Ubiquitin-mediated proteolysis and *Drosophila* embryogenesis

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Thesis presented for the degree of Doctor of Philosophy
Institute of Cell and Molecular Biology
University of Edinburgh
2000

Declaration

I declare that this thesis was composed by myself and the research presented is my own except where otherwise stated.

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2000

Acknowledgements

Thanks are due to many people for helping me out during the past few years, particularly to my supervisor, David Finnegan, for all his advice and support. Thanks also to past and present members of the group, especially Angela Dawson, Eve Hartswood and Lei Zhang. The other I.C.M.B. fly people were a constant source of help, especially Margarete Heck and Petra zur Lage. Strangers from afar responded unfailingly to my requests for reagents - I am particularly grateful to Russ Finley for the yeast two-hybrid materials and to Paul Lasko for Vasa antibodies. Finally, on the lab-assistance front, I would like to thank my three project students, Victor Simossis, Alexis Rideau and Mischa Smolle, for outrageous levels of hard work and enthusiasm.

It was a great bonus to be part of the Wellcome Trust 4-year PhD programme - thanks to all the management team, particularly Dorothy Watson, to my temporary supervisors David Price and Ian Dransfield, and to my fellow guinea pigs P'ng, James, Eleanor and Madeleine.

Members of the international anxious postgrads' guild provided much moral support and, ahem, intellectual discussion - special mention to Sinead, Ruth, Graham, Catherine and Gareth.

Finally, monumental thanks to the renowned Linux/LaTeXguru Dr Graham P. Davies, without whom this thesis would long ago have been lost to a type 137 error.

Abstract

Many cellular processes are regulated by proteolysis, the majority of which, in eukaryotic cells, is carried out by the ubiquitin system. Covalent attachment of a ubiquitin polymer to a target protein leads to degradation of the protein in the proteasome. Ubiquitination provides a means of rapidly and irreversibly eliminating an unwanted protein from the cell, and is therefore a potentially effective tool for regulating cellular behaviour. Ubiquitin-mediated proteolysis is involved in such diverse physiological functions as growth control, cell signalling, differentiation and the immune response. The aim of this research has been to investigate its role in *Drosophila* embryogenesis. Protein ubiquitination is a stepwise process carried out by three classes of enzyme known as E1s, E2s and E3s. The E1 (ubiquitin-activating enzyme), generates a thiolester linkage with a ubiquitin cysteine residue. The activated ubiquitin is then transferred to an E2 (ubiquitin-conjugating enzyme) which, with the help of an E3 (ubiquitin-protein ligase), recruits the substrate protein which is to be degraded. I examined the embryonic expression patterns of several known and novel genes encoding each type of ubiquitinating enzyme. The E2 UbcD4 is transcribed during early to mid-embryogenesis in a variety of tissues, with specific germ cell expression in stage 10 embryos. This suggested a possible role for UbcD4 in germ cell migration towards the somatic gonadal precursors. I screened for UbcD4 - interacting proteins using the yeast two-hybrid system, and identified several putative substrates for, as well as ancillary factors involved in, ubiquitination by UbcD4. These included a novel E3 of the Hect-domain family, which has an expression pattern suggesting a role in epidermal morphogenesis. In an attempt to examine the function of UbcD4 directly, I used RNA interference to disrupt UbcD4 function. The results suggest a post-germband retraction requirement for UbcD4.

Abbreviations

A_x	absorbance at x nm
A	adenine; one letter code for alanine
aa	amino acid
AP	alkaline phosphatase
ATP	adenosine triphosphate
bp	base-pair(s)
BSA	bovine serum albumin
C	one-letter code for cysteine
C-terminus	carboxy-terminus
cDNA	complementary DNA
cfu	colony forming units
Ci	curie
CM	Complete Medium
ds	double-stranded
DIG	digoxygenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	diaminoethanetetraacetic acid
EST	Expressed Sequence Tag
f	femto (10^{-15})
F	Farad
g	gram
g	relative centrifugal force
G	guanine or guanosine; one-letter code for glycine
Gal	galactose
Glu	glucose
GST	glutathione-S-transferase

GTP	guanosine triphosphate
HRP	horseradish peroxidase
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
l	litre
m	milli (10^{-3})
M	molar
mA	milli-ampere
min(s)	minute(s)
mol	mole (6.022045×10^{23} molecules)
MOPS	3-(n-morpholino)propane sulfonic acid
n	nano (10^{-9})
N-terminus	amino-terminus
nt	nucleotide
O.D.$_x$	optical density at x nm
p	plasmid designation; pico (10^{-12})
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pH	-$\log_{10}[H^+]$
RACE	rapid amplification of cDNA ends
Raff	raffinose
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second
ss	single-stranded
T	thymine or thymidine; one-letter code for threonine
TEMED	N,N,N',N'-tetramethylethyledediamine
U	unit; uracil or uridine
UTR	untranslated region
U.V.	ultra violet
V	volts
v/v	volume per volume

W	Watt
w/v	weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-galactopyranoside
Δ	deletion
μ	micro (10^{-6})
Ω	ohm
°C	degree centigrade

Amino Acids

Amino acid	3-letter code	1-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	The	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Introduction

Chapter 1

Introduction

1.1 Ubiquitin-mediated proteolysis - a historical overview

Biologists interested in regulation of cellular behaviour have generally focussed on the synthesis of proteins rather than their degradation. When Monod's group (Hogness *et al.*, 1955) showed that induced β-galactosidase does not turn over in *E. coli*, the existence of protein turnover in any cell was questioned. Then evidence was gathered for selective ATP-dependent destruction of mutant proteins (Goldberg and St. John, 1976). The importance of protein degradation in controlling enzyme levels in eukaryotes was recognized through the work of Schimke in the 1960s and 1970s, (Schimke and Doyle, 1970), but the mechanism remained unknown.

In the late 1970s, Hershko and Ciechanover were studying the selective ATP-dependent degradation of abnormal proteins in reticulocyte lysates. They found that if a lysate was separated into two fractions by DEAE-cellulose chromatography, ATP-dependent proteolytic activity was present only in fraction 1. The active component was found to be a heat stable, low molecular weight protein initially called ATP-dependent proteolysis factor 1 (APF-1) (Ciechanover *et al.*, 1978). APF-1 was later identified as a protein which had been isolated from thymus by Goldstein *et al.* (1975) and was represented in all tissues and organisms studied (Wilkinson *et al.*, 1980). This protein was called ubiquitin. When radio-

labelled ubiquitin was incubated with fraction 2 of reticulocyte lysate, it formed a covalent linkage with high molecular weight proteins (Ciechanover *et al.*, 1980). Ubiquitin had previously been observed to bind to a lysine residue on histone 2A through its C-terminus but the significance of this was not understood (Goldknopf and Busch, 1977). However when Hershko's laboratory found that ubiquitin conjugated to proteins which were good substrates of the proteolytic system, and that several ubiquitin molecules bound to each substrate protein, they proposed that a covalently-attached polyubiquitin chain tags a protein for recognition by a protease (Hershko *et al.*, 1980).

The enzymes which carry out polyubiquitination of a substrate were purified and characterised in the order of their action in the process. E1 was shown to carry out the ATP-dependent activation of the C-terminal glycine residue of ubiquitin before transfering it to a thiol site on E1 (Ciechanover *et al.*, 1981). Two further enzymes were found to be required for ubiquitin-protein ligation. E2 accepts activated protein from E1 and transfers it to a substrate in a reaction requiring the third factor, E3 (Hershko *et al.*, 1983). Ubiquitin-lysozyme conjugates were used as substrates to identify a high molecular weight ATP-dependent protease from rabbit reticulocyte lysates, the 26S proteasome (Hough *et al.*, 1986).

Recognition of the involvement of ubiquitin-mediated proteolysis in specific physiological processes began to emerge in the mid-1980s. A cell-cycle block in a mutant mouse cell line was shown to be due to a defective ubiquitin-activating enzyme (Ciechanover *et al.*, 1984), and a yeast gene essential for DNA repair was found to encode a ubiquitin-conjugating enzyme (Jentsch *et al.*, 1984). However it was not until the discovery that cyclin ubiquitination and degradation was a key event in exit from mitosis (Glotzer *et al.*, 1991) that the ubiquitin-proteasome pathway was widely recognised as an important regulatory mechanism. Other more recent processes which have been shown to be mediated by the pathway include virally-induced tumorigenesis (Scheffner *et al.*, 1990), and activation of

the transcription factor NF-κB (Palombella *et al.*, 1994). It is now clear that selective protein degradation is central to maintaining homeostasis.

In this chapter I describe the components of the ubiquitin-proteasome pathway, namely ubiquitin itself; the enzymes which control its attachment to target proteins; the proteasome; and the elements in the target proteins which confer recognition by the ubiquitination machinery. I then discuss some of the physiological processes controlled by ubiquitin-mediated proteolysis.

1.2 Components of the ubiquitin-proteasome pathway

1.2.1 Ubiquitin

Ubiquitin is the most conserved protein known in eukaryotes, with only three amino acid substitutions, all conservative, between the 76 amino acid yeast and mammalian proteins. Although absent from most prokaryotes, ubiquitin has been found in the cyanobacterium *Anabaena variabilis* (Durner and Boger, 1995) and the archaebacterium *Thermoplasma acidophilum* (Wolf *et al.*, 1993). Ubiquitin has a compact, globular structure with a protruding C-terminus, the site of ubiquitin-protein conjugation (Vijay-Kumar *et al.*, 1987). All ubiquitin genes encode fusion proteins, either a polyubiquitin or a fusion with a small carboxyl extension protein (CEP), such as the ribosomal proteins S27a or L40 (Finley *et al.*, 1989; Redman and Rechsteiner, 1989; Chan *et al.*, 1995). In yeast, polyubiquitin genes are generally stress-inducible whereas ubiquitin-CEP genes provide most of the ubiquitin during vegetative growth (Finley *et al.*, 1987). Synthesis as a ubiquitin fusion has functional significance for the ribosomal proteins, as slow growth and ribosomal defects result when they are expressed independently of ubiquitin (Finley *et al.*, 1989). It is thought that the N-terminal ubiquitin assists folding of the CEP. Both polyubiquitin and ubiquitin-CEP fusions are processed by ubiquitin C-terminal hydrolases to release mature ubiquitin (see section 1.2.3).

1.2.1.1 Polyubiquitin chains

Polyubiquitin chains formed by linking the K48 residue of one ubiquitin with the G76 of the next are the predominant means of tagging a protein for proteasomal degradation (reviewed by Pickart (1999)). There are two possible mechanisms for achieving a polyubiquitin-substrate conjugate : conjugation of a preassembled polyubiquitin chain to a substrate, and monoubiquitination of a substrate followed by extension of the chain. Both appear to operate in cells. E2-14K and E3α use monoubiquitin as the substrate to assemble polyubiquitin chains on N-end rule substrates (see section 1.2.5.1) (Chau *et al.*, 1989). The mammalian E2 E2-25K can assemble K48-linked chains from free ubiquitin *in vitro*. While it is not clear whether this occurs *in vivo*, pools of unanchored polyubiquitin chains do exist in cells (Van Nocker and Vierstra, 1993; Spence *et al.*, 1995; Haldeman *et al.*, 1995), and di- and tri-ubiquitin can be activated by E1 and transferred to E2s, and subsequently to target proteins, as efficiently as monoubiquitin (Van Nocker and Vierstra, 1993; Chen and Pickart, 1990).

It appears that K48-G76 ubiquitin chains have properties which allow them to be efficiently assembled, to persist and to be recognised by the proteasome (see Pickart (1999)). However there are at least four other lysine residues in ubiquitin which can initiate chain assembly. There is some evidence that such alternatively linked chains can also function as tags for protein degradation. Mutation of K63 in yeast leads to failure of the polyubiquitination of a small basic substrate, and to defects in the DNA repair pathway mediated by the E2 Rad6 (Spence *et al.*, 1995). The polyubiquitin binding subunit of the proteasome, S5a, does not appear to discriminate between ubiquitin chains linked through different lysines (Baboshina and Haas, 1996). It is not clear whether the existence of ubiquitin chains harbouring distinct isopeptide linkages lends an additional degree of specificity to the ubiquitination machinery. Histones, receptors and several other proteins are modified by monoubiquitination and remain metabolically stable. In

the case of receptors, the modification appears to trigger their internalisation (see section 1.3.3.2.1). The significance of histone ubiquitination is not clear.

1.2.1.2 Ubiquitin-like proteins

An number of proteins include domains with significant sequence similarity to ubiquitin. Ubiquitin-like (Ubl) domains can be divided into two classes : processed (type 1) and unprocessed (type 2). Type 1 ubiquitin-like domains have a C-terminal diglycine which facilitates processing by ubiquitin C-terminal hydrolases. Such proteins have the potential to be conjugated in a similar manner to ubiquitin. The best-studied member of this class is the mammalian protein Small Ubiquitin-related Modifier 1 (SUMO-1), which, when conjugated to the GTPase activating protein RanGAP1, targets it to the nuclear pore complex (Mahajan *et al.*, 1997; Matunis *et al.*, 1996). The enzymes which carry out sumoylation are related to, but distinct from, the ubiquitinating enzymes (Saitoh *et al.*, 1998). Type 2 Ubl domains lack the C-terminal diglycine needed for processing and are therefore an integral part of the mature protein in which they occur. In the cases of elongin B (Aso *et al.*, 1995) and Rad23 (Watkins *et al.*, 1993), the Ubl domain may act as a chaperone, similar to the role of ubiquitin in synthesis of ribosomal proteins. Several type 2 Ubl proteins can associate with the proteasome and have been implicated in regulation of ubiquitin-mediated degradation. It has been proposed that such proteins may act as a link between the ubiquitination and proteolytic machineries. This topic is discussed further in section 4.3.1.

1.2.2 The thioester cascade

The ligation of ubiquitin to a target protein is carried out in a stepwise fashion by three classes of enzyme (Figure 1.1). First of all the ubiquitin must be 'activated' by a ubiquitin-activating enzyme (E1). This reaction requires ATP hydrolysis and results in a high-energy thioester linkage between the ubiquitin C-terminal glycine and a specific cysteine residue on the E1. The ubiquitin can now be

transferred in a similar fashion to a ubiquitin-conjugating enzyme or E2. The E2 transfers the ubiquitin to the substrate with the help of additional proteins termed E3s or ubiquitin ligases. While E2s are structurally related proteins, encoded by a gene family, E3s differ widely in their structure and in their mode of action. Some accept ubiquitin as a thioester from an E2 and transfer it to a substrate, while others appear to mediate E2 - substrate interactions, and others modulate E2 activity or localisation. It has been proposed that the term E3 should apply only to those enzymes which can form a thioester with ubiquitin, and that other proteins involved in substrate selection should be termed 'ancillary factors' (Scheffner *et al.*, 1999), but this has not achieved general use. The E1 enzyme has a general function and does not discriminate between different E2s. However E2s exhibit specificity both in their co-operation with different subsets of E3s and in their recognition of substrates. E3s in turn are apparently more numerous and more diverse than E2s, and may also be more sensitive to regulation by ancillary factors. Thus the three classes of protein provide the specificity and selectivity required of protein ubiquitination.

1.2.2.1 Ubiquitin-activating enzymes

Ubiquitin activating enzymes are abundant in both cytosol and nucleus. They seem to work as homodimers (Ciechanover *et al.*, 1982) and share a conserved nucleotide binding domain with a consensus GlyXGlyXXGly motif, which is probably the ATP-binding site.

Only one functional E1, Uba1, is known in *Saccharomyces cerevisiae* (McGrath *et al.*, 1991). A similar *Saccharomyces cerevisiae* protein, Uba2, does not appear to bind ubiquitin and may instead be involved in conjugating ubiquitin-like molecules (Dohmen *et al.*, 1995). The human Uba1 homologue encodes two distinct isoforms of E1 by using alternative translational start sites (Handley-Gearhart *et al.*, 1994). Mice have two E1-encoding genes, one of which is expressed specifically in the testis (Kay *et al.*, 1991; Mitchell *et al.*, 1991). Multiple

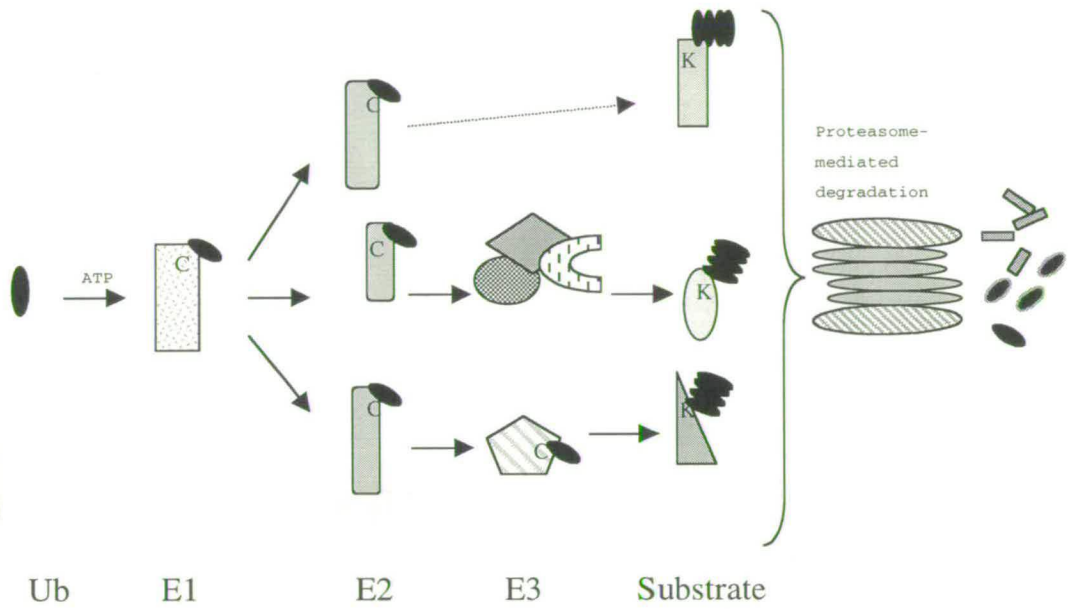


Figure 1.1: Schematic representation of the thioester cascade which results in protein polyubiquitination. Ubiquitin (Ub, black ovals) forms a high-energy thioester intermediate with a cysteine residue on the ubiquitin-activating enzyme (E1), and is transferred to a cysteine residue on one of several related ubiquitin-conjugating enzymes (E2). Finally it forms an isopeptide linkage with a lysine residue on a substrate protein. Ubiquitin transfer from the E2 to the substrate is generally mediated by a ubiquitin ligase (E3). The E3 does not necessarily bind the ubiquitin directly, and may be a single protein or a complex. Some E2s may target their substrate directly. Polyubiquitinated substrates are degraded by the 26S proteasome, yielding peptides and free ubiquitin.

E1 genes are present in wheat (Hatfield and Vierstra, 1992).

Ubiquitin activation is pivotal to ubiquitin-mediated proteolysis, which is in turn required for a wide variety of cellular processes. Both Uba1 and Uba2 are essential genes in *S. cerevisiae.* Mammalian cell lines with a thermolabile E1 display defects in cell-cycle progression and DNA replication (Mori *et al.*, 1993; Kulka *et al.*, 1988; Ciechanover *et al.*, 1984; Finley *et al.*, 1984). The human E1 isoforms display differential localisation in the cell, with the larger form (E1a) present in the nucleus and the smaller form (E1b) in the cytoplasm (Handley-Gearhart *et al.*, 1994). E1a is subject to cell-cycle dependent phosphorylation (Stephen *et al.*, 1996). However there is no clear evidence that protein ubiquitination is regulated at the level of E1 activity.

1.2.2.2 Ubiquitin-conjugating enzymes

E2s share a conserved 160 - amino acid domain called the UBC domain, which includes the 'active site' cysteine residue which forms a thioester with ubiquitin. E2s can be divided into four classes based on their structure. Class I E2s consist of the UBC domain alone, while class II and class III E2s have C-terminal and N-terminal extensions respectively, and class IV E2s have extensions at both termini. E2s share 35-40% sequence identity in the UBC domain, while the N and C-terminal extensions are highly divergent (reviewed by Jentsch *et al.* (1990)). There is some evidence that, for the class II E2s at least, the C-terminal extensions are necessary for function and may mediate substrate specificity or intracelluar localisation (Silver *et al.*, 1992; Kolman *et al.*, 1992). Certain E2s function as homodimers (Gwozd *et al.*, 1995; Pickart and Rose, 1985), or as heterodimers with other E2s (Chen *et al.*, 1993).

There are 12 ubiquitin-conjugating enzymes in *Saccharomyces cerevisiae*, which differ in their properties and intracellular localisation. Mutations in different E2s give distinct phenotypes, indicating their involvement in specific processes (reviewed by Jentsch (1992)). Only Ubc3 is essential for growth under normal

conditions, being required for cell-cycle progression (discussed in section 1.3.1). Ubc1, Ubc4, Ubc5 and Ubc7 are stress - inducible and show some functional redundancy (Jungmann *et al.*, 1993; Seufert and Jentsch, 1990; Seufert *et al.*, 1990) : it is thought that these enzymes are responsible for turnover of abnormal proteins. Ubc2/Rad6 is a DNA repair enzyme (Jentsch *et al.*, 1984). Ubc6 has a C-terminal transmembrane domain and interacts genetically with a component of the protein translocation component of the endoplasmic reticulum (Biederer *et al.*, 1996; Sommer and Jentsch, 1993), suggesting that this E2 may be part of the pathway which degrades integral membrane proteins (see section 1.3.3). Ubc10 is also membrane bound, being found in the peroxisome membrane, and is required for peroxisome biogenesis (Wiebel and Kunau, 1992).

Homologues of most yeast E2s have been found in higher eukaryotes. Yeast strains defective in *UBC4* (Zhen *et al.*, 1993; Treier *et al.*, 1992) and *UBC2/RAD6* (Koken *et al.*, 1991b) can be rescued by expression of homologues from various multicellular organisms. However expansion of the E2 family has taken place in multicellular organisms, as some yeast E2s have multiple homologues in higher organisms (Nuber *et al.*, 1996; Jensen *et al.*, 1995; Koken *et al.*, 1991b). Higher eukaryotes also possess E2s unrelated to those found in yeast. For example, there are no class IV E2s in yeast, whereas the mammalian UbcM1 has a short C-terminal and large N-terminal extension to its UBC domain (Scheffner *et al.*, 1999). E2-230K, which has two active site cysteines (Berleth and Pickart, 1996), similarly has no counterpart in yeast. The complete *Drosophila* genome encodes 39 putative E2s, and both mammals and plants have a greater number.

1.2.2.3 Ubiquitin-protein ligases

In vitro studies on substrate ubiquitination revealed that recognition of many substrates is not mediated by E1s and E2s alone. The term E3 or ubiquitin-protein ligase was coined as a general term for proteins, other than E1s or E2s, which are required for substrate ubiquitination. The first E3 identified was *S.*

cerevisiae Ubr1, the protein responsible for recognising destabilising N-terminal amino acids. The first E3 protein shown to catalyse ubiquitin transfer was the mammalian E6-associated protein (E6-AP). The identification of a conserved C-terminal domain, the Hect domain, found in many proteins which proved to have similar activity, made it possible to identify putative E3s based on their sequence alone. The factor responsible for mediating G1 cyclin ubiquitination in yeast was later found to be a complex of three proteins : Cdc53, Skp1 and the F-box protein Cdc4. Similar SCF complexes, and the related VBC complexes have now been shown to regulate other processes. Most recently, proteins bearing RING finger domains have been found to particiate in many ubiquitination events, and these may represent a fifth type of E3.

1.2.2.3.1 The N-end recognition E3, Ubr1

Artificial proteins which bear different N-terminal amino acids, but which are otherwise identical, have different *in vivo* half-lives. The N-end rule relates the stability of a protein to the identity of its N-terminal residue (reviewed by Varshavsky (1999); see section 1.2.5.1). The protein responsible for recognising proteins with destabilising N-terminal residues (N-degrons) in *S. cerevisiae* is Ubr1. Loss of Ubr1 function stabilises normally short-lived N-end rule substrates (Bartel *et al.*, 1990). Ubr1 has two independent substrate binding sites, one of which interacts with basic N-terminal residues and the other with bulky hydrophobic residues (Baker and Varshavsky, 1991). Strains with null *UBR1* mutations have mild growth defects and are unable to import di- and tri-peptides (Alagramam *et al.*, 1995). Peptides are imported by the product of *PTR2*, transcription of which is repressed by Cup9 (Byrd *et al.*, 1998). Cup9 is a shortlived protein whose degradation requires Ubr1 (Byrd *et al.*, 1998). Degradation of Cup9 is allosterically activated by binding of imported dipeptides, with N-terminal destabilising residues, to Ubr1 (Turner *et al.*, 2000). This results in a increased transcription of

PTR2, creating a positive feedback loop which accelerates peptide uptake. Ubr1 also controls turnover of Gα, part of a complex which regulates cell differentiation in response to mating pheromones (Madura and Varshavsky, 1994). E3α, the mammalian N-end rule E3, has been biochemically characterised in reticulocyte lysate (Hershko and Ciechanover, 1992). However it has not been purified and the gene has not been cloned.

1.2.2.3.2 Hect domain proteins

The only class of E3s with a well-understood ubiquitination mechanism are the Hect-domain proteins, members of which are present in all eukaryotes examined. The prototype of the family, E6-AP, was discovered as a host factor which mediates complex formation between p53 and the human papillomavirus (HPV) oncoprotein E6 (Huibregtse *et al.*, 1991). This event promotes p53 ubiquitination and degradation and contributes to HPV-associated carcinogenesis (Scheffner *et al.*, 1990; Werness *et al.*, 1990). E6-AP can ubiquitinate cellular proteins in the absence of E6 (Scheffner *et al.*, 1993). Like E1 and E2 enzymes, but unlike other classes of E3, E6-AP forms a thioester with ubiquitin (Scheffner *et al.*, 1995). A cysteine residue near the E6-AP C-terminus is crucial both for thioester formation and for p53 ubiquitination. This suggests that ubiquitin is transferred from an E2 to E6-AP, which then ubiquitinates a substrate directly.

The Hect domain is a conserved region of approximately 350 amino acids, homologous to the E6-AP C-terminal, which is found in a number of other proteins. Because the active site cysteine residue, as well as several surrounding residues, is absolutely conserved among Hect domain proteins, they are thought to be a family of ubiquitin ligases (Huibregtse *et al.*, 1995). Several members of the family, including *S. cerevisiae* Rsp5, *S. pombe* Pub-1 and rat p100, have been shown to form a thioester with ubiquitin (Huibregtse *et al.*, 1995). The Hect domain is the minimal region of E6-AP and Rsp5 necessary for thioester formation (Schwarz *et al.*, 1998). As well as the ubiquitin transferase activity, the

Hect domain contains the E2 interaction site of the enzyme. Hect domain E3s interact with a particular subclass of E2s, namely Ubc4/Ubc5 in yeast and their relatives in higher organisms (Nuber *et al.*, 1996; Scheffner *et al.*, 1994; Kumar *et al.*, 1997; Schwarz *et al.*, 1998). The specificity of the interactions between E2s and Hect-domain E3s is discussed in section 4.3.5.3.

Hect-domain proteins range from 90 kDa to over 200 kDa in size and their N-termini are highly divergent. This suggests that the enzymes are modular in structure, with substrate specificity residing in the N-terminus while the conserved C-terminus forms the core catalytic domain. Consistent with this model, the N-terminus of E6-AP directs E6 and p53 binding (Huibregtse *et al.*, 1993). The N-termini of the Nedd-4 family contain several protein-protein interaction motifs called WW domains, which preferentially interact with a small proline-rich sequence called a PY motif (Sudol, 1996). Nedd-4 downregulates the PY-containing epithelial sodium channel (ENaC) in a ubiquitin-dependent fashion(Dinudom *et al.*, 1998; Staub *et al.*, 1996).

Substrates have been identified for relatively few other Hect-domain proteins. The substrates for E6-AP ubiquitination in HPV-negative cells may include the DNA repair protein hHR23a (Kumar *et al.*, 1999), the replication protein MCM-7 (Kuhne and Banks, 1998) and the Src family tyrosine kinase Blk (Oda *et al.*, 1999). Mutations in the human gene encoding E6-AP cause the mental retardation condition Angelman's syndrome (AS) (Kishino *et al.*, 1997; Jiang *et al.*, 1999), but the link between loss of E6-AP function and AS pathogenesis is unclear. *S. cerevisiae* Rsp5 has been implicated in ubiquitin-dependent turnover of several membrane proteins, including the general amino acid permease (Hein *et al.*, 1995), the uracil permease (Galan and Haguenauer-Tsapis, 1997) and the maltose transporter (Lucero and Lagunas, 1997). Rsp5 also targets the large subunit of RNA polymerase II (Huibregtse *et al.*, 1997). *S. pombe* Pub1 targets the mitosis-activating phosphatase Cdc25 for degradation (Nefsky and Beach, 1996)

and is also implicated in the regulation of membrane permeases (Saleki *et al.*, 1997).

1.2.2.3.3 SCF complexes

CDC4 and *CDC53* were identified by mutational analysis as genes required for progression from the G1 to the S phase of the cell cycle. G1 arrest in *cdc4* and *cdc53* mutant strains was found to be caused by failure to degrade the cyclin dependent kinase (CDK) inhibitor Sic1 (Schwob *et al.*, 1994). Similarly, both Cdc53 and Grr1 were shown to be necessary for degradation of G1 cyclins (Deshaies *et al.*, 1995; Barral *et al.*, 1995). Cdc53 and Cdc4 were shown to physically associate with the E2 Cdc34 (Willems *et al.*, 1996; Mathias *et al.*, 1996). Meanwhile, a gene called *SKP1* was identified, various alleles of which caused stabilisation of either Sic1 or G1 cyclins. Further *skp1* alleles can suppress mutations in several genes including *cdc4* and *ctf13-30* (Bai *et al.*, 1996; Zhang *et al.*, 1995; Connelly and Hieter, 1996). The human Skp1 protein, after which the yeast homologue was named, was identified as part of a complex with cyclin F. Alignment of cyclin F with Cdc4 revealed a conserved motif called the F-box, which was also present in Grr1 (Bai *et al.*, 1996). Since degradation of Sic1 and G1 cyclins share the requirement for Cdc34, Cdc53 and Skp1, but need different F-box proteins (Cdc4 or Grr1), it was hypothesised that Cdc53 and Skp1 form the core of a E3 complex which associates with Cdc34 (Bai *et al.*, 1996). The model further proposed that different F-box proteins, which recognise different substrates, can dock onto the complex by binding Skp1, promoting ubiquitin transfer from Cdc34 to the substrate (Skowyra *et al.*, 1997) (Figure 1.2). No components of the SCF complex have been shown to form a covalent linkage with ubiquitin.

There is now substantial evidence in support of the idea that the SCF ubiquitin ligase can be targeted to different substrates via an array of F-box proteins. Further substrates for Cdc4-mediated degradation have been identified, such as the CDK inhibitor Far1 (Henchoz *et al.*, 1997), the kinetochore protein Ctf13

(Kaplan *et al.*, 1997) and the transcription factor Gcn4 (Meimoun *et al.*, 2000). Another F-box protein, Met30, has been shown to associate with Cdc53 and Skp1 (Patton *et al.*, 1998a), and to target the transcriptional activator Met4 (Rouillon *et al.*, 2000). The budding-yeast genome encodes 17 proteins that contain an obvious F-box motif (Patton *et al.*, 1998b). As well as their conserved Skp1-binding F-box, these proteins typically have other protein-protein interaction motifs such as WD40 motifs, which may be the substrate selection sites.

A third 'core' subunit of SCF complexes, Hrt1, has been identified. Hrt1 binds to Cdc53, Cdc34 and Cdc4, but not Skp1 (Seol *et al.*, 1999). Hrt1 contains a RING finger - a series of cysteine and histidine residues arranged to form two zinc co-ordination sites. Hrt1 potently stimulates the E3 activity of recombinant SCF complexes containing either of the F-box proteins Cdc4 or Grr1 (Seol *et al.*, 1999; Kamura *et al.*, 1999; Skowyra *et al.*, 1999). A Cdc53/Hrt1 subcomplex alone can ubiquitinate a substrate, suggesting that these two proteins define the minimal E3 whereas Skp1 is the adaptor, whose interaction with Cdc53 links the F-box protein to the core (Figure 1.2). Cdc53 is stably modified at a single site by covalent attachment of the ubiquitin-like protein Rub1/Nedd-8 (Lammer *et al.*, 1998). Although the significance of this event is not clear, it is necessary for optimal assembly or function of the SCF complex (Lammer *et al.*, 1998). As well as stimulating substrate ubiquitination, Hrt1 appears to activate Rub1 modification of Cdc53 (Kamura *et al.*, 1998).

Metazoan homologues of Cdc53 and Skp1 were initially identified in human cell lines, and shown to complex with the F-box protein Skp2 (Zhang *et al.*, 1995; Yu *et al.*, 1998). It is now clear that multicellular organisms have several Skp1 and Cdc53-related proteins (cullins). Cullins share a highly-conserved region in the Cdc34-binding C-terminus, suggesting that they all participate in ubiquitin-ligase complexes analogous to SCF complexes (Kipreos *et al.*, 1996). This has been demonstrated only for Cul-2 (Figure 1.2). Cul-2 is associated with the Skp1-

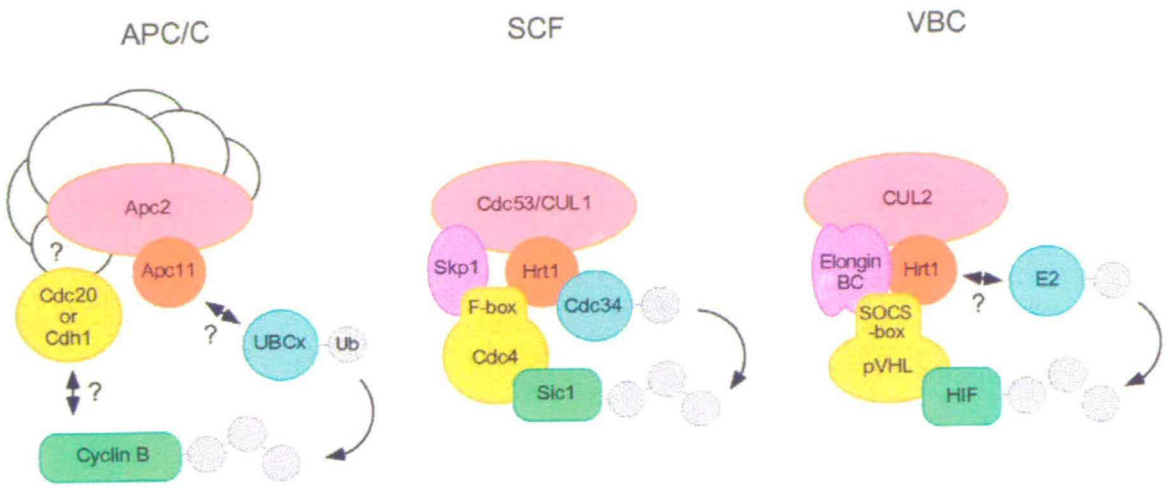


Figure 1.2: Schematic representation of the three types of multisubunit E3s. The APC, SCF and VBC complexes all feature a cullin subunit (red) and a RING-finger subunit (orange). They each use a specialised subunit for substrate recognition (yellow). SCF complexes use various different F-box proteins for this task : Cdc4 recognition of Sic1 is shown as an example. These E3s are thought to mediate ubiquitin transfer from an E2 to a substrate by bringing the two proteins into proximity. Arrows with question marks indicate interactions which are suspected but not yet proven. Taken from Zachariae and Nasmyth (1999).

related protein elongin C. Elongin C in turn binds to the ubiquitin-like protein elongin B (Pause *et al.*, 1999). Whereas Skp1 recruits F-box proteins to the SCF complex, Elongin C binds to a family of proteins sharing a sequence element called the SOCS-box. The SOCS box was identified among suppressors of cytokine signalling, but is now known to contain an elongin C binding site (Kamura *et al.*, 1998). The first SOCS box protein shown to form an E3 complex with Cul-2, elongin B and elongin C was the Von Hippel Lindau tumour suppressor protein (VHL) (Kamura *et al.*, 1998; Iwai *et al.*, 1999; Lisztwan *et al.*, 1999) - hence these E3s are referred to as VBC complexes. Independently of the identification of Hrt1 in yeast, an Hrt1 homologue called Rbx1 was identified as part of the VBC complex (Kamura *et al.*, 1999). The only putative target for VBC - mediated degradation known at present is the hypoxia-inducible factor 1 (HIF1) (Maxwell *et al.*, 1999; Cockman *et al.*, 2000). Other roles for SCF complexes in metazoans are discussed in sections 1.3.1.1 and 1.3.2.

1.2.2.3.4 The anaphase promoting complex

The anaphase promoting complex (APC) was discovered as a high molecular weight complex, which, along with E1 and a specific E2, is sufficient for cyclin degradation in clam extracts (Hershko *et al.*, 1994; King *et al.*, 1995; Sudakin *et al.*, 1995). The complex, initially called the cyclosome, was capable of acting as an E3 only when extracted from mitotic cells. Genetic studies in yeast revealed several mutants, defective in mitotic cyclin degradation, which were also unable to achieve sister chromatid separation (Irniger *et al.*, 1995; Zachariae *et al.*, 1996). Their products were found to be subunits of the APC (Zachariae and Nasmyth, 1996).

Nine vertebrate and twelve yeast APC subunits have so far been identified and the composition of the particle is conserved in eukaryotes. In most cases, their sequences give little indication of function. Some contain motifs also found in other proteins of the ubiquitin-proteasome pathway. Apc1 shares some similarity with the proteasome regulatory subunits Rpn1 and Rpn2 (Zachariae and Nasmyth, 1996), and Apc10 has a Doc domain also found in several Hect domain proteins (Grossberger *et al.*, 1999). More attention has been given to the discovery that the APC shares some architectural similarity with SCF complexes. Apc2 is a distant cullin homologue (Zachariae *et al.*, 1998; Yu *et al.*, 1998), and Apc11 is a RING-finger protein similar to Hrt1/Rbx1 (Ohta *et al.*, 1999). Like Cul1 and Rbx1, Apc2 and Apc11 interact directly (Figure 1.2). Thus it has been proposed that cullin-RING partnerships define a new superfamily of multiprotein ubiquitin ligases, possibly derived from a single ancestral E3 (Zachariae and Nasmyth, 1999).

The APC collaborates specifically with a subclass of E2s represented by Ubch10 in humans, E2-C in clam and UbcP4 in *Schizosaccharomyces pombe*, (Aristarkhov *et al.*, 1996; Yu *et al.*, 1996; Osaka *et al.*, 1997; Townsley *et al.*, 1997), but can collaborate with a number of E2s in *S. cerevisiae* (Zachariae and Nasmyth, 1999).

The ancillary factors Cdc20/Fizzy and Cdh1/Fizzy-related are crucial to regulating the substrate specificity of the APC, but is not clear whether they bind substrates directly. This topic is discussed further in section 1.3.1.

1.2.2.3.5 RING finger proteins

As well as the Hrt1 and Apc11 proteins, which are associated with multisubunit E3s, some RING finger proteins have intrinsic E3 activity. The best-studied of these is the p53 regulator Mdm2.

The rapid turnover of p53 under normal conditions is dependent both on the proteasome and on the ability of Mdm2 to bind p53 (Kubbutat *et al.*, 1997; Haupt *et al.*, 1997). The stabilisation of p53 in response to stress is largely achieved through abrogation of Mdm2 binding, either by phosphorylation of p53 (Unger *et al.*, 1999) or by sequestering Mdm2 (Weber *et al.*, 1999; Tao and Levine, 1999). Mdm2 together with E1 and the E2 UbcH5b is sufficient to ubiquitinate p53 *in vitro*, demonstrating that Mdm2 is an E3 (Fang *et al.*, 2000). Mutation of putative zinc co-ordination residues in the RING finger abolishes E3 activity *in vitro*, and also stabilises p53 in cells (Fang *et al.*, 2000).

The otherwise unrelated RING finger proteins Siah-1, BRCA-1 TRC8 and Praja1 also display the capacity to mediate ubiquitination (Lorick *et al.*, 1999). Many other RING finger proteins, for which E3 activity has not been demonstrated, associate with E2s and/or modulate the degradation of specific substrates. An example is the *Drosophila* protein Seven-in-absentia (Sina), which is involved in photoreceptor specification. Sina binds to both the E2 UbcD1 and the transcription factor Tramtrack (Ttk), causing Ttk to be ubiquitinated and degraded (Li *et al.*, 1997; Tang *et al.*, 1997). Several cellular proteins, including the nuclear body protein PML and the centromeric protein CENP-C, are targeted for degradation following infection by herpes simplex virus type 1. The viral RING finger protein Vmw110 is required for this (Everett *et al.*, 1998, 1999).

RING finger proteins have other cellular functions, such as mediation of

BRCA1 dimerisation (Meza *et al.*, 1999) and binding RNA (Lai *et al.*, 1998). The RING fingers of the proteins implicated in ubiquitination vary considerably in sequence, raising the possibility that, in addition to interacting with and activating E2s, the RING fingers may help to select substrates. Consistent with this, not all proteins which bind Mdm2 are targeted for degradation (Zeng *et al.*, 1999). The tertiary structure of a given RING finger may juxtapose a RING - associated E2 with a particular subset of proteins.

1.2.2.4 The E4 ubiquitination factor

Recently Koegl *et al.* (1999) have described a novel factor which is needed for efficient ubiquitination of a test substrate *in vitro*. E1, E2 and E3 are sufficient for conjugation of a chain consisting of 1-3 ubiquitin moieties, but this does not cause the substrate to be efficiently degraded. Assembly of a longer polyubiquitin chain requires a factor termed Ufd2 or E4. Ufd2 does not interact with the substrate directly but with its ubiquitin conjugates. Ufd2 is essential for viability only under stress conditions. The activity of Ufd2 may be antagonised by Rad23, which inhibits formation of long polyubiquitin chains and degradation of test substrates (Ortolan *et al.*, 2000). Rad23 co-precipitates with a substrate that contains a short multi-Ub chain. These studies suggest that polyubiquitin chain-promoting and chain-inhibiting factors compete to modulate protein stability.

1.2.3 Deubiquitinating enzymes

Polyubiquitination of a protein can be reversed by hydrolysis of the isopeptide bond linking the ubiquitin residues. This process is catalysed by the deubiquitinating enzymes (DUB), which are also required for processing of ubiquitin precursors. Currently there are more deubiquitinating enzymes than ubiquitinating enzymes known, with 17 in the *Saccharomyces cerevisiae* genome. Presumably this reflects a degree of functional divergence and substrate specificity, although this is not well understood. There are two classes of deubiquitinating enzyme,

ubiquitin C-terminal hydrolases (Uchs) and ubiquitin-specific proteases (Ubps).

1.2.3.1 Ubiquitin C-terminal hydrolases

The first DUBs were identified by their ability to deubiquitinate a ubiquitin fusion peptide and were members of the Uch class (Liu *et al.*, 1989; Mayer *et al.*, 1989). These are small enzymes, 40 kDa or smaller, and generally cleave ubiquitin-fusion precursors or ubiquitin with small leaving groups. They may carry out the co-translational processing of ubiquitin precursors and/or regenerate free ubiquitin from nonproductive derivatives, thereby maintaining a free pool of ubiquitin in the cell. The Uchs are unrelated to any other known proteases and share approximately 40 % sequnce identity with each other. There is a single Uch in *S. cerevisiae*, Yuh1, which is non-essential for growth under normal conditions (Liu *et al.*, 1989). This suggests that Ubps may be able to functionally substitute for Uchs. Three mammalian Uchs are known, with different tissue-specific patterns of expression (Wilkinson *et al.*, 1992). The *Drosophila uch-D* is strongly expressed in nurse cells, ovary and testis (Zhang *et al.*, 1993).

1.2.3.2 Ubiquitin-specific proteases

The larger (50-250 kDa) Ubps were cloned using a screen based on cleavage of a ubiquitin-protein fusion (Tobias and Varshavsky, 1991; Baker *et al.*, 1992). These enzymes are highly divergent but share several short consensus sequences which may form the catalytic domains (Baker *et al.*, 1992; Papa and Hochstrasser, 1993). There are 16 putative Ubps in yeast, many of which give no obvious phenotype when mutated (Hochstrasser, 1996; Baker *et al.*, 1992). Some Ubps appear to have a broad role in cleaving proteolytic intermediates which may otherwise inhibit ubiquitin-mediated proteolysis. The *S. cerevisiae* Ubp Doa4 may be responsible for the recycling of free ubiquitin after proteasomal degradation of a substrate, as a *doa4* mutant strain accumulates ubiquitin conjgated to short peptides (Papa and Hochstrasser, 1993) - see section 1.2.4.2.3. The mammalian

Ubp isopeptidase T acts specifically on unanchored polyubiquitin chains of up to four subunits (Hadari *et al.*, 1992; Wilkinson *et al.*, 1995). Mutation of the yeast homologue, Ubp13, causes proteolytic defects and accumulation of unanchored ubiquitin chains (Amerik *et al.*, 1997).

Another potential function of Ubps is the inhibition of ubiquitin-mediated proteolysis of specific substrates by deconjugation of their polyubiquitin chains. This could serve as a proofreading mechanism to prevent the degradation of inappropriately targeted proteins. This hypothesis requires discrimination by the enzymes between different substrates. Although not proven, the large number of different Ubps is consistent with this. There is evidence of substrate - specific inhibition of degradation for the *Drosophila* Ubp Fat facets (Faf). Faf is required maternally for cellularisation of embryos (Fischer-Vize *et al.*, 1992). Zygotic *faf* mutants are viable but have excessive photoreceptors in the eye (Fischer-Vize *et al.*, 1992; Huang *et al.*, 1995). Genetic interaction studies suggest that Faf antagonises proteolysis, rather than enhancing it by maintaining a pool of free ubiquitin (Huang *et al.*, 1995; Wu *et al.*, 1999). A candidate for the Faf substrate is the product of *Liquid facets (Lqf)*, mutations in which enhance the *faf* eye phenotype (Cadavid *et al.*, 2000). Lqf is the *Drosophila* homologue of vertebrate epsin, which is associated with the clathrin-mediated endocytosis complex. Faf and Lqf function in common cells to prevent ectopic photoreceptor specification (Cadavid *et al.*, 2000). Mutations in several other genes required for endocytosis, including those encoding clathrin, dynamin and adaptin, enhance the *faf* and *lqf* phenotypes (Cadavid *et al.*, 2000). These experiments suggest that the endocytosis complex is regulated by ubiquitination: specifically, that deubiquitination activates endocytosis. It is not clear whether Lqf is a direct substrate of Faf, or whether another endocytotic protein is the direct Faf target, and Lqf is simply limiting for endocytosis. If Lqf is deubiquitinated by Faf, this may increase the Lqf level and thereby promote endocytosis. Further evidence that

Ubps can regulate specific processes comes from work on yeast Ubp3, deletion of which enhances silencing of genes located near telomeres and of the silent mating-type locus (Moazed and Johnson, 1996). Ubp binds specifically to Sir4, which is required for gene silencing (Moazed and Johnson, 1996). The involvement of Ubps in growth control in mammals is implied by findings that several oncogenic mutations disrupt Ubp genes such as *tre-2* (Papa and Hochstrasser, 1993).

1.2.4 The proteasome

The 26S proteasome was first detected as a barrel-shaped structure in electron micrographs of erythrocyte extracts, and named cylindrin (Harris, 1968). During the 1980s, several investigators isolated multicatalytic, high molecular weight (700 kDa) proteases from a variety of tissues (Schmid *et al.*, 1984; Ishiura *et al.*, 1985; McGuire and DeMartino, 1986). At the same time, ubiquitin researchers were attempting to identify the protease which degrades ubiquitinated substrates. Hough *et al.* (1986) identified an activity in reticulocyte lysate which degraded ubiquitin-lysozyme conjugates, but not unmodified lysozyme. This ATP-dependent protease was found to be a 26S particle which copurified with a smaller, ATP-independent 20S protease (Hough *et al.*, 1987). The smaller enzyme was identifed as the multicatalytic protease described earlier. The larger complex appeared to consist of the 20S protease with additional subunits. It is now accepted that the 26S proteasome consists of the 20S complex, which is the catalytic core, plus the 19S regulatory complex, which confers the ability to recognise ubiquitin conjugates in an ATP-dependent manner (Figure 1.3). Both forms are present in all eukaryotes studied. A simplified 20S complex is present in many species of archaebacteria and in the actinomycete family of eubacteria.

1.2.4.1 The 20S proteasome

The crystal structure of the yeast 20S proteasome shows a cylindrical 700 kDa complex, consisting of a stack of four rings, each containing seven subunits (Groll

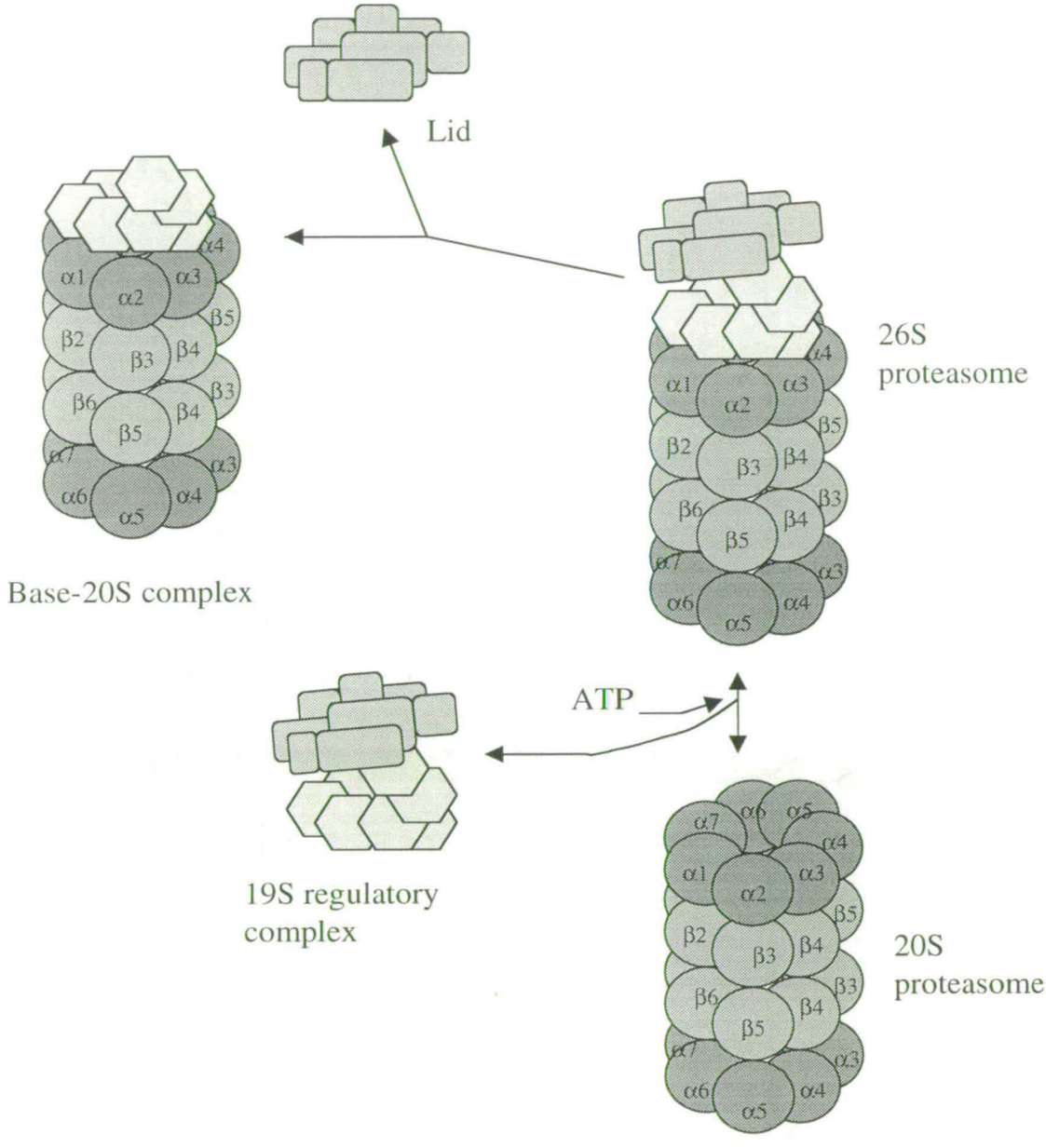


Figure 1.3: Schematic representation of the 26S proteasome and its subcomplexes. The proteolytic active site resides in the 20S proteasome, which consists of four rings of α and β subunits. The 19S regulator can bind to either or both ends of the 20S proteasome, enabling ATP-dependent degradation of polyubiquitinated proteins. The 19S regulator consists of the base and lid subcomplexes. 19S base-20S proteasome complexes have been isolated. The base supports elevated peptidase activity, but the lid appears to be required for recognition of polyubiquitinated substrates. After Ferrell *et al.* (2000).

et al., 1997). Subunits fall into two families, the α and β families, on the basis of sequence similarity and position in the complex. Each of the two outer rings of the yeast 20S proteasome contains α subunits 1-7, and each of the two inner rings contain β subunits 1-7 (Figure 1.3). The four rings encompass three cavities, two 'antechambers' between the α and β rings and a central cavity, containing the proteolytic active site, between the two β rings. The central channel through which proteins pass to reach the proteolytic cavity is 13 Å wide. The entrance to the antechambers is blocked by N-termini of the α subunits. It appears that proteins must be unfolded and actively transported to the inaccessible proteolytic site, preventing their accidental degradation. Upon reaching the active site, proteins are processively cleaved to peptides of between 4 and 25 residues (Wenzel *et al.*, 1994; Dolenc *et al.*, 1998; Nussbaum *et al.*, 1998). The proteasome can cleave a substrate at hydrophobic or aromatic residues, at basic residues and at glutamate. Each of these three activities has been assigned to one of the three catalytically active β subunits (Heinemeyer *et al.*, 1997, 1993, 1991; Enenkel *et al.*, 1994; Dick *et al.*, 1998).

Unlike eukaryotes, the archaebacterium *Thermoplasma acidophilum* has only one type of α and one type of β subunit. When expressed in *E. coli*, α subunits can spontaneously assemble into double seven-membered rings whereas β subunits cannot (Zwickl *et al.*, 1994). Co-expression of α and β subunits yields mature and active proteasomes (Zwickl *et al.*, 1992). The N-terminal propeptide of the β subunits, which prevents its proteolytic activity, is autocatalytically removed during proteasome assembly (Seemuller *et al.*, 1996). Assembly of eukaryotic proteasomes is more complex and not yet understood. It requires additional factors that are only transiently associated with the nascent complex and may act as chaperones (Schmidtke *et al.*, 1997; Ramos *et al.*, 1998). Only one of the α subunits, human C8, has been shown to spontaneously assemble into rings (Gerards *et al.*, 1997). Other α subunits cannot form rings alone but can co-

assemble into rings with C8 (Gerards *et al.*, 1998). An intermediate in proteasome assembly, consisting of all 7 α subunits and 3 β subunits has been detected (Nandi *et al.*, 1997; Schmidtke *et al.*, 1997). Incorporation of the remaining β-type subunits triggers dimerization of these precursor complexes and cleavage of the β subunit N-terminal propeptides. As well as shielding the active site, these propeptides appear to promote correct folding and incorporation of the β subunits.

1.2.4.1.1 The immunoproteasome

During an immune response in vertebrates, interferon-γ (IFN-γ) upregulates transcription of three genes encoding non-constitutive proteasomal β subunits. These proteins, termed LMP2, LMP7 and MECL, can replace the catalytically active β subunits of the 'housekeeping' proteasome to generate the 'immunoproteasome'. This is thought to be a form of the proteasome adapted for production of pathogen-derived peptides, for presentation by class I major histocompatibility complex (MHC) molecules (reviewed by Coux *et al.* (1996); Fruh and Yang (1999)) . Immunoproteasomes are preferentially localised on the cytoplasmic side of the endoplasmic reticulum (ER) (Brooks *et al.*, 2000), and their products are transported into the ER lumen for association with MHC class I molecules. It is not clear how the IFN-γ - inducible subunits alter the spectrum of peptides generated. However the structure of LMP2 suggests that the immunoproteasome may have a decreased propensity to cleave after acidic amino acids (Eleuteri *et al.*, 1997). MHC class I molecules associate poorly with peptides with acidic C-termini. Another pair of IFN-γ-inducible genes, PAα and PAβ, encode subunits of the PA28 proteasome activator (Realini *et al.*, 1994). Association of PA28 with the proteasome enhances degradation of fluorogenic substrates (Coux *et al.*, 1996) but its role in the immune response has yet to be proven.

1.2.4.2 The 19S regulatory complex

Ubiquitin-mediated proteolysis requires the ATP-dependent association of the 20S proteasome with the 19S regulatory complex to form the 26S proteasome. The 19S regulator comprises 17 subunits and is thought to have several distinct activities : recognition of polyubiquitinated substrates; recycling of free ubiquitin from the polyubiquitin chain; opening of the narrow pore into the central channel of the proteasome; unfolding of the target protein and translocation of the unfolded protein into the central channel. The *Saccharomyces cerevisiae* 19S complex can be dissociated into two subcomplexes termed the base and the lid (Glickman *et al.*, 1998a).

1.2.4.2.1 The 19S base subcomplex

The base complex consists of six different members of the AAA-ATPase family (Rpn1-6) and three non-ATPases (Rpn1, Rpn2 and Rpn10) (Glickman *et al.*, 1998b). The AAA-ATPases (ATPases associated with a variety of cellular activities) are a functionally diverse family of proteins which share a conserved 230 amino acid ATPase module (see Patel and Latterich (1998) for review). All six 19S ATPases are essential and mutate to distinct phenotypes (Rubin *et al.*, 1998). The ATPases associate *in vitro* in specific pairs (Richmond *et al.*, 1997), and the three pairs can associate to form a hexamer (Gorbea *et al.*, 2000). The base complex exhibits chaperone-like activity *in vitro* (Braun *et al.*, 1999), suggesting that the *in vivo* role of the ATPases may be to unfold the targeted proteins before translocating them into the 20S core. They may also participate in proteasome assembly. A particle called the 'modulator', which promotes formation of the 26S proteasome from the 20S and 19S complexes has been identified as the Rpt5-Rpt6 ATPase heterodimer plus an additional protein, p27 (DeMartino *et al.*, 1996; Watanabe *et al.*, 1998).

The best-studied of the non-ATPase base subunits is Rpn10 (S5a in mam-

mals). This is the only proteasomal subunit known to bind polyubiquitin (Deveraux *et al.*, 1994). There are two polyubiquitin binding sites, both located towards the C terminus (Young *et al.*, 1998; Fu *et al.*, 1998). However loss of Rpn10 function impairs degradation of only a subset of test proteins (Van Nocker *et al.*, 1996), and truncation studies show that the N-terminus rather than the C-terminus is required for Rpn10 function *in vivo* (Fu *et al.*, 1998). These data suggest that Rpn10 has functions other than polyubiquitin recognition, and that Rpn10 binding to polyubiquitin is not the only means of targeting a substrate to the proteasome. This topic is discussed further in section 4.3.1. The other non-ATPase base subunits, Rpn1 and Rpn2 have KEKE (lysine and glutamate-rich) and LRR (leucine-rich) protein-protein interaction motifs. They may serve to link the base and lid subcomplexes.

1.2.4.2.2 The 19S lid subcomplex

A complex of the 20S core and the base of the 19S complex is sufficient for ATP-dependent degradation of nonubiquitinated peptides but not for degradation of ubiquitinated proteins (Glickman *et al.*, 1998a). Therefore it is assumed that the lid is required for recognition and/or processing of a polyubiquitinated substrate prior to its degradation. Comparatively little is known about the activities of the eight lid subunits. Five of them contain a PCI domain - a sequence motif of unknown function also found in two subunits of the plant photomorphogenic regulator complex COP9 and in two subunits of the translation initiation factor eIF3 (Hofmann and Bucher, 1998). The PCI domain is discussed further in section 4.3.2.

1.2.4.2.3 A proteasomal deubiquitinating enzyme ?

While none of the 19S subunits bear any resemblance to known deubiquitinating enzymes, such an activity is associated with the proteasome. Recycling of free ubiquitin requires that a component of the proteasome can cleave the polyubiq-

uitin chain from its target. Eytan *et al.* (1993) showed that such an isopeptidase activity cofractionates with the 26S proteasome. Lam *et al.* (1997) have described an isopeptidase activity associated with the 19S complex and which disassembles the polyubiquitin chain from its distal end. Potentially this could serve as an 'editing' isopeptidase, which rescues poorly-ubiquitinated proteins from degradation. In neither case was the identity of the deubiquitinatinq enzyme discovered. Recently, the yeast deubiquitinating enzyme Doa4 has been shown to associate with the yeast proteasome (Papa *et al.*, 1999). This association is required for Doa4 function *in vivo*. *doa4* mutant cells are deficient in degradation of a variety of proteins and also accumulate ubiquitin bound to small peptides (Papa *et al.*, 1999), suggesting that Doa4 may release ubiquitin from proteolytic intermediates generated by the proteasome.

1.2.4.3 Subcellular distribution of proteasomes

Proteasomes exist in several forms and in several subcellular compartments. Yeast proteasomes accumulate almost exclusively in the nucleus, possibly reflecting their importance in cell-cycle progression (Russell *et al.*, 1999). In mammalian cells, proteasomes are found both in nucleus and cytosol, with a minor fraction associated with the ER (Palmer *et al.*, 1996; Reits *et al.*, 1997; Brooks *et al.*, 2000). Most cytoplasmic proteasomes are associated with the intermediate filaments (Palmer *et al.*, 1996). There are changes in the subcellular distribution of proteasomes in between different cell types, during cell-cycle progression and during development in lower eukaryotes (reviewed by Rivett (1998). However the functional significance of these observations is unclear.

1.2.5 Recognition signals in substrates

Many proteins have short *in vivo* half-lives, allowing for rapid adjustment of their concentrations through altering the rates of their synthesis. Other proteins are degraded only under certain conditions. Features of a protein which confer

metabolic instability are called degradation signals, or degrons. The first degrons discovered were those which govern the 'N-end rule' of protein stability. Consensus sequences recognised by specific E3s have been identified in a small number of proteins, including cyclins and I-κB. Their susceptibility to ubiquitination can be regulated by phosphorylation. The search for general features of a protein which render it susceptible to ubiquitination is currently under way. There are indications that structural elements rather that primary sequence may be recognised by some ubiquitinating enzymes.

1.2.5.1 The N-degron

The N-end rule states that the half-life of a protein can be related to the identity of its N-terminal residue. Its was first observed in *S. cerevisiae* using artificial β-galactosidase-derived substrates which were identical apart from their N-terminal residues. The E3 responsible for recognising N-end rule substrates (Ubr1/N-recognin in *S. cerevisiae*, or E3α in mammals), has been described in section 1.2.2.3.1. This section describes the degradation signal in more detail.

Ubr1 has at least two substrate binding sites (Varshavsky, 1999). The type 1 site is specific for bulky or hydrophobic amino acids (Phe, Leu, Trp, Tyr and Ile), while the type 2 site is specific for basic amino acids (Arg, Lys, His). These two groups are designated 'primary destabilising' residues. 'Secondary destabilising' residues are those which can be converted into primary destabilising residues under certain conditions : N-terminal Asp and Glu residues can be arginylated by Arg-tRNA protein transferase. Asn and Gln, the 'tertiary destabilising' residues can be deamidated by N-terminal amidohydrolase to yield Asp and Glu. The remaining amino acids are designated 'stabilising' by default, as they are not targeted by components of the N-end pathway. The strength of the interaction between N-recognin and a substrate is strengthened by the presence of an internal lysine residue on the substrate. This lysine, which is the site of ubiquitination, can be considered the second necessary component of the N-degron.

Since nascent proteins are synthesised with an N-terminal Met residue, which is stabilising, the protein must be processed to generate an N-degron. This could potentially be achieved by removal of the N-terminal Met, but the known yeast Met-aminopeptidases cleave only if the second residue is stabilising. However proteins which function in, or pass through, the ER or Golgi may bear N-terminal destabilising residues as a result of signal peptide cleavage. Thus, physiological N-end substrates may include previously compartmentalised proteins which have inappropriately been transported into the cytosol. Other potential substrates are the derivatives of viral polyproteins: the Sindbis alphavirus RNA polymerases has an N-terminal Tyr, replacement of which by a stabilising residue increases its half-life in reticulocyte lysate (de Groot *et al.*, 1991). However degradation of the only known physiological substrates for N-recognin in yeast, Gα and Cup9, does not require their N-termini (Madura *et al.*, 1993; Byrd *et al.*, 1998). Therefore N-recognin must also recognise internal degradation signals, which have not yet been identified.

1.2.5.2 The destruction box

The first specific amino acid sequence shown to confer recognition by the ubiquitination machinery was the 'destruction box', the sequence element required for proteolysis of mitotic cyclins (Glotzer *et al.*, 1991). Mitotic cyclins are targeted by the anaphase promoting complex (see section 1.3.1.2). The consensus destruction box sequence is RXALGXIXN, although A- and B-type cyclins have slightly different sequences. Mutations in the destruction box severely reduce cyclin ubiquitination and degradation (Glotzer *et al.*, 1991). The destruction box of cyclin B can function as a portable recognition element, conferring instability on fusion proteins (Glotzer *et al.*, 1991; Brandeis and Hunt, 1996; Yamano *et al.*, 1996). The cyclin A destruction box can direct ubiquitination but not degradation of a hybrid protein, suggesting that cyclin A must be bound to Cdc2 or Cdk2 in order to be degraded (Klotzbucher *et al.*, 1996). The different properties of the

two types of destruction box may influence the timing of their degradation.

Destruction boxes are also found in some other substrates of the anaphase promoting complex, such as Pds1/Cut2 (Cohen-Fix *et al.*, 1996; Funabiki *et al.*, 1997) and Ase1 (Juang *et al.*, 1997). The E3 activity of the APC is dependent on its association with Cdc20/Fizzy and Cdh1/Fizzy-related (see section 1.3.1.2). Destruction boxes may be recognised by these proteins (see section 1.3.1.2), although this has not been proven.

1.2.5.3 The KEN box

Destruction boxes are present in all known Cdc20-APC substrates but only in a subset of Cdh1-APC substrates. Pfleger and Kirschner (2000) have identified a specific sequence, called a KEN box, required for degradation of Cdh1-APC substrates lacking a destruction box. The consensus sequence is K-E-N-X-X-X-N. Like the cyclin B destruction box, the KEN box works as a transposable motif which can confer instability onto hybrid proteins (Pfleger and Kirschner, 2000).

1.2.5.4 Acidic transcriptional activation domains as degrons

Myc is a short-lived transcription factor (TF) in which the transcriptional activation domain (TAD) overlaps closely with the sequences required for its turnover (Salghetti *et al.*, 1999). Recently this correlation has been noted in several other unstable TFs (Salghetti *et al.*, 2000) The activation domains of VP-16 and E2-F1, but not of other TFs, can confer instability on hybrid proteins (Salghetti *et al.*, 2000). Like Myc, their TADs are rich in acidic residues. With synthetic transcription factors, derived from repeats of the VP-16 TAD, there is an inverse correlation between transcriptional activation potential and ability to direct ubiquitination (Salghetti *et al.*, 2000). TAD-directed protein destruction may be a common mechanism regulating transcription factor stability.

1.2.5.5 The I-κB and β-catenin recognition signals

Activation of the transcription factor NF-κB requires ubiquitin-dependent degradation of its inhibitor, I-κB (see section 1.3.2). I-κB ubiquitination occurs primarily at lysines 21 and 22, and is triggered by phosphorylation of two serine residues at positions 32 and 36. Using peptides spanning the phosphorylation site to inhibit I-κB ubiquitination, Yaron *et al.* (1997) have defined the minimal phosphorylation-dependent E3 recognition element as DSGLDS. This element directs ubiquitination of nearby lysine residues but does not itself include the site of polyubiquitination. The I-κB ubiquitin ligase which binds to this element is the F-box and WD domain protein β-TrCP (Yaron *et al.*, 1998; Spencer *et al.*, 1999; Winston *et al.*, 1999). β-TrCP also mediates ubiquitination of the transcription factor β-catenin through a short, phosphorylation-dependent recognition element similar to that in I-κB (Hart *et al.*, 1999; Winston *et al.*, 1999). The small size of the element recognised by β-TrCP raises the question of how the enzyme achieves sufficient substrate specificity. Perhaps the short phosphorylation-dependent motif is the primary recognition site, and further substrate elements are required to stabilise the interaction.

1.2.5.6 Hydrophobic regions as degrons

In an attempt to find novel degradation signals, Gilon *et al.* (1998) screened random sequences, 16-50 amino acids in length, for their ability to produce unstable fusions with reporter proteins. The stability of the proteins were then assayed in strains with mutations in various ubiquitinating enzymes, to determine which enzymes recognise which degrons. Degradation signals which are stabilised in *ubc6ubc7* mutants display no identifiable consensus sequence but share a high degree of hydrophobicity (Gilon *et al.*, 1998). The yeast transcription factor α2 is ubiquitinated by Ubc6/Ubc7. The α2 degradation signal, Deg1, is a 60-amino acid region predicted to form an amphipathic helix (Chen *et al.*, 1993; Johnson

et al., 1998). Point mutations which inactivate Deg1 cluster on the hydrophobic surface of the predicted helix (Johnson *et al.*, 1998). Taken together, these studies suggest that the Ubc6/Ubc7 ubiquitination complex recognises structural features of its substrates, specifically hydrophobic regions, rather than a primary consensus sequence. Since hydrophobic regions may be normally buried within the core of a protein, or at a protein-protein interface, this may serve to target misfolded proteins or proteins which have failed to associate with their normal partners.

1.3 Physiological processes regulated by ubiquitin-mediated proteolysis

The ubiquitin-proteasome system provides a rapid, specific and highly regulated means of changing the protein composition of a cell, and hence of regulating cellular behaviour. The research presented in this thesis investigates possible roles for the ubiquitin system in regulating development of a model multicellular organism, *Drosophila melanogaster*. While the list of known substrates for the ubiquitin system has expanded rapidly in recent years, there is still little specific information available on the role of the system in mediating developmental decisions. Instead this review focusses on three relatively well-studied groups of substrates: cell-cycle regulators, transcription factors, and membrane proteins. It highlights the importance of controlled protein turnover in controlling the relevant physiological processes, and the diseases which can result from dysregulation of ubiquitination.

1.3.1 Proteolysis as a means of regulating the cell cycle

Phosphorylation and proteolysis are the two interdependent processes which regulate the growth and division of eukaryotic cells. The cell cycle is controlled chiefly by cyclin-dependent kinases (CDKs), whose activity is dependent on forming a complex with a particular cyclin. Waves of synthesis and destruction of stage-

specific cyclins regulate CDK activity. CDK inhibitors prevent improper transition between different stages of the cycle. Ubiquitin-mediated proteolysis is crucial to rapid and timely destruction of both cyclins and CDK inhibitors at the transitions between the various phases (Figure 1.4). The phosphorylation state of cell-cycle proteins can decide their ability to be ubiquitinated. The two E3s which dominate cell cycle control are the SCF complexes and the anaphase-promoting complex (APC), previously described in section 1.2.2.3. SCF complexes catalyse ubiquitination of CDK inhibitors and G1 cyclins, as well as transcription factors and other proteins throughout the cell cycle. The APC is active from late metaphase until the next G1 phase, during which time it targets anaphase inhibitors, mitotic cyclins, spindle proteins and its own activators.

1.3.1.1 SCF complexes and cell-cycle control

Entry into S phase in yeast requires destruction of G1 cyclins (Cln1-3) and of the CDK inhibitor Sic1. As described in section 1.2.2.3.3, these proteins are targeted by the E2 Ubc3/Cdc34, and Cdc4-containing SCF complex. Recognition by the F-box protein Cdc4 is dependent on substrate phosphorylation by the CDK Cdc28, complexed to G1 cyclins (Feldman *et al.*, 1997; Skowyra *et al.*, 1997). Phosphorylated G1 cyclins themselves are targeted for ubiquitination by the F-box protein Grr1 (Patton *et al.*, 1998b). Proteolysis of the G1 cyclins Cln1 and Cln2 requires the presence of S phase cyclins, providing a mechanism for coupling synthesis of one class with the disappearance of the other (Blondel and Mann, 1996). SCF complexes also act at the G2-M transition, targeting the mitotic inhibitor Swe1 through the F-box protein Met30 (Kaiser *et al.*, 1998). Several F-box proteins are themselves turned over in an SCF-dependent manner (Zhou and Howley, 1998; Galan and Peter, 1999; Mathias *et al.*, 1999).

It is likely that the regulatory pathway described here have been conserved and elaborated in metazoans. In mammalian cells, the Cul-1/Skp1/Skp2 SCF complex regulates the abundance of cyclin D (Yu *et al.*, 1998), and Cul-3 regulates

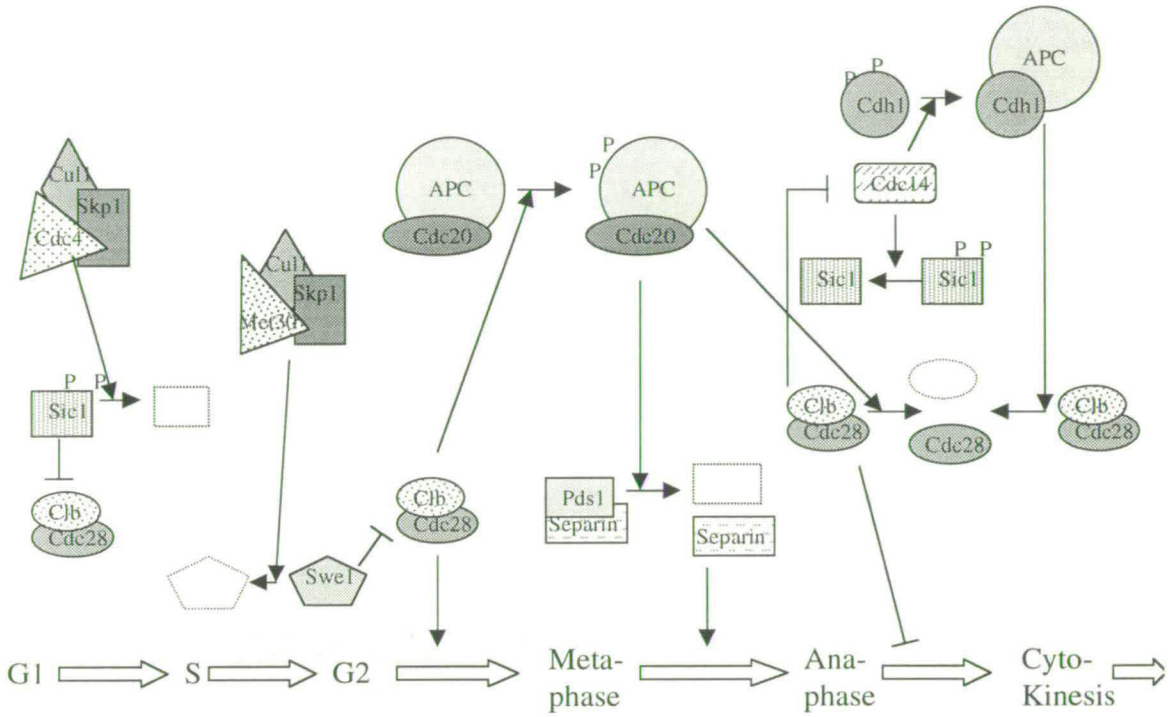


Figure 1.4: Schematic representation of the regulation of cell-cycle progression by ubiquitin-mediated proteolysis. Arrows indicate activation, a line at right angles indicates inhibition, dotted outlines indicate proteolysis and a P indicates phosphorylation. Sic1 inhibits the CDK activity of the Clb-Cdc28 complex: phosphorylation-dependent Sic1 proteolysis during G1, mediated by a Cdc4-containing SCF complex, enables progression into S phase. Similarly, G2-M progression requires proteolysis of the Swe1 CDK inhibitor : this event is mediated by a Met30-containing SCF complex. Clb-Cdc28 phosphorylates, and activates, the APC-Cdc20 complex, which then degrades the anaphase inhibitor Pds1. Pds1 proteolysis enables the separin protein to cause sister chromatid separation. APC-Cdc20 also degrades mitotic cyclins, inactivating Cdc28. This activates the Cdc14 phosphatase, which dephosphorylates Cdh1, allowing it to associate with the APC. Cdc14 also dephosphorylates Sic1, causing it to accumulate. APC-Cdh1 maintains the degradation of mitotic cyclins until after completion of cytokinesis.

the abundance of cyclin E (Singer *et al.*, 1999). Skp2 is also important in turnover of the transcription factor E2F-1, which drives the expression of several genes needed for entry into S phase. Targeting of E2F-1 may be limited by availablility of Skp2, which peaks just after entry into S phase (Marti *et al.*, 1999). Other important growth control proteins targeted by Skp2 include $p21^{CIP1/WAF1}$ (Yu *et al.*, 1998) and p27 (Sutterluty *et al.*, 1999).

1.3.1.2 Regulation of mitosis by the anaphase-promoting complex (APC)

The APC is needed for two distinct events in mitosis - execution of anaphase, and mitotic exit. Its activity is positively regulated by stage-specific association with two conserved accessory factors, Cdc20 and Cdh1 (known as Fizzy and Fizzy-related in *Drosophila*)(Schwab *et al.*, 1997; Visintin *et al.*, 1997; Fang *et al.*, 1998). Cdc20 and Cdh1 contain WD-40 repeats, which are also found in F-box proteins. It is not known whether they bind directly to APC substrates.

Cdc20 is expressed during G2 and binds the APC during late metaphase. The ability of the APC to be activated by Cdc20 depends on the phosphorylation of core subunits by polo-like kinases (Kotani *et al.*, 1998). APC-Cdc20 substrates include mitotic cyclins (Clb) and the anaphase inhibitor Pds1/securin (Visintin *et al.*, 1997). Pds1 sequesters the 'separin' protein Eps1 in an inactive complex. Destruction of Pds1 allows Eps1 to cleave the 'cohesin' protein Scc1 which in turn allows sister chromatids to separate (Ciosk *et al.*, 1998; Uhlmann *et al.*, 1999). Cdc20 protein levels decline rapidly as cells exit mitosis and enter G1. This is thought to be due to protein degradation targeted by APC-Cdh1 (Fang *et al.*, 1998; Prinz *et al.*, 1998).

Unlike Cdc20, Cdh1 is expressed throughout the cell cycle but CDK-dependent phosphorylation prevents it from binding the APC before the end of anaphase, when it is dephosphorylated by Cdc14 (Zachariae *et al.*, 1998; Visintin *et al.*, 1998). Other mechanisms help to control the relative timing of APC-Cdc20 and APC-Cdh1 activity, such as the inhibition of APC-Cdh1 activity by the APC-

Cdc20 substrates Pds1 and Clb5 (Shirayama *et al.*, 1998) and the degradation of Cdc20 by APC-Cdh1. APC-Cdh1 targets the spindle protein Ase1 (Juang *et al.*, 1997) and also maintains the degradation of Clbs intiated by APC-Cdc20 (Schwab *et al.*, 1997; Visintin *et al.*, 1997).

The formation and activity of the APC is influenced by the cAMP/protein kinase A (PKA) pathway (Yamashita *et al.*, 1996; Yanagida, 1998). Mutations in the APC component Cut4 cause hypersensitivity to cAMP and to stress-inducing heavy metals. Negative regulation of APC activity by cAMP/PKA may integrate cell cycle control with the stress response, during which proteasomal availability to ubiquitinated proteins may become limiting. Inhibition of APC may therefore suppress entry into anaphase until normal conditions are restored.

1.3.1.3 Ubiquitination and tumorigenesis

In mammalian cells, ubiquitin-mediated proteolysis regulates the activity of many additional growth control molecules, including the proto-oncoproteins c-Jun (Treier *et al.*, 1994), c-Fos (Stancovski *et al.*, 1995) and c-Mos (Ishida *et al.*, 1993). Growth control proteins can be transformed into oncoproteins by alterations which abrogate their ubiquitination. The C-terminal truncated version of c-Myb, found in murine myeloid leukaemias is less efficiently ubiquitinated, and more stable, than the full-length protein (Bies and Wolff, 1997). A tyrosine-aspartic acid substitution in the c-Kit receptor tyrosine kinase is often observed in mouse mastocytoma cell lines. The mutation upregulates Kit signalling by altering its substrate specificity, leading to the phosphorylation of an unknown intermediate protein and to the ubiquitin-dependent degradation of the protein tyrosine phosphatase Shp-1, which is a negative regulator of Kit signalling (Piao *et al.*, 1996). Other sections in this review deal with abrogation of β-catenin ubiquitination (section 1.3.2.3), and with human papillomavirus-mediated p53 degradation (section 1.2.2.3.2), which also contribute towards cancer.

1.3.2 Turnover of transcription factors by the ubiquitin system

Recently, a novel case has been described in which the ubiquitination of a transcription factor directly changes its activity. The yeast transcriptional activator Met4 is ubiquitinated by an SCF complex but remains stable: ubiquitinated Met4 can bind to promoters but cannot form functional transcription complexes, leading to repression of its target genes (Kaiser *et al.*, 2000). However ubiquitin-mediated transcriptional regulation generally involves degradation of transcription factors.

The cellular concentration of many transcriptional regulators can undergo rapid changes - this requires that the protein be short-lived. Most known cases of regulated transcription factor degradation involve the ubiquitin system (reviewed by Hochstrasser and Kornitzer (1999)). Previous sections have described the targeting of p53 by the RING finger protein Mdm2 (section 1.2.2.3.5), and of the yeast transcription factor Gcn4 by an SCF complex (section 1.2.2.3.3). This section describes the activation of NF-κB, which requires the activity of an SCF E3 both to process the inactive NF-κB precursor and to degrade an inhibitor. At least two other transcription factors, which are the terminal components of two different signalling pathways, are targeted by the same F-box protein, β-TrCP (Figure 1.5).

1.3.2.1 Regulation of NF-κB activation

NF-κB is a dimeric transcription factor belonging to the Rel family, and is involved in regulation of the immune response, development and differentiation, transformation and apoptosis. NF-κB activity is induced by a variety of stimuli including cytokines, oxidants and bacterial and viral peptides. Activation of NF-κB involves the ubiquitin-proteasome pathway at two levels : processing of the inactive precursor, and release of the mature transcription factor from a complex

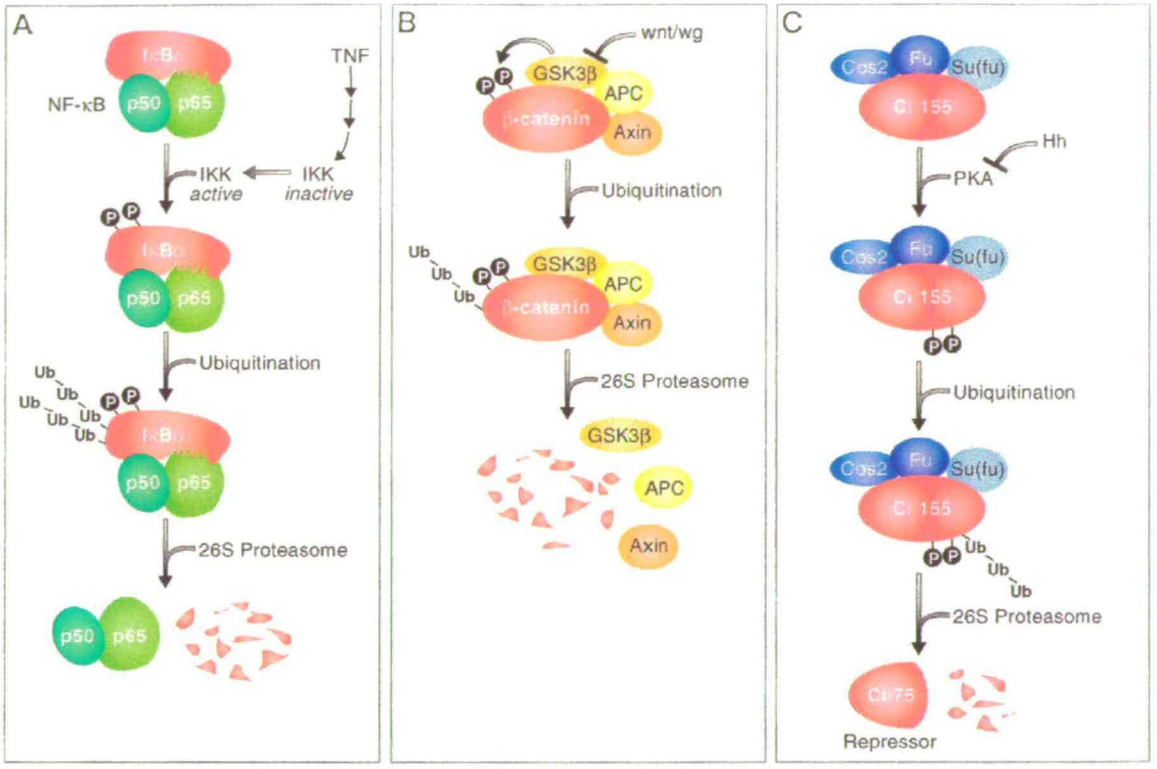


Figure 1.5: Schematic representation of the role of the ubiquitin system in the regulated degradation of I-κB and β-catenin, and in the processing of Cubitus interruptus (Ci).
(A) Degradation of I-κB. Stimulation by cytokines, such as TNF (tumour necrosis factor), activates I-κB kinase (IKK), which phosphorylates two serine residues of I-κB. This renders I-κB susceptible to polyubiquitination and proteasomal degradation, resulting in the release and nuclear translocation of NF-κB. (B) Degradation of β-catenin. In the absence of signalling through the Wnt/Wg pathway, β-catenin forms a complex with GSK3β, axin and APC. This enables GSK3β to phosphorylate β-catenin, which causes it to be ubiquitinated and degraded. The TCF/LEF transcription factor, which is the terminal component of the Wnt signal transduction pathway, requires β-catenin as a cofactor. In the presence of Wnt signalling, the GSK3β-axin-APC-β-catenin complex is dissociated, leading to accumulation and nuclear translocation of β-catenin. (C) Processing of Cubitus interruptus (Ci). In the absence of Hh signalling, Ci forms a microtubule-associated complex with Cos-2, Fu and Su(fu). PKA phosphorylates Ci, and this may target Ci for ubiquitination. Ci is cleaved in a proteasome-dependent fashion to yield a 75 kDa N-terminal fragnent, Ci75, which acts as a transcriptional repressor. Hh signalling inhibits Ci phosphorylation. Taken from Maniatis (1999).

with its inhibitor, I-κB.

1.3.2.1.1 Processing of the NF-κB precursor

NF-κB is derived from the precursor proteins p105, which undergo ubiquitin- and proteasome-mediated cleavage to generate the active subunit p50 (Palombella *et al.*, 1994). This is the only established case in which the ubiquitin-proteasome pathway achieves processing rather than complete destruction of its target, and the mechanism is not fully understood. The N-terminal signals required for p105 processing include a glycine-rich region which may serve as a 'stop' signal for proteasomal degradation (Lin and Ghosh, 1996; Orian *et al.*, 1999), two nearby lysine residues which are the sites of polyubiquitination, and a downstream acidic region that may function as an E3 recognition motif (Orian *et al.*, 1999). Although the N-terminal domain can support basal processing, C-terminal sequences appear to be important for accelerated, signal-induced processing (Belich *et al.*, 1999; Heissmeyer *et al.*, 1999; Orian *et al.*, 2000). A C-terminal 20 amino-acid motif is phosphorylated by I-κB kinase, and phosphorylation promotes recognition by the SCF-β-TrCP E3 (Orian *et al.*, 2000). However p105 lacking the β-TrCp recognition motif can be ubiquitinated by crude extracts, suggesting that p105 is targeted by multiple E3s recognising different structural motifs (Orian *et al.*, 2000).

1.3.2.1.2 Degradation of the NF-κB inhibitor, I-κB

The active NF-κB transcription factor is formed by heterodimerisation of p50 with another member of the Rel family, p65. Under resting conditions, the heterodimer is sequestered in the cytosol by binding to I-κB, which masks the nuclear translocation signal. Following stimulation, I-κB is degraded and NF-κB is released for nuclear export. The E3 which recognises and ubiquitinates I-κB is an SCF complex containing the β-TrCP F-box protein (Yaron *et al.*, 1998; Winston *et al.*, 1999). As discussed in section 1.2.5.5, recognition of I-κB is dependent on

its phosphorylation on serine residues 32 and 36. Two homologous and cytokine-activated I-κB kinases (IKKs) have been identified, termed IKKα and IKKβ (Mercurio *et al.*, 1997; Woronicz *et al.*, 1997; Zandi *et al.*, 1997). A knockout mouse lacking both IKKs shows a complete lack of NF-κB activity and dies at day 12 of embryogenesis (Li *et al.*, 2000).

The *Drosophila* transcription factor Dorsal is homologous to NF-κB, and is involved in patterning the dorso-ventral axis. Binding of Dorsal to the I-κB homologue Cactus prevents its translocation to the nucleus. Signalling through the Toll receptor results in Cactus degradation. Flies lacking the β-TrCP homologue, Slimb, fail to activate Dorsal-dependent genes such as *twist* and *snail* (Spencer *et al.*, 1999), suggesting conservation of the NF-κB activation mechanism in flies. Signal-dependent phosphorylation of Cactus is required for its degradation (Reach *et al.*, 1996). Dorsal is also phosphorylated, while bound to Cactus, and this is essential for its translocation to the nucleus (Drier *et al.*, 1999). Phosphorylation may be carried out be Pelle, a serine-threonine kinase which functions downstream of Toll, but this has not been proven.

1.3.2.1.3 β-catenin proteolysis and Wnt signalling

β-catenin is a coactivator of the transcription factor TCF/LEF, and both proteins are terminal components of the Wnt signalling pathway. In the absence of Wnt signalling, β-catenin is phosphorylated by the glycogen synthase kinase-3-β (GSK3β), a modification which renders it susceptible to ubiquitination and degradation (Orford *et al.*, 1997; Polakis, 1997). β-catenin is a poor substrate for GSK3β *in vitro*, and efficient phosphorylation is thought to require its association with the adenomatous polyposis coli tumour suppressor protein (APC) and conductin/axin (Ikeda *et al.*, 1998; Behrens *et al.*, 1998). Axin acts as a scaffold protein, bringing together GSK3β and its substrates, β-catenin and APC (Behrens *et al.*, 1998). While APC mutations stabilise β-catenin (Rubinfeld *et al.*, 1997), its role in the complex is not well understood.

Activation of Wnt signalling inhibits phosphorylation of β-catenin, via the cytoplasmic Wnt effector Dishevelled. It is thought that activated Dishevelled binds to axin and effects a change in the β-catenin-axin-APC-GSK3β complex (Salic *et al.*, 2000; Zhang *et al.*, 2000). The result is accumulation and nuclear translocation of β-catenin and transcription of downstream genes.

The first indication of the identity of the β-catenin E3 came from studies of the *Drosophila* Wingless signalling pathway. The *Drosophila* homologue of β-catenin, Armadillo, is stable in flies lacking Slimb (Jiang and Struhl, 1997), suggesting that Arm/β-catenin, like Cactus/I-κB, may be targeted by an SCF complex containing the F-box protein Slimb/β-TrCP. This has been confirmed in mammals and in *Xenopus* (Latres *et al.*, 1999; Hart *et al.*, 1999; Kitagawa *et al.*, 1999). β-catenin/TCF activity elevates the expression of β-TrCP mRNA and protein, resulting in a negative feedback loop (Spiegelman *et al.*, 2000). The upregulation of β-TrCP by β-catenin elevates NF-κB activity as it lowers β-catenin activity, providing co-ordination between the two signalling pathways.

1.3.2.2 Cubitus interruptus proteolysis and Hedgehog signalling

The *Drosophila* transcriptional regulator Cubitus interruptus (Ci), a component of the Hedgehog (Hh) signalling pathway, presents another possible case of limited processing by the ubiquitin-proteasome system. The full-length Ci protein, termed Ci155, is cytoplasmic (Aza-Blanc *et al.*, 1997). In the absence of Hh signalling, Ci155 is cleaved to produce the Ci-75 derivative, which translocates to the nucleus and functions as a transcriptional repressor (Methot and Basler, 1999). While Hh signalling prevents Ci-155 cleavage, Ci stabilisation alone cannot mimic Hh signalling (Methot and Basler, 1999). It is thought that Ci can also function as a Hh-responsive transcriptional activator (Aza-Blanc *et al.*, 1997), but the 'activator' form of Ci has not been identified.

Ci processing can be blocked by proteasome inhibitors (Ingham, 1998). Since flies mutant for the F-box protein Slimb accumulate Ci-155 (Jiang and Struhl,

1997), an SCF complex containing Slimb may be involved in the cleavage event, although this has not been proven. Hh signalling may influence Ci processing by modulating its phosphorylation state. Protein kinase A (PKA) activity is required for repression of Hh-responsive genes, and mutating each of the putative PKA sites in Ci results in its stabilisation (Chen *et al.*, 1998). The processing of Ci also depends on its subcellular localisation, which is regulated by the kinesin-like Costal-2 protein. Costal-2 forms a complex with Fused, Suppressor of Fused and Ci, which is localised to the microtubules (Robbins *et al.*, 1997). Hh signalling causes dissociation of the complex (Robbins *et al.*, 1997). Loss of Costal-2 function causes accumulation of Ci-155, suggesting that the association of Ci with microtubules may be essential for its processing (Sisson *et al.*, 1997).

1.3.2.3 β-TrCP and human disease

Dysregulation of Wnt signalling is established as a causative factor in cancer, and this can be caused by a number of different genetic defects (reviewed by Polakis (2000)). Many of these defects result in failure of β-catenin ubiquitination and degradation. The APC tumour suppressor is important in β-catenin destabilisation, but mutant forms of APC, such as those found in colorectal carcinomas, fail to downregulate β-catenin (Munemitsu *et al.*, 1995). Many human tumours and cancer cell lines have mutations in the β-catenin gene itself (Rubinfeld *et al.*, 1997; Morin *et al.*, 1997). Commonly, mutations affect the serine residues which are normally phosphorylated by I-κB kinase : these mutations abolish recognition of β-catenin by the F-box protein β-TrCP (Hart *et al.*, 1999). Axin is also required for β-catenin destabilisation. Recently, mutations in the axin gene have been found in human hepatocellular cancers and cell lines (Satoh *et al.*, 2000). All of the mutations were predicted to affect the β-catenin binding sites.

Thus β-catenin can be stabilised either by direct genetic mutation,or by the inactivation of its regulators, and this stabilisation appears to contribute towards carcinogenesis. Activation of gene transcription through LEF/TCF, which is

coactivated by β-catenin, is probably involved in transformation. The proto-oncogene c-myc is one of the targets of the APC pathway (He *et al.*, 1998), and many more of the LEF/TCF target genes are involved in growth control.

The human immunodeficiency virus type 1 (HIV-1) uses β-TrCP function to reduce the amount of its receptor molecule CD4 on the surface of infected cells. The viral Vpu protein induces formation of a complex, consisting of CD4, Vpu and β-TrCP, at the ER membrane (Margottin *et al.*, 1998). This complex targets CD4 for rapid degradation, downregulating its secretion to the cell surface and avoiding superinfection of a host cell.

1.3.3 Ubiquitin-mediated proteolysis of membrane proteins

The ubiquitination system can target secretory and membrane proteins as well as intracellular proteins. Ubiquitination is important both in 'quality control', the degradation of aberrant proteins which have been retained in the endoplasmic reticulum, and in regulating the trafficking of cell surface receptors and other plasma membrane proteins.

1.3.3.1 Degradation of ER proteins

The endoplasmic reticulum (ER) is the site at which secretory proteins and membrane proteins enter the central vacuolar system which includes the ER, Golgi apparatus, lysosomes, and plasma membrane. Protein folding and assembly into complexes occurs in the ER. Misfolded or uncomplexed proteins are retained in the ER by chaperones and eventually degraded.

The first indication that the ER integral membrane proteins could be targets for ubiquitin-mediated proteolysis came from the identification of a yeast ER-membrane-bound E2, Ubc6, which interacts genetically with a component of the ER protein import apparatus, *SEC61* (Sommer and Jentsch, 1993). A mutant form of Sec61 was subsequently confirmed to be polyubiquitinated by Ubc6 and

degraded by the proteasome (Biederer *et al.*, 1996). Studies on several aberrant proteins, implicated in human disease, showed that ER luminal proteins could also be targeted. The mutant cystic fibrosis transmembrane regulator (CFTR) encoded by the Δ508 *CFTR* allele (Ward *et al.*, 1995) and a mutant form of the prion protein (Jin *et al.*, 2000) are retained in the ER before being degraded by the cytosolic ubiquitin-proteasome pathway. Sec61 is the central component of the translocation apparatus which mediates protein import into the ER. Degradation of ER proteins requires their retrotranslocation across the membrane and this is also mediated by the Sec61 complex (Wiertz *et al.*, 1996b; Plemper *et al.*, 1997). After retrotranslocation, substrates are probably delivered directly to the proteasomes which have been identified observed to the cytoplasmic face of the ER (Palmer *et al.*, 1996; Brooks *et al.*, 2000).

While ER quality control prevents the cellular accumulation of non-functional endogenous proteins, the system can be subverted by viruses. The US2 and US11 proteins of human cytomegalovirus cause MHC class I molecules in the ER to be retrotranslocated and degraded by the proteasome (Wiertz *et al.*, 1996b,a).

1.3.3.2 Ubiquitination and cell-surface signalling.

The activity of cell-surface proteins is often regulated by controlling the level of protein localized at the plasma membrane. For example, receptors can be downregulated by endocytosis into the cell after stimulation, allowing the cell to return to an unstimulated state. Internalized receptors can either be degraded or recycled to the plasma membrane to function again. It has become clear that ubiquitination plays a key part in downregulating receptors and other cell-surface proteins. However, this does not always involve degradation by the proteasome - some ubiquitinated membrane proteins are degraded in the lysosome.

1.3.3.2.1 Ubiquitination as a means of downregulating receptors

The first evidence for ubiquitin-dependent receptor downregulation came from studies on two G-protein coupled receptors in yeast. The α factor receptor and the a-factor receptor are activated by mating pheromones, and expressed on yeast cells of opposite mating types. Ligand binding induces receptor ubiquitination, endocytosis and lysosomal degradation. Receptor internalisation requires Ubc4 and Ubc5, and the ubiquitination signal sequences on the cytoplasmic tails of the receptors (Hicke and Riezman, 1996; Roth and Davis, 1996; Roth *et al.*, 1998).

While there is no evidence for ubiquitination of G-protein-coupled receptors in metazoans, a number of mammalian receptor tyrosine kinases (RTKs) undergo ubiquitin-dependent downregulation. RTKs have a variety of roles in controlling growth and differentiation. Ligand binding induces receptor autophosphorylation and dimerisation, and also ubiquitination. Downregulation of a number of RTKs has been shown to depend on their ubiquitination (Strous *et al.*, 1996; Mori *et al.*, 1995), and in some cases this appears to be mediated by the proteasome rather than the lysosome (Mori *et al.*, 1995; Jeffers *et al.*, 1997). The signal for sorting a ubiquitinated receptor to either the proteasome or the lysosome may reside in the length or degree of branching of the polyubiquitin chain : the yeast mating pheromone receptors, which are degraded by the lysosome, are mono- or di-ubiquitinated (Hicke and Riezman, 1996).

1.3.3.2.2 Membrane protein ubiquitination and human disease

Defects in ubiquitin-mediated downregulation of plasma membrane proteins can result in various human diseases. For example, desensitisation of ErbB RTKs can cause malignant transformation. cCbl is a RING-finger protein which normally participates in ErbB ubiquitination and degradation (Levkowitz *et al.*, 1999), but its activity can be antagonised by the viral ocoprotein vCbl. The epithelial sodium channel (ENaC) is targeted for ubiquitination by the Hect domain E3

Nedd-4. Defects in ENaC regulation in human kidney cells result in Liddles's syndrome, an inherited form of hypertension (Staub *et al.*, 1996). All the mutations associated with Liddle's syndrome identified to date affect the sequences necessary for interaction with Nedd4.

1.4 Conclusion

The information presented in this chapter demonstrates that ubiquitin-mediated proteolysis can facilitate the correct spatial and temporal expression of regulatory proteins. Given that multicellular organisms have many more ubiquitinating enzymes than yeast, it seems likely that they exploit the system to control differentiation and development. However the physiological functions of most of the ubiquitinating enzymes in metazoans have not been studied. The research described in the following chapters aimed to uncover roles for the ubiquitin system in controlling *Drosophila* embryogenesis.

Materials and Methods

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Media

2.1.1.1 Bacterial media

Luria broth (L-broth): 10 g Difco Bacto tryptone; 5 g Difco bacto yeast extract; 5 g NaCl; per litre, adjusted to pH 7.2

SOC broth: 100 ml L-broth; 1 ml 1 M $MgCl_2$; 1 ml 1 M $MgSO_4$.

Luria agar (L-agar) : As Luria broth with 15 g per litre Difco agar.

Ampicillin was added to a final concentration of 100 $\mu g \cdot ml^{-1}$, or chloramphenicol to a final concentration of 34 pg/ml, or kanamycin to a final concentration of 30 $\mu g \cdot ml^{-1}$, when appropriate.

2.1.1.2 *Drosophila* media

Dundee food: 10.7 g agar; 78.6 g glucose; 44.3 g brewers' yeast; 71.4 g maize; 5.7 g live yeast; 2.7 g nipagin ; 3.2 ml propionic acid; per litre.

Grape juice agar: 22.5 g agar; 25 g sucrose; 1.5 g nipagin; 125 ml red wine concentrate; per litre.

2.1.1.3 Yeast media

YPDA: 1% (w/v) yeast extract; 2% (w/v) Bacto-peptone; 2% (w/v) glucose; 0.003% (w/v) adenine.

YMG/CAS: 0.67% (w/v) yeast nitrogen base without amino acids; 2% (w/v) casamino acids; 2%(w/v) glucose. For plates 2% (w/v) agar was added. Media was supplemented with other amino acids as required.

Complete minimal medium (CM): 0.67% (w/v) yeast nitrogen base without amino acids; 2 % (w/v) glucose or 2 % (w/v) galactose and 1 %(w/v) raffinose. For plates 2% (w/v) agar was added. For drop-out media, CM was supplemented with 0.2% (w/v) of the appropriate drop-out powder. For X-gal plates, CM was supplemented with 10 % (w/v) 10×BU salts and 4 ml 20 $mg \cdot ml^{-1}$ X-gal in dimethylformamide.

2.1.2 Materials

2.1.2.1 Solutions

Annealing buffer: 1 mM Tris pH 7.2; 1 mM EDTA.

AP detection buffer: 100 mM NaCl; 50 mM $MgCl_2$; 100 mM Tris.HCl, pH 9.5.

Boiling mix: 1 ml stacking gel buffer; 0.8 ml 25% (w/v) SDS; 0.5 ml β-mercaptoethanol; 1 ml glycerol; 0.05% (w/v) bromophenol blue.

10×BU salts: 70 g Na_2HPO_4; 30 g NaH_2PO_4; per litre.

Church buffer: 0.5 M NaPi (72 ml 1 M Na_2HPO_4; 28 ml 1 M NaH_2PO_4) ; 7 % (w/v) SDS; 1 mM EDTA.

Denaturation solution: 0.5 M NaOH; 1.5 M NaCl.

DNA extraction buffer: 0.1 M Tris.HCl, pH 9.0; 0.1 M EDTA, pH 8.0 ; 1 % (w/v) SDS)

DNA loading buffer: 20% (w/v) glycerol; 100 mM EDTA; 0.1 % (w/v) bromophenol blue.

Hybridisation solution: 50% (v/v) deionised formamide; 5× SSC; 100 $\mu g \cdot ml^{-1}$ *E. coli* tRNA; 50 $\mu g \cdot ml^{-1}$ heparin; 0.1% (w/v) Tween20; pH adjusted to 6.5 with HCl.

Injection buffer: 0.1 mM NaPi, pH 6.8; 5 mM KCl.

10×MOPS buffer: 41.86 g MOPS; 1 l 50 mM sodium acetate; 2 ml 0.5 M EDTA; adjusted to pH 7.0 with 10 N NaOH.

Neutralisation solution: 1 M Tris, pH 7.5; 1.5 M NaCl.

10×PBS: 80 g NaCl; 20 g KCl; 0.2 g Na_2HPO_4; 20 g KH_2PO_4; per litre.

Resolving gel buffer: 18.15 g Tris; 0.4 g SDS; per 100 ml, adjusted to pH 8.9 with HCl.

RNA extraction buffer: 4.7 g guanidinium thiocyanate; 0.333 ml 0.75 M sodium citrate, pH 7; 1 ml 10% (w/v) N-laurylsarcosine; 78 μl β-mercaptoethanol added immediately before use; per 10 ml.

RNA loading buffer: 50% (w/v) glycerol; 1 mM EDTA; 0.4 % (w/v) bromophenol blue.

RNA sample buffer: 10 ml formamide; 3.5 ml formaldehyde; 2 ml 10X MOPS

20×SSC: 175 g NaCl; 88 g tri-Na citrate; per litre.

Stacking gel buffer: 5.1 g Tris; 0.4 g SDS; per 100 ml, adjusted to pH 6.7 with HCl.

10×TBE: 108 g Tris; 55 g boric acid; 9.3 g EDTA; per litre.

10×TBS: 60.5 g Tris; 87.6 g NaCl; per litre, adjusted to pH 7.5 with HCl.

T.E.: 10 mM Tris; 50 mM EDTA.

TFB1: 30 ml 1 M potassium aetate, pH 7.5; 12 g RbCl; 9.9 g $MnCl_2.4H_2O$; 1.5g $CaCl_2.2H_2O$; 150 ml glycerol; per litre, adjusted to pH 5.8 with acetic acid.

TFB2: 20 ml 0.5 M MOPS pH 6.8; 1.2 g RbCl; 11 g $CaCl_2.2H_2O$; 300 ml glycerol; per litre, adjusted to pH 6.8 with KOH.

10×TGS: 30 g Tris; 144 g glycine; 10 g SDS; per litre.

Transfer buffer: 47.5 g glycine; 10 g Tris; per 3 litres.

Yeast extraction buffer: 2 % (w/v) TritonX-100; 1 % (w/v) SDS; 100 mM NaCl; 10 mM Tris.Cl pH 8.0; 1 mM EDTA pH 8.0.

Wash solution: 0.1% (w/v) SDS; 0.2×SSC.

2.1.2.2 Radioisotopes

α^{32}P-dCTP (3000 Ci/mM)

2.1.2.3 Suppliers

Oligonucleotides : MWG-Biotech and Gibco.

Enzymes: New England Biolabs, Roche and Promega.

Nucleic acid purification kits : Stratagene and Qiagen.

Chemicals: BDH and Sigma.

Radioisotopes: Amersham.

2.1.2.4 Bacterial strains

Name	Genotype	Use	Reference
DH5α	ϕ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17, (r_K^-, m_K^+, *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)*U169*	General cloning applications	Hanahan (1983)
MC1066	*galU*, *galK*, *strA*^{R}, *leuB6*, *trpC-9830*, *pyrF74::Tn5(Kn*^{r}*)hsdr*$^-$, Δ(*LacIPOZYA*)*74*	Rescue of pJG4-5 from yeast plasmid preparations in two-hybrid screen	P. Legrain, Institut Pasteur
BL21(DE3)pLysS	F-, *ompT*, *hsdS*, (r_B^-, m_B^-), *dcm*, *gal*, λ(DE3), pLysS(Cm^r)	Expression of recombinant proteins	Studier *et al.* (1990)

Table 2.1:

2.1.2.5 Yeast strains

Name	Genotype	Use	Reference
EGY48	*MAT*α, *his3*, *trp1*, *ura3,6LexAop-LEU2).*	Strain used for yeast two-hybrid screen	Gyuris *et al.* (1993)

Table 2.2:

2.1.2.6 Plasmids

Name	Description Use	Reference	
pSPT18/19	pUC 18/19 derivatives with polylinkers in opposite orientations, flanked by T7 and SP6 promoters	Production of DIG-labelled RNA probes	Roche catalogue
pGEM-T	Cloning vector with single 'T' overhangs	Cloning of PCR products	Promega catalogue
pET15b	Polylinker flanked by T7 promoter and six-histidine tag	Expression of C-terminally-tagged recombinant protein in *E. coli*	Novagen catalogue
pEG202	Yeast plasmid with LexA coding sequence downstream of polylinker	Expression of LexA fusion protein as bait in yeast two-hybrid system	Gyuris *et al.* (1993)
p18-34	Yeast plasmid carrying *lacZ* reporter gene with LexA binding sites near promoter	Screening for *lacZ* transcriptional activation due to bait-prey interactions in yeast two-hybrid screen	Gyuris *et al.* (1993)
pJK101	Yeast plasmid carrying *lacZ* reporter gene with strong promoter repressible by LexA	Testing two-hybrid bait protein for translocation to nucleus and binding to LexA operators	Gyuris *et al.* (1993)
pJG4-5	Yeast plasmid with polylinker flanked by *GAL1* promoter and acidic transcriptional activation domain coding sequence	Expression of transcriptional activator fusion proteins as prey in yeast two-hybrid screen	Gyuris *et al.* (1993)

Table 2.3:

2.1.2.7 *Drosophila* stocks

Name	Genotype	Use	Reference
Oregon R53	Wildtype	Embryo collection for expression analysis	Lindsley and Grell (1968)
Δgcl	*yw; rev390/CyO; cmp44E, rescue(w^+)/+=Δgcl-51*	Obtaining embryos lacking germ cells	T. Jongens
2535	w^{1118}; ry^{506}, Sb^1, *P[ry$^+$Δ2-3]/ TM6, Ubx*	Source of *P* element transposase in mutagenesis	A.Jarman
P1329	cn^1, *P[ry$^{+t7.2}$=PZ]Uba1^{03405}/ CyO;* ry^{506}	Carries *P* element insertion in *Duba1*	Bloomington Stock Center
P1558	*P[ry$^{+t7.2}$=PZ]l(3)002240,* ry^{506}*/ TM3,* ry^{RK}, Sb^1, Ser^1	Source of *P* element in *UbcD4* mutagenesis	Bloomington Stock Center
233	w^{1118}; *Sco / CyO*	Used in excision of *P* element from P1329	A. Jarman
232	w^{1118}; Dr^{Mio}/*TM3, ry, Ser*	Used in *UbcD4* mutagenesis crosses	A. Jarman
CyO-30	w^{1118}; *Pin/CyO, P[w^{+mC}=GAL4-Kr.C]DC4, P[w^{+mC}=UAS-GFP.S65T]DC8*	Source of GFP balancer used to identify *l(2)03405* homozygotes	Casso *et al.* (2000)

Table 2.4: For explanation of gene symbols see Lindsley and Zimm (1992).

2.1.2.8 Oligonucleotides

Name	Sequence	Comments
DUBA1-1	GAA AACTGAAT-GAGCCAGG	corresponds to positions 4189-4207 in the *Duba1* cDNA '+' strand
DUBA1-2	GAA GTC AAT TTC CCG TGG	corresponds to positions 107-124 on the *Duba1* cDNA '−' strand
DUBA1-3	TCT GAC TCG TTC CAG GAC	corresponds to positions 85-104 on the *Duba1* '−' strand
6067	CCG TCA TTA AAA CCA TGG CG	corresponds to positions 1432-1452 on the *Duba1* '−' strand
Y4104	GCG GCG TTG ACA TTT CGG	corresponds to positions 453-471 on the *Duba1* '+' strand
D4.B1	GAA TTC ATG GCG AAC ATG GCA G	forward primer used to amplify the UbcD4 coding sequence for cloning into yeast two-hybrid bait vector and *E. coli* expression vector
D4.B2	GGA TCC TAA CTG AAC AGG CCC	reverse primer used as for D4.B1
E2p1	ATC GCC GGT CCG CCT GAC CAG CCC	corresponds to positions 295 to 318 on the *UbcD4* cDNA '+' strand
E2p5	TAG GCC ACC ACT GCG TCC	corresponds to positions 563 to 546 on the *UbcD4* cDNA '−' strand
P31	CGA CGG GAC CAC CTT ATG TTA TTT CAT CATG	corresponds to 31 bp inverted repeats of P element
LEX1	CGT CAG CAG AGC TTC ACC ATT G	40 bp upstream of *EcoRI* site in pEG202 polylinker; used for sequencing two-hybrid bait
BCO1	CCA GCC TCT TGC TGA GTG GAG ATG	70 bp downstream of *EcoRI* site in pJG4-5 polylinker; used for sequencing two-hybrid prey
BCO2	GAC AAG CCG ACA ACC TTG ATT GGA G	40 bp upstream of *XhoI* site in pJG4-5 polylinker; used for sequencing two-hybrid prey
T7	GTA ATA CGA CTC ACT ATA GGG C	T7 promoter sequencing primer
M13 rev	GGA AAC AGC TAT GAC CAT G	M13 reverse sequencing primer

Table 2.5: All oligonucleotides are shown in 5′ - 3′ orientation. Restriction enzyme sites are underlined : *BamHI* = GGATCC ; *EcoRI* = GAATTC.

2.2 Methods

2.2.1 Manipulation of bacteria

2.2.1.1 Growth of *E. coli* bacterial cultures

L-broth, with antibiotic if necessary, was inoculated with a single colony using a sterile metal loop. Cells were shaken at 37°C for an appropriate length of time. 5 ml cultures were grown in 2 oz glass bottles and larger cultures were grown in conical flasks with a maximum volume 5-10 times greater than that of the culture.

2.2.1.2 Storage of *E. coli* bacterial cultures

Bacteria were stored as liquid overnight cultures or on L-agar plates, with appropriate antiobiotics, at 4°C for up to 6 weeks. For long-term storage, 0.8 ml of overnight culture was mixed with glycerol in sterile vials, snap-frozen in dry ice or liquid nitrogen and stored at -70°C. Bacteria were recovered by thawing vials and streaking cells onto appropriate agar plates.

2.2.1.3 Preparation of competent *E. coli* cells

A 5 ml overnight culture of *E. coli* was grown from a single colony. 1 ml was then used to inoculate 20 ml of L-broth and the culture was grown at 37°C, shaking, for approximately 90 minutes, until it reached an $O.D._{600}$ of 0.3-0.5. Cells were pelleted by centrifugation at 5,000 rpm for 10 minutes in a cold Sorvall centrifuge. The pellet was resuspended in 10 ml of cold TFB1. After a 30 minute incubation on ice, cells were pelleted as before and resuspended in 2 ml cold TFB2. The suspension was aliquotted into microfuge tubes, snap-frozen on dry ice and stored at -70°C.

2.2.1.4 Transformation of plasmid DNA into competent cells

100 μl of competent cells were used per transformation. The cells were thawed slowly on ice and mixed with half a ligation reaction, or 0.1 - 10 ng of plasmid DNA. The cells were incubated on ice for 30 minutes, heat-shocked in a 42°C water

bath for 60-90 seconds and chilled on ice for 2 minutes. 0.9 ml of SOC medium was added and the cells were shaken gently at 37°C for 45 min - 1 hour. 150 - 200 μl of the transformation reaction was spread on an L-agar plate containing appropriate antibiotics.

2.2.1.5 Preparation of electrocompetent *E. coli* cells

500 μl of a 5ml overnight culture was used to inoculate 50 ml of L-broth and the culture was grown to an $O.D._{600}$ of approximately 0.5. The cells were pelleted by centrifugation at 5000 rpm for 10 min at 4°C and resuspended in 50 ml ice-cold distilled water. This wash was repeated twice more before finally resuspending the cells in 1 ml ice-cold water.

2.2.1.6 Electroporation of DNA into competent cells

50 μl electrocompetent *E. coli* cells was mixed with 2 μl of a ligation reaction on ice. The mixture was transferred to a 0.2 cm electroporation cuvette and electroporated at 25 μF; 2.5 kV; 200 Ω. 1 ml of SOC medium was added and the cells were transferred to a microfuge tube and incubated at 37°C for 45 - 60 min with gentle shaking. 200 μl cells were spread on L-agar plates with appropriate antibiotics.

2.2.1.7 Small scale preparation of plasmid DNA

A single bacterial colony was inoculated into 5 ml of L-broth with antibiotic and grown for 14-16 hours at 37°C with shaking. 1.5 ml of the culture was decanted into a microfuge tube and spun down for 30 seconds in a microfuge. Plasmid DNA was isolated using the StrataPrep Plasmid Miniprep kit (Stratagene) according to the manufacturers' instructions. The method involved alkaline lysis of the cells followed by binding of DNA to a silica-based fibre matrix in a microspin cup. Impurities were removed using a wash buffer. DNA was eluted in 10 mM Tris pH 8.5.

2.2.1.8 Large scale preparation of plasmid DNA

A single bacterial colony was inoculated into 5 ml L-broth with antibiotic and grown for 8 hours at 37°C with shaking. 50 μl was used to inoculate a 25 ml culture which was grown overnight. Plasmid DNA was isolated using the Qiagen Plasmid Midi Kit according to manufacturer's instructions. The method involves alkaline lysis of cells followed by binding of plasmid DNA to anion-exchange resin under low-salt and pH conditions. Impurities were removed by a medium-salt wash. Plasmid DNA was eluted in a high-salt buffer and concentrated and desalted by isopropanol precipitation.

2.2.2 Manipulation of yeast

2.2.2.1 Growth and storage of yeast cultures

For liquid cultures, 5-20 ml YPDA or the appropriate dropout medium was inoculated with a single colony and incubated with shaking at 30°C overnight. For plate cultures, appropriate plates were streaked with a single colony and incubated at 30°C for 2-3 days until colonies formed. Yeast were stored on plates or as liquid cultures for 6-8 weeks.

2.2.2.2 Transformation of yeast with plasmid DNA

A 20 ml overnight culture of yeast was grown in the appropriate medium. The culture was diluted into 300 ml medium to an $O.D._{600}$ of 0.1 (corresponding to 2×10^6 cells/ml). The culture was grown until the $O.D._{600}$ reached 0.5, then centrifuged at $2000 \times g$ for 5 min. The pellet was resuspended in 20 ml sterile distilled water. This step and all the following manipulations were carried out at room temperature unles otherwise stated. The cells were centrifuged at $2000 \times g$ for 5 min and resuspended in 5 ml LiOAc/T.E. (10 mM Tris HCl pH 7.5, 1 mM EDTA, 100 mM lithium acetate). They were pelleted as before and resuspended in 2 ml LiOAc/T.E.. 50 μl competent cells were used in each transformation. The cells were mixed with plasmid DNA (0.1-2 μg) and carrier DNA (50 μg salmon

sperm DNA, boiled for 10 min before use). DMSO was added to 10 % (v/v). 300 μl of PEG solution (40% (w/v) PEG_{4000}, 10 mM Tris HCl pH 7.5, 1 mM EDTA, 100 mM LiOAc) was added and the tubes were inverted several times to mix the contents. After a 30 min incubation at 30°C, the cells were heatshocked in a 42°C water bath for 15 min. They were then placed in a room temperature water bath and 200 μl of the reaction was plated on the appropriate medium.

2.2.2.3 Collection of primary transformant cells

Plates containing transformant colonies were cooled at 4°C. Yeast was scraped off the plate using a glass microscope slide, and placed in a 50 ml centrifuge tube. The cells were washed twice in an equal volume of T.E., pelleting them by centrifugation at $1500 \times g$ for 5 min. The pellet was resuspended in an equal volume of glycerol, aliquoted and stored at -70°C.

2.2.2.4 Calculation of replating efficiency

Replating efficiency was calculated by thawing yeast, diluting 1/10 in Gal/Raff/CM -Ura -His -Trp medium and incubating at 30°C for 4 hours. Serial dilutions of yeast were plated on Gal/Raff/CM- Ura -His -Trp dropout plates and incubated for 2-3 days until colonies formed. The colonies were counted and the number of colony-forming units (cfu) per ml of transformed yeast was determined.

2.2.2.5 Screening of transformant cells for bait-prey interaction

10^7 cells were thawed, incubated in Gal/Raff/CM -Ura -His -Trp as described above and plated on a total of 20 Gal/Raff/CM -Ura -His -Trp -Leu plates containing X-gal. The plates were incubated at 30°C for 3-4 days until colonies appeared. Blue colonies were streaked onto fresh Gal/Raff/CM -Ura -His -Trp plates and incubated at 30°C for 3-4 days. Cells were restreaked onto Glu/CM -Ura -His -Trp plates and grown overnight. Cells were then streaked onto Glu/Xgal/CM -Ura -His -Trp, Gal/Raff/Xgal/CM -Ura -His- Trp, Glu/CM -Ura -His -Trp -Leu

and Gal/Raff/CM -Ura -His -Trp -Leu plates and incubated at 30°C for 3 days. Colonies which grew on Gal/Raff/CM -Ura -His -Trp -Leu but not on Glu/CM -Ura -His -Trp -Leu, and which appeared blue on Gal/Raff/Xgal/CM -Ura -His -Trp -Leu but not on Glu/Xgal/CM -Ura -His -Trp, were chosen for further analysis.

2.2.2.6 Small scale preparation of plasmid yeast DNA

2 ml Glu/CM -Trp medium was inoculated with a single yeast colony and incubated at 30°C overnight with shaking. The yeast was pelleted for 10 sec in a microfuge and the pellet was resuspended in 200 μl yeast extraction buffer. 200 μl glass beads and 200 μl phenol/chloroform were added before vortexing for 5 min and spinning for 5 min. 160 μl of the aqueous phase was transferred to a clean eppendorf. 500 μl ethanol and 80 μl 7.5 M ammonium actetate was added and the tubes were incubated at -20°C for 30 min before being spun in a cold microfuge for 10 min. The pellet was washed in 70% ethanol, air-dried and resuspended in 10 μl T.E..

2.2.2.7 Extraction of yeast proteins for immunoblot analysis

5 ml Glu/CM -Ura -His was inoculated with a single colony, incubated overnight at 30°C with shaking and used to inoculate a fresh 5 ml culture to $O.D._{600}$=0.15. This was shaken at 30°C until the $O.D._{600}$ reached 0.45 to 0.7. 1.5 ml of the culture was pelleted for 3 min in a microcentrifuge and resuspended in 50 μl of boiling mix. The suspension was snap-frozen on dry ice and boiled for 5 min. Large cellular debris was pelleted for 5 seconds. SDS-PAGE was performed on 20-50 μl supernatant.

2.2.3 Manipulation of *Drosophila*

2.2.3.1 Culture of *Drosophila melanogaster*

Flies were kept on Dundee food in vials or bottles at 25°C. Adults were allowed to lay eggs for 3-4 days before being removed. Progeny were placed onto new vials after another 10-11 days. Virgin females were obtained by collecting progeny every 4 hours at 25°C during the day. Vials were then stored at 18°C overnight so that virgins could be obtained the following morning.

2.2.3.2 Collection of *Drosophila* developmental stages

Embryos were collected on grape juice agar plates, spread with yeast, placed at the bottom of fly cages. The embryos were washed into a nylon mesh using a paintbrush and distilled water. Larvae were obtained by allowing eggs laid on a grape juice agar plate to hatch. Pupae and third instar 'wandering' larvae were collected from the sides of bottles using a paintbrush.

2.2.3.3 Preparation of embryos for *in situ* hybridisation and immunostaining

Embryos were dechorionated by shaking in 50% bleach in a nylon sieve for 2 min. After thorough washing in tap water they were transferred to a glass bottle containing 10 ml 3.7% formaldehyde in PBS and 10 ml heptane. The embryos were fixed for 15-20 mins at the formaldehyde/heptane interface with gentle agitation. The heptane and half the formaldehyde was removed and the bottle was filled with methanol. The embryos were shaken vigorously for 1 min in order to remove the vitelline membrane. Devitellinised embryos sank to the bottom of the tube and were collected using a plastic Pasteur pipette. They were placed into microfuge tubes, rinsed 3-4 times with methanol and stored in methanol at -20°C.

2.2.3.4 Injection of embryos with double-stranded RNA

Embryos were collected for one hour and dechorionated as described previously. Thirty embryos were placed onto a strip of double-sided sticky tape on a glass

coverslip. The coverslip was placed in a petri dish containing dried silica gel for 5-10 minutes, to desiccate the embryos. The embryos were covered with series 700 halocarbon oil (KMZ chemicals) and injected with approximately 100 - 300 pl double-stranded RNA in injection buffer. Injections were performed using a fine glass capillary (Eppendorf Sterile Femtotip) and a transjector 5246 (Eppendorf).

2.2.3.5 Preparation of *Drosophila* genomic DNA

10 adult flies were placed into a microfuge tube and frozen at -70°C for 5 min. The flies were homogenised in 200 μl of DNA extraction buffer for 3-5 min. They were incubated at 70°C for 30 min. 28 μl of 8M potassium actetate was added and the tubes were incubated on ice for 30 min. They were centrifuged in a cold microcentrifuge for 15 min. The supernatant was decanted into a fresh tube and the step was repeated to obtain a clear supernatant. 100 μl isopropanol was added and the DNA was allowed to precipitate for 10 min at -70°C. After a 5 min centrifugation, the pellet was washed in 70 % ethanol, air-dried and dissolved in 20 μl T.E..

2.2.4 DNA manipulation and detection

2.2.4.1 Digestion of DNA with restriction endonucleases

0.1-10 μg of DNA was incubated with 0.5 μl of each appropriate enzyme in the appropriate buffer for 1-12 hr at 37°C. For genomic DNA digests, the reaction was stirred with a glass rod and stored for an hour on ice prior to addition of enzyme. After 3 hours of digestion, another 0.5 μl of enzyme was added, the reaction was stirred again and allowed to proceed overnight.

2.2.4.2 Agarose gel electrophoresis of DNA

Agarose gels were made by dissolving agarose in 1 $\times$ TBE buffer to a concentration of 0.8-1.2 % (w/v), and adding 0.5 $\mu g \cdot ml^{-1}$ ethidium bromide. DNA samples were mixed with 0.1 volume DNA sample buffer. HindIII - digested λ DNA or

HaeII - digested ϕX174 DNA (New England Biolabs) was used as a size marker. Electrophoresis was carried out horizontally at approximately 10 v/cm. DNA was visualised by UV transillumination.

2.2.4.3 Recovery of DNA from agarose gels

After electrophoresis, the gel slice containing the desired DNA band was cut out. The DNA was extracted using the Qiagen Gel Extraction Kit following manufacturers' instructions. The method involves dissolving the agarose, binding the DNA to an anion-exchange resin in a microspin cup under low salt conditions, removing impurities in a medium-salt wash and eluting DNA in a high-salt wash.

2.2.4.4 Ligation of cohesive termini

Vector and insert DNA were digested with restriction enzymes to yield compatible overhanging ends. The DNAs were ligated at a vector:insert molar ratio of 1:1 - 1:10 in a 10 μl reaction using T4 DNA ligase (New England Biolabs) and ligase buffer. Ligation was carried out overnight at 16°C.

2.2.4.5 Southern blotting

After electrophoresis, the agarose gel was soaked in denaturing solution for 30 min, followed by incubation in two changes of neutralisation solution, for 25 min each time. The gel was then placed on a perspex support covered in a wick of blotting paper which was soaked in 10 × SSC. The gel was surrounded by Parafilm and covered with a nylon membrane, 3 sheets of 10 × SSC-soaked Whatman 3MM paper, two inches of paper towels and a weight such as a bottle. The transfer was allowed to proceed overnight before the membrane was rinsed in 2 × SSC and cross-linked in a Stratalinker.

2.2.4.6 Random primed radioactive labelling of DNA

DNA was labelled with α^{32}P-dCTP using the T7 Quickprime kit (Amersham) following manufacturers instructions. Briefly, approximately 50 ng of linear DNA

was mixed with random hexanucleotide primers and boiled for 5 min before adding 50 μCi α^{32}P-dCTP, 10 μl labelling mix (provided in kit) and 2 μl Klenow DNA polymerase. The reaction was incubated at 37°C for 20 min. Unincorporated nucleotides were removed using NucTrap push columns (Stratagene) following manufacturers instructions.

2.2.4.7 Hybridisation of radioactively labelled DNA probes to nucleic acid immobilised on nylon membranes

Nylon membranes, with bound nucleic acid, were immersed briefly in 2 × SSC and placed in Techme hybridisation cylinders, in a hybridisation oven prewarmed to 65°C, containing 20 ml of prewarmed Church buffer. Prehybridisation was carried out for 1-2 hours. The radiolabelled DNA probe was boiled for 5 min and added to the cylinder. Hybridisation was carried out at 65°C overnight. Membranes were washed twice for 20 minutes each in wash solution before being wrapped in Saran wrap, placed in an autoradiographic cassette and exposed to X-ray film at -70°C for 4 hours - 1 week. The film was developed in an automatic X-ray film processor. Alternatively, the membrane was exposed to a PhosphorImager screen for 4 hours or overnight. The signal was detected by scanning the screen in a PhosphorImager.

2.2.4.8 Polymerase chain reaction (PCR)

50 μl PCR reactions were set up using 1 × Taq buffer (Promega), 1.5 mM $MgCl_2$, 50 pmol each primer, 20 nmol dNTPs, and 0.5 μl Taq polymerase (Promega). The template was either 50 pg plasmid DNA, 10 ng genomic DNA or a few bacterial cells from a colony. Reactions were carried out in a Hybaid OmniGene thermal cycler. A typical PCR programme consisted of 25 - 30 cycles, with each cycle consisting of a denaturation step (94°C for 1 min), an annealing step (55°C-65°C for 30 min) and an extension step (72°C for 1 - 3 min). For genomic DNA templates, an initial denaturation period of 5 min at 94°C was included. PCR

products were purified, if necessary, using PCR purification columns (Qiagen) following manufacturers' instructions.

2.2.4.9 Cloning of PCR products

PCR products were cloned using the pGEM-T cloning kit (Promega) following manufacturer's instructions. 3 μl PCR product was mixed with 1 μl pGEM-T cloning vector, 5 μl ligation buffer and 1 μl T4 DNA ligase. The reactions were incubated at room temperature for one hour. If a proofreading polymerase had been used for PCR, 'A' overhangs were added before ligation, by incubating purified PCR product with 2 mM dATP, 1 $\times$ Taq buffer and 1 μl Taq, for 20 min at 72°C.

2.2.4.10 DNA sequencing

Automated DNA sequencing reactions were carried out using the dRhodamine sequencing kit (Perkin-Elmer) following manufacturer's instructions. Approximately 100 ng plasmid DNA was mixed with 5 pmol primer and 4 μl reaction mix. The reaction was incubated in a thermal cycler with the following programme : 1 cycle of 96°C for 10 sec; 25 cycles of 96°C for 10 sec, 50°C for 15 sec and 60°C for 4 min; one cycle of 60°C for 4 min. Sequencing products were precipitated by adding 2 μl 3M sodium acetate, pH 5.2, and 50 μl 95 % ethanol, and pelleted at in a cold microfuge for 15 - 30 min. The pellets were washed in 70 % ethanol and air-dried.

2.2.5 RNA manipulation and detection

2.2.5.1 Preparation of total RNA

50-100 mg of tissue was homogenised in 0.5 ml RNA extraction buffer on ice. 50 μl 2M sodium actetate, pH 4.0, was added, followed by 0.5 ml phenol, pH 6.7, and 0.1 ml of 49:1 chloroform/isoamyl alcohol. The tubes were vortexed and incubated for 15 min on ice. After centrifugation at 4°C for 20 min, the upper

layer was removed to a clean tube and an equal volume of isopropanol was added. The tubes were incubated at -20°C for 30 min and spun for 10 min at 4°C. The pellet was resuspended in 0.15 ml RNA extraction buffer. An equal volume of isopropanol was added and the tubes were again incubated at -20°C for 30 min and spun at 4°C for 10 min. The pellet was resuspended in 1 ml 75 % ethanol and incubated at room temperature for 10 min. After a 10 min centrifugation the supernatant was discarded and the pellet airdried and resuspended in 50 μl 10 mM Tris pH 8.0. The RNA content and purity was calculated by measuring the $O.D._{260}$ and $O.D._{280}$ of the samples.

2.2.5.2 Gel electrophoresis of RNA

To prepare a formaldehyde gel, 1.3 g agarose was dissolved in 109 ml 1 × MOPS buffer. The solution was cooled and 21 ml 37 % formaldehyde was added before pouring. Prior to loading the samples, the gel was pre-run at 50 volts for 10 min. The appropriate amount of RNA, in a volume of 12 μl, was added to 38 μl RNA sample buffer and heated to 65°C for 15 min. The samples were spun briefly and chilled on ice before addition of 4 μl RNA loading buffer. Electrophoresis was across a potential difference of 50 volts. The gel was stained in 50 $\mu g \cdot ml^{-1}$ ethidium bromide for 30 min, and destained in distilled water for several hours. RNA was visualised by U.V. transillumination. The ethidium bromide staining was omitted if Northern blotting was to be carried out.

2.2.5.3 Northern blotting

After electrophoresis, RNA gels were washed in a large excess of distilled water for one hour, then blotted as for Southern blots (section 2.2.4.5).

2.2.5.4 Preparation of DIG-labelled RNA probes

cDNAs were inserted into cloning vectors pSPT18 and pSPT19, which have multiple cloning sites in opposite orientations with respect to their T7 and SP6 pro-

moters. DIG-labelled RNA probes were produced using the DIG-RNA labelling kit (Roche) according to manufacturers instructions. Briefly, 1 μg of plasmid DNA was linearised and used in a 20 μl *in vitro* transcription reaction with either T7 or SP6 polymerase and rNTPS including DIG-labelled rCTP. For each gene to be analysed, both sense and antisense RNAs were produced. After two hours the template DNA was removed by digestion with DNAse. 2.5 μl of 4 M LiCl and 75μl of ethanol was added and the RNA was allowed to precipitate at -20°C overnight. It was then pelleted in a microcentrifuge for 15 min, washed in 70 % ethanol, airdried, resuspended in 20 μl T.E. with 1 μl RNAsin, aliquoted and stored at -70°C. 2 μl of probe was subjected to formaldehyde gel electrophoresis. DIG-labelling of the probe was verified by spotting serial dilutions onto nylon membrane, probing with anti-DIG conjugated to alkaline phosphatase (AP) and incubating the membrane in the AP substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

2.2.5.5 RNA *in situ* hybridisation on wholemount embryos

The method used is a modified version of that described by Tautz and Pfeifle (1989). Briefly, embryos prepared as described in section 2.2.3.3 were rehydrated in PTW (PBS with 0.1% Tween20) and postfixed for 15 min in 3.7% formaldehyde in PTW. The embryos were washed 5 times for 5 min each in PTW, then for 10 min in 50% PTW; 50% hybridisation solution, then for 10 min in hybridisation solution. Prehybridisation was carried out in hybridisation solution at 70°C for 1-2 hours in a water bath. Hybridisation was carried out overnight at 70°C in a water bath, using approximately 0.5 $\mu g \cdot ml^{-1}$ probe in hybridisation solution. Embryos were washed once in hybridisation solution, once in 50% hybridisation solution, 50% PTW and four times in PTW. All washes were at 70°C with no agitation. The anti-DIG alkaline phosphatase-conjugated antibody (Roche), which had been preadsorbed by incubation overnight at 4°C with fixed and rehydrated embryos, was diluted 1 in 2000 in PTW and incubated with the probed embryos for 1-2

hours at room temperature on a rolling wheel. The embryos were washed 3 times for 20 min each in PTW. They were then rinsed in AP detection solution for 2 min. 45 μl NBT and 35 μl BCIP were added to 1 ml AP detection solution and the embryos were incubated in this substrate mixture for several hours - overnight at room temperature. They were then washed several times in PTW, incubated for 2 hours in 50% glycerol, 50% PTW, and mounted upon a glass microscope slide in 80% glycerol, 20% PTW.

2.2.5.6 Production of double-stranded RNA

Single-stranded RNA was produced from both the sense and antisense strands of cDNA as for section 2.2.5.4, except that the rNTPs were used were not DIG-labelled. After removal of the template using DNase I, the RNA was extracted with an equal volume phenol/chloroform/isoamyl alcohol (25:24:1). After vortexing and 2 min centrifugation, the aqueous phase was removed to a new microfuge tube and an equal volume chloroform/isoamyl alcohol (25:1) was added. The tubes were vortexed and spun as before and the aqueous phase placed in a new tube. 0.5 $\times$ volume 7.5 M ammonium acetate and 2.5 $\times$ volume ethanol was added and the tubes were incubated at -70°C for 1 hour. The RNA was pelleted by 10 min centrifugation in a microfuge, washed in 70 % ethanol, air-dried and resuspended in 5 μl annealing buffer. The single-stranded RNA was quantitated by U.V. spectrophotometry. Equimolar amounts of each strand were mixed to a final concentration of 0.45 mM in annealing buffer. 12 μl aliquots of the mixture were boiled for 1 min and left at room temperature for 18 hours to allow annealing of the strands. 6 - 10 μg of the mixture was used for native agarose gel electrophoresis to verify that most of the RNA was in the double-stranded form. The double-stranded RNA was stored as a sodium actetate/ethanol precipitate at - 80°C until immediately before use.

2.2.5.7 Reverse transcription of total RNA

20 μl reverse transcription reactions were set up using 2 μg total RNA, 4 μl reverse transcriptase buffer (Promega), 2 μl dNTPs (10 mM each), 12.5 pmol primer, and AMV reverse transcriptase (Promega). The reactions were incubated at 55°C for one hour, then at 65°C for 10 min.

2.2.6 Protein manipulation and detection

2.2.6.1 Expression of proteins in *E. coli.*

Plasmids encoding tagged recombinant proteins under the control of a T7 promoter, were transformed into the BL21(DE3)pLysS strain of *E. coli.* A single colony was used to inoculate 100 ml L-broth with appropriate antibiotics and the culture was shaken at 80 rpm at 30°C overnight. 10 ml of this culture was used to inoculate 1 L L-broth with antibiotics and this culture was grown at 37°C with vigorous shaking, to an $O.D._{600}$ of approximately 0.5. 2 ml of 0.5 M IPTG was added and the culture was shaken for an additional 3 hours at 37°C. The cells were pelleted at 10,000 rpm for 20 minutes and stored at - 20°C. The pellet was resuspended in 10 ml Binding Buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris.Cl pH 7.9) and the cells were sonicated on ice in three bursts of 45 seconds each. The lysate was centrifuged at 12,500 rpm for 30 min to pellet insoluble proteins. Recombinant proteins containing a 6×His tag were purified by nickel affinity chromatography using His-Bind resin (Novagen) according to manufacturers' instruction. Briefly, the soluble protein extract was passed through a charged 2 ml His-Bind column at 4°C under gravity flow. The column was washed with Binding Buffer followed by Wash Buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris.Cl pH 7.9) to remove bacterial proteins. The recombinant protein was eluted in Elute Buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris.Cl pH 7.9). Fractions were analysed by SDS-PAGE. The recombinant protein was dialysed into PBS and concentrated to 1 $mg \cdot ml^{-1}$ using Vivaspin columns according to

manufacturers' instructions.

2.2.6.2 Extraction of proteins from *Drosophila* embryos.

Embryos were homogenised in Boiling Mix. Samples were boiled for 10 min before loading on an acrylamide gel. 10 μg- 100 μg was loaded per well for Western blot analysis.

2.2.6.3 Separation of proteins by SDS-PAGE

A 15% acrylamide resolving gel was prepared from 2.8 ml distilled water, 2.4 ml resolving gel buffer, and 4.8 ml 30% acrylamide. 30μl 25% ammonium persulphate and 3 μl TEMED was added to catalyse polymerisation. The stacking gel, prepared from 2.8 ml distilled water, 1.2 ml stacking gel buffer, 0.8 ml 30% acrylamide, 30 μl 25 % ammonium persulphate and 3 μl TEMED, was poured on top of this. Protein samples were mixed with an appropriate volume of 6 $\times$ boiling mix and boiled for 2 min before loading on the gel. Electrophoresis was carried out at 200 volts in 1 $\times$ TGS buffer.

2.2.6.4 Western blotting

Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Roche) in transfer buffer. Transfer was carried out in an electroblotter at 200 V for 90 min at 4°C. Detection was carried out using the Chemiluminescence Western Blotting Kit (Roche). Briefly, membranes were incubated in blocking solution (provided in kit) for 2 hours at room temperature with agitation. Primary antibody was diluted to an appropriate concentration in blocking solution and incubated with the membranes overnight at 4°C with no agitation. Membranes were washed twice in 1 $\times$ TBS, and twice in 0.5 $\times$ blocking solution, for 15 min each. HRP-conjugated secondary antibodies were added, diluted 1 in 2000-5000 in 0.5 $\times$ blocking solution, and incubated for 1 hour at room temperature. The membranes were washed four times for 15 min in 1 $\times$ TBS.

The chemiluminescent HRP substrate was added and the membranes exposed to X-ray film for 1-30 min.

2.2.6.5 Dot blots

Protein was applied directly to strips of PVDF membrane in spots of 1 μl. Detection was carried out as for Western blots.

2.2.6.6 Affinity purification of antibodies

20 μg recombinant protein was electrophoresed in each lane of an SDS-PAGE gel and transferred to nitrocellulose. The filter was blocked for one hour at room temperature, then the lanes at each end of the filter were cut off and probed with antiserum, washed and probed with HRP-conjugated secondary antibody as described in section 2.2.6.4. The bands were detected on the filter using 3-3′ diaminobenzidine (DAB) as a HRP substrate. These were used as guidance markers to cut out slices containing the band of interest from the rest of the filter. The slices were incubated with a 1 in 10 dilution of antiserum overnight at 4°C. Unbound antibodies were removed by washing 3 times for 15 minutes in PBS. Bound antibodies were eluted by incubating filter slices in 180 μl 100 mM glycine, pH 2.5 for 10 min. The antibody solution was then neutralised by adding 20 μl 1 M Tris pH 8.2.

2.2.6.7 Immunostaining of whole embryos

Embryos prepared as described in section 2.2.3.3 were rehydrated in PTW. Immunostaining was carried out using the ABC Vectastain kit (Vector Laboratories) following manufacturers' instructions. Briefly, embryos were blocked by incubating in 5% normal horse serum for 2 hours at room temperature, then incubated overnight at 4°C with the appropriate concentration of primary antibody. After washing 4 times for 15 minutes in PBST (PBS with 0.03% TritonX-100), the embryos were incubated with a prediluted biotinylated secondary antibody,

then washed and incubated with a streptavidin-HRP conjugate. After washing, the embryos were incubated in 3,3′-diaminobenzidine (DAB) until a signal was detected.

2.2.7 Computer methods

2.2.7.1 Database queries

DNA and protein sequences were retrieved from GenBank using the Entrez search engine (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed). *Drosophila* EST sequences were retrieved from the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org). Information on *Drosophila* genes was retrieved from FlyBase (http://flybase.bio.indiana.edu), GadFly (http://hedgehog.lbl.gov:8000/cgi-bin/annot/query) and GeneSeen(http://www.fruitfly.org/annot/geneseen-launch-static.html). Genes are referred to by their GenBank names, or acccession numbers where specified. Predicted *Drosophila* genes and their products are referred to by their GadFly identifiers, which begin with the letters CG.

2.2.7.2 Similarity searches.

Sequence similarity searches were carried out using the Basic Local Alignment Search Tool (BLAST). Sequences were searched against GenBank from the National Center for Bioinformatics website (http://www.ncbi.nlm.nih.gov/BLAST) and against *Drosophila* sequences from the Berkeley *Drosophila* Genome Project (http://www.fruitfly.org/BLAST). Default parameters were used in all searches.

2.2.7.3 Sequence alignments.

Protein sequence alignments were carried out using the CLUSTALW algorithm provided by the Multiple Alignment General Interface (MAGI) at the Human Genome Mapping Project website (http://www.hgmp.mrc.ac.uk). Default parameters were used in all alignments.

2.2.7.4 Primary sequence analysis

Motifs were identified in protein sequences by scanning them against the PROSITE database, and by using the Sequence Analysis Modular Architecture Research Tool (SMART). These analyses were carried out from the ExPasy website (http://www.expasy.ch).

Results

Chapter 3

Expression analysis of genes encoding components of the ubiquitination pathway

3.1 Introduction

Ubiquitination was once thought of only as a housekeeping process which mediated the turnover of damaged and misfolded proteins. While this is an essential function, it is now clear that the ability to destroy the right protein at the right time also provides the cell with a powerful regulatory mechanism. Since multicellular organisms have many more E2s and E3s than yeast, it seems likely that they exploit the regulatory power of the ubiquitin-proteasome pathway to control the processes of differentiation and development.

Drosophila melanogaster has proved a powerful model for understanding the mechanisms which control embryogenesis. In this project, I aimed to identify developmental processes in which ubiquitin-mediated proteolysis of specific proteins is used as a regulatory mechanism. I focussed on the process of embryogenesis due to the greater body of research on the molecular mechanisms of this developmental stage than of larval or pupal development. I hypothesised that E2s and E3s with roles in specific processes of embryogenesis would have correspondingly specific spatial and temporal distributions, whereas those enzymes required for housekeeping would be present in every cell. I therefore undertook an anal-

ysis of the embryonic expression patterns of genes encoding components of the ubiquitination pathway.

When the project was begun, there was a putative E1, five putative E2s and one putative E3 known in *Drosophila*. The gene encoding the E1, *Duba1*. was cloned by a degenerate PCR approach followed by screening of λ libraries (zur Lage, unpub). The first E2 identified was Dhr6/UbcD6, the *Drosophila* homologue of *S. cerevisiae RAD6* (Koken *et al.*, 1991a). It was shown to rescue the defects in U.V. resistance and U.V. - induced mutagenesis in the *S. cerevisiae rad6* deletion strain (Koken *et al.*, 1991a). The genes encoding UbcD1 and UbcD2 were identified in a hybridisation screen to identify ubiquitin-conjugating enzymes in model multicellular organisms (Matuschewski *et al.*, 1996; Treier *et al.*, 1992). They were shown to partially rescue yeast *ubc4ubc5* double mutants. Cenci *et al.* (1997) reported that mutations in *UbcD1* cause frequent end-to-end association of chromosomes during meiosis and mitosis. The *effete* gene, originally identified as a male-sterile mutation (Castrillon *et al.*, 1993), was later shown to be identical to *UbcD1*. There were no reports on the physiological role of UbcD2 in *Drosophila*. The *UbcD3/bendless* gene was discovered as a mutation which affected patterns of neuronal connections in the developing central nervous system (Oh *et al.*, 1994; Thomas and Wyman, 1984). In *bendless* mutant flies, the giant fibre axon fails to bend, and hence fails to connect with the tergotrochanter muscle. Kirby (1996) used a degenerate PCR approach to identify new *Drosophila* E2s, which resulted in the cloning of *UbcD4*.

The only known candidate for a *Drosophila* Hect-domain E3 was Hyperplastic Discs (Hyd), which controls proliferation of imaginal disk cells in the larva (Mansfield *et al.*, 1994). Supernumerary limbs (Slimb) was the only known *Drosophila* F-box protein, and was therefore a putative component of an SCF - type E3. *Slimb* was identified as a mutation which caused ectopic activation of Hedgehog and Wingless signalling (Jiang and Struhl, 1997).

I identified novel E2 and E3 - encoding genes by screening cDNA sequences deposited in the EST (Expressed Sequence Tag) database produced by the Berkeley *Drosophila* Genome Project. I have analysed the embryonic transcription pattern of these novel genes as well as many of the genes mentioned above.

3.2 Expression of the E1-encoding gene *Duba1* during development

3.2.1 Developmental Northern analysis

I isolated total RNA from flies at various stages of development : from embryos aged 0-3 hours, 3-8 hours and 8-22 hours, from larvae, pupae and adults. I probed a Northern blot of this RNA using radiolabelled *Duba1* cDNA (Figure 3.1). No transcripts were detected in 8 - 22 hour embryos. At all other stages, two transcripts were detected in approximately equal amounts. Transcript levels appeared to be highest during the first eight hours of embryogenesis.

The largest known *Duba1* cDNA (GenBank accession Y15895) is 4337 bp in size. On a Northern blot, the *Duba1* probe hybridised to a band of approximately 4.3 kb and also to a larger transcript, of approximately 5 kb. This is unlikely to represent cross-hybridisation to a similar transcript, as there are no *Drosophila* genes highly similar to *Duba1*. I carried out 5′ and 3′ rapid amplification of cDNA ends (RACE) analysis in order to investigate whether *Duba1* transcripts with extra 5′ and 3′ sequences exist.

3.2.1.1 3′ RACE analysis of *Duba1*

Total embryonic RNA was reverse transcribed using the primer RACE-1, which consists of an oligo-dT sequence preceded by an anchoring nucleotide and a 22-nucleotide sequence which is used as a priming site for subsequent PCR amplification. The 3′ ends of *Duba1* transcripts were amplified by PCR using the primers DUBA1-1 and RACE-2. DUBA1-1 corresponds to positions 4189-4207 of the

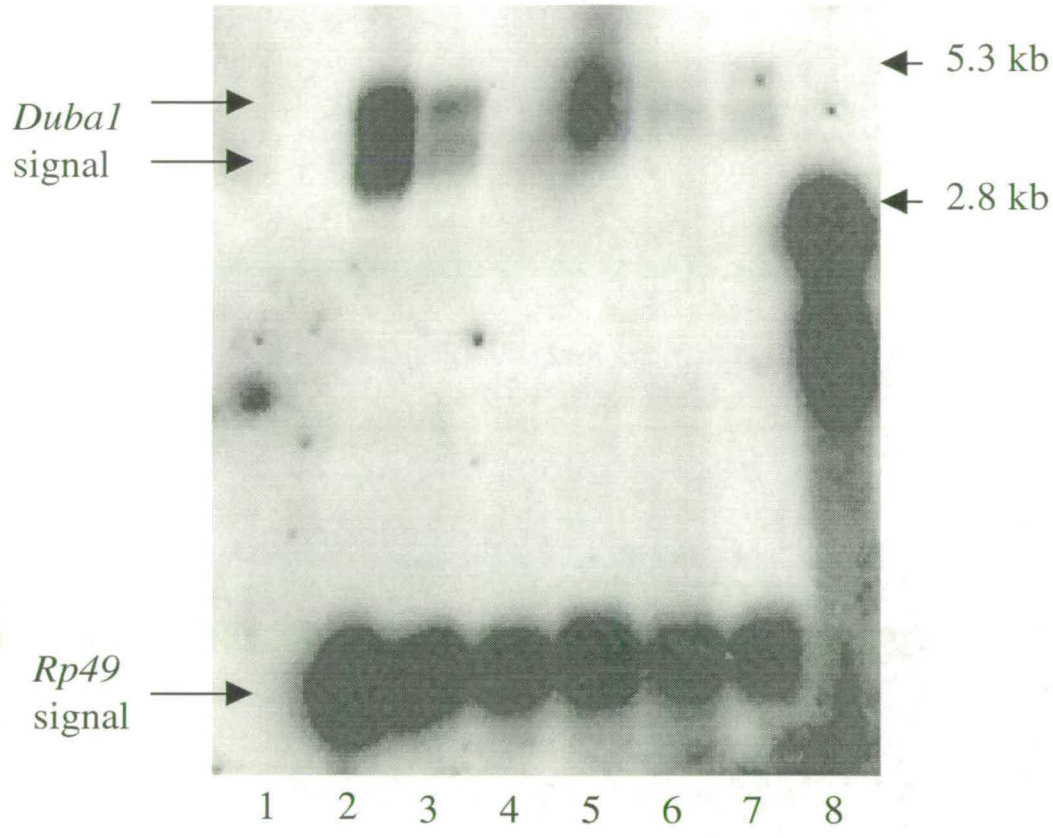


Figure 3.1: Developmental Northern analysis of *Duba1* expression.
The blot was sequentially probed with *rp49* (loading control) and *Duba1*. Each lane contains 2 μg of total RNA isolated from flies at the following stages of development : 2 - 0-3 hour embryos : 3 - 3-8 hour embryos : 4 - 8-22 hour embryos : 5 - larvae of mixed ages : 6 - pupae : 7 - adults. Lane 1 contains *E. coli* ribosomal RNA and lane 8 contains DIG-labelled RNA molecular weight markers. The arrows on the right indicate the positions of bands from the molecular weight markers after detection with AP-conjugated anti-DIG.

Duba1 'plus' strand. RACE-2 corresponds to the priming site introduced to the cDNA ends by RACE-1. The sequences of all primers are given in Table 2.5. After two rounds of amplification a band of approximately 130 bp was observed. Sequencing confirmed that this product resulted from amplification of a 4337-nt *Duba1* transcript. No larger bands were observed, either by subsequent rounds of PCR amplification or by blotting the PCR products onto nylon filters and probing using a *Duba1* probe (Figure 3.2). The larger band on the developmental Northern is therefore unlikely to reflect *Duba1* transcripts with extra 3′ sequence.

3.2.1.2 5′ RACE analysis of *Duba1*

The 5′ ends of *Duba1* cDNAs were reverse transcribed from total embryonic RNA using the reverse primer DUBA1-2, which corresponds to positions 124-107 on the *Duba1* 'minus' strand. 3′ polyA tails were added to the resulting cDNA using terminal transferase, and PCR was carried out using the primers RACE-1 (described above) and DUBA1-3, which corresponds to positions 85-104 on the *Duba1* 'minus' strand. This would be expected to yield a 104 bp product from the a *Duba1* 4.3 kb transcript. A transcript with extra 5′ sequence would yield larger products. After two rounds of amplification, two bands were observed on a gel, sized approximately 200 and 150 bp. Sequencing showed that these products derived from the 5′ end of *Duba1* and extended upstream of the reported cDNA sequence (GenBank accession Y15895). When the gel was blotted onto a nylon filter and and probed with radiolabelled *Duba1* DNA, a smear of larger and smaller products was observed (Figure 3.2).

This analyis indicates that *Duba1* transcription can initiate from a site upstream from that reported. It can be difficult to identify the true transcriptional start site using RACE analysis because secondary structure can impede reverse transcription of the full-length RNA. It is possible that two major PCR products observed both reflect true transcriptional start sites. These would give similarly-sized transcripts : the 50 bp size difference may not have been resolved

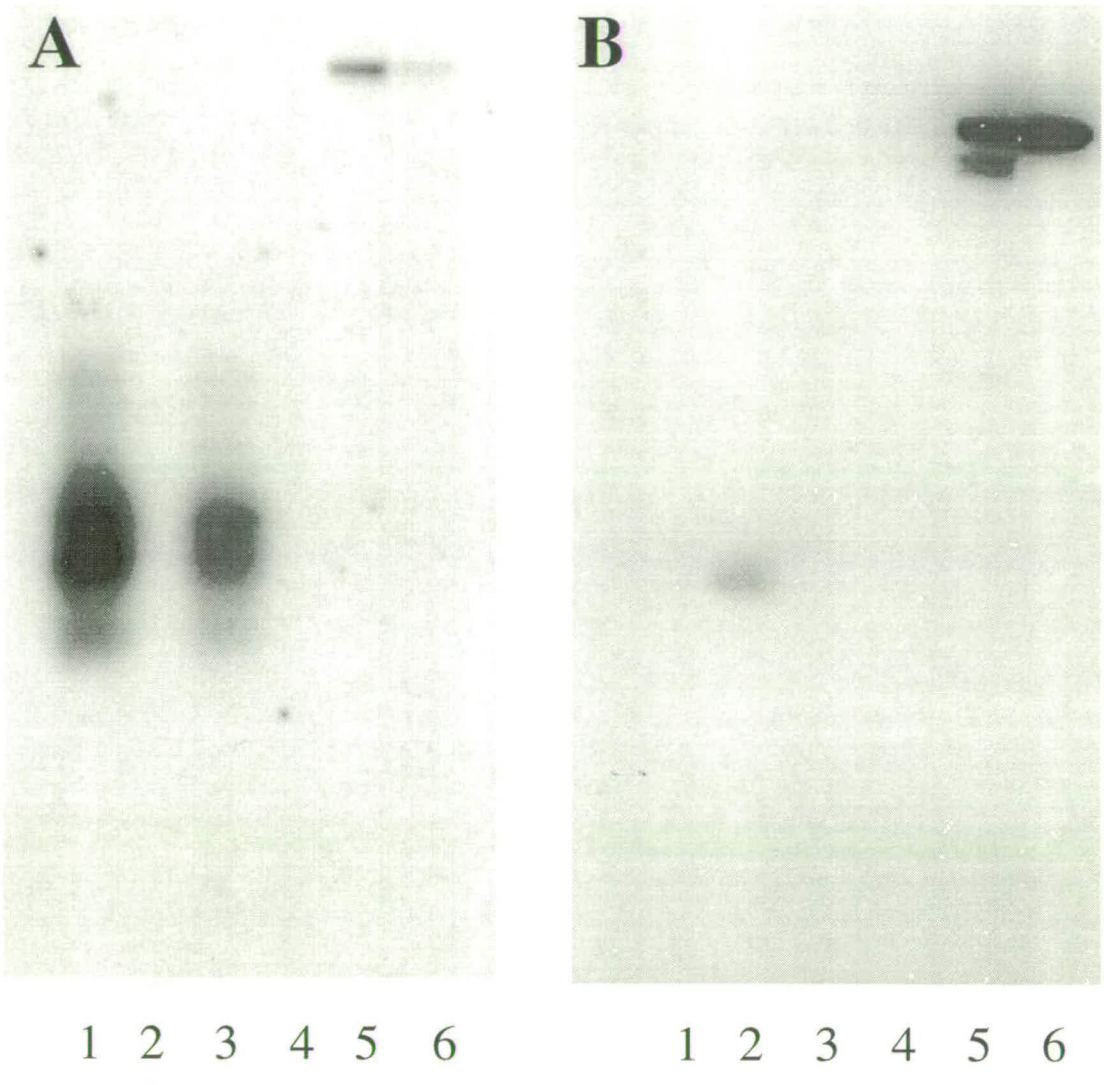


Figure 3.2: 5′ and 3′ RACE analysis of *Duba1* transcripts.
PCR products resulting from amplification of the 5′ or 3′ ends of *Duba1* cDNAs were blotted and probed with the full-length radiolabelled *Duba1* cDNA. As a negative control, amplifications were carried out on cDNA ends to which polyA tails were not added. As a positive control for hybridisation, the full-length *Duba1* cDNA in pBluescript was loaded.
A. 5′ RACE products. Lane 1 - PCR products from embryonic cDNA. Lane 2 - Negative control PCR products from embryonic cDNA. Lane 3 - PCR products from adult cDNA. Lane 4 - Negative control PCR products from adult cDNA. Lane 6 - *Duba1* cDNA. Lane 1 - HindIII-digested λ DNA.
B. 3′ RACE products. Lane 1 - PCR products from embryonic cDNA. Lane 2 - PCR products from adult cDNA. Lane 3 - negative control PCR products from embryonic cDNA. Lane 4 - negative control PCR products from adult cDNA. Lane 5 - HindIII-digested λ DNA. Lane 6 - *Duba1* cDNA.

on the Northern blot. Alternatively, the larger product may reflect a region of the transcript where reverse transcription frequently terminated due to secondary structure. If the 5 kb band on the Northern blot was a *Duba1* transcript with extra 5′ sequence, a PCR product of approximately 800 bp would be expected. It is possible that cDNA with the intact 5′ sequence was present in such small quantities that PCR products were not produced in quantities detectable on a gel. However the largest of the smear of PCR products observed after blotting and probing was approximately this size and may correspond to the full-length cDNA. Together with the Northern analysis, these results suggest that there is a *Duba1* transcriptional start site approximately 700 bp upstream of the reported site, and that the two transcripts are produced in approximately equal amounts.

3.2.2 Embryonic *in situ* hybridisation analysis of *Duba1*

In order to detect the spatial expression pattern of *Duba1* during embryogenesis, *in situ* hybridisation of a DIG-labelled antisense mRNA probe on wholemount embryos was performed (Figure 3.3). The probe was detected using anti-DIG antibodies conjugates to alkaline phosphatase (AP). When the embryos were stained with AP substrates, a purple colour was produced where the probe had hybridised to mRNA. *Duba1* sense RNA probes were used as a negative control.

Maternal *Duba1* transcripts are abundant in freshly laid eggs, decreasing slightly during cellularisation. Zygotic expression of *Duba1* is high in anterior and posterior midgut primordia and the extending germband during stages 6-10. In germband-retracted embryos, expression is high in gut and nervous system, particularly in the head. After dorsal closure, expression is highest in the foregut and nervous system, particularly the brain. *Duba1* expression is low and uniform in stage 15-17 embryos.

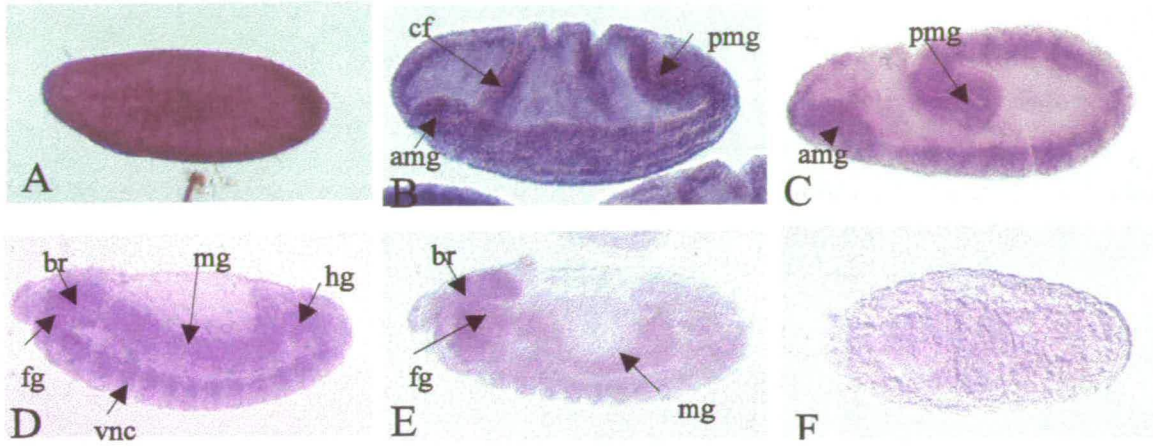


Figure 3.3: *In situ* hybridisation analysis of *Duba1* expression. Embryos are oriented with anterior to the left and dorsal uppermost.A - stage 1-3. B - stage 6. C - stage 8. D - stage 12. E - stage 14. F - stage 15 . Abbreviations: amg=anterior midgut, br=brain, cf=cephalic furrow, fg=foregut, hg=hindgut, mg=midgut, pmg=posterior midgut, vnc=ventral nerve cord.

3.2.3 Discussion

Since ubiquitination is required for housekeeping, and Duba1 is expected to catalyse an essential first step in the pathway, it is to be expected that the gene would be transcribed in all cells. I found that *Duba1* transcripts are abundant in RNA extracted from flies at all developmental stages except for 8 - 22 hour embryos. *In situ* hybridisation showed that *Duba1* transcripts were detectable in all tissues at all stages, but were extremely low in embryos which had completed dorsal closure and head involution (approximately 13 hours). There is an apparent discrepancy between the results of Northern analysis and of *in situ* hybridisation for embryos aged 8 - 13 hours. It seems likely that, in the RNA sample from 8-22 hour embryos used in the Northern, the transcripts from 8-13 hour embryos were underrepresented compared to those from 13 - 22 hour embryos, and were therefore not detected. Both results suggest there is little ubiquitination during late embryogenesis. The high level of expression in gut and nervous system may reflect particularly high levels of ubiquitin-mediated proteolysis in these tissues.

3.3 Expression analysis of known E2-encoding genes

I carried out *in situ* hybridisation analysis of the embryonic expression patterns of all five known E2-encoding genes (Figure 3.4). *UbcD1* and *Dhr6* transcripts are widely distributed, particularly in gut and nervous system primordia. Expression of *UbcD2* is detectable above background only in the cellular blastoderm and in the nervous system of stage 12-13 embryos. As previously reported, (Mansfield *et al.*, 1994), *UbcD3* is highly expressed in the embryonic nervous system, from the ventral ectodermal neurogenic region in gastrulae to the brain and central nervous system of stage 15-17 embryos. The expression pattern of *UbcD4* is described in section 3.5.

3.4 Expression analysis of novel genes encoding putative E2s and E3s

3.4.1 Identification of cDNAs encoding putative E2s and E3s from an Expressed Sequence Tag (EST) database.

I used UbcD4 and UbcD1 as query sequences in BLAST searches of six-frame translations of the EST database produced by the Berkeley *Drosophila* Genome Project (BDGP). The searches were performed in September 1997. From the resulting 'hits' with an Expect value of less than 6, I discarded ESTs which only hit one of the query sequences, or which encoded known E2s. The searches resulted in the identification of two putative E2-encoding cDNA sequences, each represented by several EST clones. I obtained a cDNA fragment representing each of the two sequences from the BDGP. The cDNAs were termed *LD06921* and *LD10212*. Both cDNAs came from a library derived from 0-22 hr embryos. The alignment of the *LD10212* and *LD06921* products with UbcD1 and UbcD4 are shown in Figure 3.5.

I used a similar method to identify novel genes encoding Hect domain proteins,

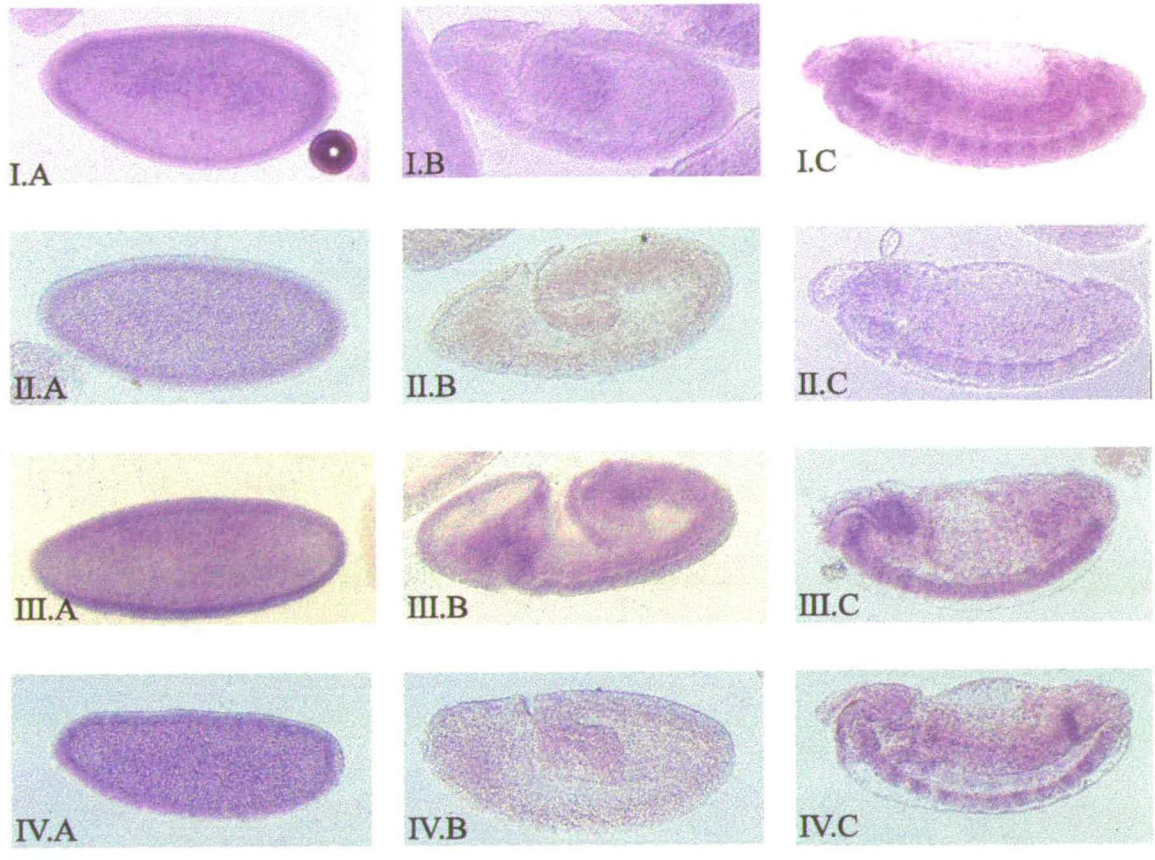


Figure 3.4: *In situ* hybridisation analysis of *UbcD1*, *UbcD2*, *UbcD3* and *Dhr6* expression.
Embryos are oriented with anterior to the left and dorsal uppermost. I.A-C : *UbcD1* expression. II.A-C: *UbcD2* expression. III.A-C : *UbcD3* expression. IV.A-C : *Dhr6* expression. A : stage 4-5 embryos. B - stage 8-9 embryos. C - stage 12-14 embryos.

which are predicted to have ubiquitin-ligase activity. I used the Hect domains of the human E6-associated protein (E6-AP), and the *S. cerevisiae* Rsp5 protein, as query sequences. Again I identified two novel cDNA sequences encoding putative E3s, and obtained representative cDNA fragments. These were termed *HL01334* and *HL02858*. Both came from a library derived from adult heads. The alignment of the *HL01334* and *HL02858* products with the C-termini of E6-AP and Rsp5 is shown in Figure 3.6.

Finally I searched for potential components of SCF complexes. By using the yeast Cdc53 and Skp1 as query sequences I identified a putative *Drosophila* homologue of each. The Skp1 homologue was encoded by EST clone *LD19283* from the embryonic cDNA library. The Cdc53 homologue was encoded by a full-length cDNA, called *lin-19* for its similarity to the *C. elegans lin19* gene. *C. elegans lin19* had since been renamed *cul1* (Kipreos *et al.*, 1996) but the similarity of the *Drosophila lin-19* to the cullin proteins had not been noted. The alignments of the *Drosophila* cullin and Skp1 homologue with the corresponding proteins from yeast, nematodes and humans are shown in Figures 3.7 and 3.8. Despite carrying out searches with several F-box proteins I failed to identify any convincing novel members of this family in *Drosophila*.

3.4.2 Embryonic *in situ* hybridisation analysis of novel E2 - and E3 - encoding genes.

In situ hybridisation analysis was performed as described previously. Both of the putative E2 genes, *LD10212* and *LD06921* are weakly but detectably expressed in gut primordia. *HL01334* and *HL02858* transcripts are present at low levels in gut and nervous system primordia from gastrulation onwards. Both the putative SCF components are transcribed at high levels in all tissues throughout embryogenesis (Figure 3.10).

Since the expression analysis of the novel E2 and E3 genes failed to indicate specific roles for them in embryogenesis, they were not investigated further.

Figure 3.5: CLUSTALW alignment of the novel E2s encoded by *LD10212* and *LD06921* with UbcD4 and Ubcd1. The full sequences encoded by the EST cDNA clones LD10212 and LD06921, and the full UbcD1 sequence, are shown. The UbcD4 sequence omits the C-terminal 50 residues. Identical residues are shaded in black and similar residues are shaded grey.

Figure 3.6: CLUSTALW alignment of the novel E3s encoded by *HL01334* and *HL-2858* with the Hect domains of E6-AP and Rsp5. The E6-AP and Rsp5 sequences shown start respectively at positions 601 and 667 of the full-length proteins and finish at the C-termini. The full sequences encoded by the EST cDNA clones *HL02858* and *HL01334* are shown. Identical residues are shaded in black and similar residues are shaded grey.

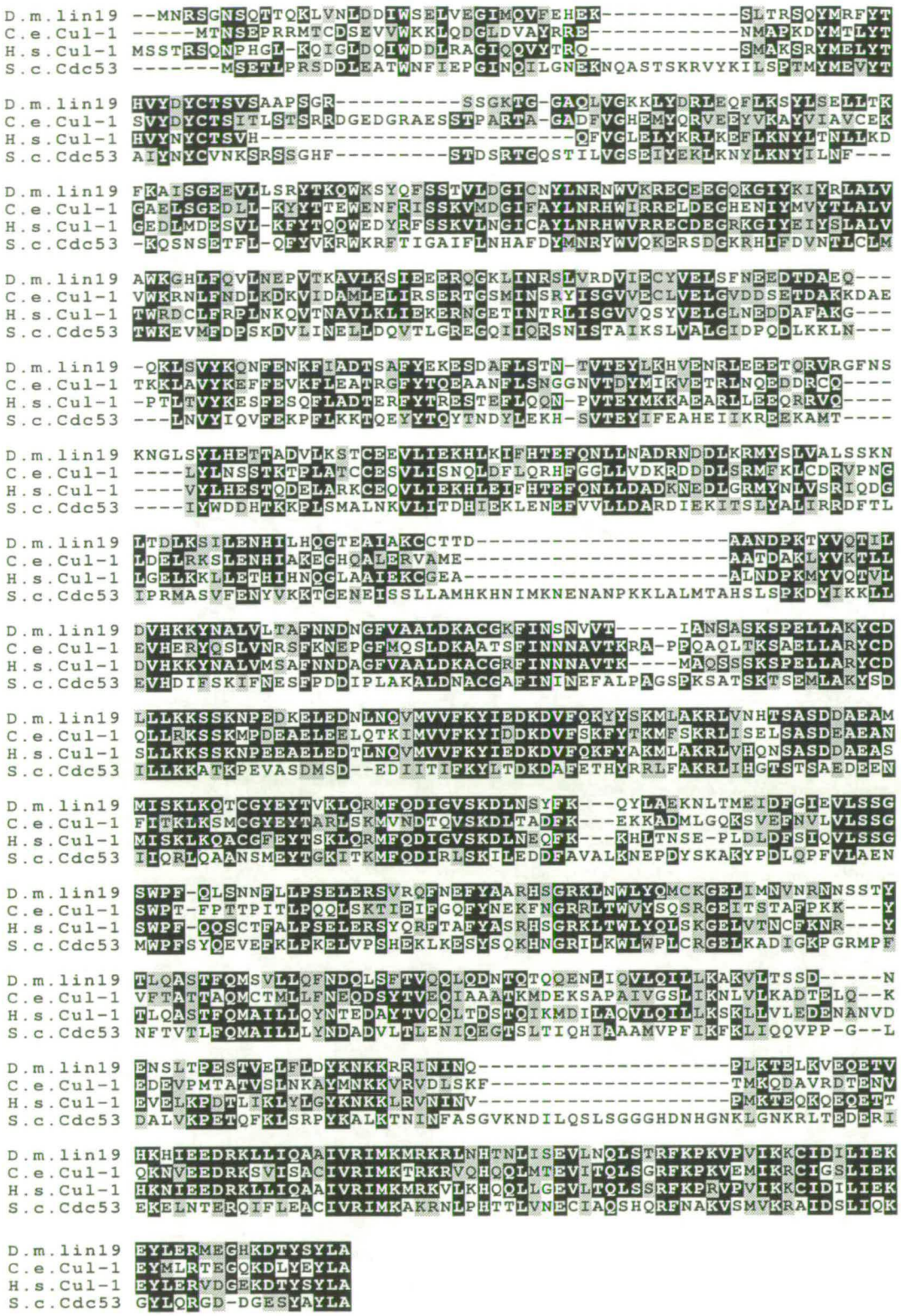


Figure 3.7: CLUSTALW alignment of the putative *Drosophila* cullin (lin19) with yeast Cdc53, *C. elegans* cul-1 and human Cul-1. Identical residues are shaded black and similar residues are shaded grey. Abbreviations : D.m.= *Drosophila melanogaster*, S.c.=*Saccharomyces cerevisiae*, C.e. = *Caenorhabditis elegans*, H.s. = *Homo sapiens*.

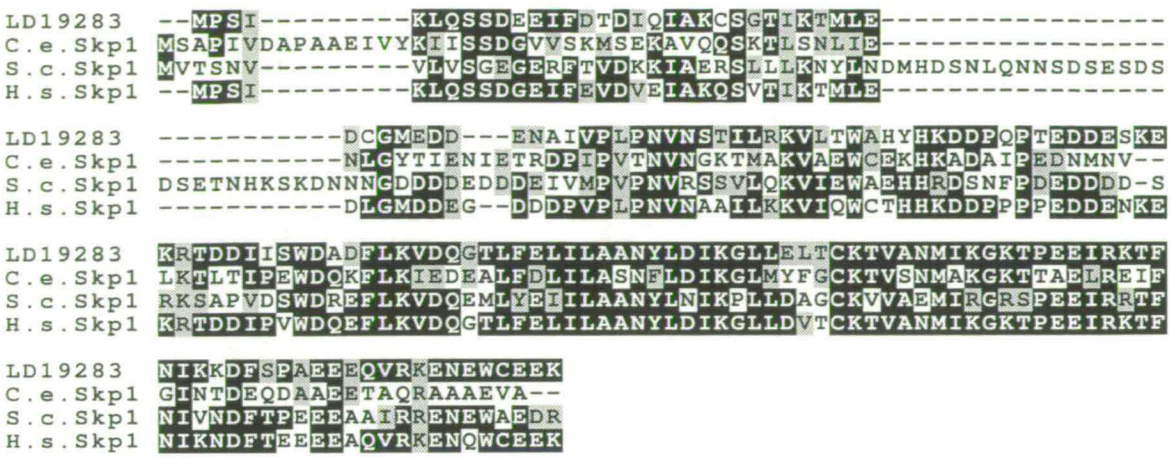


Figure 3.8: CLUSTALW alignment of the novel Skp1-related protein encoded by *LD19283* with the yeast, nematode and human Skp1 proteins. Identical residues are shaded black and similar residues are shaded grey. Abbreviations : S.c.=*Saccharomyces cerevisiae*, C.e. = *Caenorhabditis elegans*, H.s. = *Homo sapiens*.

Since this analysis was carried out, *HL02858* has been identified as *Suppressor of deltex, Su(dx)* (GenBank accession AAD38975) (Cornell *et al.*, 1999). Mutational analysis suggests that Su(dx) is a negative regulator of Notch signalling (Fostier *et al.*, 1998). It is expressed in the pupa and prepupa. HL01334 is now called HERC2 (GenBank accession AAF61856). As well as its Hect domain, it has a motif found in guanyl-nucleotide exchange factors. LD10212 and LD06921 are now called UbcD10 (GenBank accession AAF57787) and CG2257 (GenBank accession AAF46318) respectively. There is no further information on the expression patterns or physiological role of either these or of HERC2. LD01343 and LD19283 are now designated Cul-1 (GenBank accession AAD33676) and SkpA (GenBank accession AAF64674) respectively. Expression of *Drosophila cul1* in a yeast *cdc53* mutant strain rescues the G1 arrest (Filippov *et al.*, 2000). The participation of SkpA in ubiquitination has not been demonstrated. Since they are predicted to form the catalytic core of the SCF complex, rather than the substrate recognition part, it is perhaps not surprising that their expression is universal in embryos. On the other hand, multicellular organisms have multiple cullins and Skp1-related proteins, presumably reflecting some degree of functional

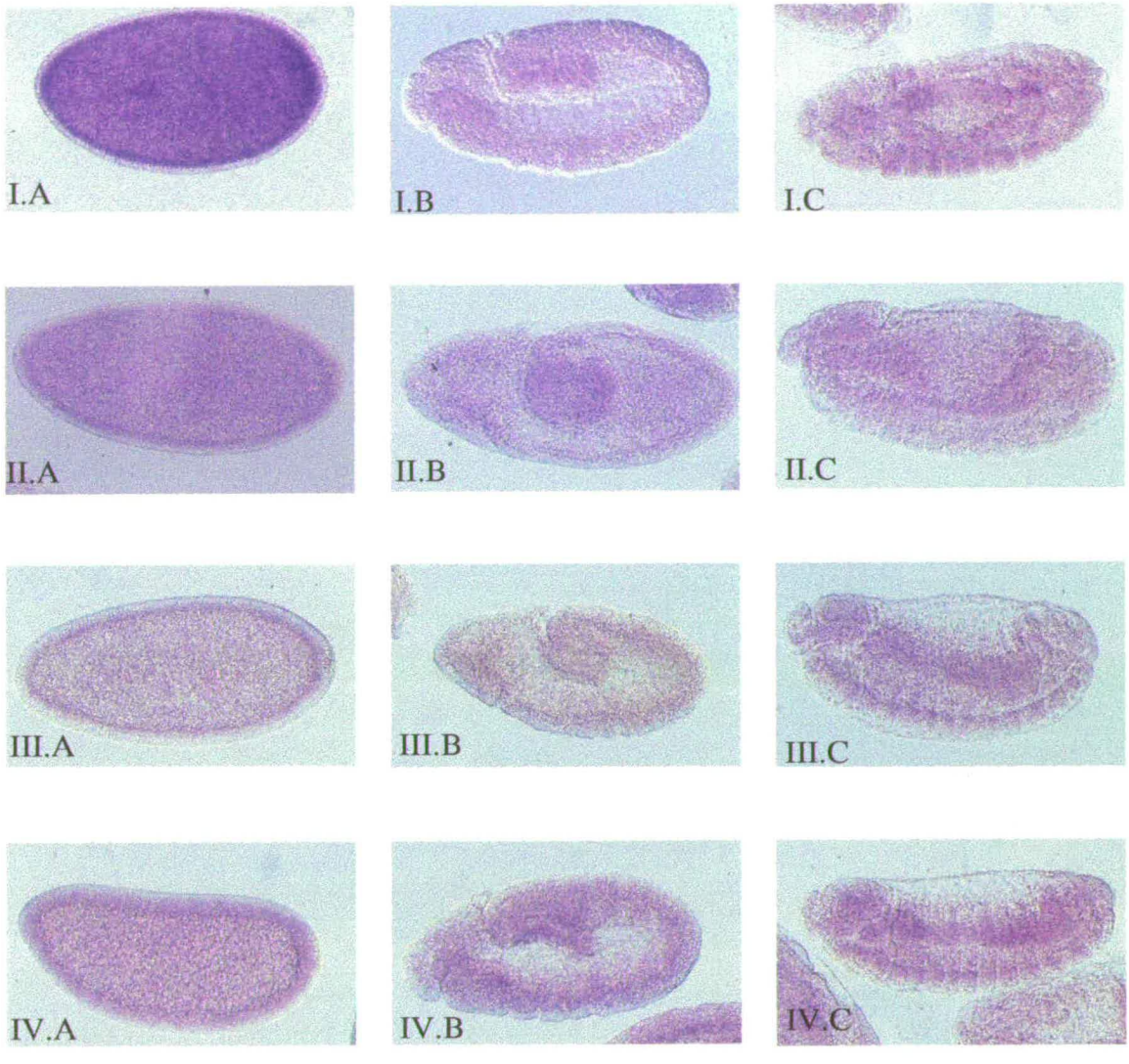


Figure 3.9: *In situ* hybridisation analysis of *LD06921*, *LD10212*, *HL02858* and *HL01334* expression in embryos.
Embryos are oriented anterior to the left and dorsal uppermost. I.A-C : *LD06921* expression. II.A-C : *LD10212* expression. III.A-C : *HL02858* expression. IV.A-C *HL01334* expression. A - stage 4-5 embryos. B - stage 8-9 embryos. C - stage 12-14 embryos.

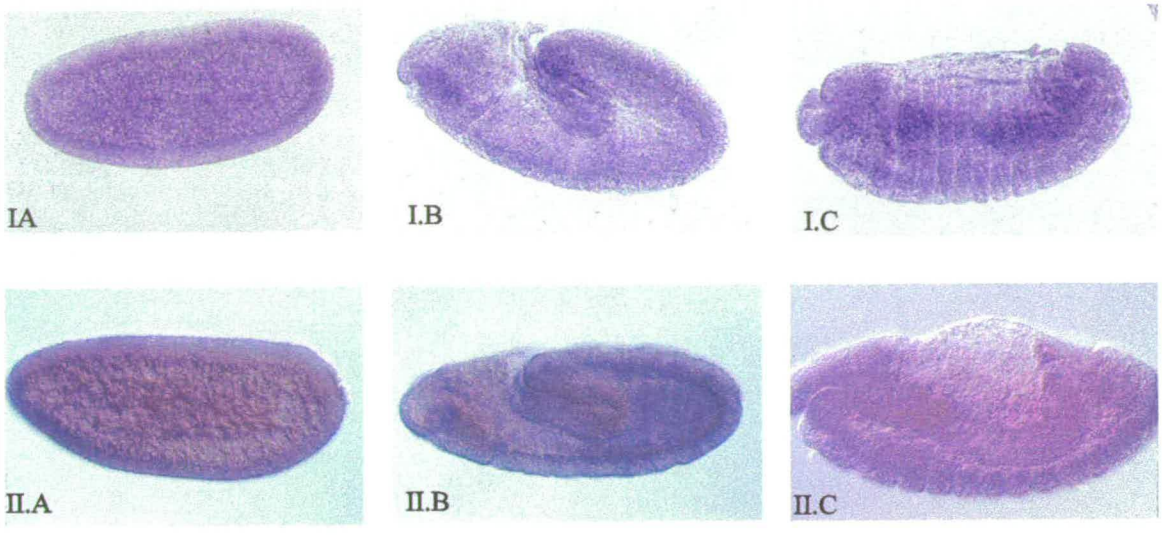


Figure 3.10: Embryonic *in situ* hybridisation analysis of *cul-1* and *skpA* expression. I.A-C : *cul-1* expression. II.A-C : *skpA* expression. A - stage 4-5 embryos. B - stage 8-9 embryos. C - stage 12-14 embryos.

diversity. The complete *Drosophila* genome encodes a total of six putative cullins and seven putative Skp1 homologues. There are no reports on their physiological roles.

3.5 Expression analysis of *UbcD4*

3.5.1 Developmental Northern analysis of *UbcD4* expression

I probed a developmental Northern blot, made as described previously, with radiolabelled *UbcD4* cDNA (Figure 3.11). The probe hybridised to two RNA bands, both of which were present only in the RNA extracted from 0-3 and 3-8 hour embryos. Probing of the blot with an *rp49* probe showed that the absence of a signal in the remaining lanes was not due to unequal loading of RNA. The two transcripts detected were approximately 1.1 kb and 600 bp in size. The larger transcript has the size predicted from the *UbcD4* cDNA (GenBank accession Y11394). The significance of the smaller band is unknown. It may represent processing of the transcript.

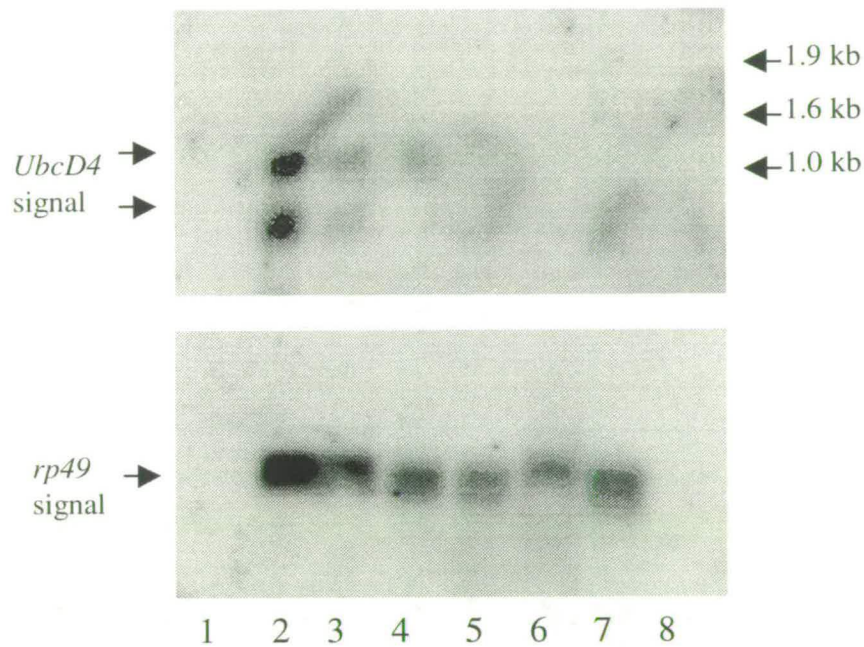


Figure 3.11: Developmental Northern analysis analysis of *UbcD4* expression. Each lane contains 2 μg of total RNA isolated from flies at the following stages of development : 2 - 0-3 hour embryos : 3 - 3-8 hour embryos : 4 - 8-22 hour embryos : 5 - larvae of mixed ages : 6 - pupae : 7 - adults. Lane 1 contains *E. coli* ribosomal RNA and lane 8 contains DIG-labelled RNA molecular weight markers. The arrows on the right show the positions of the markers after detection with AP-conjugated anti-DIG. The bottom panel shows the result of probing the same blot with *rp49*.

3.5.2 Embryonic *in situ* hybridisation analysis of *UbcD4* expression.

Hybridisation and detection of a DIG-labelled *UbcD4* RNA probe to 0-22 hour embryo revealed a dynamic expression pattern (Figure 3.12). Maternal transcripts were abundant in 0-2 hour embryos, decaying slightly as cellularisation progressed. During gastrulation, expression appears to be highest in the invaginating posterior midgut primordium, with high expression also in the cephalic furrow and ventral ectodermal neurogenic region. *UbcD4* transcripts are abundant in the anterior midgut primordium and extending germband during stage 5-7. In stage 10 embryos, expression is very low throughout most of the embryo, but high in a cluster of cells in the pocket formed by the posterior midgut primordium. In stage 11 embryos, overall expression remains low, but a group of the *UbcD4* positive cells can be observed on the dorsal side of the PMG pocket. These appear to split into two groups on either side of the ventral midline. During germband retraction, transcription reinitiates in many tissues, especially the gut and nervous system. After dorsal closure, *UbcD4* transcripts are detectable at low levels throughout the embryo.

The spatial and temporal specificity of *UbcD4* transcription suggested that it was the best candidate for a ubiquitination enzyme which mediates specific developmental decisions during *Drosophila* embryogenesis. Particularly interesting was the expression pattern during maximal germband extension. The position of the cluster of *UbcD4* positive cells in stage 10-11 embryos suggested that they may be the primordial germline or 'pole' cells.

3.5.3 Investigation of whether *UbcD4* transcription is restricted to the germ cells in stage 10-11 embryos.

The primordial germ cells cellularize at the posterior pole of the embryo during stage 5 of embryogenesis. They divide asynchronously with the soma to form a cluster of 30-35 cells. During gastrulation, the pole cells adhere to the posterior

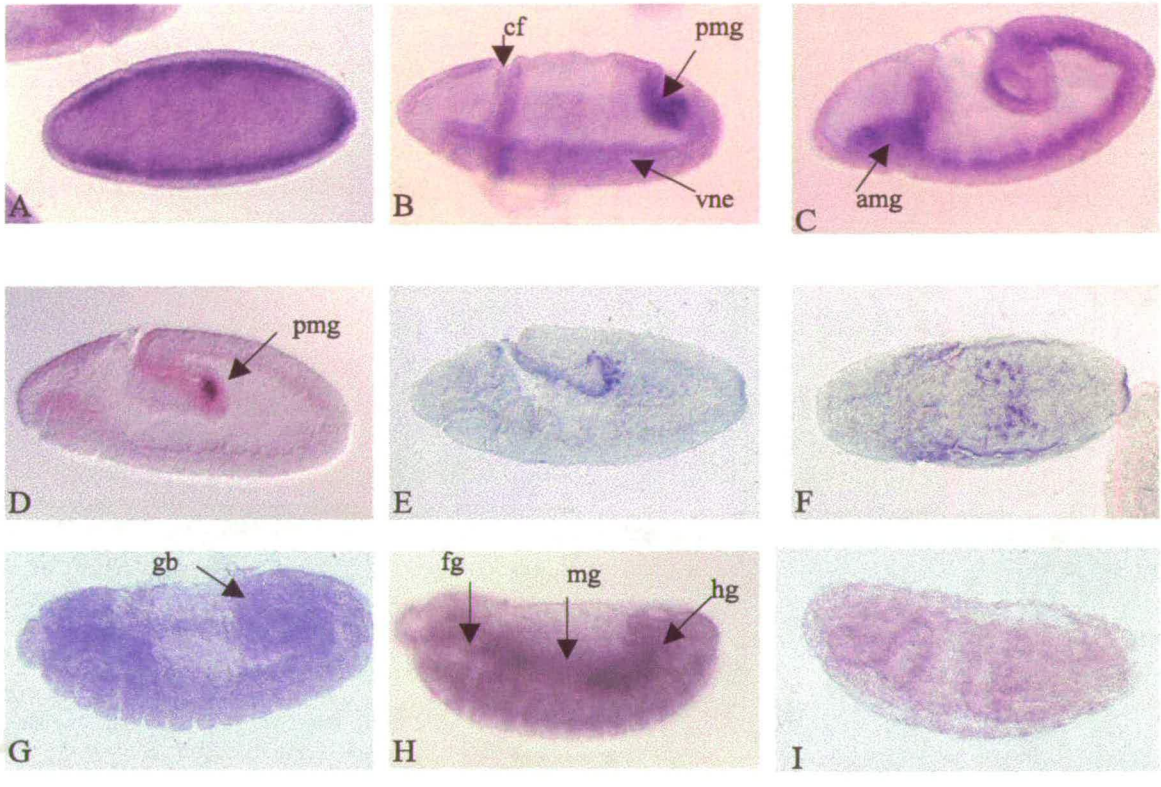


Figure 3.12: Embryonic *in situ* hybridisation analysis of *UbcD4* expression.
A : stage 4-5 embryo. B : stage 6. C : stage 7-8. D : stage 9-10. E : stage 11. F : stage 11, dorsal view. G : stage 12. H: stage 13-14. I: stage 16-17. Abbreviations: amg=anterior midgut, cf=cephalic furrow, fg=foregut, gb=germband, hg=hindgut, mg=midgut, pmg=posterior midgut, vne=ventral neurogenic ectoderm.

midgut primordium (PMG) and become incorporated into the pocket formed by PMG invagination. The pocket moves anteriorly during germband extension, to reach approximately 50% egg length. When the germband is maximally extended, during stage 10, the pole cells migrate through the wall of the PMG towards the lateral mesoderm in parasegments 11-13. They separate into two groups, one on either side of the ventral midline. During germband retraction, the germ cells associate with the mesodermal somatic gonadal precursor (SGPs) in parasegments 10-12. The SGPs and germ cells coalesce to form the gonad during stage 15.

3.5.3.1 Staining stage 10-11 embryos for *UbcD4* mRNA and Vasa protein

Vasa is a protein specifically expressed in germcells throughout embryogenesis. We carried out *in situ* hybridisation using a *UbcD4* probe, and subsequent immunostaining using anti-Vasa antibodies, on 5-7 hour embryos. The results demonstrate that the *UbcD4* positive cells in stage 10-11 embryos are also Vasa-positive, and therefore are the germline cells (Figure 3.13).

3.5.3.2 Analysis of *UbcD4* transcription patterns in embryos lacking germ cells

The Δ-*gcl* strain of flies carry a null allele of *germcell-less, (gcl)* over the balancer chromosome *CyO* (see Table 2.4). Maternal contribution of *gcl* mRNA is essential for formation of germ cell precursors. Approximately 80% of embryos derived from *gcl* homozygous females fail to form pole cells. I collected embryos from *gcl* homozygous mutant females, identified by lack of the *CyO* balancer, and performed *UbcD4 in situ* hybridisation and Vasa immunostaining. The majority of embryos were Vasa-negative and these embryos also showed no *UbcD4* expression during stage 10-11 (Figure 3.13). In the few embryos with Vasa-positive cells, these cells were also *UbcD4* positive. The expression pattern is similar to that in wildtype embryos in all other respects (data not shown).

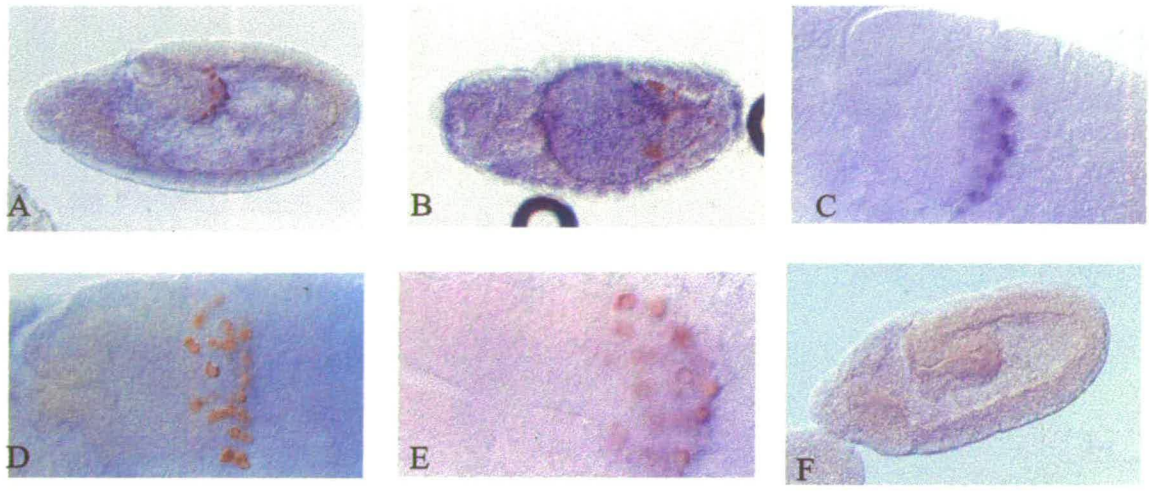


Figure 3.13: Analysis of *UbcD4* and Vasa colocalisation in stage 10-11 embryos. A,B,F: embryos photographed under 10× objective lens. C,D,E: embryos viewed under 40× objective lens. A: *UbcD4* and Vasa staining in stage 10 embryo. B: *UbcD4* and Vasa staining in stage 12 embryo, dorsal view. C: *UbcD4* staining in stage 10-11 embryo. D: Vasa staining in stage 10-11 embryo. E: *UbcD4* and Vasa staining in stage 10-11 embryo.F: *UbcD4* and Vasa staining in stage 10 embryo from *gcl* mother.

3.6 Production of recombinant UbcD4 and polyclonal antisera

The presence of transcripts in a tissue does not guarantee that the corresponding protein is also present, since its expression may be under post-transcriptional control. The distribution of a protein is therefore likely to provide a more accurate indication of function than that of an mRNA. In order to analyse the distribution of the *UbcD4* gene product, I attempted to raise polyclonal antibodies which could be used in immunostaining of embryos.

3.6.1 Production of recombinant UbcD4 in *E. coli*

A construct consisting of the *UbcD4* coding sequence flanked by *EcoRI* and *BamHI* restriction sites was synthesised by PCR and sequenced (described in section 4.2.1). This was cloned into the *E. coli* expression vector pET15b (see Table 2.3), which encodes a 'tag', including six histidine residues, upstream of the multiple cloning site. Transcription of the tag and downstream sequence is under the con-

trol of the T7 promoter, which is inducible with IPTG. The recombinant plasmid was sequenced to ensure that the tag was in frame with the *UbcD4* coding sequence. It was then transformed into the *E. coli* strain BL21(DE3)pLysS (see Table 2.1). IPTG was added to mid-log cultures derived from a single transformant. Cells were allowed to synthesise recombinant UbcD4 for three hours, then pelleted and sonicated. Recombinant UbcD4 was found primarily in the soluble fraction (Figure 3.14). The presence of the six histidines in the N-terminal tag allowed the recombinant UbcD4 to be purified by nickel chelation chromatography. Impurities were removed by washing with buffers containing progressively higher levels of imidazole. UbcD4 eluted in 350 mM imidazole. No copurifying proteins were detectable by Coomassie staining of SDS-PAGE gels (Figure 3.14). Approximately 3 mg recombinant UbcD4 was obtained per litre of *E. coli*. A mock purification was carried out on a culture to which IPTG was not added. No overexpressed products the size of UbcD4 were observed in the cell pellets, and no proteins remained bound to the nickel column after washing with 120 mM imidazole (Figure 3.14).

3.6.2 Production of polyclonal anti-UbcD4 sera in mice

Recombinant UbcD4 was dialysed into PBS and concentrated to 1 $mg \cdot ml^{-1}$. 100 μg was injected into each of 10 mice. Two mice were injected with PBS, to obtain control sera. The injections were repeated after one month and again after two months. A week after the third injection, the mice were sacrificed and bled. The injections and bleeding were performed by P. Loke at the Institute for Cell, Animal and Population Biology, University of Edinburgh.

3.6.2.1 Testing the specificity of recognition of recombinant UbcD4 by Western blotting

Preliminary testing of the antisera on dot blots and Western blots established that a dilution of 1 in 2000 was optimal for detection of recombinant UbcD4.

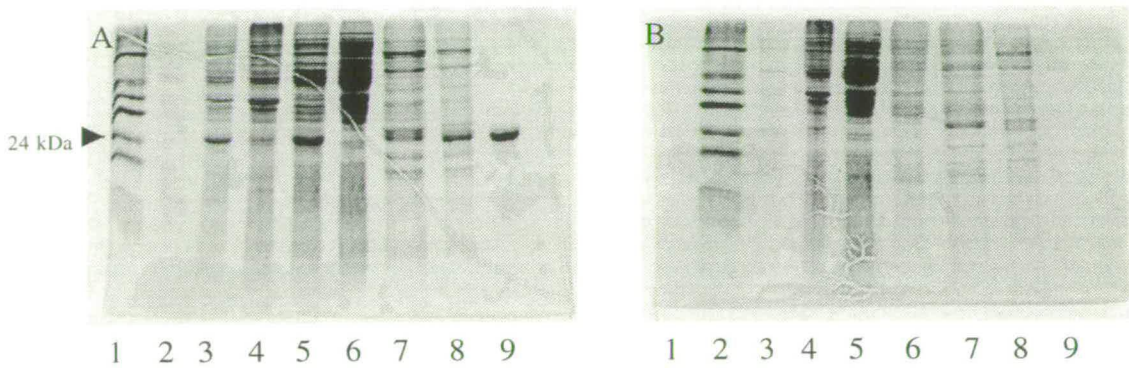


Figure 3.14: Expression and purification of recombinant UbcD4.
A. Protein fractions from cells induced with IPTG. Lane 1 - molecular weight marker. Lane 2 - total cell protein before induction. Lane 3 - total cell protein 3 hours after induction. Lane 4 - total insoluble fraction. Lane 5 - total soluble fraction. Lanes 6 - 9 are fractions of soluble proteins from nickel affinity chromatography. Lane 6 - proteins which do not bind the nickel column. Lane 7 - 10 mM imidazole wash. Lane 8 - 120 mM imidazole wash. Lane 9 - pure UbcD4 eluted in 350 mM imidazole.
B. Mock purification : protein fractions from cells in the absence of IPTG. Lane 2 - molecular weight marker. Lane 3 - total cell protein. Lanes 4 - 9 : as for A.

The control sera showed no recognition of UbcD4. The antisera from the immunised mice were not very sensitive, as less than 10 ng of UbcD4 could not be detected. No significant differences were observed between the results obtained using antisera from individual mice, and pooled antisera.

When tested against the purest fractions of UbcD4, the equivalent of the preparation which had been used for injection, the antisera detected only one band, of the size expected for UbcD4 (approximately 25 kDa). However when tested against a less pure fraction, which included some co-purifying bacterial proteins, a stronger band of a larger size was observed, in addition to the UbcD4 band (Figure 3.15). This suggests that a highly immunogenic bacterial protein was present in the injected preparation, at levels too low to be detected by Coomassie staining of SDS-PAGE gels, and that the majority of antibodies produced recognise this protein rather than UbcD4.

No recognition of endogenous UbcD4 was observed on Western blots of em-

bryonic protein extracts. A lane containing 10 μg of total embryonic protein yielded a smear of bands, while 1 μg yielded no bands (Figure 3.15). The antisera were preabsorbed on 15-20 hour embryos and used to immunostain 0-22 hour embryos. 15-20 hour embryos were used for preabsorption because, based on their mRNA levels, they would be expected to have lower levels of UbcD4 than younger embryos. No specific staining was observed in anti-UbcD4 - stained embryos compared to those stained with the control sera (data not shown).

In order to purify antibodies with high affinity for UbcD4, I cut out small slices, corresponding to the position of the UbcD4 band, from a nitrocellulose filter to which the recombinant protein preparation had been transferred. These slices were incubated with antisera. Unbound antibodies were removed by washing, and the antibodies which had remained bound to UbcD4 were eluted using an acidic glycine solution. When tested on Western blots, the antisera specifically recognised recombinant UbcD4, even when bacterial contaminants were also present. However no bands were observed in the embryonic extract (Figure 3.15). No specific immunostaining of embryos was obtained from the purified antibodies (data not shown). As the antibodies apparently failed to recognise endogenous UbcD4, possibly due to a conformational difference between the recombinant and endogenous proteins, they were not used in any further experiments.

3.7 Discussion

In general, the expression patterns of the genes analysed here give little indication that ubiquitinating enzymes commonly mediate specific developmental decisions during embryogenesis. Those genes which are transcribed in embryos generally show widespread distribution, often with slightly increased expression in gut or nervous system (e.g. *UbcD1, LD06921, HL02858*). Strong expression is generally not observed in embryos after stage 15. The transcription pattern of the E1 gene, *Duba1*, is consistent with these trends. This may give an overall indication of the

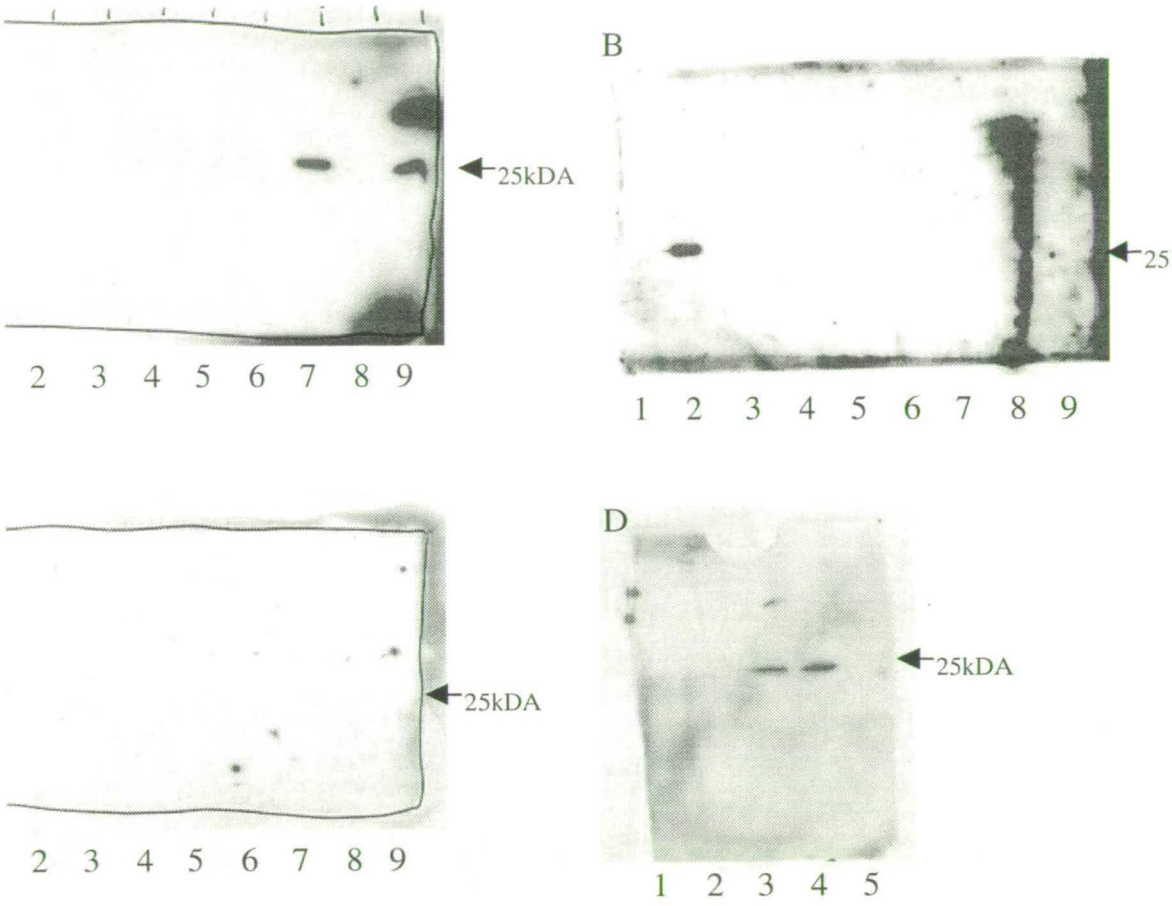


Figure 3.15: Analysis of UbcD4 antisera using Western blotting.
A : Testing pooled unpurified antisera against recombinant UbcD4. Lanes 1 - 7 contain the following amounts of purified UbcD4 : Lane 1 - 1 pg: Lane 2 - 5 pg: Lane 3 - 10 pg : Lane 4 - 50 pg : Lane 5 - 500 pg : Lane 6 - 5 ng : Lane 7 - 50ng. Lane 8 - molecular weight marker. Lane 9 : 50 ng protein from a UbcD4 preparation which includes some contaminating bacterial protein.
B : Testing pooled unpurified antisera against recombinant UbcD4 and embryonic proteins. Lane 1 - molecular weight marker. Lane 2 - 20ng UbcD4. Lane 3 - 2 ng UbcD4. Lane 4 - 200 pg UbcD4. Lane 5 - 20 pg UbcD4. Lanes 6 and 7 - empty. Lane 8 - 10 μg total embryonic protein. Lane 9 - 1 μg total embryonic protein.
C : Testing control sera against recombinant UbcD4 and embryonic proteins. Lanes as for B.
D : Testing affinity-purified UbcD4 antibodies against recombinant UbcD4 and embryonic proteins. Lane 1 - 2 μg total embryonic proteins. Lane 2 - 10 μg embryonic proteins. Lane 3 - 50 ng protein from a UbcD4 preparation containing some copurifying proteins, as for lane 9 in A. Lane 4 - 50 ng UbcD4.
The arrowheads indicate the position on the filter of the 25 kDa band of the prestained molecular weight markers.

relative ubiquitination levels in various tissues and stages, since its expression is a prerequisite for the functioning of any E2 or E3.

The exception to the trend of non-specific expression is *UbcD4*. In stage 10-11 embryos, *UbcD4* is transcribed specifically in germ cells. This is not a commonly-observed expression pattern. None of the other genes, encoding components of the ubiquitin pathway, whose expression was also analysed, showed a similar pattern. They were either not expressed in germ cells at all or, in the case of *Duba1*, transcripts were so abundant throughout the embryo that the germ cells could not be discerned. In the FlyBase database less than forty genes are reported to be transcribed in germ cells, and the majority of these do not show specific germ cell expression at any stage. Thus the presence of *UbcD4* transcripts in germcells at this stage may be functionally significant.

The most obvious process occurring in germ cells during stage 10-11 is their movement through the gut wall towards the somatic gonadal precursors in the lateral mesoderm. It is interesting to speculate on whether UbcD4-mediated proteolysis could play a role in controlling germ cell migration. Perhaps destruction of a target protein is needed for normally sessile cells to initiate movement. Alternatively, the UbcD4 target may repress a component of the signal transduction pathway which allows the germ cells to respond to guidance cues.

The molecular basis of germ cell migration is poorly understood. The third chromosome has been exhaustively screened for zygotic mutations specifically affecting gonad formation (Moore *et al.*, 1998). The genes identified fall into several classes, each of which appears to direct a discrete step in the process. *serpent* and *huckebein* are needed for pole cell invasion of the gut wall. Attachment of the germ cells to the somatic mesoderm requires *zfh-1, columbus* and *heartless*. Coalescence of germ cells with gonadal mesoderm requires *abdominal-A* and *-B*, *trithorax*, *tinman* and *fear -of-intimacy*.

In most cases, the molecular basis for the effect of the mutation on gonad

formation is not clear. However the products of two genes, *wunen* and *columbus*, appear to directly guide movement of germ cells. *columbus*, which is expressed in gonadal mesoderm, encodes a homologue of HMG-CoA reductase (HMGCR) (Van Doren *et al.*, 1998). Ectopic expression of *columbus/hmgcr* can redirect germ cell migration to the novel site of expression (Van Doren *et al.*, 1998). The loss-of-function *hmgcr* mutations which disrupt germ cell migration are all in the catalytic domain (Van Doren *et al.*, 1998). These observations suggest that germ cells are attracted by a factor produced by HMGCR enzymatic activity. HMG-CoA reductase synthesises mevalonate, which in mammals is a cholesterol precursor. In *Drosophila*, mevalonate is a precursor of various lipid biosynthetic and protein modification pathways. It is not yet known which pathway produces the signal attracting germ cells. The *wunen* product, a phospatidic acid phosphatase type 2 (PAP2) (Zhang *et al.*, 1997), is also putatively involved in lipid metabolism. Misexpression experiments show that Wunen activity appears to repel germ cells (Zhang *et al.*, 1997). The significance of the finding that both *wunen* and *columbus* encode proteins with putative roles in lipid metabolism is not clear. It is possible, for example, that the same lipid-derived signal is produced by *columbus*-expressing cells and degraded by *wunen* - expressing cells, or vice versa. Alternatively they could produce two independent guidance cues with opposite effects on germ cells.

It is remarkable that the screen performed by Moore *et al.* (1998) failed to identify any genes, required for gonad development, which function in the germ cells. Possibly such mutations would have a maternal effect only. Maternal contribution of two factors, *nanos (nos)* and *polar granule component (pgc)*, is required for pole cell migration. *pgc* produces a noncoding RNA (Nakamura *et al.*, 1996). Nos is a zinc-finger protein which acts as a posterior morphogen but which has a separate role in germline development. Lack of germline Nos activity causes pole cells to remain clustered on the distal surface of the gut instead of migrating dorsally (Forbes and Lehmann, 1998). Nos presumably exerts its effect on germ

cell migration via its role as a regulator of transcription.

Studies of other cell migration events in *Drosophila* embryos may shed light on the molecular mechanisms of germ cell migration. These events include the migration of tracheal precursor cells during formation of the respiratory system, and the migration of mesodermal precursor cells during gastrulation. Signalling by fibroblast growth factor receptors, such as Breathless and Heartless, has been implicated in both systems (reviewed by Forbes and Lehmann (1999)).

Two features which distinguish the germline from the soma during early to mid-embryogenesis are its lack of mitotic activity and its transcriptional quiescence. Newly-formed pole cells divide asynchronously until cellularisation, when their number has reached approximately thirty. From this point until their incorporation into the gonad, they do not divide further. The arrest seems to be at the G2-M transition (Su *et al.*, 1998). Whereas zygotic transcription begins in the soma at the blastoderm stage, pole cells in the early embryo shut down RNA polymerase II (RNAP II) - dependent transcription (Seydoux and Dunn, 1997). This correlates with the absence until stage 7 of a specific RNAP II phosphoepitope which is present in transcriptionally active somatic cells (Seydoux and Dunn, 1997). In addition to its role in migration, Nanos has been implicated in downregulating both transcription and mitosis in pole cells. In pole cells lacking Nos activity, several genes normally only expressed in the gonad are prematurely expressed (Seydoux and Dunn, 1997; Kobayashi *et al.*, 1996). nos^{-} pole cells also continue dividing after the cellular blastoderm stage, a defect which is presumably due to transcriptional deregulation (Seydoux and Dunn, 1997). It is not clear whether Nos is needed for regulation only of specific genes or whether it also plays a role in the global repression of RNAP II transcription. However it is striking that the inappropriate presence of proteins normally found only in the soma, or in the germline after gonadal coalescence, can cause multiple defects in germ cells at stage 10-11. In this context it is particularly interesting that the general

repression of RNAP II may be relieved at stage 7, but that many genes appear to remain specifically repressed until gonad formation. Perhaps ubiquitin-mediated proteolysis has a role to play in destruction of unwanted proteins, including cell-cycle proteins, whose premature appearance in germ cells would disrupt their development.

Chapter 4

Identification of proteins which interact with UbcD4

4.1 Introduction

Protein-protein interactions are an intrinsic part of nearly every cellular process, including ubiquitin-mediated proteolysis. An essential aspect of studying the function of a protein of interest is to identify other proteins which interact with it. In the case of a ubiquitin-conjugating enzyme, this approach may identify other components of the pathway which mediate target ubiquitination, as well as the targets themselves.

Traditionally, the tools available to study interactions have been based on biochemistry (reviewed by Phizicky and Fields (1995)). More recently, a variety of methods have been developed which allow the screening of libraries for genes or fragments of genes whose products interact with a test protein. These have the great advantage that the cDNA encoding the interacting protein is immediately available. The technique which has had the greatest impact on interaction cloning methodology is the yeast two-hybrid system (Chien *et al.*, 1991; Fields and Song, 1989). This system detects protein-protein interactions via the activation of reporter gene expression. It relies upon the modular structure of many eukaryotic transcription factors, which are composed of a DNA binding domain and a transcriptional activation domain. The two domains need not not be cova-

lently linked and can be brought together by the interaction of other proteins to which they are fused. The two hybrid sreen requires the construction of hybrid genes that encode a DNA-binding domain fused to a target protein (the 'bait'), and an activation domain fused to a test protein (the 'prey'). Interaction between the bait and prey reconstitutes a functional transcription factor which stimulates expression of reporter genes. Nutritional selection can be included by using a biosynthetic gene as a reporter (Figure 4.1).

The selection procedure used in the yeast two-hybrid system facilititates the screening of a large number of coding sequences. It is also more sensitive than biochemical methods, due to overexpression of the hybrid proteins and amplification of the signal by the production of multiple stable reporter enzymes from each transcript. Unlike biochemical methods which require multiple washing steps, a high rate of dissociation does not preclude detection of an interaction.

The are several disadvantages to the yeast two-hybrid system. Some bait proteins, when fused to a DNA binding domain, are themselves capable of activating transcription. The expression levels, posttranslational modifications, folding, stability, and nuclear localisation of the fusion proteins can all affect the results. Another limitation is the frequent detection of spurious interactions ('false positives'). The system can identify proteins which, although capable of interaction, never associate in the relevant organism because they are normally present in different cells, or in different cellular compartments, or at different times. Many false positives seem to be dependent on promoter context, and so their occurrence can be reduced by scoring for two reporter genes with minimal sequence similarity in their promoters. There are many possible reasons why genuine interactors may not be recovered ('false negatives'). Prey proteins expressed as fusions may be unable to act as transcriptional activators due to their folding and/or stability. Certain fusion proteins may be toxic to the yeast cell. As a result of these limitations, the magnitude of the interaction detected in a two-hybrid screen can-

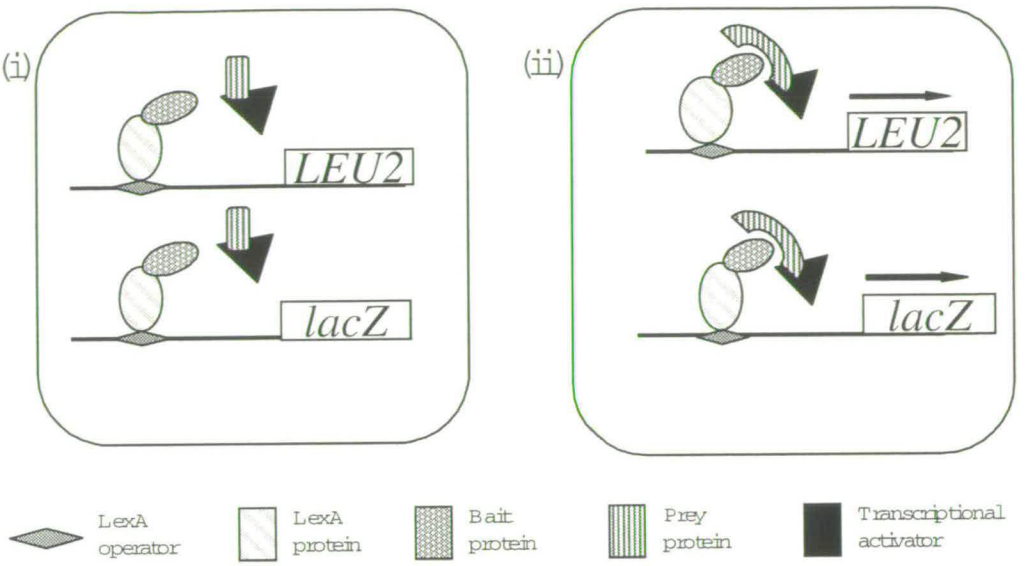


Figure 4.1: Schematic representation of the LexA-based interaction trap. The yeast strain used has a chromosomal *LEU2* gene, and a plasmid-borne *lacZ* gene with LexA operator sites directly upstream. A 'bait' protein fused to the LexA DNA-binding domain is coexpressed with a library of 'prey' proteins fused to a transcriptional activation domain. The bait-LexA hybrid protein binds the LexA operators but, in the absence of an interaction with prey protein, cannot activate transcription of the reporter genes (i). Interaction between bait and prey reconstitutes a transcriptional activator at the *LEU2* and *lacZ* promoters (ii).

not be correlated with its biological significance nor with the affinity of the two wild-type proteins in the relevant organism. Nonetheless the technique provides a powerful and versatile tool for identifying potentially important protein-protein interactions.

The first two-hybrid system was based on the yeast Gal4 DNA-binding domain (Fields and Song, 1989). An alternative system, developed by Gyuris *et al.* (1993), utilises the *E. coli* LexA protein and a yeast strain which has a dual *lacZ LEU2* reporter system responsive to transcriptional activation through the *LexA* operator. It has the important advantage that the screen can be performed in a $GAL4^+$ strain, and therefore the prey library can be expressed from the *GAL1* promoter. By testing whether reporter activation is dependent on galactose, mutational events which lead to reporter activation can be distinguished

from two-hybrid interactions.

The yeast two-hybrid system has proved invaluable in dissecting protein-protein interactions involved in ubiquitin-mediated proteolysis. Although homology-based cloning can be used to identify putative E3s within a specific family, interaction-based approaches must be taken to identify novel classes of E3 or ubiquitination 'ancillary proteins'. The increasing awareness that RING-finger proteins are common players in ubiquitination is partly a result of yeast two-hybrid screens. These include screens which set out to find substrates and/or cognate E3s for E2s of interest, and identified novel RING finger proteins (Moynihan *et al.*, 1999; Lorick *et al.*, 1999), as well as screens which used medically important RING finger proteins as bait and found that the basis for their effects lie in their participation in the ubiquitin pathway. For example, Yokouchi *et al.* (1999) reported that the tumour-suppressing activity of c-Cbl is due to its ability to promote the ligand-induced ubiquitination of the epidermal growth factor receptor, via an interaction with the E2 UbcH7.

Yeast two hybrid screens were used to identify the components of human SCF complexes. Using hCul1, the closest known Cdc53 relative in humans, as bait, Lyapina *et al.* (1998) isolated hSkp1, confirmed the association *in vivo* and showed that the two proteins formed an SCF-like complex *in vitro* with the F-box protein Skp2. Cenciarelli *et al.* (1999) searched for further human F-box proteins using hSkp1 as a bait, and isolated a total of 26. A modified version of the yeast two-hybrid system was used to elucidate protein-protein interactions involved in the formation of VBC complexes, which require the presence of more than two components (Pause *et al.*, 1999). Elongins B and C, when coexpressed in yeast, form a stable complex which interacts with the von Hippel-Lindau tumour suppressor protein (VHL). Only when all three of these proteins are expressed can a cullin, hCUL-2, associate to form a heterotetrameric complex. By using truncated proteins in the system, Pause *et al.* (1999) were able to show that the

elongin BC dimer acts as a bridge between the cullin and the putative substrate recruitment subunit VHL, reminiscent of the interactions which make up the SCF complex.

Similar screens have been used to dissect physical interactions between E2s and E3s. Closely related E2s show high specificity in their interactions with various Hect-domain proteins, and the degree of interaction correlates with the abilities of the various E2-E3 combinations to ubiquitinate substrates (Kumar *et al.*, 1997). Putative targets can be identified by using E3 proteins as baits. Whereas the mammalian Hect-domain E3 E6-AP was originally identified through its interaction with the viral E6 protein and subsequent ubiquitination of p53, two-hybrid screens have more recently revealed that its targets in uninfected cells include Src tyrosine kinases and the DNA repair protein HHr23a (Kumar *et al.*, 1999; Harris *et al.*, 1999; Oda *et al.*, 1999).

4.2 The UbcD4 interaction trap

I used the yeast two-hybrid system to screen for proteins which interact with UbcD4. UbcD4 is a class II ubiquitin-conjugating enzyme. These enzymes consist of the conserved catalytic UBC domain with a C-terminal extension. Some class II E2s demonstrate E3-independent substrate ubiquitination *in vitro*. Their C-termini are highly divergent, suggesting that they may be involved in selection of specific substrates. Since UbcD4 may interact directly with its substrates in the absence of a cognate E3, a yeast two-hybrid screen may identify putative UbcD4 substrates. Other UbcD4-interacting proteins would be expected to include its cognate E3, and any ancillary factors which mediate either E3 recognition, substrate ubiquitination or proteasomal docking of the E2-E3-substrate complex.

The steps involved in the UbcD4 interaction trap are summarised in Figure 4.2.

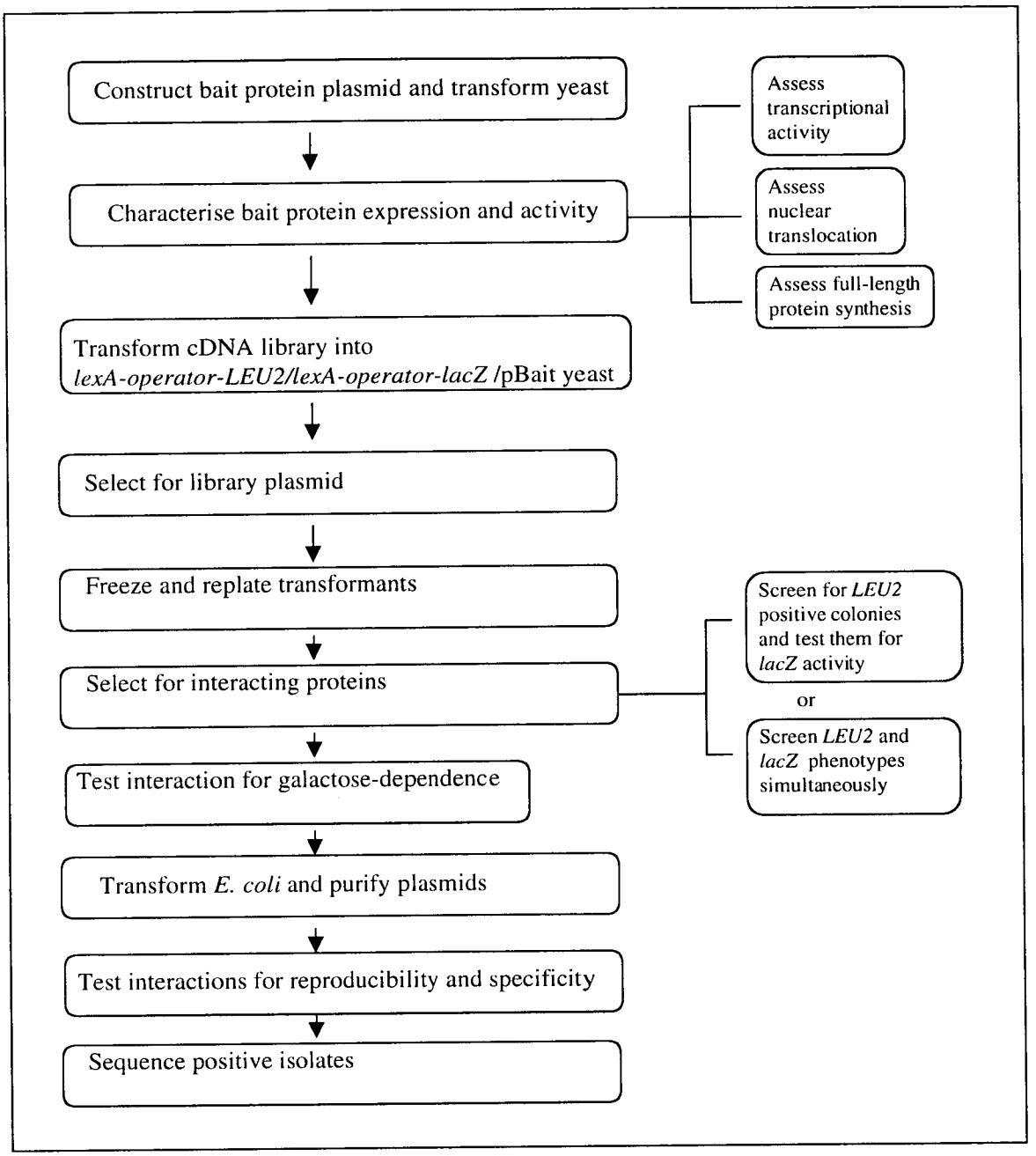


Figure 4.2: Flow chart for performing an interaction trap

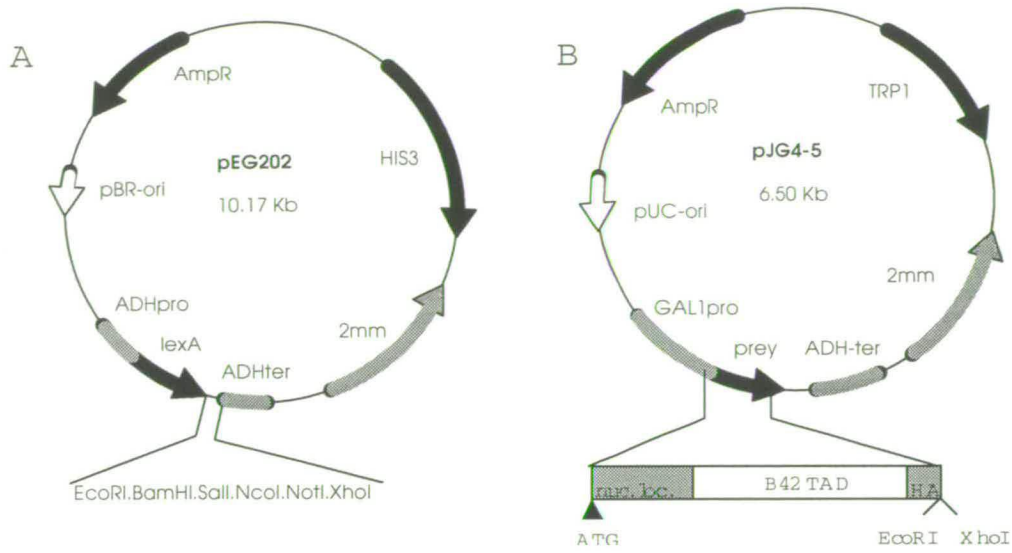


Figure 4.3: Maps of the plasmids used to make hybrid proteins for the UbcD4 interaction trap. A: pEG202 LexA fusion plasmid. *UbcD4* was cloned into the *EcoRI-BamHI* site. B: pJG4-5 B42 transcriptional activation domain (B42 TAD) fusion plasmid. A library of *Drosophila* cDNAs was cloned into the pJG4-5 *EcoRI-XhoI* site. Abbreviations: ADHpro = alcohol dehydrogenase promoter, ADHter = alcohol dehydrognase terminator, AmpR = ampicillin resistance gene, GAL1pro = *GAL1* promoter, HA = haemagglutinin epitope, HIS3 = *HIS3* selectable marker, nuc. loc. = nuclear localisation signal, pBRori = pBR322 origin of replication, pUCori = pUC origin of replication, TRP1 = *TRP1* selectable marker, 2mm = 2 μM origin of replication.

4.2.1 Construction of the *UbcD4* bait plasmid

Oligonucleotides complementary to the beginning and end of the *UbcD4* coding sequence were synthesised (D4.B1 and D4.B2 - see Table 2.5). The 'plus' and 'minus' strand oligonucleotides incorporated restriction sites for *EcoRI* and *BamHI* respectively. The coding sequence was amplified by PCR using Vent polymerase. A single PCR product of the expected size, approximately 600 bp, was obtained. Single 'A' overhangs were added by incubation with Taq polymerase and dATP, and the product was cloned into the pGEM-T cloning vector, which has single 'T' overhangs. The inserts of the transformants were sequenced until an insert with no mutations was identified. This was then subcloned into the *EcoRI/BamHI*-digested bait vector pEG202 (Figure 4.3). The insert was again sequenced to verify that the *UbcD4* sequence was in frame with that of *LexA*.

4.2.2 Preliminary tests on the LexA-UbcD4 hybrid protein

The bait construct was transformed into the EGY48 yeast strain (see Table 2.2) and various tests of its suitability were performed. The *lacZ* reporter plasmid used in the two-hybrid screen, p18-34, was transformed into EGY48 along with the bait vector. The transformants were plated on medium lacking leucine, and on medium containing X-gal, in order to ensure that the bait hybrid protein was unable to stimulate transcription of *LEU2* or *lacZ*. In both tests, the bait hybrid protein demonstrated no transcriptional activation ability. The pJK101 reporter was used to verify that the bait protein was able to enter the nucleus and bind the *LexA* operators. This reporter has a constitutively active *lacZ* reporter with *LexA* operator sites positioned such that binding of a *LexA* fusion protein represses its transcription. Colonies transformed with pJK101 alone were $lacZ^+$, whereas yeast transformed with both the bait vector and pJK101 were $lacZ^-$. Proteins were extracted from bait- expressing yeast and analysed by Western blotting using a LexA antibody. A band of the expected size, approximately 47kDa (22kDa LexA and 25kDa UbcD4), was observed after chemiluminescent detection, demonstrating that the full- length bait fusion protein is stable in yeast. These tests established that the LexA-UbcD4 bait and the EGY48 yeast strain were a suitable combination for use in a yeast two-hybrid screen.

4.2.3 Primary screening for bait-prey interactions

A library of *Drosophila* embryonic cDNAs fused to the B42 transcriptional activation domain, in the pJG4-5 plasmid, was obtained as a gift from R. Finley (see Figure 2.3). The hybrid genes are expressed from the *GAL1* promoter. The library had been generated from 3.5×10^6 independent transformants, with inserts sized 0.3 - 1.5 kb. EGY48 yeast, which had already been transformed with the bait and p1834 *lacZ* reporter plasmid, was transformed with 50 μg library

DNA, yielding 5.6×10^6 colonies. The yeast was scraped off the plates, washed, resuspended in glycerol and stored at -70 °C.

An aliquot of the transformed yeast was thawed and the replating efficiency was calculated. Sufficient yeast to yield 5×10^7 colony-forming units was thawed and incubated in galactose/raffinose medium without uracil, histidine or tryptophan (Gal/Raff/CM -Ura -His -Trp). Galactose was used to induce expression of the prey proteins. Raffinose was included because yeast grows poorly when galactose is the only carbon source, and raffinose does not cause catabolite repression of the *GAL1* promoter. After induction of library expression, the cells were plated on a total of 34 Gal/Raff/CM -Ura -His -Trp -Leu plates, to select for colonies with expression of the *LEU2* reporter gene. A total of 209 colonies, which grew in 3 days, were streaked onto master plates and then replica plated onto Glu/CM -Ura -His -Trp -Leu, Glu/Xgal/CM -Ura -His -Trp, Gal/Raff/Xgal/CM -Ura -His -Trp and Gal/Raff/CM -Ura -His -Trp -Leu to identify colonies which exhibited a galactose- dependent $lacZ^+LEU2^+$ phenotype. Only three such colonies were identified. In order to increase the efficiency of the screen, another 5×10^7 colony-forming units were plated on Gal/Raff/Xgal/CM -Ura -His -Trp -Leu, so that activation of both the *lacZ* and *LEU2* reporters could be assayed simultaneously. 54 blue colonies were obtained after 4 days incubation. 34 of these were found to have a reproducible galactose-dependent $lacZ^+LEU2^+$ phenotype. Plasmid DNA was isolated from these colonies and electroporated into the MC1066 strain of *E. coli*, which is $ura^-his^-trp^-$ (see Table 2.1). trp^+ transformants were selected and screened by PCR using the primers BCO1 and BCO2 (see Table 2.5), which flank the multiple cloning site on the library plasmid pJG4-5. PCR screening was repeated until at least three pJG4-5^+ colonies had been identified, and the sizes of the PCR products were compared. This was to ensure that each original yeast colony had only contained one prey plasmid. pJG4-5 plasmid DNA was isolated from the *E. coli*.

4.2.4 Reproducibility and specificity tests

In order to eliminate 'false positives', the specificity and reproducibility of the bait-prey interactions were tested. Reproducibility was tested by introducing the bait and reporter plasmids into EGY48 yeast and then transforming these cells with each of the putative UbcD4-interacting prey plasmids. The transformants were streaked onto Gal/Raff/CM -Ura -His -Trp -Leu and Gal/Raff/Xgal/CM -Ura -His -Trp. A total of 15 LexA-UbcD4/prey transformants were found to have a reproducible $LEU2^+$ phenotype. 12 of these also had a reproducible $lacZ^+$ phenotype. It was also necessary to test whether the interaction was specific to a UbcD4 bait, or whether it also occurred with an unrelated LexA hybrid protein. The reproducible UbcD4-interacting prey plasmids were co-transformed into EGY48 cells containing the reporter plasmid and a bait plasmid in which LexA was fused to the Bicoid homeodomain. 5 prey plasmids produced the $lacZ^+LEU2^+$ phenotype in combination with the LexA-Bicoid bait, and were discarded. Of the reproducible and specific UbcD4-interacting preys, those which produced activation of both reporters were classified 'strong' or 'medium' interactors depending on the strength of *lacZ* activity. Those which activated only the *LEU2* reporter, which has more *LexA* operators than *lacZ*, were classified 'weak' interactors (Figure 4.4). The inserts of the plasmids were then sequenced and their identities determined by BLAST searches (see Table 4.1).

4.3 Analysis of the putative UbcD4-interacting proteins

Four of the 'strong interactor' plasmids contained inserts encoding part of the polyubiquitin-binding subunit of the proteasome, Pros54. Of these, at least three had come from different plates in the primary yeast transformation and therefore represented independent identifications of the interaction. Two independently-identified 'strong interactor' plasmids had inserts representing the same novel

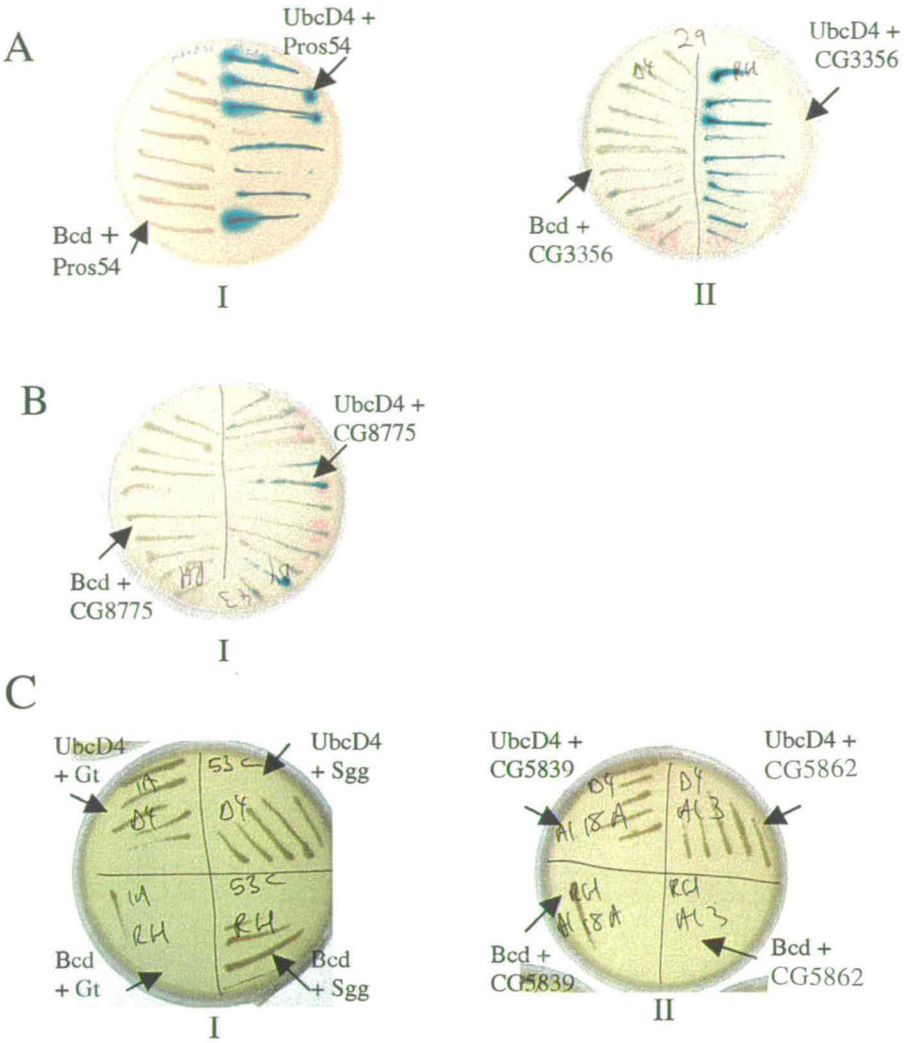


Figure 4.4: Specificity tests on UbcD4-interacting proteins.
A. Strong interactors. The prey proteins Pros54 (I) and CG3356 (II) are co-expressed with either the Bcd bait or a UbcD4 bait. Individual colonies were streaked onto Gal/Raff/X-gal/CM -Ura -His -Trp. Only colonies expressing the UbcD4 bait produce a blue colour.
B. Medium interactor. The prey protein CG8775 was co-expressed with either the Bcd bait or the UbcD4 bait. Colonies were streaked onto Gal/Raff/X-gal CM -Ura -His -Trp. Only colonies expressing the UbcD4 bait produce a blue colour.
C. Weak interactors. The prey proteins Gt, Shaggy (Sgg), CG5839 and CG5862 were co-expressed with either the UbcD4 or Bcd baits, and colonies were streaked onto Gal/Raff/X-gal/CM -Ura -His -Trp -Leu. In the case of Gt, CG5862 and CG5839, colonies expressing UbcD4 can grow better than those expressing Bcd. Sgg is an example of a prey which interacts with both baits.

Gene	Gene product	Hits	*lacZ*	*LEU2*
Pros54	Proteasome subunit	3	+++	+++
CG3356	Ubiquitin ligase	2	+++	+++
CG8775	Putative zinc metallopeptidase	1	+	++
CG5839	Putative zinc metallopeptidase	1	-	+
Giant	Transcriptional repressor	1	-	+
CG5862	Novel protein with PCI motif	1	-	+

Table 4.1: UbcD4-interacting proteins. The third column indicates the number of independently-identified plasmids which encoded each protein. The fourth column indicates the strength of the blue colour produced on X-gal medium by colonies co-expressing the relevant prey with the UbcD4 bait. The fifth column indicates the level of growth on medium lacking leucine by the same colonies.

gene, *CG3356*, which encodes a putative Hect-domain E3. The 'medium interactor' *CG8775*, also encoded a novel protein, in this case with a putative zinc protease motif and similarity to mammalian aminopeptidase A. The three 'weak interactors' had inserts representing different genes. One encoded the transcriptional repressor Giant. The other two encoded novel proteins. One of these, CG5839, had a putative zinc protease motif and similarity to insect aminopeptidase N. The other predicted protein, CG5862, was significantly similar only to uncharacterised ORFs in *C. elegans* and *A. thaliana* in BLAST searches. A PROSITE search revealed a PINT/PCI motif which may indicate an association with the proteasome.

4.3.1 UbcD4 interacts with the polyubiquitin-binding subunit of the proteasome.

The most frequently-identified UbcD4 interactor is Pros54, the *Drosophila* homologue of the mammalian S5a and the yeast Rpn10 proteins. This is the only proteasomal subunit which has been shown to bind ubiquitin chains with high affinity (Deveraux *et al.*, 1994). S5a has two polyubiquitin binding sites, PUbS1 and PUbS2, which interact preferentially with chains of between 4 and 8 ubiquitin residues (Young *et al.*, 1998). Pros54 is 52% identical to S5a (Figure 4.5). Haracska and Udvardy (1997) have shown that polyubiquitin binds to two regions

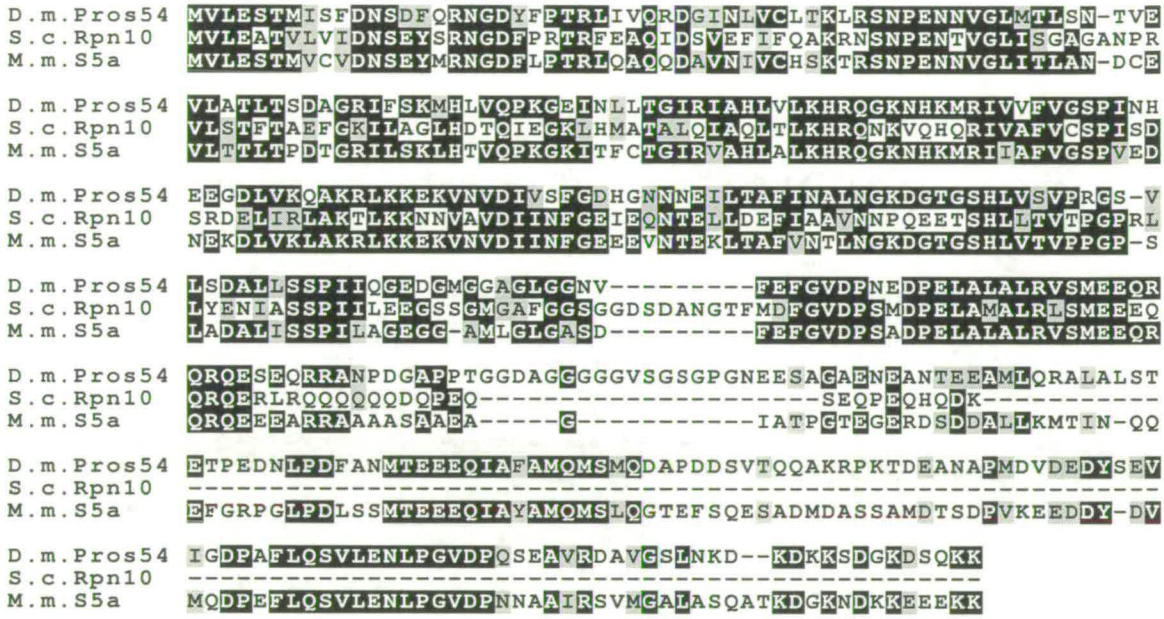


Figure 4.5: CLUSTALW alignment of the *Drosophila* Pros54 protein with yeast Rpn10 and mouse S5a. The ubiquitin-binding region PUbS1 spans residues 196-241 on the mammalian protein and includes the GVDP tetrapeptide conserved between all S5a homologues. The second ubiquitin-binding region, PUbS2, spans residues 263-307 of the mammalian protein and is only present in S5a homologues from higher eukaryotes. Abbreviations: D.m.=*Drosophila melanogaster*, S.c.=*Saccharomyces cerevisiae*, M.m.=*Mus musculus*

of Pros54, which include the motifs corresponding to PUbS1 and PUbS2. It is not clear which part of Pros54 binds UbcD4, as the shortest Pros54 fragment identified in the two-hybrid screen lacks only the N-terminal 55 amino acids of the 396 amino acid protein.

Although Rpn10 is the only subunit which has been shown to bind polyubiquitin, it is not absolutely required for proteasomal docking of a ubiquitinated substrate. Yeast strains lacking Rpn10 are impaired in the degradation of only a subset of test substrates, and do not exhibit the phenotypes characteristic of proteolysis - defective proteasomal mutants (Van Nocker and Vierstra, 1993). Another potential delivery mechanism is an interaction between the E2-E3-substrate complex and the proteasome. Several recent studies demonstrate such interactions.

A significant proportion of the yeast E2 Ubc4 in the cell is associated with the proteasome (Tongaonkar *et al.*, 2000). Consistent with the requirement of this E2 for the stress response, the interaction between Ubc4 and the proteasome is increased following heat stress. Purified proteasomes can conjugate ubiquitin to a test protein, demonstrating the presence of active ubiquitination enzymes. Two yeast E3s, Ubr1 and Ufd4, interact directly with specific subunits of the 19S particle of the proteasome (Xie and Varshavsky, 1999). In coimmunoprecipitation and GST-pulldown experiments, Ubr1 binds to Rpn2 and to the ATPases Rpt1 and Rpt6, while Ufd4 binds Rpt6. These results suggest that interactions between the E3 and the 19S particle mediate delivery of the substrate to the chaperone-like subunits of the 26S proteasome.

Direct interactions between ubiquitination enzymes and the proteasome have not been reported in multicellular organisms. However, recent research indicates that such associations may be mediated by members of the type 2 family of ubiquitin-like proteins, which contain a non-cleavable and non-transferable ubiquitin-like domain. A complex containing proteasomal subunits, the E3 E6-

AP, and hPlic-2 has been detected in human keratinocytes (Kleijnen *et al.*, 2000). hPlic-2, which contains a non-cleavable ubiquitin-like domain, was identified by its ability to interact with E6-AP in a yeast two-hybrid screen. Overexpression of full-length hPlic-2 proteins interferes with the *in vivo* degradation of p53 and I-κB, indicating a functional involvement of hPlic with ubiquitin-mediated proteolysis. hPlic-2 may provide a link between the protein ubiquitination and degradation machineries. E6-AP interacts with another protein containing a Ubl domain, hHR23a (Kumar *et al.*, 1999). hHR23a and hHR23B are human homologues of the yeast nucleotide excision repair (NER) protein Rad23. Functional data, and co-immunoprecipitation with the proteasome, have implicated hHR23 and Rad23 in regulation of ubiquitin-mediated proteolysis (Hiyama *et al.*, 1999; Lambertson *et al.*, 1999; Schauber *et al.*, 1998). In a yeast two-hybrid screen, hHR23A and hHR23B interact specifically with the polyubiquitin-binding proteasomal subunit S5a (Hiyama *et al.*, 1999).

Taken together, these studies raise the possibility that direct and indirect association between ubiquitination enzymes and proteasomal subunits might couple polyubiquitination of a substrate with its degradation. It is not clear whether ubiquitination enzymes are constitutively present in the proteasome or whether the association is subject to regulation, nor whether substrate polyubiquitination is a prerequisite for docking of a ubiquitination enzyme onto the proteasome. The finding that UbcD4 can interact with the *Drosophila* S5a homologue is the first demonstration of direct association between an E2 and the proteasome in multicellular organisms.

4.3.2 UbcD4 interacts with a novel protein which may associate with the proteasome

One of the three weak interactors identified in the screen is the product of the *CG5862* gene. The predicted CG5862 amino acid sequence includes a nuclear localisation signal and a PINT domain. The PINT domain, also known as a

PCI domain, is a conserved region of unknown function found in the C-terminal region of several regulatory components of the 26S proteasome : yeast Rpn3, Rpn5, Rpn6, Rpn7and Rpn9; and mammalian p55, p58 and p44 (Hofmann and Bucher, 1998). The motif is also found in translation initation factor 3 subunits, and in other proteins (Hofmann and Bucher, 1998). All of the characterized proteins containing PINT domains are parts of larger multi-protein complexes. The complete PINT domain is approximately 200 residues, but the highest conservation is found in the C-terminal half. Several PINT-containing proteins, including CG5862, have no significant similarity to the N-terminal half of the domain. The only sequences which are detectably similar to CG5862 on a BLAST search are putative proteins in *A. thaliana* and *C. elegans* (Figure 4.6).

In situ hybridisation analysis reveals a complex expression pattern for CG5862 in embryos (Figure 4.7). Until completion of germband retraction, CG5862 transcripts are abundant throughout the embryo. In late blastoderms and early gastrulae, expression is particularly high in the yolk nuclei, which remain in the centre of the embryo after most of the nuclei have migrated to the periphery. During germband extension, strong staining is observed in a layer of cells along the inside of the germband, which may be the neuroblasts. During germband retraction, CG5862 expression appears in the mature salivary glands. It remains high in the salivary gland until the end of embryogenesis, whereas the rest of the embryos shows little CG5862 expression after stage 14.

Is the CG5862 product a putative substrate for UbcD4-mediated degradation, or does it facilitate proteasomal docking of a UbcD4-substrate complex ? The presence of the PINT domain indicates that it may associate with the proteasome. It is becoming increasingly clear that proteasome composition is highly dynamic, with various factors reported to associate transiently and substoichiometrically. As discussed previously, proteins which can associate with both the ubiquitination and proteolytic machineries may facilitate coupling of the two processes. Perhaps

```
CG5839          ERKEHEEYLKM-KAAFSVEEEG-FEEGDADDQDNLLADFIQYIRDN-KVVVLEDLAVAFK
A.t.AL035680    EEAAALEFDKW-KGEFSVDAEG-TTEEVQGGNQDLLSEFVEYIKKQ-KCVPLEDLAAEFH
C.e.ZK1236.7    ERKEHEEYLAM-KASFAIEEEG-TDAIEGEEAENLIRDFVDYVKTN-KVVNIDELSSHFG
S.c.Rpn3        DLKKFTSTITK-YKQLLLKDD--TYQLCVRLRSNVIKTGIRIISLTYKKISLRDICLKLN
S.c.Rpn7        DYASYFPYLLETYANVLIPCKYLNRHADFFVREMRRKVYAQLLESY-KTLSLKSMASAFG

CG5839          LKTQQVIDRIQN------------------LQADGTL--------TGVIDDRGKFIYVSE
A.t.AL035680    LRTQVSSGYIFM------------------WKTKEYLLSWLSRRLSGVMDDRGKYIYISM
C.e.ZK1236.7    LKSEDAVNRLQH------------------FIEEGLV--------QGVMDDRGKFIYISD
S.c.Rpn3        LDSEQTVEYMVSRAIRDGVIEAKINHEDGFIETTELLNIYDSEDPQQVFDERIKFANQLH
S.c.Rpn7        VSVAFLDNDLGK------------------FIPNKQL--------N-CVIDRVNGIVETN

CG5839          KELLAVAKFIKQRGRVSIAELAESSNNLINLTPISAGGGEASS---------
A.t.AL035680    EEMNAVADYIKRQGRVSISHLASKSNQFIDLEPKVQHQLTEEISGMEEISVS
C.e.ZK1236.7    EEFAAVAKFINQRGRVSIHEIAEQSNRLIRLETPSAAE--------------
S.c.Rpn3        DEYLVSMRYPEDKKTQQNEKSENGENDDDTLDGDLMDDMSDISDLDDLGFL-
S.c.Rpn7        RPDNKNAQYH-----LLVKQGDGLLTKLQKYGAAVRLTGSDRV---------
```

Figure 4.6: CLUSTALW alignment between the PINT domains of *Drosophila* CG5862 and its closest relatives, *A. thaliana* (A.t.) AL035680 and *C. elegans* (C.e.) ZK1236.7, and the *S. cerevisiae* (S.c.) proteasomal subunits Rpn3 and Rpn7. The sequences shown start at positions 176, 156, 113, 355 and 300 of the five proteins respectively, and extend to the C-termini.

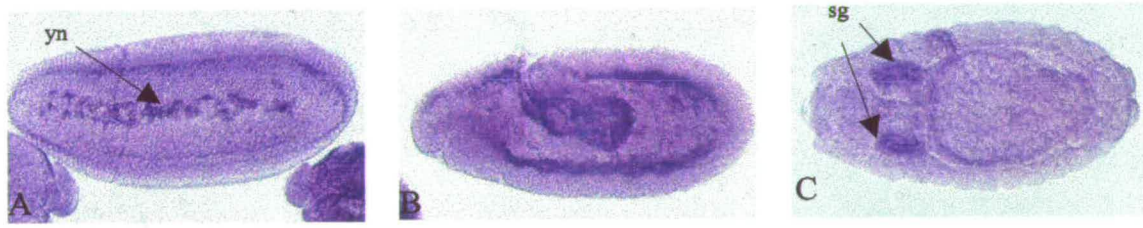


Figure 4.7: Embryonic *in situ* hybridisation analysis of *CG5862* expression. A - stage 5 embryo. B - stage 10 embryo. C - stage 12 - 13 embryo. Abbreviations: sg = salivary glands, yn = yolk nuclei

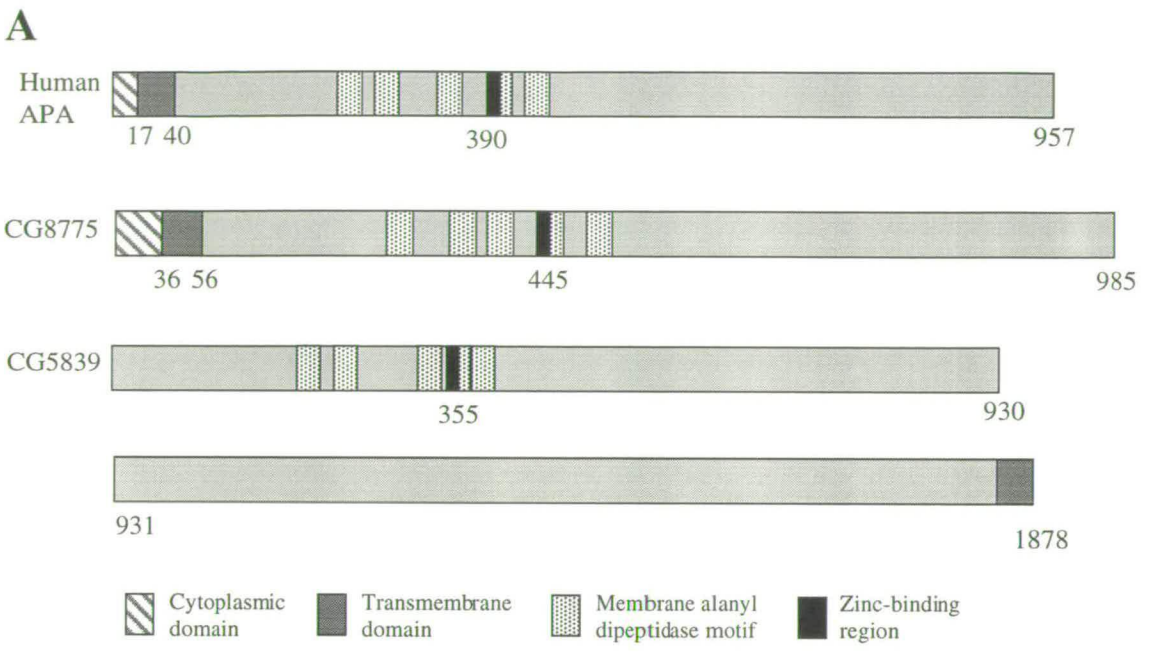
CG5862 fulfills this function for UbcD4-mediated proteolysis.

4.3.3 Two UbcD4 interactors are gut-specific putative zinc metallopeptidases

One of the plasmids encoding a strong interactor had an insert corresponding to the final 583 bp of the *CG8775* gene. A weak interactor had an insert corresponding to the final 880 bp of the *CG5839* gene. Both these predicted genes encode putative zinc metallopeptidases, as they contain the zinc-binding motif common to all such proteins (Rawlings and Barrett, 1995). This motif is defined as HEXXH, where the two histidines are the zinc-binding residues and the glutamate is the active site residue. It can be defined more stringently as [GSTALIVN]-x(2)-H-E-[LIVMFYW]-DEHRKP-H-x-[LIVMFYWGSPQ]. Zinc-dependent metallopeptidases can be classified into 21 distinct families. Based on their sequence similarity to known zinc metallopeptidases, CG5839 and CG8775 both fall into the M1 (membrane alanyl dipeptidase) family, which includes insect and mammalian aminopeptidase N (APN) and mammalian aminopeptidase A (APA). This family share a 'membrane alanyl dipeptidase signature motif' consisting of 5 elements, the fourth of which contains the zinc-binding site.

4.3.3.1 Sequence analysis of CG8775 and CG5839

CG8775 is 38% identical and 55% similar to human aminopeptidase A (APA). APA is a 957 aa cell-surface protein consisting of a short N-terminal cytoplasmic domain followed by a transmembrane region and a large extracellular domain, which includes the zinc-binding site. The predicted product of CG8775 is a similar size, 985 amino acids. The sequence of the CG5839 protein is most similar to insect aminopeptidase N (APN). Both insect and mammalian aminopeptidase N are approximately 980 amino acids long. The CG5839 product is almost twice as large, 1878 aa, with 49% identity between the first 930 and the second 948 aa. The C-terminal half of the protein is slightly more similar to aminopepti-



B

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H.s.APA     1 RKSFPCFDEPNKKATYTISITHP--KEYGALSNMP---VAKEESVDDKWTRTTFEKSVPM
CG8775      1 RQAFPCFDEPALKAQFTITVARPSGDEYHVLSNMP---VAS-EYVDGDITEVTFAETVPM
H.s.APN     1 RKSFPCFDEPAMKAEFNITLIHP--KDLTALSNMLPKGPSTPLPEDPNWNVTEFHTTPKM
CG5839      1 RHAFPCYDEPARRANFTITIHHD--PSYTAISNMP---VNTAATSSG---VTAFQTTPKM

H.s.APA    56 STYLVCFAVHQFDSVKR-ISNSGKPLTIYVQPEQKHT--AEYAANITKSVFDYFEEYFAM
CG8775     57 STYLAAFVVSDFQYKETTVEGTSIALKVYAPPAQVEK--TQYALDTAAGVMAYYINYFNV
H.s.APN    59 STYLLAFIVSEFDYVEK-QASNGVLIRIWARPSAIAAGHGDYALNVTGPILNFFAGHYDT
CG5839     53 STYLVAFIVSDFESTTG--ELNGIRQRVFSRKGKQDQ--QEWALWSGLLVESSLASYFGV

H.s.APA   113 NYSLPKLDKIAIPDFGTGAMENWGLITYRETNLLYDPKESASSNQQRVATVVAHELVHQW
CG8775    115 SYALPKLDLVAIPDFVSGAMENWGLVTFRETALLYDESTSSSVNKQRVAIVVAHELAHQW
H.s.APN   118 PYPLPKSDQIGLPDFNAGAMENWGLVTYRENSLLFDPLSSSSSNKERVVTVIAHELAHQW
CG5839    109 PFALPKLDQAGIPDFSAGAMENWGLATYREQYMWWNKQNSTINLKTNIANIIGHEYAHMW

H.s.APA   173 FGNIVTMDWWEDLWLNEGFASFF
CG8775    175 FGNLVTMNWWNDLWLNEGFASFL
H.s.APN   178 FGNLVTIEWWNDLWLNEGFASYV
CG5839    169 FGDLVSIKWWTYLWLKEGFATLF
```

Figure 4.8: Sequence analysis of the putative aminopeptidases CG8775 and CG5839.

A. Diagram showing the relative positions of the cytoplasmic and transmembrane domains, and of the membrane alanyl dipeptidase and zinc-binding motifs, in human aminopeptidase A, CG8775 and CG5839. CG5839 is shown split into two domains, which are 49% identical to each other. Human APN has a similar organisation to APA and is not shown here.

B. CLUSTALW alignment of the regions of human APN and APA, and the two putative *Drosophila* aminopeptidases, containing the M1 family signature. The sequences aligned are residues 228-422 of human APA, 281-477 of CG8755, 218-416 of human APN and 197-387 of CG5839. The HEXXH zinc-binding motif begins at position 166 of H.s. APA in the alignment shown here. Abbreviation: H.s.=*Homo sapiens*, APA=aminopeptidase A, APN=aminopeptidase N.

dase N than the N-terminal (33% and 29% identical to *Lymantria dispar* APN respectively). Figure 4.8 shows the results of sequence analysis of CG8775 and CG5839 using SMART (Sequence Analysis Modular Architecture Research Tool) to predict transmembrane domains and a PROSITE scan to locate the M1 family signature and zinc-binding motifs.

BLAST searches of the *Drosophila* genome using CG8775 and CG5839 as query sequences suggests that there are ten other putative members of the M1 zinc metallopeptidase family in flies. One of these, CG5845, has a similar size and structure to CG5839. The remainder are a similar size to CG8775, with the zinc-binding region in a similar position.

4.3.3.2 *In situ* hybridisation analysis of CG8775 and CG5839 expression in embryos.

In situ hybridisation analysis shows that both metallopeptidase genes are transcribed in the gut (Figure 4.9). CG8775, the aminopeptidase A homologue, is highly expressed in the oesophagus of stage 12 embryos. Expression is also detectable in the hindgut at this stage. The oesophageal expression alone persists until the end of embryogenesis. Apart from maternal transcript in the blastoderm, expression is not detectable at any earlier stage of embryogenesis. The aminopeptidase N homologue CG5839 is expressed in the hindgut of stage 15-17. There is no maternal transcript in the blastoderm and no detectable expression at any stage before stage 15.

4.3.3.3 Discussion

Both aminopeptidase A and N cleave the N-terminal residues from substrate proteins. Aminopeptidase A releases glutamate, and to a lesser extent, aspartate, while aminopeptidase N releases alanine. Their best-studied roles are in the catabolic pathway of the renin-angiotensin system in mammals (reviewed by Ardaillou (1997)). In brain and kidney, aminopeptidase A hydrolyses the N-

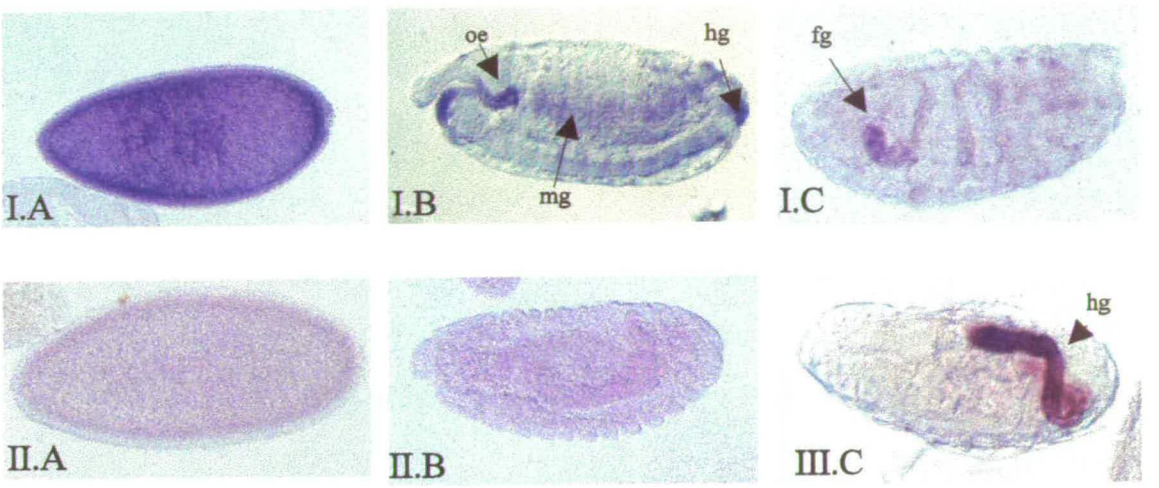


Figure 4.9: Embryonic *in situ* hybridisation analysis of the putative metallopeptidase genes *CG8775* and *CG5839*
I.A -C : *CG8775* expression. II.A-C: *CG5839* expression. A - stage 5 embryo. B - stage 12-13 embryo. C - stage 14-15 embryo. Abbreviations: fg=foregut, hg=hindgut, mg=midgut, oe=oesophagus

terminal aspartate of angiotensin II to generate angiotensin III. Aminopeptidase N hydrolyses the N-terminal arginine of angiotensin III to generate angiotensin IV. Angiotensin III is the main effector of vasopressin release, and hence increase in blood pressure. Aminopeptidase A is also present in blood cells and has been proposed to play a role in early B-cell differentiation. Aminopeptidase N is present in the intestine, where it can act as a receptor for human coronavirus 229E (Yeager *et al.*, 1992). In insects such as *Manduca sexta* and *Bombyx mori* aminopeptidase N is found in the midgut brush-border membrane, where it can act as the receptor for the *Bacillus thuringiensis* toxin (Hua *et al.*, 1998).

Although *Drosophila* does not appear to have an angiotensin homologue, it has two putative angiotensin-converting enzyme (ACE) homologues, members of the M2 zinc metallopeptidase family. ACE occurs in two forms in mammals. Somatic ACE (sACE) consists of two highly similar domains while testicular ACE (tACE) is identical to the C-terminal domain of sACE. sACE converts angiotensin I to angiotensin II, while the physiological role of tACE is unknown. The *Drosophila* homologues, AnCE (Cornell *et al.*, 1995) and Acer (Tatei *et al.*, 1995), are single-

domain enzymes similar to tACE. Only AnCe is capable of cleaving angiotensin I (Houard *et al.*, 1998). Both enzymes are expressed during pupal development, although at different stages (Houard *et al.*, 1998). During embryogenesis, *AnCE* is expressed in the amnioserosa, dorsal vessel (heart primordium) and midgut (Tatei *et al.*, 1995). Acer is expressed in the embryonic midgut (Taylor *et al.*, 1996). A *P* element insertion in *AnCE* causes homozygotes to die during larval or pupal development, and transheterozygotes for two different lethal alleles exhibit male sterility, suggesting that AnCE may be required for contractions of the heart, gut or testes (Tatei *et al.*, 1995).

It seems likely that CG5839 and CG8775 are involved in processing peptide signals which regulate gut development or function. Their interaction with UbcD4, if it occurs in *Drosophila* embryos as well as in the two-hybrid system, may represent E3-independent substrate selection by UbcD4. The regions of the aminopeptidases which interact with UbcD4 in the two-hybrid system are predicted to be extracellular, raising the question of how ubiquitination of the mature protein could take place. Perhaps UbcD4 could interact with the aminopeptidases prior to their localisation to the cell membrane.

4.3.4 UbcD4 interacts with the gap protein Giant

The remaining weak UbcD4 interactor identified was the C-terminal 350 amino acids of the Giant (Gt) protein. *giant* is a gap gene and encodes a 448 amino acid basic leucine zipper -type transcriptional repressor (Capovilla *et al.*, 1992).

The embryonic *gt* expression pattern is shown in Figure 4.10. Early in development, two broad stripes are observed at approximately 25% and 75% egg length, with two additional stripes appearing towards the anterior at the cellular blastoderm stage. Expression in several patches of cells in the head persists throughout embryogenesis.

The function of Gt in patterning the early embryo has been well established.

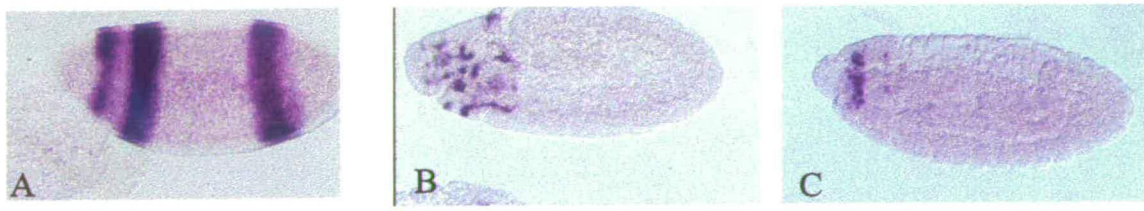
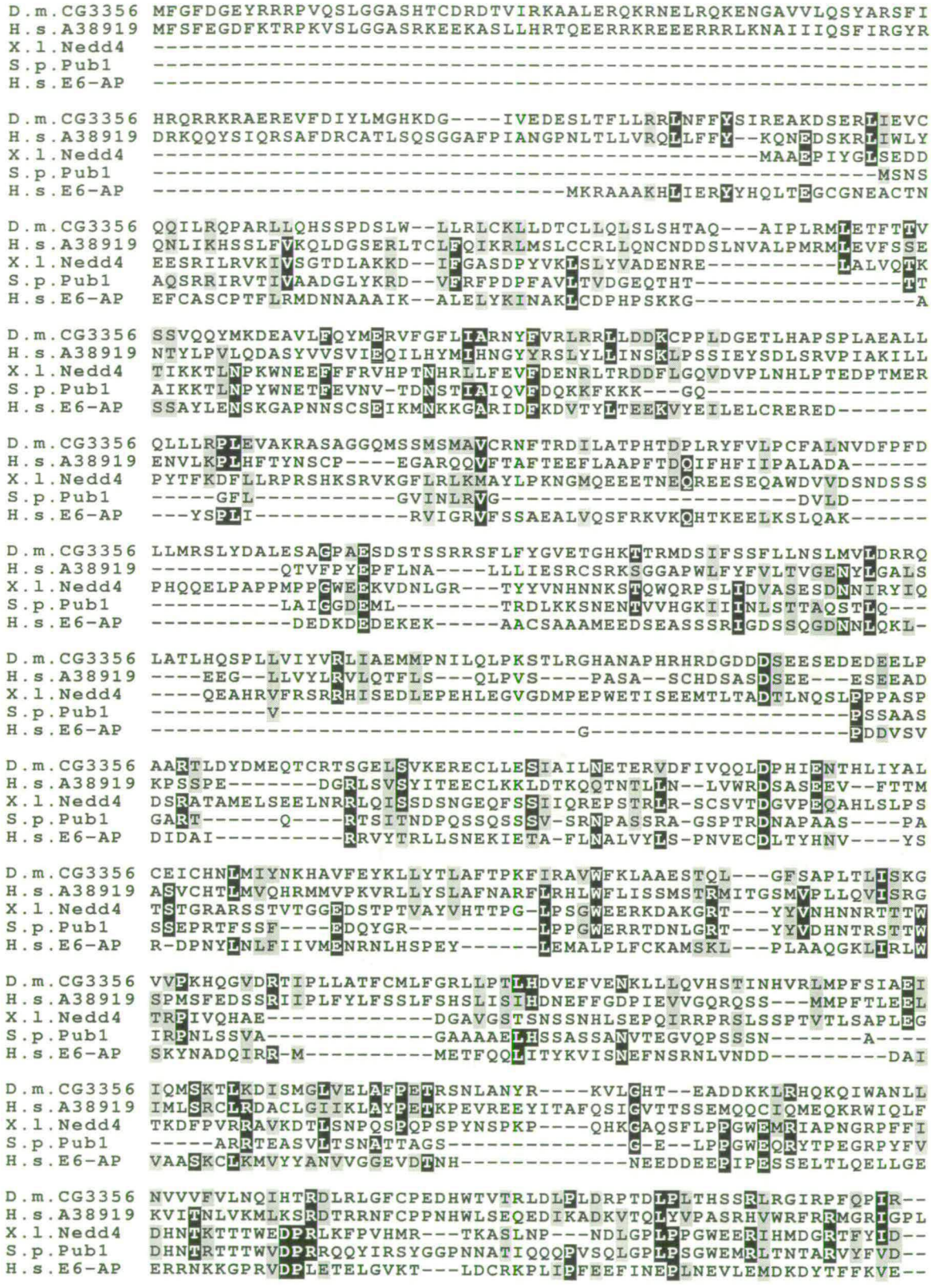


Figure 4.10: Embryonic *in situ* hybridisation analysis of *giant* expression. A. stage 5 embryo. B - stage 10 - 11 embryo

Transcriptional repression by Gt defines the expression domains of the pair- rule genes *even-skipped, fushi tarazu* and *paired* as well as the gap genes *kruppel* and *hunchback* (Wu *et al.*, 1998). Its transcription pattern suggests it may be required for head morphogenesis at later stages, and this is confirmed by mutational analysis. *gt* mutants display head defects including deletion of the posterior compartment of the labial segment (Petschek and Mahowald, 1990) and failure to complete head involution (Mohler *et al.*, 1989). The anterior compartments of abdominal segments 5 -7 are also deleted and there is extensive cell death in the epidermal components of the embryo (Petschek and Mahowald, 1990).

4.3.5 UbcD4 interacts with a putative E3 of the Hect-domain family.

Two independent UbcD4-interactors represented a region of approximately 500 bp from the 3' end of the *CG3356* gene. The amino acid sequence encoded by this region is similar to the C-termini of several E3 enzymes of the Hect-domain family. All members of the family share a conserved 350 amino acid C-terminal region defined by its homology to the mammalian E6-AP carboxy terminus. Unlike some other E3s, Hect domain proteins are capable of forming a covalent linkage with ubiquitin and of transferring ubiquitin to specific substrates. The Hect domain is responsible for binding E2s and for catalysing ubiquitin transfer. The N-termini of these enzymes are highly divergent and are thought to be responsible for substrate selection.


D.m.CG3356 MFGFDGEYRRRPVQSLGGASHTCDRDTVIRKAALERQKRNELRQKENGAVVLQSYARSFI
H.s.A38919 MFSFEGDFKTRPKVSLGGASRKEEKASLLHRTQEERRKREEERRRLKNAIIIQSFIRGYR
X.l.Nedd4 ----------
S.p.Pub1 ----------
H.s.E6-AP ----------
D.m.CG3356 HRQRRKRAEREVFDIYLMGHKDG----IVEDESLTFLLRRLNFFYSIREAKDSERLIEVC
H.s.A38919 DRKQQYSIQRSAFDRCATLSQSGGAFPIANGPNLTLLVRQLLFFY--KQNEDSKRLIWLY
X.l.Nedd4 ----------MAAEPIYGLSEDD
S.p.Pub1 ----------MSNS
H.s.E6-AP ----------MKRAAAKHLIERYYHQLTEGCGNEACTN
D.m.CG3356 QQILRQPARLLQHSSPDSLW--LLRLCKLLDTCLLQLSLSHTAQ---AIPLRMLETFTTV
H.s.A38919 QNLIKHSSLFVKQLDGSERLTCLFQIKRLMSLCCRLLQNCNDDSLNVALPMRMLEVFSSE
X.l.Nedd4 EESRILRVKIVSGTDLAKKD--IFGASDPYVKLSLYVADENRE----------LALVQTK
S.p.Pub1 AQSRRIRVTIVAADGLYKRD--VFRFPDPFAVLTVDGEQTHT----------TT
H.s.E6-AP EFCASCPTFLRMDNNAAAIK--ALELYKINAKLCDPHPSKKG----------A
D.m.CG3356 SSVQQYMKDEAVLFQYMERVFGFLIARNYFVRLRRLLDDKCPPLDGETLHAPSPLAEALL
H.s.A38919 NTYLPVLQDASYVVSVIEQILHYMIHNGYYRSLYLLINSKLPSSIEYSDLSRVPIAKILL
X.l.Nedd4 TIKKTLNPKWNEEFFFRVHPTNHRLLFEVFDENRLTRDDFLGQVDVPLNHLPTEDPTMER
S.p.Pub1 AIKKTLNPYWNETFEVNV-TDNSTIAIQVFDQKKFKKK---GQ----------
H.s.E6-AP SSAYLENSKGAPNNSCSEIKMNKKGARIDFKDVTYLTEEKVYEILELCRERED-------
D.m.CG3356 QLLLRPLEVAKRASAGGQMSSMSMAVCRNFTRDILATPHTDPLRYFVLPCFALNVDFPFD
H.s.A38919 ENVLKPLHFTYNSCP----EGARQQVFTAFTEEFLAAPFTDQIFHFIIPALADA------
X.l.Nedd4 PYTFKDFLLRPRSHKSRVKGFLRLKMAYLPKNGMQEEETNEQREESEQAWDVVDSNDSSS
S.p.Pub1 -----GFL----------GVINLRVG----------DVLD------
H.s.E6-AP ---YSPLI----------RVIGRVFSSAEALVQSFRKVKQHTKEELKSLQAK------
D.m.CG3356 LLMRSLYDALESAGPAESDSTSSRRSFLFYGVETGHKTTRMDSIFSSFLLNSLMVLDRRQ
H.s.A38919 ----------QTVFPYEPFLNA----LLLIESRCSRKSGGAPWLFYFVLTVGENYLGALS
X.l.Nedd4 PHQQELPAPPMPPGWEEKVDNLGR---TYYVNHNNKSTQWQRPSLIDVASESDNNIRYIQ
S.p.Pub1 ----------LAIGGDEML--------TRDLKKSNENTVVHGKIIINLSTTAQSTLQ---
H.s.E6-AP ----------DEDKDEDEKEK-----AACSAAAMEEDSEASSSRIGDSSQGDNNLQKL-
D.m.CG3356 LATLHQSPLLVIYVRLIAEMMPNILQLPKSTLRGHANAPHRHRDGDDDSEESEDEDEELP
H.s.A38919 ----EEG--LLVYLRVLQTFLS---QLPVS----PASA--SCHDSASDSEE---ESEEAD
X.l.Nedd4 ----QEAHRVFRSRRHISEDLEPEHLEGVGDMPEPWETISEEMTLTADTLNQSLPPPASP
S.p.Pub1 ---------V----------PSSAAS
H.s.E6-AP ----------G----------PDDVSV
D.m.CG3356 AARTLDYDMEQTCRTSGELSVKERECLLESIAILNETERVDFIVQQLDPHIENTHLIYAL
H.s.A38919 KPSSPE--------DGRLSVSYITEECLKKLDTKQQTNTLLN--LVWRDSASEEV--FTTM
X.l.Nedd4 DSRATAMELSEELNRRLQISSDSNGEQFSSIIQREPSTRLR-SCSVTDGVPEQAHLSLPS
S.p.Pub1 GART-------Q-----RTSITNDPQSSQSSSV-SRNPASSRA-GSPTRDNAPAAS----PA
H.s.E6-AP DIDAI----------RRVYTRLLSNEKIETA-FLNALVYLS-PNVECDLTYHNV----YS
D.m.CG3356 CEICHNLMIYNKHAVFEYKLLYTLAFTPKFIRAVWFKLAAESTQL---GFSAPLTLISKG
H.s.A38919 ASVCHTLMVQHRMMVPKVRLLYSLAFNARFLRHLWFLISSMSTRMITGSMVPLLQVISRG
X.l.Nedd4 TSTGRARSSTVTGGEDSTPTVAYVHTTPG-LPSGWEERKDAKGRT---YYVNHNNRTTTW
S.p.Pub1 SSEPRTFSSF----EDQYGR----------LPPGWERRTDNLGRT---YYVDHNTRSTTW
H.s.E6-AP R-DPNYLNLFIIVMENRNLHSPEY------LEMALPLFCKAMSKL---PLAAQGKLIRLW
D.m.CG3356 VVPKHQGVDRTIPLLATFCMLFGRLLPTLHDVEFVENKLLLQVHSTINHVRLMPFSIAEI
H.s.A38919 SPMSFEDSSRIIPLFYLFSSLFSHSLISIHDNEFFGDPIEVVGQRQSS---MMPFTLEEL
X.l.Nedd4 TRPIVQHAE----------DGAVGSTSNSSNHLSEPQIRRPRSLSSPTVTLSAPLEG
S.p.Pub1 IRPNLSSVA----------GAAAAELHSSASSANVTEGVQPSSSN--------A----
H.s.E6-AP SKYNADQIRR-M----------METFQQLITYKVISNEFNSRNLVNDD---------DAI
D.m.CG3356 IQMSKTLKDISMGLVELAFPETRSNLANYR----KVLGHT--EADDKKLRHQKQIWANLL
H.s.A38919 IMLSRCLRDACLGIIKLAYPETKPEVREEYITAFQSIGVTTSSEMQQCIQMEQKRWIQLF
X.l.Nedd4 TKDFPVRRAVKDTLSNPQSPQPSPYNSPKP----QHKGAQSFLPPGWEMRIAPNGRPFFI
S.p.Pub1 -----ARRTEASVLTSNATTAGS----------G-E--LPPGWEQRYTPEGRPYFV
H.s.E6-AP VAASKCLKMVYYANVVGGEVDTNH----------NEEDDEEPIPESSELTLQELLGE
D.m.CG3356 NVVVFVLNQIHTRDLRLGFCPEDHWTVTRLDLPLDRPTDLPLTHSSRLRGIRPFQPIR--
H.s.A38919 KVITNLVKMLKSRDTRRNFCPPNHWLSEQEDIKADKVTQLYVPASRHVWRFRRMGRIGPL
X.l.Nedd4 DHNTKTTTWEDPRLKFPVHMR---TKASLNP---NDLGPLPPGWEERIHMDGRTFYID--
S.p.Pub1 DHNTRTTTWVDPRRQQYIRSYGGPNNATIQQQPVSQLGPLPSGWEMRLTNTARVYFVD--
H.s.E6-AP ERRNKKGPRVDPLETELGVKT---LDCRKPLIPFEEFINEPLNEVLEMDKDYTFFKVE--

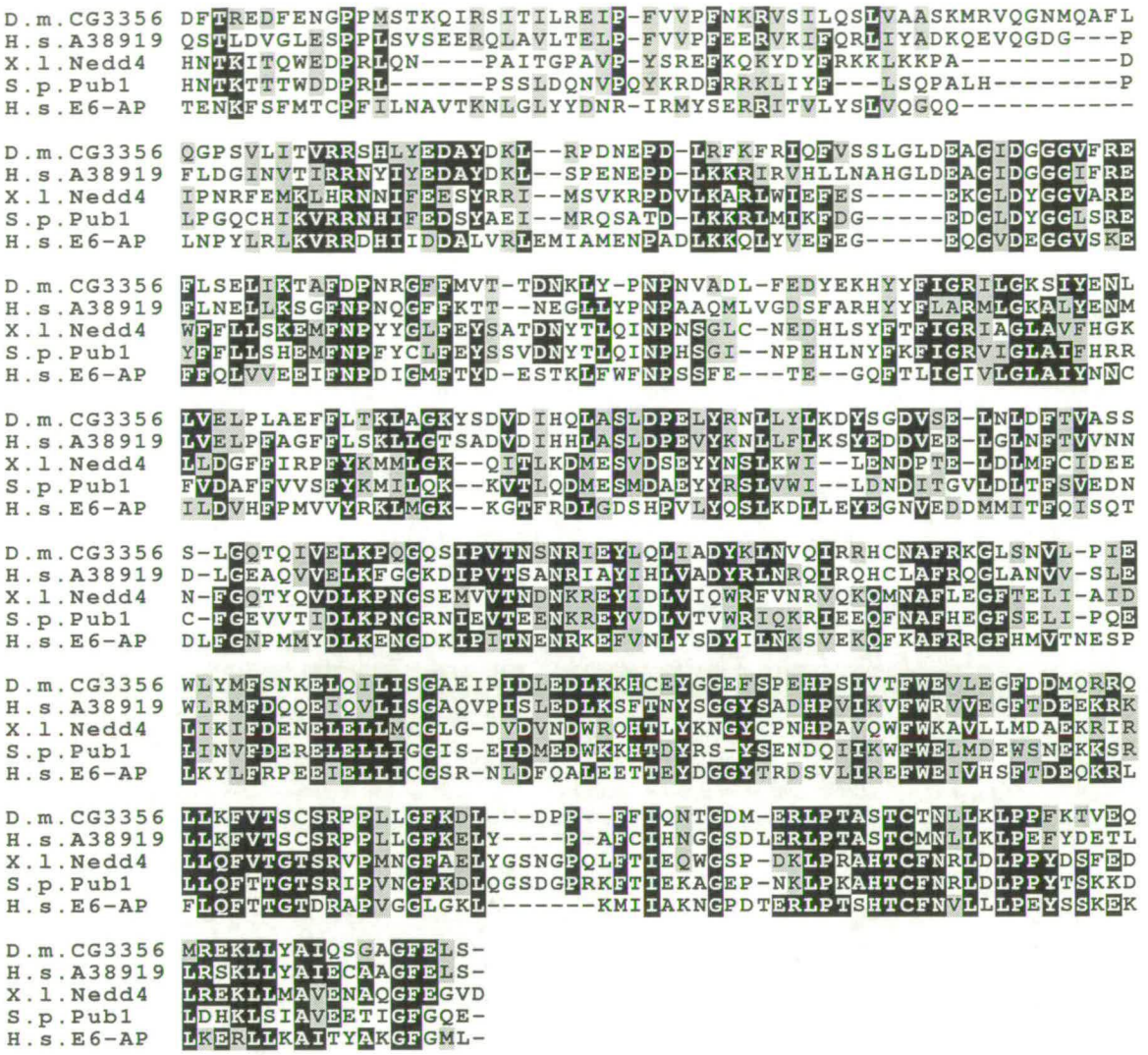


Figure 4.11: (continued from previous page). Alignment of the CG3356 protein with its closest relative, A38919 and with the Hect-domain E3s E6-AP, Nedd-4 and Pub1. Identical residues are shaded black and similar residues shaded grey. Abbreviations : H.s. = *Homo sapiens* , X.l. = *Xenopus laevis*, S.p. = *Schizosaccharomyces pombe.* The Hect-domain consists of the C-terminal 350 amino acids.

4.3.5.1 Sequence analysis

Overall, the amino acid sequence of CG3356 is most similar to that of a human hypothetical protein, A38919. Identity between A38919 and CG3356 is 35% over the entire protein and 54% in the Hect domain. The next most similar sequences are putative proteins from *A. thaliana, S. pombe, C. elegans* and another *Drosophila* ORF, CG5087. Although these gene products are similar in size to A38919 and CG3356, there is no significant similarity for the first 600 amino acids. The alignment of the full-length CG3356 protein with A38919 and with the known Hect-domain E3s E6-AP, Nedd-4 and Pub1 is shown in Figure 4.11.

4.3.5.2 Expression analysis

Developmental Northern analysis of *CG3356* expression shows that two transcripts are present in RNA from all stages of development (Figure 4.12). The larger transcript is approximately 3.5-4 kb, the size expected from its predicted 3588-bp cDNA sequence in GadFly. This band is present at low abundance. The smaller band, which is highly abundant in all stages, is approximately 1.5 kb. It may represent cross-hybridisation to a related transcript, although BLAST searches do not detect any strong similarity between *CG3356* and a gene of this size. Alternatively, it may be due to processing of the *CG3356* transcript. Interestingly there are two cDNA sequences in GenBank representing the closest relative of CG3356, human A38919; one encodes the full-length protein (GenBank accession BAA02799.1) and a shorter version encodes the C-terminal 415 amino acids (GenBank accession AC004975.1). Perhaps this truncated protein is a product of a processed transcript.

In situ hybridisation reveals a complex and highly dynamic expression pattern for *CG3356* in embryos (Figure 4.13). Maternal transcripts in the blastoderm appear localised to both poles. During gastrulation, expression is highest in the invaginating posterior midgut primordium, ventral furrow and cephalic furrow. In

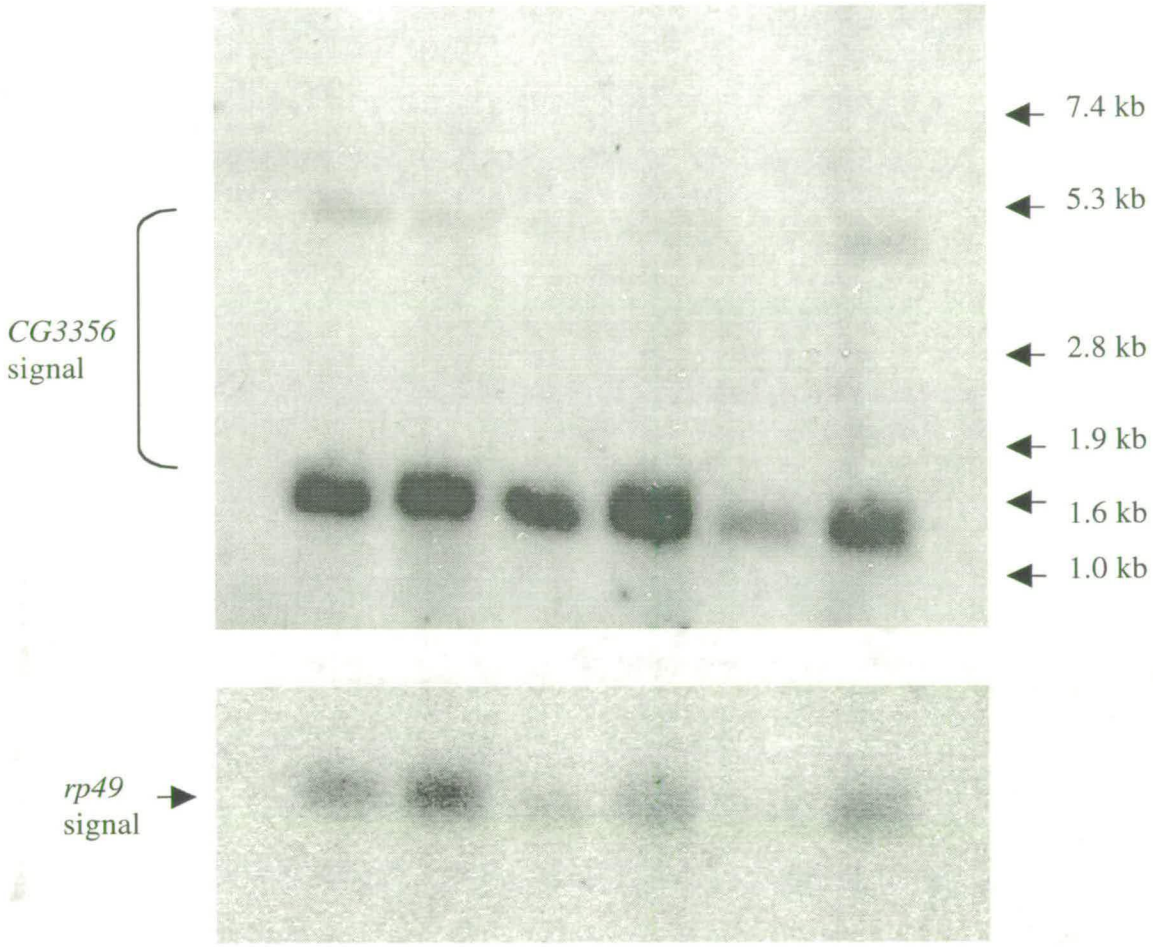


Figure 4.12: Developmental Northern analysis of *CG3556* expression. Each lane contains 2 μg of total RNA isolated from flies at the following stages of development : 2 - 0-3 hour embryos : 3 - 3-8 hour embryos : 4 - 8-22 hour embryos : 5 - larvae of mixed ages : 6 - pupae : 7 - adults. Lane 1 contains *E. coli* ribosomal RNA and lane 8 contains DIG-labelled RNA molecular weight markers. The arrowheads indicate the sizes and positions on the filter of the molecular weight markers after detection with AP-conjugated anti-DIG. The bottom panel shows the result of probing the same blot with *rp49*.

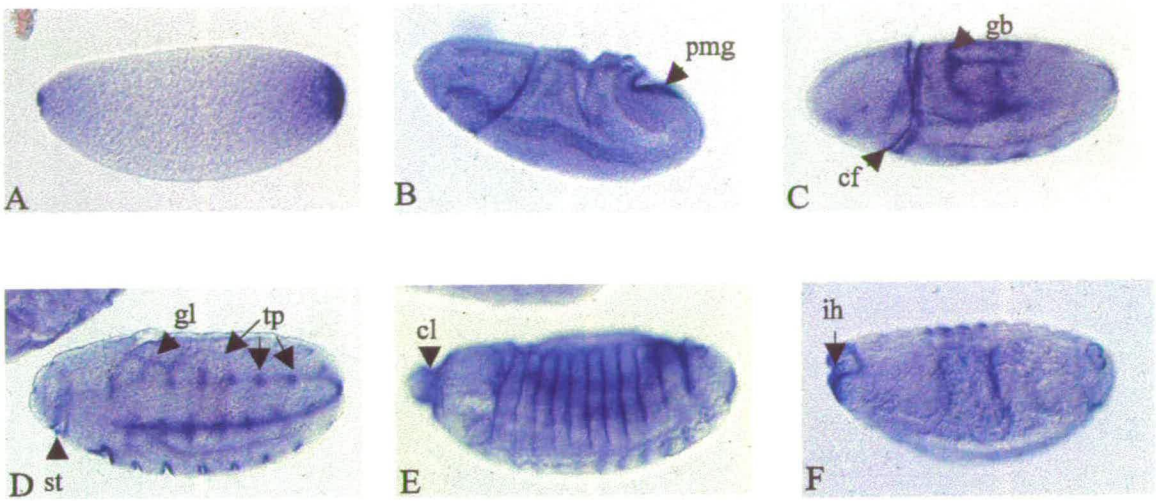


Figure 4.13: Embryonic *in situ* hybridisation analysis of *CG3356* expression. A - stage 1-3. B - stage 6. C - stage 8. D - stage 10-11. E - stage 12-13. F - stage15. Abbreviations: cf = cephalic furrow, cl = clypeolabrum, gb = germband, gl = gnathal lobes, ih = involuting head, pmg = posterior midgut, st = stomodeum, tp = tracheal pits, pmg=posterior midgut.

stage 8-9 embryos, high expression is seen at the extending tip of the germband. At stage 10-11, expression is weak in most of the embryo, with *CG3356* positive regions in the stomodeum, the gnathal lobes, along the ventral midline and in the invaginating tracheal pits. At stage 12, *CG3356* expression is strongest in the newly-formed segmental furrows and in the clypeolabrum. After this stage, transcription fades in all regions except in the head. When head involution has been completed, *CG3356* expression is no longer detectable.

One notable feature of the embryonic *CG3356* expression pattern is that, from stage 10 onwards, expression seems to be highest in epidermal tissues as they undergo morphogenetic movements. The major morphogenetic movements include head involution, formation of the tracheal pits, mesectoderm involution and formation of the segmental furrows. The earliest events of head involution occur at stage 10. These are the invagination of the foregut primordium (stomodeum) and the formation of three gnathal segments (mandible, maxillary and labial) and the anteriormost head segment (clypeolabrum). After dorsal closure has been completed, the clypeolabrum moves to the interior of the embryo. The tracheal system

develops from the invagination of defined clusters of epidermal cells during stage 10-11. By stage 12, the embryo becomes subdivided into 16 metameric units, corresponding with deep grooves in the epidermis, the segmental furrows. All these tissues exhibit high levels of *CG3356* expression at various points between stages 10 and 15. In each case, expression does not persist past the stage at which the relevant structure is forming. In younger embryos, expression is more widespread and is found in endodermal regions also, for example in the posterior midgut primordium. The extending germband consists of both ectodermal and endodermal tissues. However the correlation between *CG3356* expression and morphogenetic movement is seen in all embryos after the blastoderm stage.

4.3.5.3 Discussion

The expression pattern of *CG3356* suggests that, in embryos, it may have a role in regulating morphogenesis, particularly in the epidermis. Perhaps a CG3356 substrate is involved in a process common to foregut, tracheal and segmental furrow formation. There are a number of genes affecting formation of all these tissues, primarily members of the segment polarity class such as *decapentaplegic* and *18-wheeler*. However Northern analysis shows that it is also transcribed in larvae, pupae and adults. This suggests either that CG3356 has multiple targets, or that its target is present throughout all stages. The sequence of CG3356 gives no indication of the identity of its substrate. There are no identifiable protein-protein interaction motifs, and the Hect-domain proteins most similar to CG3356 have not been studied.

Unlike *CG3356*, *UbcD4* is transcribed only in embryos. In embryos, the expression patterns of the two genes share some region of overlap, for example the cephalic furrow and the extending germ band, but have clear differences also. In particular, *CG3356* expression is not detectable in the germ cells. This indicates that UbcD4 must be able to recognise substrates in the absence of CG3356, either independently or by co-operating with a different E3. Similarly, CG3356 must

co-operate with additional E2s.

E2s can be divided into subclasses according to their functional co-operativity with different types of E3. The E2s which interact with Hect-domain E3s share a conserved phenylalanine residue at approximately position 63 in the UBC domain (Nuber and Scheffner, 1999). This residue is present in UbcD4. Individual E2s within this family co-operate preferentially with different subsets of Hect domain proteins, and this is due to selective physical interactions between the E2 and the Hect domain. For example, the human E3 E6-AP interacts specifically with the closely related E2s UbcH7 and UbcH8 in a yeast two-hybrid screen, and also co-operates specifically with these E2s in ubiquitin transfer (Kumar *et al.*, 1997). The structure of the E6-AP - UbcH7 complex reveals the determinants of E2-E3 specificity (Huang *et al.*, 1999) (Figure 4.14). The E2-binding groove on the Hect domain is only moderately conserved between different E3s. UbcH7 has two Hect-binding loops termed L1 and L2. L1 contacts the Hect domain at five residues, four of which are well conserved between Hect-specific E2s. However three of the four Hect-binding residues in L2 are variable, suggesting that this region could determine the specificity of co-operation between E2s and Hect-domain E3s. Overall, UbcD1 is the *Drosophila* E2 most similar to UbcD4. Two of its L2 Hect-binding residues are identical to the corresponding residues in UbcD4. However the *Drosophila* E2 most like UbcD4 in L2 is Alt1/CG4502. Three of the Hect-binding residues are identical, and the fourth is similar, between the two enzymes (Figure 4.14). Alt1 also has the conserved phenylalanine residue in L1 which classifies it as a Hect-specific E2. Perhaps Alt1 is one of the other E2s which can co-operate with CG3356.

4.4 Discussion

The lack of suitable antibodies meant that none of the interactions described here could be shown to occur in embryos. Other methods of testing interactions share

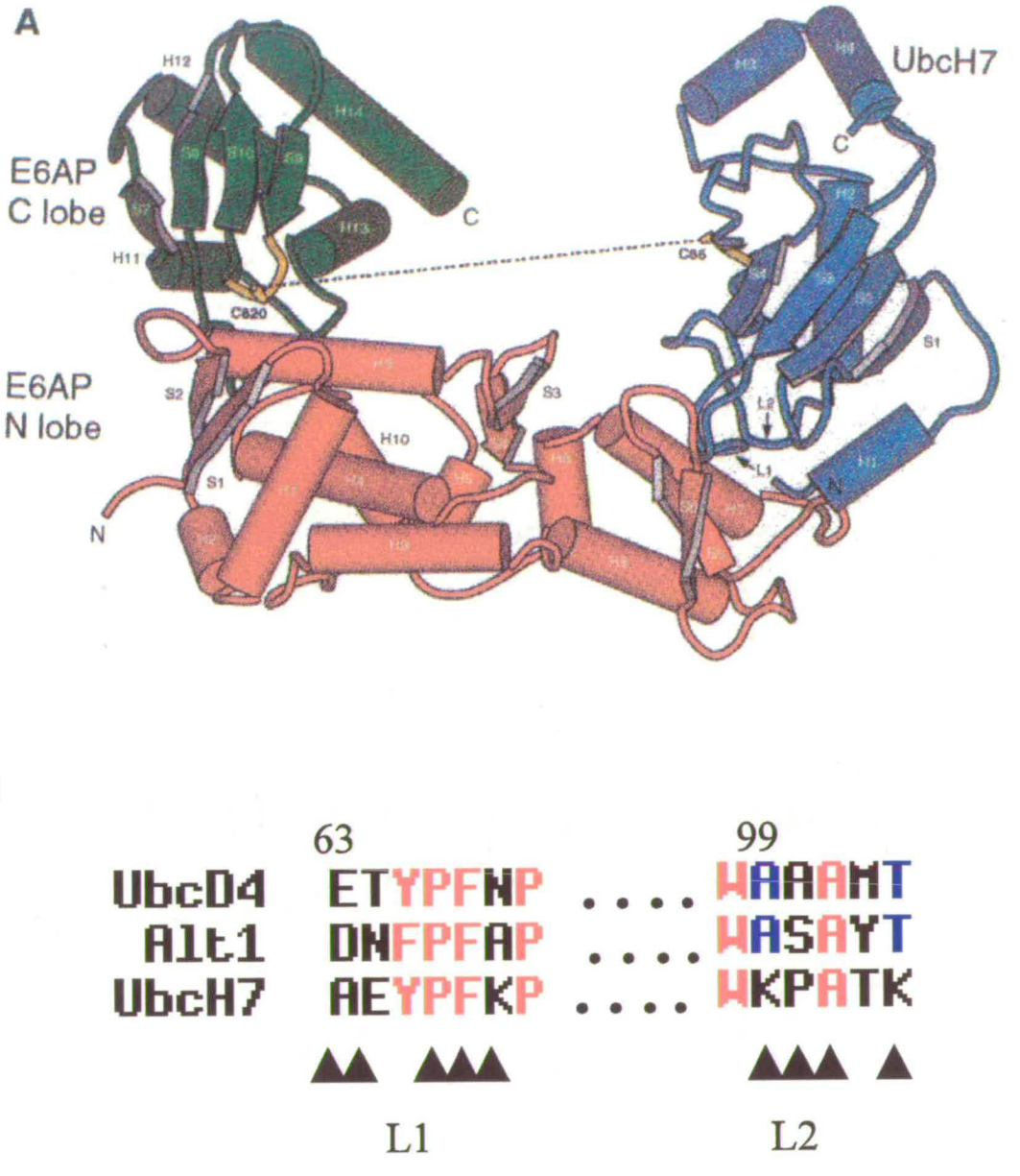


Figure 4.14: The interaction between E2s and Hect domain E3s.
A. The E6-AP Hect domain - UbcH7 complex. The E6-AP N-terminal lobe and C-terminal lobe are coloured red and green respectively. UbcH7 is coloured blue. The two active-site loops are yellow. The Hect-binding loops of UbcH7 are labelled L1 (residues 57-65) and L2 (residues 95-100). The dotted line indicates the open line of sight between the active-site cysteines of UbcH7 and E6-AP. Taken from Huang *et al.* (1999).
B. Alignment of the UbcH7 L1 and L2 sequences with the corresponding residues in UbcD4 and Alt1/CG4502. Residues identical in all three proteins are in red, those identical in two proteins are in blue. Arrowheads indicate the residues in UbcH7 which contact the E6-AP Hect domain. The numbers indicate the positions of L1 and L2 in the UbcD4 sequence.

the disadvantage of the two-hybrid system, in that they allow interactions between proteins which may never encounter one another in the embryo. However, the screen succeeded in uncovering proteins with which UbcD4 can interact reproducibly and specifically. Two of the six interactors identified, the proteasomal subunit Pros54 and the E3 CG3356, are components of the ubiquitin - proteasome pathway and a third, CG5862, has a domain which suggests it may associate with the proteasome. These proteins are therefore good candidates for ancillary factors in UbcD4-mediated degradation. The remaining three interactors, the metallopeptidases CG8775 and CG5839 and the transcriptional repressor Giant, may be UbcD4 substrates. Can any information on the possible role of UbcD4 be inferred from its interactors ? Since most are novel proteins, this is difficult. However it is notable that four of the six interactors have embryonic expression patterns which suggest post-germband retraction roles in the head and foregut.

gt is transcribed in specific groups of cells in the head and stomodeum, and is required for formation of both tissues. Foregut is really an extension of the gnathal segments of the head and is determined by the same genes (*spalt*, the head gap genes including *gt*, and the *Antennapedia-complex* genes *Deformed* and *Sex combs reduced*). The expression pattern of *gt* in the head is dynamic and highly specific. Perhaps ubiquitin-mediated proteolysis is required as well as transcriptional regulation to ensure that Gt is eliminated from particular cells when necessary. *CG3356* is expressed in the stomodeum, gnathal lobes and clypeolabrum. *CG5862* and *CG8775* are specifically transcribed in the salivary glands and oesophagus respectively. *CG5839* is the exception, being transcribed in the hindgut. *UbcD4* is strongly but not specifically expressed in head and foregut at this stage.

The results of the screen yield little information on possible roles for UbcD4 in germ cell development. A putative UbcD4 substrate in germ cells need not be specifically transcribed in germ cells - it may be a widely-expressed protein whose

destruction is required in migrating germ cells. However presence in the germ cells is obviously a prerequisite. Neither the E3 *CG3356*, nor the putative metallopeptidases *CG8775* and *CG5839*, nor *gt*, are transcribed in germ cells. *CG5862* transcripts are not present in mature gonads, but are abundant throughout the embryo at the stage when germ cells migrate. There are many reasons why the screen may have failed to identify the UbcD4 substrate in germ cells. Interactions which required an intact N-terminus in the prey protein may have been missed, as many of the library cDNAs had incomplete 5′ ends. The mRNA may be rare, resulting in underrepresentation of the cDNA in the library, or recognition may require an E3 or other cellular factor. Proof that UbcD4 is involved in germ cell development, or in head and foregut development, requires mutational analysis.

Chapter 5

Mutational analysis of genes encoding ubiquitinating enzymes.

While gene expression and protein-protein interaction data can give information on specific processes in which protein ubiquitination is involved, direct proof of biological function requires mutational analysis. This chapter describes experiments which aimed to disrupt the function of various genes encoding ubiquitinating enzymes, and to analyse the resulting defects in embryos. The techniques used were a) *P* element mutagenesis and b) RNA interference.

5.1 *P* element mutagenesis

5.1.1 Introduction

Spontaneous mutations in *Drosophila* are often due to mobile genetic elements. The *P* element was discovered through its association with male recombination and with 'hybrid dysgenesis' - a collective term for the defects found in the offspring of crosses between females from certain laboratory stocks and males from wild strains. The phenomenon is due to the mobilisation of *P* elements in the germline of the hybrid offspring. The discovery of *P* elements, and their use in insertional mutagenesis, has been reviewed by Grigliatti (1998).

Complete *P* elements consist of the transposase coding sequence flanked by 31 bp inverted repeats. Defective elements carry deletions in the transposase gene and cannot transpose autonomously. However if the ends of the element are

present, they can be mobilised if transposase is provided in *trans*. The transposase gene consists of four exons. In somatic tissue, the third intron is not removed by splicing, hence transposition occurs only in the germline.

P elements transpose by a 'cut and paste' mechanism, in which the donor element is excised and reinserted into a recipient site. They display a preference for inserting into the 5′ UTR of genes. Excision of a *P* element leaves behind a double-strand DNA break which is repaired using either the sister chromatid or the homologous chromosome as a template. If the sister chromatid is used, the end result of transposition is a net gain of one *P* element copy. Aberrant repair can lead to various types of imprecise excision, leaving either insertions or deletions at the original site of insertion. Thus *P* elements can cause mutations both by inserting into genes and by imprecise excision

These features of *P* element transposition have been adapted to create a method for insertional mutagenesis. Two important modifications have been the separation of the source of transposase from the mobile element, and the development of defective elements which carry selectable markers and bacterial plasmid sequences. The most frequently used type of element consists of *P* element flanking sequences within which is a wild-type eye colour marker gene, a *lacZ* gene, an antibiotic resistance gene and *E. coli* origin of replication. The *P* element flanking sequences are capable of being acted on by exogenously-supplied transposase. The eye colour gene, usually *rosy (ry)* or a *white (w)* derivative, allows detection of flies carrying the element. The *lacZ* gene is under the control of the weak *P* element promoter and its expression is sensitive to the effects of nearby promoters - this is useful in 'enhancer trap' screens for genes expressed in particular tissues. The plasmid sequences allow rescue of part of the element, along with its flanking sequence, by digestion, ligation and transformation into *E. coli* of fly genomic DNA.

The source of transposase is a stably-integrated *P* element in which the third

intron is deleted, causing somatic as well as germline production of transposase. Crossing flies carrying this element, known as *P[ry$^+\Delta$2-3]*, with flies carrying a marked transposable *P* element produces F1 flies in which transposase and transposable element are both present. Mutagenised chromosomes are 'cloned' by producing stocks from individual F2 flies. Flies carrying the potentially mutagenised chromosome but not the transposase source are selected, so that any insertions produced are stable. Mutations in novel genes, which function in a particular process, can be detected by screening for specific phenotypes. Flanking sequences can be directly cloned by plasmid rescue of the *P* element. Insertions in or near a specific characterised gene can be detected by PCR amplification of genomic DNA using a *P* element-specific primer and a gene-specific primer.

The *Drosophila* Genome Project provides a series of cytologically mapped *P* element insertions which disrupt genes essential for viability. In many cases, the sequence flanking the insertions has been determined. Searches of the *P* element flanking sequences in FlyBase failed to reveal any uncharacterised insertions in E2 or E3 - encoding genes. However a putative insertion in the E1 gene, *Duba1*, had previously been identified by Dr. P zur Lage. I analysed the phenotypes resulting from disruption of *Duba1*.

5.1.2 Analysis of an insertion in *Duba1*

A *P* element insertion mapping to the same polytene band as *Duba1* (46A1 -A2 on the second chromosome) was identified in FlyBase by P. zur Lage. The allele caused by the insertion was called *l(2)03405*. A stock carrying the allele, P1329, was obtained from the Bloomington Stock Centre (see Table 2.4). All P1329 adults are heterozygous for *l(2)03405*, indicating that homozygosity is lethal. The allele is balanced by the *CyO* chromosome.

Using primers complementary to the *P* element inverted repeats (P31 - see Table 2.5) and to *Duba1* sequences (Y4104 and 6067 - see Table 2.5), I amplified

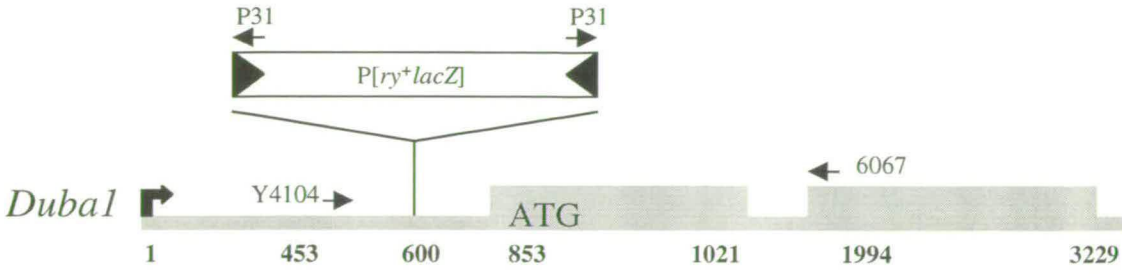


Figure 5.1: The *P* element insertion in allele *l(2)03405* of *Duba1*. The *Duba1* transcription unit, up to the end of the second exon, is shown in grey (from GadFly entry FBan0001782). The bent arrow indicates the transcription start site and wide areas represent exons. The position of the translation initiation codon (ATG) is shown. The *P* element is in white, with black triangles representing the terminal inverted repeats. Numbers in bold represent positions relative to the predicted transcriptional start site. Arrows represent the primers used in determining the position of the insertion.

the region flanking the insertion. Sequencing the PCR products revealed that the *P* element was positioned at nucleotide 600 of the cDNA, 253 nucleotides upstream of the translation initiation codon (see Figure 5.1).

5.1.2.1 Excision of the *P* element from *l(2)03405* to generate viable revertants

The mutant allele in stock P1329, *l(2)03405*, is a recessive lethal. In order to show that the *P* element insertion, rather than a separate mutation, is responsible for the lethality, I excised the element using the method described by Torok *et al.* (1993) (see Figure 5.2). Four lines were obtained, each of which yielded viable homozygotes for the *l(2)03405* derivative allele. Amplification and sequencing of *Duba1* from each line revealed that excision of the *P* element was precise. While this result shows that the inviability of *l(2)03405* homozygotes requires the presence of the *P* element, it does not prove that the phenotype is due to disruption of *Duba1* expression. Proving this would require rescue of the phenotype by providing P1329 flies with a *Duba1* transgene. This experiment was not performed.

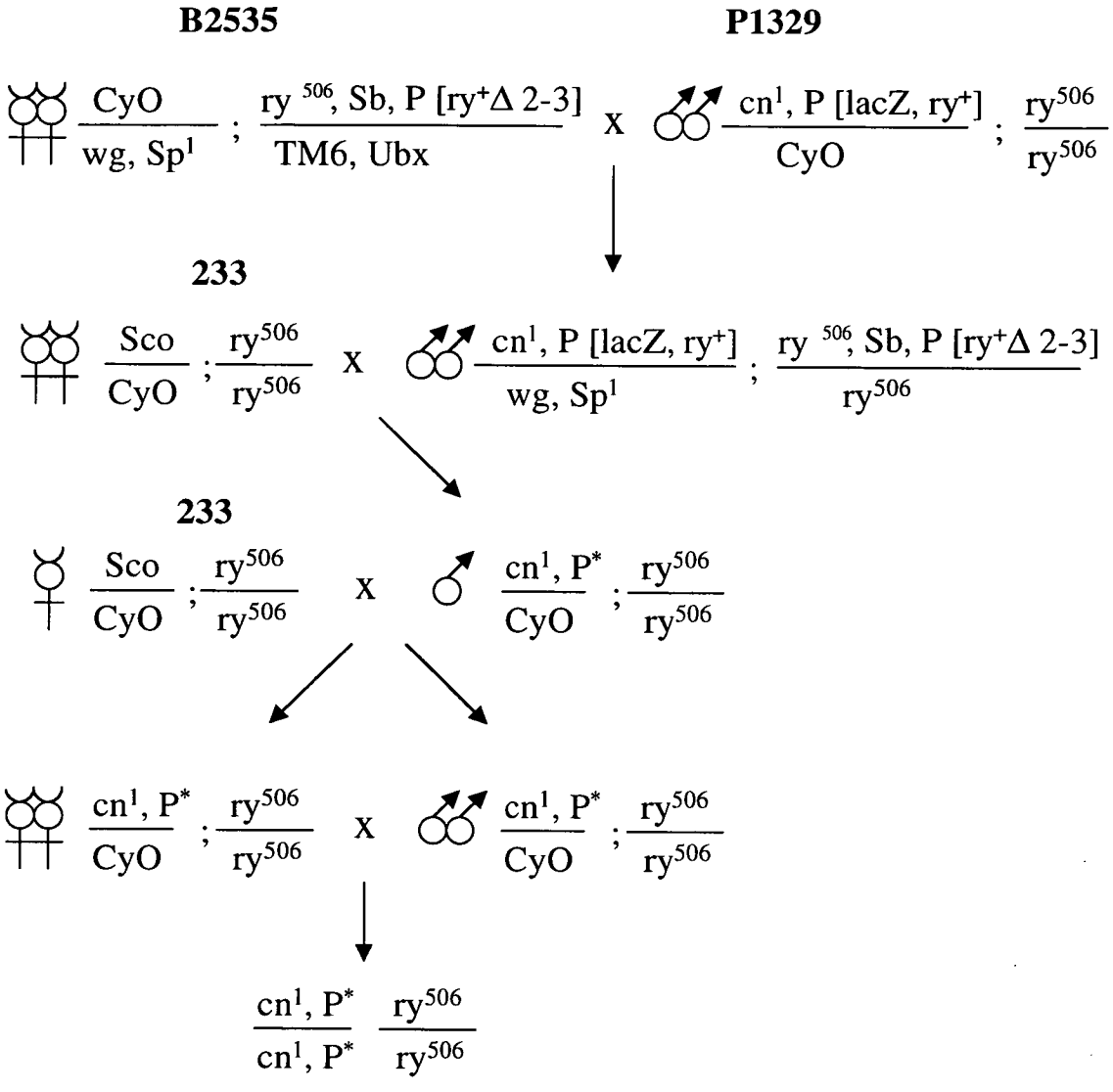


Figure 5.2: Scheme for excision of the *P* element from the *l(2)03405* allele of *Duba1*. Stock numbers are in bold. P1329 males were mated en masse to virgin females from the stock B2535, which carry the *P[ry+Δ2-3]* transposase gene. The male progeny which carried both the *P* element and the transposase gene (Sb^-, Ubx^+,ry^+) were mated to virgin females of the genotype *Sco/CyO;ry506*. Male progeny in which excision of the *P* element had occurred (Cy^-, ry^-, Sco^+, Sb^+, Sp^-) were crossed individually to virgin *Sco/CyO ; ry506* females. Progeny carrying the chromosome from which the *P* element had excised (P*) were intercrossed, yielding Cy^+ flies which were homozygous for the excision site. Abbreviations: *Cy = Curly, Sb = Stubble, Ubx = Ultrabithorax, ry = rosy, Sco = Scutoid, Sp = Sternopleural.*

5.1.2.2 Analysis of lethal phase of *l(2)03405* homozygotes.

In order to determine whether *l(2)03405* homozygotes can complete embryogenesis, 200 P1329 embryos were scored for their ability to hatch. 30% of P1329 embryos failed to hatch, compared to 7% in the wildtype strain Oregon R53. Flies were generated which carried a second chromosome, with either *l(2)03405* or *CyO*, in an Oregon R53 background. 11% of embryos failed to hatch in the *l(2)03405/+* strain, and 32% in the *CyO/+* strain. These data suggest that the approximately 25% higher level of embryonic lethality in P1329 compared to wildtype is solely due to the inviability of *CyO* homozygote embryos. *l(2)03405* homozygote embryos appear to complete embryogenesis.

In order to identify *l(2)03405* homozygote larvae, and monitor their development, I generated flies carrying *l(2)03405* over the balancer chromosome *CyO-30* (see Table 2.4). This is a second chromosome carrying *CyO*, a UAS-Green Fluorescent Protein (GFP) transgene, and a GAL4 transgene under the control of the *Kruppel* promoter (Casso *et al.*, 2000). The GFP transgene is expressed at low levels in embryos and first instar larvae, and at higher levels in second and third instar larvae and pupae. *l(2)03405* homozygotes can be identified by their lack of green fluorescence. Pupae and wandering larvae collected from the sides of a bottle of flies were all GFP^+ (100 out of 100 scored in each case) (Figure 5.3). When embryos were collected for one hour and allowed to develop at 25 °C, GFP^- larvae appeared to develop successfully to third instar, but not to pupate (Figure 5.3). *l(2)03405* is therefore a third instar larval lethal allele of *Duba1*.

5.1.2.3 The *P* element insertion in *l(2)03405* does not completely abolish expression of *Duba1*

In situ hybridisation analysis of *Duba1* expression was carried out on embryos from the P1329 stock. *Duba1* expression was not completely abolished in any embryos. Figure 5.4 shows embryos representative of the lowest levels of *Duba1* staining, which are presumed to be *l(2)03405* homozygotes. *Duba1* expression

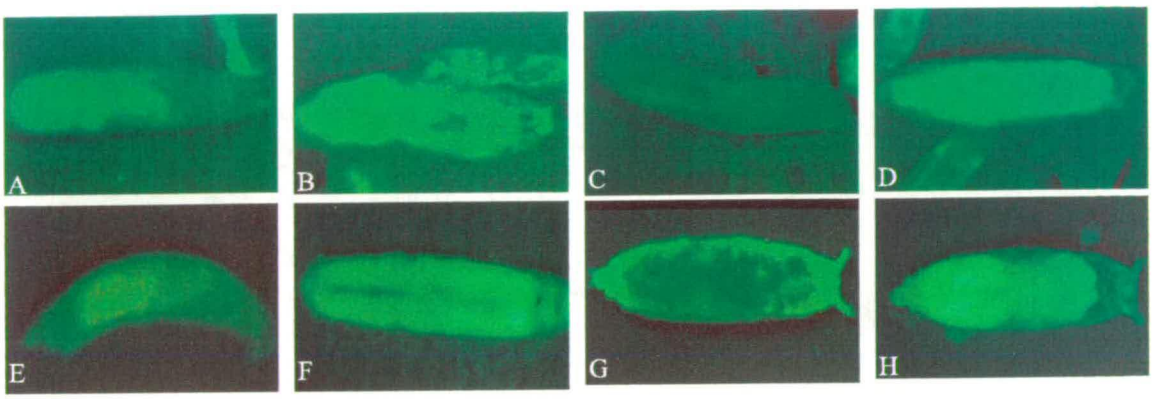


Figure 5.3: Identification of *l(2)03405* homozygote larvae by lack of GFP expression. All individuals are from a stock carrying *l(2)03405/CyO-30* except for E and G, which are from Oregon R53. Anterior is to the right. A and B: Second instar larvae, GFP^- and GFP^+ respectively. C and D: Third instar larvae, GFP^- and GFP^+ respectively. E and F: Wandering larvae. All *l(2)03405/CyO-30* wandering larvae were GFP^+ (F). E shows the level of autofluorescence in a wildtype (Oregon R53) wandering larva. G and H: pupae. All *l(2)03405/CyO-30* pupae were GFP^+ (H). G shows the level of autofluorescence in a wildtype pupa.

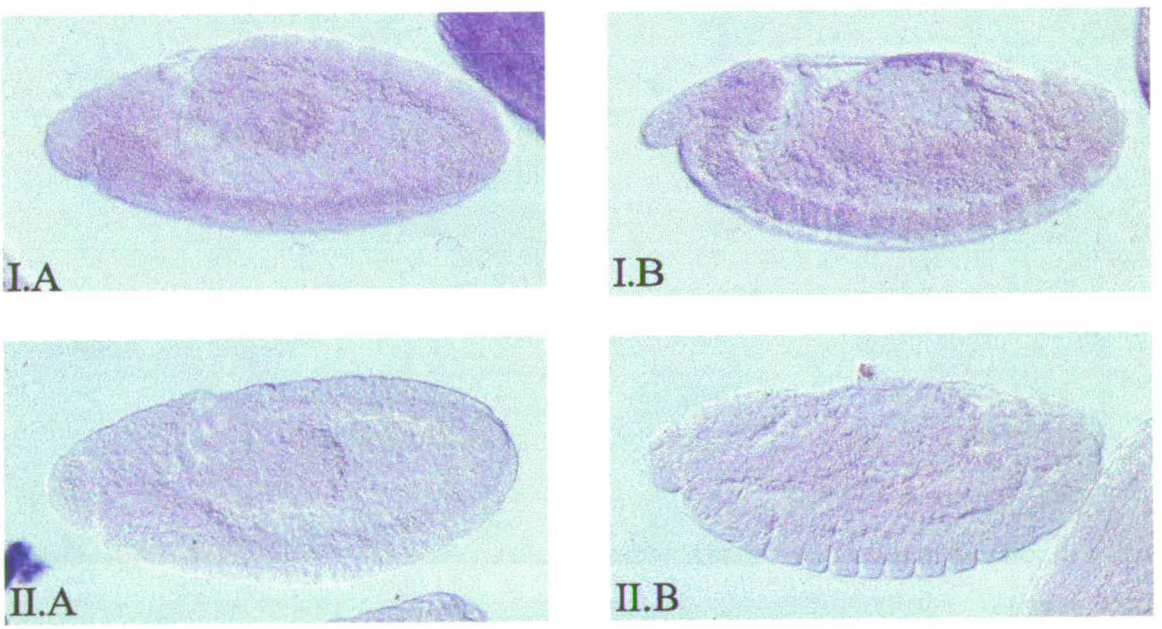


Figure 5.4: *In situ* hybridisation analysis of *Duba1* expression in embryos from the P1329 strain. The embryos shown in I.A and I.B are representative of those with the lowest levels of *Duba1* expression, and are therefore putative *l(2)03405* homozygotes. The embryos in II.A. and II.B were probed with *Duba1* sense RNA, and show background levels of staining. A - stage 9. B - stage 12. C -stage 15.

is clearly detectable above background staining. This indicates that *l(2)03405* is a hypomorphic allele of *Duba1*, reducing but not abolishing its expression in embryos. The embryos with lowest *Duba1* expression display no obvious abnormalities.

5.1.2.4 Discussion

Since at least one ubiquitin-conjugating enzyme, UbcD1, mutates to embryonic lethality, and Duba1 is expected to be essential for UbcD1 function, it is perhaps surprising that *l(2)03405* homozygotes can complete embryogenesis. However, it appears that these embryos express a low level of *Duba1* mRNA, which may be sufficient for normal function. In addition, the Duba1 produced from maternal transcripts may perdure throughout embryogenesis. The inviability of larvae homozygous for *l(2)03405* may reflect their requirement for a greater amount of Duba1, and/or degradation of the maternal protein. Alternatively the *P* element may disrupt a larva-specific enhancer of *Duba1*. The possibility that the *P* element also interferes with a neighbouring gene, essential for larval viability, cannot be discounted.

5.1.3 *P* element mutagenesis of *UbcD4*

As discussed in chapter 3, the expression pattern of *UbcD4* makes it a good candidate for a ubiquitination enzyme which regulates specific embryogenic processes, germ cell migration in particular. In order to prove its developmental function by mutational analysis, I attempted to create a *P* element insertion in *UbcD4* by 'local jumping'.

5.1.3.1 Introduction

Local jumping is based on the observation that transposing *P* elements preferentially insert close to their original position. Estimates of the magnitude of the effect vary. In a transposition experiment performed by Golic (1994), almost 80%

of transpositions were to sites so close to the parental element that they could not be resolved cytologically. However Tower *et al.* (1993) estimate that approximately 20% of transposing elements insert 2-128 kb from the original element, and that many more insert inside the orginal element. Nevertheless it is clear that the probabilility of mutating a specific gene can be significantly enhanced by mobilising a *P* element located close to it.

5.1.3.2 Experimental procedure

The map position of *UbcD4* when this experiment was carried out was estimated as 67C05-11 (Kirby, 1996). The element in the FlyBase database which mapped closest to this was inserted in the *l(3)02240* allele at position 67C04-05, and carried in stock P1558. This was chosen as the starting element for an attempted *UbcD4* mutagenesis. The mutagenic scheme is shown in Figure 5.5. Stocks were screened for insertions in *UbcD4* by carrying out PCR amplification on genomic DNA from pools of 20 flies. Separate reactions were carried out with the *P* element - specific P31 primer and each of two *UbcD4*- specific primers, E2p5 and E2p1 (see Figure 5.6). Control reactions had been performed to establish that such a scheme could detect one insertion-carrying fly in a pool of 20.

5.1.3.3 Results and discussion

Almost 1400 stocks carrying possible new insertions were created and screened. No insertions in *UbcD4* were detected. The failure to jump an element into *UbcD4* was not due to lack of transposase production, as flies in which the element had excised were observed. One stock was created which enabled a PCR product of approximately 500 bp to be amplified in both P31 - E2p5 and P31 - E2p1 reactions. The product was also amplified in reactions with P31 alone and was presumably due to reinsertion of the *P* element 500 bp from its starting position. This event demonstrates that functional transposase was produced and that the method used was capable of detecting insertions. However if local transposition

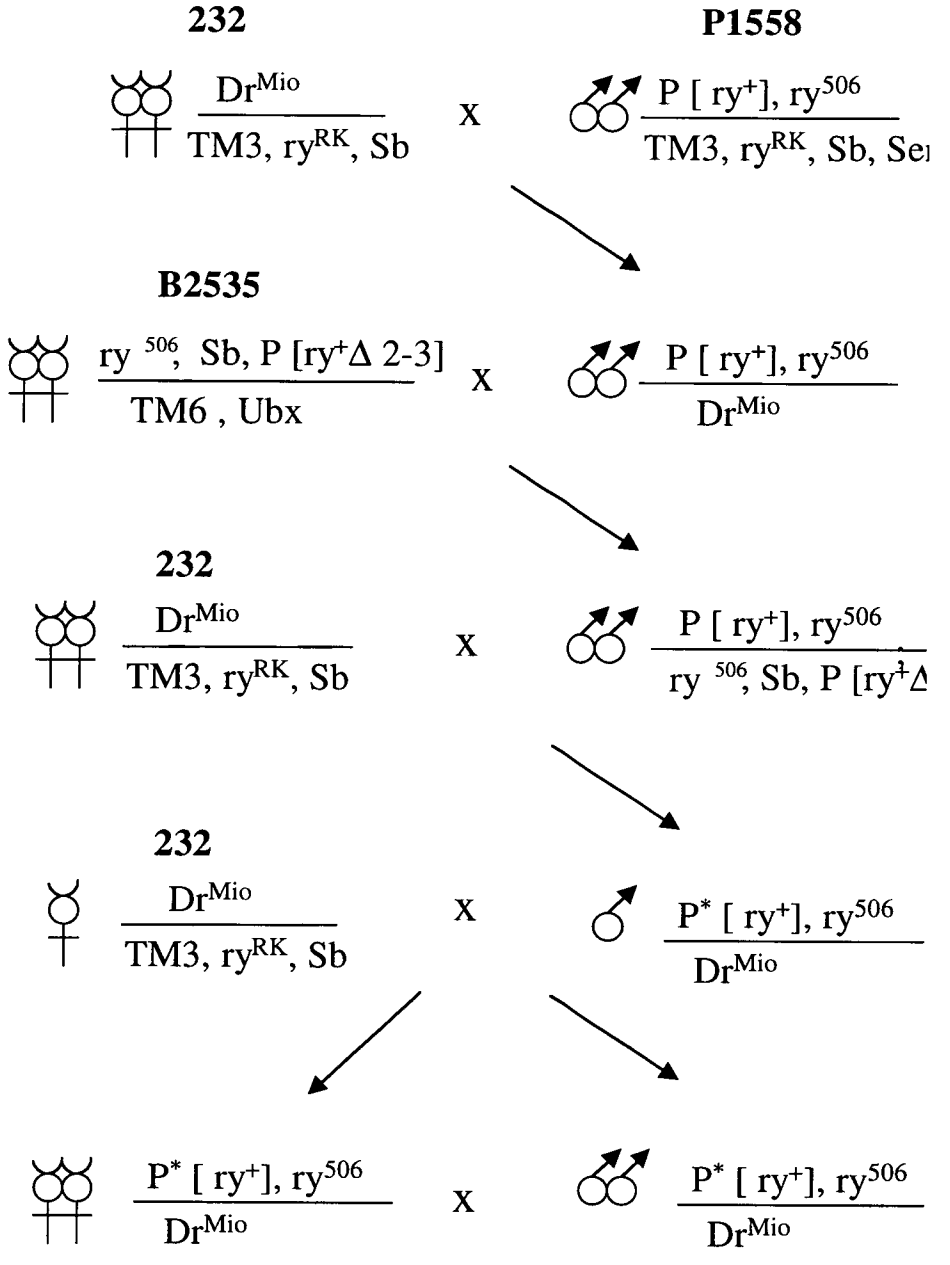


Figure 5.5: Scheme to mobilise the *P* element in strain P1558 to create an insertion in *UbcD4*. Stock numbers are written in bold. P1558 males were mated en masse to virgin females of the genotype $Dr^{Mio}/TM3$, *ry*, *Sb*. The male *P[lacZ*, $ry^+]/Dr^{Mio}$ progeny were mated to B2535 females which carry the $P[ry^+,\Delta 2\text{-}3]$ transposase gene. F2 flies which carried both the *P* element and the transposase gene (Sb^-, Ubx^+, w^+) were mated to virgin females of the genotype $Dr^{Mio}/TM3$, *ry*, *Sb*. Male progeny which lacked the transposase gene (Sb^+, Dr^{Mio}) were crossed individually to virgin $Dr^{Mio}/TM3$, *ry*, *Sb* females. ry^+, Dr^{Mio} progeny were intercrossed, yielding stocks of flies carrying each potentially mutagenised third chromosome. Abbreviations: *Cy = Curly, Sb = Stubble, Ubx = Ultrabithorax, ry = rosy, Sco = Scutoid, Ser = Serrate, Dr = Drop.*

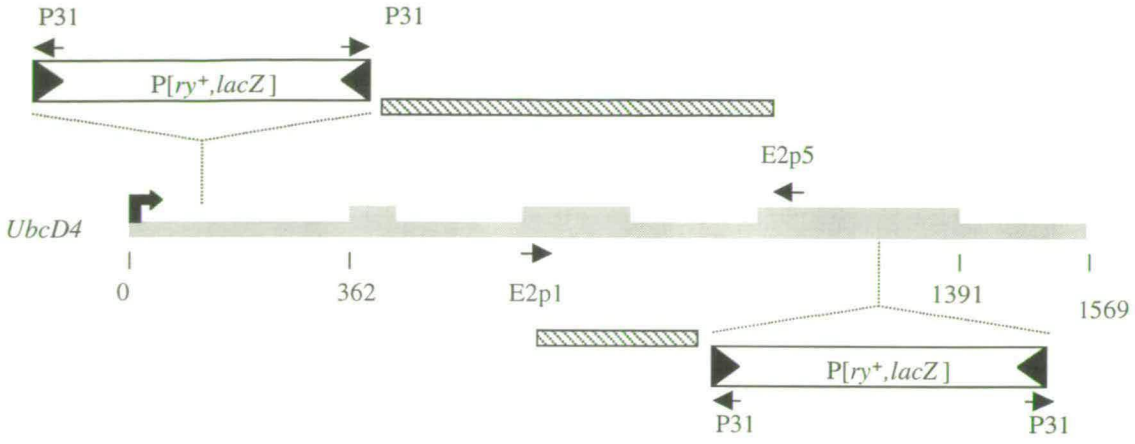


Figure 5.6: Strategy for screening mutagenised stocks for insertions in *UbcD4*. The grey box represents the *UbcD4* transcription unit (GadFly entry FBgn0015321) and the wide areas represent exons. The bent arrow indicates the predicted transcription start site. Arrowheads show the positions of the primers used in the screen. Numbers represent positions relative to the transcription start site. The shaded boxes represent the PCR products which would be generated by *P* element insertions at various positions in the gene.

was occurring efficiently, it might be expected that more than one such event should have been detected. It is probable that *UbcD4* was outside the zone of enhanced transposition (approximately 130 kb) from *l(3)02440*. Since the completion of the genome sequence, the map position of *UbcD4* has been revised from 67C05-11 to 67B11-13. *l(3)02440* maps to 67C04-05. According to the GeneSeen database, this represents a distance of approximately 800 kb.

P elements insert nonrandomly but the basis for their specificity is not understood. They display a preference for euchromatic sites over heterochromatic (Berg and Spradling, 1991) and a tendency to insert at the 5′ ends of genes (Spradling *et al.*, 1995). Local sequence composition also affects the probability of insertion. While a consensus sequence cannot be obtained, insertion sites tend to be GC rich (O'Hare and Rubin, 1983; Liao *et al.*, 2000). Structural parameters such as DNA bendability, B-DNA twist and protein-induced deformability deviate significantly from random at insertion 'hot spots', arguing that *P* elements recognise aspects of DNA structure rather that sequence composition (Liao *et al.*, 2000). Given our

lack of understanding of *P* element insertion site specificity, it is difficult to say whether the region in which *UbcD4* is located is suitable for *P* element insertion.

5.2 Double-stranded RNA-mediated interference with *UbcD4* expression

5.2.1 Introduction

Double-stranded RNA-mediated interference is a recently-discovered method to disrupt gene function in a number of organisms. The phenomenon was discovered by *C. elegans* workers attempting to silence expression of a specific gene using antisense RNA. Double-stranded RNA (dsRNA) was found to be several orders of magnitude more effective than either sense or antisense strands alone (Fire *et al.*, 1998). RNA interference is now a standard method of analysing gene function in *C. elegans* (reviewed by Fire (1999)). Introduction of dsRNA corresponding to either the UTR or coding region of a particular gene can silence the endogenous gene, producing a phenocopy of a null mutation. The dsRNA can be introduced by injection or by feeding worms on *E. coli* which have been engineered to express the appropriate dsRNA. Interference can be inherited by the progeny of the treated worms. The effect can cross cell boundaries and appears to be amplified, since a small number of molecules of dsRNA can completely silence a gene. RNAi has also been demonstrated in plants, zebrafish, mice and *Drosophila* (reviewed by Sharp (1999)).

RNAi can be produced in a cell-free extract of *Drosophila* syncytial blastoderms, and this system is being used to investigate its mechanism (Tuschl *et al.*, 1999; Zamore *et al.*, 2000). Silencing is caused by degradation of the cognate mRNA, within the sequence spanned by the dsRNA (Tuschl *et al.*, 1999; Zamore *et al.*, 2000). Pieces of dsRNA 20-25 nucleotides in length appear to be intermediates in the process (Zamore *et al.*, 2000). The current model for dsRNA-mediated mRNA degradation (reviewed by Bass (2000)) proposes that dsRNA is targeted

by a dsRNA endonuclease and cleaved into small fragments. These 20-25mers are thought to bind to a ribonuclease, possibly the same enzyme which cleaves the dsRNA, and to act as a template for sequence-specific cleavage of the endogenous mRNA. This could proceed by exchange of the mRNA with the dsRNA sense strand and cleavage of the mRNA at the same site, regenerating the nuclease bound to a short piece of dsRNA. This model could account for the apparently catalytic nature of the interference. It is possible that the signal is also amplified by an RNA-dependent RNA polymerase.

In *Neurospora*, *C. elegans* and plants, introduction of a transgene causes post-transcriptional silencing of the endogenous gene. This phenomenon shares some features with RNAi (reviewed by Birchler *et al.* (2000)). In plants and *Neurospora*, some cases of silencing are achieved by elimination of specific transcripts (Cogoni and Macino, 1997; Voinnet *et al.*, 1998). Small RNA fragments corresponding to the affected gene are observed specifically in plants whose transgenes induce silencing of the endogenous gene (Hamilton and Baulcombe, 1999). Silencing induced by a repetitive transgene array in the *C. elegans* germline can persist after the transgene has been removed, implicating a non-DNA interfering factor (Dernburg *et al.*, 2000). The sequence requirements for silencing suggest that this factor may be RNA (Dernburg *et al.*, 2000). Genetic analysis has identified at least nine *C. elegans* genes required for efficient RNAi (Ketting *et al.*, 1999; Tabara *et al.*, 1999; Smardon *et al.*, 2000; Grishok *et al.*, 2000). Some are only required for RNAi, while *rde-2* and *mut-7* are also needed for transgene-mediated gene silencing (Dernburg *et al.*, 2000) and for germline silencing of transposition (Ketting *et al.*, 1999; Tabara *et al.*, 1999). It has therefore been suggested that these phenomena use different mechanisms to generate the same interfering agent, which activates a common silencing pathway.

Injection of *Drosophila* embryos with dsRNA potently phenocopied null alleles of *twist* (Misquitta and Paterson, 1999), *fushi tarazu* and *even-skipped* (Kennerdell

and Carthew, 1998), although the strength of interference varied between individuals and with dose of injected dsRNA (Kennerdell and Carthew, 1998). Interference with *tramtrack* showed that the effect could persist, as Tramtrack functions at stage 11-12, approximately 6 hours after the time of injection. However RNAi was not equally effective at silencing all genes : for *hedgehog* and *wingless*, interference was localised to the site of injection (Kennerdell and Carthew, 1998). Currently the technique has only been used to study gene function in embryos or in cultured cells (Clemens *et al.*, 2000; Jones *et al.*, 2000), since the silencing does not persist to later developmental stages (Misquitta and Paterson, 1999). However a method to express dsRNA as an extended hairpin-loop in transgenic flies has recently been developed (Kennerdell and Carthew, 2000). Using this system, silencing can be stably inherited and induced at any developmental stage.

RNAi is increasingly widely used to investigate the function of *Drosophila* genes for which no mutant is available (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999; Clemens *et al.*, 2000; Jones *et al.*, 2000). I injected embryos with double-stranded *UbcD4* RNA in an attempt to silence *UbcD4* expression. I examined the effect of the dsRNA on the embryos' ability to complete embryogenesis, and on the ability of the pole cells to migrate from the posterior midgut to the mesoderm.

5.2.2 Experimental procedure

Sense and antisense *UbcD4* RNAs were synthesised by *in vitro* transcription. Equimolar amounts of the two strands were mixed and annealed (Figure 5.7). The dsRNA was stored as an ethanol precipitate and dissolved in injection buffer, at a concentration of approximately 2 μM, immediately before use.

Embryos were collected for one hour before being dechorionated, dessicated and injected with either *UbcD4* dsRNA or with injection buffer alone. The embryos were therefore syncytial blastoderms aged approximately 30 - 90 minutes

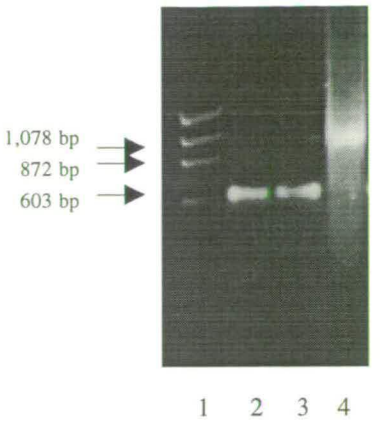


Figure 5.7: Preparation of *UbcD4* double-stranded RNA. Aliquots of sense and antisense *UbcD4* RNA, and of a mixture of the two strands which had been allowed to anneal, were electrophoresed on a 1.2 % agarose gel. 1 - *HaeIII*-digested ϕX174 DNA, 2 - sense *UbcD4* RNA, 3 - antisense *UbcD4* RNA, 4 - double-stranded *UbcD4* RNA. The arrows indicate the sizes of the ϕX174 bands.

at the time of injection. The injection of buffer alone was performed so that any defects caused by the experimental procedure could be distinguished from those caused by the dsRNA. The volume of dsRNA or buffer injected was approximately 100 - 300 pl, as estimated from the diameter of a drop injected into halocarbon oil. This corresponds to 0.2 - 0.6 fmoles of dsRNA per embryo. 0.2 fmoles dsRNA per embryo is the recommended starting dose for an RNAi experiment (Kennerdell and Carthew, pers. comm.).

Two series of injections were performed. In the first, 400 embryos each were injected with *UbcD4* dsRNA and with buffer alone. The embryos on two coverslips (60 embryos) were incubated at 25°C for 24 hours to determine the percentage which hatched. After this time, unhatched embryos were collected and fixed. The remaining embryos were incubated at 18°C for various periods up to 16 hours, before being fixed and manually devitellinised. Timepoints of 12 - 16 hours were used to allow the embryos to reach stage 9-11, when pole cell migration normally occurs. Shorter incubation periods were used to examine the effect of dsRNA on gastrulation and germband extension. In order to assess whether *UbcD4* expression had been disrupted by injection of dsRNA, *in situ* hybridisation

was performed. The embryos were subsequently stained with Vasa protein to visualise their germ cells.

In the second series, 400 embryos each were again injected with *UbcD4* dsRNA and with buffer. Again, 60 embryos were incubated at 25 °C for 24 hours to determine their ability to hatch. The remaining embryos were incubated for 12-15 hours at 25 °C before being fixed, devitellinised and stained for *UbcD4* mRNA and for Vasa protein as before. After this period, embryos would be expected to have reached stage 12-16 of embryogenesis. This experiment was designed to investigate whether dsRNA-injected embryos had a defect in late embryogenesis.

5.2.3 Results

Figure 5.8 shows the number of embryos on each of four coverslips (30 embryos per coverslip) which had failed to hatch 24 hours after injection with either *UbcD4* dsRNA or injection buffer. Embryos injected with *UbcD4* dsRNA showed a consistently lower ability to hatch than those injected with buffer alone.

The mortality of the buffer-injected embryos may be due to a variety of factors. Insufficiently dessiccated embryos, or embryos injected with too blunt a needle, leak cytoplasm at the time of injection : since the cytoplasm contains morphogens, this can impair embryogenesis. Over-dessiccated embryos undergo 'funnelling' at the time of injection, which may cause morphological abnormalities. Embryos covered with too much halocarbon oil may receive insufficient oxygen, while those covered with too little oil may dry out. While these conditions would be expected to vary between batches of embryos injected at different times, the hatching rate of buffer-injected embryos showed little variability between coverslips.

The injection procedure would also be expected to contribute to the mortality observed in dsRNA - injected embryos. However, as they showed a consistently lower viability than control embryos, it seems likely that the *UbcD4* dsRNA itself impairs embryogenesis.

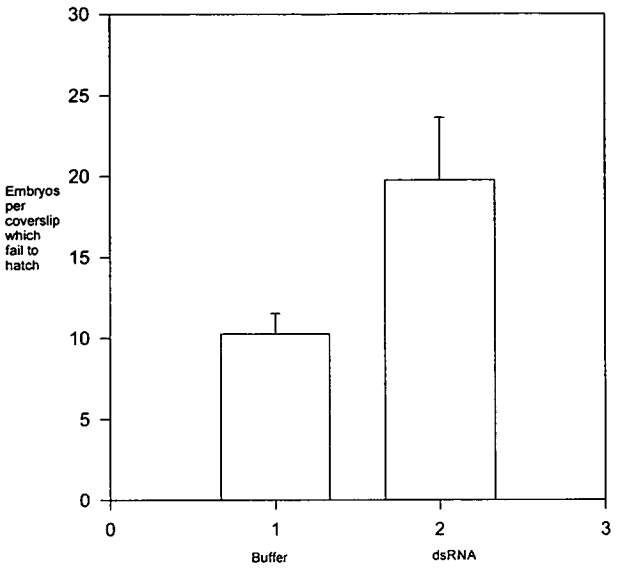


Figure 5.8: Hatching counts for embryos injected with *UbcD4* double-stranded RNA and with injection buffer alone. 4 coverslips of embryos (30 embryos per coverslip) were injected with either *UbcD4* dsRNA or with injection buffer alone then incubated at 25 °C for 24 hours. The first bar shows the average number of embryos per coverslip which had failed to hatch after injection with buffer and the second bar shows this number for *UbcD4* dsRNA - injected embryos. The error bars show the standard deviations.

While normal *UbcD4* staining was observed in control embryos which had not been through the injection procedure, only background levels of staining were observed in both dsRNA - treated and buffer - treated embryos. This implies that some aspect of the injection or manual devitellinisation procedure renders the embryos unsuitable for *in situ* hybridisation and makes it impossible to verify that injection with dsRNA causes degradation of the *UbcD4* mRNA.

No morphological defects were observed in embryos undergoing gastrulation, germband extension or early germband retraction (Figure 5.9, I.A - I.C). However a high proportion (approximately 60 %) of embryos undergoing later germband retraction appeared morphologically abnormal. Commonly the amnioserosa was displaced anteriorly, covering part of the head (Figure 5.9, I.D - I.E). This was not seen in control embryos at the same stage (Figure 5.9 II.A and II.B) although in some control embryos the amniserosa did bulge considerably, possibly due to squashing of the embryo during the experimental procedure.

Abnormalities were also seen in post-retracted embryos. Dorsal closure and head involution normally occur simultaneously during stages 14 and 15. However, in *UbcD4* dsRNA - treated sample, many embryos appeared to have completed head involution without completing dorsal closure. The embryos shown in Figure 5.9, I.G. and I.H., have the head morphology of a stage 15-17 embryo but the edge of the germ band is visible and part of the amnioserosa is exposed. Similar defects were observed in approximately half the dsRNA-treated embryos but not in the buffer-injected embryos. Figure 5.9 I.I and II.C show embryos from the dsRNA - treated sample and control sample respectively, with the normal appearance of a stage 15-16 embryo.

It is not clear whether the affected embryos can eventually achieve dorsal closure and continue development. The embryos which had failed to hatch after 24 hours varied in appearance. Most of the control embryos showed no identifiable structures, suggesting that embryogenesis had not progressed past the time of injection. The remainder appeared to have developed to stage 17, but failed to hatch (data not shown). These had not shown any muscular movement when examined prior to fixing and were probably either dead or paralysed, rather than merely delayed. Approximately the same proportion of dsRNA-injected embryos had no structures, and some also appeared to be normal stage 17 embryos which had failed to hatch (Figure 5.9, I.J). Others seem to have arrested development slightly earlier. The embryo in Figure 5.9, I.K. has a completed head involution but its midgut appears to lack the constrictions which normally appear at stage 15. Figure 5.9. I.L shows an embryo with the midgut appearance characteristic of stage 15 embryos but with an apparently malformed head. Approximately a quarter of the unhatched dsRNA-injected embryos appeared to have arrested at stage 15 or 16, compared to only one of the controls. However the numbers of embryos sucessfully retrieved and fixed were small (30 dsRNA injected embryos and 18 controls), therefore no firm conclusions can be drawn from these results.

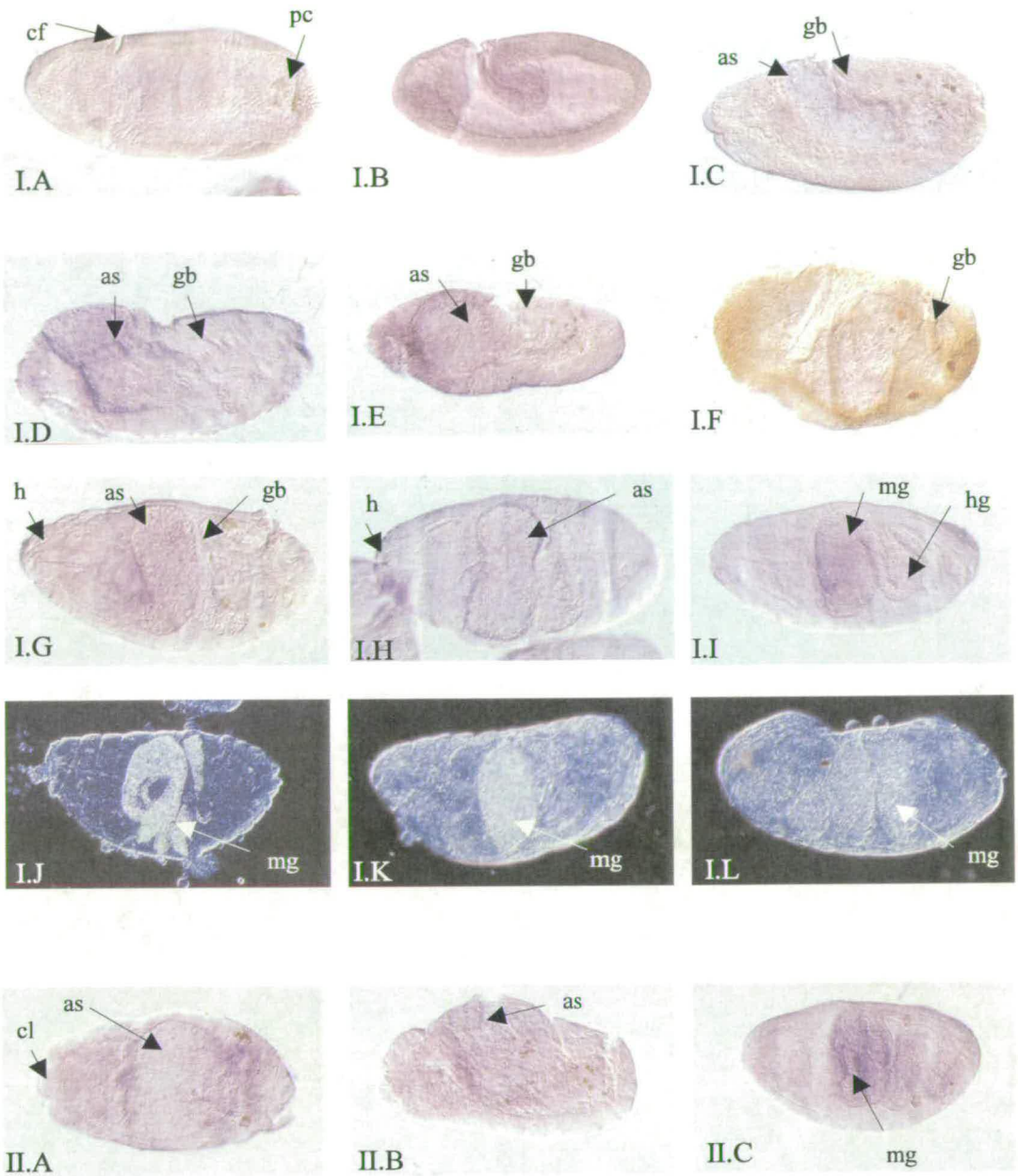


Figure 5.9: Effects of injection with double-stranded *UbcD4* RNA on embryonic development. The embryos shown in panels I.A to I.L were injected with *UbcD4* dsRNA. Those in panels II.A to II.C were injected with injection buffer alone.Embryos I.A-C, I.E-G and II.A-B were subjected to *UbcD4in situ* hybridisation and Vasa immunostaining. Embryos I.D, I.H, I.I. and II.C were subjected to *UbcD4in situ* hybridisation alone. Embryos I.J - I.L were embryos which had failed to hatch after 24 hours - they were fixed and devitellinised but not stained. These embryos are viewed in dark-field for better visualisation of internal structures. All embryos are oriented with anterior to the left. Those in panels I.B - I.E, I.J - I.L, and II.B - II.C, are viewed laterally. The remainder are viewed dorsally. Abbreviations :as = amnioserosa, cf = cephalic furrow, cl = clypeolabrum, gb = germband, h = head, hg = hindgut, mg = midgut, pc = pole cells.

No significant defects in pole cell movements were observed in dsRNA- treated embryos. Pole cells associated with the posterior midgut primordium during gastrulation and germband extension (Figure 5.9, I.A - I.B), and migrated posteriorly towards the mesoderm during early germband retraction (Figure 5.9, I.C, I.E). They then divided into two groups on either side of the ventral midline, and coalesced (Figure 5.9, I.F, I.G). In some embryos, a number of pole cells remained associated with the posterior midgut (Figure 5.9, I.F), but this was also observed in some buffer - treated embryos (data not shown).

5.2.4 Discussion

Due to the failure of the *UbcD4 in situ* hybridisation on both dsRNA and buffer - injected embryos, it is not possible to determine whether silencing of *UbcD4* expression was achieved. The lower hatching rate of dsRNA - treated embryos than of control embryos implies that embryogenesis was impaired by the dsRNA. However, a third of the dsRNA - injected embryos survived. The extent of *UbcD4* silencing may have varied between embryos due to injection of different doses of dsRNA. Although other workers report potent interference using 0.1 fmoles dsRNA per embryo, and detectable interference with doses as low as 0.01 fmoles (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999), the sensitivity of the interference may vary between genes. Another possibility is that the dsRNA may have failed to spread through the embryo, resulting in *UbcD4* silencing localised to the site of injection. This phenomenon has been observed with several genes including *engrailed*, *wingless* and *frizzled* (Kennerdell and Carthew, 1998). While all embryos were injected from the posterior end, the precise site of injection may have varied considerably.

One aim of this experiment was to examine whether *UbcD4* expression is required for pole cell migration from the posterior midgut to the mesoderm at stages 10-12. The results provide no evidence for this. The timing and direction

of pole cell movement appeared to be unaffected by injection of dsRNA. This cannot be taken as clear evidence against a role for *UbcD4* in pole cell migration, as it is not certain that silencing in the pole cells was achieved. As previously stated, silencing may have been localised to the injection site. In addition, the timing of injection meant that the pole cell membranes were formed whereas the remainder of the embryo was syncytial. Although dsRNA is able to cross the membranes of cultured cells, (Clemens *et al.*, 2000) , it is not clear how efficient this process is in the embryo.

Injection of *UbcD4* dsRNA had no effect on stages 3 - 11 of embryogenesis. All the deformities observed were in embryos of stage 12 or later. Anterior displacement of the amnioserosa during germband retraction, and delay of dorsal closure until after completion of head involution, were specifically present in a high proportion of dsRNA - treated embryos. Unhatched embryos in dsRNA-treated and control embryos included embryos which had apparently died at the time of injection, and embryos which had reached stage 17 but failed to hatch. However the dsRNA-treated sample specifically included embryos which had arrested at stage 15 - 16 with possible gut or head abnormalities. Although inconclusive, these results suggest a specific requirement for UbcD4 in morphogenetic events from stage 12 until completion of embryogenesis. It is difficult to say which defects may be directly due to loss of UbcD4 function. However, *UbcD4* mRNA is abundant in stage 12-14 embryos but less so in stage 15-17 embryos. If this reflects the relative requirement for UbcD4 during those stages, perhaps the defects in germband retraction and dorsal closure are directly due to interference with *UbcD4* expression, and the later defects are indirect.

Germ band retraction is the process by which the germband recedes to the posterior of the embryo and the amnioserosa, a membraneous structure, spreads out to cover the whole of the dorsal surface. Dorsal closure is the process by which lateral ectodermal cells elongate dorsalward, spread over the amniserosa

and ultimately fuse at the dorsal midline to form the dorsal epidermis. Both processes are primarily driven by changes in cell shape and packing, rather than in cell number or arrangement (Martinez Arias, 1993; Glise *et al.*, 1995).

Dorsal closure is the better-studied of the two movements. 'Dorsal-open' mutants fall into two classes (reviewed by Noselli and Agnes (1999)). One class consists of members of the Jun amino-terminal kinase (JNK) signalling pathway,such as *basket* and *hemiopterous.* The other class are involed in regulating aspects of cell architecture such as the cytoskeleton (*zipper, cappucino*) and gap junctions (*coracle, canoe*). The link between the signalling cascade and changes in the cytoskeleton may be provided by the Rho subfamily of small Ras-related GTPases (Magie *et al.*, 1999). The ligand which activates JNK signalling has not been identified. Germband retraction seems to be driven by a signal from the amnioserosa (Lamka and Lipshitz, 1999): this depends on amnioserosal expression of *u-shaped, pebbled/hindsight* and *serpent.* Expression of a dominant-negative version of the Rho-related Rac protein causes defects in germband retraction (Harden *et al.*, 1995). This raises the possibility that dorsal closure and germband retraction depend on similar downstream pathways, driven by small GTPases, which transduce external signals to the cytoskeleton. If UbcD4 is involved in both processes, perhaps it acts in such a common pathway.

Discussion

Chapter 6

Discussion

Targeted proteolysis has several advantages as a biological control mechanism. It changes the protein composition of a cell rapidly and irreversibly. It provides a way of limiting protein function, so that changes in patterns of gene expression can have an effect. The pathway is modular in nature, consisting of a specific recognition process and a non-specific degradation process. Thus it can be easily adapted to new roles - a substrate can be modified for recognition by a ubiquitinating enzyme, without the need to adapt a new protease. Systematic genetic and biochemical analysis of ubiquitinating enzymes in yeast has revealed the central role of ubiquitin-mediated proteolysis in controlling cell behaviour. In many cases, this information has been successfully extrapolated to higher organisms. However comparatively little is known of the role of the ubiquitin-proteasome pathway in regulating processes specific to multicellular organisms. The research presented here aimed to identify developmental roles for the ubiquitin-proteasome pathway, using *Drosophila* embryogenesis as a model system.

The overall importance of ubiquitination to the *Drosophila* embryo may be gauged by studying the ubiquitin activating enzyme, which carries out the first step in all ubiquitinating reactions. Identification of specific roles requires study of E2s and E3s, which target different subsets of proteins for degradation.

6.1 The overall importance of ubiquitination in *Drosophila* embryogenesis

Duba1 is abundantly transcribed at most developmental stages. This is not surprising, since ubiquitin-mediated proteolysis is a housekeeping as well as a regulatory mechanism. It is also expected to be required for progression through the cell-cycle. However, the expression pattern of *Duba1* in embryos does not correlate well with patterns of cell division. For example, it is abundantly expressed in stage 10-14 embryos, which are largely quiescent. It is particularly highly expressed in the germband of gastrulating embryos, and in gut and nervous system primordia during later stages. This may indicate an increased level of ubiquitin-mediated proteolysis in these tissues. In contrast, *Duba1* mRNA is scarce in stage 15 - 17 embryos. The major activity during these stages is condensation of the central nervous system - presumably ubiquitination has little or no part to play in this. The identification of a *P* element insertion in *Duba1* did not prove the importance of the process to embryogenesis, since the allele was weak and homozygous mutants died as third instar larvae. However it suggests that a low level of *Duba1* expression is sufficient for normal functioning of the ubiquitin pathway in embryos.

Overall, the expression patterns of the E2 and E3-encoding genes studied did not suggest specific developmental roles, with most genes having fairly uniform temporal and spatial expression throughout the embryo (see Chapter 3). In agreement with the *Duba1* results, several genes were expressed most highly in gut and/or nervous system primordia (*UbcD1, UbcD2, UbcD3, UbcD10, Su(dx), HERC-2*). No genes showed high expression during stages 15-17.

Compared to the rapid accumulation of information on the roles of protein ubiquitination in other systems, *Drosophila* embryogenesis remains relatively underexplored in this regard. The roles of UbcD1 in photoreceptor development and chromosome segregation, and of UbcD3/Bendless in axon guidance, were de-

scribed in Chapter 3. The only more recent report on a *Drosophila* E2 shows that Courtless, the Ubc7 homologue, is involved in male courtship behaviour and spermatogenesis (Orgad *et al.*, 2000). Of the *Drosophila* E3s, the roles of Hyd, Su(dx), and Slimb in proliferation control and signal transduction have been described in previous chapters. More recently, a Hect-domain E3 which interacts genetically and biochemically with Courtless, and which is required for axon guidance, has been discovered (A. Myatt, pers. comm.). The recent completion of the *Drosophila* genome sequence, which revealed many novel E2s and E3s, should facilitate more rapid progress.

6.2 Specific roles of the E2 UbcD4 in embryogenesis

The E2 UbcD4 was chosen for further analysis due to its strong and specific expression in embryos. Northern analysis showed that *UbcD4* mRNA was not present in larvae, pupae or adults, making a housekeeping role unlikely. Like many other E2s, it is highly expressed in the germband of stage 5 - 9 embryos and in gut and nervous tissue of stage 12 - 14 embryos. However, during stages 10 and 11, *UbcD4* expression is limited to the pole cells, the precursors of the germline. Subsequent experiments aimed to identify binding partners for UbcD4, and to examine the consequences of its loss of function, with particular interest in confirming a germline role for the enzyme.

The UbcD4 - interacting proteins identified were primarily candidates for ancillary factors in UbcD4 substrate selection, including an E3, the proteasomal subunit Pros54 and a novel protein whose PCI domain suggests possible proteasomal association. The candidate substrates identified were two aminopeptidases and the transcription factor Giant. These results must be interpreted with caution, since the interactions were not confirmed to occur in the embryo. In addition, it is not clear whether an E2 would be expected to interact strongly

with its substrate in the absence of regulatory factors. Nevertheless, the yeast two-hybrid system has been successfully used by other workers to identify E2 and E3 substrates, and the interactions were specific and reproducible.

The most direct way to examine the developmental role of a gene is by mutational analysis. Since no *UbcD4* mutants were available, the technique of RNA interference was used to silence *UbcD4* expression in embryos. The lack of UbcD4 antibodies, and the failure of *in situ* hybridisation analysis, meant that it is not certain whether and to what extent silencing was achieved. However the preliminary results indicate a requirement for UbcD4 in the main morphogenetic events of stages 11-14, germband retraction and dorsal closure.

6.3 Conclusions

The three different approaches taken to study UbcD4 function (expression analysis, interaction trap and RNA interference) do not identify a common role for the protein. The interaction trap did not identify a clear candidate for a germcell UbcD4 target protein. The PCI-domain protein is widely expressed in stage 10 - 11 embryos, and may be present in germ cells. Presumably *Pros54* is expressed in germ cells, since proteasomes are ubiquitous. None of the other UbcD4 interactors showed detectable expression in germ cells. If the interactions are physiologically relevant, they indicate that UbcD4 targets may be important in development of ectodermally-derived gut tissues: the aminopeptidases are only expressed in either fore- or hindgut, while Giant is involved in both head and foregut formation. The PCI-domain protein is highly expressed in salivary glands, which are foregut derivatives. The putative E3 deviates from this pattern, however, having a complex expression pattern which seems to correlate with morphogenetic movements of the ectoderm.

The RNA interference results also failed to support a requirement for UbcD4 in pole cell migration. However, although the pole cells in dsRNA injected em-

bryos successfully reached their target in the mesoderm, it is not known whether they were functionally normal. UbcD4 may be involved in another aspect of germcell development, such as regulating transcriptional quiescence. In addition, there may be some functional redundancy between UbcD4 and another E2. It is not clear whether foregut development was affected by dsRNA, since these structures were not easily visualised in the injected embryos. While the defects in germband retraction and dorsal closure were the only obvious effects of *UbcD4* dsRNA injection, they may be indirect consequences of a more subtle developmental failure.

6.4 Future work

The availability of UbcD4 antisera should allow the results obtained in these experiments to be clarified. The protein expression pattern may differ from that of the mRNA, giving a better indication of gene function. UbcD4 antibodies togther with antibodies raised against its putative interactors would facilitate confirmation that the interactions occur in the embryo. *In vitro* ubiquitination assays using purified proteins could be used to distinguish UbcD4 substrates from ancillary factors involved in substrate recognition. Anti-UbcD4 staining of dsRNA-injected flies would show whether and to what extent silencing was achieved. Another possible means of confirming the results obtained with dsRNA injection would be to generate a line of flies expressing a hairpin-loop *UbcD4* RNA. This approach would avoid the defects observed in injected embryos which are solely due to the experimental procedure.

It is possible that specific developmental roles for the ubiquitin system may be identified more easily by studying E3s rather than E2s, as they probably target a smaller number of substrates. The novel Hect-domain E3 identified in the UbcD4 two-hybrid screen has a highly regulated expression pattern in embryos and is an excellent candidate for further study.

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