enzyme has proven elusive, further insights into the E1 mechanism have come from structural and biochemical analysis of the E1 like enzyme responsible for activation of the ubiquitin like protein (Ubl), Nedd8 (Walden, Podgorski et al. 2003; Walden, Podgorski et al. 2003; Huang, Walden et al. 2004), as well as analogies with the bacterial MoeB and ThiF enzymes. The Nedd 8 activating enzyme consists of two subunits, APPBP1and Uba3. MoeB and ThiF function with orthologs of ubiquitin, MoaD and ThiS, and play roles in bacterial molybdopterin and thiamin biosynthesis, respectively (Lake, Wuebbens et al. 2001; Rudolph, Wuebbens et al. 2001; Wang, Xi et al. 2001). The structure and associated mutational analyses reveal that the activation of ubiquitin and Ubl is a two-step mechanism as described above in which the C terminus of ubiquitin or Ubl is first adenylated and then ubiquitin or Ubl is transferred from the adenyl group to an E1 cysteine. The common feature of E1s for ubiquitin and all Ubl is a region of sequence homology to MoeB and ThiF which is required for adenylation, suggesting that the common function associated with all those E1s is the C-terminal adenylation of ubiquitin or a Ubl. The cocrystal structures of MoeB and ThiF complex and of the APPBP1and Uba3 complex revealed that this sequence homology region adopts a Rossmann fold, a structure frequently found in nucleotide binding proteins (Rossmann, Moras et al. 1974; Lake, Wuebbens et al. 2001; Walden,

Podgorski et al. 2003). In addition to playing a role in nucleotide binding, this sequence homology region is also involved in dimerization. The structural homology between the two heterodimer complexes extends beyond the region of significant sequence homology, and also includes the C-terminal part of the MoeB sequence, which is involved in binding to MoaD. This raises the possibility that even distally related E1s may bind to their cognate Ubls via a similar structure.

Ubiquitin activating enzyme is a very abundant protein localized in both the cytoplasm and the nucleus. Genes encoding E1 for ubiquitination have been cloned from different species, including yeast (*UBA1*) (McGrath, Jentsch et al. 1991), wheat (*UBA1*) (Hatfield, Callis et al. 1990) and human (*UBE1*) (Zacksenhaus and Sheinin 1990; Handley, Mueckler et al. 1991). Most organisms have a single gene encoding the E1 enzyme, which appears to be an essential gene in all eukaryotes (Zacksenhaus and Sheinin 1990; Jentsch, Seufert et al. 1991; McGrath, Jentsch et al. 1991). Isoforms of mammalian E1 enzymes may be present in some cell types (Cook and Chock 1992; Shang, Deng et al. 2001), as well as multiple phosphorylated states of E1 (Cook and Chock 1995; Stephen, Trausch-Azar et al. 1996). However, biochemical characteristics have not been shown to differ among any of these alternative products. Phosphorylation has been reported to regulate localization of E1 to the nucleus (Stephen, Trausch-Azar et al. 1996; Stephen, Trausch-Azar et al. 1997).

1.2.1.2. Ubiquitin conjugating enzyme-E2

Ubiquitin conjugating enzymes, E2s, are encoded by a gene family and differ in their properties and intracellular localization (Jentsch, Seufert et al. 1990). Each E2 must have specificity for E1, as well as for one or more E3s. This specificity is generally encoded within the ~150–200 amino acid residues of the highly conserved catalytic domain,

although in some cases N- and/or C-terminal extensions appear to be important as well. Within the catalytic domain of E2, there is an active site cysteine residue required for the thioester linkage of E2 and ubiquitin, mutation of which results in inactivation of the E2 (Sung, Prakash et al. 1991; Sommer and Jentsch 1993; Seufert, Futcher et al. 1995).

There are 13 E2s in *S. cerevisiae*, and even more in higher organisms, reflecting multiple orthologs to the yeast E2s (Jensen, Bates et al. 1995; Rajapurohitam, Morales et al. 1999) as well as the evolution of new E2s (Wefes, Mastrandrea et al. 1995; Liu, Haas et al. 1996; Hauser, Bardroff et al. 1998). Different E2s have distinct biological functions through interaction of different E2s with specific E3s, and is most evident from genetic studies in yeast (Hochstrasser 1996). Mutations of different E2s in yeast lead to distinct phenotypes, indicating that those E2s have different functions. For example, mutants in yeast *CDC34* (*UBC3*) gene are defective in the transition from G1 to the S phase of the cell cycle (Goebl, Yochem et al. 1988). Mutations of *UBC2* (*RAD6*) cause a variety of phenotypes, including extreme vulnerability to DNA damaging agents; deficiencies in sporulation, meiotic recombination and induced mutagenesis; and enhanced rates of mitotic recombination and spontaneous mutagenesis (Friedberge, Siede et al. 1991). Turnover of short lived proteins is markedly reduced in *ubc4/ubc5* mutants (Seufert and Jentsch 1990)

The three dimensional structure of E2 has been determined by X ray crystallography (Cook, Jeffrey et al. 1993; Worthylake, Prakash et al. 1998; Jiang and Basavappa 1999). The structure of E2 charged with ubiquitin (Miura, Klaus et al. 1999) and the structure of E2/E3 cocrystals (Huang, Kinnucan et al. 1999; Zheng, Wang et al. 2000) have also been characterized. The active core domain of E2 folds into a conserved structure with four α helices and a four stranded antiparallel β sheet. The active site cysteine is located in a

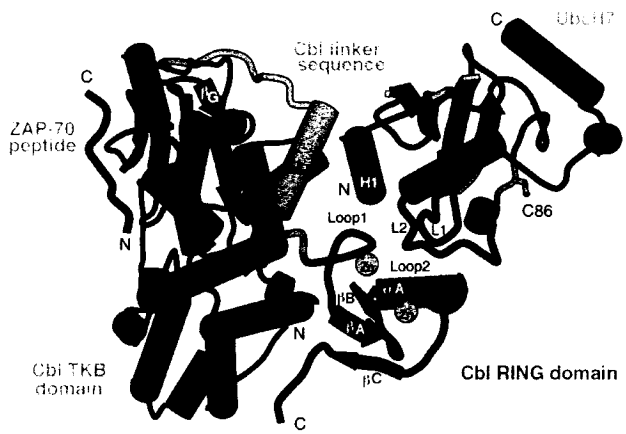
cleft between two loops. Not surprisingly, many of the most highly conserved E2 residues surround the active site cysteine (Cook, Jeffrey et al. 1993; Worthylake, Prakash et al. 1998; Jiang and Basavappa 1999). Some of these residues interact with ubiquitin. There are two major E3 families, one containing a conserved HECT domain and the other possessing a conserved RING domain. Both of these conserved domains are required for binding of E2s, as revealed by deletion analysis (Schwarz, Rosa et al. 1998; Seol, Feldman et al. 1999; Skowyra, Koepp et al. 1999). Certain E2s, such as UbcH7, work with E3s from both of the two E3 families, although the catalytic modules of the two E3 families are distinct in sequence and structure (Huang, Kinnucan et al. 1999; Borden 2000; Zheng, Wang et al. 2000). UbcH7 efficiently binds to the HECT domain enzyme E6AP (Nuber, Schwarz et al. 1996; Huang, Kahana et al. 1997; Kumar, Kao et al. 1997) and to several RING domain E3s, including c-Cbl which ubiquitinates several membrane receptors (Moynihan, Ardley et al. 1999; Yokouchi, Kondo et al. 1999; Zheng, Wang et al. 2000). The crystal structures of the UbcH7-E6AP HECT domain and UbcH7-c-Cbl RING domain complexes (Huang, Kinnucan et al. 1999; Zheng, Wang et al. 2000) reveal that the E2-E3 contacts involve many of the same E2 residues in both complexes (Fig. 6, A-B). Most of these residues are located in two loops at the end of the UbcH7 β sheet. From these findings it is easier to understand how the presence of a few divergent surface residues of E2 could modulate the binding properties of a particular E2 to an E3.

1.2.1.3. Ubiquitin protein ligase-E3

E3 catalyzes the final step of ubiquitination to link ubiquitin to an ε-amino group of a Lys residue on the substrate protein or on ubiquitin itself to form a ubiquitin chain. E3s range from single polypeptides to large complexes in which distinct subunits are

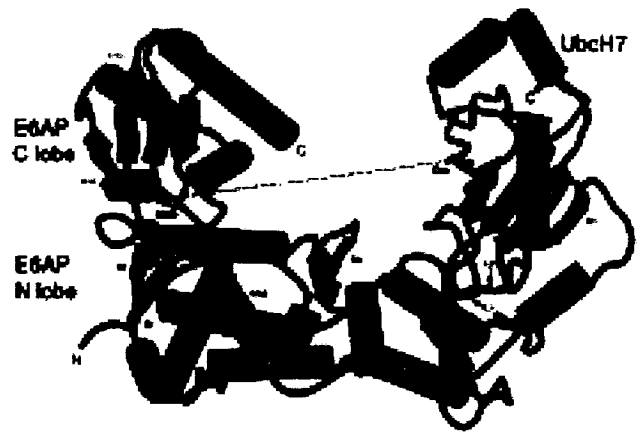
Fig. 6

A



Zheng N. et al, *Cell,* 2000 Aug 18;102(4):533-539

B



Huang L. et al, *Science,* 1999 Nov 12; Vol. 286 (5443): 1321 – 1326.

Fig. 6. Crystal Structures of E2/E3 complexes. A. Overall view of the ternary complex of the TKB and the RING domains of c-Cbl, UbcH7, and the substrate, phosphorylated ZAP-70 Peptide. The TKB domain is colored green, the RING domain red, and the linker region of c-Cbl yellow. UbcH7 is colored in cyan and its active site cysteine in orange. The two zinc ions are shown as gray spheres. B. Orthogonal views of the overall U-shaped structure of the E6AP HECT domain-UbcH7 complex. The E6AP HECT domain N lobe (consisting of 12 helices and six strands), C lobe (six helices and four strands), and UbcH7 (four helices and four strands) are colored in green, red, and cyan, respectively. The active site loops of the E2/E3 are colored yellow. The UbcH7 active-site loop consists of residues 70 to 101. The dotted line indicates the open line of sight between the active-site cysteines of E6AP and UbcH7 (Huang, Kinnucan et al. 1999).

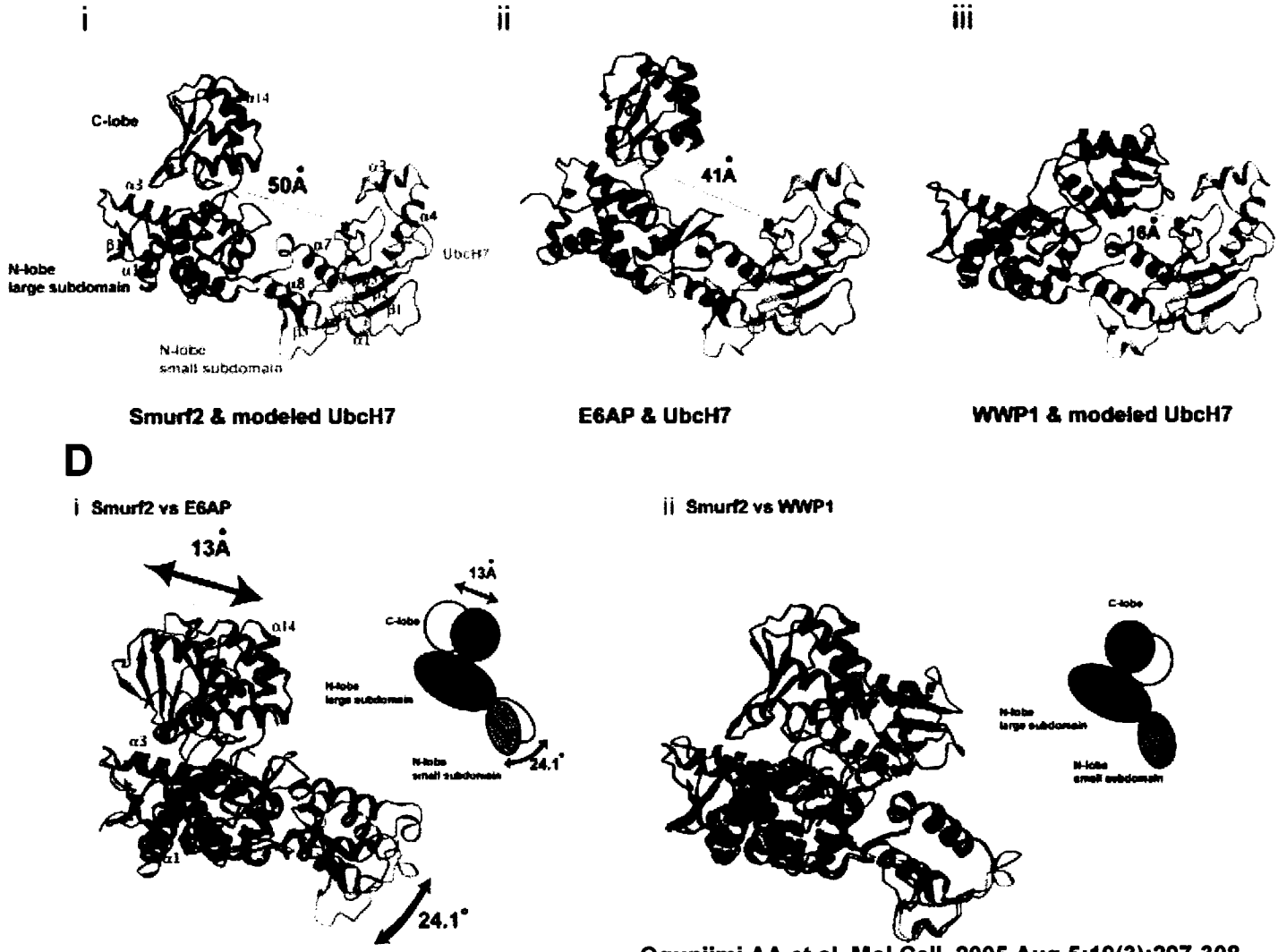


Fig. 6. C. Overall structure of Smurf2 HECT domain with modeled UbcH7 (yellow). (Ci) The E2, UbcH7 was docked on the Smurf2 HECT domain based on E6AP-UbcH7 complex structure. Smurf2 HECT domain revealed an open L-shaped conformation for the two lobes, N- and C-terminal. The N-terminal lobe (residues 369–624) consists of two subdomains: the large subdomain in red (residues 369–514 and 597–624) and the small subdomain that contains the E2 binding groove in pink. The two subdomains of the N-terminal lobe are connected by short linker sequences between residues 515–517 and 593–596. The C-terminal lobe (residues 628–741, colored blue) contains the catalytic Cys-716. The structure of E6AP-UbcH7 complex with the C lobe (blue) and the N lobe (red and pink) is shown in panel (Cii). In panel (Ciii), the structure of WWP1 with modeled UbcH7 revealed a T-shaped conformation of WWP1 with the N lobe colored red and pink and the C-lobe in blue. D. Superimposed ribbon diagrams of Smurf2 HECT (red-pink-blue) showing the motion of the C lobe (blue) and the E2 binding lobe (pink) about the hinge region when compared to (Cii) E6AP (cyan) and (Ciii) WWP1 (cyan). Schematics of the superimposed structures are represented to the right of each ribbon diagram. Note the rotation of the E2 binding lobe by 24.1° and the translation of the C lobe by 13 Å when compared to E6AP (i) (Ogunjimi, Briant et al. 2005).

responsible for substrate recognition and for ubiquitin conjugation (Fig. 7). Multisubunit E3s are often organized in a modular fashion in which substrate recognization subunits can be readily exchanged to facilitate the specific recognition of diverse substrates (Fig. 7, C-D).

A given E3 usually binds its cognate substrate(s) through a structural motif known as a ubiquitination signal (Chau, Tobias et al. 1989; Hochstrasser 1996; Varshavsky 1997; Gilon, Chomsky et al. 1998; Hershko and Ciechanover 1998; Deshaies 1999; Koepp, Harper et al. 1999; Laney and Hochstrasser 1999; Page and Hieter 1999; Tyers and Jorgensen 2000). Ubiquitination signals on target proteins can be genetically encoded such as various destruction motifs, or can be acquired such as by phosphorylation, by binding to an adapter protein, or by protein damage due to fragmentation or oxidation (Laney and Hochstrasser 1999). Destruction motifs are short sequences of amino acids that are necessary and sufficient to direct their ubiquitination. The first identified ubiquitination signal in this category were N-end rule proteins whose ubiquitination rate depends strongly on the identity of the N-terminal residue (Bachmair and Varshavsky 1989). A number of other sequences have since been identified. The RING domain containing E3, the Anaphase Promoting Complex (APC), can recognize several such motifs, such as the D Box (Glotzer, Murray et al. 1991; Koepp, Harper et al. 1999; Page and Hieter 1999; Owens and Hoyt 2005), the Ken box (Pfleger and Kirschner 2000) and the A box (Castro, Vigneron et al. 2002; Littlepage and Ruderman 2002). Hydrophobic surfaces have also been shown to lead to the degradation of certain proteins (Gilon, Chomsky et al. 1998; Johnson, Swanson et al. 1998; Laney and Hochstrasser 1999).

The ubiquitination of many cellular regulators has been shown to be regulated by phosphorylation. These include: cyclins and cyclin kinase inhibitors; other

Fig. 7

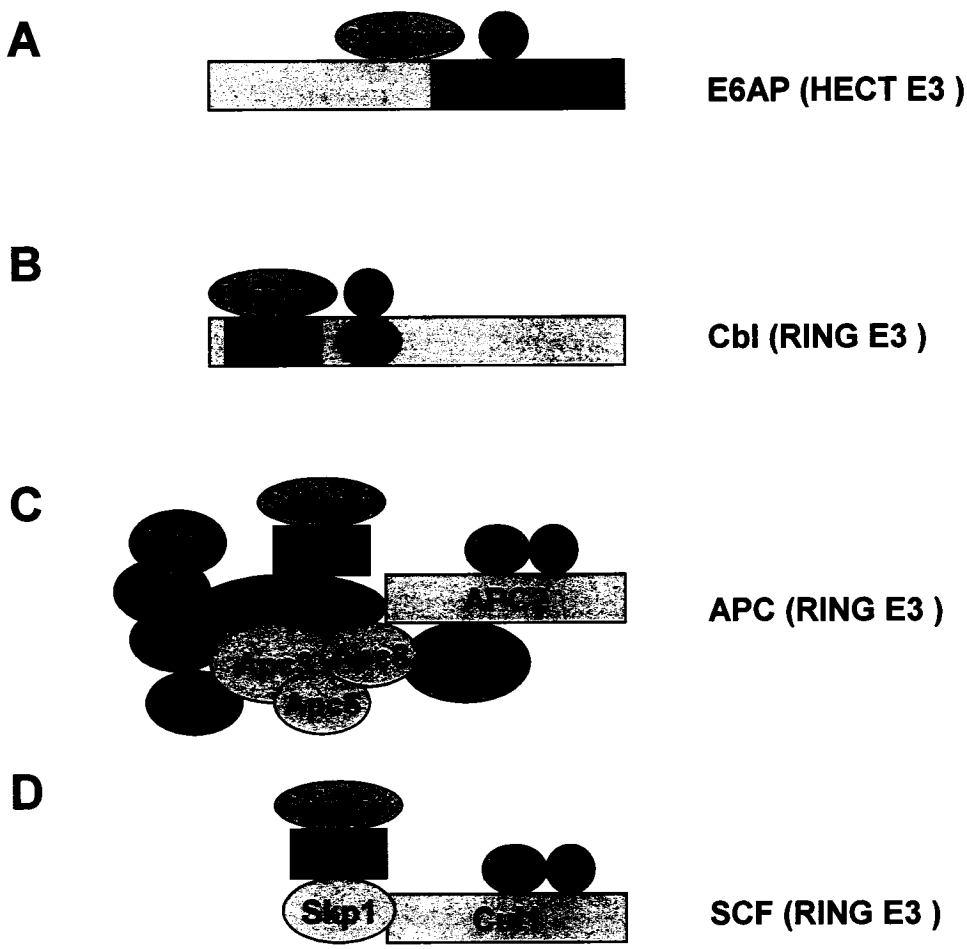


Fig. 7. Diagram of representative enzymes of different ubiquitin protein ligases (E3s). A-B, monomer forms of E3; C-D, multisubunit E3 complexes.

checkpoint regulators such as cdc6p (replication) and Swe1p (budding); signal transduction components such as Smads, TGFβ, Cb1, numerous cell surface receptors and transporters, and proteins regulating transcription such as IκBα, β catenin, p53, Jun, and even RNA polymerase (Koepp, Harper et al. 1999; Laney and Hochstrasser 1999). The RING domain containing E3, Skp1-Cul1-F box protein complex, requires its substrates to be phosphorylated before ubiquitination (Cardozo and Pagano 2004).

For damaged and misfolded proteins, interaction with chaperones and the cytoskeleton appears to induce their degradation. Abnormal proteins in the cytoplasm are bound to the microtubule network and localized to the centrosome along with many of the enzymes of the pathway (Johnston, Ward et al. 1998; Stumptner, Heid et al. 1999). Misfolded proteins in the ER are shuttled back to the cytoplasm by retrograde transport and ubiquitinated and degraded there (Plemper and Wolf 1999).

Ubiquitination sites on the substrate can be specific or nonselective. Potential ubiquitination sites have been analyzed on c-Jun (Hou, Cenciarelli et al. 1994; Treier, Staszewski et al. 1994), the T cell receptor ζ subunit (Hou, Cenciarelli et al. 1994), and the encephalomyocarditis (EMC) virus 3C protease (Lawson, Gronros et al. 1999). In each case, all of the single lysine to arginine mutants retained nearly the same level of ubiquitination as that of the wild type, suggesting that their ubiquitination are not site specific. In contrast, Mdm2, a RING E3, promotes its own degradation via the ubiquitination of a specific lysine residue (Buschmann, Fuchs et al. 2000). The ubiquitination site on histone H2A has been mapped to the highly conserved residue Lys 119 (Nickel, Roth et al. 1987; Wang, Wang et al. 2004). Histone H2B is also specifically ubiquitinated on Lys 120 in human H2B and Lys123 in yeast H2B, respectively (Thorne, Sautiere et al. 1987). The transcription factor MyoD and the latent membrane protein 1 of

Epstein-Barr virus are polyubiquitinated at a noncanonical site, the N terminus, as suggested by ubiquitination of a Lys-less mutants of each protein (Breitschopf, Bengal et al. 1998; Aviel, Winberg et al. 2000). In both cases, N-terminal residues of both proteins are sufficient and necessary for promotion of ubiquitin conjugation and subsequent degradation of the polyubiquitinated protein by the 26S proteasome. Very interestingly, ubiquitination can occur on proteins lacking accessible lysines or an accessible N terminus as well (Cadwell and Coscoy 2005). MHC I molecules (*M*ajor *H*istocompatibility *C*omplex) are ubiquitinated by MIR1 (the *M*odulators of *I*mmune *R*ecognition) (Ishido, Wang et al. 2000). One recent study shows that MIR1 can stimulate ubiquitination on a cysteine residue in the intracytoplasmic tail of MHC I molecules, lacking lysine residues in the intracytoplasmic domain, and promotes downregulation of the MHC I molecules (Cadwell and Coscoy 2005).

As described before, most known E3s are characterized into two families, HECT E3s and RING E3s, which are introduced in details in the following sections.

1.2.1.3.1. RING E3s

RING (*R*eally *I*nteresting *N*ew *G*ene) E3s are defined by the presence of multiple Cys and His residues that coordinate two zinc ions to form a globular E2 binding domain (Petroski and Deshaies 2005). RING E3s bind both the ubiquitin charged E2 and the substrate, thus facilitating direct attack of the substrate Lys on the E2 linked ubiquitin (Joazeiro and Weissman 2000; Zheng, Wang et al. 2000; Weissman 2001; Zheng, Schulman et al. 2002).

There are ~500 RING finger proteins in databases and they are involved in diverse cellular processes (Deshaies 1999; Borden 2000; Joazeiro and Weissman 2000).

Although it is not known if all RING finger proteins are E3s, it is clear that there is a larger family of RING E3s than HECT E3s.

1.2.1.3.1.1. Typical RING E3s

Typical RING E3s includes single subunit and multiprotein complex RING E3s (Deshaies 1999; Page and Hieter 1999; Joazeiro and Weissman 2000; Tyers and Jorgensen 2000) (Fig. 7, B-D).

One group of typical and well studied single subunit E3s is the Cbl family. The Cbl proteins comprise a highly conserved family of RING E3s that play a central role in the downregulation of signaling cascades involving receptor and nonreceptor tyrosine kinases (Thien and Langdon 2001; Dikic, Szymkiewicz et al. 2003; Duan, Reddi et al. 2004). There are three mammalian Cbl proteins, c-Cbl, Cbl-b and Cbl-c (also known as Cbl-3), encoded by separate genes. Structurally, all Cbl proteins have a N-terminal tyrosine kinase binding (TKB) domain for substrate binding and a RING finger domain to recruit the E2 (Fig. 6A, 7B). The C-terminal portion includes a proline rich region in c-Cbl and Cbl-b, but this region is rudimentary in Cbl-c/Cbl-3. Also, present in the C-terminus of c-Cbl and Cbl-b, but absent in Cbl-c/Cbl-3 are a number of tyrosine residues which can be phosphorylated by protein tyrosine kinases, a ubiquitin associated domain (UBA) and leucine zipper (LZ). Cbls regulate the signaling process through ubiquitination of the plasma membrane receptors and various downstream signaling components.

The Cbl proteins are regulated by an increasingly complex network to ensure the accurate effects of Cbl proteins on signaling. First, the ubiquitin protein ligase activity of c-Cbl is regulated. The E3 activity of c-Cbl is negatively regulated by domains present in the N-terminal half of the protein (the TKB and linker helix domains between TKB and

RING) and this negative regulation is removed when the protein is phosphorylated on tyrosine residues, which alters the conformation of c-Cbl (Kassenbrock and Anderson 2004). Cbl dependent ubiquitination and degradation of the receptor tyrosine kinase, Fyn, is dependent on the kinase activity of Fyn (Ghosh, Reddi et al. 2004). Secondly, the stability of Cbl proteins is regulated. Autoubiquitination of Cbls is regulated by activation of the receptor substrate EGFR (*E*pidermal *G*rowth *F*actor *R*eceptor) (Ettenberg, Magnifico et al. 2001), by receptor tyrosine kinases c-Src (Bao, Gur et al. 2003), or by CD28 costimulation in T cell activation (Zhang, Bardos et al. 2002). Cbl proteins can also be ubiquitinated by other E3s, such as Nedd4 and Itch, two WW domain HECT E3s (detail in 1.2.1.3.2, HECT E3s) (Magnifico, Ettenberg et al. 2003). Moreover, the activity of Cbls can be regulated through interacting of Cbls with other signaling proteins (Feshchenko, Smirnova et al. 2004; Kowanetz, Crosetto et al. 2004). Finally, there is a level of regulation by deubiquitination of the Cbl substrates (Gesbert, Malarde et al. 2005; Mizuno, Iura et al. 2005). Since Cbls play an important role in downregulation of activated tyrosine kinases to terminate signaling, defects in the regulatory mechanisms that result in impaired Cbl function are implicated in pathological conditions such as immunological and malignant diseases (Roussel, Shurtleff et al. 1990; Muthuswamy, Gilman et al. 1999; Peschard, Fournier et al. 2001; Mancini, Koch et al. 2002; Wu, Tu et al. 2003).

The cocrystal structure of c-Cbl bound to a cognate E2, UbcH7, and an 11 residue peptide containing the c-Cbl recognition sequence from the ZAP-70 shows how the RING domain recruits the E2 (Zheng, Wang et al. 2000) (Fig. 6A). The RING domain of c-Cbl is anchored onto the TKB domain by interacting with the four helix bundle of TKB. UbcH7 adopts an α/β structure characteristic of the E2 fold and binds to c-Cbl with one

end of its elongated structure. The crystal structure reveals that c-Cbl binds UbcH7 using both its RING domain and its linker helix. Two UbcH7 loops pack in and around a shallow groove on the c-Cbl RING domain and a UbcH7 helix interacts with the c-Cbl linker helix. The UbcH7 active site cysteine is located on the side of the complex opposite from where the ZAP-70 recognition peptide binds and is separated by ~60 Å from the peptide. UbcH7 and the TKB and RING domains of c-Cbl are tightly associated by interactions across multiple interfaces. These involve conserved c-Cbl residues. The structure reveals a rigid coupling between the peptide binding and the E2 binding domains and a conserved surface channel leading from the peptide to the E2 active site, suggesting that the precise relative position and orientation of the substrate binding and E2 binding domains are important for ubiquitination.

Important multisubunit RING E3s include the APC/C (*A*naphase *P*romoting *C*omplex or cyclosome) and the SCF (*S*kp1–*C*ullin–*F* box) complex (Fig. 7, C-D). APC was identified through a genetic screen for mutants defective in cyclin destruction in budding yeast (Irniger, Piatti et al. 1995) and from two different biochemical studies in clam and *Xenopus* egg extracts which screened for the E3 ligase required for cyclin degradation (King, Peters et al. 1995; Sudakin, Ganoth et al. 1995). This approximately 1500 kDa complex is a multisubunit E3 that contains at least 11 subunits. The catalytic core consists of the adaptor protein Apc2 and a RING containing protein Apc11 (Fig. 7C). Substrate recognition is mediated by two activators, Cdc20 or Hct1p/Cdh1p (Page and Hieter 1999; Castro, Vigneron et al. 2002). The APC functions predominantly to induce progression and exit from mitosis by inducing proteolysis of different cell cycle regulators. These include securin proteins (Pds1p in budding yeast and Cut2p in fission

yeast), whose destruction is required for the separation of sister chromosomes at anaphase (Cohen-Fix, Peters et al. 1996; Funabiki, Kumada et al. 1996; Ciosk, Zachariae et al. 1998) and mitotic cyclins, such as cyclin B, which must be destroyed before mitotic exit (Glotzer, Murray et al. 1991; Irniger, Piatti et al. 1995; King, Peters et al. 1995; Sudakin, Ganoth et al. 1995). APC activity must therefore be tightly regulated to ensure that the timing and order of mitotic events are strictly maintained. The works of several groups have shown that APC activity is regulated at multiple levels, including APC subunit phosphorylation (King, Peters et al. 1995; Lahav-Baratz, Sudakin et al. 1995; Peters, King et al. 1996; Shirayama, Zachariae et al. 1998; Kotani, Tanaka et al. 1999), selective association with substrates via Cdc20p or Hct1p/Cdh1p (Schwab, Lutum et al. 1997; Visintin, Prinz et al. 1997) and the spindle assembly checkpoint, which ensures that duplicated chromosomes are properly aligned and attached to spindles before being separated at anaphase (He, Patterson et al. 1997; Huang, Kahana et al. 1997; Fang, Yu et al. 1998; Chung, Shin et al. 2000; Hwang, Venkatasubrahmanyam et al. 2003).

The SCF E3 ubiquitin ligase family was discovered through genetic analysis of cell cycle progression in the budding yeast *Saccharomyces cerevisiae* (Patton, Willems et al. 1998). The core complex of these multisubunit enzymes is composed of the Cdc53/Cul1 scaffold protein, Skp1 adaptor and the Rbx1 (also known as Roc1 or Hrt1) RING domain protein (Fig. 7D). The Skp1 adaptor binds to a family of F box proteins that recognize specific substrates for ubiquitination (Haas, Bright et al. 1988; Patton, Willems et al. 1998; Deshaies 1999). In particular, the F box proteins recognize phosphorylated SCF targets. The SCF controls cell proliferation and cell cycle, principally at the G1/S and G2/M boundaries, by inducing the degradation of different critical regulators such as cyclin A and E (Krajewski, Bodrug et al. 1995; Zhang, Kobayashi et al. 1995; Connelly

and Hieter 1996; Skowyra, Koepp et al. 1999; Strohmaier, Spruck et al. 2001) and CDK inhibitors p21, p27 and Sic1 (Schwob, Bohm et al. 1994; Sudakin, Ganoth et al. 1995; Yamanaka, Hatakeyama et al. 2000; Spruck, Strohmaier et al. 2001; Verma, Aravind et al. 2002; Bornstein, Bloom et al. 2003). The SCF can also control gene transcription. For example, SCF can indirectly activate NFκB mediated transcription by ubiquitination and downregulation of the IκB αsubunit, or directly inhibit β catenin mediated transcription by ubiquitination and downregulation of β catenin (Yaron, Hatzubai et al. 1998; Winston, Strack et al. 1999; Deng, Wang et al. 2000; Liu, Stevens et al. 2001). Some complexes analogous to the SCF complex have been described. These complexes retain the RING domain protein Rbx1, but have different cullin homology domain containing proteins and adapter proteins that recognizing substrates. For example, VHL ('von Hippel-Lindau') tumor suppressor protein is a E3 complex analogous to SCF. In VHL complex, the VHL protein, analogous to F-box protein, is linked to a Cul2/Rbx1 module by the ubiquitin like Elongin B and Skp1 like Elongin C adaptor proteins (Duan, Pause et al. 1995; Kibel, Iliopoulos et al. 1995). Recent structural insights into SCF like complexes have begun to illuminate aspects of substrate recognition and catalytic reaction mechanisms (Min, Yang et al. 2002; Zheng, Schulman et al. 2002; Orlicky, Tang et al. 2003). The crystal structure of the Cul1-Rbx1-Skp1-F box protein Skp2 complex generates a 50 Å gap between the structure of Skp2 substrate adapter and the E2 active site. The space could be bridged by the substrate such that it is now ready for ubiquitination by the E2 Cdc34 bond to Rbx1.

1.2.1.3.1.2. Special RING E3 family members

Ufd2 promotes elongation of polyubiquitination chains and so was referred to as an E4 enzyme (Koegl, Hoppe et al. 1999; Skowyra, Koepp et al. 1999). U box is a motif identified in Ufd2. This U box has an overall structure closely related to that of the RING

finger (Makarova, Aravind et al. 2000) and has been shown to be sufficient for both E2 binding and polyubiquitination in the absence of any other known E3 (Hatakeyama, Yada et al. 2001; Jiang, Ballinger et al. 2001). Thus, U box containing proteins are a subfamily of RING E3 family.

PHD domain is another structure closely related to that of the RING finger (Pascual, Martinez-Yamout et al. 2000; Capili, Schultz et al. 2001). PHD domain containing proteins have recently been shown to bind ubiquitin loaded E2 and promote polyubiquitination (Boname and Stevenson 2001; Coscoy, Sanchez et al. 2001; Henry, Wyce et al. 2003). Some of these E3s have important functions on transcription (Aasland, Gibson et al. 1995), immune system (Boname and Stevenson 2001; Coscoy, Sanchez et al. 2001; Coscoy and Ganem 2003) and MAP kinase pathways (Henry, Wyce et al. 2003).

1.2.1.3.2. HECT E3s

The second main E3 family is the HECT domain containing E3s. The HECT domain was first identified through studies of the degradation of the p53 tumor suppressor caused by an oncogenic form of papilloma virus in cervical cancer cells (Scheffner, Huibregtse et al. 1993). Virus induced degradation of p53 depends on the HPV *E6* gene product and a ~100 kDa host cellular protein E6AP (*E6* Associated Protein). E6 and E6AP form a complex that functions as a p53 specific E3 which mediates degradation of p53 by ubiquitination (Scheffner, Huibregtse et al. 1993). E6AP is the catalytic component of the ligase and requires a cysteine residue in the C-terminus for the activity. The C-terminus of E6AP was found to contain a sequence around the essential cysteine, conserved in a number of other proteins. This conserved ~350 residue C-terminal region became known as a HECT domain (Homologous to E6AP C-Terminus). It contains the strictly conserved cysteine residue positioned ~35 residues upstream of the C-terminus

(Huibregtse, Scheffner et al. 1995; Scheffner, Nuber et al. 1995). The HECT domains are 35–45% identical. The active site Cys residue of HECT E3 forms a thioester linkage with ubiquitin which is transferred from a cognate E2, prior to attack of the substrate Lys residue (Huibregtse, Scheffner et al. 1995; Scheffner, Nuber et al. 1995) (Fig. 6 B-D, 7A). An isolated HECT domain interacts tightly with the E2 and forms a thioester with ubiquitin. Deletion of the HECT domain does not affect substrate binding (Huibregtse, Yang et al. 1997; Kumar, Talis et al. 1999; Qiu, Joazeiro et al. 2000), suggesting that the unique N-terminus of each HECT domain E3 interacts with specific substrate(s). However, one HECT domain cannot substitute for another (Schwarz, Rosa et al. 1998), probably due to the HECT domain itself contributing to the interaction with the substrate to some extent or to the specificity of the E2 (Huibregtse, Scheffner et al. 1993).

To gain insight into the biochemical mechanism of ubiquitin transfer mediated by this family of ubiquitin ligases, the interaction of HECT domain and E2 was studied by determining the structures of the UbcH7–E6AP HECT complex (Huang, Kahana et al. 1997) (Fig. 6B) and UbcH7-Smurf–HECT complex (Ogunjimi, Briant et al. 2005) (Fig. 6, C-D). The HECT domain is L-shaped, the short arm of which is formed by the final 100 residues of E6AP, while the long arm is formed by the N-terminal part of the HECT domain (Huang, Kahana et al. 1997). The E2 binds at one end of this long arm. The active site cleft, defined by the side chain of the catalytic cysteine residue, is at the junction of the two arms of E6AP (Huang, Kahana et al. 1997). The most highly conserved residues of the HECT domain are within and around the cleft. Both of these studies of the E2-E3 HECT domain reveal separations of 41–50 Å between the active site Cys residues of the E2 and E3 (Fig. 6, B-C) (Huang, Kahana et al. 1997; Ogunjimi,

Briant et al. 2005), which is surprisingly far apart for the transfer of ubiquitin from the active Cys residue of E2 to that of E3.

However, the crystal structure of the HECT domain of human ubiquitin ligase WWP1/AIP5 shows that, relative to E6AP, rotation about a hinge region results in a predicted separation of only 16 Å between the E2 and E3 Cys residues (Verdecia, Joazeiro et al. 2003) (Fig. 6C). Continued rotation along the same direction could bring these two residues to a distance that might reasonably bridge the gap. Such proximity would allow the chain to be built up at the E3 active site through successive transfers of E2 bound ubiquitin to the E3 bound ubiquitin or to a E3 bound ubiquitin chain (Fig. 6D). This model is supported by the observation that synthesis of Lys48 linked diubiquitin catalyzed by the E6AP–HECT domain occurs through the reaction of two thioester bound ubiquitins: one linked to the E2 and the other linked to the E3 (Zhong, Gao et al. 2005). However, another HECT domain E3, KIAA10, uses a different mechanism of chain synthesis than E6AP (Zhong, Gao et al. 2005). KIAA10 synthesizes the chain as a free entity: a noncovalently bound acceptor ubiquitin or ubiquitin chain is the substrate for sequential ubiquitin transfer events. The HECT domain may therefore specify both the linkage of a polyubiquitin chain and the mechanism of its assembly.

Screening the human genome database suggests that there are ~90 HECT domain E3s, the majority of which are functionally uncharacterized. The remaining structure outside of the C-terminal HECT domain likely plays roles in targeting the HECT E3 to substrates or for other purpose such as subcellular localization. A number of domains have been recognized in these N-terminal regions. In particular is a group of ligases with a protein kinase C related C2 domain and multiple WW domains. The C2 domain functions in lipid binding and thus targets proteins to the plasma membrane. The WW

domain represents two highly conserved tryptophan (W) residues, which are spaced 20–22 amino acids apart. Some of these WW domains bind phosphoserine or phosphothreonine (Henry, Wyce et al. 2003), while the others bind to proline rich motifs, including the PPXY (Nguyen, Turck et al. 1998), PY, PPLP and PGM motifs (Kilinc, Guvel et al. 2004).

1.2.1.3.2.1. HECT domain E3s with C2 and WW domains

Members of this subfamily of the HECT domain E3 are involved in different cellular functions, such as endocytosis, DNA damage response, protein precursor processing, ion transportation and cell cycle. Rsp5, the best characterized HECT E3 in this subfamily, is an essential *Saccharomyces cerevisiae* yeast enzyme. WW domains of Rsp5 are required for the recognition of its substrates. Rsp5 has been shown to be required for endocytosis of membrane proteins. It is recruited to plasma membrane via its C2 domain and recognizes a set of plasma membrane permeases and receptors, including uracil permease (Fur4p) (Galan, Moreau et al. 1996), a general amino acid permease (Gap1p) (Hein, Springael et al. 1995) and a receptor for α mating pheromone (Ste2p) (Hicke, Zanolari et al. 1998). Ubiquitination of these substrates is required for subsequent endocytosis (Rotin, Staub et al. 2000). Rsp5 has also been shown to regulate gene transcription through mediating ubiquitination and degradation of Rpb1, the large subunit of RNA polymerase II (Pol II LS), upon induction of DNA damage (Huibregtse, Yang et al. 1997; Beaudenon, Huacani et al. 1999). In murine and human cells, Rpb1 is also a substrate of Nedd4, the murine/human ortholog of Rsp5 (Beaudenon, Huacani et al. 1999). Finally, Rsp5 is involved in processing of the transcription factors Spt23 and Mga2. Rsp5 ubiquitinates endoplasmic reticulum bound precursor forms of Spt23 and Mga2 (Hoppe, Matuschewski et al. 2000). This ubiquitination induces a limited processing event,

catalyzed by the proteasome, which releases the active Spt23 and Mga2 for translocation to the nucleus.

E3s in this subfamily have also been shown to be involved in modulating ion transport. The epithelial Na^+ channel protein ENaC is a substrate of Nedd4 and is endocytosed upon ubiquitination (Schild, Lu et al. 1996; Staub, Dho et al. 1996; Staub, Gautschi et al. 1997). The localization of Nedd4 to membranes through its C2 domain is required for ENaC ubiquitination (Plant, Lafont et al. 2000). PPXY motifs of ENaC is recognized by WW domains of Nedd4 (Staub, Gautschi et al. 1997). Certain *ENaC* mutations that predispose to the inherited disorder known as Liddle syndrome involve mutations of these PPXY motifs (Schild, Lu et al. 1996; Staub, Dho et al. 1996). In transfected cells, such mutations abrogate Nedd4 dependent ubiquitination and internalization of ENaC (Staub, Gautschi et al. 1997), resulting in an increased number of channel molecules in the plasma membrane. This deregulated ENaC turnover results in increased Na^+ reabsorption and contributes to the hypertension seen in Liddle syndrome.

1.2.1.3.2.2. HECT domain E3s without C2 and WW domains

HECT E3s of this subfamily are involved in multiple cellular processes and pathways, including DNA replication, gene transcription, cell proliferation, cell signaling, nucleotide excision repair, mRNA export and spermatogenesis. E6AP, the first ever identified HECT domain E3, mediates ubiquitination and degradation of p53 in cervical carcinoma cells by forming a complex with the HPV *E6* gene product (Scheffner, Huibregtse et al. 1993).

E6AP also plays important roles in cell signaling, nucleotide excision repair and DNA replication in uninfected cells. E6AP selectively ubiquitinates the activated form of Src family tyrosine kinases Blk and Src and targets them for degradation by the 26S

proteasome (Harris, Shoji et al. 1999; Oda, Kumar et al. 1999). The nucleotide excision repair factor HHR23A is also ubiquitinated by E6AP in a cell cycle dependent manner (Kumar, Talis et al. 1999). In addition, Mcm7, a subunit of the replication licensing factor, is another substrate of E6AP (Kuhne and Banks 1998). Mutations in the *E6AP* gene cause Angelman syndrome, an inherited condition characterized by severe mental retardation (Kishino, Lalande et al. 1997; Matsuura, Sutcliffe et al. 1997). It is not known if Angelman syndrome is a result of losing any of the E6AP functions illustrated above.

EDD is a HECT E3 that appears to be involved in spermatogenesis. It is the mammalian ortholog of Hyd (Callaghan, Russell et al. 1998), which is the product of *Drosophila melanogaster* hyperplastic disc gene *Hyd* and has a critical role in control of cell proliferation during development (Mansfield, Hersperger et al. 1994). Mutation of *Hyd* in Drosophila results in sterility and defects in germ tissue morphology (Mansfield, Hersperger et al. 1994). Rat Edd (RAT100) is expressed predominantly in germ cells and precisely regulated during spermatogenesis within the testis (Muller, Rehbein et al. 1992; Oughtred, Bedard et al. 2002). *EDD* family genes (*Edd, hyd, and Rat100*) encode highly conserved, large, predominantly nuclear proteins (approximately 300 kDa) that contain a HECT domain, a RING like zinc finger domain, a UBA domain and a poly(A) binding protein (PABP) like domain near the C terminus (Kwon, Reiss et al. 1998; Deo, Sonenberg et al. 2001; Wilkinson, Seeger et al. 2001; Henderson, Russell et al. 2002). PABP binds to the mRNA 3' poly(A) tail and stimulates recruitment of the ribosome to the mRNA at the 5' end. PABP activity is tightly controlled by its inhibitor, PABP-interacting protein 2 (Paip2). EDD was found recently to stimulate the turnover of Paip2 in the cell (Yoshida, Yoshida et al. 2006). This function of EDD is negatively regulated by PABP (Yoshida, Yoshida et al. 2006). This mechanism serves as homeostatic

feedback to control the activity of PABP in cells. Amplification and overexpression of *EDD* occurs frequently in several cancers, including breast and ovary cancers (Clancy, Henderson et al. 2003; Fuja, Lin et al. 2004). Truncating mutations of *EDD* are also observed in gastric and colon cancer with microsatellite instability (Mori, Sato et al. 2002).

Tom1 is a HECT E3 that is implicated in cell cycle, mRNA export and gene transcription. Tom1 (*T*rigger *o*f *m*itosis) is a 3268 amino acid *Saccharomyces cerevisiae* protein containing two domains with unknown function at the N-terminus, DUF 908 (*D*omain with *U*nknown *F*unction) and DUF 913. It was identified by a genetic study of genes involved in the G2/M transition (Utsugi, Toh-e et al. 1995). Cells that have the HECT domain of Tom1 deleted display temperature sensitivity and growth arrest at the G2/M transition of the cell cycle (Utsugi, Hirata et al. 1999). Furthermore, Tom1 is required for efficient mRNA export (Utsugi, Hirata et al. 1999; Duncan, Umen et al. 2000). Mutations in *tom1* predicted to abolish ubiquitin ligase activity block efficient export of Nab2p and mRNA, causing Nab2p–mRNA complexes to accumulate in a punctuate pattern coincident with the nuclear pore complex, while the subcellular distribution of several other hnRNP proteins is not affected (Duncan, Umen et al. 2000). Null mutation studies show Tom1 has a role in transcriptional regulation as well (Saleh, Collart et al. 1998). These effects of Tom1 are likely mediated through the ADA transcriptional coactivator complexes. Ubiquitination appears essential for the regulation by Tom1 since a cysteine residue for thioester bond formation with ubiquitin is required for transcriptional regulation by Tom1. Tom1 associates with the ADA proteins and is required for the ubiquitination of a 210 kDa protein, likely SPT7p, that coimmunoprecipitates with the ADA components (Saleh, Collart et al. 1998). In the

absence of Tom1, the normal associations of the ADA proteins with SPT3p and the TATA binding protein are reduced, which results in transcription defects (Saleh, Collart et al. 1998). Finally, gene deletion studies reveal Tom1 is required for full induction of the general stress and heat shock responses (Utsugi, Hirata et al. 1999; Sasaki, Toh-e et al. 2000).

UreB1 (*U*pstream *r*egulatory *e*lement *B*inding protein) is another HECT E3 of this subfamily that is involved in gene transcription. UreB1was originally identified as a 310 amino acid HECT protein that has a role in transcriptional regulation through direct DNA binding (Gu, Ren et al. 1994; Gu, Irving et al. 1997). UreB1 was later predicted by cDNA sequences to be a much larger protein named LASU1 (gi:22090626). The E3 ligase described in this thesis turns out to be the same protein as LASU1 (gi:60549639 for human, gi:60549637 for mouse), but even longer than as described in the sequence database (gi:22090626). We designated this E3 as $E3^{Histone}$/LASU1. Detailed results are in the following chapters. Interestingly, the complete $E3^{Histone}$/LASU1sequence (gi:60549639 for human, gi:60549637 for mouse) also contains unknown function domains DUF 908 and DUF 913 at the N terminus as yeast Tom1, suggesting that $E3^{Histone}$/LASU1 could be mammalian ortholog of Tom1.

1.2.2. Proteasome

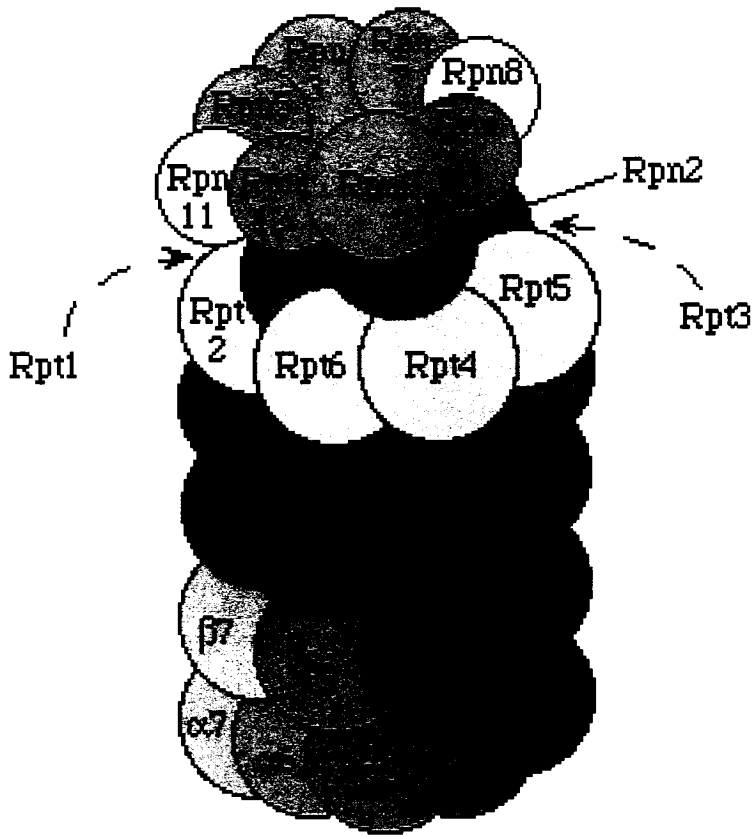
Proteins ligated to Lys 48 linked polyubiquitin chains are degraded by the 26S proteasome complex. This complex consists of a 20S proteasome and two 19S regulatory particles, one at each end of the core (Voges, Zwickl et al. 1999).

The 20S proteasome is a 700 kDa complex composed of a cylindrical stack of four heptameric rings, each of which contains seven subunits (Fig. 8). The two identical inner rings are made up of structurally similar subunits and designated β type. Three of these

subunits, β1, β2 and β5, are catalytically active, and their distinct active sites are directed toward the inner chamber. The two identical outer rings are composed of α type subunits. Access to the chamber of the 20S proteasome is limited by the two narrow openings in the outer rings that are occluded by N-terminal strands of these α type subunits.(Rivett 1989; Orlowski 1990; Rechsteiner, Hoffman et al. 1993; Hilt and Wolf 1996; Groll, Ditzel et al. 1997; DeMartino and Slaughter 1999; Orlowski and Wilk 2000; Zwickl, Baumeister et al. 2000).

The 20S proteasomes are extremely inefficient in degrading ubiquitinated substrates unless one or two multisubunit 19S regulatory particles bind to the ends of the 20S proteasome cylinder, forming the 26S proteasome. These regulatory complexes are able to recognize Lys 48 linked ubiquitin chains and therefore bind proteasome substrates. In all eukaryotic cells, the 19S regulatory complex is made up of two main classes of subunits: the non-ATPases (Rpn1 to Rpn3 and Rpn5 to Rpn12) and the ATPases (Rpt1 to Rpt6) (Glickman, Rubin et al. 1998) (Fig. 8). The 19S regulatory complex consists of two subcomplexes: the base subcomplex which contains all six ATPase subunits and three non-ATPase subunits, and the lid subcomplex which contains at least eight non-ATPase subunits (Glickman, Rubin et al. 1998) (Fig. 8). Among the non-ATPases, Rpn11 (POH1 in humans) is a deubiquitinating enzyme (belongs to the JAMM family of DUBs, details in 1.2.3 deubiquitinating enzyme) and is required for deconjugation of polyubiquitin chains from the substrate protein (Verma, Aravind et al. 2002; Yao and Cohen 2002). The 19S regulatory complex appears to initiate unfolding of the substrates and their translocation into the proteolytic chamber of the 20S proteasome, where they are broken down into peptides of 3-25 amino acids.

Fig. 8



http://www.genome.jp/dbget-bin/get_pathway?org_name=hsa&mapno=03050

Fig. 8. Diagram of proteasome (one 19S proteasome and one 20S proteasome). The 19S regulatory particle can be divided into a base and a lid. The base contains AAA ATPases Rpt1-6 (Green), non-ATPase Rpn 1, 2 (Purple) and Rpn 10 (Red). The lid is composed of eight subunits, Rpn3, 5, 6, 7, 9, 12 (Orange), Rpn8, 11 (Cyan). The 20S proteasome is represented by 4 stacked, 7-member rings representing alpha (Brick colour) and beta (Blue) proteasome subunits.

A distinct complex is formed by binding of a heptameric 11S regulator (PA28 activator) to both ends of the 20S proteasome (Dubiel, Ferrell et al. 1992; Ma, Slaughter et al. 1992). This binding leads to a rearrangement of the α subunit chains resulting in widening of the two openings in the outer rings, thereby facilitating access of some substrates to and egress of proteolytic products from the proteolytic chamber (Whitby, Masters et al. 2000). Formation of this complex leads to an acceleration of the degradation of small peptides, but not proteins, in an ATP independent manner. There is also evidence for the existence of a hybrid complex in which the 20S core proteasome is capped by the 19S regulatory complex at one end and an 11S regulator at the other end (Tanahashi, Murakami et al. 2000).

1.2.3. Deubiquitinating enzyme

Ubiquitin conjugation can be countered by the action of deubiquitinating enzymes (DUBs), which remove the ubiquitin moiety from the protein substrate (Wilkinson 1997; D'Andrea and Pellman 1998; Wilkinson 2000; Wing 2003; Soboleva and Baker 2004). DUBs are proteases that cleave the isopeptide bond at the C-terminal Gly76 residue of ubiquitin, either removing ubiquitin from the substrate or processing the polyubiquitin chain (Fig. 5). There are five classes of DUBs based on conserved sequence motifs: ubiquitin specific protease (USP), ubiquitin C-terminal hydrolase (UCH), Otubain protease (OTU), Machado-Joseph disease protease (MJD), and JAB1/MPN/Mov34 metalloenzyme (JAMM). All of those classes except JAMM are cysteine proteases containing the catalytic triad, Cys, His and Asp. The human genome project has led to identification of approximately 95 putative DUBs, including 58 USP, 4 UCH, 5 MJD, 14 OUT, and 14 JAMM domain containing genes (Nijman, Luna-Vargas et al. 2005). DUBs are involved in various biological processes, such as cell growth, differentiation, immune

function, and memory consolidation; mutation of DUBs are implicated in the pathogenesis of diseases such as cancer (Wilkinson 1997; Chung and Baek 1999; Fischer 2003; Soboleva and Baker 2004).

UCH and USP are the two major families of DUBs. The UCHs are papain like thiol proteases with a 230 amino acid core catalytic domain, whereas the core catalytic domain of UBPs is ~350 amino acids (D'Andrea and Pellman 1998). USP is the largest family of DUB. USPs have conserved Cys-box and His-box, which are required for proteolytic cleavage (Wilkinson 1997; Hu, Li et al. 2002). USPs mainly differ by insertions between the catalytic boxes or by having divergent N- or C- terminal extensions, all of which may be involved in substrate selectivity (Wilkinson 1997; Lin and Wing 2000).

Ataxin-3 and a number of Ataxin-3 like proteins were identified by a bioinformatics search as possible ubiquitin proteases (Burnett, Li et al. 2003; Scheel, Tomiuk et al. 2003). They are classified into the MJD subfamily of DUB, characterized by a domain called the Josephin domain. The cysteine in the active site is required for the DUB enzyme activity *in vitro* (Burnett, Li et al. 2003). The sequence similarity is low between the catalytic domain of Ataxin-3 and other DUBs, but NMR structures reveal that the catalytic triad is conserved (Mao, Senic-Matuglia et al. 2005; Nicastro, Menon et al. 2005).

The OTU (*O*varian *tu*mor) related proteases were predicted by a bioinformatics analysis as thiol proteases (Makarova, Aravind et al. 2000). The OTU gene may regulate the localization and translation of certain RNA transcripts and is involved in the development of the *Drosophila melanogaster* ovary (Steinhauer, Walsh et al. 1989; Goodrich, Clouse et al. 2004). Otubain-1, Otubain-2 and Cezanne, OTU DUBs, have been shown to display DUB activity *in vitro* (Balakirev, Tcherniuk et al. 2003; Evans,

Smith et al. 2003). For most OTU proteases, their physiological role *in vivo*, including their putative role as DUBs, remains to be investigated.

JAMMs are DUBs which are metalloproteases. A subunit of the proteasome, Rpn11/POH1 was the first identified JAMM (Maytal-Kivity, Reis et al. 2002; Verma, Aravind et al. 2002). AMSH (*A*ssociated *M*olecule with the *SH*3 domain of STAM), another protein with the MPN+/JAMM motif, was also found to have deubiquitinating activity (McCullough, Clague et al. 2004).

1.2.4. Functions of ubiquitination

With the many substrates targeted by the ubiquitin pathway, it is not surprising to find that this pathway is responsible, directly or indirectly, for regulation of many biological systems, including DNA repair (Pastushok and Xiao 2004), transcription (Desterro, Rodriguez et al. 2000; Yang, Li et al. 2004; Dhananjayan, Ismail et al. 2005), membrane protein trafficking (Marmor and Yarden 2004; d'Azzo, Bongiovanni et al. 2005; Rubin, Gur et al. 2005), ER quality control (Hampton 2002; Ye 2005), cell signaling pathways (Moustakas, Souchelnytskyi et al. 2001; Daniel, Torok et al. 2004; Hammond, Carter et al. 2004; Rome, Meugnier et al. 2004; Strous, dos Santos et al. 2004; Wojcikiewicz 2004; Chen, Kon et al. 2005; Laine and Ronai 2005), cell cycle (Vodermaier 2004; Hershko 2005), ion transport (Rotin, Staub et al. 2000), skeletal muscle wasting (Lecker 2003; Taillandier, Combaret et al. 2004; Attaix, Ventadour et al. 2005; Cao, Kim et al. 2005; Wing 2005), synaptic plasticity (Schwartz, Goldfinger et al. 1993; DiAntonio and Hicke 2004; Yi and Ehlers 2005), and the immune system (Ben-Neriah 2002; Liu 2004; Mueller 2004; Liu, Penninger et al. 2005). Dynamic modification of proteins by ubiquitin enables reversible switches between different functional states (Pickart and Eddins 2004; Varshavsky 2005).

1.2.5 The ubiquitin system in spermatogenesis

A variety of elements involved in the ubiquitin system have been detected in the testis (Kay, Ashworth et al. 1991; Baarends, Hoogerbrugge et al. 1999; Bebington, Doherty et al. 2001; Sutovsky, Terada et al. 2001). The ubiquitin is expressed in the round spermatids and mature sperm in roosters (Agell and Mezquita 1988), bulls (Sutovsky, Moreno et al. 2000), and mice (Tipler, Hutchon et al. 1997). Ubiquitin system is activated during spermatogenesis (Baarends, Roest et al. 1999; Baarends, van der Laan et al. 2000; Bebington, Doherty et al. 2001; Sutovsky 2003). In testis, the enzymes for ubiquitin conjugation, E1 (Kay, Ashworth et al. 1991; Mitchell, Woods et al. 1991), E2 (Wing and Jain 1995; Roest, van Klaveren et al. 1996; Wing, Bedard et al. 1996; Rajapurohitam, Morales et al. 1999; Sutovsky, Moreno et al. 2000) and E3 (Mitchell, Woods et al. 1991; Dickins, Frew et al. 2002; Oughtred, Bedard et al. 2002; Kwon, Xia et al. 2003; Lorenzetti, Bishop et al. 2004; Liu, Oughtred et al. 2005; Nishito, Hasegawa et al. 2006) are present. Our lab has been studying the rodent homologs of UBC4/5, which in yeast are essential for the degradation of short lived and abnormal proteins (Seufert and Jentsch 1990). UBC4/5 have been shown to be responsible for the activation of ubiquitin conjugation during spermatogenesis (Rajapurohitam, Morales et al. 1999). There are multiple isoforms of these homologs in higher eukaryotes. In mammals, three rat orthologs (Wing and Jain 1995; Wing, Bedard et al. 1996) and four human orthologs of UBC4/5 (Scheffner, Huibregtse et al. 1994; Jensen, Bates et al. 1995; Rolfe, Beer-Romero et al. 1995), have been identified. Two of the rat orthologs are widely expressed in rat tissues, consistent with their fundamental cellular function, while one isoform, UBC4-testis which although having more than ninety percent amino acid identity with the other isoforms, is expressed in the testis only (Wing, Bedard et al. 1996). UBC4-testis

is absent in early life, but is induced during sexual maturation. It is induced at the stage when histones are degraded during spermatogenesis. Although this property of UBC4-testis suggests it may be required for spermatid maturation, mice lacking the UBC4-testis gene show normal spermatogenesis and fertility, but only a minor defect, a delay in postnatal testis development (Bedard, Hingamp et al. 2005).

Polyubiquitin chain binding protein Mcb1 is expressed in testis and there is a testis specific longer Mcb1 transcript in mouse testis (Pusch, Jahner et al. 1998). DUBs are also activated during spermatogenesis. Ubiquitin C-terminal hydrolase L-1 is essential for the early apoptotic wave of germinal cells and for sperm quality control (Kwon, Kikuchi et al. 2003; Kwon, Wang et al. 2004; Kwon, Mochida et al. 2005). The ubiquitin C-terminal hydrolase PGP 9.5 is expressed in both the testis (Chen, Kon et al. 2005) and the epididymis (Santamaria, Martin et al. 1993; Fraile, Martin et al. 1996). Deletions in the ubiquitin specific protease *USP9* result in infertile males with defective spermatogenesis (Sun, Skaletsky et al. 1999; Van Landuyt, Lissens et al. 2001; Lee, Song et al. 2003). Proteasomes are present in testis (Mochida, Tres et al. 2000; Wojcik, Benchaib et al. 2000; Bialy, Ziemba et al. 2001). A testis specific homolog of human 19S proteasome subunit, TPB-1, is present in manchettes and in the cytoplasmic lobe of rat elongating spermatids (Rivkin, Cullinan et al. 1997). There are two testes specific 20S proteasome genes expressed during specific stages of spermatogenesis in *Drosophila* (Yuan, Miller et al. 1996). The importance of proteasomes in normal spermatogenesis has been highlighted by the suggestion that C5, a 20S proteasome subunit, may be a candidate for the hybrid sterility 1 gene of inbred mice (Trachtulec, Mnukova-Fajdelova et al. 1997). In addition, spermatozoa from patients with varicocele associated sterility show abnormal

localization of the proteasome to sperm chromatin and large residual bodies compared to normal spermatozoa (Ziemba, Bialy et al. 2002).

Unique expression of different elements of the ubiquitin system in developing germ cells and maturing spermatozoa suggests that different phases of mammalian spermatogenesis probably require different specialized activities of the ubiquitin system. There is very likely a direct link between ubiquitination and other cellular events such as meiosis, gene transcription/silencing, spermatid remodeling, and apoptosis or elimination of defective cells during spermatogenesis. The ubiquitin system is highly regulated and activated during spermiogenesis (Rajapurohitam, Morales et al. 1999). This may be related to the massive breakdown of cytoplasmic and nuclear proteins during this last phase of spermatogenesis.

Development of mature spermatozoa involves the significant reduction of mitochondria. It appears that mitochondria are degraded during spermatogenesis and in fertilized eggs (Sutovsky, Moreno et al. 1999). The colocalization of ubiquitin with mitochondria in spermatids and mature sperm suggests the ubiquitination of the spermatid mitochondria (Sutovsky, Moreno et al. 1999; Sutovsky, Moreno et al. 2000). Ubiquitin tagged proteins in the mammalian sperm mitochondria may target sperm mitochondria for destruction in the egg cytoplasm after fertilization, and may therefore explain the exclusive maternal mitochondria in cells of the offspring. Both lysosomal and proteasomal proteolysis have been implicated in such targeted degradation of the sperm mitochondria inside the fertilized oocyte (Sutovsky, Moreno et al. 2000). A recent study (Thompson, Ramalho-Santos et al. 2003) has shown that the mitochondrial membrane protein prohibitin is one of the ubiquitinated substrates and may play a role in the regulation of mitochondrial inheritance and sperm quality control.

Centrosome reduction is a hallmark of spermatid elongation (Manandhar, Sutovsky et al. 1998). The centrosome must be reduced during spermatogenesis to avoid its doubling in the successive generation. In a somatic cell, like in an immature spermatid, the centrosome is composed of two centrioles and a halo of pericentriolar, microtubule nucleating material. After fulfilling its role in generating the sperm axoneme, this centrosome is either completely removed (mouse, rat, hamster, probably most other rodents) or reduced to a single inactive centriole (other mammals, including humans) (Sutovsky P 1999). Ubiquitin immunoreactivity can be detected in the centrosomal part of the human and rhesus sperm tail (Sutovsky 2003). Proteasomal subunits were also detected inside or near the sperm centriole (Mochida, Tres et al. 2000; Wojcik, Benchaib et al. 2000; Bialy, Ziemba et al. 2001). Ubiquitin system may therefore be involved in centrosome reduction.

A key event during spermatogenesis is the degradation of histones. Histones are found ubiquitinated during spermatogenesis (Agell, Chiva et al. 1983; Agell and Mezquita 1988; Chen, Sun et al. 1998; Baarends, Hoogerbrugge et al. 1999). Ubiquitination may be required for somatic histones replacement by testis specific histones during meiosis and the subsequent substitution of most histones by transition proteins which are later replaced by protamines during spermiogenesis, both of which result in chromatin reorganization. Ubiquitination of histones may also play an important role in meiosis as described in detail in the later section, "the role of histone ubiquitination in spermatogenesis".

1.3. Histone ubiquitination

Histones are small basic proteins that bind to DNA in chromatin. The most fundamental structural unit of chromatin and the first level of DNA organization is the

nucleosome core particle, which consists of 146 bp of DNA wrapped around an octamer of very basic proteins called histones. Each nucleosome core has a core histone octamer which consists of two copies of each of the histones: H2A, H2B, H3 and H4. A $(H3–H4)_2$ tetramer associates with two H2A–H2B dimers core on each side of the tetramer (Arents, Burlingame et al. 1991; Pruss 1995; Luger, Mader et al. 1997; Ramakrishnan 1997). A fifth histone, H1, protects an extra 20 bp of DNA that links the nucleosome. The evolutionarily conserved histones on nucleosomes have a globular C-terminal domain critical to nucleosome formation and a flexible N-terminal tail that protrudes from the nucleosome core which is accessible to enzymatic machinery responsible for posttranscriptional modification. Histone tails are highly flexible and dynamic. For years, histones were assumed to be merely inert structural components, a scaffold for the DNA in nucleosome formation, despite the fact that the histone tails are subject to many posttranslational modifications such as acetylation, methylation, phosphorylation, ADP ribosylation, and ubiquitination (Strahl and Allis 2000; Ausio, Abbott et al. 2001; Berger 2001). Now it has become increasingly recognized that posttranslational modifications of the histones are a key factor in chromatin dynamics and folding, which has fundamental impact on many cellular processes including DNA replication and repair, transcriptional activation and silencing, gene expression, and cell cycle progression (Garcia-Ramirez, Dong et al. 1992; Hansen and Ausio 1992; Moore and Ausio 1997; Wang, He et al. 2001). Compared to extensive functional and structural studies on histone acetylation (Roth, Denu et al. 2001), methylation (Zhang and Reinberg 2001; Lachner and Jenuwein 2002), and phosphorylation (Cheung, Allis et al. 2000), much less is known about histone ubiquitination (Ausubel 1993; Jason, Moore et al. 2002; Zhang 2003).

Different histones are capable of being ubiquitinated, including H2A (Nickel, Roth et al. 1987; Bonner 1988; Wang, Wang et al. 2004), H2B (West and Bonner 1980; Nickel, Roth et al. 1987; Thorne, Sautiere et al. 1987; Robzyk, Recht et al. 2000), H1(Pham and Sauer 2000), H3 (Chen, Sun et al. 1998) and histone H2A variants H2A.1, H2A.2, H2A.Z and H2A.X (West and Bonner 1980). Histone H2A was the first protein ever identified to be ubiquitinated (Goldknopf, Taylor et al. 1975). Most studies by far have concentrated on the ubiquitination of histones H2A and H2B (uH2A and uH2B), which are the most abundant ubiquitinated histones. In eukaryotic cells, 10 to 15% of total histone H2A is ubiquitinated (West and Bonner 1980), while 10% of histone H2B is ubiquitinated in yeast (*Saccharomyces cerevisiae*) (Robzyk, Recht et al. 2000) and 1.5% of histone H2B is monoubiquitinated in higher eukaryotes (West and Bonner 1980). The ubiquitin on uH2A and uH2B is exchanged via conjugation and deconjugation and is in equilibrium with free ubiquitin within the nucleus (Matsui, Seon et al. 1979; Wu, Kohn et al. 1981; Carlson and Rechsteiner 1987).

The ubiquitination site on histone H2A has been mapped to the highly conserved residue Lys 119 (Nickel, Roth et al. 1987; Wang, Wang et al. 2004). The majority of uH2A is in monoubiquitinated form; however, polyubiquitinated H2A has also been detected in many tissues and cell types (Nickel, Roth et al. 1987). In H2B, the ubiquitinated site is also on a specific lysine residue, Lys 120 in human and Lys 123 in yeast (Thorne, Sautiere et al. 1987). So far, only monoubiquitinated H2B and di-ubiquitinated H2B have been reported (Nickel, Roth et al. 1987; Davie, Lin et al. 1991). The ubiquitination site on H3 or H1 has not been determined.

Low levels of different polyubiquitinated histones have been identified in many tissues, including trout liver and testes, human breast cancer cells, bovine thymus, and

tetrahymena (Davie and Nickel 1987; Nickel, Roth et al. 1987; Davie and Murphy 1990; Jasinskiene, Jasinskas et al. 1995). Among all histones, the highest level of polyubiquitination is seen with histone H2A where tetra- and hexa-ubiquitinated forms have been identified (Davie and Nickel 1987; Nickel, Roth et al. 1987).

1.3.1. Enzymes involved in histone ubiquitination

Different E2s and E3s have been identified as capable of ubiquitinating different histones. Two budding yeast proteins, Rad6 and Cdc34, have been shown to ubiquitinate H2B *in vitro* even without the presence of E3 (Jentsch, McGrath et al. 1987; Goebl, Yochem et al. 1988). However, *in vivo* in yeast only Rad6 appears to be indispensable for H2B ubiquitination (Robzyk, Recht et al. 2000). HR6B (*H*uman homolog of *R*ad*6*) and its nearly identical homolog HR6A are the mammalian homologs of yeast Rad6. They are multifunctional proteins with ubiquitin conjugating activity and essential roles in postreplication DNA repair (Roest, van Klaveren et al. 1996; Baarends, Hoogerbrugge et al. 1999; Adegoke, Bedard et al. 2002; Shekhar, Lyakhovich et al. 2002; Baarends, Wassenaar et al. 2003; Lyakhovich and Shekhar 2004; Roest, Baarends et al. 2004; Kavakebi, Hausott et al. 2005). They can partially complement mutations of yeast Rad6 *in vivo* and are able to ubiquitinate histones *in vitro* without any E3 (Wing, Dumas et al. 1992). Just like mHR6B knockout mouse embryonic fibroblasts, mHR6A-deficient cells appear to have normal DNA damage resistance properties (Baarends, Roest et al. 1999; Roest, Baarends et al. 2004). Neither animals lacking both proteins nor females with only one intact mHR6A allele are viable, suggesting it is important to keep at least one functional mHR6A or mHR6B allele in all somatic cell types (Roest, Baarends et al. 2004). The different phenotype is that mHR6B knockout males show a severe spermatogenic defect (Baarends, Roest et al. 1999), while mHR6A knockout males are

normally fertile, but mHR6A knockout females fail to produce offspring despite a normal ovarian histology and ovulation (Roest, Baarends et al. 2004). Taken together these observations suggest that HR6A and HR6B have redundant but possibly also dose dependent roles in somatic cell types and germ line cells in mammals. Recent studies have identified Bre1, a Rad6 associated RING finger protein, as the E3 ligase for H2B ubiquitination (Hwang, Venkatasubrahmanyam et al. 2003; Wood, Krogan et al. 2003) (Table 2). Two putative orthologs of Bre1 have also been identified in humans (Hwang, Venkatasubrahmanyam et al. 2003). The Mdm2 oncoprotein, a RING domain E3 known to ubiquitinate p53, has been recently shown to interact directly with histones and promote *in vitro* monoubiquitination of histones H2A and H2B (Minsky and Oren 2004). Moreover, Mdm2 induces H2B monoubiquitination *in vivo*, suggesting that Mdm2 could be another E3 catalyzing H2B monoubiquitination (Table 2).

Several enzymes capable of histone H2A ubiquitination have also been reported. *In vitro* studies show that Rad6 is capable of ubiquitinating both H2A and H2B with similar kinetics (Haas, Reback et al. 1991). However, it is unknown if Rad6 supports ubiquitination of H2A as it does for H2B *in vivo*. uH2A is not detectable in the budding yeast *Saccharomyces cerevisiae* (Robzyk, Recht et al. 2000), which prevents the identification of the relevant E2 and E3 enzymes for H2A ubiquitination in yeast. In mouse, H2A ubiquitination level appears to be strongly correlated with the level of HR6B during spermatogenesis (Baarends, Hoogerbrugge et al. 1999). However, there is no defect in the overall pattern of ubiquitinated H2A during mouse spermatogenesis in HR6B knockout mice (Baarends, Hoogerbrugge et al. 1999), suggesting that HR6B may not be the major E2 for H2A ubiquitination *in vivo*. Functional redundancy between HR6A and HR6B could be another explanation. Recently, an E3 ubiquitin ligase

Table 2. All identified E3s that mediate histone ubiquitination

	Types of histone	E2	Types of E3	Mono- or poly-ubiquitination	*in-vitro* proof	*in-vivo* proof	Regulation of transcription
NP95	All	UbcH5B	Ring domain	Mono-/ poly-?	+	+	Activation?
$TAF_{II}250$	H1	?	Ring domain	Mono-	+	+	Activation
Bre1	H2B	Rad6	Ring domain	Mono-	+	+	Activation/ Repression
dRing1A / 1B	H2A	UbcH5C	Ring domain	Mono-	+	+	Repression
Mdm2	H2A/H2B	UbcH5C	Ring domain	Mono-	+	+	Repression
BRCA1/ BARD1	All, except H1	UbcH5A/ UbcH5B	Ring domain	Mono-/ poly-	+		

complex, hPRC1L (*H*uman *P*olycomb *R*epressive *C*omplex *1* *L*ike), has been identified that is specific for histone H2A ubiquitination (de Napoles, Mermoud et al. 2004; Fang, Chen et al. 2004; Wang, Wang et al. 2004). The complex is composed of several Polycomb group proteins including Ring1, Ring2, Bmi1 and HPH2. hPRC1L monoubiquitinates nucleosomal histone H2A at lysine 119 (Table 2). Reducing the expression of Ring2 results in a dramatic decrease in the level of ubiquitinated H2A in cells.

Histone H1 is also ubiquitinated. It has been reported that TAFII250, a component of the general transcription factor TFIID, possesses ubiquitin activating/conjugating activity for H1 *in vitro* (Pham and Sauer 2000) (Table 2). This is the first report that E1 and E2 activities can be contained within the same protein. Point mutations on TAFII250 that abolish the H1 ubiquitination activity *in vitro* also lead to decreases in the level of ubiquitinated H1 in the Drosophila embryo (Pham and Sauer 2000), suggesting that H1 may represent a real *in vivo* target for TAFII250.

Np95, a RING E3 that is important in regulating cell cycle, has been found to bind histones directly and ubiquitinate histones as an E3 ligase, with a remarkable preference for histone H3 (Citterio, Papait et al. 2004) (Table 2). A novel protein domain, the SRA-YDG domain contained in Np95, is indispensable both for the interaction with histones and for chromatin binding *in vivo* and is required for the E3 ubiquitin ligase activity of Np95 on histone ubiquitination *in vitro.*

Human BRCA1/BARD1 heterodimeric complex has been shown to be capable of monoubiquitinating histones *in vitro*, including the variant histone H2AX (Mallery, Vandenberg et al. 2002) (Table 2).

1.3.2. Deubiquitinating enzymes for histones

Ubiquitin can be added and removed from histones as with other reversible posttranslational modifications, such as acetylation and phosphorylation. Therefore, the levels of histone ubiquitination are determined by the availability of free ubiquitin and of the enzymatic activities involved in adding or removing the ubiquitin moiety from histones. Several deubiquitinating enzymes have been identified that negatively regulate histone ubiquitination. It has been demonstrated that yeast Ubp8 is a stable and stoichiometric subunit of the Spt-Ada-Gcn5-acetyltransferase (SAGA), a complex that mediates histone acetylation and is required for the expression of a subset of genes, and Ubp8 has a role in H2B deubiquitination *in vitro* and *in vivo* (Henry, Wyce et al. 2003; Daniel, Torok et al. 2004). Mutation of *Ubp8* results in the accumulation of ubiquitinated H2B (Henry, Wyce et al. 2003). Furthermore, SAGA complex purified from wild type strain, but not from *ubp8* deletion strain, can deubiquitinate H2B *in vitro*. The deubiquitination function of Ubp8 is specific to H2B but not to other histones. Thus SAGA is a complex that possesses both a histone deubiquitination function and a histone acetylation function. Potential Ubp8 orthologs exist in various species from plants to humans. It remains to be determined whether these Ubp8 orthologs deubiquitine H2B.

The ubiquitin protease Ubp10 is another DUB that targets H2B for deubiquitination in yeast (Emre, Ingvarsdottir et al. 2005). Ubp10 serves to maintain low level of H2B Lys123 ubiquitination proximal to telomeres. Very recently it was reported that Drosophila ubiquitin specific protease 7 (USP7) also deubiquitinates uH2B (van der Knaap, Kumar et al. 2005). USP7 forms a heteromeric complex with guanosine 5'-monophosphate synthetase (GMPS). The USP7-GMPS complex catalyzes the selective deubiquitination of histone H2B, but not H2A. Mutation studies show that USP7 binding to GMPS is required for histone H2B deubiquitination.

1.3.3. Functions of histone ubiquitination

Most ubiquitination of histones does not seem to lead to their increased turnover or degradation (Seale 1981; Wu, Kohn et al. 1981). Regulatory roles of histone ubiquitination and deubiquitination include gene transcription, cell cycle and spermatogenesis (Jason, Moore et al. 2002; Zhang 2003; Emre and Berger 2004; Kao, Hillyer et al. 2004).

1.3.3.1. The role of histone ubiquitination in gene transcription, activation and silencing

1.3.3.1.1. Role of H2A ubiquitination

Ubiquitination of histones has been linked to the complex processes that regulate eukaryotic gene transcription. However, the cellular factors that mediate this histone modification during the processes of transcriptional activation are not well characterized. Very recently, some of the important roles of histone ubiquitination on gene transcription have been determined. Evidence from early studies suggested that H2A ubiquitination may participate in gene activation. It has been reported that transcriptionally active *Hsp 70* genes contain up to 50% uH2A, whereas nucleosomes of nontranscribed DNA contain only one uH2A per 25 nucleosomes (Levinger and Varshavsky 1982). Furthermore, diubiquitinated H2A was found to be preferentially enriched around the first exon of the actively transcribed mouse dihydrofolate reductase (DHFR) gene (Barsoum and Varshavsky 1985). Both uH2A and uH2B were found to be enriched around transcriptionally active sequences in bovine thymus, chicken erythrocyte, and *Tetrahymena* macronuclei (Nickel, Roth et al. 1987). In contrast, recent studies showed a link of H2A ubiquitination with gene repression (Wang, Wang et al. 2004) and to the inactive X chromosome (de Napoles, Mermoud et al. 2004; Fang, Chen et al. 2004).

hPRC1L/ PRC1 positively regulate Polycomb silencing through H2A ubiquitination via its E3 ubiquitin ligase activity (de Napoles, Mermoud et al. 2004; Fang, Chen et al. 2004; Wang, Wang et al. 2004). dRing, which is a RING domain subunit of hPRC1L, colocalizes with ubiquitinated H2A at the PRE (*P*olycomb *r*esponse *e*lement) and promoter regions of the *Drosophila Ubx* gene in wing imaginal discs and localize on the inactive X chromosome. Removal of dRing in SL2 tissue culture cells by RNA interference resulted in loss of H2A ubiquitination accompanied by derepression of *Ubx* (Wang, Wang et al. 2004). Similarly, analysis of Ring1B null embryonic stem (ES) cells revealed extensive depletion of global uH2A levels (de Napoles, Mermoud et al. 2004). It is remained to determine if inactive X chromosome is actived in Ring1B null ES cells. Also Ring1B mediated H2A ubiquitination is likely involved in the initiation of both imprinted and random X inactivation (Fang, Chen et al. 2004). These studies suggest that the function of H2A ubiquitination on gene transcription may be gene dependent.

TAFII250 mediates histone H1 monoubiquitination and is likely to be required for activation of transcription (Pham and Sauer 2000). TAFII250 is the central subunit within the general transcription factor TFIID. In the Drosophila embryo, inactivation of the TAFII250 E1/E2 activity reduces the cellular level of monoubiquitinated histone H1 and the expression of genes targeted by the maternal activator Dorsal. Thus, TAFII250 mediated H1 ubiquitination may contribute to the process of directing activation of eukaryotic transcription. Interestingly, TAFII250 mutations that impair the E1/E2 activity do not cause a general transcriptional defect, suggesting that H1 ubiquitination may participate in transcriptional regulation of a subset of genes such as those controlled by the transcriptional factor Dorsal (Pham and Sauer 2000).

1.3.3.1.2. Role of H2B ubiquitination

More has been learned about histone H2B monoubiquitination. Mdm2 mediated H2B monoubiquitination is implicated in transcriptional repression rather than activation (Minsky and Oren 2004). Mdm2 was originally determined to ubiquitinate and mediate p53 degradation under normal conditions (Michael and Oren 2003). It now appears that there is another form of regulation in which Mdm2 associates with p53 inducible genes in a p53 dependent manner to repress them through monoubiquitination of H2B on Lys120 and/or Lys125 (Minsky and Oren 2004). Rad6 mediated ubiquitination of H2B is involved in transcription, including gene activation (Kao, Hillyer et al. 2004) and repression (Turner, Ricci et al. 2002; Carvin and Kladde 2004), as well as telomeric and *HMR* gene (one of the silent (HM) mating type loci) silencing (Huang, Kahana et al. 1997). Thus, the function of H2B Lys123 ubiquitination on gene transcription is gene specific. The ability of Rad6, in cooperation with the RING domain E3 Bre1, to ubiquitinate H2B is specifically required for these transcriptional activities (Huang, Kahana et al. 1997; Sun and Allis 2002; Turner, Ricci et al. 2002; Henry, Wyce et al. 2003; Hwang, Venkatasubrahmanyam et al. 2003; Wood, Krogan et al. 2003; Carvin and Kladde 2004; Kao, Hillyer et al. 2004). Rad6 is recruited to gene promoters (Wood, Krogan et al. 2003) in a Bre1 dependent manner (Kao, Hillyer et al. 2004), resulting in increased H2B ubiquitination (Henry, Wyce et al. 2003; Kao, Hillyer et al. 2004).

So far, several studies have shown that H2B monoubiquitination and deubiquitination cross talk to methylation of histone and tightly regulate gene transcription through this mechanism. Ubiquitination of H2B is required for methylation of histone H3 on both Lys4 (Dover, Schneider et al. 2002; Sun and Allis 2002) and Lys79 (Briggs, Xiao et al. 2002; Ng, Xu et al. 2002). Either H2B Lys123Arg substitution or RAD6/BRE1 deletion abolishes detectable methylation (Hwang, Venkatasubrahmanyam et al. 2003; Wood,

Krogan et al. 2003). However, methylation of H3 on Lys36 does not require uH2B (Briggs, Xiao et al. 2002; Ng, Xu et al. 2002). The function of histone ubiquitination on transcription is likely mediated by the cross talk with methylation, since there are strong parallels between the alterations in uH2B and methylation of H3. Similar to uH2B, methylation of Lys79 and Lys4 on H3 by the methyltransferase Dot1 and Set1, respectively, are implicated in silencing (Nislow, Ray et al. 1997; Singer, Kahana et al. 1998; Briggs, Bryk et al. 2001; Bryk, Briggs et al. 2002; Feng, Wang et al. 2002; Krogan, Dover et al. 2002; Ng, Feng et al. 2002; van Leeuwen, Gafken et al. 2002), gene activation (Noma and Grewal 2002; Santos-Rosa, Schneider et al. 2002), and repression (Carvin and Kladde 2004).

H2B deubiquitination both positively and negatively regulates gene transcription, as can H2B ubiquitination. Ubp8 deubiquitinates H2B in the context of SAGA *in vitro* and *in vivo* (Henry, Wyce et al. 2003; Daniel, Torok et al. 2004). Consistent with previous studies (Henry, Wyce et al. 2003; Kao, Hillyer et al. 2004), this confirms a link between H2B ubiquitination and SAGA complex dependent gene activity. Ubp10 has a broader role than Ubp8, helping to maintain quiescence of certain regions within the yeast genome (Emre, Ingvarsdottir et al. 2005). Ubp10 targets H2B for deubiquitination on Lys123, resulting in low H3 Lys4 and Lys79 methylation, thus helping to localize Sir2 to the telomere for telomeric silencing. Ubp10 also similarly regulates gene silencing on the rDNA locus, a second silenced domain. Deletion of *UBP10* leads to increased uH2B, specifically at a telomere proximal region, and expression of a normally strongly silenced gene within this affected region is increased in the absence of Ubp10 (Emre, Ingvarsdottir et al. 2005). Compared to Ubp8 which is the SAGA associated H2B deubiquitinating enzyme involved in gene activation, Ubp10 shows mainly telomeric and

gene silencing functions. Drosophila USP7, another H2B specific deubiquitinating enzyme, contributes to epigenetic silencing of homeotic genes by Polycomb (van der Knaap, Kumar et al. 2005).

Interestingly, both ubiquitination and deubiquitination of H2B are required for the proper activation of SAGA dependent genes (Henry, Wyce et al. 2003). The intriguing fact is that H3 Lys4 trimethylation transactivated by H2B ubiquitination occurs at the 5′ end of the ORF early during transcript elongation (Santos-Rosa, Schneider et al. 2002; Ng, Ciccone et al. 2003), while H3 Lys36 methylation occurs primarily later during transcript elongation (Krogan et al. 2003b; Xiao et al. 2003). As described above, H2B ubiquitination is required for H3 Lys4 and Lys79 methylation, but not for H3 Lys36 methylation. In fact while ubiquitination is required for methylation of H3 on Lys4, deubiquitination is required for methylation of H3 on Lys36. Thus sequential ubiquitination and deubiquitination of H2B establishes the correct balance among H3 methylations required for proper gene activation (Henry, Wyce et al. 2003; Wyce, Henry et al. 2004). Both the histone deubiquitination function and the histone acetylation function of SAGA complex are required for the role of SAGA in transcriptional activation of certain genes. Histone ubiquitination is increased early in gene activation and then, in contrast to histone acetylation, is rapidly cleaved by deubiquitination. The failure to remove ubiquitin results in the failure to progress from H3 Lys4 methylation to H3 Lys36 methylation, and thus blocks gene activation (Henry, Wyce et al. 2003).

In summary, histone ubiquitination and deubiquitination have emerged as key regulatory signals for gene transcription. Similar to histone acetylation, ubiquitination regulates two distinct aspects of transcriptional regulation: gene specific activation and

silencing in yeast. In contrast to the role of histone acetylation in transcription, both adding and removing ubiquitin is required for gene activation by SAGA in yeast.

1.3.3.2. The role of histone ubiquitination in cell cycle

The levels of H2A and H2B ubiquitination change during the cell cycle (Wu, Kohn et al. 1981). Both uH2A and uH2B have been reported to disappear during the G_2 to M phase transition when chromatin becomes condensed, but reappear as chromatin decondenses at the M to G_1 transition (Wu, Kohn et al. 1981). Because the ubiquitin molecule is as big as half of the size of core histones, it was believed that incorporation of ubiquitinated histones into the nucleosome would impact nucleosome structure and hamper chromatin folding. However, biochemical studies reported so far have failed to demonstrate a major role for histone ubiquitination in nucleosome core particle assembly and nucleosome folding (Moore, Jason et al. 2002).

Mutation of the conserved site of H2B monoubiquitination is shown to confer defects in mitotic cell growth and meiosis, suggesting H2B monoubiquitination regulates cell cycle and cell division (Robzyk, Recht et al. 2000). Recently, it is reported that Np95 may regulate cell cycle by ubiquitinating core histones with a preference for H3 (Citterio, Papait et al. 2004). Np95 (Fujimori, Matsuda et al. 1998; Hopfner, Mousli et al. 2000) is a cell cycle regulated protein that is expressed at the G_1/S boundary and in S phase and is absent in G_0 and G_1 (Fujimori, Matsuda et al. 1998; Uemura, Kubo et al. 2000; Miura, Watanabe et al. 2001; Bonapace, Latella et al. 2002). Np95 is absolutely required for the G1/S transition (Muto, Kanari et al. 2002). Np95 has been determined to be tightly bound to chromatin *in vivo* and it binds to histones *in vivo* and *in vitro* (Citterio, Papait et al. 2004). Thus, Np95 may regulate cell cycle through specific histone ubiquitination.

1.3.3.3. The role of histone ubiquitination in spermatogenesis

The presence of uH2A during spermatogenesis has been well documented for a number of years (Agell, Chiva et al. 1983; Agell and Mezquita 1988). uH2A has been found during spermatogenesis in different species, such as the rooster (Agell, Chiva et al. 1983), trout (Nickel, Roth et al. 1987), rat (Chen, Sun et al. 1998), and mouse (Baarends, Hoogerbrugge et al. 1999). However, it remains unclear how histone ubiquitination regulates spermatogenesis.

There are two peaks of H2A ubiquitination during spermatogenesis (Hendriksen, Hoogerbrugge et al. 1995; Baarends, Hoogerbrugge et al. 1999). Since it has been reported that histone modification alters nucleosome stability and thereby may facilitate processes that require access to DNA (Li, Nagaraja et al. 1993), ubiquitination of histone H2A during pachytene may facilitate replacement of somatic histones by the testis specific isotypes that are synthesized in pachytene (Poccia, Simpson et al. 1987; Meistrich 1989; Drabent, Bode et al. 1996). The other peak of H2A ubiquitination occurs post-meiotically during nuclear elongation and condensation phases of spermatogenesis (Hendriksen, Hoogerbrugge et al. 1995), when histones are replaced by transition proteins (Mills, Van et al. 1977; Meistrich 1989). Ubiquitinated histone H2As are at the highest levels at this stage during rooster (Agell, Chiva et al. 1983) and mouse (Baarends, Hoogerbrugge et al. 1999) spermatogenesis. Thus, ubiquitination of H2A is likely required for its degradation, but this is yet to be proved.

H2B ubiquitination could be important for spermatogenesis. Mutation of *rad6* in *Saccharomyces cerevisiae* shows a meiotic prophase arrest (Robzyk, Recht et al. 2000; Yamashita, Shinohara et al. 2004). The *rad6* mutant exhibits multiple phenotypes during meiosis. RAD6 is required for efficient formation of double strand breaks (DSBs) in meiotic recombination. Similarly, the disruption of the ligase Brel as well as substitution

of the ubiquitination site of histone H2B also reduces some DSB formation, suggesting that Rad6-Bre1, through ubiquitination of histone H2B, is necessary for efficient recruitment and/or stabilization of a DSB forming machinery. Therefore H2B ubiquitination may be indispensable for meiotic recombination during spermatogenesis. Knock out of the gene for mouse HR6B generates a sole phenotype, male infertility (Roest, van Klaveren et al. 1996; Baarends, Hoogerbrugge et al. 1999; Baarends, Wassenaar et al. 2003). In *HR6B* knockout mice, spermatogenesis appears to start up normally. The first prominent morphological signs of defective spermatogenesis become visible when the spermatids differentiate during the first wave of spermatogenesis. The number of elongating and condensing spermatids is reduced in *HR6B* knockout compared to wild-type mouse testes, and the nuclear morphology is highly abnormal (Roest, van Klaveren et al. 1996). However, the overall pattern of histone ubiquitination remains unchanged in the*HR6B* knockout mice (Baarends, Hoogerbrugge et al. 1999). Thus, the exact role of HR6B mediated H2B ubiquitination in mammalian spermatogenesis remains to be determined.

Ubiquitinated histone H3 and testes specific H3 (TH3) were first identified in a study of rat spermatogenesis, with the highest level in elongated spermatids (Chen, Sun et al. 1998). Overall, the role of histone ubiquitination during spermatogenesis remains largely unknown. However, based on the above described studies, it is probably involved in gene silencing, meiosis, specific gene induction and degradation of the histones.

1.4. Summary

- Upon degradation in early and late elongated spermatids, histones are replaced by transition proteins, which in turn are replaced by protamines;

- The replacement of histones by protamines is required for chromatin condensation;
- Since ubiquitination is activated during spermatogenesis, and ubiquitinated histones have been detected in different species, the degradation of histones during the haploid spermatid maturation may be mediated by the ubiquitin mediated degradation pathway.
- Inactivation of mouse HR6B leads to defective spermatogenesis but does not appear to prevent histone degradation, suggesting histones may be degraded in a HR6B independent ubiquitination pathway.
- Activation of ubiquitination during spermatogenesis is dependent on a specific E2, UBC4. UBC4-testis is induced in late round and early elongating spermatids in which histones are degraded.

1.5. Objective of the project

The objective of this project is to study the mechanism of histone degradation during spermatogenesis, specifically, to identify an E3 that polyubiquitinates histones during spermatogenesis.

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Chapter 2

Characterization of $E3^{Histone}$, a Novel Testis Ubiquitin Protein Ligase Which Ubiquitinates Histones

Running title

Characterization of a Ligase which Ubiquitinates Histones

2.1. Abstract

During spermatogenesis a large fraction of cellular proteins is degraded as the spermatids evolve to their elongated mature forms. In particular, histones must be degraded in early elongating spermatids to permit chromatin condensation. Our laboratory previously demonstrated activation of ubiquitin conjugation during spermatogenesis. This activation is dependent on the ubiquitin conjugating enzyme (E2) UBC4 and a testis particular isoform, UBC4-testis, is induced when histones are degraded. Therefore we tested whether there are UBC4 dependent ubiquitin protein ligases (E3s) that can ubiquitinate histones. Indeed a novel enzyme, $E3^{Histone}$, was found that could conjugate ubiquitin to histones H1, H2A, H2B, H3 and H4 *in vitro*. Only the UBC4/UBC5 family of E2s supported $E3^{Histone}$-dependent ubiquitination of histone H2A, and of this family, UBC4-1 and UBC4-testis are the preferred E2s. We purified this ligase activity 3600 fold to near homogeneity. Mass spectrometry of the final material revealed the presence of a 482 kDa HECT domain containing protein, which was previously named LASU1. Anti-LASU1 antibodies immunodepleted $E3^{Histone}$ activity. Mass spectrometry and size analysis by gel filtration and glycerol gradient centrifugation suggested that $E3^{Histone}$ is a monomer of LASU1. Our assays also show that this enzyme is the major UBC4-1 dependent histone ubiquitinating E3. $E3^{Histone}$ is therefore a HECT-domain E3 that likely plays an important role in the chromatin condensation that occurs during spermatid maturation.

2.2. **Introduction**

Spermatogenesis is a complex developmental process during which stem cell spermatogonia are transformed into highly differentiated spermatids (Clermont 1993). This transformation can be divided into three phases. The first phase is the proliferative phase in which spermatogonia undergo successive mitotic divisions. Subsequently, in the meiotic phase, spermatogonia are transformed into spermatocytes in which the genetic material undergoes homologous recombination and two sequential cell divisions, resulting in haploid spermatids. In the final spermiogenic phase, the spermatids are transformed into cells structurally equipped to reach and fertilize the eggs. During spermiogenesis, each immature spermatid develops an acrosome and tail, reorganizes its mitochondria and loses most of its cytoplasm.

Proteolysis plays an important role in these developmental phases. In the first two phases, proteolysis is essential in regulating cell cycle. Proteolysis also appears important for the third spermiogenic phase. During this cellular remodeling of haploid spermatids, many proteins are degraded. Histones are among the key proteins that undergo proteolysis (Meistrich 1989). Upon degradation in early and late elongated spermatids, histones are replaced by transition proteins which in turn are replaced by protamines (Meistrich 1989). The substitution of histones by protamines is essential to permit the condensation of chromatin into the narrow head of the compact and elongated mature spermatid. The mechanisms underlying the degradation of histones remain unclear. Previous work suggests that many proteins are removed in a cytoplasmic droplet that is phagocytosed by the adjacent Sertoli cells (Russell and Clermont 1976; Morales 1985). However, the ubiquitin system appears to be involved in this loss of cellular proteins. Indeed, ubiquitinated histones have been detected in the testis of different species (Agell, Chiva et

al. 1983; Nickel, Roth et al. 1987; Chen, Sun et al. 1998; Baarends, Hoogerbrugge et al. 1999). During rooster (Agell, Chiva et al. 1983) and mouse (Baarends, Hoogerbrugge et al. 1999) spermatogenesis, ubiquitinated histone H2A are at the highest levels before the replacement of histones by protamines in late spermatids. During trout germ cell maturation, the levels of monoubiquitinated histone H2B decreased while those of doubly ubiquitinated histone H2B increased (Nickel, Roth et al. 1987). Ubiquitinated H3 was first identified in the study of rat spermatogenesis, with the highest level in elongated spermatids (Chen, Sun et al. 1998). The results from these studies suggest that ubiquitination plays an important role in histone replacement.

Conjugation of ubiquitin to proteins requires the sequential cooperation of three enzymes (rev. in (Hershko and Ciechanover 1998; Pickart 2001)). The ubiquitin activating enzyme (E1) hydrolyses ATP to convert ubiquitin into an activated form, which is covalently linked at its carboxyl terminus to a cysteine residue of the E1 enzyme via a high energy thioester linkage. The activated ubiquitin molecule is then transferred to the second enzyme of this pathway, a ubiquitin conjugating enzyme (E2), and the activated form is maintained through the formation of a thioester linkage with a cysteine residue of the E2 enzyme. The third enzyme in the process, the ubiquitin protein ligase (E3), supports the transfer of ubiquitin to substrates.

E3 is the key to specificity of ubiquitin conjugation as it recognizes substrates (rev. in (Hershko and Ciechanover 1998; Pickart 2001)). There are two major classes of E3 enzymes. One class is the RING finger containing E3s. This type of E3 functions as a scaffold protein that binds both the E2 and the substrate, permitting ubiquitin to be transferred from the E2 to the substrate. The RING finger domain chelates two Zn^{2+} ions, and is essential for interaction with the E2. The other class of E3 enzymes is the HECT

domain containing E3s. These E3s support ubiquitination in a process that requires two steps. First, ubiquitin is transferred from E2 to the E3 via a thioester linkage with a cysteine residue in the HECT domain, and then ubiquitin is transferred from the E3 to the substrate. This family of E3s therefore requires a free thiol group for its function.

The E3 covalently links the ubiquitin moiety by its C-terminal residue to the ε-amino group of an internal lysine residue of the target protein (Hershko and Ciechanover 1998; Pickart 2001) or in some cases to the α-amino group at the N-terminus of the protein (Ciechanover and Ben-Saadon 2004). Monoubiquitination has important functions as a signal (rev. in (Hicke 2001)). Monoubiquitination of membrane proteins signals their internalization to endosomes that traffic to lysosomes for degradation (Rotin, Staub et al. 2000; Hicke 2001). Histone monoubiquitination regulates gene transcription. Monoubiquitination of histone H2B in yeast results in telomeric gene silencing by stimulating the methylation of histone H3 on lysine 4 and lysine 79 (Sun and Allis 2002), while monoubiquitination of histone H3 in yeast results in activation of transcription (Briggs, Xiao et al. 2002). For most ubiquitination though, a polyubiquitin chain is usually formed after the linkage of ubiquitin to the substrate protein (Hershko and Ciechanover 1998). When linked via the lysine 48 residue of each ubiquitin, these chains usually target the attached protein to the 26S proteasome for degradation (Chau, Tobias et al. 1989). Chains consisting of non-lysine 48 linkages appear to have non-proteolytic functions. For example, lysine 63 linked ubiquitin chains can mediate DNA repair and signaling via the NF-κB pathway (Spence, Sadis et al. 1995; Hofmann and Pickart 1999; Deng, Wang et al. 2000; Spence, Gali et al. 2000).

Previous work in our laboratory supports a role for ubiquitination during spermatogenesis. The rate of ubiquitin conjugation increases during the first wave of spermatogenesis that occurs in early postnatal life (Rajapurohitam, Morales et al. 1999). This increase in conjugation appears dependent on the UBC4 family of E2s. UBC4 is induced during spermatogenesis (Rajapurohitam, Morales et al. 1999). This activation is at least partly due to induction of UBC4 isoforms. UBC4 is highly expressed in the testis and it is widely expressed in many different tissues. A particular testis specific UBC4 isoform, UBC4-testis, is induced in round spermatids and early elongated spermatids (Wing, Bedard et al. 1996).

To further explore the role of this UBC4 dependent ubiquitination during male germ cell development, we have been identifying UBC4-dependent E3s expressed in the testis. Since histones are known to be ubiquitinated and degraded in early elongated spermatids when UBC4-testis is induced, we hypothesized that histones may be substrates of a UBC4 dependent E3. Therefore we used ^{125}I-histone H2A as a substrate to biochemically screen for UBC4 dependent E3 activity in testis extracts. Interestingly, a HECT domain E3 was identified to be able to conjugate ubiquitin to different core histones. We now describe the characterization of this new E3.

2.3. Methods

Iodination of proteins-The chloramine-T method was used to label bovine ubiquitin with $Na^{125}I$ to a specific radioactivity of 3000 cpm/pmol, and histones H1, H2A, H3 and H4 (Boehringer Mannheim) to 375,000 cpm/μg. Unincorporated ^{125}I was removed by passing the reaction products over a Sephadex G25 column.

Preparation and quantification of enzymes-E1 was isolated from rabbit liver by ubiquitin affinity chromatography as previously described (Haas and Bright 1988). Bacterially expressed recombinant UBC4-1, UBC4-testis, $E2_{14K}$, UBC7, UbcH6, UbcH7 and UbcH10 proteins were prepared as described previously (Wing, Dumas et al. 1992; Wing and Jain 1995; Nuber, Schwarz et al. 1996; Wing, Bedard et al. 1996; Townsley, Aristarkhov et al. 1997; Bastians, Topper et al. 1999; Lin and Wing 1999). UBC4-1, UBC4-testis and $E2_{14K}$ enzymatic activity were quantified by measuring the initial release of radioactive pyrophosphate following incubation in the presence of (γ-^{32}P) ATP, ubiquitin and E1 (Haas and Rose 1982). The remaining E2s were quantified using a thioester assay (Oughtred, Bedard et al. 1998) (see below). Recombinant GST fused to the RING domain of the E3 ARNIP (GST-ARNIP) was expressed in *E.coli* and purified by glutathione Sepharose beads (Beitel, Elhaji et al. 2002).

Conjugation Assay-To measure the conjugation of ubiquitin to histone substrates, the reaction mixture contained in a final volume of 20 μl: 10 μl of the fractions from each protein purification step, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 2 mM $MgCl_2$, 2 mM ATP, 0.5 units pyrophosphatase, 12.5 mM phosphocreatine, 2.5 units of creatine kinase, 50 nM E1, 30 μM ^{125}I-histone H2A and 250 nM of UBC4-1 or UBC4-testis. All ubiquitin

conjugation reactions used to screen fractions for $E3^{Histone}$ activity were initiated with 25 μM of reductively methylated ubiquitin (RMUb), prepared and quantified as described (Hershko and Heller 1985), and incubated for 1 hour at 37°C. Reaction products were separated by SDS PAGE, and the results were detected by autoradiography.

For quantification of $E3^{Histone}$ activity, the peak fractions from each purification step were tested in the conjugation assay at 30 °C for only 10 min. Under these conditions of shorter time and lower temperature, the assay was linear with respect to the amount of enzyme. $E3^{Histone}$ activity was quantified by adding up the intensity of each band multiplied by the number of ubiquitin moieties linked to histone H2A in that band.

Conjugation assays which tested the ability of different E2s to support E3-mediated ubiquitination of histone H2A contained E2 enzymatic concentrations between 150-300 nM. The conjugation assay comparing different E2s or ^{125}I-histone types were carried out under quantification conditions (i.e. 30 °C for 10 min).

In the assays of testing the substrate specificity (Fig. 1C) and E2 specificity of $E3^{Histone}$ (Fig. 1D) and testing the different results of $E3^{Histone}$ mediated ubiquitination of histone H2A with wild type ubiquitin (Ub) and methylated ubiquitin (MeUb) (Fig. 1B), the $E3^{Histone}$ used were purified with two anion exchange columns, Q-Sepharose and Mono Q columns, and a gel filtration column, Superdex 200 column (See *$E3^{Histone}$ purification* in methods for details).

For the conjugation assay with ARNIP, a RING-finger E3, the components and their concentrations were the same as those with $E3^{Histone}$ except that the E3 ARNIP was used at a concentration of 1 μM, 2 μl of 50 μM ^{125}I-ubiquitin was used to start the reaction, and there was no histone H2A. Since there was no exogenous substrate, autoubiquitinating

activity was measured. The reaction was incubated at 37°C for one hour. Reaction products were resolved by SDS-PAGE, and detected by autoradiography.

Thioester Assay-The components for a 10 µl reaction were as follows: 1 µl of 10x thioester buffer (0.5 M Tris-HCl pH 7.5, 0.1 M $MgCl_2$, 20 mM ATP, 5 mM DTT), 1 µl inorganic pyrophosphatase (0.5 units/µl) (Sigma) and 1 µl of E1 (1 µM). 1 µl of 50 µM ^{125}I-ubiquitin was used to start the reaction. The reaction was incubated at 37°C for 1 min. Laemmli sample buffer without 2-mercaptoethanol was used to stop the reaction and the products were separated by SDS PAGE at 4 °C. The gel was dried and results were detected by autoradiography.

Preparation of testis extracts-Testes from Sprague-Dawley rats (200 g, Charles River Laboratories) were sliced and homogenized at 4 °C with a Potter-Elvehjem tissue grinder (Fisher) in 5 volumes of ice-cold homogenization buffer (50 mM Tris, pH 7.5, 1 mM DTT). Bovine testes were homogenized in 3 volumes of homogenization buffer using a Waring blender. The homogenate was centrifuged at 10,000 g for 15 min., and the supernatant at 100,000 g for one hour.

Bovine $E3^{Histone}$ purification-The 100,000 g supernatant was subjected to purification by the following steps. After each step, the fractions were assayed as described above to detect their ability to support UBC4-dependent conjugation of ubiquitin to histone H2A. The peak fractions were pooled for the next step.

The 100,000 g supernatant was loaded onto a 50 mm x 35 cm DEAE-cellulose (Whatman DE-52) column, equilibrated with the homogenization buffer. Bound proteins

were stepwise eluted with 0.1 M, 0.2 M and 0.3 M NaCl in 50 mM Tris, pH 7.5, 4 °C, 1 mM DTT. Most of the $E3^{Histone}$ eluted with buffer containing 0.2 M salt.

The $E3^{Histone}$ activity collected was precipitated by 0-50% ammonium sulfate precipitation. The pellet was redissolved in and dialyzed against homogenization buffer.

The dialyzed sample was then loaded onto a 26 mm x 20 cm quaternary amine anion exchange column (Pharmacia Amersham Biotech, Q Sepharose) equilibrated with the homogenization buffer. Bound proteins were eluted with a 0-0.5 M NaCl gradient (in 50 mM Tris, pH 7.5, 1 mM DTT).

Peak fractions containing $E3^{Histone}$ activities eluted at ~ 0.4 M NaCl. They were pooled and dialyzed against homogenization buffer containing 0.7 M $(NH_4)_2SO_4$, and loaded onto an 8 mm x 7.5 cm Phenyl 5PW column. Bound proteins were eluted with a 0.7 M - 0 M $(NH_4)_2SO_4$ gradient in 50 mM Tris, pH 7.5, 1 mM DTT.

The peak fractions eluting at ~ 0.24 M $(NH_4)_2SO_4$ were pooled, dialyzed overnight against homogenization buffer and then loaded onto a 5 mm x 5 cm quaternary amine anion exchange column (Pharmacia Amersham Biotech, Mono Q) equilibrated with the homogenization buffer. Retained proteins were eluted with the same gradient as for the Q Sepharose chromatography noted above.

The peak fractions eluting at ~ 0.4 M NaCl were then concentrated using Centriplus 10 concentrators (Amicon). The concentrated eluate was further purified on a 10 mm x 30 cm gel filtration column (Superdex 200, Pharmacia Amercham Biotech).

The peak fractions were pooled and centrifuged on a 10%-40% glycerol gradient in 10 mM Tris-Cl, pH 7.5. The centrifugation was performed at 28,000 rpm in a SW 40Ti rotor (Beckman) for 16 hours at 4 °C.

Free thiol group requirement test-To test if $E3^{Histone}$ might be a HECT domain E3, we assessed whether a free thiol group was required for activity. Partially purified enzyme following the Superdex 200 chromatography step for the conjugation assay was treated with 5 mM N-ethylmaleimide (NEM) on ice for 5 min, and then 2.5 mM DTT was added to quench the remaining NEM. To confirm the effectiveness of the NEM treatment, 1 pmol of E1 was similarly treated and then subjected to a thioester assay for activity. To confirm that there was sufficient quenching of NEM by DTT, buffer was treated with the same amount of NEM and DTT prior to use in an assay of E1 for thiolester activity.

Zn^{2+} ion requirement test-To test if the $E3^{Histone}$ might be a RING domain E3, we assessed whether divalent cations were required for the activity. Glycerol gradient fraction #4 or 20 pmol of recombinant GST-ARNIP were treated with 10 mM EDTA at 4°C overnight. $MgCl_2$ 10 mM was added to bind the residual EDTA prior to use in a conjugation assay. The treated E3s were then tested in conjugation assays. To confirm the function of Zn^{2+}, the $E3^{Histone}$ containing fraction, HIC fraction #55 or 20 pmol of recombinant GST-ARNIP were treated with a relatively zinc specific chelator TPEN, 3.2 mM. The assay was performed as above.

Mass spectrometry analysis-Following glycerol gradient centrifugation, the fractions were analyzed for $E3^{Histone}$ activity and protein composition by both native gel and SDS-PAGE. Prominent bands comigrating with $E3^{Histone}$ activity were excised and analyzed at the Harvard Microchemistry Facility. The proteins in the bands were digested with trypsin, after which the peptides were resolved and analyzed by microcapillary reverse-phase HPLC, directly coupled to the nano-electrospray ionization source of an ion trap

mass spectrometer. These MS/MS spectra were then correlated with known sequences using the algorithm Sequest developed at the University of Washington (Eng 1994), and programs developed at Harvard University (Chittum, Lane et al. 1998). MS/MS peptide sequences were then reviewed for consensus with known proteins and the results manually confirmed for fidelity.

Antibody design, production and purification –A fragment encoding 185 amino acid residues on the N-terminal part of LASU1 was amplified by RT-PCR using testis mRNA as a template. The amplified nucleotide fragment was cloned into pET15b vector (Novagen) and expressed in *E. coli* BL21. The expressed protein contained a N-terminal His tag fusion and was purified on a Ni^{2+} column. Rabbits were injected with the protein in Freund's adjuvant, and after two boosts the serum was collected and the antibody purified on a column containing GST fused to the protein fragment.

Immunoblotting and immunoprecipitation-Rabbit sera against LASU1 diluted 1000 times or purified LASU1 antibody diluted to 2.7 μg/ml were used for immunoblotting on PVDF membranes (Millipore). Immunoblots were visualized by chemiluminescence using the ECL detection system (Amersham). Antibody against the 20S proteasome (Affiniti) was used to detect the 20S proteasome subunits.

To immunoprecipitate LASU1 from $E3^{Histone}$ containing fractions, an equal volume of LASU1 antiserum or pre-immune serum and protein A beads were incubated at 4°C for 2 hours as described elsewhere (King, Peters et al. 1995). Beads were washed three times in immunoprecipitation (IP) buffer, 20 mM Tris-HCl pH 7.5 at 4°C, 1% NP40, 50 mM NaCl, protease inhibitor cocktail (Roche), then incubated with an equal volume of

$E3^{Histone}$ containing fraction at 4°C for 2 hours. Beads were washed as before. The pellet and the supernatant were assayed for $E3^{Histone}$ activity as described above.

2.4. **Results**

Identification and characterization of E3^{Histone} Since a specific E2, UBC4, is induced in elongating spermatids when histones are removed from spermatids (Rajapurohitam, Morales et al. 1999), we used ^{125}I-histone H2A as a substrate and UBC4 as the E2 to screen biochemically for a ubiquitin protein ligase that can ubiquitinate histones (Methods). When this assay was applied to a rat testis extract (Fig. 1A), multiple high molecular weight forms of histone H2A were generated (lane 2), compared to the negative control (lane 1) which was an assay done in the absence of the testis extract. These results indicated the presence of an E3 in the testis that can ubiquitinate the histone. We carried out the assay with reductively methylated (RM) ubiquitin, as it resulted in easily visible and quantifiable monoubiquitinated forms of H2A. In contrast, use of wild type ubiquitin resulted in a high molecular weight smear at the top of the gel (Fig. 1B, lane 1 compared with lane 3). The presence of multiple bands when RMUb was used must represent monoubiquitination of histone H2A on multiple lysine residues. This suggests that at least *in vitro* this ubiquitination is not site specific on histone H2A. Since the substrate of the reaction, ^{125}I-histone H2A, showed a strong signal whose shadow rendered difficult observation of the 8kDa higher monoubiquitination band on film, we typically ran the substrate off the gel. Our more recent assays used a phosphorimager which does not generate a large shadow because of a shorter path (no intensifying screen) to the detection plate. One picture was shown with the substrate kept on the gel by detecting with phosphorimager (Fig. 1B). To characterize further this E3, the testis extract was fractionated on a Mono Q anion exchange column. The E3 activity eluted at ~0.4 M salt (Fig. 1A, lanes 5, 6). This E3 activity was designated as E3^{Histone}.

Since all of the different types of histones are degraded during spermatogenesis, we tested if this E3 can ubiquitinate other histones besides histone H2A (Fig. 1C). Histones H1, H2B, H3 and H4 were radiolabeled and incubated in the presence of E1, UBC4, RM-Ub with or without $E3^{Histone}$. High molecular weight forms of the histones were observed for all the histone types tested, but only in the presence of $E3^{Histone}$, indicating that $E3^{Histone}$ could ubiquitinate all core histones tested here *in vitro*.

Since E3s generally interact with specific E2s, we tested the ability of different E2s to support ubiquitination of histone H2A (Fig. 1D). Of the E2s tested, only UBC4-1, UBC4-testis and UBC H7 supported $E3^{Histone}$ dependent ubiquitination. These three E2s belong to the UBC4/UBC5 family of E2s.

$E3^{Histone}$ is a HECT domain E3. There are two major families of E3s, RING finger E3s and HECT domain E3s. To assess whether $E3^{Histone}$ might be a HECT domain E3, we tested whether $E3^{Histone}$ activity requires the presence of a free thiol group (Fig. 2A left). $E3^{Histone}$ was treated with N-ethylmaleimide (NEM) to inactivate thiol groups. Following addition of dithiothreitol (DTT) to quench the excess NEM, the treated $E3^{Histone}$ was reassayed for E3 activity (Fig. 2A left, lane 3). After the treatment, $E3^{Histone}$ lost its activity as did E1 when similarly treated and tested in a thioester assay as a positive control (Fig. 2A left lane 3 & right lane 3). The loss of activity was not due to inactivation of E1 or E2 in the assay from incomplete quenching of NEM by DTT as identical treatment of a mock sample with NEM and DTT did not affect the activity of subsequently supplemented E1 in a thioester assay (Fig. 2A right, lane 4). Thus, this E3 could require a free thiol group for its function and may therefore be a HECT domain E3. However, at this point we can not rule out the possibility that under the conditions employed the NEM may have reacted with other nucleophiles besides thiol groups. Since

RING finger motifs chelate Zn^{2+} ions, we tested $E3^{Histone}$ for the requirement of divalent cations by incubating it with the chelater EDTA or a relatively zinc specific chelator TPEN (Fig. 2B). Following incubation, the remaining EDTA or TPEN was bound by adding excess Mg^{2+} to avoid intereference by the chelators of the E1 mediated activation of ubiquitin, which requires Mg^{2+}. The treated enzyme was reassayed in the conjugation assay. $E3^{Histone}$ activity was unaffected by EDTA treatment (Fig. 2B, lane 4) or TPEN treatment (Fig. 2B, lane 12). The enzymatic activity of the $E3^{Histone}$ was lower in the TPEN study due to the use of a different fraction. To confirm that this treatment was effective at chelation, we applied the identical protocol to a sample of ARNIP (Fig. 2B), a known RING finger E3 (Beitel, Elhaji et al. 2002). Indeed ARNIP autoubiquitination activity was lost upon treatment (Fig. 2B, lanes 8, 16), indicating that the negative result with $E3^{Histone}$ was valid. These data together suggest that $E3^{Histone}$ is probably a HECT domain E3.

LASU1 is a component of $E3^{Histone}$. To identify this new E3, we purified it to near homogeneity by the sequential application of different protein purification methods (details are in the Methods). To obtain an adequate amount of purified enzyme, we started with an extract prepared from bovine testes. After the multi-step purification (Fig. 3A), the specific enzyme activity was 3600 fold that of the crude extract (Fig. 3B). The precipitation of the activity by relatively low concentrations of ammonium sulfate suggested that the enzyme was probably large in size. This was indeed confirmed by the gel filtration chromatography step which suggested a molecular mass of ~600 kDa for this enzyme (Data not shown and Fig. 7A). Therefore, we subjected it to a final step of purification by centrifugation on a glycerol gradient. The fractions from this last purification step were assayed for $E3^{Histone}$ activity (Fig. 4A) and the protein components

analyzed by denaturing and native gel electrophoresis (Fig. 4B, 4C). Native gel electrophoresis revealed a single major band (Fig. 4B). This band was cut and analyzed by tandem mass spectrometry (MS). SDS-PAGE revealed a number of bands ranging from ~30 kDa to >180 kDa (Fig. 4C). Several bands on this denaturing gel appeared to comigrate closely with $E3^{Histone}$ activity (Fig. 4A) and so were also cut and analyzed by tandem MS. Interestingly, analysis of the band from native gel electrophoresis (Table 1) identified 68 peptides corresponding to a human 370 kDa HECT domain containing protein previously named LASU1 (Gu, Dubner et al. 1995; Gu, Irving et al. 1997). This appeared to be the dominant protein in the band and indicated that LASU1 was likely to be $E3^{Histone}$ or a part of $E3^{Histone}$.

Interestingly, the second largest number of peptides in the native band corresponded to an unnamed protein (gi|26340188) whose C-terminal 356 amino acids are the same as amino acid residues 41-397 in the N-terminal part of LASU1 (Fig. 5A). Searching the EST data base identified other independent sequences of LASU1 in this region and revealed that the first 40 amino acids of LASU1 were miscoded because of a frame shift caused by an extra G at base 266 of the previous DNA sequence of LASU1. Therefore the C-terminal 396 amino acids of the unnamed protein are exactly the same as the N-terminal 396 amino acids of LASU1. Furthermore, using the LASU1 and unnamed protein sequences to probe the mouse genome database indicated that the DNA encoding these two putative proteins are adjacent to and overlapping each other on the X chromosome. Northern blot analysis of testis mRNA with a probe against the overlapping region detected only a single band > 12 Kb in size (unpublished data). Finally, the cDNA clone #4017134 (Invitrogen) contains the overlapping region of these two proteins, and non-overlapping sequences of the unnamed protein and LASU1 on the N terminus and C

terminus, respectively. All of these independent pieces of evidence suggested very strongly that the unnamed protein and LASU1 are parts of the same protein. The complete human LASU1 has 4374 amino acids, and predicts a molecular weight of 481.9 kDa, and a pI of 4.87. Mouse LASU1 has 4377 amino acids, and predicts a molecular weight of 482.7 kDa, and a pI of 4.86.

Mass spectrometric analysis of the bands from the SDS-PAGE gel (Fig. 4C) revealed that all three bands contained LASU1 and that the two larger ones also contained the unnamed protein. Those bands likely contain degradation fragments of LASU1.

Domain analysis of this protein showed that it has a classical HECT domain at the C-terminal end (Fig. 5A). In addition a UBA and a UIM domain, both known to function in ubiquitin binding are found in the overlapping region between the unnamed protein and LASU1. A WWE domain was also identified and this domain has been associated with proteins involved in regulation of ubiquitin dependent proteolysis (Aravind 2001). Finally, two domains with unknown function, DUF908 and DUF913, were identified in the N-terminal region located in the unnamed protein (Fig. 5A). Interestingly, most of the other proteins that contain these two domains appear to be related to the 374 kDa *S. cerevisiae* HECT domain ligase TOM1 (Duncan, Umen et al. 2000) (Fig. 5B, 5C). TOM1 and the complete LASU1 share high degrees of similarity in these domains and in the HECT domain, but are relatively poorly conserved elsewhere showing only 17.3% and 34.7% overall amino acid identity and similarity respectively.

To confirm that $E3^{Histone}$ indeed contains LASU1, an antibody was raised against a His tagged fragment of LASU1 containing residues 353-538 of the LASU1 sequence (residues 1367-1552 of the complete protein). The antibody was affinity purified on a column bearing the fragment fused to GST. This antibody was tested for its ability to

immunoprecipitate $E3^{Histone}$ (Fig. 6A). The antibody, but not the pre-immune serum, bound $E3^{Histone}$ activity confirming that LASU1 is a part of $E3^{Histone}$. Since UBC4 appears to be the cognate E2 for $E3^{Histone}$, we tested for the presence of UBC4 in the immunoprecipitated LASU1 (Fig. 6B). UBC4 was not detectable in the pellet, suggesting that the interaction is transient which is consistent with UBC4-Ub thioester acting as a substrate in the reaction.

$E3^{Histone}$ appears to be a monomeric protein. To determine if $E3^{Histone}$ is a monomeric protein or multisubunit complex, we estimated the molecular mass of this E3. Since purifying the protein resulted in significant degradation, we chromatographed a crude testis extract on a Superdex 200 gel filtration column (Fig 7A). The peak of $E3^{Histone}$ eluted at fraction #19. When compared to the elution positions of protein standards, this corresponded to a molecular mass of ~600 kDa. Immunoblotting revealed that LASU1 protein comigrated as expected with $E3^{Histone}$ activity.

Since gel filtration chromatography is not a precise method of sizing such large proteins, density gradient centrifugation was also used to estimate the molecular mass of $E3^{Histone}$. Glycerol gradient centrifugation was performed on $E3^{Histone}$ and also on thyroglobulin (667 kDa) and the 20S proteasome (700 kDa) which were used as standards (Fig. 7B). The peak of thyroglobulin was in fraction #13, the peak of the 20S proteasome was in Fraction #13 and 14, while that of $E3^{Histone}$ was detected in fraction #11. Thus $E3^{Histone}$ is smaller than the 20S proteasome (700 kDa) and thyroglobulin (667 kDa), supporting a size of ~600 kDa.

To determine if $E3^{Histone}$ contains other subunits besides LASU1, the anti-LASU1 antibody was covalently linked to protein A beads by DMP, and the covalently linked antibody was used to immunoprecipitate $E3^{Histone}$ from a crude rat testis extract. The

proteins immunoprecipitated by the antibody were resolved by SDS PAGE. Several protein bands that were in the pellet immunoprecipitated by the antibody but not by the rabbit IgG control were cut and analyzed by mass spectrometry (data not shown). There was only one protein, heat shock protein 70 (HSP70), that was present in the proteins identified in the $E3^{Histone}$ band on native gel electrophoresis (Fig. 4B). However, in both cases, the numbers of peptides ascribed to HSP70 were small and to date we have not been able to confirm this finding by western blot analysis using anti-HSP70 antibody of the $E3^{Histone}$ immunoprecipitated using anti-LASU1 A (data not shown). On the other hand, all of LASU1 comigrated with $E3^{Histone}$ (Fig. 7A and 7B). Taken together, these data suggest that $E3^{Histone}$ probably consists simply of the 482 kDa protein LASU1.

$E3^{Histone}$ is the major UBC4-1 dependent E3 that ubiquitinates histone H2A in the testis. So far two different Ring domain E3s, Np95 (Citterio, Papait et al. 2004) and BRCA1/BARD1 (Mallery, Vandenberg et al. 2002), have been found to ubiquitinate histone H2A *in vitro* by cooperation with UBCH5B, the human homolog of UBC4-1. Therefore we tested whether other UBC4-dependent E3s besides $E3^{Histone}$ are present in the testis. We fractionated a testis extract on the Superdex 200 gel filtration column (Fig. 7A) and glycerol gradient (Fig. 7B), and screened fractions for their ability to ubiquitinate histone H2A in the presence of UBC4-1. In both cases, we did not find any significant activity in fractions other than the ones containing $E3^{Histone}$. Therefore, $E3^{Histone}$ appears to be the major UBC4-1 dependent E3 in the testis that ubiquitinates histone H2A.

2.5. **Discussion**

During spermatid maturation, several chromatin rearrangements occur. Initially, the histones are replaced by transition proteins which in turn are finally replaced by protamines (Oliva and Dixon 1991). Histones are still degraded when genes encoding transition proteins are inactivated in the mouse indicating that the loss of histones is not due to simply displacement by the transition proteins (Yu, Zhang et al. 2000). Ubiquitination of several histones is increased just prior to their degradation (Meistrich 1989) suggesting that ubiquitin dependent proteolysis plays an important role in histone replacement. In this paper, we have identified and characterized a new testis ubiquitin protein ligase, $E3^{Histone}$, which is an excellent candidate ligase for mediating ubiquitination of histones. It ubiquitinates all core histones *in vitro* (Fig. 1A, 1B). With wild type ubiquitin, $E3^{Histone}$ produced much higher molecular weight forms of histone than when MeUb was used (Fig. 1B), indicating that $E3^{Histone}$ can form polyubiquitin chains on histones, thus conferring the potential for recognition and degradation by the proteasome. Its preferred interaction with UBC4 would also be consistent with such a role as UBC4 isoforms are induced in early elongating spermatids (Rajapurohitam, Morales et al. 1999) when histones begin to be replaced. Indeed, the induction of UBC4 may be a mechanism by which histone ubiquitination and degradation is initiated and regulated.

Mass spectrometric analysis identified $E3^{Histone}$ as a HECT domain containing ligase, previously named LASU1. This was consistent with our observations that $E3^{Histone}$ activity was abolished by the sulfhydryl reactive compound N-ethylmaleimide, but not by the divalent cation chelator EDTA (Fig. 2). In addition, our EST sequence analysis corrected a frame shift inducing sequencing error present in the previous sequence of LASU1, thus resulting in a much longer N-terminus that includes what was hitherto a

protein of unknown function. Remarkably, mass spectrometric coupled to bioinformatics analyses of this bovine enzyme was able to identify 88 peptides matching the human LASU1 or mouse unnamed protein sequence. This suggests a significant degree of conservation of sequence amongst these species which was confirmed by observation of 97.7% and 98.5% identity and similarity respectively between mouse and human sequences present in the EST databases. All other proteins identified in the analyses had fewer than seven matching peptides suggesting that LASU1 was the dominant protein in this enzyme. None of the other identified proteins appeared to be a potential ligase. The predicted molecular weight of 482 kDa was reasonably close to the estimated molecular mass of ~600 kDa of $E3^{Histone}$ considering the limited prediction of mass estimation by chromatography for such a large protein. And the enzyme bound to anion exchange columns as would be expected for a protein with an acidic pI. All of these observations together argue strongly that LASU1 is $E3^{Histone}$. The latter findings also suggest that $E3^{Histone}$ functions as a monomeric protein, but the mass determinations are not precise enough to rule out the presence of an additional small subunit(s), particularly nonstoichiometrically in subsets of the enzyme molecules.

Interestingly, LASU1 was found expressed in many different tissues at both mRNA and protein levels, and more highly expressed in testis, brain, lung and kidney (unpublished data). $E3^{Histone}$ could therefore have other important roles in different tissues besides its function on chromatin condensation in elongated spermatids. It is possible that it may play a role in ubiquitination of histones in other cells. However, in most cells, ubiquitination of histones appears to modify function rather than target for degradation. In eukaryotic cells, 10-15% of total histone H2A is ubiquitinated (West and Bonner 1980), while 10% of histone H2B is ubiquitinated in yeast (Robzyk, Recht et al. 2000) and 1.5%

of cellular histone H2B is monoubiquitinated in higher eukaryotes (West and Bonner 1980). The function of this ubiquitination remains largely unknown except for a few examples. Monoubiquitination of histone H2B in *S. cerevisiae* leads to methylation of histone H3 on its lysine 4 and lysine 79 residues which in turn causes telomeric gene silencing (Briggs, Xiao et al. 2002; Sun and Allis 2002). This ubiquitination is mediated by the RING domain containing E3, Bre1, which relies on Rad6p, as its cognate E2 (Robzyk, Recht et al. 2000; Hwang, Venkatasubrahmanyam et al. 2003; Wood, Krogan et al. 2003). Inactivation of a mouse homolog of Rad6p leads to defective spermatogenesis but does not appear to prevent histone degradation. Furthermore, monoubiquitination of histone H1 in Drosophila by $TAF_{II}250$, another RING finger containing protein, result in transcriptional activation (Pham and Sauer 2000). These results taken together support a link between histone ubiquitination and gene transcription. In addition, two other ubiquitin protein ligases also belonging to the RING domain E3 family, BRCA1/BARD1 (Mallery, Vandenberg et al. 2002) and Np95 (Citterio, Papait et al. 2004), have been recently shown to promote ubiquitination of histone *in vitro*.

In this paper, we have identified for the first time, a HECT domain containing E3 that ubiquitinates histones. Besides a role in mediating histone degradation, this ubiquitination could also be involved in transcriptional regulation. Indeed, previous studies with the rat homolog of LASU1 suggested that it may function as a DNA-binding transcriptional regulator (Gu, Dubner et al. 1995; Gu, Irving et al. 1997). The most similar other known E3s are *S. cerevisiae* Tom1 and E3s closely related to Tom1. Tom1 mutants are pleiotropic and include defects in transcription that appear to be mediated through ADA coactivator proteins (Saleh, Collart et al. 1998) as well as defects in export of mRNA (Utsugi, Hirata et al. 1999; Duncan, Umen et al. 2000; Sasaki, Toh-e et al. 2000).

Suppressors of tom1 mutants include STO1/G4p2/STM1 a basic protein partially homologous to histone H1 that can bind to G4 nucleic acids (Frantz and Gilbert 1995; Utsugi, Toh-e et al. 1995; Nelson, Musso et al. 2000). Thus, Tom1 appears to interact with basic nucleic acid binding proteins and in that way is similar to our demonstrated ability of LASU1 to interact with and ubiquitinate histones.

LASU1 is here demonstrated to be one of the largest E3s to be characterized to date. Like other large E3s such as the 374 kDa Tom1 (Utsugi, Toh-e et al. 1995) and 300 kDa EDD (Oughtred, Bedard et al. 2002), the large size likely permits it to interact with a variety of substrates and to exert a large number of functions which remain to be defined.

Table 1

Reference No.	Protein name	Species	Peptide Number	Present in SDS Bands
gi|22090626	HECT domain protein LASU1	Homo sapiens	68	A,B,C
gi|26340188	unnamed protein product	Mus musculus	20	B,C
gi|10436857	unnamed protein product	Homo sapiens	7	A
gi|25140230	similar to tripeptidyl peptidase II	Homo sapiens	7	B
gi|1061310	valyl-tRNA synthetase		7	B
gi|2493459	Protein kinase C substrate, 60.1 kDa protein, heavy chain (PKCSH) (80K-H protein)		3	A
gi|28478776	similar to Elongation factor 1-gamma (EF-1-gamma) (eEF-1B gamma)	Mus musculus	7	
gi|28189194	similar to pancreatic tumor-related protein	Bos taurus	3	
gi|417844	VALYL-TRNA SYNTHETASE (VALINE--TRNA LIGASE) (VALRS),		2	
gi|27807311	vesicle docking protein p115	Bos taurus	5	
gi|232037	Elongation factor 1-gamma (EF-1-gamma) (eEF-1B gamma)		3	
gi|12804891	Similar to tubulin, beta 5	Homo sapiens	4	
gi|6015101	Endoplasmin (94 kDa glucose-regulated protein) (GRP94)		5	
gi|25021127	similar to transitional endoplasmic reticulum ATPase (EC 3.6.1.-) [validated] - rat	Mus musculus	2	
gi|4204880	heat shock protein	Homo sapiens	3	
gi|11265337	hypothetical protein DKFZp434K0126.1 - human (fragment)		3	
gi|1174593	TUBULIN ALPHA-2/ALPHA-4 CHAIN		2	
gi|12847562	unnamed protein product [Mus musculus]		2	

Table 2. MS/MS analysis of the band isolated from the native acrylamide gel (Fig. 4B). Proteins also detected in the tandem mass spectrometry analysis's reports of bands A, B or C from the denaturing gel (Fig. 4C) are indicated.

Figure Legends

Fig. 1: Identification and initial characterization of $E3^{Histone}$. A, A soluble testis extract was chromatographed on a Mono Q anion exchange column. The 100,000 g supernatant of the rat testis extract (load), water as a negative control (Ctl-) and eluted fractions were tested for the ability to support conjugation of ubiquitin to ^{125}I-labelled histone H2A in the presence of E1 and UBC4. Lane 3-8 are the fractions eluting at approximately 0.4 M NaCl that contained the activity. B, $E3^{Histone}$ (lane 1 & 3) or water (Ctl-) was used for the ubiquitin conjugation assay of ^{125}I-labelled histone H2A with wild type ubiquitin or methylated ubiquitin. C, Different ^{125}I-labelled histones were tested in the conjugation assay in the presence (+) or absence (-) of $E3^{Histone}$. D, $E3^{Histone}$ mediated ubiquitination was tested in the presence or absence (ctl-) of the indicated E2s. A-D, Reaction products were resolved by SDS-PAGE and detected by autoradiography. *, background bands. Arrows show single monoubiquitination form of histone H2A. Note that in panels A, C, D, the free histone substrates were ran out of the gels because they created large shadows when the gels were exposed to film with the use of intensifying screens. B-D, the source of $E3^{Histone}$ is described in *Conjugation Assay* in Methods.

Fig. 2: $E3^{Histone}$ is probably a HECT domain containing E3. A, $E3^{Histone}$ requires a free thiol group for its E3 activity. Left, In the conjugation assay, $E3^{Histone}$ was first treated with or without N-ethylmaleimide (NEM). Excess NEM was then quenched by DTT, and the other components of the conjugation assay were added. Products of the assay were resolved by SDS PAGE and detected by autoradiograghy. Right, To confirm that both the NEM treatment and quenching by DTT were adequate, E1 was treated sequentially with

NEM/DTT (lane 3) or added subsequently to buffer that had been similarly treated with NEM/DTT (lane 4), and then assayed for its ability to form a thioester bond with ^{125}I-ubiquitin. Reaction products were resolved by SDS PAGE at 4°C under non-reducing conditions and detected by autoradiography. B, $E3^{Histone}$ does not require divalent cations for its function. $E3^{Histone}$ was treated with EDTA (lane 3 & 4) or TPEN (lane 11 & 12). Remaining chelators were then quenched with excess Mg^{2+} (lane 4 and lane 12) or not (lane 3 and lane 11) prior to assaying for ubiquitination of histone H2A. To confirm the efficacy of the treatment, the RING finger E3 ARNIP was similarly treated, and then assayed for its autoubiquitination ability by incubation with E1, UBC4-1 and ^{125}I-ubiquitin (lane 5-8 & lane 13-14). Products were resolved by SDS PAGE and detected by autoradiography.

Fig. 3: Purification of $E3^{Histone}$ from bovine testis extract. A, $E3^{Histone}$ purification scheme. The salts used to elute proteins in each step are shown on the right of the purification tree. Arrows indicate the salt concentration at which the enzyme eluted. Buffers used were as described in Methods. **B,** Protein Purification Table. Aliquots of the pooled fractions from each protein purification step were assayed under quantification conditions (see Methods) to measure $E3^{Histone}$ activity. The results were detected by phosphorimager and quantified as described in the methods.

Fig. 4: Analysis of the fractions from the last purification step. A, Fractions from the glycerol gradient centrifugation were assayed for $E3^{Histone}$ activity. As described in Methods, MeUb was used to start the reaction. The results were detected by autoradiography. B, Protein fractions were resolved on a 5 % native acrylamide gel and

detected by colloidal blue staining. C, Protein fractions were resolved by 7%-15% gradient SDS PAGE and detected by colloidal blue staining. Arrows in panels B and C show the bands cut for tandem mass spectrometric analysis.

Fig. 5: Structure of LASU1. A. Domain analysis of LASU1 and the unnamed protein. The region used to generate an anti-LASU1 antibody is shown. B. Alignment of DUF908. C. Alignment of DUF913. In B and C, shown are the conserved residues in the domain. gi:16944653, related to TOM1 protein; gi:22832284, CG8184-PB; gi:26327257, unnamed protein product; gi:22532851, Hypothetical protein Y67D8C.5; gi:3176689, Contains similarity to ubiquitin carboxyl-terminal hydrolase 14; gi:2440180, SPAC19D5.04; gi:927738, Tom1p; gi:23499033, ubiquitin-protein ligase 1; gi:8778329, F14J16.10.

Fig. 6: $E3^{Histone}$ contains HECT domain protein LASU1. A. $E3^{Histone}$ was immunoprecipitated with pre-immune serum (PIS) or anti-LASU1 antiserum (Ab). Left, Western blot of the indicated sample with anti-LASU1 antibody. Right, Samples were assayed for $E3^{Histone}$ activity. B. UBC4 was not co-immunoprecipitated with $E3^{histone}$. Crude testis extract was immunoprecipitated with the LASU1 antibody, and then the supernatants and pellets were subjected to immunoblotting with anti-LASU1 antibody or anti-UBC4 antibody. Multiple bands in the LASU1 blot likely represent degradation products of LASU1 during the procedure. CTL, the same amount of sample before immunoprecipitation (IP). S, supernatant. P, pellet.

Fig. 7: $E3^{Histone}$ appears to be a monomeric protein. A, Separation of a crude testis extract using a Superdex 200 gel filtration column. Top, Fractions were assayed for $E3^{Histone}$ activity as described in Fig. 1. Elution positions and molecular weights of the protein standards are shown. Bottom, Western blot of the fractions with the anti-LASU1 antibody. Arrow shows the LASU1 band. B, Separation of a crude testis extract by glycerol gradient centrifugation. The sample or purified 20S proteasome or thyroglobulin were applied to a 4%-40% glycerol gradient and subjected to the centrifugation at 30,000 rpm in a SW 40Ti rotor for 20 hours at 4 °C. Top, Fractions from 4%-40% glycerol gradient centrifugation were assayed for $E3^{Histone}$ activity. Bottom, Detection of protein standards and LASU1. 20S proteasome and LASU1 were detected by western blotting, respectively. Thyroglobulin was detected by Commassie blue staining.

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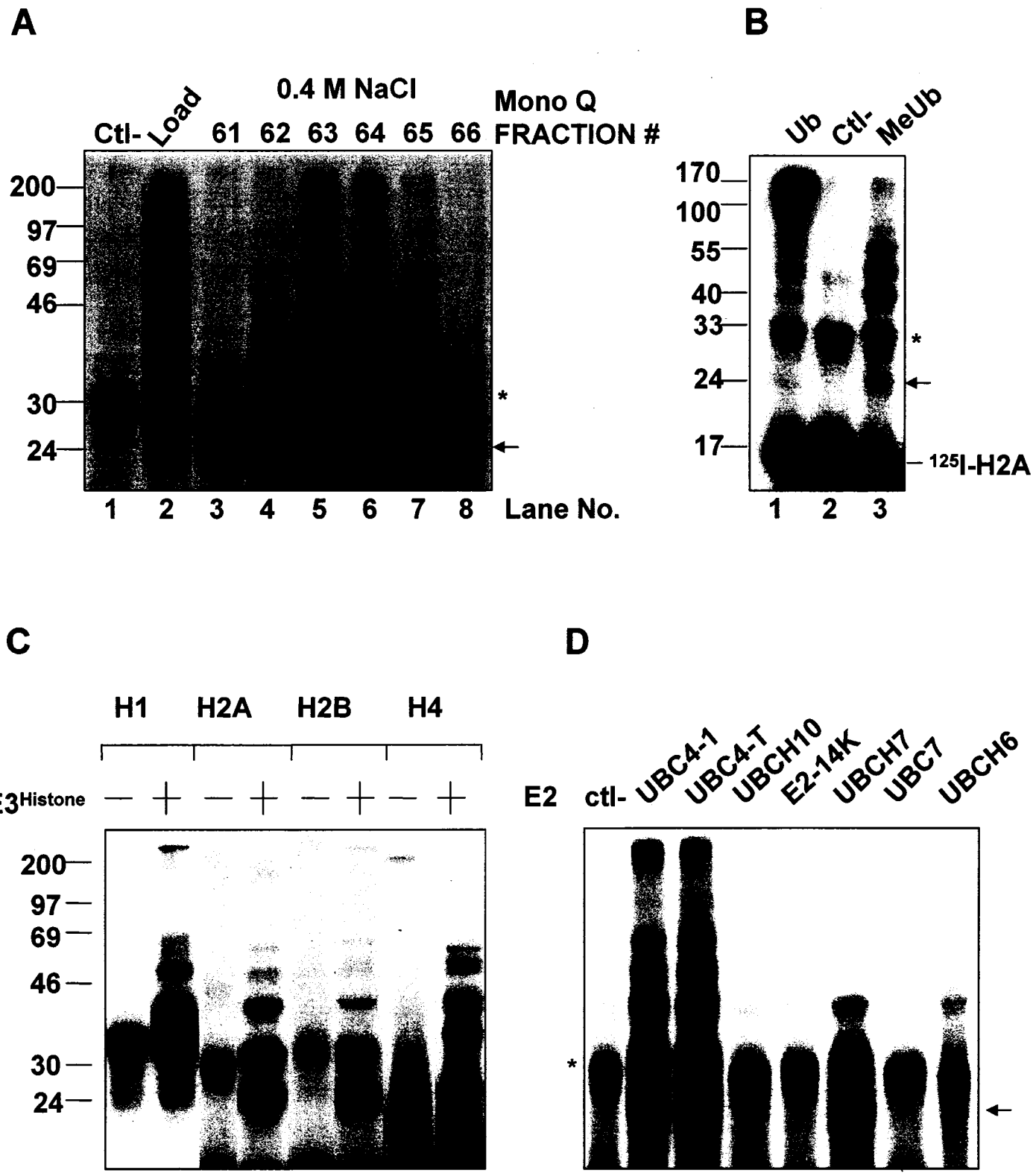
Fig. 1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H4
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UBCH6
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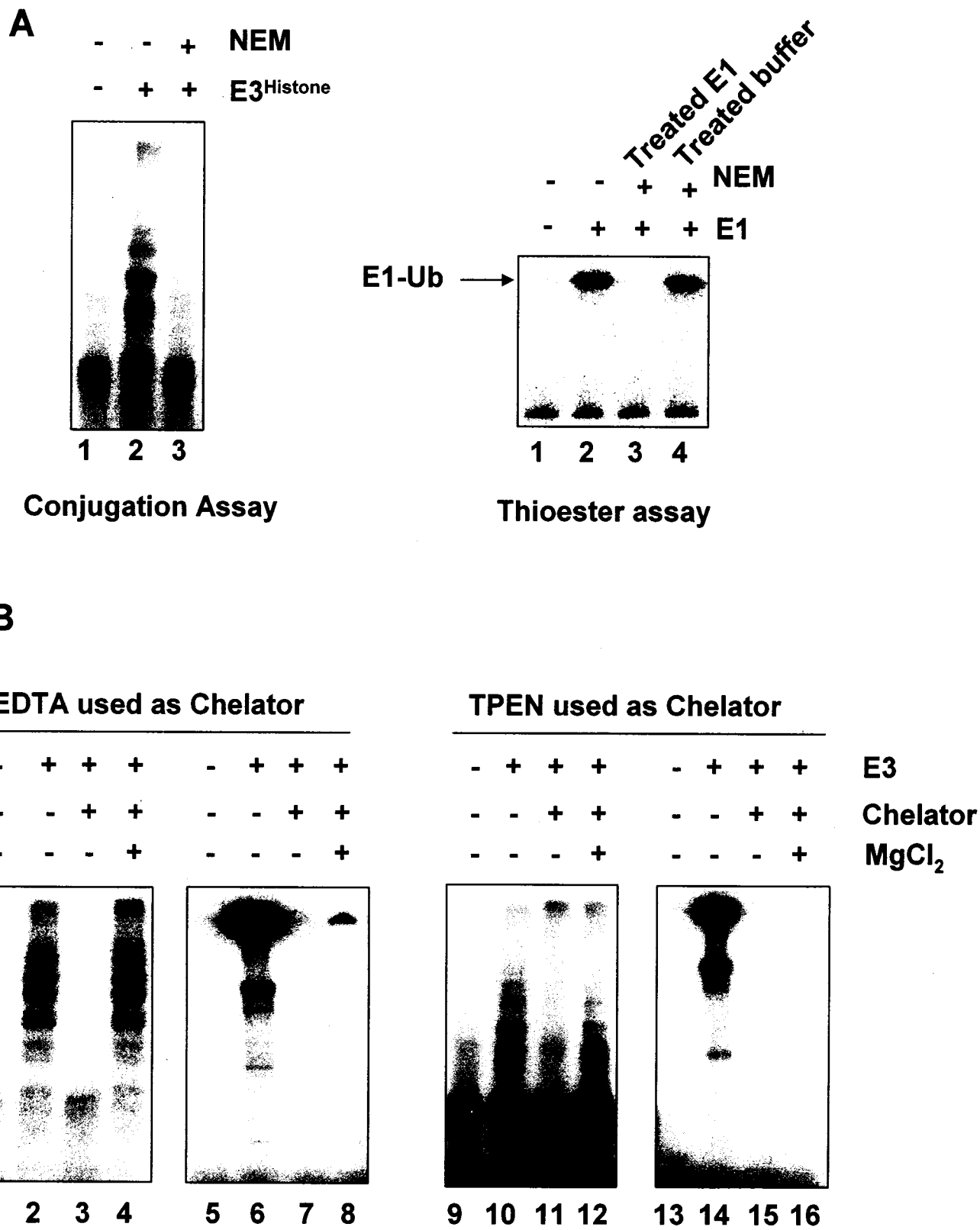
Fig. 2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1 2 3 4
Thioester assay
B
EDTA used as Chelator
TPEN used as Chelator
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- - - + - - - + - - - + - - - + MgCl2
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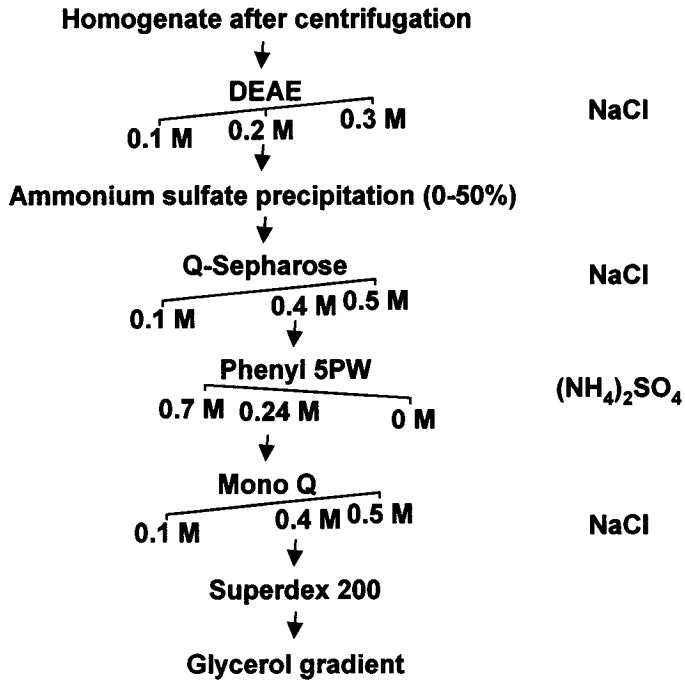
Fig. 3

A

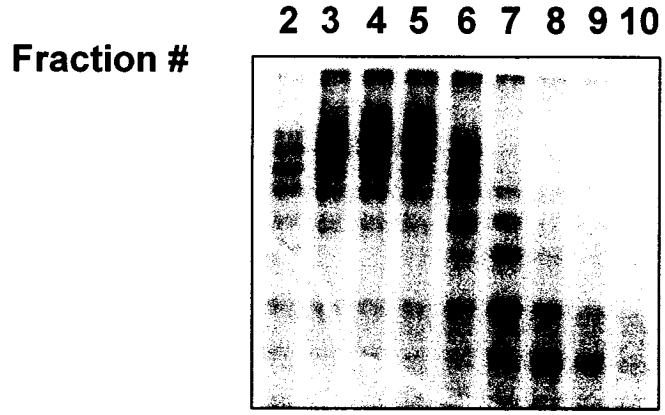


B

	Homogenate	Ammonium sulfate precipitation	Q-Sepharose	Phenyl 5PW	Mono Q	Superdex 200	Glycerol gradient
Protein concentration (g/l)	5.96	6.56	0.33	0.19	0.30	0.74	0.13
Enzyme activity ($\times 10^9$ cpm/l)	62.9	156	227	1415	3553	8361	4680
Volume (ml)	1125	225	135	19.5	4	0.5	0.27
Total protein (mg)	6705	1475	44.55	3.71	1.20	0.37	0.035
Total enzyme activity ($\times 10^9$ cpm)	70.8	35.1	30.6	27.6	14.2	4.2	1.3
Specific enzyme activity ($\times 10^{10}$ cpm/g)	1.06	2.38	68.8	744	1184	1130	3611

Fig. 4

A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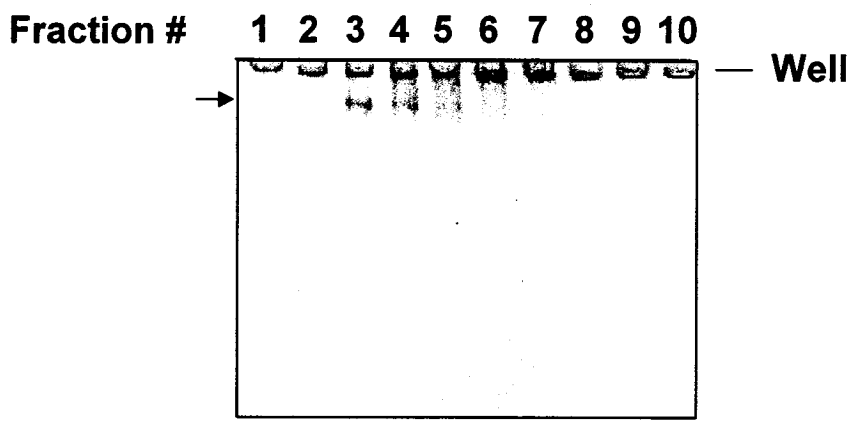
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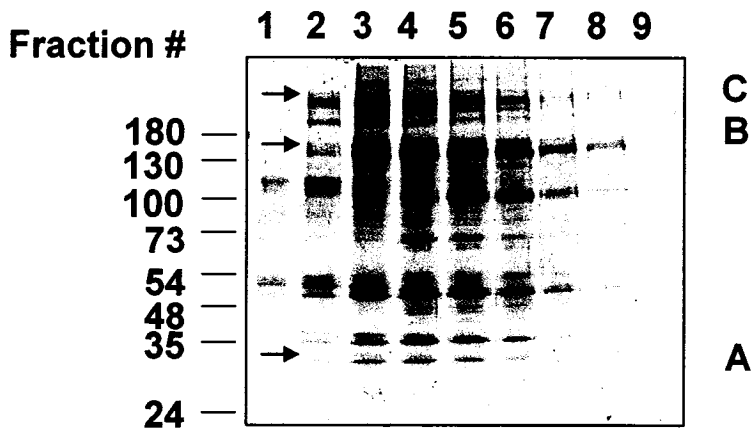
C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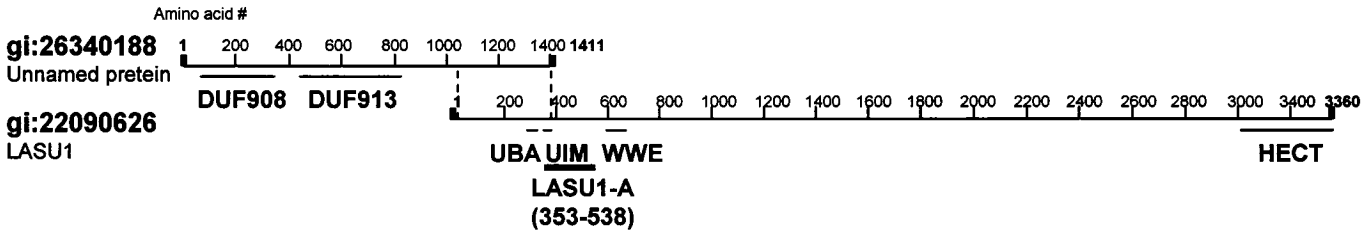
A

Fig. 5

A



B

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LASU1          86 KEQLKMLLL-AVLNFTALLIEYSFSRHLYSSIE-HLTTLLASSDMQVV---LAVLNLLYVFSKRSNY---LGSDKRTPLLTRLQHLAE---GKENGFGLAECCRDLQ 187
gi 16944653   117 EDGDCQLLV-AILKFTRMLLEHCGNRSIYASSH-HIDRLLNSPYLEVQ---AAALEVGLELAQRYNA---LLANEYNIDLEKVQLLAR---ASMYANDLKALASSRP 271
gi 22832284    87 QETDVTLLL-EVLNFTTLLIEHSFSRHLYNSIE-HLTVLLSSQNMDIV---LAVLNLLYMFSKRSNF---LPFEKKELLIVKLFNIAE---DPNYGLSLKDCCIGEP 188
gi 26327257    12 KEQLKMLLL-AVLDFTALLIEYSFSRHLYSSIE-HLTTLLASSDMQVV---LAVLNLLYVFSKRSNY---LGSDKRTPLLTRLQHLAE---GKENGFGLAECCRDLQ 113
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LASU1         188 MLKYP---LHFEFYADPG---EKRTTSNTLHYIHIEQLDKIS---LTKMYSIPKDRQMLLFTHIRLAHGFSNHRK---QARLHAISILVYS---ALQESANSILYN- 303
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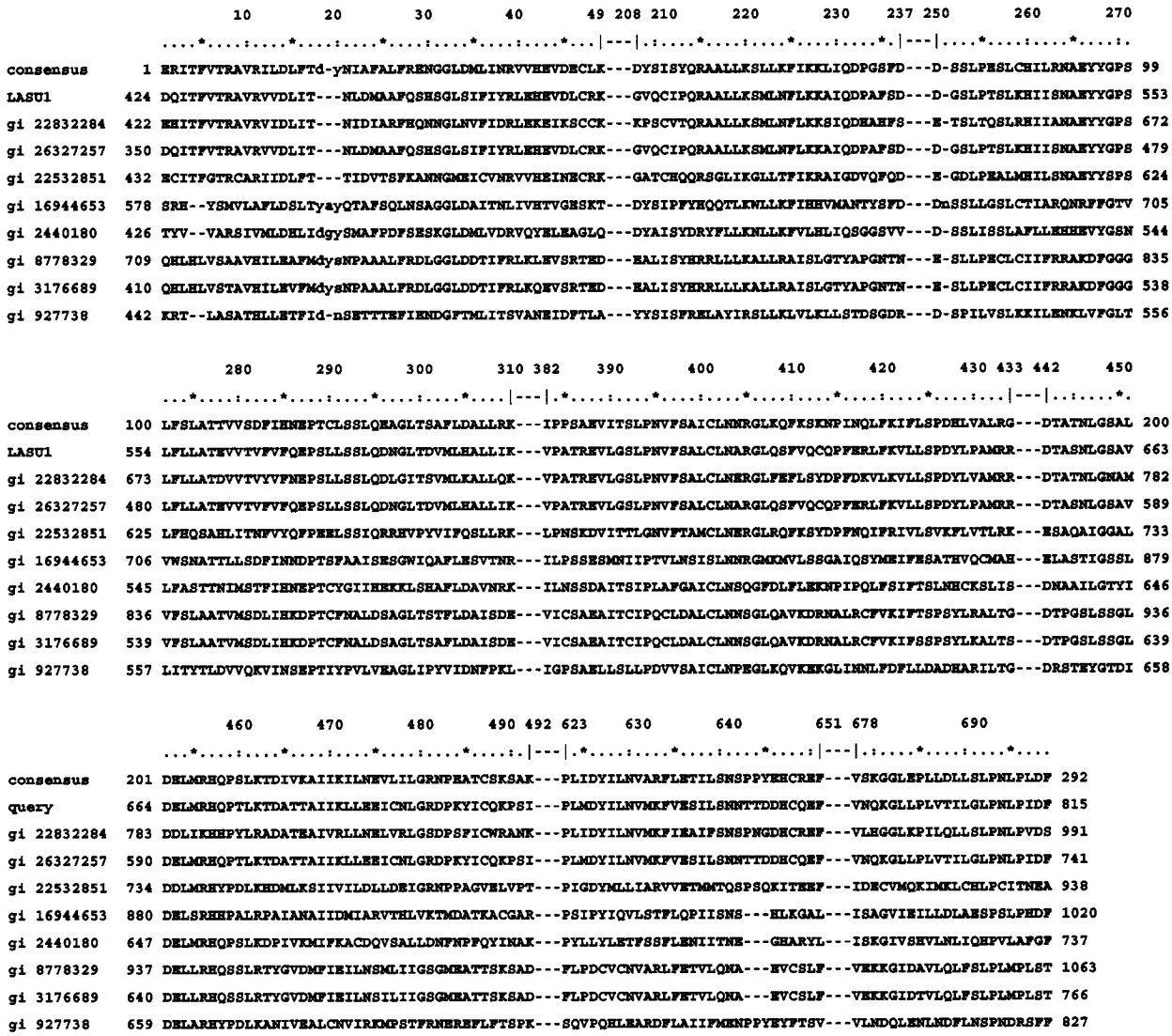

Fig. 6

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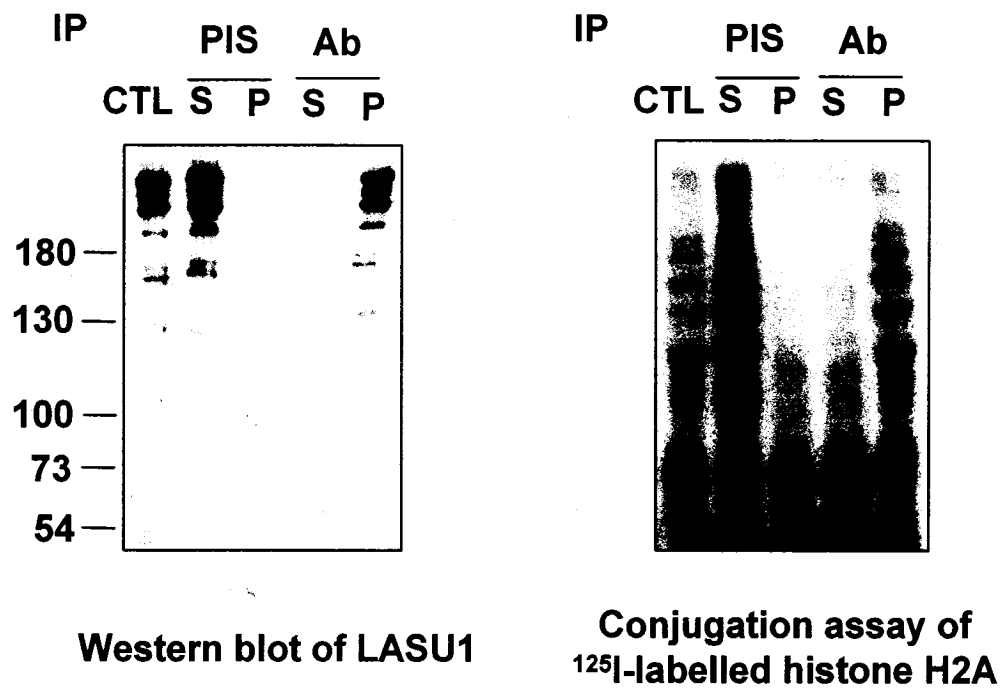

Western blot of LASU1

Conjugation assay of ^{125}I-labelled histone H2A

B

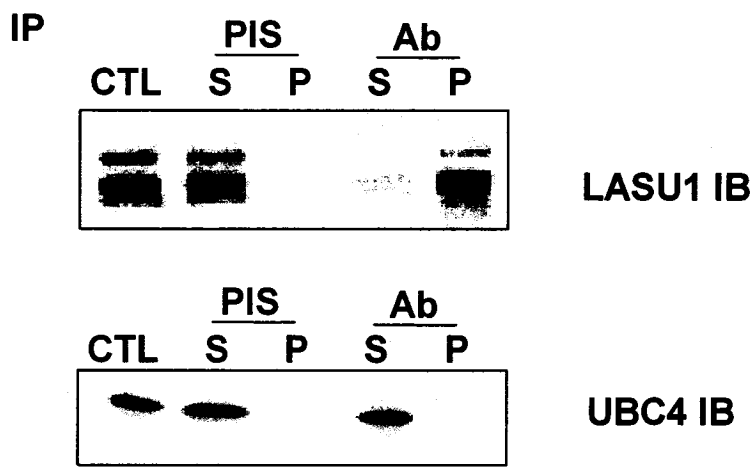

Fig. 7

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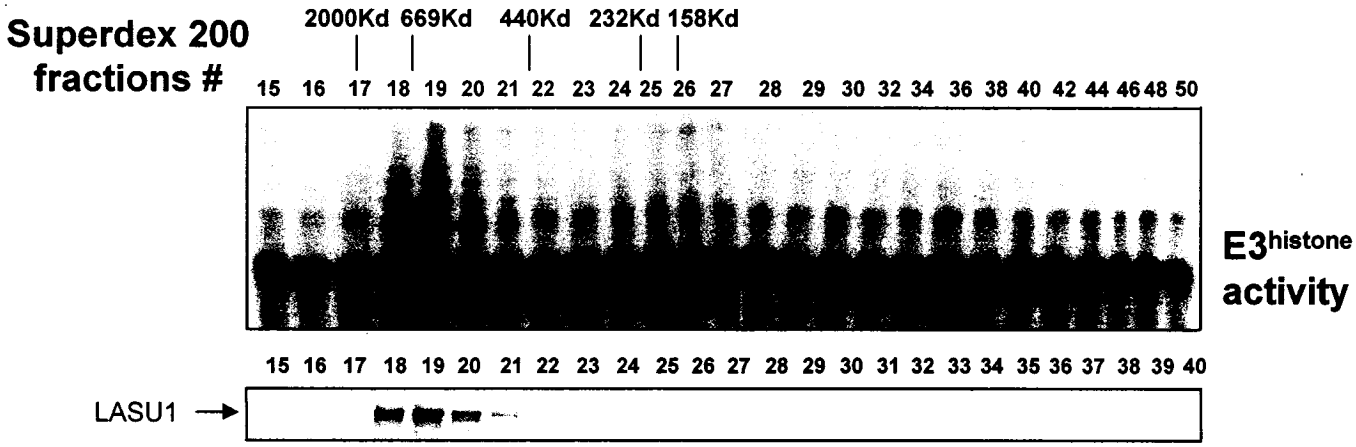
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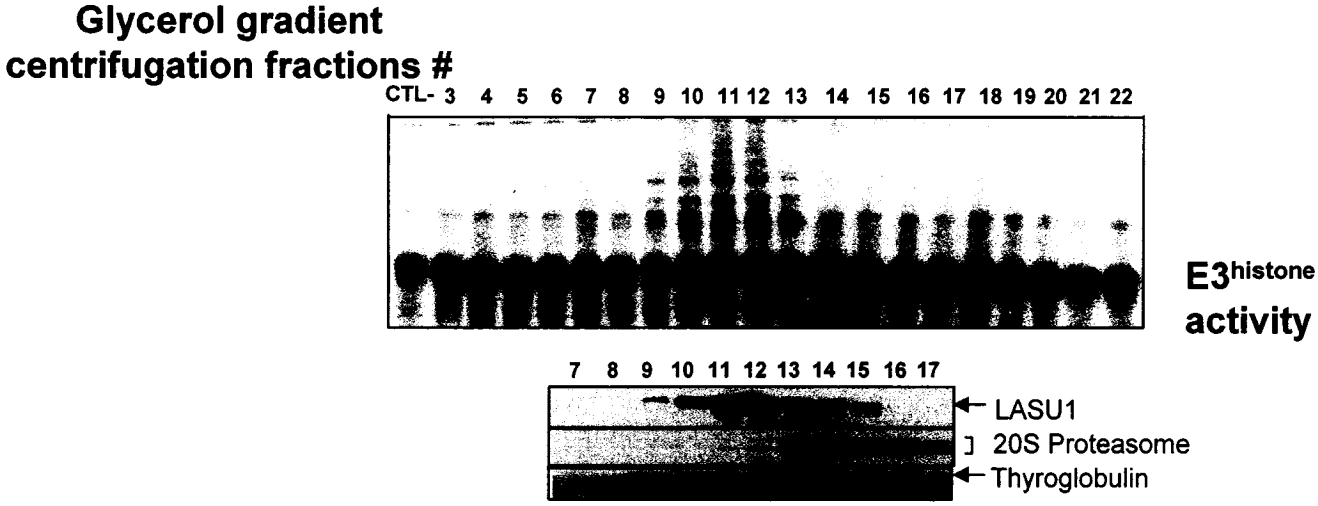
LASU1

B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E3histone activity
7 8 9 10 11 12 13 14 15 16 17
LASU1
20S Proteasome
Thyroglobulin

Bridge of Chapter 2 & 3

In Chapter 2, we identified $E3^{Histone}$, a monomer of HECT domain containing protein LASU1, as the major Ubc4-dependent E3 ligase in the testis for histone ubiquitination *in vitro*. $E3^{Histone}$ is therefore likely involved in histone degradation during spermatogenesis which is required for the normal maturation of spermatids. Since histones are degraded specifically in late round and early elongating spermatids, to further evaluate the possible function of LASU1 on histone degradation, we determined which cell types in the testis express $E3^{Histone}$/LASU1 in this chapter. Since $E3^{Histone}$/LASU1 is a novel E3 ligase with other substrates such as Mcl-1 and p53, we also determined its distribution among different tissues.

Chapter 3

Expression of the ubiquitin protein ligase, $E3^{Histone}$ /LASU1/ Mule/ARF-BP1, in Rodent Tissues and Its Regulation during Spermatogenesis

Running title

Expression of $E3^{Histone}$ /LASU1/Mule/ARF-BP1

3.1. Abstract

We recently purified the ubiquitin protein ligase, $E3^{Histone}$/LASU1 (Mule/ARF-BP1) from the testis and found that it stimulated ubiquitination of all the core histones *in vitro* (Liu, Oughtred et al. 2005). Since histones are ubiquitinated at various stages of spermatogenesis, we studied the expression of $E3^{Histone}$/LASU1 during this process. During the first wave of spermatogenesis in the neonatal rat, $E3^{Histone}$/LASU1 mRNA was highly expressed at day 10 and gradually decreased with age. Levels of the protein rise and fell, peaking at day 20. Consistent with these findings, LM (Light microscopy) and EM (Electro microscopy) immunohistochemistry determined that $E3^{Histone}$/LASU1 was highly expressed in nuclei from spermatogonia to mid-pachytene cells. The strongest expression was in nuclei from leptotene to mid-pachytene cells, suggesting that $E3^{Histone}$/LASU1 may be involved in histone ubiquitination in spermatocytes. There was no obvious staining of $E3^{Histone}$/LASU1 in spermatids when histones are ubiquitinated and degraded. $E3^{Histone}$/LASU1 was also widely expressed in different rodent tissues at both mRNA and protein levels. It was mainly expressed in the cytoplasm, except in neuronal cells of the brain and in early germ cells of the testis, where it was expressed in the nucleus. In most tissues, $E3^{Histone}$/LASU1 was expressed in epithelia. These results suggest that $E3^{Histone}$/LASU1 may play a role in chromatin modification in spermatocytes, but also has functions in other tissues.

3.2. Introduction

Spermatogenesis is the process of producing haploid spermatids, one of the most highly specialized cell types, in the seminiferous tubules of the testis. It is a complex developmental process involving mitotic proliferation, meiotic divisions and extensive haploid spermatid cell remodeling. It starts with successive mitotic divisions of the stem cell spermatogonia to produce diploid spermatocytes. After two meiotic divisions of spermatocytes, haploid round spermatids are formed. These round spermatids are transformed through significant remodeling into highly differentiated spermatozoa.

The prophase of the first meiotic division, including leptotene, zygotene, pachytene and diplotene, are very long compared to the subsequent phases of meiotic divisions. It can occupy up to 90% of the entire duration of meiosis. It starts from leptotene cells, in which the chromatin begins to condense. In zygotene cells, chromatin has been condensed into chromosomes, which begin to be paired in an apparatus called the synaptonemal complex. In pachytene cells, the chromosomes are fully paired. Genetic recombination or crossing over occurs at this stage, resulting in the exchange of distal portions of the paired chromosomes. Histones appear to be highly ubiquitinated in pachytene cells (Chen, Sun et al. 1998; Baarends, Hoogerbrugge et al. 1999). In diplotene cells, the paired chromosomes are separated. Diplotene phase and the following meiotic divisions are short lasting. Haploid spermatids are formed after two meiotic divisions, and then undergo a remodeling process.

Protein degradation is involved in haploid spermatid remodeling. The mature spermatid loses almost all of its cytoplasm, and the nuclear volume shrinks to less than 5% of that of somatic cells (Sawada, Ochi et al. 1975). The chromatin is extremely

compact in the nuclei of mature spermatids. Histones are replaced by transition proteins in late round and early elongating spermatids (Meistrich 1989). The substitution of histones by transition proteins and subsequently by protamines permits chromatin condensation. When genes encoding transition proteins are inactivated in the mouse, histones are still degraded, indicating that the loss of histones is not simply due to displacement by the transition proteins (Yu, Zhang et al. 2000). It remains unclear how histones are degraded. Many proteins are removed in a cytoplasmic droplet that is phagocytosed by the adjacent Sertoli cells during the remodeling of spermatids (Russell and Clermont 1976). But the ubiquitin proteasome pathway may also play an important role in this process (Rajapurohitam, Morales et al. 1999). Ubiquitinated histones have been detected in the testis of various species (Agell, Chiva et al. 1983; Nickel, Roth et al. 1987; Chen, Sun et al. 1998; Baarends, Hoogerbrugge et al. 1999). Ubiquitination is activated during development of the testis and the rate of conjugation of ubiquitin to proteins appears to peak at the stage when histones are replaced by transition proteins (Rajapurohitam, Morales et al. 1999). Thus, histones may be degraded by the ubiquitin proteasome degradation pathway.

Ubiquitination requires sequential cooperation of three enzymes, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin protein ligase (E3). Of these, E3 plays the important role of recognizing substrates and catalyzing the final step of ubiquitination-transferring ubiquitin from E2 to the substrate. Increased ubiquitination occurs during spermatogenesis and appears due to activation of a UBC4 dependent pathway of conjugation (Rajapurohitam, Morales et al. 1999). Expression of UBC4 appears maximal in late round and early elongating spermatids when the degradation of

histones occurs. Thus, we searched for a ligase that would polyubiquitinate histones. We recently purified a novel E3, which may be the E3 ligase for histone ubiquitination in spermatogenesis (Liu, Oughtred et al. 2005). This E3, $E3^{Histone}$/LASU1, is ~500 kDa HECT domain containing protein. It polyubiquitinates all the core histones *in vitro*. Furthermore, it is the major UBC4-dependent histone-ubiquitinating E3 in testis (Liu, Oughtred et al. 2005). Finally, UBC4-1 and UBC4-testis, E2s required for the activation of ubiquitination during spermatogenesis, are the preferred E2s for $E3^{Histone}$ /LASU1 dependent ubiquitination of histone. This ligase is therefore a good candidate E3 ligase required for histone ubiquitination during spermatid maturation.

To further verify the possible function of $E3^{Histone}$ /LASU1 in the testis, in this paper we studied the regulation of expression of this E3 during spermatogenesis. Three recent studies have identified two additional substrates for $E3^{Histone}$/LASU. The same ligase was identified as Mule (*M*cl-1 *u*biquitin protein *l*igase *E*3) (Zhong, Gao et al. 2005) or LASU1 (Warr, Acoca et al. 2005) for ubiquitination of the antiapoptotic protein Mcl-1. One other study identified it as ARF-BP1 (*ARF* tumor suppressor *b*inding *p*rotein*1*) and demonstrated that it can ubiquitinate the tumor suppressor protein p53 (Chen, Kon et al. 2005). Since $E3^{Histone}$ /LASU1 has these other substrates, we therefore also surveyed the expression of $E3^{Histone}$ /LASU1/ Mule/ARF-BP1 in different tissues and determined its subcellular localization.

3.3. **Material and methods**

Preparation of tissue extracts- Male Sprague-Dawley rats or male CD-1 mice (Charles River Laboratories) were decapitated under anesthesia. The indicated tissues were isolated quickly and homogenized using a Potter-Elvehjem homogenizer (except for heart and skeletal muscles in which a polytron tissue disruptor was used) in 50 mM Tris, pH 7.5, 1 mM dithiothreitol (DTT), 0.25 M sucrose, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin at 4 °C (5 ml/g tissue). The homogenates were centrifuged at 10,000 *g* for 10 min, and then the supernatant was centrifuged at 100,000 *g* for 60 min. The final supernatants were stored at −80 °C until use.

RNA analysis-RNA was prepared from rat tissues by the guanidium thiocyanate CsCl method (Ausubel 1993). Expression of $E3^{Histone}$/LASU1 was determined by RNA blotting (Northern analysis). Samples of RNA (20μg) were resolved on 1% formaldehyde-containing agarose gels followed by transfer to nylon membranes and cross-linking with UV light. The membranes were hybridized with ^{32}P labeled probes, washed, and then subjected to autoradiography. Single stranded cDNA probes were prepared by PCR based amplification using $E3^{Histone}$/LASU1 containing plasmid DNA as template and a reverse primer (primer for N-terminal probe: 5' gccacttgattcaaccacc 3' (complementary to nt 4638-3656 of the full length $E3^{Histone}$/LASU1, Gene bank accession No. AY929611), primer for C -terminal probe: 5' gaatgggtcaagggaggatg 3' (complementary to nt 11792-11811 of $E3^{Histone}$/LASU1).

Preparation of tissue sections-Male Sprague-Dawley rats or CD-1 mice were exsanguinated under isoflurance anaesthesia. Indicated tissues were isolated and fixed in periodate-lysine-paraformaldehyde solution (PLP solution: 10 mM sodium periodate (Sigma), 75 mM L-lysine (Sigma), 2% paraformaldehyde (W/V) (Sigma)) at 4 °C for 24 hours. After fixation the samples were washed twice with PBS and then stored in PBS solution prior to paraffin embedding. Embedded samples were sectioned at 4 μm (bone was decalcified prior to paraffin section).

$E3^{Histone}$/LASU1 antibodies-LASU1-A antibody was prepared as described (Liu, Oughtred et al. 2005) against amino acid residues 1367 to 1552 of the human $E3^{Histone}$/LASU1 sequence (identical sequence in mouse $E3^{Histone}$/LASU1). Commercial LASU1 antibodies BL671 and BL672 from Bethyl Laboratories were also used. The epitopes recognized by BL671 and BL672 map to residues 1939 to 1989, and 3764 to 3814, respectively, of human $E3^{Histone}$/LASU1. The mouse ortholog is highly conserved at both sites with only two amino acids differences from the human sequence.

Light microscopic immunohistochemistry-Tissue sections were re-hydrated and heated in 1% sodium citrate at 70 °C for 30 minutes to expose the epitopes. Specimens were incubated with $E3^{Histone}$/LASU1 primary antibody (10 μg/ml), at room temperature overnight. For negative controls, antigen neutralized antibody was used. After washing with high salt and low salt washing buffers (50 mM Tris-HCl, pH 7.6, 500 mM NaCl (high salt), 150 mM NaCl (low salt), 0.01% Tween-20), the sections were incubated with

biotinylated goat anti-rabbit IgG (Sigma) at room temperature for 45 minutes. After three washes, the Vectastain ABC-AP (Vector Laboratories, UK) alkaline phosphatase detection reagent was applied according to the manufacturer's instructions. Samples were treated with Vector methyl green nuclear counterstain and mounted with Kaiser's glycerol jelly.

Electron microscopic immunocytochemistry-Rat testes were fixed by a 10-min perfusion through the abdominal aorta with 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer. Tissue was then embedded in Lowicryl K4M as previously described (Oko, Hermo et al. 1993). Ultrathin sections were mounted on 300-mesh Formvar-coated nickel grids. Each grid containing numerous sections was floated on a drop of 20 mM Tris-buffered saline (TBS) (pH 7.4) containing 10% goat serum and then incubated for 1 h with BL671 antibody in TBS. The grids were washed three times for 5 min each in TBS containing 0.5% Tween 20 and then incubated for 1 h with colloidal gold (diameter, 10 nm)-conjugated goat anti-rabbit antibody (diluted 1:20 in TBS). The sections were washed three times for 5 min each in TBS containing 0.05% Tween 20 and then once in distilled water. They were counterstained with uranyl acetate in 30% ethanol for 2 min followed by lead citrate for 30 s. As a negative control, the LASU1 antibodies were preincubated with the peptide antigen for 1 h at 37°C before being used on the sections. Electron micrographs were obtained on a Philips 400 electron microscope.

Immunofluorescence-FR3T3 fibroblasts were grown on coverslips, and then fixed with paraformaldehyde solution (4% paraformaldehyde, pH 7.4) at room temperature for 10

minutes. After three washes with PBS, fixed cells were permeabilized with 0.2% Triton X100 (in PBS) at room temperature for 10 minutes. $NaBH_4$ (0.5 mg/ml in PBS) was then applied to decrease the background. Following blocking of non-specific binding sites by incubation with 3% inactivated normal goat serum with 1% Triton X100 at room temperature for one hour, the cells were exposed to anti-LASU1-A antibody (6.3 μg/ml), for one hour. Following washing, cells were incubated with second antibody conjugated to FITC (Sigma F-1262), at room temperature for one hour. After washing, DAPI (2 μg/ml) was applied to stain the nuclei. Coverslips were mounted with Moviol and the signals detected using a fluorescence microscope.

3.4. **Results**

Developmental regulation of expression of E3^{Histone}/LASU1 Since histones are ubiquitinated and degraded in early elongating spermatids, but also found to be ubiquitinated in primary spermatocytes (Agell, Chiva et al. 1983; Nickel, Roth et al. 1987; Chen, Sun et al. 1998; Baarends, Hoogerbrugge et al. 1999), we explored the regulation of expression of E3^{Histone}/LASU1 during spermatogenesis. We first examined the regulation of expression of E3^{Histone}/LASU1 at the levels of both mRNA and protein during the first wave of spermatogenesis between birth and sexual maturation. Using probes complementary to either the N terminal part or C terminal part of E3^{Histone}/LASU1 mRNA, we observed a single transcript above 10 kb, consistent with the size of E3^{Histone}/LASU1 cDNA coding sequence of 13.1 kb. Therefore there is no apparent alternative splicing of E3^{Histone}/LASU1 mRNA. E3^{Histone}/LASU1 mRNA was highly expressed in testis of young rats (Fig. 1A). Quantification of the Northern blots showed that levels of E3^{Histone}/LASU1 mRNA gradually decreased from day 10 to day 30 of life, after which it remained constant at low levels. E3^{Histone}/LASU1 protein levels increased from day 10, appeared to peak at day 20, and then gradually decreased to day 35 (Fig. 1B). These results suggest that E3^{Histone}/LASU1 protein levels may be highly expressed in late pachytene or diplotene spermatocytes, which are the most advanced germ cells present at day 20 of life.

To verify this, immunohistochemistry studies were used to detect E3^{Histone}/LASU1 expression in rat testis sections (Fig. 2). E3^{Histone}/LASU1 was mainly expressed in the nuclei of spermatogonia and early spermatocytes (Fig. 2A, right panel). No staining was detected with pre-absorbed antibody (Fig. 2A, left panel). Detection under high

magnification showed $E3^{Histone}$/LASU1 is expressed in nuclei from spermatogonia to mid-pachytene cells. More mature germ cells did not express $E3^{Histone}$/LASU1 in the nucleus (Fig. 2B-C). The expression of $E3^{Histone}$/LASU1 in nuclei of primary spermatocytes was also detected by EM immunocytochemistry (Fig. 2D). Shown is the staining of the nucleus of a pachytene cell (Fig. 2D). The summary of the staining of $E3^{Histone}$/LASU1 and germ cells expressing $E3^{Histone}$/LASU1 are shown in (Fig. 2E-F). The expression of $E3^{Histone}$/LASU1 was stronger in the nuclei of primary spermatocytes than in nuclei of spermatogonia. Amongst spermatocytes, $E3^{Histone}$/LASU1 expression was highest in the nucleus of leptotene cells and early- to mid-pachytene cells (Fig. 2C). There appears a discrepancy between this result and that of Fig. 1. The developmental western blot of testis extract showed the highest expression of $E3^{Histone}$/LASU1 at day 20 after birth (Fig. 1). Although the most advanced germ cells at this stage are late pachytene cells and diplotene cells, the more abundant cells are primary spermatocytes earlier than mid pachytene. Since by LM immunohistochemistry, these cells have the highest expression of $E3^{Histone}$/LASU1, this may explain $E3^{Histone}$/LASU1 protein maximally expressed in the nucleus of leptotene and early- to mid-pachytene cells (Fig. 2B-C), while western blot determined highest expression of $E3^{Histone}$/LASU1 protein at day 20 after birth (Fig. 1B). There is no obvious staining detected in spermatids or in Sertoli cells. (Fig. 2, B-C).

$E3^{Histone}$/LASU1 is widely expressed in different tissues. Since the same protein has been shown to ubiquitinate Mcl-1 (Warr, Acoca et al. 2005; Zhong, Gao et al. 2005) and p53 (Chen, Kon et al. 2005), we tested whether $E3^{Histone}$/LASU1 is expressed in other

tissues and determined the subcellular localization in those cells. $E3^{Histone}$/LASU1 mRNA is widely expressed in different rat tissues (Fig. 3A), but more highly expressed in heart, brain and young rat testis.

$E3^{Histone}$/LASU1 protein is also widely expressed in different rat tissues (Fig. 3B). Because $E3^{Histone}$/LASU1 is a very large protein, it is easily degraded during preparation of the homogenates. As a result, some of the tissues did not show the whole $E3^{Histone}$/LASU1 band, but only the degradation band (Fig. 3B). To determine the cell type and subcellular localization of $E3^{Histone}$/LASU1, different tissue sections were subjected to immunohistochemistry (Fig. 4). Antibodies against both the C- and N-termini of $E3^{Histone}$/LASU1 respectively were used to confirm the results. In all tissues except in testis (as in discussion), the two antibodies gave the same results (Fig. 4). First we detected the expression of $E3^{Histone}$/LASU1 in different tissues of the immune system. In the spleen, $E3^{Histone}$/LASU1 is expressed in B lymphocyte dependent regions (Fig. 4A, upper panels). However, in these regions, $E3^{Histone}$/LASU1 is not expressed in the lymphocytes, but mainly in the cytoplasm of non-lymphocytes in splenic nodules. In thymus, $E3^{Histone}$/LASU1 is expressed in medullary regions- again not in lymphocytes, but in epithelial cells and (Fig. 4A, middle panels). It is mainly expressed in the cytoplasm. In bone marrow, $E3^{Histone}$/LASU1 is expressed in the cytoplasm of a limited number of cells (Fig. 4A, lower panels).

In abdominal tissues, $E3^{Histone}$/LASU1 is highly expressed in the renal tubules of the kidney, both proximal and distal tubules (Fig. 4B, upper panels). It is also expressed in hepatocytes in liver (Fig. 4B, lower panels). In both cases, $E3^{Histone}$/LASU1 was expressed in the cytoplasm. In muscle tissues, $E3^{Histone}$/LASU1 is expressed in cytoplasm

of both skeletal muscle and cardiac muscle cells (Fig. 4C). In brain, $E3^{Histone}$/LASU1 is expressed in Purkinje cells in the cerebellum (Fig. 4D, upper panels). It is mainly expressed in cytoplasm and may also be partially expressed in the nucleus. Surprisingly, in neurons of various parts of the cerebrum, $E3^{Histone}$/LASU1 is mainly expressed in the nucleus (Fig. 4D, lower panels).

From these tissue studies, $E3^{Histone}$/LASU1 was found to be mainly expressed in the cytoplasm of most mouse tissues, except in brain and testis. In neuronal cells in the cerebrum and early germ cells in the testis, $E3^{Histone}$/LASU1 is expressed mainly in the nucleus. Also $E3^{Histone}$/LASU1 was found mainly expressed in epithelia (kidney, spleen, thymus (Fig. 4); intestine, lung, breast (data not shown)).

Subcellular localization of $E3^{Histone}$/LASU1 To further explore the subcellular expression of $E3^{Histone}$/LASU1, we evaluated $E3^{Histone}$/LASU1 expression in cultured cells by using immunofluorescence (Fig. 5A). In FR3T3 fibroblasts (Fig. 5A) and HEK 293 cells (data not shown), $E3^{Histone}$/LASU1 is mainly expressed in the cytoplasm with a small amount of signal being localized in the nucleus. Western blot of cell fractionated samples of cytoplasm and nucleus also revealed the presence of most $E3^{Histone}$/LASU1 in cytoplasmic fractions (Fig. 5B).

3.5. **Discussion**

The $E3^{Histone}$/LASU1 ligase has recently been implicated to be involved in the ubiquitination of several important proteins including the anti-apoptotic protein Mcl-1 (Warr, Acoca et al. 2005; Zhong, Gao et al. 2005), the tumor suppressor p53 (Chen, Kon et al. 2005), and histones (Liu, Oughtred et al. 2005). However, the regulation of this ligase remains poorly defined. In this report we have, for the first time, characterized extensively and in detail the expression of this important ligase during spermatogenesis and in various tissues.

In the testis, we observed that $E3^{Histone}$/LASU1 expression was highly regulated with expression detected mainly in spermatogonia and spermatocytes. This suggests that the enzyme may be involved in the ubiquitination of histones that occurs in spermatocytes. During spermatogenesis, histones appear to be highly ubiquitinated in pachytene cells (Chen, Sun et al. 1998; Baarends, Hoogerbrugge et al. 1999) and also in early elongating spermatids before histones are replaced by transition proteins (Agell, Chiva et al. 1983; Agell and Mezquita 1988; Chen, Sun et al. 1998; Baarends, Hoogerbrugge et al. 1999). In early-pachytene cells, ubiquitinated H2A is found mainly limited to the condensed and transcription inactive XY chromatin of the sex body, an inactive nuclear structure that contains the heterochromatic X and Y chromosomes, distributes to the entire nucleus in mid-pachytene cells and concentrates again to the sex body in late-pachytene cells (Baarends, Hoogerbrugge et al. 1999). Since it has been reported that histone modification alters nucleosome stability and thereby may facilitate processes that require access to DNA (Li, Nagaraja et al. 1993), ubiquitination of histone H2A during pachytene may facilitate replacement of somatic histones by the testis specific isotypes

that are synthesized in pachytene cells (Poccia, Simpson et al. 1987; Meistrich 1989; Drabent, Bode et al. 1996). Thus, $E3^{Histone}$/LASU1 expressed in the nucleus of pachytene cells may be involved in the replacement of somatic histones by ubiquitination, and it may thereby be involved in extensive chromatin reorganization occurring in this stage through mediating histone ubiquitination.

In addition, it is now well established that histone ubiquitination can regulate transcription of specific genes (Zhang 2003). In these cases, the histones are monoubiquitinated. In vitro, $E3^{Histone}$/LASU1 clearly manifests polyubiquitination of histones, but it is possible that in an in vivo context, the localization of enzyme and substrate in the presence of other regulatory proteins and/or deubiquitinating enzymes may limit the activity of $E3^{Histone}$/LASU1 to monoubiquitination.

Although our original hypothesized function for $E3^{Histone}$/LASU1 was a role in the polyubiquitination and degradation of histones in early elongating spermatids, no expression of $E3^{Histone}$/LASU1 was detected in spermatids by immunohistochemistry using the LASU1-A antibody generated in our laboratory. However, because of the relative insensitivity of immunohistochemistry, we cannot exclude the possibility that $E3^{Histone}$/LASU1 is expressed at low levels in spermatids. Indeed to date, we have found it expressed in all cultured cell lines that we have tested. Furthermore, Mcl-1 and p53, substrates of $E3^{Histone}$/LASU1, are essential cellular proteins expressed in most of cells (Chen, Kon et al. 2005; Warr, Acoca et al. 2005; Zhong, Gao et al. 2005). Therefore it is very likely that $E3^{Histone}$/LASU1 itself is also a widely expressed enzyme. Using commercial antibodies derived against two distinct peptides from the enzyme, we did detect staining of the acrosomes of late round and early elongating spermatids (steps 2-8)

in addition to the staining of spermatogonia and spermatocytes observed with our own antibody. Preabsorption of the commercial antibodies with antigen eliminated the acrosomal staining supporting that this staining was specific. However, when used in EM immunohistochemistry, these antibodies did not label the acrosomes of spermatids (data not shown), but did label the nuclei of spermatocytes. This in conjunction with the known propensity of the acrosome to be a site of non-specific binding of antibody has led us to take the conservative interpretation that $E3^{Histone}$/LASU1 is not present in spermatids. Ultimately, to evaluate more precisely the role of $E3^{Histone}$/LASU1 in spermatids or in spermatocytes will require generation of conditional knockout mice using promoters that will activate expression of cre recombinase in haploid (protamine driven cre) (Steger 1999) and diploid germ cells (Sycp-1 driven cre) (Vidal, Sage et al. 1998; Sage, Martin et al. 1999), respectively.

Our data showed that $E3^{Histone}$/LASU1 was mainly expressed in the cytoplasm of most tissues, except in early germ cells in the testis and neuronal cells in cerebrum where it was mainly expressed in the nucleus (Fig. 4). $E3^{Histone}$ /LASU1 was mainly expressed in epithelia (Fig.4 and data not shown). These results indicate that $E3^{Histone}$ /LASU1 must have other functions besides its role in spermatogenesis. As described earlier, $E3^{Histone}$ /LASU1 acts on Mcl-1 and p53. Thus, the functions of $E3^{Histone}$ /LASU1 in cytoplasm is likely to be linked to downregulation of Mcl-1 and p53 through ubiquitination, while in nucleus $E3^{Histone}$ /LASU1 may be involved in the regulation of histones and p53 as well.

The subcellular localiztion of $E3^{Histone}$ /LASU1 in different tissues may be regulated by the modulated exposure of the nucleus localization signal of $E3^{Histone}$ /LASU1, which is located at the middle of the protein distal to the WWE domain (Chen, Kon et al. 2005).

Since this E3 is involved in apoptosis (Chen, Kon et al. 2005; Warr, Acoca et al. 2005; Zhong, Gao et al. 2005), it would be interesting to test if $E3^{Histone}$/LASU1 is shuttled between nucleus and cytoplasm under apoptotic stimuli.

$E3^{Histone}$/LASU1 is one of the largest HECT domain E3 ligases known. Therefore it likely has many more substrates besides histones, Mcl-1 and p53. Indeed, inactivation of $E3^{Histone}$/LASU1/Mule/ARF-BP1 suppresses the growth of p53 null cells in a manner reminiscent of ARF induction (Chen, Kon et al. 2005), suggesting that there are additional substrate(s) that mediate the function of this E3 on cell growth. Identification of these other substrate(s) of $E3^{Histone}$/LASU1 will enlarge our understanding of the functions of this E3.

Figure legends

Fig. 1 Expression of $E3^{Histone}$/LASU1 during postnatal development of rat testis. **A.** mRNA from the testes of rats at different ages (days) were used in Northern Blot with probes against 5'- or 3'-ends of $E3^{Histone}$/LASU1. $E3^{Histone}$/LASU1mRNA was quantified and normalized to the level of 18S RNA in the same lane. Means ± standard error are shown (n=3 for each time point). **B.** Rat testes homogenate at different ages (days) were used in Western Blot with LASU1-A antibody. $E3^{Histone}$/LASU1protein was quantified and normalized to the level of β-Actin in the same lane. Means ± standard error are shown. (n=3 for each time point, shown is a representative blot) * indicates the major degradation product of $E3^{Histone}$/LASU1 found in the extracts.

Fig. 2 Immunohistochemistry of rat testis. **A.** Shown are cross sections of tubules from an adult rat (3-4 month old). Right, staining with antibody against C-terminal region of $E3^{Histone}$/LASU1. Left, Negative control in which the antibody was pre-absorbed with the antigen. (Magnification 10X) **B.** Magnification of the stained sections. Left panel, magnification (20X) image; Right panel, magnification (40X) image: red arrow shows nuclear staining of spermatogonia; blue arrow shows nuclear staining of spermatocyte (pachytene cell). **C.** Magnified image of the right panel in B. The stages of the seminiferous tubules are indicated. Arrows indicate same germ cells as in B. **D.** Detection of nuclear expression of $E3^{Histone}$/LASU1 in pachytene cell by EM immunocytochemistry. The black spots are the gold particles indicating the positive staining of $E3^{Histone}$/LASU1. The nucleus shows blurry background. **E.** Summary of the expression of $E3^{Histone}$/LASU1 during rat spermatogenesis. $E3^{Histone}$/LASU1 was expressed in nucleus of germ cells

from early spermatogonia to mid-pachytene cells (red continuous lines). The stages with highest expression of $E3^{Histone}$/LASU1 were shown by heavier lines. **F.** Summary of the expression of LASU1 in different germ cells.

Fig. 3 Expression of $E3^{Histone}$/LASU1 in different rat tissues. **A.** Detection of $E3^{Histone}$/LASU1 mRNA by Northern Blot. **B.** Detection of $E3^{Histone}$/LASU1 protein expression by Western Blot with $E3^{Histone}$/LASU1 antibody. * indicates the major degradation product of $E3^{Histone}$/LASU1.

Fig. 4 Immunohistochemistry staining of different mouse tissues using two different $E3^{Histone}$/LASU1 antibodies. Expression of $E3^{Histone}$/LASU1 is identified as the red product of the alkaline phosphatase reaction. The nuclei are identified as blue products of the Vector methyl green reaction. **A-D.** Left, negative controls with preabsorbed antibody; Middle, detection with LASU1-A antibody; right, detection with BL672 antibody. **A.** Expression of $E3^{Histone}$/LASU1 in different tissues of immune system. Upper, $E3^{Histone}$/LASU1 is expressed in cytoplasm of non-lymphocytes in spleen; Middle, $E3^{Histone}$/LASU1 is expressed in cytoplasm of epithelial cells in the medullary region of the thymus; Lower, $E3^{Histone}$/LASU1 is expressed in cytoplasm of some cells in bone. **B.** Expression of $E3^{Histone}$/LASU1 in cytoplasm of renal tubules and hepatocytes in kidney and liver, respectively. **C.** Expression of $E3^{Histone}$/LASU1 in cytoplasm of muscle cells. **D.** Expression of $E3^{Histone}$/LASU1 in brain. Upper, $E3^{Histone}$/LASU1 is expressed in

cytoplasm of Purkinje cells; Middle, $E3^{Histone}$/LASU1 is expressed in nucleus of neuronal cells in cortex.

Fig. 5 Subcellular localization of $E3^{Histone}$/LASU1 in culture cells. **A.** FR3T3 fibroblasts were analyzed by immunofluorescence using LASU1-A antibody. DAPI staining shows nucleus. **B.** Western blot of fractionated cell cytoplasm and nucleus using LASU1-A antibody and H2A antibody as a nuclear marker.

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Fig. 1

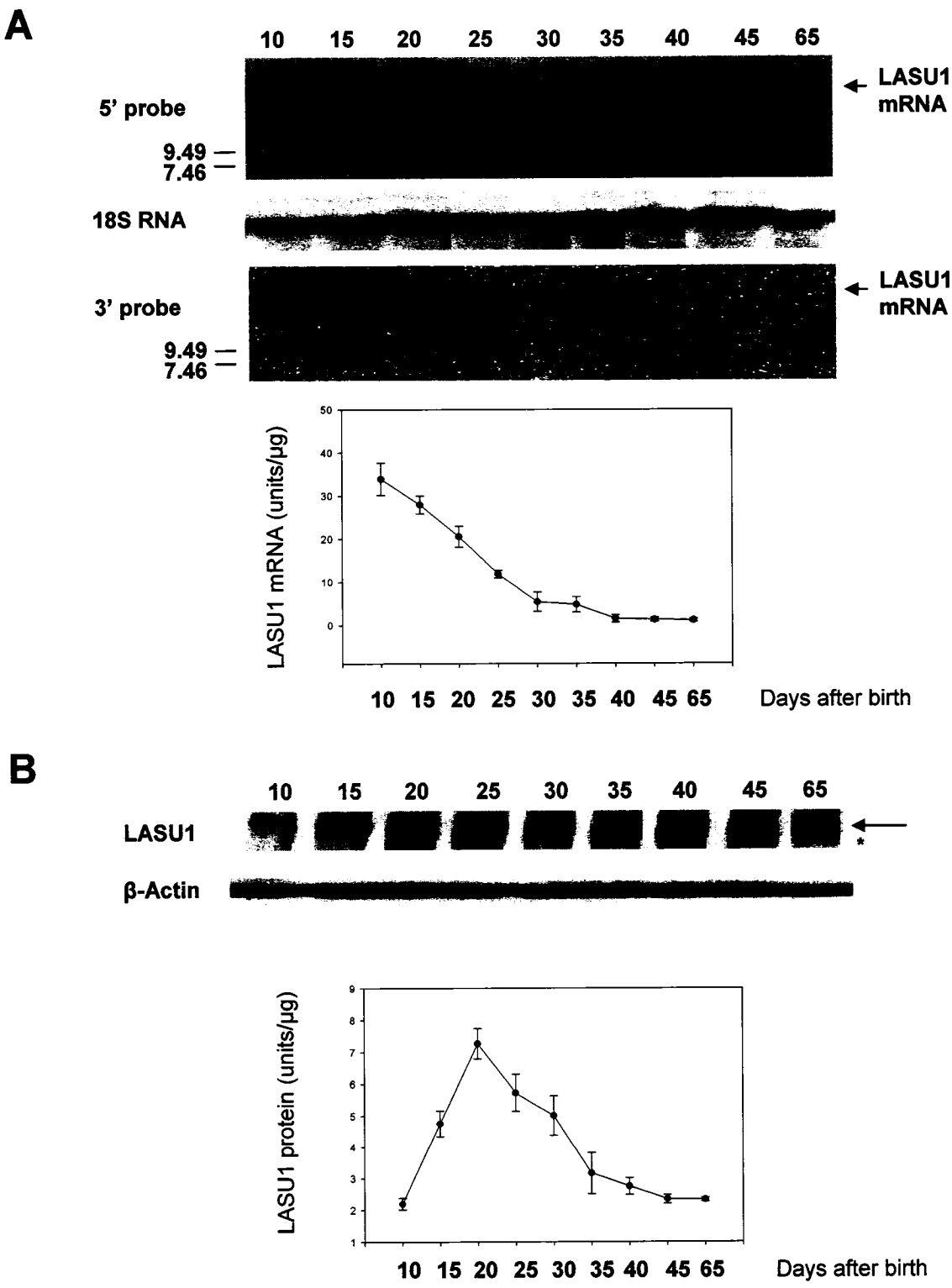
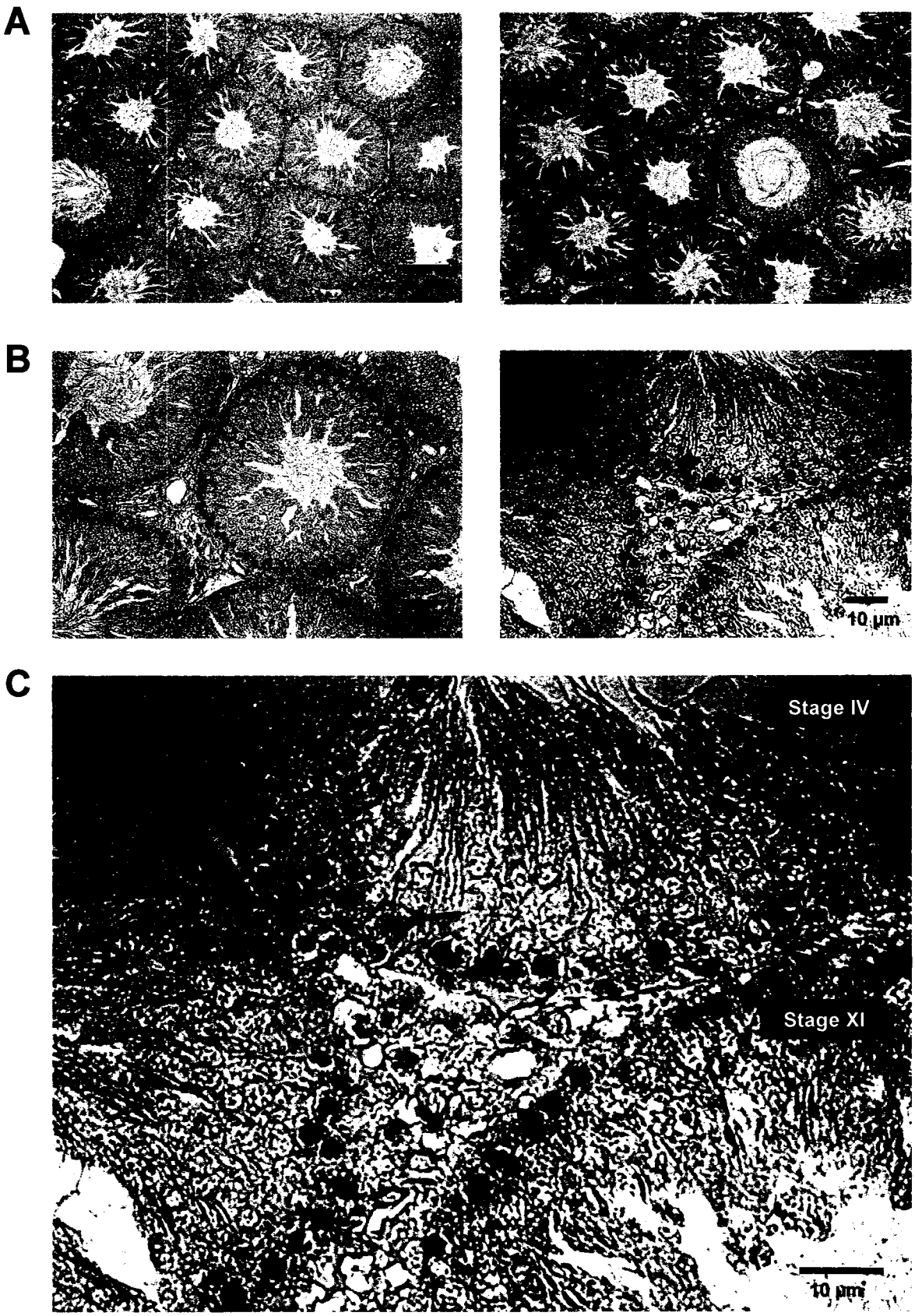
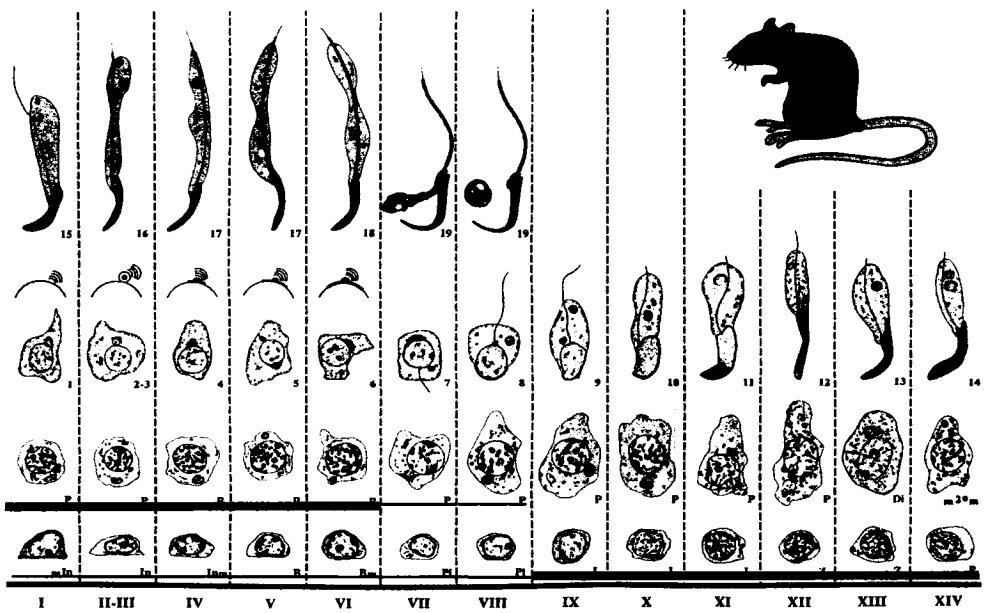

A
10 15 20 25 30 35 40 45 65
5' probe
9.49
7.46
LASU1 mRNA
18S RNA
3' probe
9.49
7.46
LASU1 mRNA
LASU1 mRNA (units/µg)
50
40
30
20
10
0
10 15 20 25 30 35 40 45 65
Days after birth
B
10 15 20 25 30 35 40 45 65
LASU1
*
β-Actin
LASU1 protein (units/µg)
9
8
7
6
5
4
3
2
1
10 15 20 25 30 35 40 45 65
Days after birth

Fig. 2



D

E

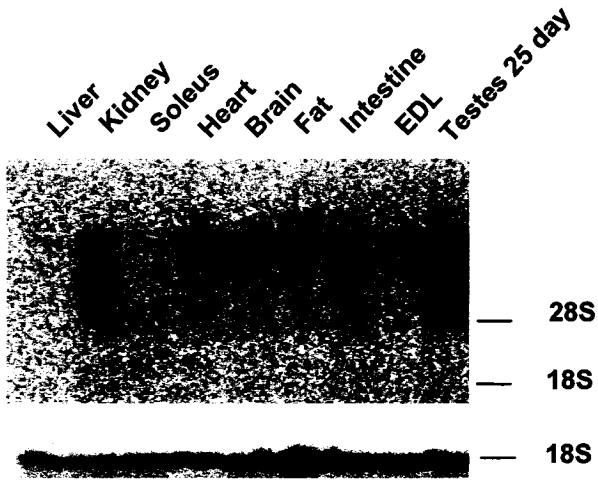


F

Type of germ cells			Intensity of staining
Spermatogonia	A		+/-
	Intermediate		+
	B		+
Spermatocytes	Proleptotene cells		+
	Leptotene cells		++
	Zygotene cells		++
	Pachytene cells	Pre-	++
		Mid-	++/+
		Late-	-
	Diplotene cells		-
Spermatids	Step 1-19		-

Fig. 3

A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Kidney
Soleus
Heart
Brain
Fat
Intestine
EDL
Testes 25 day
28S
18S
18S

B

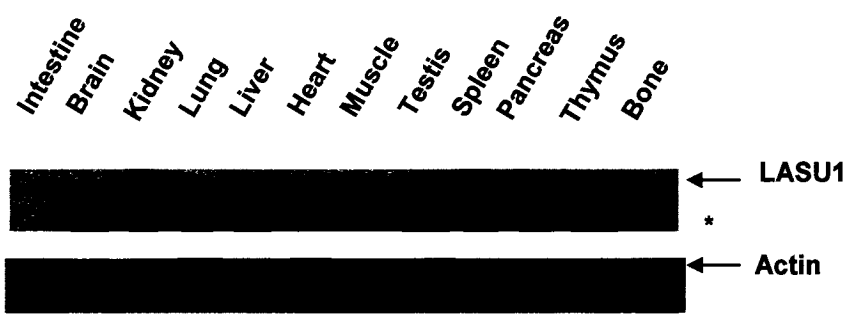
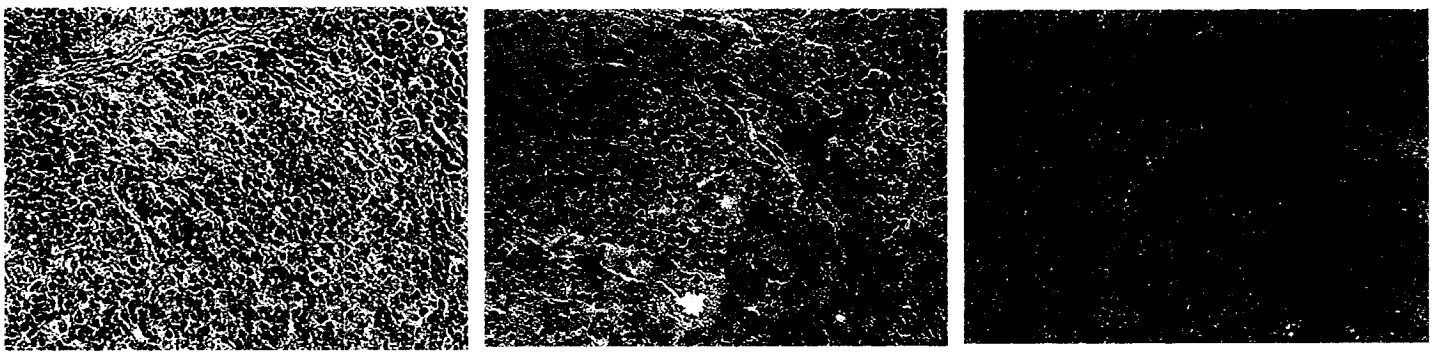

Intestine
Brain
Kidney
Lung
Liver
Heart
Muscle
Testis
Spleen
Pancreas
Thymus
Bone
LASU1
*
Actin

Fig. 4

A

Spleen



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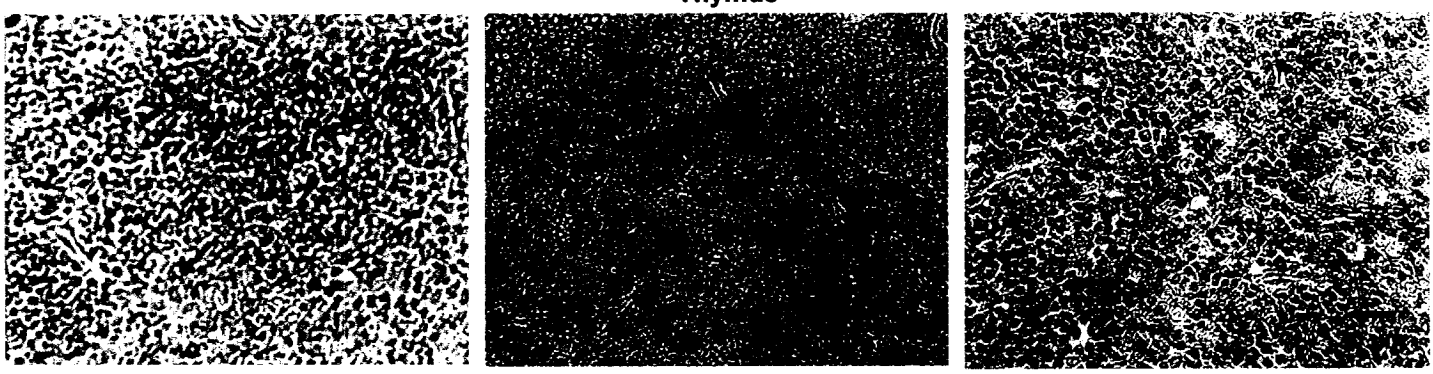
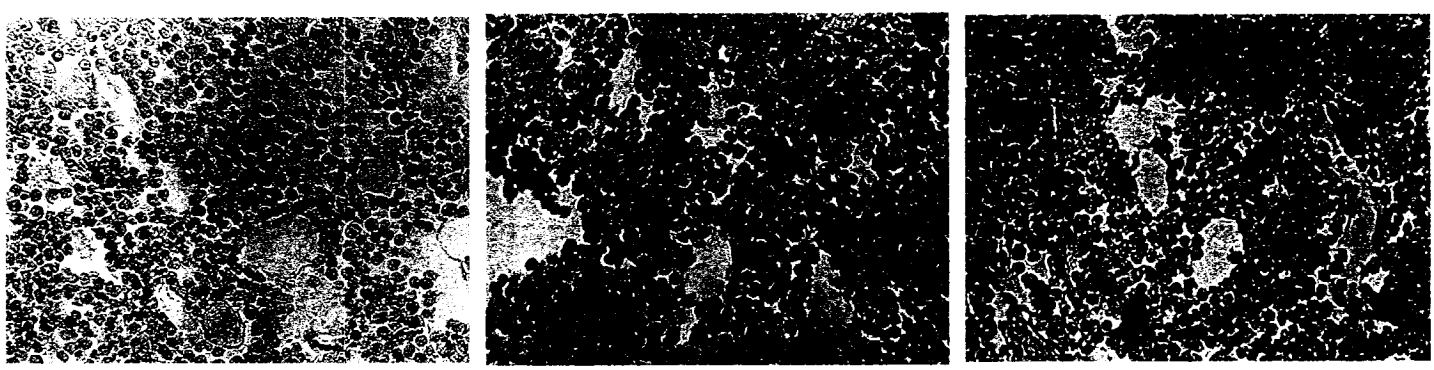
Thymus



CTL- LASU1-A Ab BL672

Bone marrow



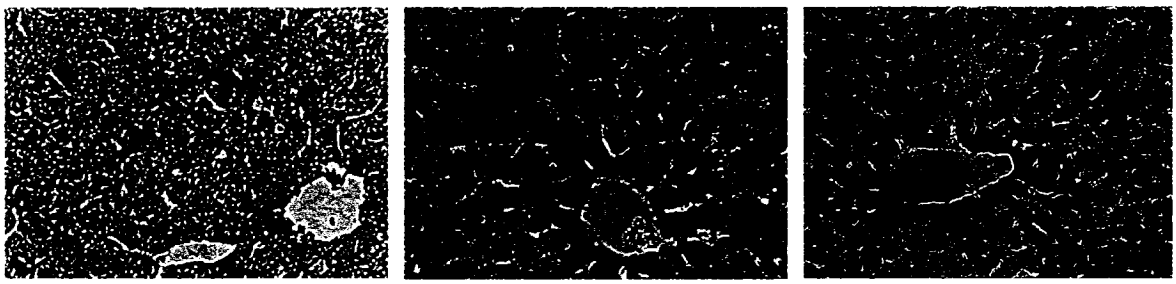
CTL- LASU1-A Ab BL672

B

Kidney

CTL- LASU1-A Ab BL672

Liver



CTL- LASU1-A Ab BL672

C

Skeletal muscle

CTL- LASU1-A Ab BL672

Heart

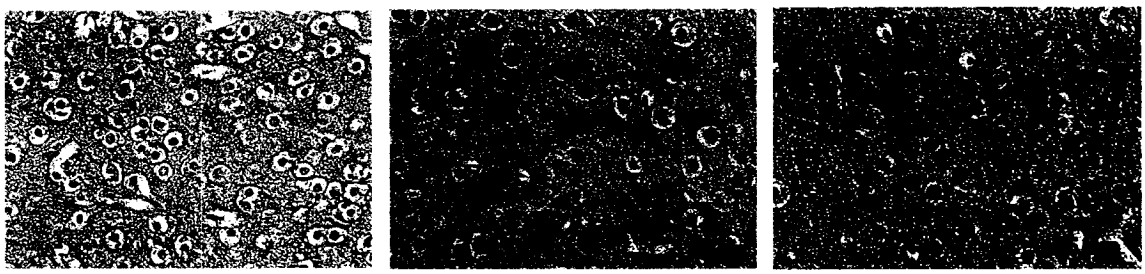
CTL- LASU1-A Ab BL672

D

Cerebellum

CTL- LASU1-A Ab BL672

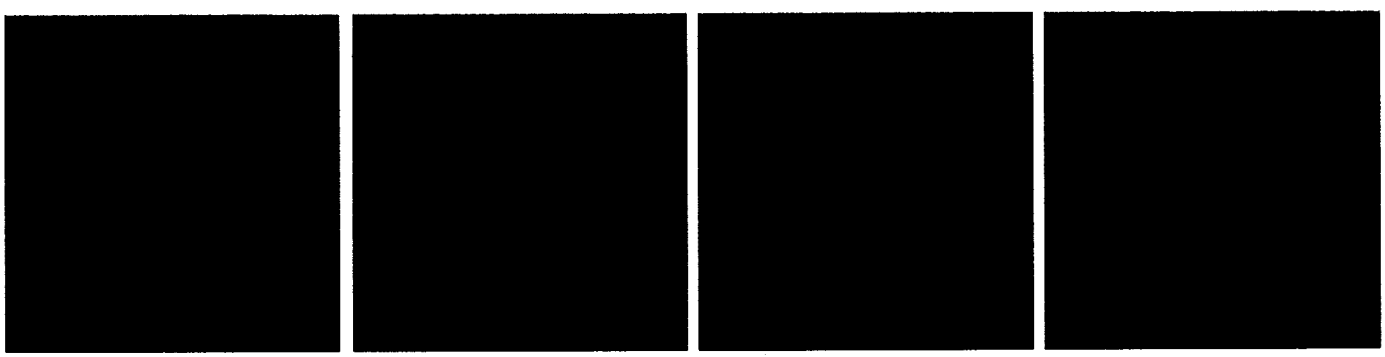
Cortex



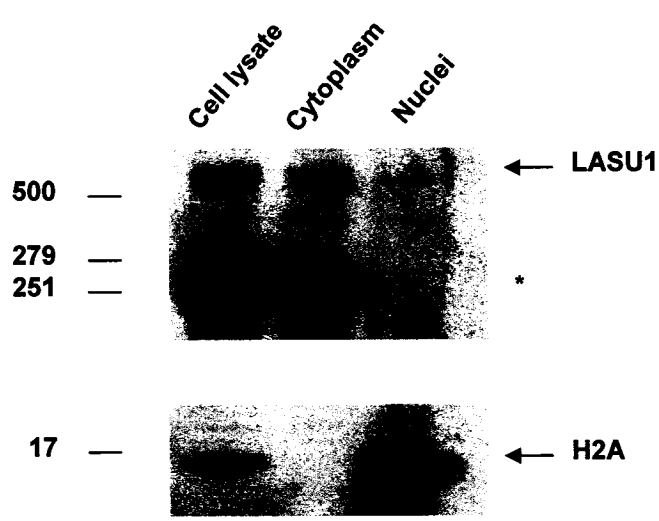
CTL- LASU1-A Ab BL672

Fig. 5

A


CTL-
LASU1-A Ab
DAPI
Merge

B


Cell lysate
Cytoplasm
Nuclei
500
279
251
17
LASU1
*
H2A

Bridge of Chapter 3 & 4

In Chapter 3, we showed that $E3^{Histone}$/LASU1 is widely expressed in various rodent tissues. Previous studies suggest that $E3^{Histone}$/LASU1 may regulate gene transcription. It is known that monoubiquitination of histones can regulate gene transcription. Therefore, in this chapter we tested if $E3^{Histone}$/LASU1 mediates histone ubiquitination in somatic cells. Our collaborator determined that $E3^{Histone}$/LASU1 contains a BH3 domain, a conserved domain originally described in Bcl-2, and that $E3^{Histone}$/LASU1 specifically interacts with the proapoptotic protein Mcl-1 which is degraded by the ubiquitin proteasome pathway. To demonstrate if Mcl-1 is a substrate of $E3^{Histone}$/LASU1, we also tested the ability of purified $E3^{Histone}$/LASU1 to ubiquitinate Mcl-1 *in vitro*.

Chapter 4

Evaluation of the roles of $E3^{Histone}$/LASU1 on the ubiquitination of histones in somatic cells and of Mcl-1 *in vitro*

4.1. Abstract

$E3^{Histone}$/LASU1 ubiquitinates histones *in vitro* and may regulate the ubiquitination and degradation of histones during spermatogenesis (Liu, Oughtred et al. 2005). Since $E3^{Histone}$/LASU1 was found widely expressed in different tissues (Chapter 3), there must be other functions of this E3 other than its role in spermatogenesis. $E3^{Histone}$/LASU1 was previously suggested to affect gene transcription. Since histone monoubiquitination can regulate gene transcription, we tested using siRNA approaches if $E3^{Histone}$/LASU1 ubiquitinates histones in somatic cells. Depletion of $E3^{Histone}$/LASU1 by siRNA did not result in any changes of the levels of free or ubiquitinated histones H2A, H2B, H3 and H4.

Bioinformatics analysis revealed the presence of a BH3 region in $E3^{Histone}$/LASU1 protein sequence. The BH3 region is a conserved region of Bcl-2 that is essential for heterodimerization among Bcl-2 family members. $E3^{Histone}$/LASU1 was shown to specifically interact with Mcl-1, a BH3 containing antiapoptotic protein that is degraded by the ubiquitin proteasome degradation pathway. We therefore tested whether Mcl-1 is a substrate of $E3^{Histone}$/LASU1. Indeed, purified $E3^{Histone}$/LASU1 was able to stimulate polyubiquitination of Mcl-1 *in vitro*. $E3^{Histone}$/LASU1 may therefore regulate cell apoptosis by mediating the degradation of Mcl-1.

4.2. Introduction

Although histone H2A was the first protein ever identified to be ubiquitinated (Goldknopf, Taylor et al. 1975), the precise mechanism and functional significance of histone ubiquitination still remain unclear. Only very recently has the function of monoubiquitination of histone H2B become defined (Emre and Berger 2004). As described in detail in Chapter 1 (1.3. Histone Ubiquitination), monoubiquitination of histone H1, H2A and H2B has been shown to be capable of regulation of gene transcription (Bignell, Warren et al. 2000; Jason, Moore et al. 2002; Emre and Berger 2004; Caron, Boyault et al. 2005; Chen, Kon et al. 2005), and may also be involved in cell cycle (Citterio, Papait et al. 2004) and in gene silencing, meiosis, and specific gene induction during spermatogenesis (Baarends, Hoogerbrugge et al. 1999; Sassone-Corsi 2002; Lewis, Abbott et al. 2003; Sutovsky 2003). Polyubiquitination of histones has also been demonstrated in the testis. The function of histone polyubiquitination remains unknown, but is likely to be important in mediating the loss of histones during spermiogenesis.

Our studies described in Chapter 2 identified a novel ubiquitin protein ligase, $E3^{Histone}$/LASU1, which may mediate histone ubiquitination and degradation during spermatogenesis (Liu, Oughtred et al. 2005). $E3^{Histone}$/LASU1, purified from testis, showed ubiquitin protein ligase activity for different core histones *in vitro* (Liu, Oughtred et al. 2005). Although $E3^{Histone}$/LASU1 was purified from testis, we also observed that this E3 was expressed widely in different tissues (Chapter 3), suggesting that there must be other functions of $E3^{Histone}$/LASU1 besides its possible role in spermatogenesis. Early studies worked on a fragment of $E3^{Histone}$/LASU1, UreB1, which only contains the HECT domain (Gu, Ren et al. 1994; Gu, Dubner et al. 1995; Gu, Irving et al. 1997).

UreB1 has a DNA binding domain and specifically binds to the upstream regulatory element (URE) that contains the consensus initiator sequence in the promoter of the rat prodynorphin gene (Gu, Ren et al. 1994). A gene reporter assay determined that UreB1 suppresses gene expression by binding to URE (Gu, Irving et al. 1997). This result suggests that $E3^{Histone}$/LASU1 might be a transcriptional regulator. In support of this, Tom1, which appears to be an ortholog of $E3^{Histone}$/LASU1 in yeast, also has a role in transcriptional regulation as shown by null mutation studies (Saleh, Collart et al. 1998). In the absence of TOM1p, the normal associations of the transcriptional regulator ADA with the TATA-binding protein (TBP) are reduced. TBPs are DNA binding proteins that are required for gene initiation. The action of TOM1p is likely mediated through ubiquitination since mutation of critical Cys in the HECT domain results in similar changes in transcription as the null mutation. Similarly, $E3^{Histone}$/LASU1 may have a role in gene transcription in mammals. Since monoubiquitination of histones is known to regulate gene transcription (Bignell, Warren et al. 2000; Jason, Moore et al. 2002; Emre and Berger 2004; Caron, Boyault et al. 2005; Chen, Kon et al. 2005), and $E3^{Histone}$ /LASU1 stimulated ubiquitination of core histones *in vitro* (Liu, Oughtred et al. 2005), we reasoned that $E3^{Histone}$ might regulate gene transcription via monoubiquitinating histones in somatic cells. Therefore we inhibited the expression of $E3^{Histone}$/LASU1 protein by using siRNA to test if the levels of free histones and/or the ubiquitination of histones are affected.

Interestingly, a screen of a protein database using a bioinformatics program identified $E3^{Histone}$/LASU1 as a BH3 region containing protein (Warr, Acoca et al. 2005). BH3 region is a conserved region of Bcl-2 that is required for the interaction of some Bcl-2 family members (Shibue and Taniguchi 2006). Furthermore, Shore and his colleagues

determined that $E3^{Histone}$/LASU1 specifically interacts with Mcl-1, but not with Bcl-2 or with Bcl-xl. Mcl-1 is an BH3 containing antiapoptotic protein (Edwards, Derouet et al. 2004; Le Gouill, Podar et al. 2004; Michels, Johnson et al. 2005), which sequesters Bak, a proapoptotic protein, under normal conditions (Willis, Chen et al. 2005). Upon apoptotic stimulus, prosurvival Mcl-1 is targeted for rapid degradation by the ubiquitin proteasome pathway, and this degradation is required to initiate apoptosis (Cuconati, Mukherjee et al. 2003; Nijhawan, Fang et al. 2003). Mcl-1 is therefore a good candidate substrate of $E3^{Histone}$/LASU1.

Thus, in this chapter, we studied if histones are substrates of $E3^{Histone}$/LASU1 in somatic cells and if Mcl-1 is also a substrate of this E3 *in vitro*.

4.3. Methods

siRNA mediated depletion of $E3^{Histone}$/LASU1-Cells used for siRNA transfection were 30-40% confluent. The transfection protocol was according to the manufacturer's instructions (Invitrogen Plus Reagent). The final siRNA concentration was 20 nM. Cells were split the day after the transfection and harvested after another two days. siRNA oligonucleotide C was designed from human $E3^{Histone}$/LASU1 nt 12556-12674 (mouse $E3^{Histone}$/LASU1 nt 12665-12683). siRNA oligonucleotide F was designed to be complementary to human $E3^{Histone}$/LASU1 nt 9003-9021 (mouse $E3^{Histone}$/LASU1 nt 11909-11928 with one mismatch at 11916). Control siRNA is a non-targeting siRNA which is not complementary to any of the known human and mouse genes (Dharmacon).

Preparation of histone enriched protein samples-Cells were trypsinized from the cell culture plate, collected by centrifugation at 500 g, 4°C for 5 min, washed in 10 ml cold PBS buffer and then frozen in liquid nitrogen and stored at -80°C until processed. Cells were lysed by thawing on ice and resuspended in 1 ml cold PBS with protease inhibitor cocktail (Roche). After incubation for 10 min on ice, the nuclei were collected by centrifugation as before. The supernatant was the cytosolic fraction. The pellet was washed once with the same buffer. The pellet containing the nuclei was then resuspended in 1 ml 0.2 M H_2SO_4 and incubated on ice for 30 min to extract the histones. The insoluble debris were removed by centrifugation at 13,000g in a microcentrifuge for 5 min. The supernatant containing the histones was transferred to a new eppendorf tube. Trichloroacetic acid (TCA) was added to 25% of the final solution and incubated on ice for 30 min to precipitate proteins. The pellet containing the precipitated histones was

centrifuged at 13,000g in a microcentrifuge for 20 min. The pellet was washed with 1 ml acetone and incubated on ice for 10 min to eliminate TCA. After centrifugation again 13,000g in a microcentrifuge for 20 min, the pellet containing purified histones was air-dried and resuspended in 20 µl 0.1 M Tris-Cl buffer (pH 7.6). The histone enriched samples were then subjected to western blot and probed with antibodies against different histones (Upstate) and anti-ubiquitin antibody (Sigma), respectively.

In vitro ubiquitination of Mcl-1-The reaction mixture contained the following in a final volume of 20 µl: 50 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol (DTT), 2 mM $MgCl_2$, 2 mM ATP, 0.5 U of pyrophosphatase, 12.5 mM phosphocreatine, 2.5 U of creatine kinase, 50 nM E1, 250 nM UBC4-1, 10 µl of purified $E3^{Histone}$/LASU1 (Liu, Oughtred et al. 2005), 50 µM MG132, 2 µM of ubiquitin aldehyde and 360 nM of purified recombinant Flag-Mcl-1-His_6, with the small C-terminal transmembrane domain deleted. Ubiquitin conjugation reactions were initiated with 23 µM GST-Ub (Boston Biochem), incubated for 1 h at 37°C, and then stopped by 2 mM N-ethylmaleimide (NEM). Mcl-1 was isolated by affinity chromatography by Ni^{2+} beads (Invitrogen), eluted with 350 mM imidazole and resolved by SDS–PAGE, followed by transfer to PVDF membranes. The membranes were then subjected to western blot and probed with anti-Flag antibody (Sigma) to detect Mcl-1 and with anti-GST antibody (Upstate) to detect ubiquitin, respectively.

4.4. Results

Depletion of $E3^{Histone}$/LASU1 resulted in impaired cell growth. To detect the function of $E3^{Histone}$/LASU1 in somatic cells, we depleted $E3^{Histone}$/LASU1 in HEK 293 cells by siRNA (Fig. 1). Compared with transfection without siRNA oligo (Mock) and control siRNA treatment (CTL-), $E3^{Histone}$/LASU1 protein was dramatically decreased with $E3^{Histone}$/LASU1 siRNA treatment (LASU1) (Fig. 1A upper panel). Levels of actin were detected as a loading control. To demonstrate if $E3^{Histone}$/LASU has a role in cell growth, we determined the changes in cell number upon $E3^{Histone}$/LASU1 depletion. When 25 nM $E3^{Histone}$/LASU1 siRNA oligo C was used, the cell number decreased after three days to nearly half of the controls, while treatment with 50 nM of siRNA C resulted in decrease of the cell number to one third of the controls. Similarly, when cells were treated with 50 nM siRNA F, the cell number decreased to half of the controls (Fig. 1B). These dramatic decreases in cell number upon $E3^{Histone}$/LASU1 depletion suggests that $E3^{Histone}$/LASU1 may be required for cell proliferation or may inhibit cell death.

Depletion of $E3^{Histone}$/LASU1 did not result in any changes in the levels of free or ubiquitinated histones. Ubiquitination of histones can regulate gene transcription and cell proliferation. To test if that may be a reason for the decrease in cell number upon depletion of $E3^{Histone}$/LASU1, we measured levels of ubiquitinated histones under the same conditions (Fig. 1C, 1D). Since western blot of the whole cell lysate using anti-histone antibodies yielded only faint signals, we used acid extraction of nuclei to obtain histone enriched fractions which were then subjected to western blot to detect various histones (Fig. 1C) and ubiquitin (Fig. 1D), respectively. In the western blot detecting H2A under three different conditions (Fig. 1C, upper left panel), there were no changes in the levels of free H2A (the strong band of approximately 17 kDa). The bands, just

below 24 kDa, likely represent monoubiquitinated H2A and were not changed either. The upper blurry bands could represent multiubiquitinated H2A or be cross reactive with other proteins. The monoubiquitinated H2A detected by a specific ubiquitinated H2A (uH2A) antibody showed no changes either (Fig. 1A lower panel). Thus, the levels of free and ubiquitinated H2A were not changed upon depletion of $E3^{Histone}$/LASU1. In the western blot detecting H2B (Fig. 1C, upper right panel), there were no changes in the level of the free H2B, the strongest band below 17 kDa. The band a bit higher then the 24 kDa marker would be monoubiquitinated H2B and showed no dramatic changes either. The bands between 17 kDa and 24 kDa and the bands below H2B are likely background bands and did not change either. In the western blot detecting H3 and H4, respectively (Fig. 1C, lower panels), there were no obvious ubiquitinated H3 or ubiquitinated H4 bands detected. The arrows showed free H3 and H4 whose levels were not changed upon depletion of $E3^{Histone}$/LASU1. The faint blurry bands in those two panels are likely background bands. In summary, there were no obvious changes in H2A, H2B, H3 or H4 ubiquitination, neither were there any changes in the levels of free histones, suggesting that $E3^{Histone}$/LASU1 does not mediate histone degradation in HEK 293 cells. To confirm these results, we also probed the same set of samples with anti-ubiquitin antibody (Fig. 1D). The band just below the 24 kDa marker is likely to be monoubiquitinated histone(s), while the band just below 33 kDa marker is likely diubiquitinated histones (Fig. 1D). There are no obvious changes of ubiquitinated histones under three different conditions. The bands between the markers of 17 kDa and 11 kDa are most likely generated from cross reaction with the abundant free histones (Fig. 1D). Taken together, inhibition of $E3^{Histone}$/LASU1 protein expression did not affect the levels of free or ubiquitinated core histones.

$E3^{Histone}$/LASU1 can ubiquitinate Mcl-1. To determine if $E3^{Histone}$/LASU1 is an E3 ubiquitin ligase for Mcl-1, we performed an *in vitro* ubiquitination assay using purified bovine $E3^{Histone}$/LASU1 (Liu, Oughtred et al. 2005) and recombinant Flag-Mcl-1-His_6 lacking the C-terminal transmembrane domain which is a very small fragment. The assay mixture contained E1, E2 (rat Ubc4-1/human UBC H5B), $E3^{Histone}$/LASU1, Mcl-1, GST-ubiquitin, and an ATP regeneration system. Reaction products were isolated by Ni^{2+} chromatography, resolved on SDS-PAGE and subsequently subjected to western blot with anti-Flag antibody to detect Mcl-1(Fig. 2A) and with anti-GST antibody to detect ubiquitin (Fig. 2B). Addition of $E3^{Histone}$/LASU1 resulted in Mcl-1 polyubiquitination (lane 4 of Fig. 2A, 2B), indicating that Mcl-1 is a substrate of $E3^{Histone}$/LASU1 *in vitro*. The detection of some faint higher molecular weight bands in the absence of $E3^{Histone}$/LASU1 (lane 2 of Fig. 2A, 2B) may reflect the ability of Ubc4-1 to ubiquitinate Mcl-1; however, the presence of $E3^{Histone}$/LASU1 dramatically stimulated the polyubiquitination of Mcl-1 (lane 4 of Fig. 2A, 2B). These findings support the role of $E3^{Histone}$/LASU1 as a ligase for Mcl-1.

4.5. Discussion

In this study we demonstrated that $E3^{Histone}$/LASU1 is required for normal cell growth. However, these effects were not associated with any changes in levels of free or ubiquitinated histone H2A, H2B, H3 or H4. Thus, $E3^{Histone}$/LASU1 does not appear to be required for ubiquitination of histones in somatic cells. However, because it is known that ubiquitination of histones is cell cycle dependent (Wu, Kohn et al. 1981), it is possible that $E3^{Histone}$/LASU1 functions on histone ubiquitination at a specific stage of cell cycle, such as M/G1 boundary. Further studies need to be done in synchronized cells to more conclusively determine if $E3^{Histone}$/LASU1 is involved. Plasmids expressing tagged histones may also be employed to amplify the possible effects.

Since proteasome inhibitor inhibits the degradation of Mcl-1 (Cuconati, Mukherjee et al. 2003; Nijhawan, Fang et al. 2003), Mcl-1 is triggered for degradation by the ubiquitin proteasome pathway. Purified $E3^{Histone}$/LASU1 showed ubiquitin protein ligase activity on Mcl-1 *in vitro* (Fig 2). Moreover, Shore and his colleagues determined that the level of Mcl-1 is upregulated upon depletion of $E3^{Histone}$/LASU1 protein in Hela cells, further confirming that $E3^{Histone}$/LASU1 is responsible for degradation of Mcl-1. These results are consistent with those in the recent publication from Wang's group (Zhong, Gao et al. 2005). Interestingly, another group reported that $E3^{Histone}$/LASU1/Mule/ARF-BP1 is also an important regulator of the ARF tumor suppressor (Chen, Kon et al. 2005). In p53 positive cells, $E3^{Histone}$/LASU1/Mule/ARF-BP1 appears to be an E3 ligase for p53 ubiquitination. As is well known, Mcl-1 is an antiapoptotic protein (Edwards, Derouet et al. 2004; Le Gouill, Podar et al. 2004; Michels, Johnson et al. 2005), while p53 has proapoptotic functions (Levine 1997; Brooks and Gu 2006; Pietsch, Humbey et al. 2006). The intriguing question remaining is how this E3 ligase, $E3^{Histone}$/LASU1/Mule/ARF-

BP1, functions by having both proteins with opposite functions as substrates. One possible explanation is that the subcellular localization of the E3 could control its substrate accessibility and E3 activity (Shmueli and Oren 2005). But the results of those two studies are contradictory with respect to the effect of depletion of $E3^{Histone}$/LASU1 on cell response to apoptotic stimuli (Chen, Kon et al. 2005; Zhong, Gao et al. 2005). The biological impact of this E3 is possibly regulated in subtle ways, leading to very different outcomes even under superficially similar conditions (Shmueli and Oren 2005). These two studies used different methods to delete $E3^{Histone}$/LASU1/Mule/ARF-BP1 expression in cells, siRNA (Chen, Kon et al. 2005) versus hnRNA (Zhong, Gao et al. 2005). These two methods may have depleted $E3^{Histone}$/LASU1 to different levels and thereby yielded distinct results. In our study, we actually observed cell number decreasing upon depletion of $E3^{Histone}$/LASU1, consistent with the results from Gu's group (Chen, Kon et al. 2005). Further studies are needed to determine the precise function and mechanism of this E3 on cell apoptosis and proliferation.

Since $E3^{Histone}$/LASU1 has different substrates with different functions, some of which are even opposite to each other, and with the functions of this E3 likely to be cell type specific, it would be interesting to study the regulation of this E3 more precisely under these conditions.

Figure legend:

Fig. 1. Depletion of $E3^{Histone}$/LASU1 leads to decreased cell growth, but no change in histone ubiquitination. A. HEK 293 cells were transfected with control siRNA (CTL-), $E3^{Histone}$/LASU1 siRNA (LASU1) or no siRNA (Mock). Cytosolic fraction (Methods) was subjected to western blot with LASU1-A antibody. Actin was used as a loading control. Histone enriched samples were prepared (as in Methods) and were blotted with anti-uH2A antibody (Upstate). Asterisk shows the major degradation product of $E3^{Histone}$/LASU1. B. Cell number decreased upon $E3^{Histone}$/LASU1 depletion. Shown are results with different concentration of siRNA C or F. Means ± standard deviation are shown (n=3 for each point). C. Histone enriched samples were blotted with anti-H2A, H2B, H3 and H4 antibodies (Upstate), respectively. Arrows indicate different free histones and ubiquitinated histones. D. Histone enriched samples were blotted with anti-ubiquitin antibody (Sigma).

Fig. 2. $E3^{Histone}$/LASU1 ubiquitinates Mcl-1 *in vitro*. E1, E2, and an ATP regenerating system were incubated in the presence or absence of purified bovine $E3^{Histone}$/LASU1, recombinant Flag -Mcl-1-His6, and GST-ubiquitin at 37°C for 1 h. Reaction products were recovered with Ni^{2+} beads. Proteins eluted with 350 mM imidazole were resolved by SDS–PAGE and the products visualized by immunoblotting with (A) anti-Flag antibody, or (B) anti-GST antibody.

Baarends, W. M., J. W. Hoogerbrugge, et al. (1999). "Histone ubiquitination and chromatin remodeling in mouse spermatogenesis." Dev Biol **207**(2): 322-33.
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Fig. 1

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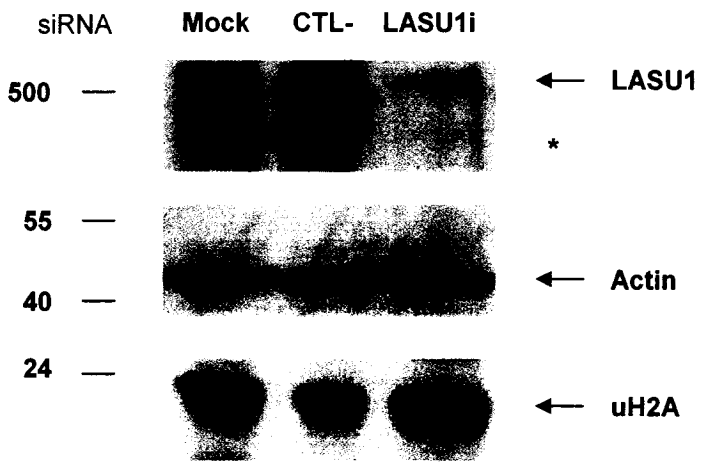
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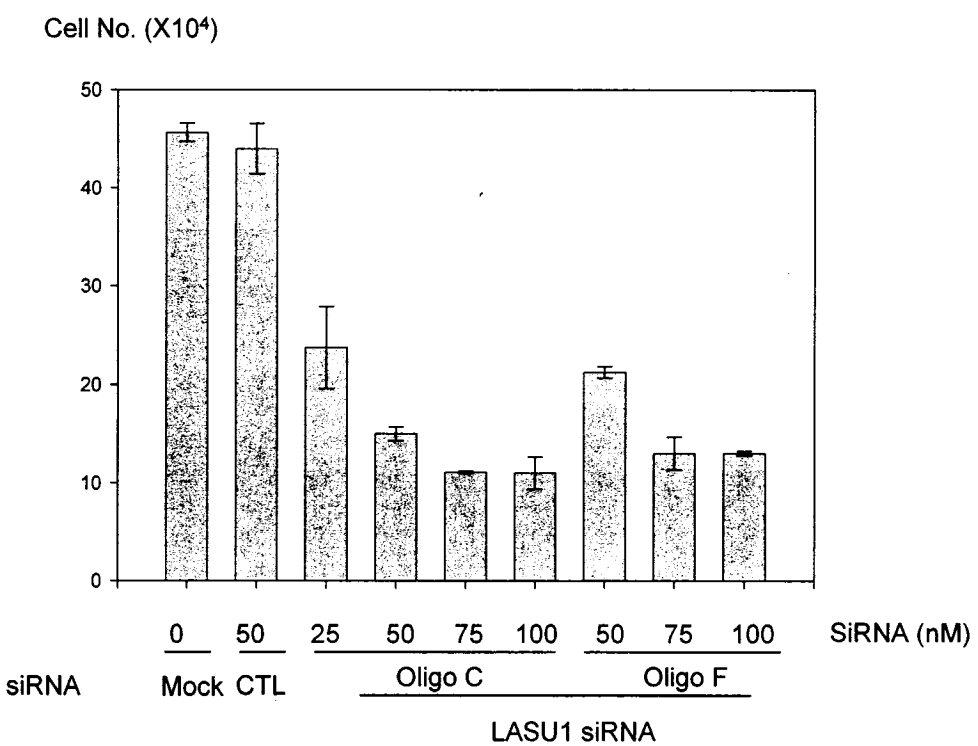
24
LASU1
*
Actin
uH2A

B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20
10
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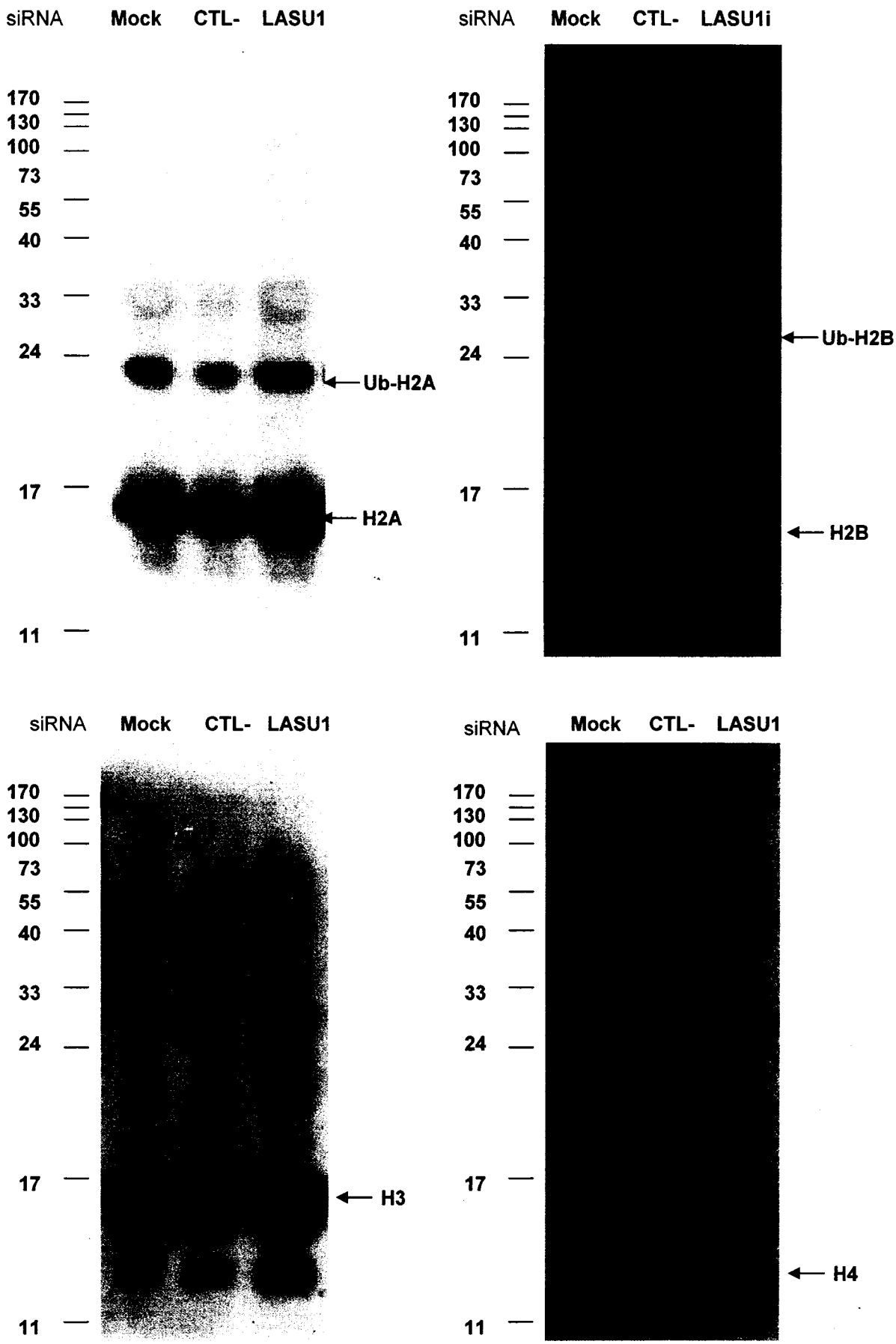
siRNA
Mock
CTL
Oligo C
Oligo F
LASU1 siRNA

C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siRNA
Mock
CTL-
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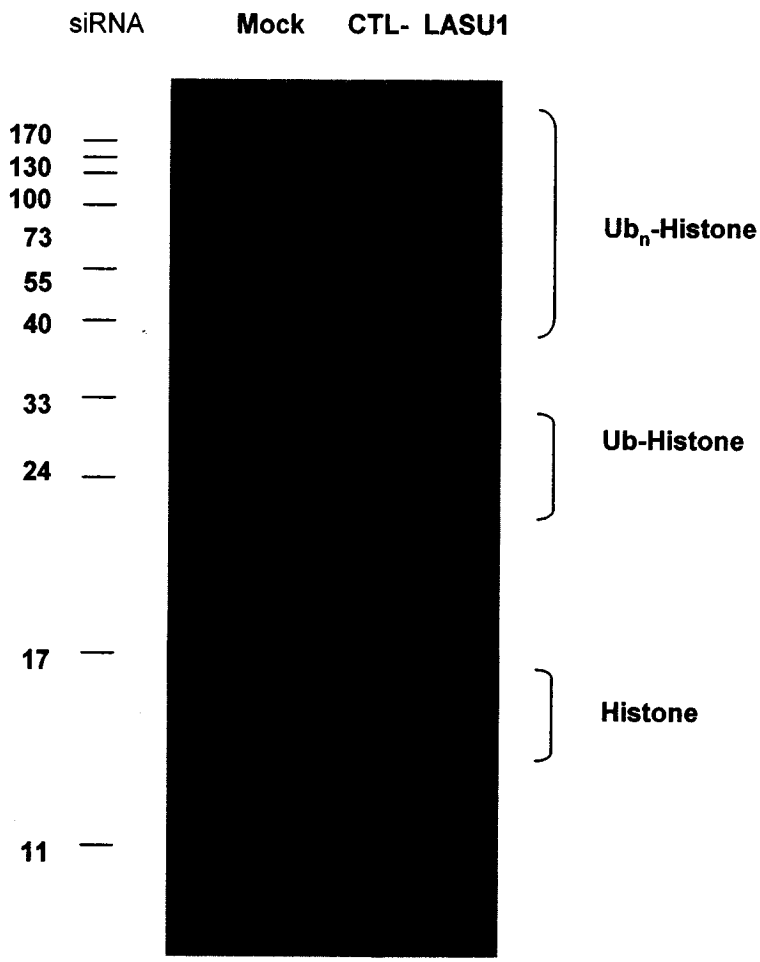
H4

D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Mock
CTL-
LASU1
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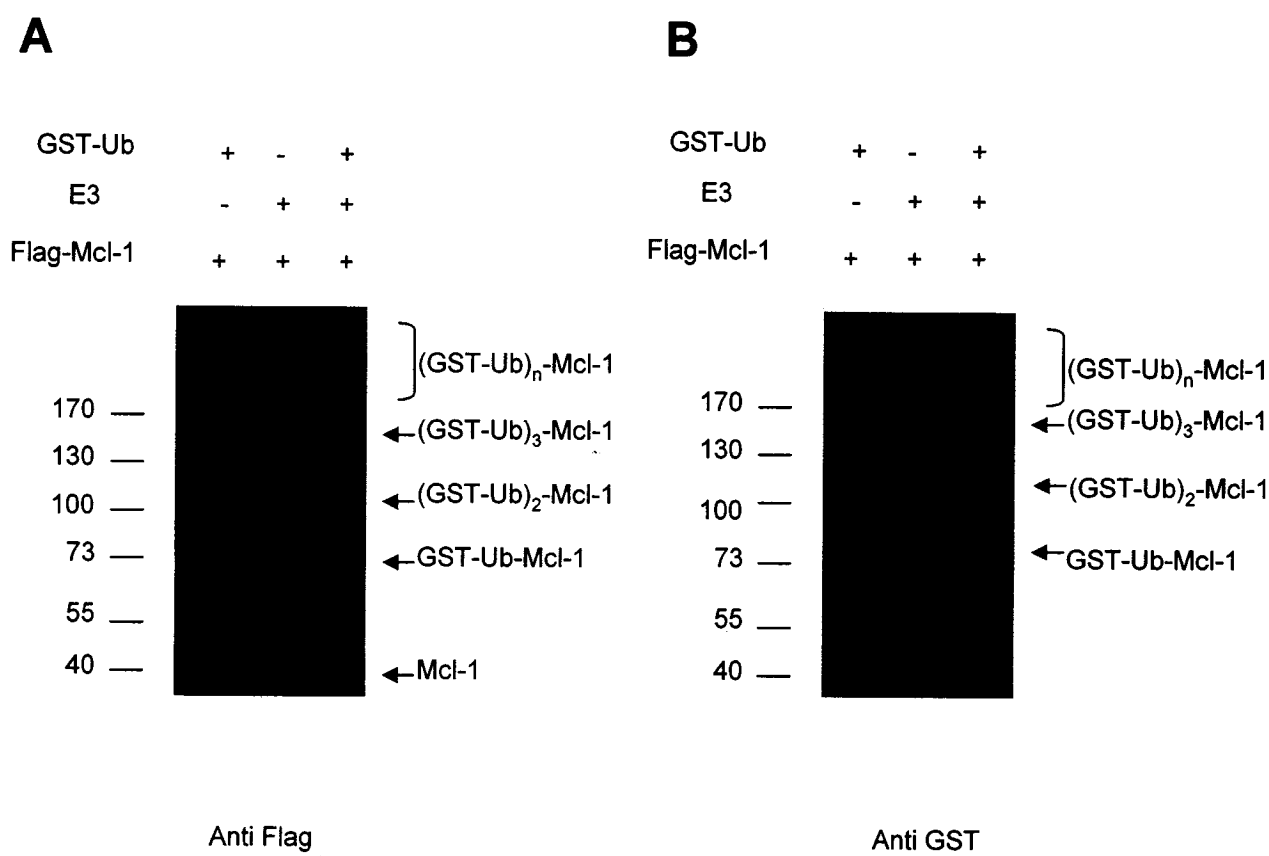
Ub$_n$-Histone
Ub-Histone
Histone
Anti-Ub

Fig. 2


A
GST-Ub + - +
E3 - + +
Flag-Mcl-1 + + +
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130
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(GST-Ub)3-Mcl-1
(GST-Ub)2-Mcl-1
GST-Ub-Mcl-1
Mcl-1
Anti Flag
B
GST-Ub + - +
E3 - + +
Flag-Mcl-1 + + +
170
130
100
73
55
40
(GST-Ub)n-Mcl-1
(GST-Ub)3-Mcl-1
(GST-Ub)2-Mcl-1
GST-Ub-Mcl-1
Anti GST

Chapter 5

General discussion and original contribution

5.1. General discussion

In this thesis, I have identified and characterized a novel ubiquitin protein ligase, $E3^{Histone}$/LASU1. Structurally (Chapter 2), this enzyme is 482 kDa in size and therefore one of the largest HECT domain containing E3 enzymes known. Previous work had identified the enzyme only as incomplete carboxy-terminal fragments (Gu, Ren et al. 1994; Gu, Irving et al. 1997). This work identified the complete sequence showing that it is a composite of what was previously thought to be two genes, LASU1 (gi|22090626) and unnamed protein product (gi|26340188). Like other members of this family, the HECT domain is located at the carboxy terminal end of the molecule. The remaining N-terminal sequences contain a number of sequence motifs, UBA, UIM, WWE, BH3 and at the N-terminal DUF 908 and DUF 913 domains (two domains with unknown function) (Liu, Oughtred et al. 2005; Warr, Acoca et al. 2005; Zhong, Gao et al. 2005). UBA (Hofmann and Bucher 1996) and UIM (Young, Deveraux et al. 1998; Hofmann and Falquet 2001) domains are ubiquitin interacting domains. UIM and UBA domains have been found in many proteins and are able to bind to both monoubiquitin and polyubiquitin chains (Hicke, Schubert et al. 2005). UIMs and UBA domains may play a role in adding multiple ubiquitin moieties onto the substrates of $E3^{Histone}$/LASU1, and may also mediate $E3^{Histone}$/LASU1 autoubiquitination. WWE domain is named after its most conserved residues and is predicted to mediate specific protein-protein interactions in the ubiquitin and ADP-ribose conjugation systems (Aravind 2001). WWE domain may be essential for recruitment of the substrates or regulators. BH3 region is Bcl-2 homology region 3, which has been characterized in many proteins (Huang, Kahana et al. 1997; Lutz 2000). The BH3 region is essential for heterodimerization among Bcl-2 family proteins (Zha, Aime-Sempe et al. 1996; Diaz, Oltersdorf et al. 1997) and appears to promote cell death (Lutz

2000). $E3^{Histone}$/LASU1 recruits Mcl-1 for polyubiquitination via its BH3 region (Warr, Acoca et al. 2005; Zhong, Gao et al. 2005). DUF 908 and DUF 913 are domains with unknown functions. Interestingly, searches of the protein database reveal that all the proteins containing DUF 908 and DUF 913 domains are very large proteins and most of them are HECT domain containing proteins. Therefore it would be interesting to determine the functions of two domains. Currently yeast two hybrid studies with those two domains as baits are ongoing in our laboratory to identify the interacting proteins, which are possible substrates of this E3 or adaptor proteins required for the functions of $E3^{Histone}$/LASU1.

$E3^{Histone}$ appears to be a monomer of LASU1 based on mass spectrometry analysis of the enzyme and size analysis by gel filtration and glycerol gradient centrifugation (Chapter 2). However, we cannot completely rule out the presence of additional small subunit(s), particularly nonstoichiometric ones in subsets of the enzyme molecules. We are currently cloning $E3^{Histone}$/LASU1 into expression vectors. If we are able to express the recombinant protein of LASU1 using baculovirus for example, we will be able to answer this question by testing if LASU1 alone has the E3 ligase activity for histones *in vitro*.

With respect to regulation, this thesis made a number of new observations. Although widely expressed in many tissues, $E3^{Histone}$/LASU1 is preferentially expressed in specific cell types and in epithelia in various tissues (kidney, spleen, thymus (Chapter 3); intestine, lung, breast (data not shown)). We also described for the first time developmental regulation of the enzyme in the testis showing that it is mainly expressed in the nuclei of spermatogonia and early spermatocytes.

Within the cell, its location can also be regulated. In most tissues, $E3^{Histone}$/LASU1 was mainly expressed in the cytoplasm, except in neuronal cells of the brain and in early germ cells of the testis, where it was expressed in the nucleus. Since $E3^{Histone}$/LASU1 has a nuclear localization signal, there may be a binding protein that regulates the presence of $E3^{Histone}$/LASU1 in nucleus or cytoplasm by exposing or hiding the nuclear localization signal of $E3^{Histone}$/LASU1. The localization in the nucleus or the cytoplasm likely determines the function of $E3^{Histone}$/LASU1 by modulating accessibility to the substrates and/or by binding different positive or negative regulators in each compartment. For example, such regulation may determine the ability of $E3^{Histone}$/LASU1 to ubiquitinate cytoplasmic Mcl-1 or nuclear p53.

Functionally, we have identified this enzyme as the major UBC4-dependent histone ubiquitinating ligase in the testis. As discussed above, the high expression of $E3^{Histone}$/LASU1 in nuclei of spermatogonia and primary spermatocytes suggested that this enzyme may be involved in the chromatin modifications that occur during mitosis in spermatogonia and meiosis in spermatocytes. Although LASU1-A antibody did not detect the expression of $E3^{Histone}$/LASU1 in the germ cells in which histones are degraded, $E3^{Histone}$/LASU1 may still be expressed at a low level and may therefore still play a role in the loss of histones during spermiogenesis. These propositions will be further tested using transgenic models. Two different kinds of $E3^{Histone}$/LASU1 knockout mice will be generated with this E3 deleted either specifically in germ cells later than pachytene cells or in all germ cells. Protamine genes are transcribed in step 1 to step 4 spermatids (Steger 1999). Therefore the promoter of protamine genes could be used to express Cre, when the Cre-LoxP system is used to generate testis specific knockout of $E3^{Histone}$/LASU1 to study its function on histone degradation. In addition, a dominant negative $E3^{Histone}$/LASU1

transgenic mouse model bearing a mutation of the active site cysteine to alanine can be generated to confirm that the E3 ligase activity of E3^{Histone}/LASU1 is required. The same set of studies can be done with E3^{Histone}/LASU1 deleted or mutated in all germ cells.

The widespread expression raised the possibility that this enzyme might be involved in general histone ubiquitination and thereby have a role in the histone code. Such a role could have been an explanation for the defect in cell growth seen upon silencing expression of E3^{Histone}/LASU1 (Chapter 4). However, inhibition of E3^{Histone}/LASU1 protein expression in HEK 293 cells showed no changes in the level of histone ubiquitination (Chapter 4). It has been shown that histone ubiquitination is regulated through cell cycle (Wu, Kohn et al. 1981). Since ubiquitinated H2B and ubiquitinated H2A variants are absent from isolated metaphase chromosomes, but they are present in G1 phase, E3^{Histone}/LASU1 may specifically function at M/G1 boundary. It would be interesting to test how depletion of E3^{Histone}/LASU1 in synchronized cells at M/G1 boundary affects ubiquitination of histone H2A and/or H2B. Also, further studies could use plasmids expressing tagged histones to maximize the effects.

Work by other groups has clearly identified important non-histone related functions of E3^{Histone}/LASU1 in the cell. Mcl-1 and p53 have been identified as physiological substrates of the enzyme (Chen, Kon et al. 2005; Warr, Acoca et al. 2005; Zhong, Gao et al. 2005). Those three independent studies have revealed that E3^{Histone}/LASU1 regulates the cell response to stimulation of apoptosis (Chen, Kon et al. 2005; Warr, Acoca et al. 2005; Zhong, Gao et al. 2005). Therefore unregulated E3^{Histone}/LASU1 may result in carcinogenesis. Future studies are needed to determine if there are mutation(s) of the *E3^{Histone}/LASU1* gene in cancers, or if E3^{Histone}/LASU1 activity is altered in cancers,

especially in carcinoma, since this enzyme has been shown to be expressed primarily in epithelia (Chapter 3).

The large size of $E3^{Histone}$/LASU1 indicates that the complete inventory of functions and regulation of this enzyme remain to be determined. Our work has provided molecular tools that will enable the necessary cellular and transgenic studies to arrive at this goal.

5.2. Original contribution

This thesis work has identified and characterized a novel ubiquitin protein ligase, $E3^{Histone}$/LASU1/Mule/ARF-BP1.

- $E3^{Histone}$/LASU1 can ubiquitinate all the core histones *in vitro*;

- UBC4/5 family E2s appeared to be the preferred E2s supporting $E3^{Histone}$/LASU1-dependent histone ubiquitination;

- $E3^{Histone}$/LASU1 appears to be a monomer of the HECT domain protein LASU1, nearly 500 kDa;

- $E3^{Histone}$/LASU1 is the major UBC4 dependent E3 for histone ubiquitination in the testis;

- The mRNA of $E3^{Histone}$/LASU1 is highly expressed at the start of the first wave of spermatogenesis;

- During the first wave of rat spermatogenesis after birth, $E3^{Histone}$/LASU1 protein peaks at day 20 when the most advanced germ cells are late pachytene cells and diplotene cells;

- $E3^{Histone}$/LASU1 protein is highly expressed in nucleus of early primary spermatocytes;

- $E3^{Histone}$/LASU1 is widely expressed in various tissues at both mRNA and protein levels;

- $E3^{Histone}$/LASU1 is mainly expressed in cytoplasm in most tissues, except in early germ cells and neuronal cells where it is mainly expressed in nucleus;

- $E3^{Histone}$/LASU1 is widely expressed in different epithelia;

- $E3^{Histone}$/LASU1 stimulates polyubiquitination of Mcl-1 in vitro;

- Deletion of $E3^{Histone}$/LASU1 protein expression did not change the levels of free histones and ubiquitination of histones in HEK 293 cells.

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5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes, indicate training date.
Josée-France Villemure	Medicine	Research Associate	
Nathalie Bédard	Medicine	Technician	
Marie Plourde	Medicine	Technician	

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

Adenovirus are classified as a risk group 2 agent.
Adenovirus (serotype 5) deleted for E1 and E3 genes (Ad-dE1/-dE3). The replication negative form of this Cre recombinase expressing virus will be grown and purified by the lab personnel of Dr B. Posner, McGill University, who has a biohazards protocol.

ii) the procedures involving biohazards

We plan to use adenovirus as a vector to transfect Cre recombinase into the liver of TSG101 fl/fl mice (6-7 weeks). The Cre recombinase will induce the excision of a part of the TSG101 gene and consequently inactivate the gene. We will evaluate the consequences of inactivation of TSG101 gene on insulin receptor endocytosis and insulin signaling.
Mice will be injected intravenously (tail vein) with recombinants adenovirus [~10E9 plaque forming units (p.f.u.) in saline, total volume of 0.2ml]. Three days post-injection, mice will be starved for 16 hours. The fourth day, mice will be injected intra-peritonealy with glucose (Solution 20% glucose in saline, 2g glucose/kg, max 2ml). At time 0, 15, 30, 60 and 120 minutes post-injection, blood samples will be collected to measure glycemia. Six hours following glucose injection, insulin (0.75U/kg in saline solution, max 2ml) will be injected intra-peritonealy. At time 0, 15, 30, 60 and 120 min post-injection, blood sample will be collected to measure the glycemia. Then mice will be provided with food normally for a minimum of 6 hours and re-starved for 12-16 hours.
The fifth day, mice will be injected intra-veinously (tail vein) with insulin (0.75U/kg in saline solution. max 0.2ml). Fifteen minutes post-injection, mice will be sacrified and liver, spleen and gastroc muscle will be collected and weighted. A part of the liver will be used for histological analyses and the other part will be homogenized and used for biochemical analyses (insulin receptor signaling by in vitro kinase assays and protein characterization by Western blotting techniques).

iii) the protocol for decontaminating spills

Our procedure is established according to the McGill Laboratory Biosafety Manual.
a) Prevention: Intraveinal injection of animals with adenovirus will be conducted in a closed room under a Class II biological safety cabinet. The manipulators will wear appropriate protective equipment (goggles, gloves, mask and gown). Sacrifice, surgery and homogenization of livers will take place in the Class II biological safety cabinet. The infected materials will be transported in sealed centrifuge tubes using secondary sealed containers.

b) Spill Response Procedure: our experiments involve small volumes (less than 50 ml) and thus if spills occur, these will be small. At each step, centrifuge tubes will be opened in the Class II biological safety cabinet. The working area will be covered with bench paper. If any spill occurs, the contaminated area will be covered with a towel soaked with 1% Sodium Hypochlorite. If any clothing is contaminated, it will be removed and autoclaved.

c) Clean-up Procedure:
- 1% Sodium Hypochlorite will be poured around the perimeter of the spill
- Furniture and equipment that have been splashed will be wiped dowm
- Forceps will be used to pick up and transfer contaminated materials into an autoclave bag of container
- Waste and clean-up ustensiles will be autoclaved

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards?

All manipulations involve small volumes of virus (less than 10 ml of concentrated virus). The highest concentration of virus used will be 10E10 p.f.u./ml.

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Yes.

9. What precautions will be taken to reduce production of infectious droplets and aerosols?

a) Every adenovirus preparation will be tested for their content of the Ad5 revertant replicative form using a PCR assay to detect the presence of E1/E3 genes. This procedure is able to detect one particle of replication conpetent virus per 10E6 virus particles [Lochmuller et al., (1994) Human Gene Therapy, 5:1485-1491]. IF revertant are detected such a positive preparation will be destroyed and discarded.

b) Animals injection with adenovirus will be performed in a Class II biological safety cabinet.

c) Animal sacrifice, surgical procedures, homogenization of livers and the opening of centrifuge tubes will be performed in a Class II biological safety cabinet.

10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.

No

11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.

No

12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Strathcona Anatomy Building	W202	Nuaire	NU0425-600	16810TT	Feb. 24th 2004
740 Dr Penfield	2110	Forma	1286	101248-1961	Oct. 14th 2004