Identification and characterisation of new isoforms of the proto-oncogene c-Cbl and its new roles in apoptosis and cell proliferation

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Identification and characterisation of new isoforms of the proto-oncogene *c-Cbl* and its new roles in apoptosis and cell proliferation

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I dedicate this work to members of my family for their unconditional support during all these years

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last but not at least: *to Émilie* and *her family*

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Part I

Introduction

Abbreviations:

MEF - Mouse Embryo Fibroblast

IP - ImmunoPrecipitation

co-IP - co-ImmunoPrecipitation

GST - Gluthatione-S Transferase

EGF(-R) - Epidermal Growth Factor (Receptor)

TGF-β(-R) - Transforming Growth Factor β (Receptor)

(R-)TK - (Receptor) Tyrosine Kinase

PI3K - Phosphoinozitol-3 Kinase

PKC - Protein Kinase C

RF - Ring Finger

UBA - Ubiquitin Associated

LZ - Leucine Zipper

Pro-rich - Proline Rich

GEF - Guanidine Exchange Factor

PGC - Primordial Germ Cell

SCY - spermatocyte

STD - spermatid

DTT - Di-Thio-Threitol

upH_2O - ultra-pure water

ROS - **R**eactive **O**xygen **S**pecies

DISC - **D**eath **I**nducing **S**ignalling **C**omplex

pY - phosphorylated Tyrosine(Y)

SH2 - **S**rc **H**omology domain **2**, binds phosphorylated tyrosine (pY) residues

SH3 - **S**rc **H**omology domain **3**, recognises PXXP or PXXXP amino acid motifs

all nucleotide sequences are 5'–>3' unless indicated otherwise

Chapter 1

The *c-Cbl* proto-oncogene

1.1 Introduction

The proto-oncogene *c-Cbl* was first described in 1989., as the cellular homologue of the *v-cbl* proto-oncogene[91] - an oncogene responsible for the transforming capacity of the murine retrovirus, Cas-NS-1, causing early B-lineage lymphomas[92].

These early studies identified *c-Cbl* as a 130 kDa nuclear protein expressed mostly in the testis and the thymus, *v-Cbl* being a truncated form of c-Cbl where all C-terminal and some of the distal N-terminal sequences are missing(*Figure* 1.1). The locus encoding the *c-cbl* gene was mapped to chromosome 9 of the mouse genome[134], while other studies explored cell-cycle specific expression[93] or the structural difference between normal and the

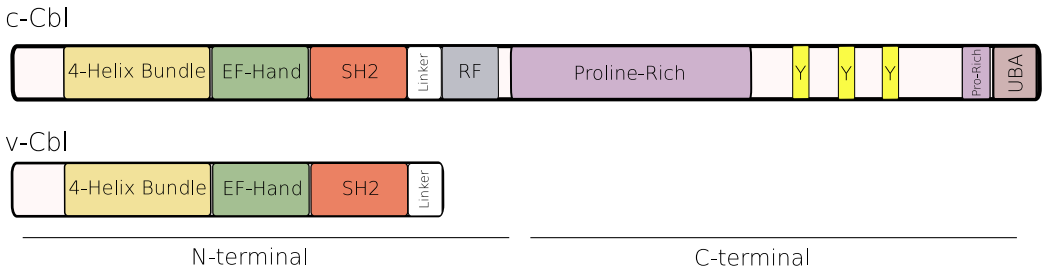


Figure 1.1: c-Cbl general structure

oncogenic forms of c-cbl[13][12].

*c-Cbl*s function remained a mystery until the middle of the 1990's when first papers described it being phosphorylated on tyrosine residues following T-cell Receptor (TCR)[35] or Epidermal Growth Factor Receptor (EGF-R) activation[127]. It's tyrosine-phosphorylation and association with c-Abl in c-Abl induced tumours[5] and its role as a negative regulator of receptor tyrosine kinase signalling during vulval development in *C. elegans[175]* further strengthened the idea that *c-Cbl* is a protein involved in signal transduction rather than being a transcription factor. As only *v-Cbl*, but not *c-Cbl*, was ever found to enter the nucleus, the idea of *c-Cbl* being a transcription factor has been abandoned.

With the discovery of *Cbl-b[79]* and *Cbl-c* (also known as *Cbl-3)*[78], *c-Cbl* became the principal member of the Cbl family proteins (*Figure 1.5)*. *Cbl-b* differs only slightly from *c-Cbl* with it's N-terminal part being practically identical, only the PRO-rich parts of the C-terminal show slight differences in their ligand preferences[79]. *Cbl-c* differs the most - while retaining fully functional Tyrosine-Kinase Binding (TKB) and Ring Finger (RF) domains, most of the C-terminal part is missing[78].

c-Cbl was first identified in the mouse, the identification of it's highly conserved human counterpart from T-cell leukaemia cell lines soon followed[13]. In Drosophila, two isoforms have been found, *D-Cbl* (resembling *v-Cbl* and *cbl-c*)[61] and *D-cblL* (a *c-cbl* homologue)[138]. We should also mention here SLI-1, the Caenorhabditis homologue of *c-Cbl*, that contributed a lot to our early understanding of *c-Cbl* function and the discovery of its E3-ubiquitin ligase function [99].

Its association with activated receptor and non-receptor tyrosine kinases and its E3-Ubiquitin ligase function explained well its negative effect on RTK an TK activated signalling events by directing activated tyrosine kinases to lyzosomes or proteosomal degradation[143]. To complicate things even more, *c-Cbl* was found capable to act as judge, deciding the fate of tyrosine kinases and other binding partners: condemn them to degradation by the proteosome or let them be recycled[100].

Oddly enough, *c-Cbl* knock-out mice don't show strong phenotypic changes - they are viable, of normal appearance. Upon closer histological inspection one can discover mild hyperplasias[119] and reduced fertility[39]. This relatively weak phenotype might be due to overlaps in *c-Cbl* and *Cbl-b* function - although their respective knock-outs are viable with mild phenotypes, the pre-natal lethality of the *c-Cbl/Cbl-b* double knock-out genotype illustrates best that although they might compensate for the lack of each other, but the complete loss of *Cbl* function is not compatible with life[121].

From the beginning of the 21st century, research concerning *c-Cbl* followed multiple paths. The best studied function of *c-Cbl* is it's negative regulatory role in RTK[159] and immunoreceptor signalling[107], now thought to be part of the first wave of events that lead to signal attenuation or extinction from activated receptors[160].

We should mention studies dealing with *c-Cbl*s role in the regulation of glucose uptake by stimulating IR-triggered GLUT4 transporter translocation to the plasma membrane[135][102]. These results alone would not justify a claim that c-cbl has a strong overall influence on glucose metabolism, were it not for results showing *c-cbl* knock-out mice having an overall increased rate of metabolism and altered insulin responsiveness[117].

Another interesting field of study is *c-Cbl*s role in osteoclast function - it has been shown that c-cbl has an important role in osteoclast migration[24] and bone resorption[157] by being a negative regulator of the Src tyrosine kinase. It was also in osteoclasts that *c-Cbl* was first shown to associate directly with the pro-apoptotic Bcl-2 family member protein *Bim* [1].

c-Cbl serves in many cases only as a scaffold-providing adaptor protein : due to it's size and it's capability to interact with a great palette of other proteins[33], it is the ideal candidate to act as the core of large multiprotein complexes. A good example for this is *c-Cbl*s role in the assembly of **F**ocal **A**dhesion **C**omplexes, where its E3 ligase function is not needed at all[144]. It also participates in the formation of clathrin-coated pits and their fusion with early endosomes by acting as a scaffolding protein and not as an E3-ligase[144].

Interestingly enough, no research has been done on c-Cbls role in the testis. With the exception of the initial publication describing *c-cbl*[91], no research has been published concerning *c-Cbl*s role in the testis even though it was already mentioned in this first paper that *c-Cbl* is present as abundantly in testis than in thymus [91]. This situation changed with the publication describing *c-Cbl*s role in androgen-dependent apoptosis in germ cells, at the end of 2005[39].

After 16 years of research on *c-Cbl* our understanding of c-cbl function grew a lot, but we still ignore the process how v-cbl or other c-cbl mutants[159] cause oncogenic transformation. Hopefully the oncoming era of systems biology[144] will finally reveal one of the most interesting mysteries surrounding c-cbl.

1.2 Structure and function

c-Cbl is traditionally divided into an N-terminal and C-terminal half. This division is not the result of mere dualist reductionism, but a division reflecting the obvious differences in the functionality and evolution of the two parts.

The N-terminal half is like a well-organised toolbox, containing a Tyrosine-Kinase Binding (TKB) domain to bind activated receptor and non-receptor tyrosine kinases, and a Ring Finger (RF) domain with E3-Ubiquitin ligase function to promote the ubiquitilation of bound interacting proteins (*Figure* 1.1, 1.2). We have to remark that *c-Cbl* participates mostly - but not exclusively[1] - in the ubiquitilation of its N-terminal TKB binding partners (*Figure* 1.2) [159][144].

The C-terminal half is more like a big store-room of tools that fulfil various functions, the nature of possible protein-protein interaction sites - to our present knowledge - lack a central organising idea. There are multiple tyrosines, serines and threonines subject to phosphorylation and more than a dozen of PXXP and PXXXP motifs, capable of binding a wide array of

[1] results exist indicating *c-Cbl* mediated degradation of the tyrosine kinase *Fyn*[4]

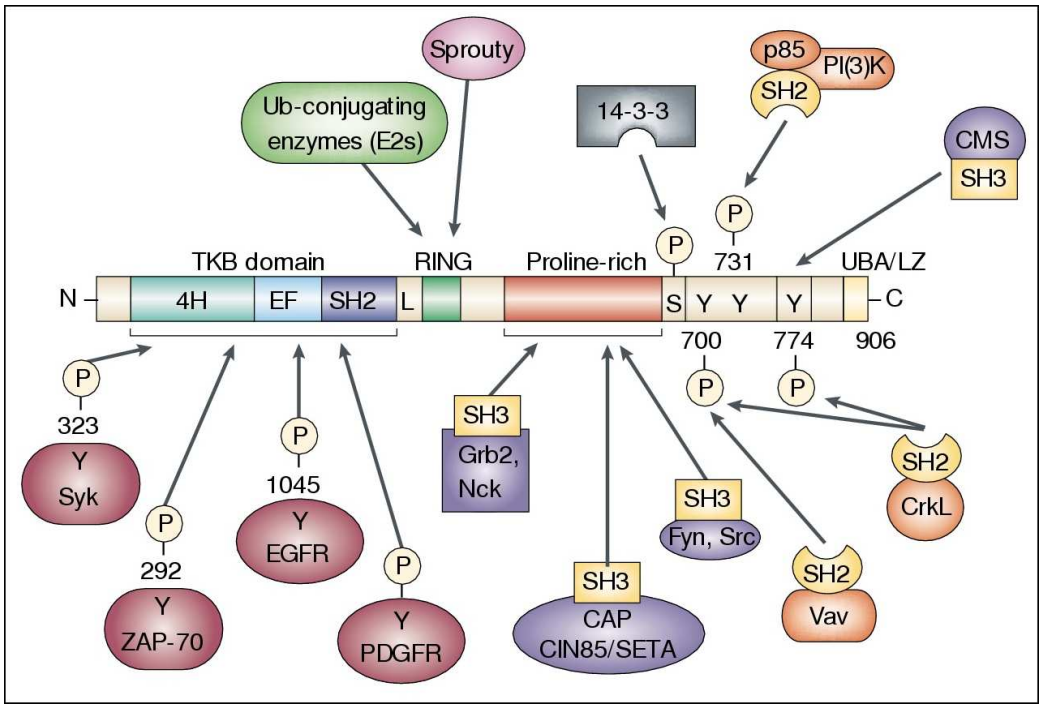


image from Thien *et al.*[159]

Figure 1.2: interacting partners of *c-Cbl*

interacting partners (*Figure* 1.1, 1.2). The Ubiquitin Associated (UBA) and Leucine-Zipper (LZ) domains (normally found in transcription factors) at the very C-terminal end give the *coup de grâçe* to perplexed observers trying to guess it's function.

On the next few pages I will first describe the functional domains if c-Cbl, then discuss the differences between Cbl family members with the domain structure differences in sight. A review of the evolution of this protein family will terminate this chapter.

1.2.1 N-terminal part of c-Cbl

The first half of *c-Cbl* shows remarkable sequence conservations among vertebrate species - for the ClustalW alignment of fish, amphibian, bird and mammalian *c-Cbl* sequences see *Figures* 10.2 and 10.3 - indicating that func-

Domain	Amino Acids
N-Terminal TKB	*46-357*
4-Helix Bundle	46-177
EF-hand-like 1	212-220
EF-hand-like 2	229-240
SH2 - atypical	267-341
Linker	342-380
RING Finger	381-420
Asp/Glu-rich acidic I	357-476
Proline Rich	477-688
Asp/Glu-rich acidic II	689-834
UBA	856-895
Total Length	906

Table 1.1: Functional Domains of c-Cbl and their relative positions in the amino acid sequence

tions associated with this part of *c-Cbl* appeared long ago, and conserved their importance up to present day. The very high level of sequence conservation ends about 14 amino acids after the RING-finger domain with a PXXP motif[2] at the end (*Figure* 10.3).

Tyrosine Kinase Binding (TKB) domain

Including the region between amino acids 48-347, this is the most conserved part of the protein, and also the part that was initially found as the transforming sequence in the Cas-B-L retrovirus. Early studies were almost entirely focused on this N-terminal part of the c-Cbl protein that was further justified by the fact that this is the only part well conserved in worms, insects and vertebrates.

The TKB domain is in fact the composition of three smaller domains that act in a coordinated fashion to produce a pocket capable of phosphotyrosine-binding:

[2]the exact sequence is **PFDP**

4-Helix Bundle (44 - 175) Made up of amino acids 44 - 175 arranged in four long helices, it shows similarity to various, functionally unrelated proteins like cytochrome-c and interleukin-5.

The loop connecting the first two helices (AB loop) was found to contribute to the pY-binding pocket of the SH2 domain. Disrupting the structure of this loop by substituting serine in position 80 with aspartate (S80D) or proline in position 82 to alanine (P82A) eliminated ZAP-70 binding in both cases[114]

EF-Hand (176-260) Including amino acids 176 through 260, classically known as a Ca^{++} binding domain. Contains two α - helices that surround the calcium atom, inducing a change in the conformation of the protein. One example for other EF-hand containing proteins: *calmodulin*. This functional domain is made up of two α-helices, EF-hand-like 1 and 2, made up of amino acids 210-218 and 227-238, respectively.

When mutating amino acids important in the co-ordination of calcium binding, *c-Cbl*s capacity to bind the ZAP-70 kinase is either eliminated (E240S) or markedly reduced (N229Q).[114]

N-terminal SH2 (265-339) Compromised of amino acids from 265 to 339, contains a universally conserved "FLVRES" amino acid sequence where the substitution of the arginine to lysine in position 294 (R294L) will abolish *c-Cbl*s ability to bind ZAP-70. The G306E mutation will produce the same result. These substitutions correspond to the mutations discovered in the *Caenorhabditis* SLI-1 impairing vulval development. [114].

The capacity to bind tyrosine-phosphorylated proteins needs the coordinated participation of all three above listed domains. The particularity of the SH2 domain present in the N-terminal half of *c-Cbl* is that it needs the nearby 4H domain to form the pY binding socket [114].

Linker Region (342-380) Situated between the TKB and RING-finger domains, it contains two conserved tyrosine residues at positions Y368 and

Y371. The phosphorylation of these sites was reported to be necessary for *c-Cbl*s E3-ligase function[99] and the oncogenicity of mutants lacking these phosphorylation sites[5] could be well explained with the incapability to down-regulate activated tyrosine kinases like EGF-R [172] or Src[173]. The fact that the Y371 residue is not exposed to the solvent in E2-bound *c-Cbl* suggested that its phosphorylation plays only a structural role[181] has been since refuted as the Y368E/Y371E mutants show constant E3 activity, probably because the negatively charged residues of glutamic acid mimic phosphorylated tyrosines[155].

RING-Finger (381-418)

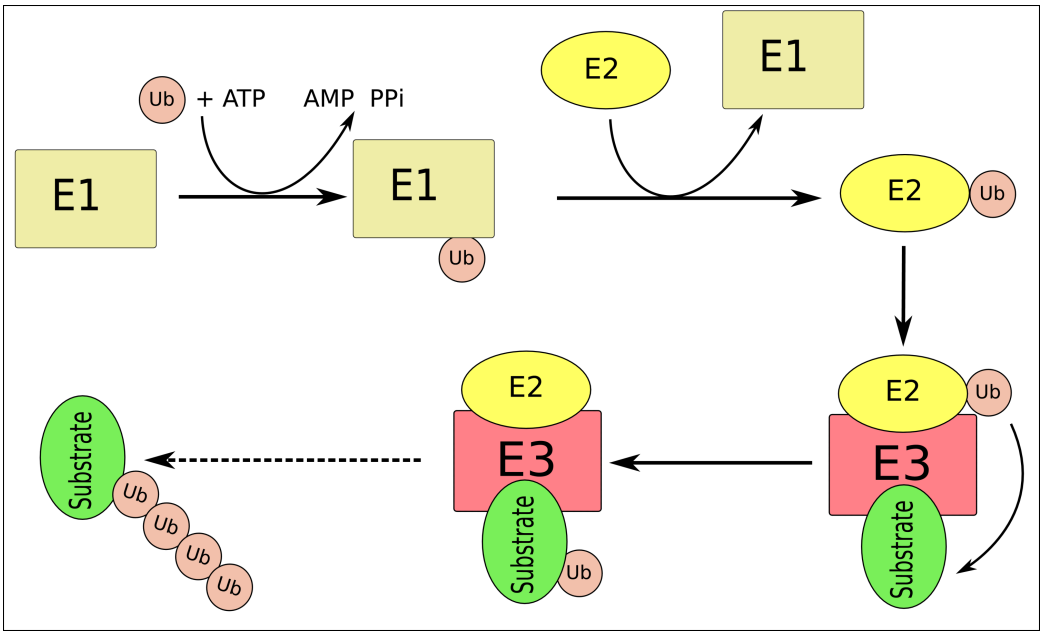
The RING[3]-Finger domain was first described as a novel cysteine-rich motif in a number of previously unrelated proteins[48] in the early 90's. With it's seven conserved cysteines and one conserved histidine residue[4] it is capable of binding two Zn^{2+}atoms, distinguished from a tandem repeat of Zn-finger motifs only in it's special way of binding the zinc atoms: Zn^{2+}atoms are bound by the first-third and second-fourth pairs of ligands[5], forming one compact Zn-binding domain[14].

It is a functional domain of proteins with E3-Ubiquitin ligase activity, the third element of the protein ubiquitilation chain. The transfer of ubiquitin to proteins starts with it's activation: ubiquitin is linked through a thio-esther bond to a Ubiquitin-activating (E1) enzyme. From this E1 protein it will be transferred to a **Ub**iquitin-**C**onjugating (Ubc or E2) protein, bound again by a thio-esther bond. The transfer of ubiquitin to the target protein will take place in a { E2-Ubiquitin + target protein + Ubiquitin-ligase (E3) } complex, where the E3 Ubiquitin ligase will provide the link - thus the specificity - between E2-Ubiquitin and the protein to be ubiquitilated (*Figure* 1.3) [70].

[3]"RING" is an acronym for ***R****eally* ***I****nteresting* ***N*** *ew* ***G*** *ene*

[4]zinc ligands are present in the C-C-C-H-C-C-C-C order, corresponding to a CCCH and a CCCC Zn-finger motif[14]

[5]before the description of the RING-finger motif, Zn-finger domains were only known to bind the zinc atoms with all four ligands present in a sequential order



c-Cbl corresponds to the enzyme marked E3

Figure 1.3: The process of ubiquitilation

c-Cbl can mediate the ubiquitilation of numerous interacting partners. One of the best known example is EGFR - the activated receptor is down-regulated through *c-Cbl* mediated ubiquitilation, what can happen either through direct binding of *c-Cbl*[168][99][77] or by having the Grb2 adaptor protein recruit *c-Cbl* to the activated EGFR[167]. In the first case c-Cbl binds pY residues on the activated receptor with its N-terminal tyrosine-kinase binding domain. In the second example, c-Cbl binds with proline rich regions to SH3 domains of Grb2, both of them linked to the activated EGFR through the SH2 domain of Grb2. The result is the ubiquitination of activated EGFR in both cases.

Apart of EGFR, *c-Cbl* mediates the ubiquitination of a great number of other receptor and non-receptor tyrosine kinases. A non-exhaustive list of them is presented in *Table* 1.2, further references can be found in the review articles by *Swaminathan et al.*[155] and *Thien et al.*[159].

Receptor Tyrosine Kinases	**Non-receptor TKs**
Platelet-Derived Growth Factor, PDGF-R	Syk
Vascular Endothelial Growth Factor, VEGF-R	Fyn
Fibroblast Growth Factor, FGF-R	Lck
Insulin Receptor, IR	Src
Hepatocyte Growth Factor, HGF-R	Lyn
Colony Stimulating Factor, CSF-1	c-Abl

Table 1.2: tyrosine kinases ubiquitilated by c-Cbl

Sites	Position	Function
TKB domain	46-357	pY binding, docking site for Syk, ZAP-70, EGF-R, PDGF-R
Proline Rich	477-688	docking site for Grb2, CIN85, CAP, Fyn, Src
Tyrosine	700	docking site for CrkL, Vav
Tyrosine	731	docking site for p85 subunit of PI3K
Tyrosine	774	docking site for CrkL
Serine	619, 623, 639, 642	docking site for 14-3-3 proteins

Table 1.3: Sites of protein-protein interaction

1.2.2 C-Terminal part of c-Cbl

Tyrosine Residues

The three conserved tyrosine residues of the C-terminal (*Table* 1.3) link *c-Cbl* either the cytoskeleton or to MAPK/Erk[6] signalling.

The later is done by the binding of the adaptor CrkL to tyrosines Y700 and Y774. CrkL was described to bring together the C3G GEF with its G-protein Rap1[7], responsible for the maintenance of T-cell anergy and the blockade of IL-2 production in the absence of co-stimulation[15].

The link with the actin cytoskeleton and focal adhesions is established by the binding of Vav to Y700 and PI3K to Y731[144] and can explain for example why *c-Cbl* is important in osteoclast migration and bone resorption[157][24].

[6]Erk stands for **E**xtracellular signal **r**egulated **k**inase

[7]a small G-protein like Ras, activating MAPK signalling pathways

Serine Residues

The serine residues of *c-Cbl* (*Table* 1.3) are known to recruit 14-3-3 proteins upon phosphorylation. Serine phosphorylation of *c-Cbl* was reported after activation of neutrophil granulocytes through β2-integrins[112] or after CD42 stimulation of human T-cells[130]. In both cases PKC mediated the phosphorylation, without activating the E3-ligase function of *c-Cbl* . This required tyrosine phosphorylation by Src, independently of PKC, showing how *c-Cbl* can influence the intracellular localisation of an interacting partner (when only serine phosphorylated) or direct it to proteosomal degradation (both serine and tyrosine phosphorylation) [112].

Proline - Rich regions

C-terminal *c-Cbl* sequences are very rich in proline, the number of interacting partners is close - if not exceeding - to two dozens[144]. They show great variation in function, we find tyrosine kinases like Abl, v-Src, Syn or Fyk, adaptor proteins like Grb2 or Nck, the phospholipase PLCγ and the **C**bl **A**ssociated **P**rotein (CAP) binding with their SH3 domains.

As the binding of SH3 domain to proline rich sequences doesn't need any prior modification (like phosphorylation) of the target sequence, some of these associations are constant while others need the recruitment of the interacting partners into close proximity.

Leucine Zipper

A putative Leucine Zipper (LZ) motif is present at the very C-Terminal of *c-Cbl*. The deletion of the last 50 amino acids, encompassing the LZ domain, will completely eliminate *c-Cbls* capacity to dimerise. It has been also shown that the deletion of the LZ motif will result in a significantly lower tyrosine phosphorylation of *c-Cbl* by the activated EGF-R in HEK293 cells[8]. Interestingly enough, this LZ motif is not found by any automated

conserved-domain search available free on the Internet like the one propose by the NCBI-NIH website (*personal observation*).

UBA (863-902)

45 amino acid long protein domain, found in a great variety of proteins. It is made up of three tightly-packed alpha helices, stabilised by non-polar amino acids and interacts with the hydrophobic surface on the five-stranded beta-sheet of ubiquitin. The UBA domain can be positioned internally or at the C-terminal end, or in some rare cases, in both locations[8]. It's presence is not closely associated with a distinct protein family, UBA domains have been found in proteins involved in the ubiquitin/proteosome pathway, DNA excision-repair, and cell signalling via protein kinases[62][158].

The UBA domain present in *c-Cbl* is situated at the very C-terminal end, similarly to what one can find in *Cbl-b*, but it's exact role has not yet been elucidated. There are contradicting results concerning the capacity of the UBA domain of c-Cbl to bind to ubiquitin or ubiquitilated proteins - *Davies et al.* had shown that only *Cbl-b*, and not *c-Cbl,* binds high molecular weight (HMW) poly-ubiquitilated proteins and mono-ubiquitin constitutively[28]. These results contradict previously published results of *Kleijnen et al.* who used an UBA-GST fusion protein to precipitate poly-ubiquitilated proteins[85], although they did not test the monoubiquitin-binding capacity of their UBA-GST fusion protein.

At present, *c-Cbl* is known to play key roles in receptor and non-receptor TK regulation, T-cell receptor signalling and determining the fate of interacting proteins, either directing them towards endosomal sorting or proteolitic degradation by the 26S proteosome[144]. To fulfil it's function as an E3-Ubiquitin ligase, the C-terminal UBA domain is completely dispensable[101].

[8]the human homologue of the yeast Rad23A protein - involved in nucleotide excision-repair - contains both an internal and a C-terminal UBA domain

1.2.3 *c-Cbl* as an E3-Ligase

It took six years after the initial discovery of *v-cbl* to find out that it associates with activated TCR[35] or EGFR[127]. Evidence that *c-Cbl* regulates RTK signalling came, however, from studies of the vulval development of the nematode *Caenorhabditis elegans*[73][175] (more about SLI-1 in *subsection* 1.3.4).

Ever since, *c-Cbl* was described to mediate:

- monoubiquitination

  The adaptor CIN85, binding *c-Cbl* quickly after EGF-R activation, was shown to be mono-ubiquitinated by *c-Cbl* at the same time as EGF-R was polyubiquitinated, showing the capacity of *c-Cbl* to both direct a target protein to degradation and influence the endosomal sorting of another. CIN85 mutants delaying EGFR degradation had also shown impaired ubiquitination, showing the interdependence of these two functions [58].

- poly-ubiquitination

  Early publications mentioned either "ubiquitination" or "polyubiquitination" of EGFR by *c-Cbl* but rarely - if ever - was an effort made to clarify if its really polyubiquitination or monoubiquitination in multiple positions[9]. Since 2003 it is known that EGFR is indeed poly-monoubiquitinated for internalisation, but non-receptor tyrosine kinases like Syk[133] still need to be polyubiquitinated prior degradation.

- monoubiquitination in multiple positions

  The activated EGFR becomes auto-phosphorylated and recruits *c-Cbl* either directly to pY1045[167] or through Grb2 - *c-Cbl* interactions

[9] This is understandable if we look at the time-line of ubiquitin-related research: the link between ubiquitin and proteasomal degradation was described only in 1989[108] and monoubiquitination of receptors as a signal for internalisation was first described only in 2000 [149].

where Grb2 binds the activated receptor with its SH2 domain and *c-Cbl* with it(s) SH3 domain(s)[65]. This is followed by multiple mono-ubiquitination of EGF-R, which will target it to degradation[155].

of other proteins.

The above outlined mode of action, namely: binding an activated RTK, mediating its multiple - monoubiquitination and as a consequence, down-regulating the receptor initiated signal, seems to be the same other RTKs bound by *c-Cbl* (*Table* 1.2).

As for activated non-receptor tyrosine kinases, their down regulation correlates well with their ubiquitination. *c-Cbl* mediated degradation will not always amount to important differences in detectable protein levels as the percentage of activated and degraded TKs often represents only a small fraction of the total amount present in cells, as it has been demonstrated for the interaction between the Syk TK [133].

1.2.4 *c-Cbl* and Endocytosis

The role of *c-Cbl* played in endocytosis is closely connected to its E3-ligase function, a intact TKB and RING domains need to be present. *c-Cbl* can bind the activated receptor directly through its TKB domain (like pY1045 of EGFR[168]) or through the adaptor Grb2 [106][34] (*Figure 1.4/a-b*).

This is followed by the monoubiquitination of the activated receptor in multiple positions and its recruitment to clathrin-coated pits (*Figure 1.4/c-d*). While the disruption of the RF domain of *c-Cbl* prevented only the endocytosis activated EGFR [69], the combined presence of c-Cbl and Grb2[10] was necessary for the translocation of EGFR to clathrin-coated pits and following this, to endosomes (*Figure 1.4/e-f-g*)[65].

After internalisation into endosomes, there are two possible paths open to the EGFR: either remain associated with *c*-Cbl ubiquitilated - this will direct

[10]a c-Cbl - {Grb2 - SH2} fusion protein was shown to rescue the block of endocytosis of EGFR in Grb2 depleted cells[65]

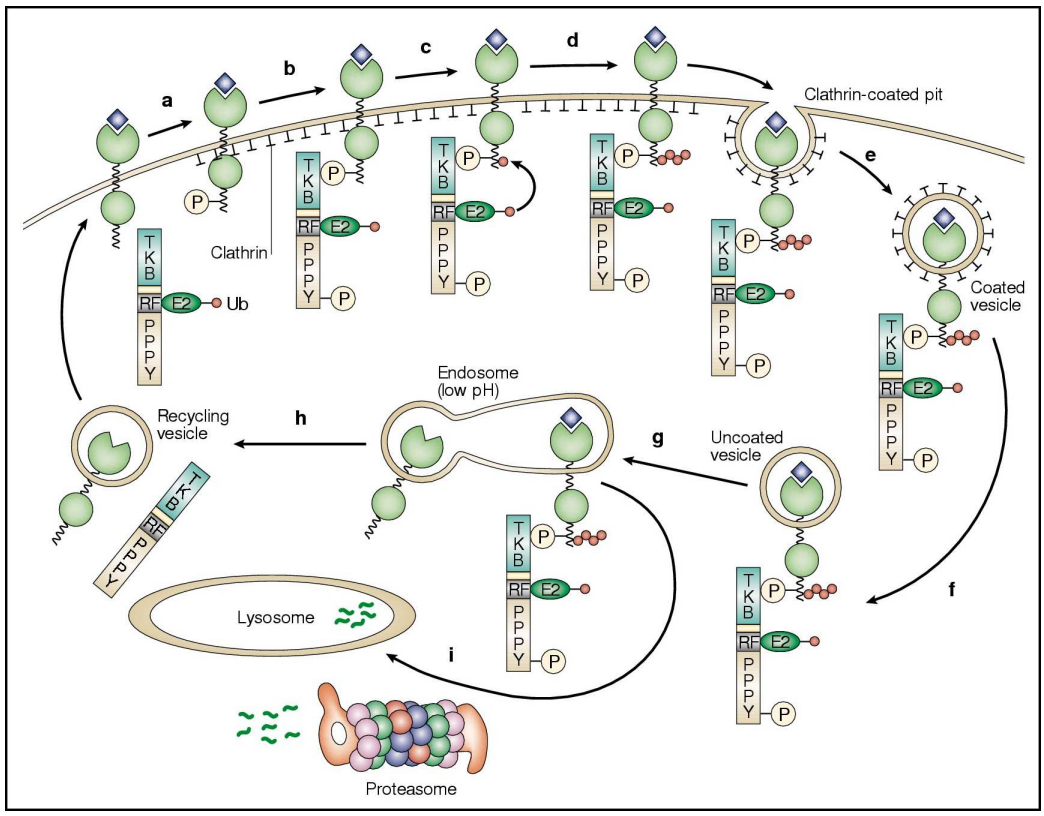


image from Thien *et al.*[159]

Figure 1.4: c-Cbl and the down-regulation of activated RTKs

it to degradation, or part with c-Cbl and ubiquitin and get recycled to the membrane (*Figure 1.4/i-h*) [65] [155].

These results suggest that although EGFR becomes mono-ubiquitinated quickly after its activation, its translocation to coated pits and endosomes will depend on its association with adaptor proteins like Grb2, while *c-Cb*l mediated ubiquitination and their association will be important in deciding if the receptor will be degraded by the proteasome or recycled to the membrane [56].

A third possibility - bypassing Grb2 altogether - also exists: the EGFR- *c-Cbl* complex might translocate to clathrin-coated pits by binding CIN85, an adaptor protein interacting with the endophilins[11] [156][155].

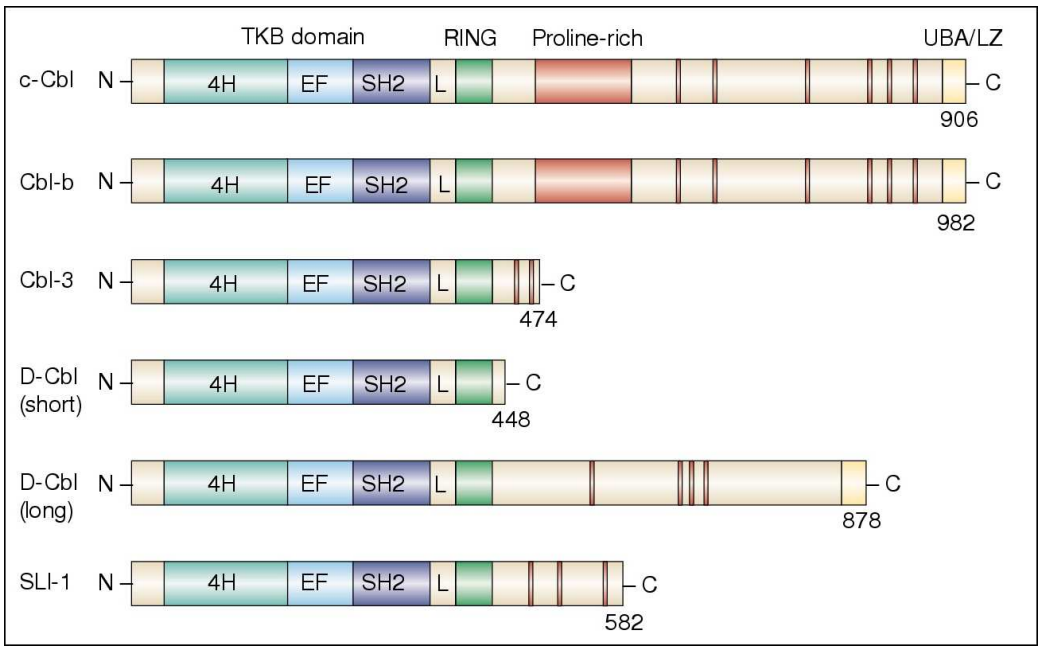
1.2.5 Proliferation and apoptosis

First described as capable of oncogenic transformation[91][92], it soon became evident that *c-Cbl* should rather be called a tumour suppressor due to its capacity to negatively regulate RTK/TK signalling.

A good example to illustrate of its tumour suppressor role is the reversal of the oncogenic transformation of murine fibroblast by the Src-family kinase Hck. In this model, membrane localised and ectopically expressed *c-Cbl* was found capcable to reverse the oncogenic transformation of murine fibroblast expressing the Hck tyrosine kinase [64]. This, again, needs intact RF and TKB domains showing the generalised role of the N-terminal sequences of *c-Cbl*.

An interesting finding was described by Sinha *et al.*, 2001, where the oncogenic *v-Cbl* form was transfected in to GM-CSF dependent myeloid cell line 32Dcl3 and was found to rescue the cells from apoptosis upon GM-CSF withdrawal[152]. GM-CSF also failed to induce the differentiation of 32Dcl3 cells expressing *v-Cbl*[152]. These cells had also shown elevated baseline activity of Lyn, Syk and PI3K with no change in*c-Cbl* phosphorylation levels. Interestingly, Bcl-2 levels remained elevated in *v-Cbl* expressing cells after

[11] endophilins are regulatory components of clathrin-coated vesicles



from Thien *et al.[159]*

Figure 1.5: other Cbl family members

withdrawal of GM-CSF and this change was not due to increased transcription or reduced ubiquitilation, providing the first clues to possible interactions between *c-Cbl* and the family of Bcl-2 proteins [152].

The direct interaction of *c-Cbl* and Bcl-2 family members - and its direct involvement in apoptosis - was first shown by Akiyama *et al.*, 2003[1]. They demonstrated increased levels of Bim[12] and subsequent induction of apoptosis in osteoclasts after withdrawal of M-CSF where the reduced ubiquitilation of Bim was regulated through direct interaction between Bim and *c-Cbl*[1].

1.3 Other Cbl Family Members

To our present knowledge, there are three members in the Cbl family of proteins: *c-Cbl*, *Cbl-b* and *Cbl-3* (or *Cbl-c*). As it can be seen on *Figure 1.5*,

[12]a BH3-only pro-apoptotic Bcl-2 family member, for more details please refer to *Subsection* 3.2.1

they share a highly conserved N-terminal half enclosing the TKB and RF domains, separated by the so-called "Linker" region. The C-terminal part is less conserved, even completely missing in Cbl-3, while showing different ligand binding preferences in the case of *c-Cbl* and *Cbl-b*.

As they contributed a lot to our understanding of *c-Cbl* function, we also included two orthologues in this overview: D-Cbl and SLI-1.

1.3.1 Cbl-3

The smallest member of the Cbl protein family, weighting only 52.5 kDa. Although it has both of the N-terminal domains - the TKB and RING-finger domains - found in other Cbl proteins , its C-terminal is very short in comparison, it terminates abruptly after a short stretch of PRO-Rich sequences, missing all the Tyrosine, Serine and Threonine residues, the UBA and Leucine-Zipper and the other PRO-Rich domains (*Figure* 1.5).

Epithelial cells of the epidermis, intestines and the respiratory tract are the principal sites of Cbl-3 expression, although it is expressed at very low levels in almost all tissues[78][54]. This is markedly different from the expression patterns of c-Cbl and Cbl-b whose expression often overlaps, like in tissues of lymphoid origin.

The TKB and RF domains have been found functional, as Cbl-3 is recruited to the activated EGFR and can effectively reduce resulting Erk-2 activation *in vitro*. The proline rich region at the C-terminal end was also found to bind Lyn and Crk[78].

In spite of it's well defined expression pattern and it's implication in major signalling pathways, Cbl-3 knock-out mice were found to be completely normal and viable, with no abnormalities in epithelial development. More interestingly, the kinetics of Erk-1 and Erk-2 activation following EGF stimulation was identical in Cbl-3^{KO} and Cbl-3^{WT} primary keratinocytes[54]. This can be explained by the presence of endogenous c-Cbl and Cbl-b in this cell type, but raises questions about the importance of Cbl-3 *in vivo*.

Sequence comparisons also show that Cbl-3 is closer to SLI-1 and D-Cbl than to it's mammalian family members c-Cbl and Cbl-b. It's apparent redundancy *in vivo* and it's close ties to ancient forms of the Cbl family suggest that Cbl-3, once the only representative of the mammalian Cbl family[122], is now slowly loosing it's importance to it's younger brothers.

1.3.2 Cbl-b

Slightly bigger that *c-Cbl*, it possesses an N-terminal almost 100% identical to that found in *c-Cbl*, and a C-terminal slightly longer with enough variance in key regions to assure modified ligand specificity in SH3 and SH2 domain mediated interactions (*Figure* 1.5).

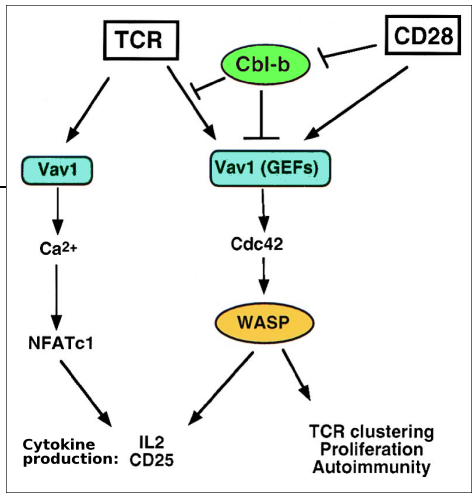


image from Krawczyk *et al.* [86]

Figure 1.6: The role of *Cbl-b* in T-cell activation

The first publication about Cbl-b appeared in 1995, describing a protein highly similar to c-Cbl, albeit slightly bigger in size. It was also predicted to bind to various proteins involved in signal transduction through numerous SH3 domains present in its C-terminal half. It was found to be expressed in significant quantities in cells of haematopoietic origin and in epithelial cells of the mammary gland and at much lower level in a great variety of tissues[79].

First articles discussing it's function spoke both about negative and positive regulatory roles. It was first described as the negative regulator of c-Jun N-terminal kinase activation by forming a trimeric complex with the activated RTK and Vav-GEF[20] Later it was found to be rapidly phosphorylated in response to TCR activation and it's over-expression was enough to increase the expression of the transcription factor NFAT, an indicator of activated T-cells[180].

From this point on (end of the 90's), research on Cbl-b is divided into two major fields, one studying it's association with RTKs and MAPK signalling, the other examining it's role in the context of the immune system.

The interaction of Cbl-b interaction with RTKs and the results of this association follows the scheme that as of c-Cbls. Within a few minutes of receptor activation Cbl-b becomes tyrosine-phosphorylated and can be co-immunoprecipitated with the receptor. This association is followed rapidly by the ubiquitilation of the receptor, resulting in the attenuation or complete extinction of the signal. If Cbl-b is over-expressed, signal-down-regulation occurs more rapidly while Cbl-b^{KO} cells show prolonged activation of downstream MAPKs when examining their phosphorylation state[43][42]. In these studies the RTK studied is EGFR - with the exception of a recently published paper describing negative regulation of activated cKIT by *Cbl-b*[177][13], interaction of *Cbl-b* with other RTKs has not been studied in detail.

A more fruitful area of research concerning Cbl-b is immunology[14]. Although both c-Cbl and Cbl-b had been shown play a role in TCR-mediated signalling[103], Cbl-b's role seems more important *in vivo* by setting the limit for lymphocyte activation[179].

It is known that activation of *naive* T-cells require two signals - one is provided by the antigen-MHC complex recognised by the TCR-CD3 complex[15], while the *co-stimulatory*[16] signal is given by the CD28 receptor. In Cbl-b^{KO}thymocytes T-cell activation becomes uncoupled from CD28, resulting in the deregulation of lymphocytes and the appearance of autoimmunity in Cbl-b^{KO} mice (*Figure* 1.6)[23][67]. As already mentioned, Cbl-b inhibits Vav

[13]once more, Cbl-b is examined side-by-side with c-Cbl, showing overlapping function in cKIT regulation

[14]although c-Cbl and Cbl-b are co-expressed in thymocytes, their respective roles seems to differ considerably

[15]The TCRc consists of a TCR dimer and a CD3 hexamer. TCR molecules do not posses intrinsic tyrosine-kinase activity, they rely on cytosolic Zap70, Lyn and Fck tyrosine kinases instead. These TKs are recruited through ITAM motifs of TCRc-associated proteins to activate various signalling pathways like Nfκb and various MAPK cascades, just to mention the most important ones.

[16]In the absence of this second signal, T-cells will fail to proliferate and will either become insensitive for further stimulation or enter apoptosis. Under normal condition this mechanism serves to protect the individual from autoimmunity.

under normal circumstances, this inhibition is maintained until CD28 activation when Cbl-b is directed to degradation[20][179]. Cbl-b has also been described to interact with a large *palette* of tyrosine kinases from the Syk (Syk, Zap-70) and Src (Fyn, Lck) family kinases[41], directly responsible for the tyrosine kinase activity associated with activated TCRcs.

Recent advances in the field report Cbl-b's involvement with TGFβ dependent regulation of T-cell function, too. Cbl-b^{KO}effector T-cells failed - or rather *resisted* - to convert to regulatory T-cells in the presence of TGF-β[17]. Cbl-b^{KO} mice were also able to mount an effective immune response against TGF-β secreting EL-4 tumour cells, demonstrating the relevance of these findings *in vivo* [170].

We can conclude that although the great similarity between *Cbl-b* and *c-Cbl* they do posses separate roles physiologically and these differences are due to the differences in their C-Terminal protein sequences and the pattern of their expression in different tissues and cell types.

1.3.3 D-Cbl, *Drosophila*

The first *Drosophila* orthologue of *c-Cbl* (*Figure* 1.5) to be found was ***D-Cbl***[111][61], a 448 amino-acid protein, expressed during embryonic development. Although it has well conserved TKB and RF domains compared to *SLI-1* or *c-Cbl*, the RF domain is followed only by 40 amino acids before terminating, leaving *D-Cbl* devoid of all proline rich sequences.

The other *Drosophila* orthologue, ***D-CblL***, was identified as a splice variant of *D-Cbl*. It consist of 878 amino acids, possessing not only the historically well-conserved N-terminal structures but a C-terminal UBA domain and six PXXP motifs, defining a Pro-rich domain[138].

Both isoforms were found to bind and negatively regulate the Drosophila EGFR, which controls photoreceptor-neuron cell (R-cell) development. D-

[17]As TGFβ was found to shift the balance between effector and regulatory T-cells in the favour of the later, TGFβ-secreting tumours are more aggressive - they reduce the number effector T-cells thus the immune system is less effective - if effective at all? - in eliminating them[52]

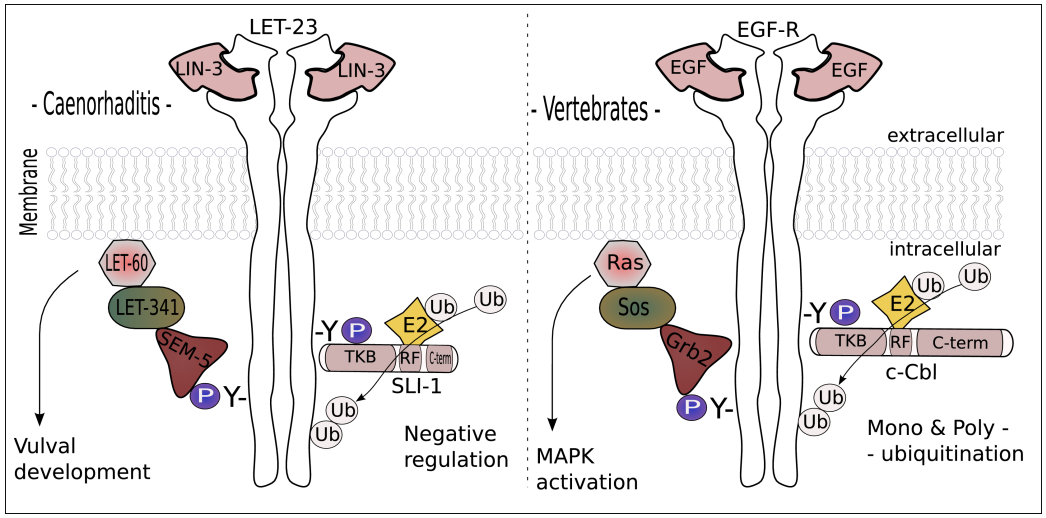


Figure 1.7: conserved E3-ligase function of c-Cbl

Cbl was shown to have an dose-dependent inhibitory effect on the development of the compound eye[111] while the *D-CblL* mutant *Dv-Cbl* (corresponding to the oncogenic form *v-Cbl*) induced melanotic tumours when combined with a constitutively active Ras1 background[138], acting as a dominant-negative oncogene.

1.3.4 SLI-1, *Caenorhabditis elegans*

The c-Cbl orthologue present in *C. elegans* is called SLI-1 (standing for "**Suppressor** of **LI**neage **1**"), involved in the regulation of vulval development - a developmental process coordinated by the *C. elegans* orthologue of the EGFR signalling pathway. It consists of the two N-terminal domains, the TKB and RF domains, and a short stretch of low-complexity, proline-rich region at the very C-terminal end (*Figure* 1.5).

SLI-1 (standing for "**Suppressor** of **LI**neage **1**") was discovered as a result of screening for suppressors of hypomorphic mutations of *let-23* (orthologue of EGFR), resulting in missing or underdeveloped vulvae. Inactivating SLI-1 mutations were able to rescue hypomorphic - but not null! - alleles of let-23, restoring normal vulval development, while duplication of this locus

further enhanced the hypomorphic let-23 phenotype (*Figure* 1.7) [175] [73]. A subsequent study had shown that the C-terminal proline-rich region is not necessary for let-23 regulation [174] and pointed at the TKB and RF domains as responsible for SLI-1 functionality.

These results were the first to show the inhibitory role of c-Cbl in tyrosine kinase signalling, a role well-conserved throughout the whole animal kingdom.

Chapter 2

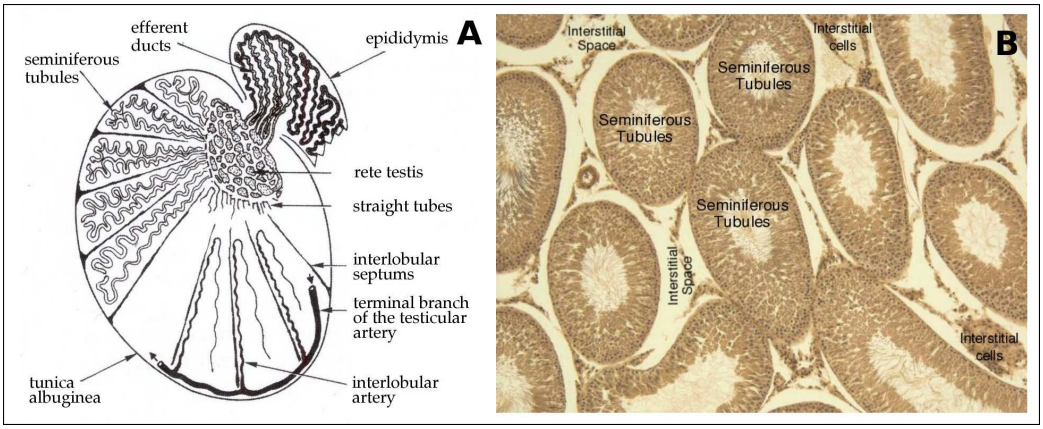
Testis

2.1 Introduction

Spermatogenesis starts at the onset of puberty and male individuals of mammalian species will produce 5-25 million gametes per day per each gram of testicular mass[66] from that day on until the end of their lives[1].

The incessant production of such a huge number of cells is a complex and finely regulated process, demanding a similarly well define organ that can provide the necessary infrastructure - the testis. It is a paired organ, in mammals it usually placed outside the abdominal cavity, this rather exposed emplacement is necessary to ensure a temperature 4-5°C inferior in the testes compared to normal body temperature as the process of spermatogenesis can fully terminate only at this lower temperature.

In this chapter I would like to briefly discuss the structure of the testis, the process and regulation of spermatogenesis and how the former affects the latter during normal male reproductive function.



A - from Dadoune & Demoulin *et al., [66]*
B - Victoria College Biology Department, Course Supplement, *100x*[165]

Figure 2.1: structure of the testis

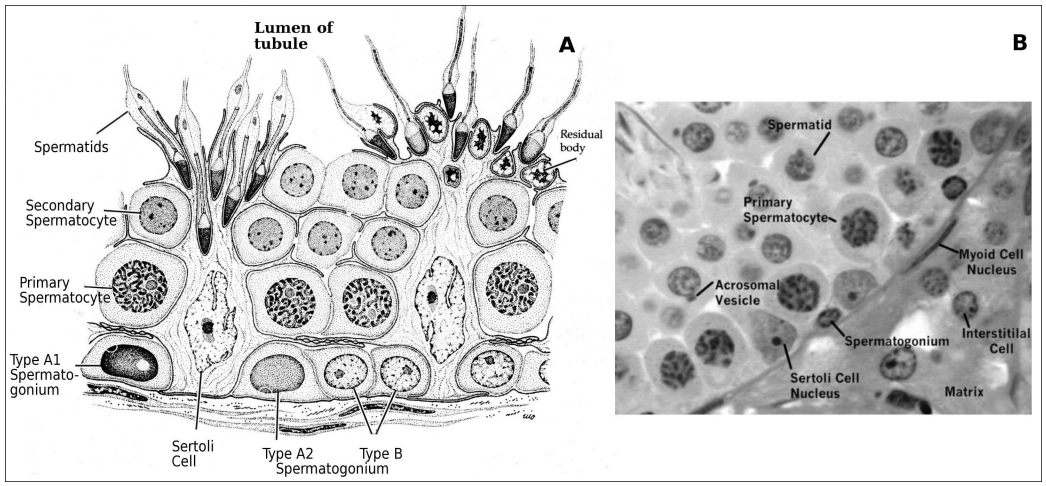
2.2 Structure

The testes are covered by three main layers of tissue - the skin, the *tunica vaginalis* and the *tunica albuginea.*

After descending from the abdominal cavity, testes find their final place in the *the scrotum*, in a loose sack formed by the skin. Under this out-most layer we can find the *tunica vaginalis* - it is the extension of the *peritoneum* - loosely covering the testes. The body of the testis is firmly enveloped by the *tunica albuginea*, a tough, fibrous tissue made of collagen and - sometimes - smooth muscle cells. As the inner lining of the tunica albuginea is rich in blood vessels, it is distinguished by the name *tunica vascularis (Figure 2.1/a)* [140] [166] [146].

The *tunica albuginea* readily protrudes into the volume of the testis, forming *septa* that separate the volume of the testis into hundreds of compartments, each containing one ore more (usually 1-4) *seminiferous tubules,* the place of spermatogenesis. The remaining space between the seminiferous tubules and the *septa* is called *interstitial space*, containing nerves, lymph and blood vessels and *interstitial cells (Figure 2.1/b)* [140][83][166].

[1]the rate and length of spermatogenesis shows a decline with age[71]



A - from Dym *et al.,* 1977[38]
B - Temkin *et al.,* Developmental Biology Picture Atlas, *400x* [116]

Figure 2.2: Seminiferous tubule cross-section

2.2.1 Interstitial space

The interstitial space is made up of a loose connective tissue. The quantity of this varies between species, it can make up as much as 40% of total testicular tissue, in humans it represents between 25-30% (*Figure 2.1/b*).

It is rich in blood and lymph vessels and contains lots of nerve endings also - they terminate here as they cannot enter the lumen of the seminiferous tubules. The cellular elements of the interstitial space are *Leydig cells*, organised as more or less tightly packed islands (in rodents they are more tightly associated than in bigger mammals) and free cells: fibroblasts, lymphocytes, mastocytes and macrophages (*Figure 2.2/a,b*)[140].

The best-known cell type from the above mentioned is the *Leydig cell.* They are responsible for testosterone production which is crucial not only for normal spermatogenesis[148] but in the development of secondary male characteristics during after puberty.

2.2.2 Seminiferous tubules

Male gametogenesis takes place in these heavily convoluted, 150-300 μm wide tubules.

Wall of the tubules The volume of these tubules is lined with a basal lamina made of type IV collagen and heparan-sulphate. The exterior surface of this basal lamina is covered by one or more layers of *peritubular cells* - contractile myoïd cells rich in myosin and actin, cross-linked between each other by cadherine. Under paracrine-endocrine regulation (they don't appear to be innervated), they contract at regular intervals and propel mature spermatozoïds towards the epididymis. These peritubular cells are in return covered by another layer of type IV collagen and fibronectin fibres, followed by a final layer of endothelial cells.

Lumen of the tubules The volume of the tubules contains Sertoli and germ cells (*Figure 2.2/a,b*).

Sertoli cells are of somatic origin, with multiple roles in spermatogenesis. They are in intimate contact with germ cells all through the process of spermatogenesis. It is known that each Sertoli cell can support only a certain number of developing germ cells[118]. Sertoli cells secrete a great number of different substances into the lumen of the tubules to satisfy the special needs of developing spermatids, like their dependency on lactate as primary energy source[74][16], or the androgen binding protein (ABP) that ensures the high intraluminal testosterone concentrations necessary for GC maturation[145].

It is also the Sertoli cells that form and maintain the *blood-testis barrier*. This barrier - resembling a lot to the blood-brain barrier - is supposed to protect developing germ cells from the harmful effects of xenobiotics[120] and to reduce the chance of autoimmune reactions[2][136][140]. Sertoli cells

[2]Male germ cells and their antigens - with the exception of spermatogonia - are not present when tolerance toward self evolves and male animals are capable of producing

form a network of tight junctions that together with the basal lamina of the tubules and the layer of myoid cells enveloping it blocks access of common macromolecules to the lumen of the seminiferous tubules. Small hydrophobic molecules - which could pass through the plasma membrane - are removed by ABC-transporters present in the luminal capillary endothelium, in myoid cells and on the basolateral surface of Sertoli cells[3][7].

Germ cells are all bound to Sertoli cells by N-cadherin[124] and galactosyltransferase[132] molecules displayed on their respective surfaces. Their development - spermatogenesis, and maturation of haploid spermatids into spermatozoïds - spermiogenesis also takes place while connected to their nurse cells.

The position of primordial germ cells - spermatogonia - is adjacent to the inner wall of the seminiferous tubule and as they develop they move further and further towards the lumen where they pass the blood-testis barrier when entering the *pre-leptotene* spermatocyte I stage. From this point on, their only means of communication with the "outside world" is through the Sertoli cells[55][83].

We discuss the properties of germ cells and their development more in detail in *Section 2.3*.

2.3 Development of Germ Cells

2.3.1 Origin of germ cells

Male individuals are born with germ stem cells, spermatogonia that lay dormant until the individual reaches sexual maturity. Upon entering adulthood,

antibodies against their own gametes - in case the blood-testis barrier is damaged, chronic inflammation may reduce the fertility of the individual

[3]Sertoli cells express the MRP-1 protein while myoid and epithelial cells express the MDR-1 transporter

production of germ cells starts and goes on either in a cyclical fashion or continuously, until the death of the individual.

Future germ cells become separated early on during embryonic development from future somatic cells, they can be identified in many mammalian species early on during embryonic development as AP-positive cells of the yolk-sac endoderm. These primordial germ cells (PGCs) will then migrate to the gonadal ridge, the precursor of the future gonad, by passing through the hind-gut mesenterium.

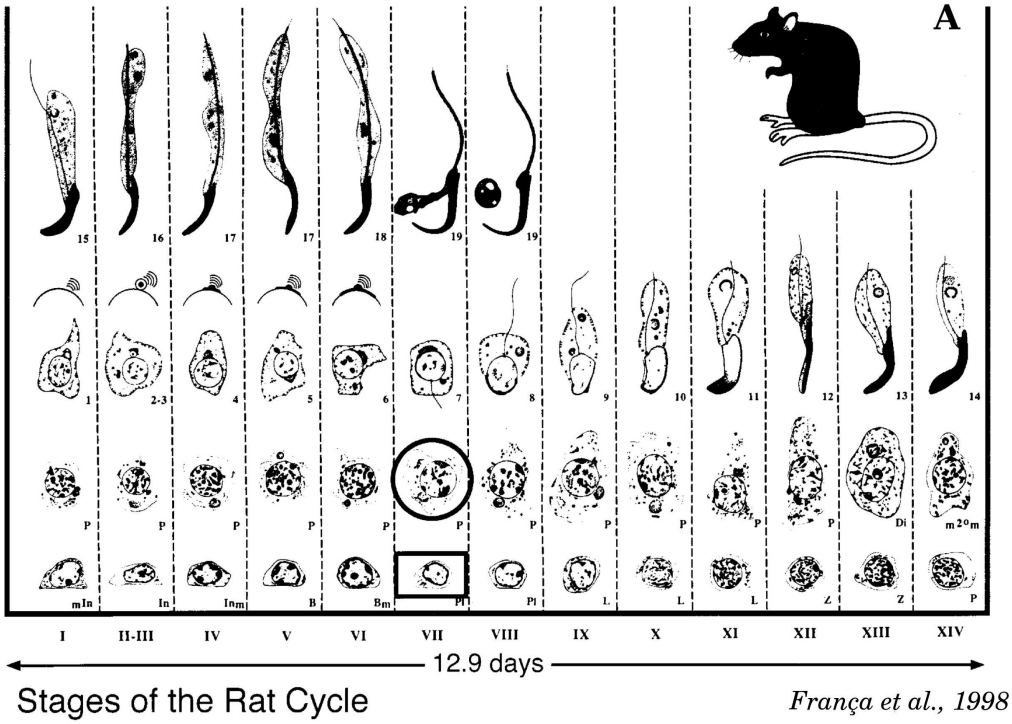
Once at the genital ridge they will be move inside the forming gonadal chords, from this point on they are referred to as *spermatogonia*. Although PGCs divide actively during their migration, spermatogonia enter G_1 phase mitotic arrest soon after their integration into the future testicular chords. This arrest will last until the onset of spermatogenesis at puberty [94].

2.3.2 Spermatogenesis

The process of spermatogenesis - the development of mature spermatozoïds from gametogonia - needs a considerable length of time to reach completion as developing germ cells proceed through its numerous steps. The total length of this process is constant and species-specific (53.2 days in rat, 74 days in men[66]) although it has a tendency of becoming longer at older ages (*Figure 2.3/b*).

Another important property of spermatogenesis is the interval at which new spermatogonia enter spermatogenesis. The length of this period is usually much shorter than the duration of spermatogenesis, being 13.3 days in rats and 16 days in men[66]. These periods are called the *cycles of the seminiferous epithelium* or *spermatogenic cycles*.

Germ cells complete multiple spermatogenic cycles before reaching the end of their development, moving further towards the lumen of the tubules with each completed cycle, forming multiple layers of germ cells at different stages of development. Their position relative to the wall of the seminiferous tubule indicates how far they have progressed in their development (*Figure* 2.2/a,



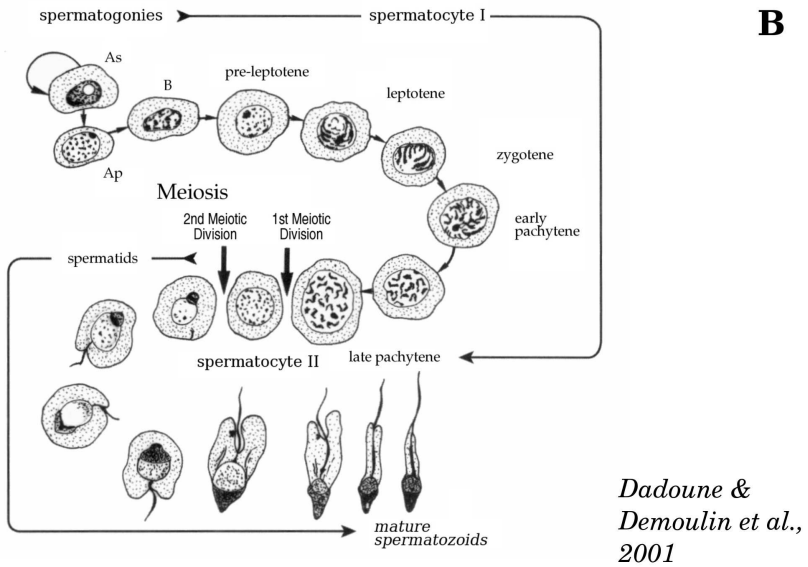


Figure 2.3: Stages of spermatogenesis

Figure 2.3/a). The duration of the spermatogenic cycle is determined by the genotype of the germ cells and does not depend on regulation by Sertoli cells.[46].

The cycles of seminiferous epithelium can be further divided into stages based on morphological differences between cells, looking at all the layers of developing germ cells simultaneously. An example: if we were looking at rat seminiferous epithelium in stage IX, we would see leptotene spermatocytes in the first layer, pachytene spermatocytes in the second layer and spermatids beginning elongation in the innermost layer of the seminal epithelium, but no elongated spermatids - they already completed their development at stage XVIII (*Figure 2.3/a*).

The different stages of the spermatogenic cycle show not only morphological but physiological differences, too. The most important is the fact that androgens are capable to influence spermatogenesis at stages VI-VIII (in rat), the other stages are considered "androgen independent".

Spermatogenesis starts with the mitotic division and differentiation of type A spermatogonia into interconnected daughter cells - first into type B spermatognia, then into primary spermatocytes. The later differentiate through meiotic division into spermatids (*Figure* 2.3). Although each rat spermatogonia entering spermatogenesis could theoretically give rise to 4096 mature spermatozoïds [46], the final number of daughter cells is much lower as many of them die with programmed cell death either while type A spermatogonia (to regulate the density of spermatogonia entering spermatogenesis in a given part of the seminiferous epithelium) or right before the first meiotic division (if there is not enough testosterone to fully support spermatogenesis).

Spermatocytes can be referred to as stage I (before the first meiotic division) or stage II (between the first and second meiotic division). Stage II spermatocytes are seldom seen as the interval between the two meiotic divisions is less than a day while stage I spermatocytes are easily visible, often spending more than a whole seminal cycle at the pachytene stage (*Figure* 2.3)[66][46].

Round spermatids, the results of the second meiotic division and already possessing a haploid(n), single set of chromosomes, don't divide any further but

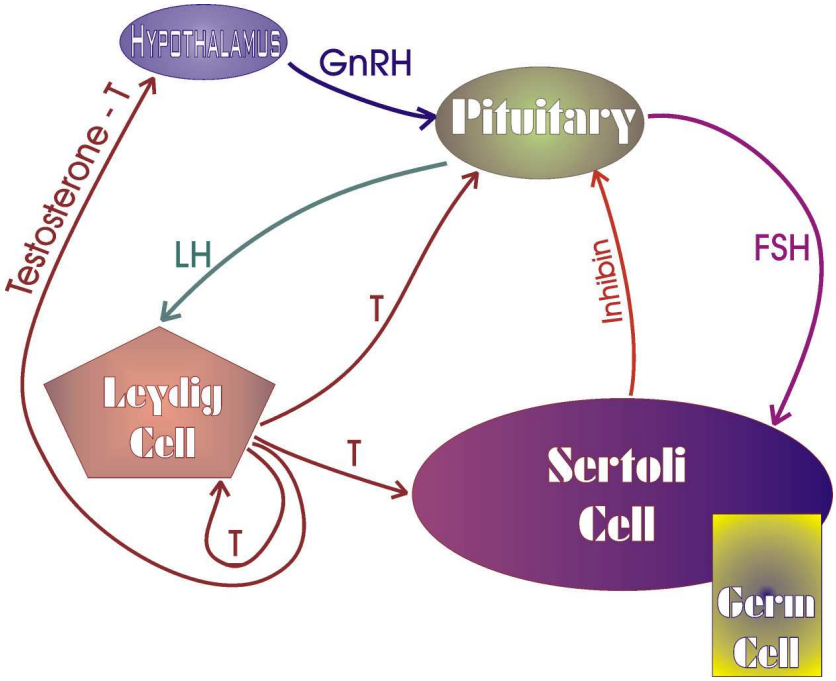


Figure 2.4: Endocrine regulation of spermatogenesis

start the process of *spermiation*. Spermiation ends with the transformation of round spermatids into fully mature, elongated spermatozoïds.

2.3.3 Hormonal regulation

Germ cell development is under the strict regulation of the hypothalamo - pituitary axis of the endocrine system. The main actors of the regulation and maintenance of spermatogenesis are the **G**o**n**adotropin **R**elease **H**ormone (GnRH), **L**uteinizing **H**ormone (LH), **F**ollicle-**S**timulating **H**ormone (FSH), testosterone and the gonadal peptide Inhibin.

Gonadotropin Release Hormone, GnRH GnRH is a decapeptide hormone, synthesised as a larger pro-hormone and cleaved enzymatically to reach it's mature form. It's production is suppressed until the onset of puberty, when it's secretion starts in a pulsatory manner. It promotes LH and FSH secretion from the anterior lobe of the pituitary gland, the frequency - that

can vary between once an hour to once a day in women, staying constant in men - of subsequent GnRH releases modulating the LH:FSH ratio[115].

In the total absence of GnRH - like in hypogonadal*(hpg)* mice[4] - animals are infertile with small gonads[125]. This phenotype[5] was readily reproduced by the selective ablation of pituitary gonadotropes, showing that the *hpg* phenotype is indeed due to the lack of FSH and LH and not due to the absence of GnRH[80].

It's production is both under negative and positive regulation. GnRH release is promoted by a group of neurons acting as a "pacemaker"[6] while the frequency of release is modulated by sensory input, stress and circadian rhythms[147]. Negative regulation is done through gonadal steroids and inhibins, although the inhibitory effect of gonadal steroids is indirect as there are no steroid receptors on GnRH secreting cells[147].

Luteinizing Hormone, LH Produced by the anterior lobe of the pituitary gland, the principal target of LH in the male reproductive system are the Leydig cells, activation of the LH-R by LH will provoke the production of testosterone through the activation of cAMP signalling pathway[68][7].

LH-R deficient male mice show marked reduction in size of their accessory sex organs, coupled with very low levels of testosterone and abdominal testes. Adult-type Leydig cells are missing and the number of testicular macrophages[8] is reduced. The size and area of the seminiferous tubules is reduced, too, probably due to lower cell number : although the numbers

[4] *hypogonadal* mice are GnRH deficient due to the partial deletion of the GnRH gene and consequent splicing problems. *Hpg* mice are sterile with undetectable levels of FSH or LH and as such they provide an ideal system to study the hormonal regulation of reproductive function[153]

[5] the term *hypogonadal* is also used as a medical term in human medicine to describe the failure of the gonads and/or of the pituitary gland and the hypothalamus to produce the necessary sex hormones and ensure correct reproductive function[137]

[6] situated in the *arcuate nucleus*

[7] since the cAMP signalling can be constitutively activated by forskolin, measuring testosterone production after forskolin treatment in whole testes or testis slices is an elegant method to assess for the presence or absence of functional Leydig cells[178]

[8] they differentiate from monocytes infiltrating the testis, the process is thought to be dependent on the presence of Leydig cells

of Sertoli cells, spermatogonia and early spermatocytes are unchanged, the numbers of germ cells reaching later stages of development is reduced. Late spermatocyte count is lowered only slightly, round spermatid numbers are markedly lower and elongated spermatids are missing altogether - the process of spermatogenesis clearly fails to reach completion in these KO animals[178].

These phenotypic changes are mostly reversible - full-length spermatogenesis with mature sperm, albeit with a diminished sperm count can be achieved - by the administration of testosterone[154], indicating that the principal target of LH in the testis are the Leydig cells and that LH is essential for a quantitatively and qualitatively full spermatogenesis through the maintenance of intra-testicular testosterone levels.

LH production is regulated through multiple feedback loops, the most important being the negative feedback by testosterone: it inhibits both LH and GnRH production[115].

FSH Like LH, it is released from the anterior lobe of the pituitary gland upon GnRH stimulation. It's role in the male reproductive systems remained mostly unknown until the early 1990's, when researchers started to make use of either transgenic models like FSH-R and FSH-β subunit knock-out mice[141] or *hpg* mice.

Work by *Singh et al.* - using the *hpg* model - points out that although FSH is not needed for a qualitatively complete spermatogenesis[151], it is indeed very important in early postnatal life to promote the proliferation of Sertoli cells[2]. Treating normal, healthy male rats with FSH during pre-pubertal life resulted in a dramatic increase in Sertoli and germ cell numbers, increasing spermatogenic capacity once reaching adulthood[110]. FSH treatment of *hpg* mice restored testis size and Sertoli cell numbers similar to what is observed in wild type animals[150]. The phenotype of FSH-R knock-out - *aka FORKO* - male mice, showing an average of 50% reduction in testis size, further supports the importance of FSH in determining testis size[88].

Additional roles of the FSH - FSH-R system include the regulation of tubular fluid secretion by Sertoli cells[88], control of mature sperm shape, quality

and vitality[88][57][32], maintenance of testosterone and Androgen Binding Protein(ABP) levels[89] and influencing the onset of puberty[87]. Results from LH-R knock-in mice suggest that FSH can alone maintain the development and proliferation of spermatogonia and spermatocytes[98], while LH and testosterone are important for the completion of spermatogenesis.

It is important to notice that most of the above listed results were obtained using FORKO mice, while FSH-β subunit knock-out male mice are fertile and show no abnormality apart of reduced testis size[90], indicating that in the FSH/FSH-R system the key element is indeed the receptor.

FSH production is regulated through a negative-feedback loop where feedback is provided by the protein inhibin, blocking FSH release in the pituitary gland and it is produced by the FSH-stimulated Sertoli cells [3].

Testosterone Produced by Leydig cells in response to LH stimulation, it is the single most important steroid hormone in male gametogenesis, capable alone to initiate and maintain qualitatively full spermatogenesis (for structure see *Figure* 2.5)[151]. It doesn't act on developing germ cells directly (germ cells don't even need a functional androgen receptor to complete their development[72]) but through Sertoli cells and was shown to be the sole Leydig-cell produced factor necessary for normal spermatogenesis[148]. Interestingly, the presence of testosterone is needed only during a few stages (stages VI-VIII) of spermatogenesis[109][81].

Even though testosterone act indirectly on spermatogenesis, any interference with testosterone signalling has severe consequences for spermatogenesis. LH-R knock-out[98], Leydig-cell ablation by EDS [148] or hypophysectomy [47] all result in testosterone deficiency and animals show the arrest of spermatogenesis at spermatocyte or at round spermatid stage, most probably caused by increased apoptosis of germ cells in androgen-sensitive stages of development [171]. This phenotype is readily rescued by the administration of exogenous testosterone[63].

Testosterone secreted by Leydig cells is also important to achieve the high intraluminal androgen concentration in the seminiferous tubules and it reg-

ulates the permeability of the blood-testis barrier [113].

Testosterone's role is not confined to the regulation of spermatogenesis, it is also responsible for the correct development of secondary sex organs, male-specific physical appearance and behaviour. Testosterone is already produced by the Leydig cells of the foetal testis in the developing embryo, this is important for the correct development of the male accessory sex organs[50]. Foetal and early postnatal exposition to testosterone is necessary for the development of a masculanised central nervous system [123] and the imprinting of male-specific behaviour like song production in birds [50] or normal copulatory behaviour and successful ejaculation in mice [104].

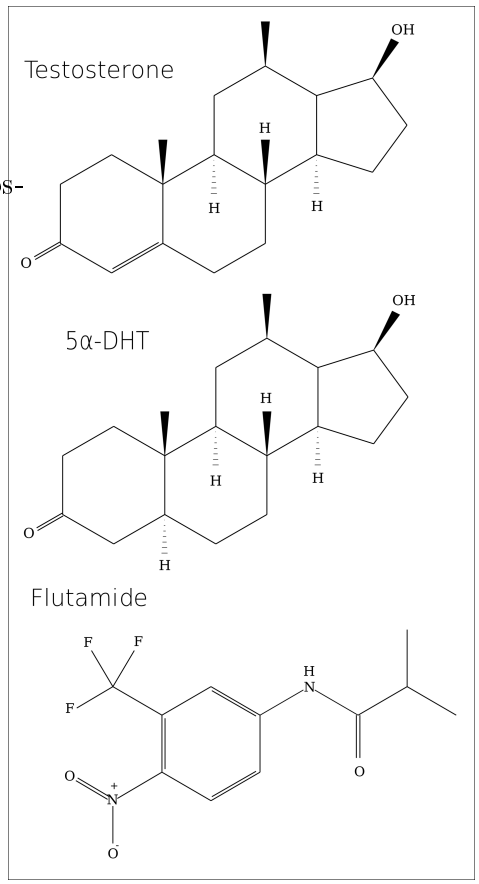


Figure 2.5: Chemical Structure of Androgens

We have to mention that outside the testis most tissues react to 5-α **D**i-**H**ydro **T**estosterone (DHT, for structure see *Figure* 2.5) and not testosterone itself[9], with the 5-α reductase enzyme doing the conversion right inside the target cells[169]. Without a functional 5-α reductase, external male genitalia and prostate will not develop but give place to corresponding female organs[125] - this and many more results suggest that "pure" testosterone act rarely in a direct fashion outside the testis.

Inhibin A glycoprotein hormone produced by Sertoli cells[10] in response to FSH stimulation, important element of the negative feedback loop regu-

[9] notable exceptions are skeletal muscle and fat, the role of 5-α reduction is not yet clear in their metabolism[59]

[10] also produced in granulosa cells of the ovary, in the placenta and in the brain[115]

lating FSH production. In castrated Rhesus monkey models LH levels can be lowered to normal by the administration of testosterone, but FSH levels remain high due to the absence of inhibin produced by the gonad[36][115].

Chapter 3

Apoptosis

3.1 Introduction

Cell death occurring during normal embryonic development was first described by *Vogt, C.* in 1852, then forgotten and re-discovered many times until 1951 when *Glückmann* discussed it in a review that attracted somewhat more attention from the scientific community[163]. Independent studies in the 1960's lead by Saunders and Lochskin can be considered as the turning point for research concerning apoptosis.

Saunders[142] had shown the necessity of waves of *"massive cell death"* for the formation of the *radius* and the *ulna* and the separation of fingers. At the same time, *Lochskin* was studying the cytology of *"programmed cell death"* in insects[105], characterised by cell shrinkage, chromatin condensation and blebbing of the plasma membrane. Because of these morphological markers *Kerr* referred to this cell death process as *"shrinkage necrosis"* until he proposed the term *apoptosis*[1] in a review[82].

Apoptosis research profited a lot from the introduction of *C. elegans* as a new model animal in the 1970's. The relative ease of genetic manipulation and the possibility to trace the fate of each of it's 1090 somatic cells - of

[1] from ancient Greek, meaning *"falling off of leaves from tree in autumn"*

which 131 cells undergo programmed cell death during development - made it an ideal model to study the underlying genetics[163].

Despite of the morphological similarities, apoptosis in vertebrates and programmed cell death in invertebrates were not considered to be related until human Bcl-2 was described to inhibit developmental cell death in *C. elegans*[164]. This had pointed out that the underlying cellular machinery is highly conserved among animal *phyla* and that results from the genetic dissection of programmed cell death in *C. elegans* can be used in vertebrate models, too.

Ever since, research concerning apoptosis exploded, producing more than 2% of all scientific publications in the field of biology by 2003[95]. As it is not in our intentions to summarise our present knowledge concerning apoptosis, we will restrict ourselves to a brief overview of the two major apoptotic pathways described up to date.

3.2 Apoptotic Pathways

3.2.1 Proteins involved

Death Receptors Receptors possessing a cytosolic Death Domain (DD) like Fas, TNF-R, Trail-R 1 & 2 (aka DR4 and DR5), belonging to the TNF-R family of receptors, are also known as Death Receptors(DR).

DD containing adaptor proteins They contain both a DD and a Death Effector Domain (DED). While they are bound to the activated death receptors through their DDs, they also recruit DED containing pro-caspases so that the Death Inducing Signalling Complex (DISC) can be formed.

Caspases A family of **C**ysteine - **ASP**artic Acid Prote**ASES**, playing a central role in apoptosis. The family counts more than a dozen members in

mammals, all of them synthesised in an inactive, *"pro-caspase"* form. During activation, the pro-caspase form is cleaved twice to yield a pro-domain (initially N-terminal), a large (situated after the pro-domain) and a small subunit (C-terminal end). The active enzyme is formed as a hetero-tetramer of two small and two large subunits. Based on their pro-domains, caspases can be grouped as follows:

- **initiator caspases** - they posses a large, >90aa pro-domain, containing either a DED or CARD (caspase recruitment domain) domain. Initiator caspases are recruited to protein complexes depending on their pro-domains prior to activation. Most prominent members of this group are caspase-8 / caspase-10 (containing a DED) and caspase-2 / caspase-9 (containing a CARD)[95][49]
- **effector caspases** - their pro-domains are short, they are most often activated by initiator caspases. Once activated they cleave a wide range of substrates resulting in the morphology associated with apoptosis. Caspase-3, -6 and -7 are the effector caspases[95][49]
- **caspases involved in cytokine maturation** - caspases 1,4 and 5 are involved in cytokine maturation[49]

Apoptosome A key element of the intrinsic apoptotic pathway, this ~1 MDa protein complex is created by the assembly of seven dATP, Caspase-9,Apaf-1 and *cytochrome c(CytC)* molecules. Upon it's release from mitochondria, *CytC* will bind constitutively to Apaf-1 (this association is stable without any additional co-factors under physiological conditions). In the presence of dATP seven of these {*CytC*, Apaf-1} complexes will assemble to form the basis of the apoptosome to which at least seven pro-caspase-9 molecules might be recruited[2]. The assembled holoenzyme - resembling a

[2]it is not yet clear if pro-caspase molecules bind to *CytC*-Apaf-1 complexes before of after apoptosome assembly

seven-spoked wheel and often referred to as the "wheel of death" - will then cleave pro-caspase-3, initiating apoptosis[3] [176][183].

Bcl-2 family proteins A family of pro- and anti-apoptotic proteins, named after the first member discovered[162]. Proteins belonging to this family posses 1-4 **B**cl-2 **H**omology (BH) domains and in most cases and additional **T**rans-**M**embrane (TM) domain, facilitating membrane insertion into the outer membrane of the mitochondria. The domains follow the BH4 - BH3 - BH1 - BH2 - TM order, starting from the N-terminal end. They are divided into two large groups based on their effect on apoptosis:

- **Anti-apoptotic** - the presence of the BH4 domain - often referred to as *"protective BH4 domain"* - clearly distinguishes them from their pro-apoptotic relatives. Bcl-2, Bcl-$X_L$and Bcl-w are the most well know members of this group. They block apoptosis by forming hetero-dimers with multi-domain pro-apoptotic family members thus sequestering them from pro-apoptotic family members.
- **Pro-apoptotic** - ~ Bcl-2 proteins are further divided into two groups:
  - multi-domain

    The only difference respective to the anti-apoptotic family members is the lack of the BH4 domain. Their present BH3 - BH1 - BH2 domains form a hydrophobic pocket that can bind the BH3 domains of both anti- and pro-apoptotic family members. They seem to be the stable component of hetero-dimers which will have a pro- nor anti-apoptotic depending on the other partner.
  - BH3-only

    The only conserved domains present are the BH3 and the TM domain (like Bim and Bik) or only the BH3 domain (Bid and

[3]there are results indicating that caspase 9 activation in the apoptosome might rather depend on the proximity of multiple pro caspase-9 molecules than on the actual cleavage of caspase-9 zymogens, as apoptosome-associated caspase-9 was found to be more effective in caspase-3 activation that activated caspase-9 homodimers[22]

Bad) . Proteins of this group have to bind to a multi-domain pro-apoptotic family member to be effective.

- **Regulation of apoptosis by Bcl-2 family members** - most researchers agree that it is the balance between anti- and BH3-only pro-apoptotic Bcl-2 proteins that decides the type of signal propagated. If anti-apoptotic family members are in excess, it will be mostly them forming dimers with multi-domain anti-apoptotic family members, favouring survival . If BH3-only pro-apoptotic proteins get in excess, it will be them forming dimers with multi-domain pro-apoptotic proteins, thus activating the apoptotic pathway. Although the mechanism by which they can promote *cytochrome c* release from mitochondria is not clear, there are indications that Bcl-2 family proteins can themselves form pores in the outer membrane of the mitochondria. Other hypotheses include a co-operative role with other mitochondrial trans-membrane-proteins like VDAC (**V**oltage-**D**ependent **A**nion **C**hannel, shown to interact with caspase-8 cleaved Bid), where they can cause membrane swelling and the eventual disruption of the mitochondrial outer membrane - followed by *cytochrome c* release - by perturbing the Ca^{++}homoeostasis of the organelle.

Inhibitor of Apoptosis(IAP) Family Proteins poses sing 1-3 **B**aculoviral **I**AP **R**epeats (BIR) belong to this family[4]. Prominent members of this family are XIAP, c-IAP1, c-IAP2 and survivin, all capable of inhibiting apoptosis by interacting with caspases 3,7 and 9.

IAP proteins inhibit the intrinsic apoptotic pathway through their interaction with caspase-9 and caspase-3, the later also serving as a way to block receptor mediated apoptosis. Blocking the proteolitic activity of caspases 3 and 7, IAP proteins also prevent the proteolitic positive-feedback loop where terminal effector caspases 3 and 7 cleave and activate initiator caspases 8 and 10[31].

[4]an additional carboxyl-terminal RING domain can also be present, although it's presence for apoptosis suppression might not be necessary

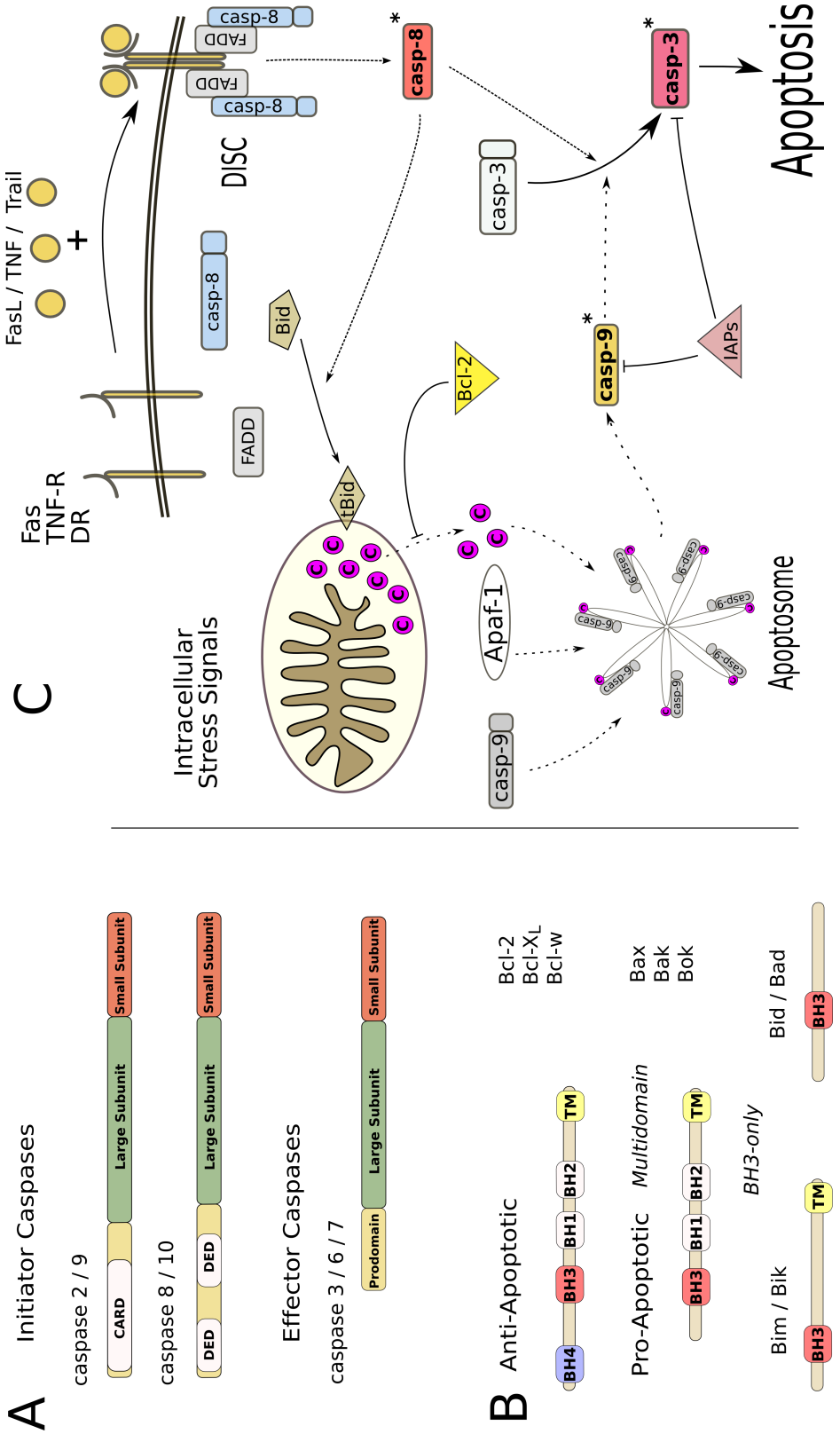


Figure 3.1: Extrinsic and Intrinsic Apoptotic Pathways

3.2.2 Receptor - mediated / Extrinsic apoptosis

This apoptotic pathway is activated upon ligand binding by death receptors like Fas or TNF-R (belonging to the TNF-R family), poses sing a cytosolic Death Domain (DD). Activation is followed by receptor trimerization and the recruitment of adaptor proteins like FADD (Fas Associated Death Domain protein) poses sing a Death Effector Domain (DED). These DED domains bind other DED domains present in initiator caspases like caspase-8 and caspase-10. Once the inactive form of caspase-8 (pro-caspase 8) is recruited to the Fas-FADD complex, the Death Inducing Signalling Complex (DISC) is formed. The multiple pro-caspase-8 proteins present in the DISC will cleave (activate) each other, the resulting active caspase-8 hetero-tetramers will in their turn activate effector caspases like caspase-3 (they can also cleave Bid thus initiating a cross-talk with the mitochondrial pathway of apoptosis)[95].

3.2.3 Mitochondrial / Intrinsic Apoptosis

This apoptotic pathway is activated by intracellular stress - like excessive DNA damage, high level of ROS and the presence of cytotoxic drugs.

The turning point of this pathway is cytochrome-c release from mitochondria, although the exact mechanism of cytochrome-c liberation is yet to be precised. Present models include both pore formation in the outer mitochondrial membrane by VDAC, ANT(Adenine Nucleotide Translocator) and pro-apoptotic Bcl-2 family members; and loss of the mitochondrial membrane potential causing the swelling and eventual rupture of the mitochondrial outer membrane[84]. This is often referred to as Mitochondrial Permeability Transition and it can be provoked by pro-apoptotic Bcl-2 family members like Bax[76] but also blocked or at least delayed by Bcl-2 itself[97][84].

Once cytochrome-c has been released into the cytosol, apoptosome assembly and caspase-9 activation will follow. The intrinsic apoptotic pathway can still be blocked though through the action of the IAP family proteins, as they can occupy the substrate binding site of caspases.[27]

The inhibitory effect of IAPs on caspase activation can be countered by Smac/Diablo, released from the mitochondria simultaneously with cytochrome-c. By binding to IAP proteins they can displace them from caspase-IAP complexes and allow apoptosis to proceed[84].

3.2.4 Perforin / Granzymes (the Shortcut)

Natural Killer (NK) cells and cytotoxic T-cells eliminate virus infected cells by secreting tightly-packed, perforin-granzyme[5] containing granulae in their proximity. Once in contact with the target cell's membrane, perforins will form pores through which granzymes can enter and cleave their substrates, initiating apoptosis. A good example to illustrate how granzymes work is Granzyme B. It directly cleaves both caspase-3 and Bid (among other substrates), quick-starting the apoptotic machinery by bypassing the extrinsic pathway and activating the intrinsic one[27].

3.3 Apoptosis induction

3.3.1 H_2O_2

Although hydrogen peroxide is present constantly at low levels and it is essential for oxidative processes in living cells, but its production and the biochemical reactions it is involved in are normally confined to the *peroxisome*[6] and the *mitochondria*[21]. Outside these organelles it behaves as a **R**eactive **O**xygen **S**pecies (ROS) as it quickly decomposes into water and free oxygen, the later much too reactive for living cells. This problem is solved by the

[5] Granzymes are a family of serine-proteases, with three family members described so far: Granzyme A, B and C.

[6] *β-oxidation* is essential for the degradation of fatty acids; the synthesis of *plasmalogens*, a class of phospholipids essential in the correct myelinisation of neurons also occurs in the peroxisome

presence of the various antioxidants[7] and the enzyme *catalase*, turning two peroxide molecules into water and a molecule of oxygen [53]:

Spontaneous decomposition:
$H_2O_2 \rightarrow H_2O + O^{2-}$
With catalase present:
$2\ H_2O_2 \rightarrow H_2O + O_2$

When it comes to inducing apoptosis, H_2O_2 seems to act through the ceramide pathway: increasing concentrations of H_2O_2 in the cytoplasm will activate the *sphingomyelinase* enzyme, converting sphingomyelins to the sphingolipid *ceramide* and its derivatives[51]. Ceramide will then induce the liberation of cytochrome c from the mitochondria[8], activating the mitochondrial pathway of apoptosis (see "intracellular stress signals", *Figure 3.1/c*) [97].

3.3.2 Etoposide

Etoposide is an anticancer drug used in the treatment of a wide range of neoplasms[9]. The target of etoposide is the Toposisomerase II enzyme, responsible for reducing tension in the structure of double stranded DNA during replication : it cleaves and re-ligates one double-stranded branch while passing through the other. In the presence of etoposide the enzyme gets blocked bound to the cleaved DNA strand, unable to re-ligate the two ends and thus introducing double-strand breaks[19].

When the number of double-stranded DNA breaks reach a critical level, it will trigger the activation of the DNA dependent Protein Kinase (DNA-PK) multiprotein-complex. DNA-PK activates p53 , starting a cascade of events

[7] these molecules - like *vitamin E* in lipid membranes or *ascorbic acid* and *glutathione* in the cytoplasm - act as preferential proton donors and react with the ROSs before they can oxidise other macromolecules like DNA, proteins or lipids

[8] this again can be blocked by Bcl-2 showing that H_2O_2activates the classical mitochondrial pathway to apoptosis[97]

that lead to the increased transcription and translation of Bax. The translocation of Bax to the mitochondrial membrane will be the final step triggering the release of cytochrome c and activating the mitochondrial pathway to apoptosis (*Figure 3.1/c*)[76].

3.3.3 Flutamide

Used in the treatment of prostate hyperplasias since the 1970's[9], it is a competitive inhibitor of testosterone and 5α-DHT at the androgen receptor. It is also a non-steroidal antiandrogen (*Figure* 2.5), showing no cross-reaction with other steroid receptors[11].

Although flutamide treatment is known to induce apoptosis in germ cells, we cannot call it pro-apoptotic in the strictest sense of this term.

First of all, it provokes apoptosis by interfering with the normal functioning of the endocrine system (it is also often used in models of endocrine disruption to examine the effect of anti-androgens[120]) - it is not really flutamide, but the absence of androgens that provokes the suicide of pachytene spermatocytes.

Secondly, it induces apoptosis in germ cells but does so indirectly as it must act through the **A**ndrogen **R**eceptor (AR) expressing Sertoli cells. Direct interaction between flutamide and germ cells is further hampered by the blood-testis barrier[7], greatly reducing its access to the luminal side of the seminiferous tubules[120].

It is known that in the testis of rats treated with flutamide *in utero* key players of the mitochondrial pathway of apoptosis are chronically deregulated. Effector caspases 3 and 6 (both the pro-caspase and cleaved forms)[129] and pro-apoptotic Bcl-2 family members Bax are up-regulated[17], while the expression of anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-w is diminished[17]. Other changes involve the decreased expression of *c-Cbl* just

[9]Since the end of the 1990's it has been replaced with bicalutamide which has less side-effects[11]

to mention one, and all these changes result in an elevated frequency of apoptosis at the pachytene spermatocyte stage[10].

3.4 Summary - Apoptosis

It has already been described that there is a protein associated with the early phases of apoptosis in glucocorticoid treated thymocytes. This protein shows a close resemblance to the proto-oncogene *c-Cbl*, abundantly expressed in pachytene spermatocytes - cells that respond to flutamide treatment, or exocrine disruption, with apoptosis.

Our hypothesis was that we should be able to find this apoptosis-related protein, CARP-90, in the testis of flutamide treated mice and provide evidence that it is in effect an isoform or splice variant of the *c-Cbl* proto-oncogene and it plays a regulatory role in the apoptosis and/or proliferation of male germ cells during their androgen-sensitive phase of development.

[10]Similar phenomenon was observed in the male offspring of rats treated *in utero* with the antiandrogen vinclozolin . F1 male offspring developed numerous fertility disorders with age and this phenotype was transmitted to untreated males of F2, F3 and F4 generations [6].

Part II

Materials & Methods

Chapter 4

Protocols

4.1 Protein Related

Protein Isolation

~ from cells and tissues was done using Lysis Buffer. Samples were kept on ice all the time.

Cells from cell culture were rinsed prior the addition of lysis buffer (1ml lysis buffer per ϕ10cm Petri dish), then scraped and incubated on a rocking platform for 15'@4°C. Lysates were pre-cleared by centrifugation for 15'@4°C with 800g. Supernatant was saved for further work.

Frozen tissues were homogenised in 1.5ml Eppendorf-tubes using plastic pestles over a period of 15'. Following homogenisation, samples were pre-cleared by centrifugation for 15'@4°C with 20000g. Supernatant was saved for further work.

In both cases, protein content was determined using the BCA-method.

Bicinchoninic Acid Protein Quantification

Each sample tube quantified contained 0.1ml 0.1% SDS, 0.8ml BCA - $CuSO_4$ and protein sample diluted in 0.1ml Lysis buffer (1 ml final volume). New

calibration curve was prepared for each quantification session, with 0 - 20 - 50 - 80 - 100 μg/ml BSA controls (0 - 20 - 50 - 80 -100 μl 1 mg/ml BSA per control). Samples were diluted 20-50 times based on their initial concentration. Step - by - step quantification process was as follows:

- dilute samples / BSA controls in 0.1 ml lysis buffer
- add 0.1 ml 0.1% SDS
- add 0.8 ml BCA - $CuSO_4$solution (20 μl 4% $CuSO_4$/ ml BCA)
- thorough end-to-end mixing, followed by immediate transfer to 37°C water bath for 30'
- quantification by measuring extinction at 562nm

Immuno-Precipitation & Co-IP

For each IP or co-IP, 200-500 μg freshly extracted and pre-cleared protein extract was incubated in the presence of 1-3 μg antibody overnight at 4°C on a rocking platform. Purified IgG corresponding to the species of origin of the precipitating antibody was used as negative control. Antibody - protein complexes were immobilised using Protein A/G linked agarose beads (*SantaCruz Cat.#sc-2003*) for 30 minutes at 4°C. Agarose beads were sedimented with centrifugation using a benchtop centrifuge (10"@400g), supernatant was removed and beads were washed three times using lysis buffer. After discarding the supernatant of the last washing step, precipitated proteins were separated from agarose beads by incubation in boiling water bath for 3 minutes in the presence of one bead volume 2xSDS protein loading dye. Denatured samples were spun down and proteins were separated using one-dimensional PAGE.

Western Blot

Proteins were separated using standard discontinuous PAGE, the blotting of proteins onto membranes was done using Tris-Glycine transfer buffer.

Following the transfer of proteins, membranes were blocked at least 30' at RT, using 5% non-fat dry milk dissolved in TBS-T. Primary antibodies were incubated o/n at 4°C. Secondary antibodies were incubated 2-4h at 4°C. Antibodies used only once (all secondary antibodies and most primary antibodies) were diluted in 1% Dry-milk - TBS-T. Re-used primary antibodies were diluted in sterile-filtered 0.5% BSA - 0.025% Na-azide - TBS-T, stored at 4°C.

Washing steps: 2x5' in TBS-T after primary antibody; 1x5' in TBS-T - 2x5' in TBS after secondary antibody.

Western Blots were revealed using kits based on **e**nhanced **c**hemi**l**uminescence (ECL) from KovaLab, Pharmacia-Amersham and Pierce.

4.2 Nuclei Acid Related

Agarose Gel Electrophoresis (DNA)

dsDNA - plasmids, PCR products were separated using 0.8-2% agarose gels, using 0.5x SB as migration buffer[18].

RNA was separated using MOPS-Formaldehyde 1.2% agarose gels.

Poly-Acrylamid Gel Electrophoresis (PAGE)

Separation of proteins using discontinuous PAGE was done according to the protocols present in Amersham Bioscience's Protein Electrophoresis technical manual.

Separation of nucleic acids / PCR products was done using 8% AA gels, with 0.5X TBE as migration buffer.

RNA isolation

~ was done using Invitrogen's TRIzol Reagent, following vendor-supplied protocol.

Genomic DNA isolation (from parts of mouse embryos)

limbs of 13.5 days old embryos were digested in Tail-buffer using Proteinase K for 4h@55°C. Following phenol-chloroform extraction, genomic DNA was ethanol-precipitated, dried and re-suspended in upH_2O.

Genotyping Mice/MEFs for *c-Cbl*

Genotype of mice/MEFs regarding *c-Cbl* was verified using PCR with genomic DNA as template. Two sets of primers were used, cbl-10 & cbl-11 to detect $WT^{c\text{-}Cbl}$ and cbl-13 & loxP to detect $KO^{c\text{-}Cbl}$ genotype, producing 273bp and 306bp PCR products, respectively.

PCR program:

- 3 x { 94°C 3' - 60°C 2' - 72°C 1' }
- 30 x { 94°C 1' - 60°C 1' - 72°C 1' }
- 1 x { 94°C 1' - 60°C 1' - 72°C 10' }

Primers, 5'-3'

cbl-10 GAC GAT AGT CCC GTG GAA GAG CTT TCG ACA

cbl-11 CCT AAG TGG TAG GAT TAT AAT TGC AAG CCA CCA C

cbl-13 TCC CCT CCC CTT CCC ATG TTT TTA ATA GAC TC

loxP TGG CTG GAC GTA AAC TCC TCT TCA GAC CTA ATA AC

Reverse Transcription

~ was done using the M-MLV Reverse Transcriptase (*Invitrogen Cat#28025-013*), using reaction buffer and DTT supplied by vendor, random hexamers were used to prime the reverse-transcription. In brief, 2 μg RNA was reverse-transcribed in the presence of 1x First-Strand Buffer, 10mM DTT, 0.5mM dNTP, 200ng random hexamers, 40 units of RNase-OUT and 200 units of M-MLV in a volume of 20 μl. RNA was first denatured at 65°C for 5' then chilled on ice prior the addition of M-MLV. Reverse transcription was allowed to go on for 50'@37°C, it was terminated by heat-inactivation of the enzyme at 70°C for 15'. The reactions mixture was filled up with water to a final volume of 50 μl (2.5-fold dilution) to be used as template in subsequent PCR reactions.

PCR

1U Taq polymerase (*Promega, Cat#M1661*) was used per 20 μl PCR reaction with reaction buffer and $MgCl_2$ included with the enzyme. Composition of a PCR was as follows:

Component	**Final concentration**
Taq Polymerase	1U
Forward & Reverse Primers	500 nM each
Taq Reaction Buffer	1x
$MgCl_2$	1.5 mM
dNTP	0.2 mM

PCR Primers used to amplify *c-Cbl* sequences

- Primers designed to match individual exons:

*these sequences correspond to primers marked "F" (forward); "R" primers are the reverse-complement of their respective "F"-marked pair.

primer	@bases in mRNA	Sequence
7F	1113-1143	TGG ACG AAA TCA AAA TCC TGA CCT GAC AGG T
8F, 8R	1214-1243	AGA TTG AGC CCT GTG GAC ACC TCA TGT GCA
9F, 9R	1439-1467	TCT TCA TGA TGA AGG AGT TGG CAG GTG CCA
10F, 10R	1536-1565	ACT TGA CCT TCT ACA GCA GCG AGC ACC TGT
11F, 11R	1674-1701	ACC CCC TCC AGA CCG GCC TTA CTC TGT T
12F, 12R	1990-2018	AGC AGC CCA GTA GCA GGT CCA GAG AGT GA
13F, 13R	2087-2111	TGC CAA AAC TGC CAC CTG GGG AGC A
14F, 14R	2264-2298	TGT ATA ACA TCC AGT CCC AAG CAC TGT CTG TAG CA
15F, 15R	2438-2467	ACA TCT CCA ATG CCA GCT CCT CCT TTG GCT
16R	2517-2540	AAT GGT TTG GGG GGC CGC TCA GGA ACT

Table 4.1: Primers designed to individual exons

primer	@base in mRNA	Tm	Sequence
F1256	1256	60°C	GCAGTGGCAGCCTATTAAGG
F1428	1428	60°C	AGTGCCACCAAGACTTGACC
R1630	1630	60°C	CGTCTCTGAGGCCTTGTTTC
R1780	1780	60°C	CTCTCCCATTCCAAGGATCA
R2390	2390	60°C	TCACCATCCAAAGACAACCA

Table 4.2: Primers amplifying half or entire C-terminal of *c-Cbl*

All the primers were selected such that they have a melting point (Tm) between 63-65°C, GC content between 40-60% and A ot T bases at the 3' end. Each primer was verified using Blast to correspond without base mismatch to rat and mouse sequences while not recognising other mRNAs. The sequences listed in *Table 4.1* correspond to primers marked "F" (forward); "R" primers are the reverse-complement of their respective "F"-marked pair.

- Primers designed to amplify the whole or part of C-terminal *c-cbl* transcripts

4.3 Cell Culture

MTT cell proliferation assay

~ was used following vendor's recommendations, in brief: 5 mg/ml MTT (*Sigma Cat#M5655*) stock solution (freshly dissolved in sterile 1x PBS before use) was added to cells in volume equal to 10% of the cell culture medium volume. After exactly one hour of incubation, cells were removed from the incubator, culture medium was discarded and the formazan crystals were solubilised for 15-30'@RT using iso-propanol acidified with 0.1N HCl. To quantify results, extinction at 570nm and 690nm was determined using Bio-Rad SmartSpec 3000 spectrophotometer.

MEF Isolation

Pregnant female mice are sacrificed at 13.5 days into gestation. Embryos are first removed together with the uterus, to be dissected individually. Each embryo is rinsed multiple times in DMEM before removing limbs, tail, brain and internal organs *(these discarded tissues can be used for genotyping, if needed)*. The remaining tissues are first eminced, then digested in Trypsin-EDTA for 30' at 37°C. Tryptic digestion is stopped by the addition of equal amount of FCS. The resulting cellular mass is passed through a 19" needle to further dissociate cells and transferred to T25 cell culture flask, grown in MEF Culture Medium

MEF Immortalisation

Following the NIH 3T3 immortalisation protocol[161], in brief: primary MEFs were passaged every three days, re-seeding 3x10^5 cells / ϕ6cm Petri dish. Cell proliferation was assessed by comparing initial cell number seeded (N0) to the number of cells harvested three days later (N3). When the N3/N0 ratio climbed over 3 for cells beyond passage 20 (p20), the cell line was considered immortalised.

Elutriation

Elutriation was done as previously described[60]. In brief, five to ten (depending on age) male rats were sacrificed and their testes removed. After removing the *tunica albuginea*, the seminiferous tubules were cut to short pieces with a sterile scalpel, then washed at least five times and decanted, thus getting rid of interstitial cells. This was followed by enzymatic digestion using Trypsine-EDTA (DNase was added if needed) for 45 minutes @32°C with slow agitation using a magnetic stirrer. Up to this point all handling was done in sterile 1xPBS with Ca^{2+} and Mg^{2+} while the following steps are carried out using 1xPBS w/o Ca^{2+}, Mg^{2+}.

The digested tubules were passed through a 10ml pipette multiple times, the debris was retained by filtering through a rough sieve. The resulting solution rich in peritubular, Sertoli and germ cells was then washed multiple times (suspended in PBS, centrifuged 5'@500g@RT after which supernatant was discarded and the whole washing process was repeated using the pellet) then filtered through a fine sieve and glass-wool to remove most of the mature spermatozoids.

Centrifugal elutration and enrichment of germ cells was done following Le Magueresse-Battistoni *et al.*, 1997.[96]

DAPI

- prior DAPI staining, cells should be pelleted on glass slides, f.e. using Cytospin, following manufacturer's protocols
- after the addition of DAPI, samples should be sheltered from direct sunlight / strong sources of light
- nuclear morphology can be observed using 350nm illumination
- at least 300 cells were counted each time when determining the precent of cells showing nuclear fragmentation

Fixation	-	Fixing Solution	10'@RT
Rinse	2x	1x PBS	-
DAPI labelling	-	DAPI Working Solution	30' with gentle agitation
Rinse	2x	1x PBS	use PBS well in excess
		Mounting	

Fixing Solution 3 : 1 solution of Ethanol : 100% Acetic acid

DAPI Working Solution 0.4 μg/ml (1 : 25000) dilution of DAPI in PBS

Immuno-Fluorescence

outline of IF protocoll used:

Wash	2x	PBS	5'@RT each
Fixation	-	Methanol	2'@-20°C
Wash	2x	TBS	5'@RT each
Permeabilization	-	0.5% Tween-20 in TBS	10'@RT
Block	-	Antibody Dilution Buffer, *ADB*	30'@RT
Primary Antibody	-	in ADB	3h@RT
Wash	3x	0.1% Tween-20 in TBS	10'@RT each
Secondary Antibody	-	in ADB	2h@RT
Wash	3x	0.1% Tween-20 in TBS	5'@RT each
		Mounting	

Bioinformatics

Multiple alignments were done using ClustalW[25].

Prediction of protein-protein interaction sites was done using Scansite[126].

Chapter 5

Materials

Tris, Glycine, Boric Acid, NaCl, EDTA, Tween-20 and 20% SDS have been bought from EUROMEDEX. Bicinchoninic Acid was purchased from Sigma (#B9643). Cell culture plasticware was ordered from either Corning-Costar or Falcon (Becton-Dickinson).

ADS - Antibody Diluting solution: 0.1% Tween-20, 0.5% BSA diluted in TBS

TBS - Tris-Buffered Saline: 100mM Tris-Cl pH7.6, 150mM NaCl

TBS-T - TBS with 0.1% Tween-20

TBE - Tris - Boric Acid - EDTA: prepared as 5x Stock Solution (54g Tris - 27.5g Boric Acid - 20ml 0.5M EDTA, pH8.0 - filled up to 1l final volume with deionised water)

EDTA - prepared as 0.5M stock solution, pH8.0 (186.1 g EDTA·Na_2· $2H_20$ ad 1l upH_20, pH8.0 set with cc NaOH)

PBS - Phosphate-Buffered Saline :

Lysis-buffer - slightly modified RIPA buffer: 50 mM Tris-Cl pH7.6 - 150 mM NaCl - 1% Triton-X 100 - 2 mM EDTA - Protease Inhibitor Cocktail(Roche) - Phosphatase Inhibitors: 2mM Na_3VO_4, 2 mM NaF

Tail-buffer 50 mM Tris pH8.0 - 100 mM EDTA - 100 mM NaCl - 1% SDS

MCM - MEF Culture Medium : DMEM - 1x MEM Non-Essential Amino Acids - 2 mM L-Glutamine - Penicillin/Streptomycin - Nystatine - 10% FCS

Discontinuous PAGE of proteins:

Tank Buffer 0.192M Glicine - 0.025M Tris - 0.1% SDS

Tris - Glicine Transfer Buffer 0.192M Glicine - 0.025M Tris - 20% Methanol

Agarose Gel Electrophoresis

Sodium - Boric Acid (SB), 20x 0.2M NaOH, pH set at 8.0 using boric acid.

MOPS, 10x 0.2M MOPS (pH7.0) - 20mM sodium acetate - 10 mM EDTA

MOPS-Formaldehyde gel is prepared by boiling the agarose in 1x MOPS and adding 10% Formaldehyde just before pouring the gel. 1x MOPS is used as tank buffer.

Cell Culture reagents have been bought from Gibco, unless indicated otherwise :

DMEM(High-Glucose) Cat. #41966-029

MEM 100x Non-essential Amino Acids Cat. #1140-035

Fetal Calf Serum Cat. #10500-064

10x PBS, ϕCa^{2+}/ Mg^{2+} Cat. #14200-067

1x Trypsin-EDTA Cat. #25300-054

DMSO Cat. #D2650, *Sigma*

Nystatine, 10000 U/ml Cat. #N1638, *Sigma*

L-Glutamine, 200mM Cat. #G7513, *Sigma*

Penicillin / Streptomycin, 200x Cat. #1670249, *ICN*

Lipofectamine Cat. #182929-011, *Invitrogen*

Part III

Results

Chapter 6

Testis

Research concerning c-Cbl has been overlooking one of it's principal organs of production: the testis. Anyone can verify this by a simple PubMed search:

"cbl[title/abstract] AND testis[title/abstract]"

The number of publications returned as of current date - February, 2007 - equals **three**, where the first publication is the original paper by *Langdon et al.* describing the discovery of c-Cbl[91], the last is our recently published work[39] and the second article only mentions a *cbl-1* gene. We can thus conclude that research regarding *c-Cbl* simply neglected the testis, already mentioned by *Langdon et al.* as one of the principle places of *c-cbl* expression[91].

6.1 c-Cbl expression is limited to germ cells

Our first step to examine c-cbl expression in testis was by using basic immunohistochemistry methods on paraffin-embedded sections of whole rat testis. c-Cbl was detected using the C-15 rabbit polyclonal antibody and as it can be clearly seen on *Figure* 6.1, it is exclusively expressed in germ cells. Pachytene spermatocytes show very strong marking (*Figure* 6.1/*a*) but *c-Cbl* is also

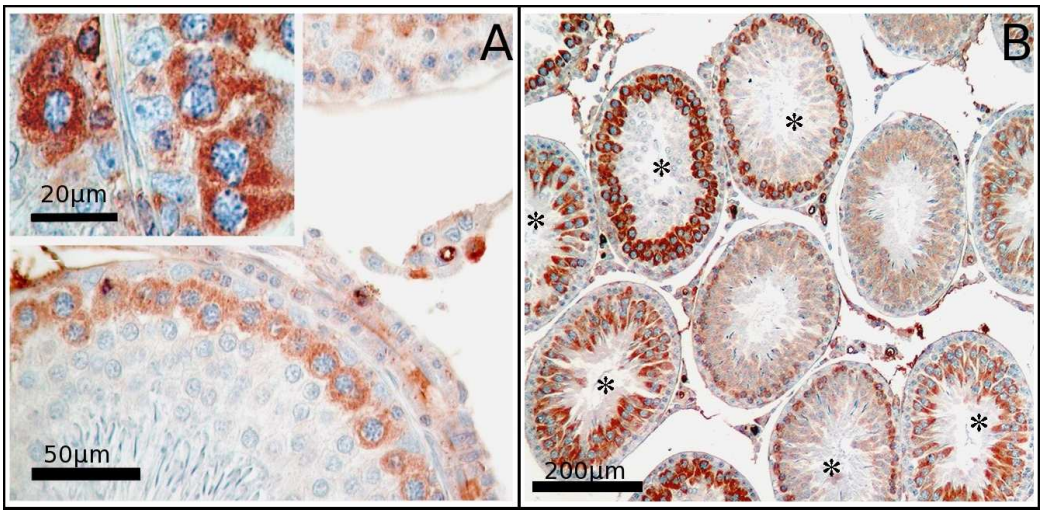


Figure 6.1: *c-Cbl* IHC on whole testis cross-section

expressed in spermatogonia albeit to a much smaller extent (*Figure* 6.1/*a*, *inset*).

When looking at more seminiferous tubules at once, it becomes apparent that *c-Cbl* marking is not uniform in all of them. This marking is the manifestation of the spermatogenic cycle moving through the seminiferous epithelium - while seminiferous tubules containing pre-pachytene germ cells are not marked with the c-Cbl antibody, tubules containing pachytene or post-pachytene germ cells show a positive marking (*Figure* 6.1/*b*). As the process of spermatogenesis at this stage depends on the presence of intra-testicular testosterone, this later observation suggests that *c-Cbl* expression in testis is also androgen-dependant[140][39].

Examining its expression at different ages it is apparent that *c-Cbl* as protein appears in the developing testis somewhere between 8 and 15dpn (*Figure 6.2/a*) although its transcript is already present at the age of 2 days post-natal (*Figure 6.2/b*). Its expression peaks at 30dpn, staying at a constant high afterwards both at mRNA and protein level (*Figure 6.2/a,b*).

Among the different germ cell types only spermatogonia are affected by radiation [75]. Strong γ-irradiation of the testes wipes out spermatogonia, they will gradually die off within a week and their disappearance will propagate

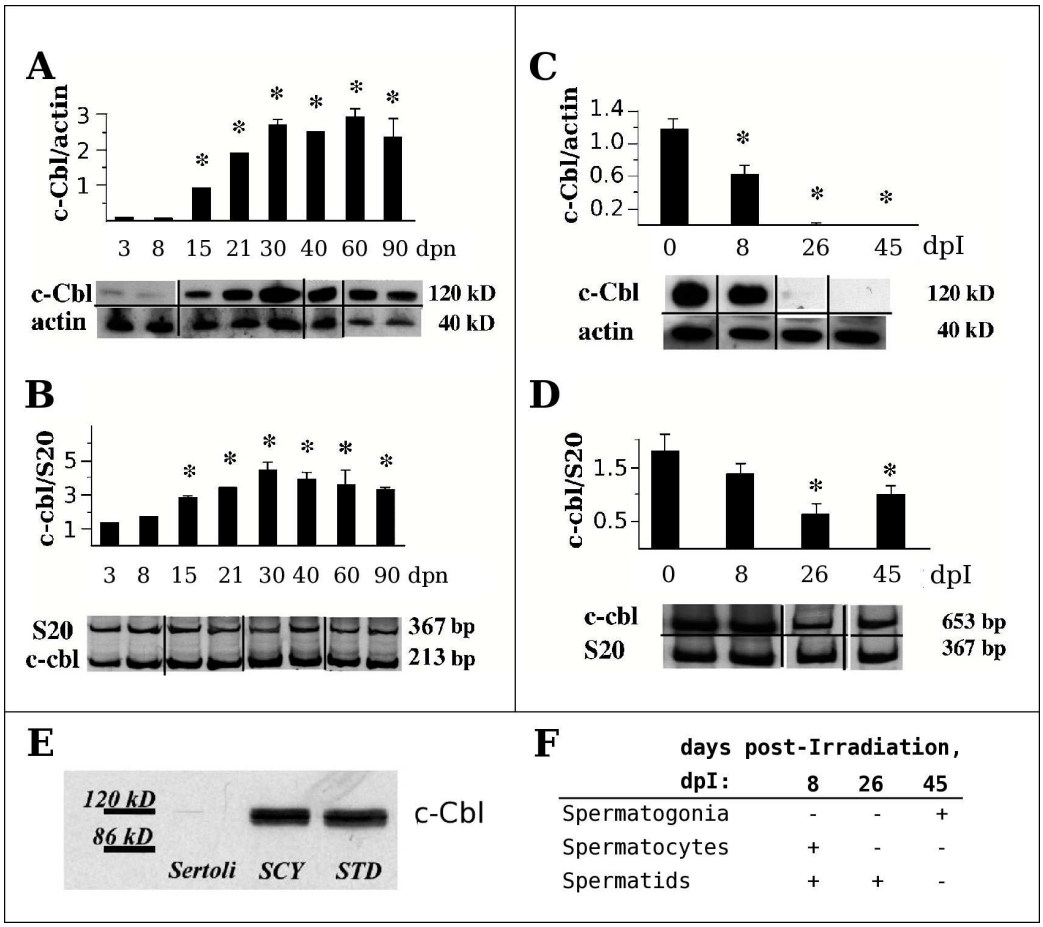


Figure 6.2: c-Cbl expression in testis in function of the age and after γ-irradiation

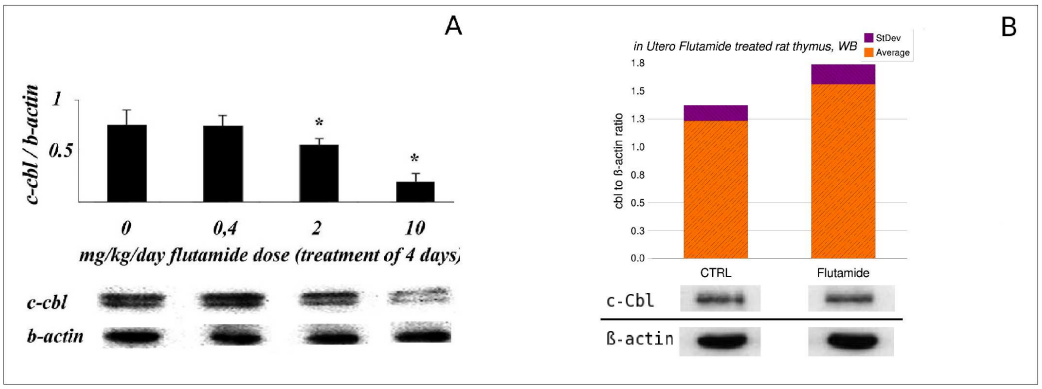


Figure 6.3: c-Cbl expression in testis

to their would-be daughter cells - 26 days after the irradiation spermatocytes disappear and at day 45 there are no more spermatids neither, while surviving spermatogonia start repopulating the tubules (*Figure 6.2/f)*[140].

When irradiating the testis of rats, *c-Cbl* expression started to drop 8 days after γ-irradiation and its presence became undetectable using Western Blot after 26 days(*Figure 6.2/c*). Although *c-Cbl* transcripts were still present, their levels dropped significantly 26 and 45 days after irradiation, too(*Figure 6.2/d*).

To further verify the results obtained using IHC and Western Blot, we examined the expression of *c-Cbl* in different cell types of the lumen of the seminiferous tubule. Pachytene spermatocytes and round spermatids were obtained using *elutriation*4.3 while Sertoli cells were obtained from 20 days old rats. *c-Cbl* was detected in both spermatocyte and round spermatid fractions but not in Sertoli cells, with spermatids showing *c-Cbl* levels comparable to that found in spermatocytes(*Figure 6.2/e*).

6.2 c-Cbl expression is regulated by testosterone

Treating rats with increasing doses of flutamide, we could observe a marked decrease in *c-Cbl* protein levels when using a dose of 10mg/kg/day(*Figure 6.3/a*). Although flutamide is known to induce apoptosis among pachytene

spermatocytes - cells expressing *c-Cbl* the most - this decrease is unlikely to be due to dropping spermatocyte numbers, as this dose of flutamide is known not to yet induce massive cell death in the testis of treated animals[129][17][39].

It was demonstrated that androgens have the opposite effect in thymus than in testis - while they inhibit the apoptosis of developing germ cells, they accelerate it in thymocytes[128][37]. Thus we were interested to see how flutamide treatment affect *c-Cbl* expression where it was the most studied, in the thymus. As it can be seen in *Figure 6.3/b*, there is a slight but significant over-expression of *c-Cbl* in flutamide treated animals.

Chapter 7

Isoforms

7.1 Looking for CARP-90

The CARP-90 (c-Cbl apoptosis-related protein 90kDa) was described to appear in the nucleus and cytoplasm of thymocytes upon apoptosis induction by hydrocortizon treatment *in vitro*[30]. These results have been reproduced by *in vivo* experiments, provoking apoptosis by γ-irradiation or by the injection of anti-CD3 antibody - both potent inducers of thymocyte apoptosis[26].

Corsois et al. also suggested - after some preliminary mapping using different antibodies - that CARP-90 might indeed be a splice variant of $p120^{c\text{-}Cbl}$, as CARP-90, cbl-b and c-cbl shared a number of common epitopes the N- and C-terminal ends[26]. This would not have been extraordinary as others have already reported *c-Cbl* splice variants in fruitfly[138] and in the mouse[10].

Initial efforts to immunoprecipitate CARP-90 from the thymus or testis of glucocorticoid treated rats and obtain its amino acid sequence were fruitless, forcing us to turn our attention towards an mRNA - RT-PCR based approach. As the N-terminal sequences were subject to much attention - often investigating single amino-acid changes! - ever since the discovery of this proto-oncogene, it was obvious that any, yet undiscovered, sequence variations can be harboured only by the much less conserved C-terminal region.

exon:	start@base:	end@base	size, bp:	Amino Acids:
8	1186	1266	81	364-390
9	1267	1470	204	391-458
10	1471	1602	132	459-502
11	1603	1980	378	503-628
12	1981	2075	95	629-659
13	2076	2216	141	660-706
14	2217	2314	98	707-739
15	2315	2494	180	740-799
16	2495	2808	313	800-896

Table 7.1: c-Cbl exons (*Mus musculus)*

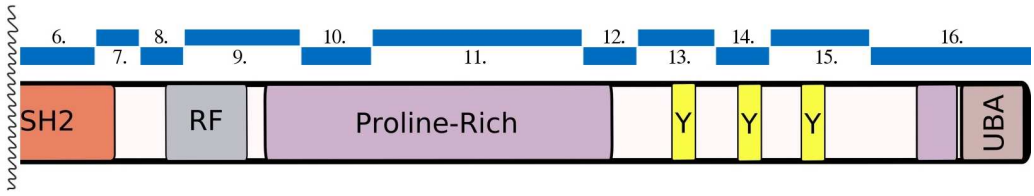


Figure 7.1: *c-Cbl*s C-Terminal exon structure

7.1.1 RT-PCR screening of the C-terminal of c-Cbl transcripts

We have designed forward and reverse primer-pairs to exons 7-16 of c-Cbl, taking care that these primers recognise sequences identical in both mouse and rat c-Cbl and they have similar melting temperatures. For initial screening we chose to use cDNA reverse transcribed from pachytene spermatocyte mRNA, the most abundant source of c-Cbl we could find. In order to choose our primers we first had to examine the exact genomic organisation of *c-cbl* and break down the mRNA sequence into a series of sequences, corresponding to each exon (*Table* 7.1, N-Terminal exons omitted).

Starting with exon 8, we have aligned these sequences to indicate their position relative to the functional domains of *c-Cbl*(*Figure* 7.1).

The sequence of primers used for screening are listed in *Table* 4.1. Possible

	F7	**F8**	**F9**	**F10**	**F11**	**F12**	**F13**	**F14**
R9	354	253	—	—	—	—	—	—
R10	452	351	126	—	—	—	—	—
R11	588	487	262	165	—	—	—	—
R12	905	804	579	482	344	—	—	—
R13	998	897	672	575	437	121	—	—
R14	1185	1084	859	762	624	308	211	—
R15	1354	1253	1028	931	793	477	380	203
R16	1427	1326	1101	1004	866	550	453	276

Table 7.2: expected size of PCR products(bp)

combination of forward and reverse primers, with expected product sizes are shown in *Table* 7.2.

When screening *c-cbl* transcripts with primers amplifying regions that span two exons, we have found that exon 10 is missing in a considerably big population of *c-cbl* mRNA, amplified by the PCR reaction at comparable levels with the expected PCR product. As you can see on *Figure 7.2/a*, the F8-R12 primer combination produces two distinct bands, the bigger band corresponding to the expected size of 487bp while the smaller band *(*indicated by *an arrow*) having a molecular weight of ~340bp[1]. This unexpected PCR product can be found with other primer combinations like F9-R11 (*see Figures 7.2/b*, 7.3)

The difference - approximately 140bp - is very close to the size of exon 10 (132bp, *see Table* 7.1). The bands have been sequenced to ascertain their identity, the obtained sequence affirmed that both PCR products are *c-Cbl* mRNA amplified, with nucleotide sequences corresponding to *c-cbl* exon 10 - referred to as *deletion 1*, or simply $\Delta 1$ from now on - missing from the smaller band.

As a protein translated from a *c-cbl* mRNA missing the $\Delta 1$ sequences would be only ~40 amino acids shorter and only ~5kDa lighter, we concluded that

[1]You might notice on *Figure* 7.2/*a*, that the F9-R12 primer combination did not amplify anything and nor did the R12 primer work when paired with the forward primer F107.2/*b*. The reason behind this is unknown to us, it was probably simple "bad luck" as all the other - 17 - primers performed well.

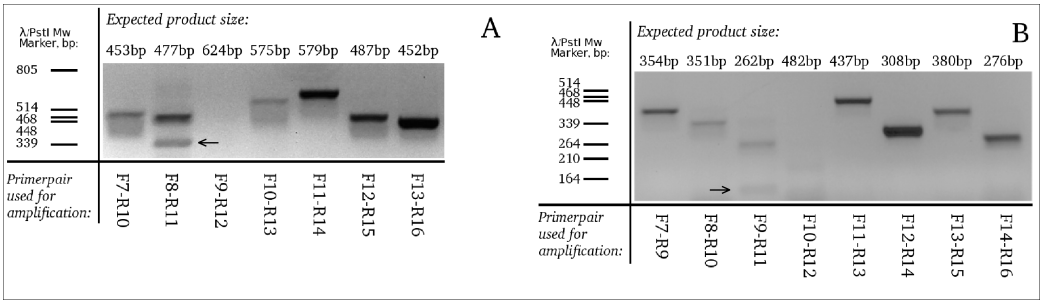


Figure 7.2: Screening c-Cbl transcripts' C-terminal using RT-PCR

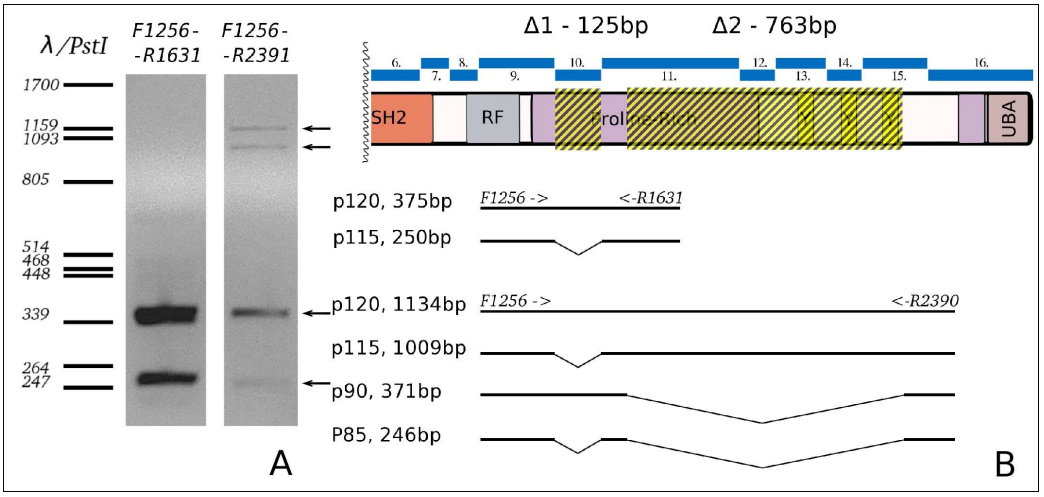


Figure 7.3: four c-Cbl isoforms amplified at the same time

this can not be identical to CARP-90 and continued our research further, with a different set of primers amplifying either the whole C-terminal or only half of it at a time.

With these primers - they are listed in *Table* 4.2 - we were not only capable of reproducing our previous results but also managed to amplify two more alternatively spliced forms of *c-cbl* mRNA, all four forms present simultaneously in an RT-PCR where reverse-transcribed total RNA from pachytene spermatocytes was used as template.

After the verification of their respectice sequences, these unexpected PCR products turned out to be two additional isoforms of *c-Cbl*, one harbouring a new, 763bp deletion (called $\Delta 2$ from now on), while the smallest one con-

taining both deletions Δ1 and Δ2. After the translation of these sequences we mapped the Δ1 deletion to amino acids 475-518, while the Δ2 deletions corresponds to the region between amino acids 533-768.

The position of the deletions within the amino acid sequence of *c-Cbl* aligned from multiple species can be seen in *Figures* 10.3, 10.4 and 10.5. Please notice that during the alignment process the position of amino acids in the sequence might change when sequences are shifted to be aligned.

Based on the expected size of the translated protein, they were named p115$^{c\text{-}Cbl}$ (carrying the Δ1 deletion), p90$^{c\text{-}Cbl}$ (Δ2 deletion) and p85$^{c\text{-}Cbl}$ (both Δ1 and Δ2 deletions). For greater consistency, from now on we will refer to WT$^{c\text{-}Cbl}$as p120$^{c\text{-}Cbl}$.

7.2 *In vivo* study of p115$^{c\text{-}Cbl}$

We examined the occurrence of these new *c-Cbl* isoforms both as mRNA transcripts and proteins in different species and in other organs than the testis. As p90$^{c\text{-}Cbl}$ and p85$^{c\text{-}Cbl}$ was impossible to find anywhere else (results not shown) - not even in RT-PCR! - we concluded that their expression is most likely limited to pachytene spermatocytes and we turned our attention towards p115$^{c\text{-}Cbl}$, the most abundant isoform discovered.

7.2.1 Expression in various organs & species

We examined the expression of p120$^{c\text{-}Cbl}$ and p115$^{c\text{-}Cbl}$ using RT-PCR in samples of mouse, rat and human origin. When comparing the relative abundance of the two isoforms in mouse organs, it was apparent that p120$^{c\text{-}Cbl}$ is transcribed in almost all tissues examined and it is most expressed in the testis as described already by *Langdon et al.*[91]. p115$^{c\text{-}Cbl}$ was also amplified from most samples (although only in trace amounts) while in testis it was present in amounts comparable to that of p120$^{c\text{-}Cbl}$ (*Figure 7.4/a*).

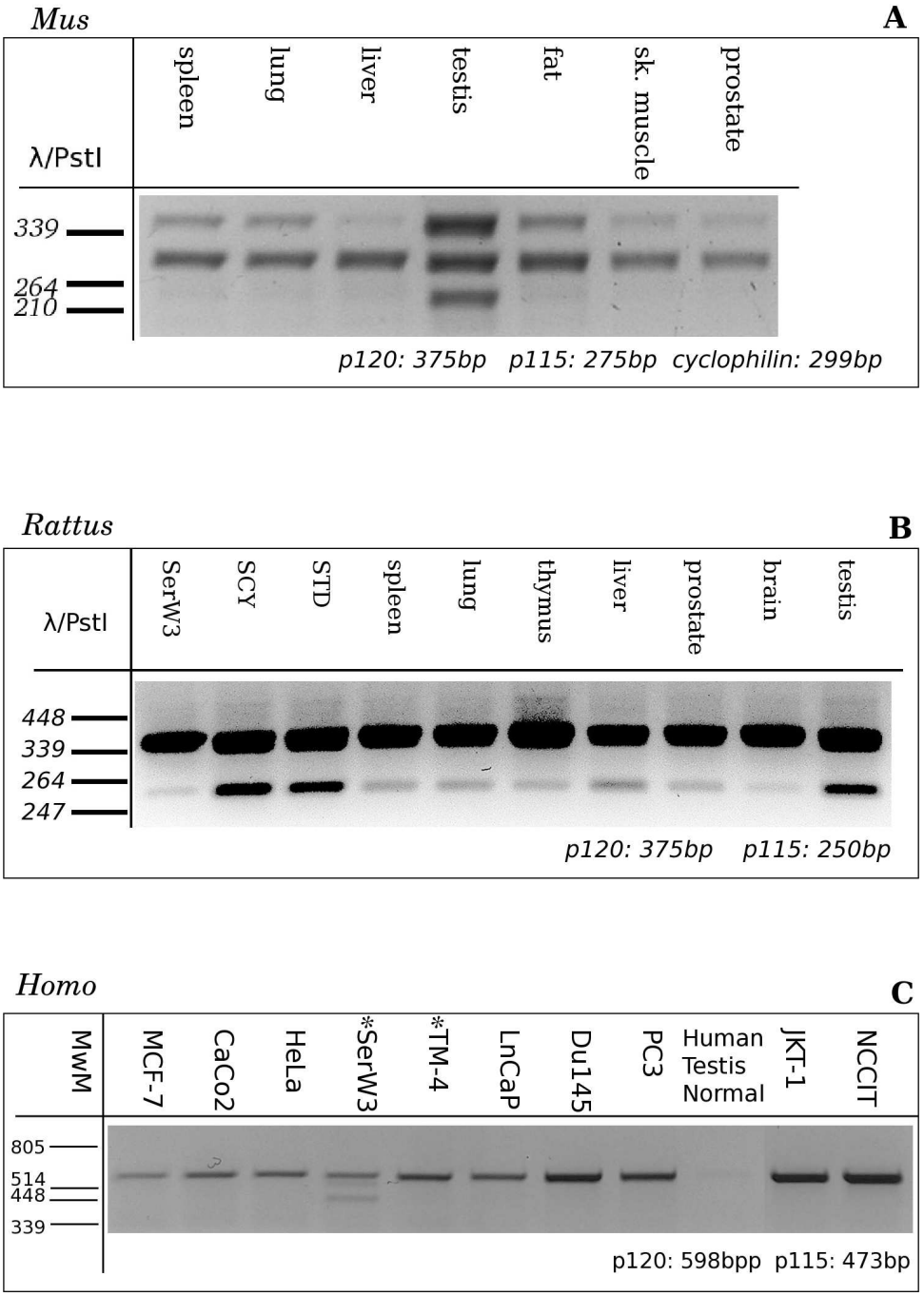


Figure 7.4: RT-PCR screening for the presence of p115$^{c\text{-}Cbl}$ in various samples of mouse, rat and human origin

When using RNA isolated from rat tissues, separated germ cell fractions or from the SerW3 cell line [2] as template for RT-PCR, p115$^{c\text{-}Cbl}$ appeared in considerable quantity only in spermatocyte, spermatid and whole testis RT-PCRs (*Figure 7.4/b*). The relevance and validity of these differences between different tissues regarding p115$^{c\text{-}Cbl}$ expression is further supported by the fact that while the amplification of the p120$^{c\text{-}Cbl}$ product reached saturation, the p115$^{c\text{-}Cbl}$ band still showed great variation in intensity.

To avoid the ethical and legal problems raised by the use of human samples we worked on cell lines of human origin[3], already present in our lab, and not protein extracts from real human tissues, to examine the possible presence p115$^{c\text{-}Cbl}$. "Normal Human Testis" refers to RNA extracted from biopsies of healthy human testes, a kind gift of Mme Catherine Deschildre. As we can see on *Figure 7.4/c*, none of these cell lines expressed p115$^{c\text{-}Cbl}$, the only sample positive was the SerW3 cell line, producing similar p120$^{c\text{-}Cbl}$/p115$^{c\text{-}Cbl}$ratios as when amplified together with other rat samples (*Figure 7.4/b*).

7.2.2 p115$^{c\text{-}Cbl}$/p120$^{c\text{-}Cbl}$ ratio is not constant

P^{33}- RT-PCR In order to produce more accurate results, we repeated the amplification of p120$^{c\text{-}Cbl}$ and p115$^{c\text{-}Cbl}$ from rat tissues using semi-quantitative radioactive RT-PCR with cyclophilin A as an internal control.

The expression of p120$^{c\text{-}Cbl}$ and p115$^{c\text{-}Cbl}$ show an interesting difference - while p120$^{c\text{-}Cbl}$ is already well present at the age of 20dpn, p115$^{c\text{-}Cbl}$ mRNA is scarce. The expression of both isoforms show a marked increase during the period between 20-30dpn but while the expression of p120$^{c\text{-}Cbl}$ reaches a *plateau* soon afterwards 30dpn (*Figure* 7.5/*a*), p115$^{c\text{-}Cbl}$ levels continue to

[2] SerW3: immortalised rat Sertoli cell line[131]

[3] MCF-7: breast adenocarcinoma; CaCo2: colon adenocarcinoma; HeLa: cervical carcinoma; LnCaP: prostate carcinoma, androgen-dependent; Du145: brain metastasis of prostate carcinoma, hormone-independent; PC3: prostate carcinoma, hormone-independent; JKT-1: human seminoma; NCCIT: intermediate between seminoma and embryonal carcinoma;

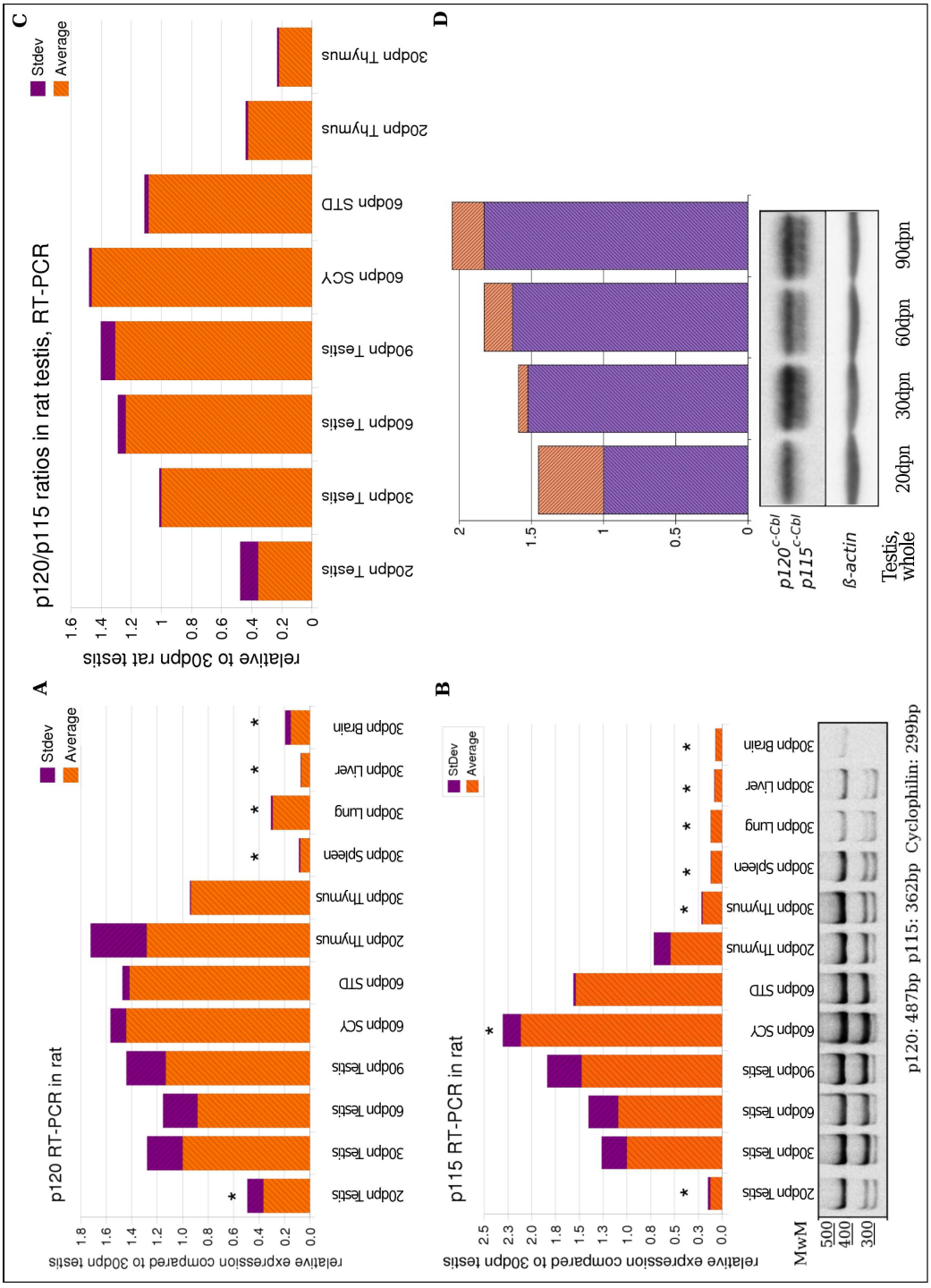


Figure 7.5: p120$^{c\text{-}Cbl}$: p115$^{c\text{-}Cbl}$ ratio is not constant in testis

grow until the age 90dpn[4]. Between all samples, it is always in spermatocytes that *c-Cbl* expression is the highest (*Figure 7.5/a-b*).

When comparing the ratio of p120$^{c\text{-}Cbl}$ and p115$^{c\text{-}Cbl}$ in the testis of animals at the age of 20, 30, 60 and 90dpn we can see an upward trend - the relative abundance of p115$^{c\text{-}Cbl}$grows quickly at the onset of spermatogenesis and continues to grow until 90dpn (*Figure 7.5/c*).

While the trend of *c-Cbl* expressions with age points toward growth in the testis, it is exactly the opposite what we can observe in the thymus. Both p120$^{c\text{-}Cbl}$ and p115$^{c\text{-}Cbl}$ show a decrease in expression with p115$^{c\text{-}Cbl}$ diminishing more rapidly between the ages 20 and 30 days post-natal (*Figure 7.5/a-b-c*).

We also observed a steady but low level of expression for both isoforms in the lung, spleen, liver and brain (*Figure 7.5/a-b*), indicating that p115$^{c\text{-}Cbl}$ is not restricted to the testis and can be easily detected in various tissue.

Western Blot Using gradient gels we were able to separate the two isoforms in Western Blot. As shown on *Figure /d*, p115$^{c\text{-}Cbl}$ is barely present compared to p120$^{c\text{-}Cbl}$ in 20dpn testis protein extract but gives a strong, separate band in samples taken from older animals (*Figure 7.5/d*).

[4]we cannot say if the trend continues beyond this age as the experiment was not done on animals older than 90 days

Chapter 8

Isoforms studied *in silico*

As *c-Cbl* is reputed for the large number of interacting partners - its role played often depends on the interacting partner indeed - we wanted to gain a deeper understanding of how the lack of sequences encompassed by the $\Delta 1$ or $\Delta 2$ deletions could alter *c-Cbl*s interaction with its partner proteins.

We used the on-line available software Scansite (http://scansite.mit.edu) [126] to look for predicted docking sites of interacting partners.

8.1 $\Delta 1$ deletion

The sequence used for motif scan - corresponding to the sequence of the $\Delta 1$ deletion - was as follows:

1 - 10	11 - 20	21 - 30	31 - 40
KVERPSSPFS	MAPQASLPPV	PPRLDLLQQR	APVPASTSVL

41 - 44

GTAS

In *Table* 8.1 you can see the proteins predicted to have a binding site when using the "high stringency" option for the research. The "Score" column means that among all binding sites examined by the software, there is only this percentage of sites that are closer to the hypothetical "ideal" binding site of the protein examined.

Protein	Binding Domain	Sequence	Position	Score
Abl	SH3	MAPQASL**P**PVPPRLD	P18	0.150%
Grb2	SH3	MAPQASL**P**PVPPRLD	P18	0.104%
Crk	SH3	MAPQASL**P**PVPPRLD	P18	0.199%

Table 8.1: predicted interaction sites in the Δ1 deletion

Due to the high stringency of research, all the sites found correspond well to the expected amino acid sequence, with Grb2 getting the best scores. Although the results of SCANSITE should be interpreted with caution, the fact that all these proteins were already described to bind *c-Cbl*[159], with Grb2 binding both to proximal and mid- C-terminal proline-rich regions[34], makes these findings rather convincing.

8.2 Δ2 deletion

The sequence corresponding to the sequence of the Δ2 deletion is as follows:

1 - 10	11 - 20	21 - 30	31 - 40
PIPPTLRDL**P**	**P**PPP**P**DRPYS	VGAETRPQRR	PL**P**CTPGDCP
41 - 50	51 - 60	61 - 70	71 - 80
SRDKL**P**PVPS	SRPGDSWLSR	TIPKVPVATP	NPGDPWNGRE
81 - 90	91 - 100	101 - 110	111 - 120
LTNRHSLPFS	LPSQMEPRAD	VPRLGSTFSL	DTSMTMNSSP
121 - 130	131 - 140	141 - 150	151 - 160
VAGPESEHPK	IKPSSSANAI	YSLAARPLPM	PKLPPGEQGE
161 - 170	171 - 180	181 - 190	191 - 200
SEEDTE**Y**MTP	TSRPVGVQKP	EPKRPLEATQ	SSRACDCDQQ
201 - 210	211 - 220	221 - 230	231 - 240
IDSCTYEAMY	TIQSQALSVA	ENSASGEGNL	ATAHTSTGPE
241 - 250	251 - 254		
ESENEDDGYD	VPKP		

The list of scansite results is much longer of course, some of them were omitted for brevity:

Protein	Binding Domain	Sequence	Position	Score
PDGFR	SH2	ESEEDTE**Y**MTPTSRP	Y167	0.056%
Crk	SH2	ESEEDTE**Y**MTPTSRP	Y167	0.040%
Nck	SH2	ESEEDTE**Y**MTPTSRP	Y167	0.069%
Abl	SH2	ESEEDTE**Y**MTPTSRP	Y167	0.027%
p85	SH2	ESEEDTE**Y**MTPTSRP	Y167	0.156%
Src	SH3	PPTLRDL**P**PPPPPDR	P10	0.036%
p85	SH3	PPTLRDL**P**PPPPPDR	P10	0.076%
Nck	2nd SH3	PPTLRDLP**P**PPPPDR	P11	0.062%
PLCγ	SH3	DLPPPPP**P**DRPYSVG	P15	0.096%
p85	SH3	RPQRRPL**P**CTPGDCP	P33	0.030%
Src	SH3	RPQRRPL**P**CTPGDCP	P33	0.007%
Grb2	SH3	CPSRDKL**P**PVPSSRP	P46	0.159%

Note: p85 refers to the p85 subunit of the PI3K

Table 8.2: predicted interaction sites in the Δ2 deletion

Again, if we compare the results with those already present in scientific literature, we can notice that PDGFR and Nck was never shown to bind *c-Cbl* through this tyrosine while for Crk, Abl and the p85 subunit of PI3K the results are probably correct[159][144]. We have to remark that Crk was shown to bind both through its SH2 or its SH3 domains and a binding site for the Crk SH3 domain was already predicted in the Δ1 deletion (*Table* 8.1). The correct prediction of the binding site for its SH2 domain in the Δ2 deletion would mean that there could be a difference between *c-Cbl* isoforms to recruit Crk.

Another interesting finding is the predicted Grb2 SH3 binding site. Donovan *et al.* already mapped the strongest Grb2 binding site between amino acids 481-528 of $p120^{Cbl}$, mentioning also that longer C-terminal parts with more prolins present can render this interaction even stronger [34]. The predicted binding site for the Grb2 SH3 domain in deletion Δ1 should correspond to the site mentioned by Donovan et al. while the second site found within the sequence ofΔ2 might correspond to the region that contributes to Grb2 binding, facing us with a similar situation as for Crk: the different isoforms of *c-Cbl* might show great differences in their interaction with Grb2 and

consequently with EGFR and all other proteins they bind to with the help of Grb2. This is what we decided to find out using a Grb2-GST fusion protein, results can be seen in 9.3 *Grb2-GST pull-down*.

Chapter 9

In vitro study of isoforms

As the newly found *c-Cbl* isoforms are present in relative abundance only in germ cells, a cell type notoriously difficult to propagate in cell culture, we sought other possibilities to explore the role of these isoforms *in* vitro. We also wanted to use a cell line that doesn't express $p120^{c\text{-}Cbl}$to eliminate any interference from the part of endogenous *c-Cbl*. As we had access to $KO^{c\text{-}Cbl}$ mice we decided to use mouse embryo fibroblast prepared from these mice for further experiments.

9.1 $KO^{c\text{-}Cbl}$ and $WT^{c\text{-}Cbl}$ MEFs

Due to the difficulties of working with isolated germ cells, we decided to continue working using an *in vitro* system devoid of endogenous *c-Cbl* to investigate the divergent properties of the isoforms. Having $KO^{c\text{-}Cbl}$[1] and $WT^{c\text{-}Cbl}$ mice at our disposition, we decided on using Mouse Embryo Fibroblasts (MEFs), either primary or immortalised using the NIH 3T3 protocol[161].

Having established immortalised $KO^{c\text{-}Cbl}$ and $WT^{c\text{-}Cbl}$ MEFs also enabled us to further investigate *c-Cbl*s possible role regarding proliferation and apoptosis to which know direct evidence have been found yet.

[1] a kind gift of M. Naramura, NIH, Bethesda, USA

9.1.1 Proliferation with Serum Withdrawal

We passaged MEFs every three days for a period of ten passages with different concentrations of Fetal Calf Serum (FCS) in 6cm Petri dishes, seeding 3*10^5 cells each time and counting the number of cells harvested three days later[2]. Cells were cultured in the presence of 10, 5 or 1% FCS; cumulative cell number and the number of cell divisions between passages were calculated for each cell line and each serum concentration.

It was remarkable that while WT$^{c\text{-}Cbl}$ proliferation increased in a linear fashion when kept in the presence of increasing concentrations of FCS (number of cell divisions / passage: 1% FCS: two; 5% FCS: three; 10%: four), KO$^{c\text{-}Cbl}$proliferation changed between the extremes. These cells barely divided once every 72 hours in the presence of 1% FCS (at the end of passage 10) but they divided faster in the presence of 5% FCS than WT$^{c\text{-}Cbl}$with 10% serum (*Figure 9.1/a*).

Looking at cumulative cell numbers produced by the two cell lines we see a similar picture: when cultured in 1% FCS, the line representing KO$^{c\text{-}Cbl}$starts a slight downwards turn by the end of passage 1O (reaching a direction parallel to the X-axis would mean it stopped proliferating altogether) but it grows as fast in the presence of 5% FCS as the WT$^{c\text{-}Cbl}$MEFs in the presence of 10% (*Figure 9.1/b*).

9.1.2 Etoposide and H_2O_2 treatment

To see if their respective sensibility to apoptotis differs, we treated WT$^{c\text{-}Cbl}$ and KO$^{c\text{-}Cbl}$ MEFs with both etoposide or H_2O_2.

KO$^{c\text{-}Cbl}$ cells were more sensitive to H_2O_2induced apoptosis than their wild-type counterparts. The number of cells showing nuclear fragmentation with DAPI staining (*Figure 9.2/a*) and the levels of activated caspase-3 in Western Blots (*Figure 9.3*) both show an increased susceptibility to apoptosis due to

[2]this indeed corresponds to the classic 3T3 protocol, seeding **3***10^5 cells and harvesting them **3** days later

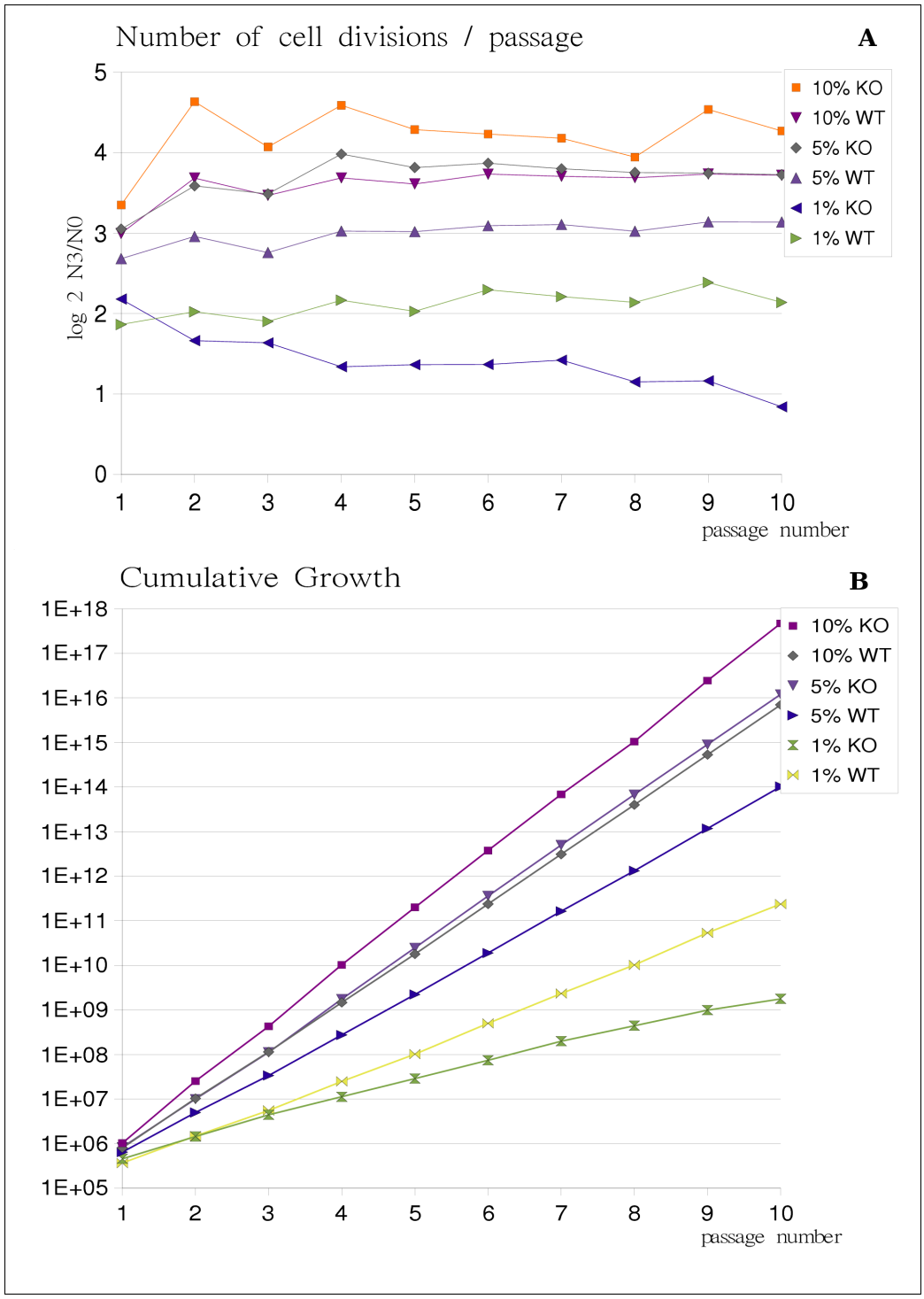


Figure 9.1: FCS gradient proliferation assay of immortalised WT$^{c\text{-}Cbl}$ and KO$^{c\text{-}Cbl}$ MEFs

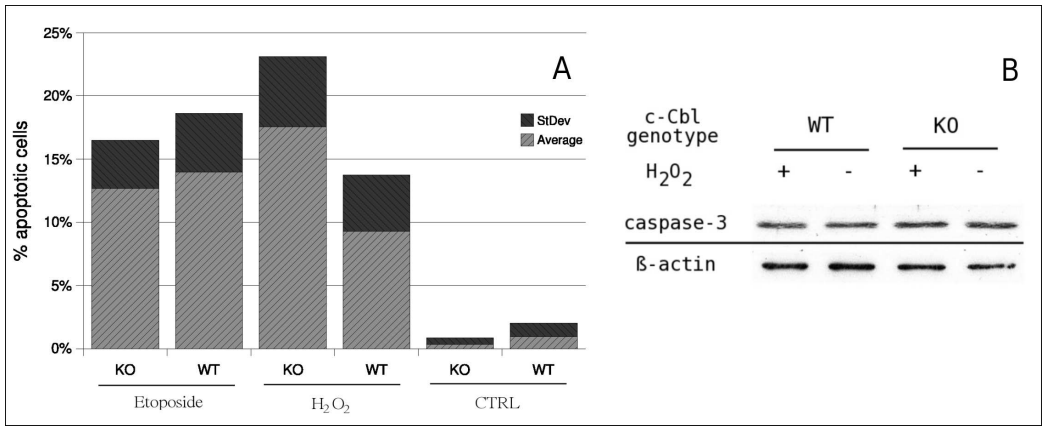


Figure 9.2: etoposide and H_2O_2treated MEFs, WT$^{c\text{-}Cbl}$ and KO$^{c\text{-}Cbl}$ - nuclear fragmentation (DAPI)

oxidative stress. A simultaneous decrease in the amount of anti-apoptotic proteins xIAP and cIAP-2 was also observed in H_2O_2-treated cells while cIAP-1 levels remained the same (*Figure 9.3*).

The difference in activated caspase 3 levels in H_2O_2 treated cells were clearly not due to a differential expression of pro-caspase 3 as it can be see in *Figure 9.3* that both cell line cells expressed pro-caspase 3 at similar levels regardless of treatment by H_2O_2.

The frequency of nuclear fragmentation in WT$^{c\text{-}Cbl}$ or KO$^{c\text{-}Cbl}$ cells did not show significant differences after etoposide treatment (*Figure 9.2/a*). Only the expression of cIAP-2 and XIAP dropped slightly, the later was detected in both cell lines (*Figure 9.3*).

9.2 Expression in KO$^{c\text{-}Cbl}$ MEF

To have a more convenient method of studying *c-Cbl* isoforms than co-culture of Sertoli and germ cells, we decided to use transient transfection to over-express these isoforms in our KO$^{c\text{-}Cbl}$ MEFs thus creating an easy-to-use *in vitro* model.

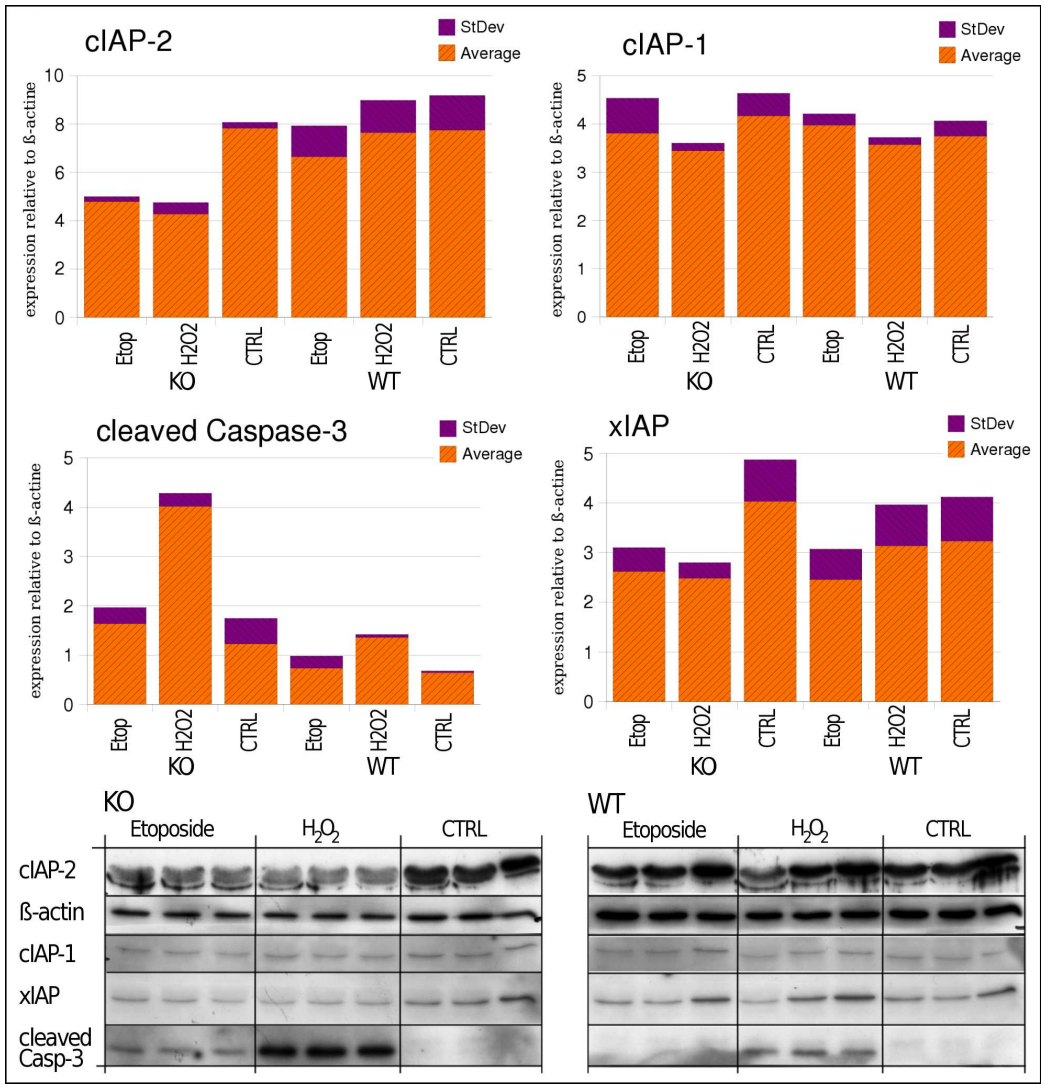
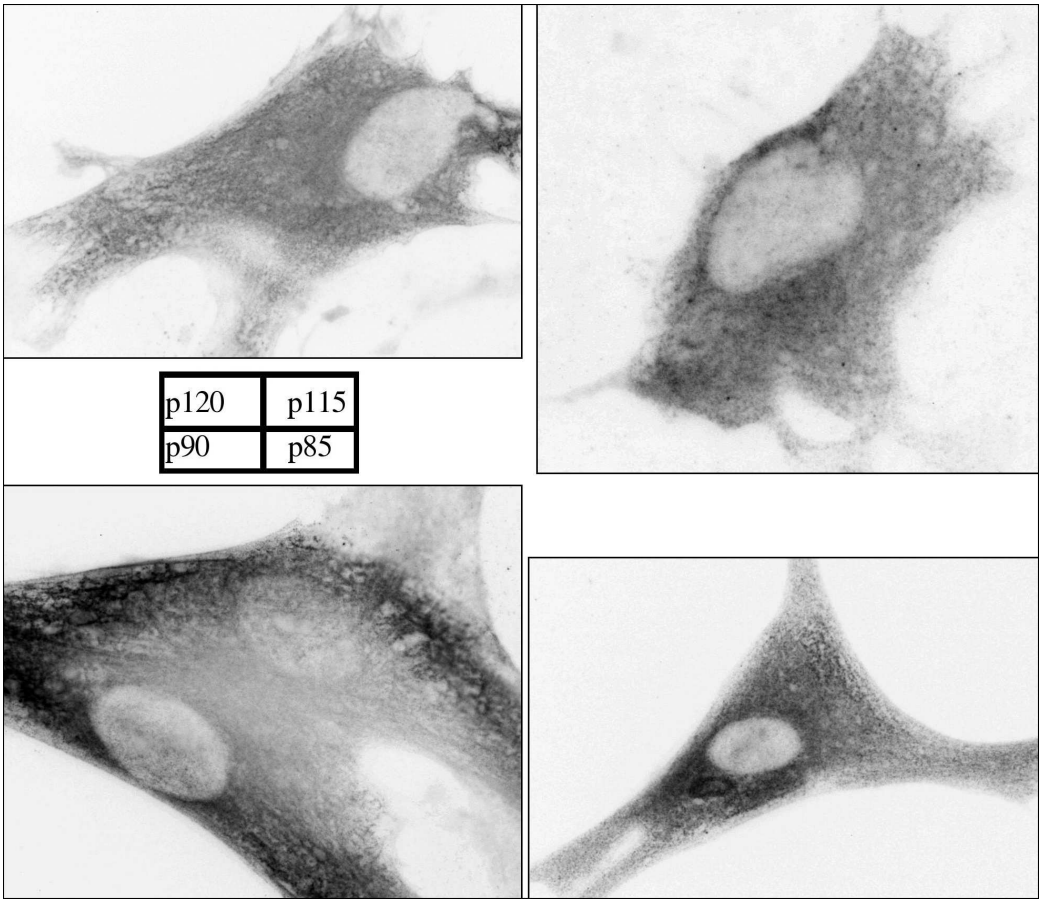


Figure 9.3: apoptosis induction in immortalised WT$^{c\text{-}Cbl}$ & KO$^{c\text{-}Cbl}$ MEFs



(a) 100x, oil immersion

Figure 9.4: cellular localisation of c-Cbl isoforms

9.2.1 localisation, IF

c-Cbl is known to be a cytoplasmic protein, occasionally located in the proximity of internal or the plasma membranes when binding membrane-bound partners like RTKs. The first thing we wanted to see whether we can see a difference in the respective sub-cellular localisation of the different isoforms.

As it can bee seen in *Figure* 9.4, there is no marked difference in the localisation of these isoforms. The same pattern is observed in all cases - they are missing from the nucleus and show a strong staining of the cytoplasm. A pattern resembling cytoskeletal association is present, most visible with p90$^{c\text{-}Cbl}$ (*Figure* 9.4, *lower left*).

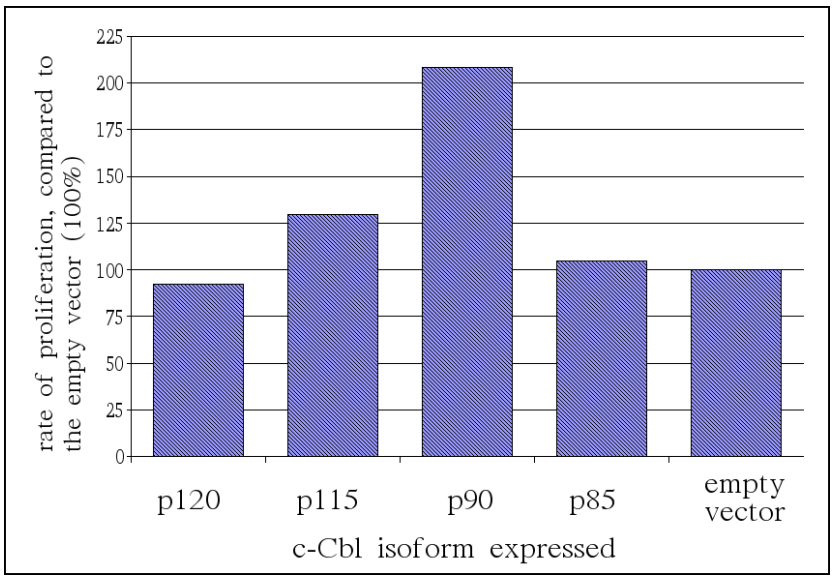


Figure 9.5: p90$^{c\text{-}Cbl}$ promotes proliferation

9.2.2 Proliferation

Trans-gene expression following transient transfection diminishes within a week, so observing the proliferation of KO$^{c\text{-}Cbl}$ cells transfected with isoforms over a period of multiple passages was not possible. We chose to use MTT instead to compare the proliferation of transfected cells in the period between 24 and 48 hours post-transfection (ptf)[3].

As we can see on *Figure* 9.5, the growth rate of cells transfected with p120$^{c\text{-}Cbl}$, p115$^{c\text{-}Cbl}$, p85$^{c\text{-}Cbl}$ or the empty vector did not show considerable difference. p90$^{c\text{-}Cbl}$ expressing cells, on the other hand, had shown a proliferation rate twice as fast on average.

9.2.3 Etoposide and H_2O_2 treatment

As we could already see in *subsection* 9.1.2, KO$^{c\text{-}Cbl}$ MEFs have been less tolerant against oxidative stress than their wild-type counterparts. We wanted

[3]We choose this time frame based on previous experiments where were optimising the transient transfection of KO$^{c\text{-}Cbl}$ MEFs (experiments not shown). Trans-gene expression was found to be high both 24h and 48h ptf but already falling 96h ptf.

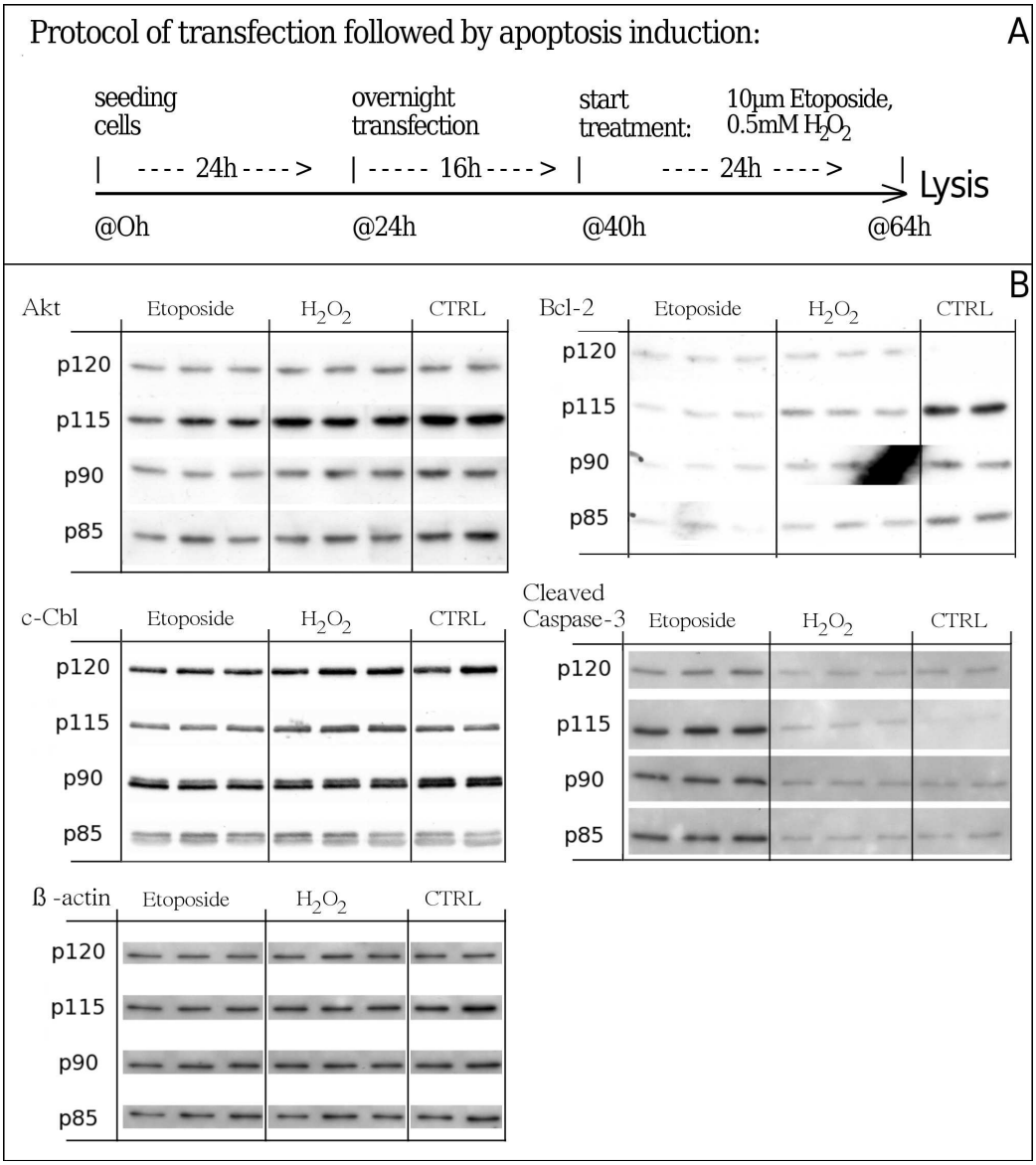


Figure 9.6: isoforms can influence effector caspase activation induced by etoposide treatment

to examine if such differences can be found between mouse embryo fibroblast transiently transfected with the different isoforms of *c-Cbl*. As the proliferation of immortalised KO$^{c\text{-}Cbl}$ MEFs was too rapid to let us set up a model for apoptosis induction and subsequent 24h incubation with H_2O_2 or etoposide, we chose to use primary KO$^{c\text{-}Cbl}$ MEFs at passage 4 for the experiments.

The scheme of the experiment is presented in *Figure 9.6/a* : we seeded KO$^{c\text{-}Cbl}$ MEF @p4 in the afternoon, started the transfection the afternoon of the following day and added etoposide or $H_2O_2$the morning of the second day. Cells were lysed after 24 hours of incubation with the apoptosis inducing drugs, transfected but untreated cells were used as control for each isoform.

The expression of *c-Cbl* isoforms and β-actin, used as internal control protein, did not show any variation between treated or control cells as we can see on *Figure 9.6/b*.

Akt is markedly over-expressed in p115$^{c\text{-}Cbl}$ transfected cells (*Figure 9.6/b top-left*), and it is also p115$^{c\text{-}Cbl}$ expressing cells that show the highest level of caspase-3 activation upon treatment with etoposide (*Figure 9.6/b middle-right*). This is accompanied by strong Bcl-2 expression in p115$^{c\text{-}Cbl}$ positive control cells, but Bcl-2 expression is reduced to the level of what we can find in cells expressing other isoforms (*Figure 9.6/b top-right*).

The expression of Akt and Bcl-2 does not show marked differences between the rest of the isoforms although it is worth noticing the slight decrease in Bcl-2 levels when comparing control and treated cells, with more difference between etoposide treated and control than H_2O_2 treated and control cells (*Figure 9.6/b*).

Caspase-3 activation upon etoposide treatment shows an increasing trend when comparing isoforms in the following order: p120$^{c\text{-}Cbl}$ $>$ p85$^{c\text{-}Cbl}$ $=$ p90$^{c\text{-}Cbl}$ $>$ p115$^{c\text{-}Cbl}$; there is also a low, activated caspase 3 signal detected in H_2O_2 treated cells comparable to what we can see in control cells, probably due to the general toxicity of the lipofectamine used for the transfection (*Figure 9.6/b middle-right*).

9.3 Grb2-GST Pull-Down

Results obtained *in silico* suggested that not all of the isoforms will retain their capacity to bind Grb2, as both p115$^{c\text{-}Cbl}$and p90$^{c\text{-}Cbl}$ miss one of the two predicted Grb2 binding sites while p85$^{c\text{-}Cbl}$ misses them both (*for details please refer to Chapter 8*).

We performed a GST pull-down assay using Grb2-GST fusion protein on the lysates of KO$^{c\text{-}Cbl}$ MEFs expressing only one isoform at a time. A brief explanation of the experiment (see also *Figure 9.7/a*): cells were transfected 24 hours after seeding in 6-well plates. Transfection was done overnight, followed by a change to fresh medium containing either 10% FCS (no serum starving) or 0.1% FCS (serum starved cells). Cells were lysed 24 hours after the change of medium, this was preceded by the addition of 100 ng/ml EGF (murine) 5 minutes before lysis where indicated. In each case, 100 μl of the cleared lysate was retained for Western Blotting and the rest was used for the pull-down experiment.

To be assured that eventual differences in Grb2-GST pull-down results are not due to differences in transfection efficiencies, we checked the level of expression of *c-Cbl* isoforms in each lysate used for a pull-down assay. As we can see on *Figure 9.7/b*, the expression of transfected isoforms shows no great variance with the exception of cells neither serum starved nor treated by EGF.

To eliminate the possibility of detecting nonspecific associations between GST and *c-Cbl* or the agarose beads as a false positive signals, each pull-down experiment was done with a GST control in duplicate ("GST CTRL" lanes, *Figure 9.7/b,c*).

The amount of *c-Cbl* isoforms pulled down by Grb2 show a different picture though. Although p85$^{c\text{-}Cbl}$was incapable - as expected - to bind to Grb2 (*Figure 9.7/c*), there was no significant difference between the relative quantities of p120$^{c\text{-}Cbl}$, p115$^{c\text{-}Cbl}$ and p90$^{c\text{-}Cbl}$pulled down (*Figure 9.7/c*).

There is a variation though between pull-downs in serum starved, serum starved treated with EGF or control lysates. The quantity of *c-Cbl* isoforms

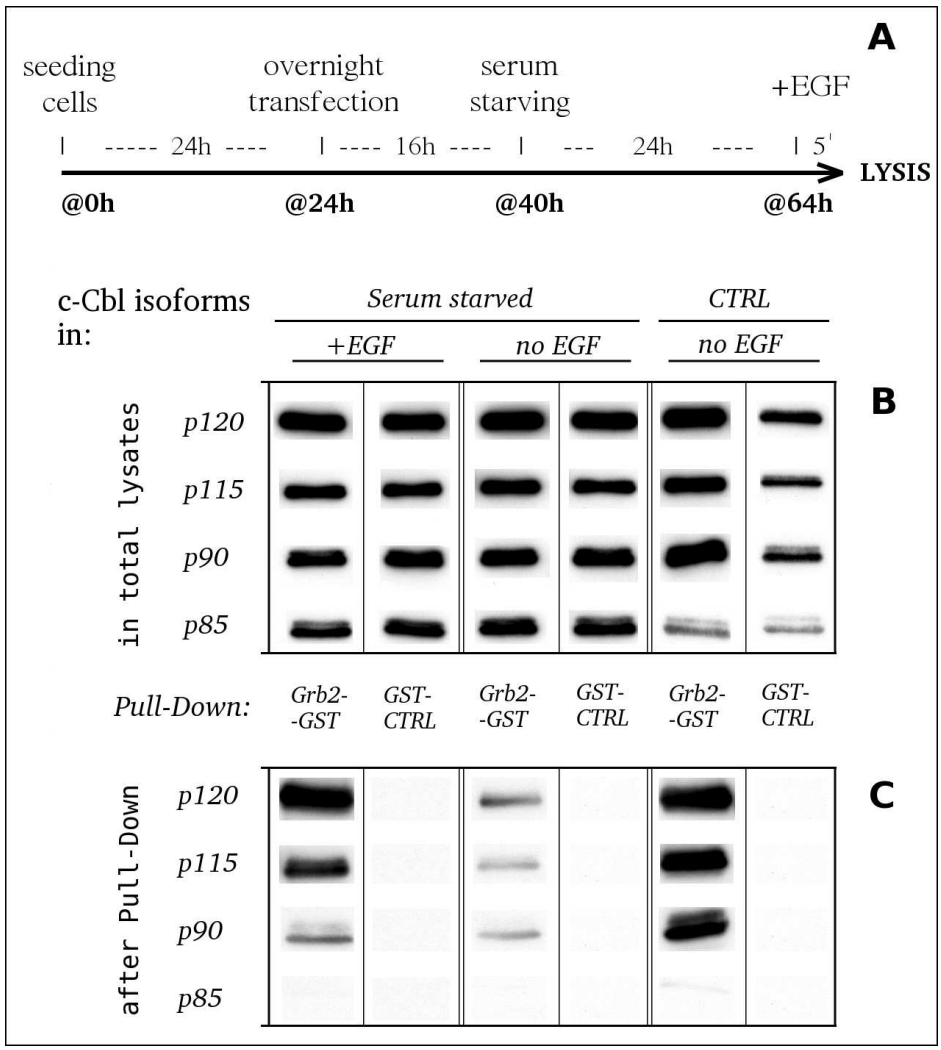


Figure 9.7: Grb2-GST pull-down of isoforms expressed in KO$^{c\text{-}Cbl}$MEFs

binding to Grb2-GST decreased in serum-starved cells, a change reversible - completely in case of p120 and p115, while the quantity of p90 pulled down falls somewhere between control and untreated serum-starved cells - by the addition of EGF a few minutes prior lysis (*Figure 9.7/c*).

There was no sign of tyrosine phosphorylation on any of the expressed isoforms, neither in Western blots nor in pull-down experiments (results not shown).

Part IV

Discussion

Chapter 10

Discussion

10.1 *c-Cbl* expression in testis

The high level of expression of *c-Cbl* in the testis was already mentioned in the original paper by *Langdon et al.* [91], showing an abundant 3.5kDa mRNA transcript in testis. Since we know that the testis is a very well organised organ with a great number cell types separated both spatially and functionally, it was interesting to see that the abundant *c-Cbl* transcript is indeed restricted to a distinct group of germ cells, right before the first meiotic division (*Figures* 6.1, 6.2) and this high level of expression is not achieved until sexual maturation.

The expression of *c-Cbl* is constantly on the rise until the age of 30-35 days post-natal[1] (*Figure* 6.2/*a,b*) which corresponds well with the increase in the number of spermatocytes relative to the other cell types making up the testis. Pachytene spermatocytes are not present in the developing testis until 18 dpn and as they don't proceed to the secondary spermatocyte and spermatid stages for a period of approximately 13 days, their relative numbers will increase continuously until approx. 32 days post-natal, supporting well the results obtained by Western blot and IHC, localising the expression of *c-Cbl* to the testis.

[1] in rats

γ-irradiation experiments further support the above results: *c-Cbl* expression is decreasing 8 days after and is already disappeared 26 days after the γ-irradiation of rat testes (*Figure* 6.2*/c, WB*). This is due to gradual disappearance of developing germ cells, starting with spermatogonia (the cells sensitive to radiation-induced apoptosis [44]) and propagating towards cells in more and more advanced stages of development (*Figure* 6.2*/c, f*; illustration of spermatogenesis can be seen on *Figure 2.3*).

Results obtained from rats treated with the antiandrogen flutamide revealed that the expression of *c-Cbl* is regulated by testosterone, as in flutamide treated animals the expression of *c-Cbl* decreased (*Figure 6.3/a*). This transcriptional regulation of *c-Cbl*s expression is not restricted to the testis however, as we could observe a small but significant increase of its expression in the thymus of flutamide treated rats (*Figure 6.3/b*). The decrease of *c-Cbl* is not due to massive cell death neither as the effective dose of flutamide used (10 mg/kg/day) induces onl the apoptosis of individual pachytene spermatocytes [129].

Although the effect of androgens have exactly the inverse effect on *c-Cbl*'s expression in the thymus, this is of no surprise. Androgens are known to protect pachytene spermatocytes from apoptosis, up-regulating its expression in these cells, while testosterone was already described to *accelerate* thymocyte apoptosis [128] and it does down-regulate *c-Cbl*s expression in the thymus. This coincidence raises the idea putting a hypothetical link between testosterone-dependent apoptosis and *c-Cbl*.

It is known that pachytene spermatocytes make up a very special group of developing germ cells - those that are responsive to androgens, found in seminiferous tubules at stages VII-IX of spermatogenesis [140] [39]. If testosterone is missing or its action is blocked by anti-androgens like flutamide, pachytene spermatocytes will undergo massive apoptosis [129]. This wave of programmed cell death is mediated through the mitochondrial pathway of apoptotis and involves the up-regulation of pro-apoptotic Bcl-2 family member Bax and the down-regulation of anti-apoptotic Bcl-2 and Bcl-w proteins [17], resulting in elevated levels of active caspase-3 and caspase-6 [129] [17].

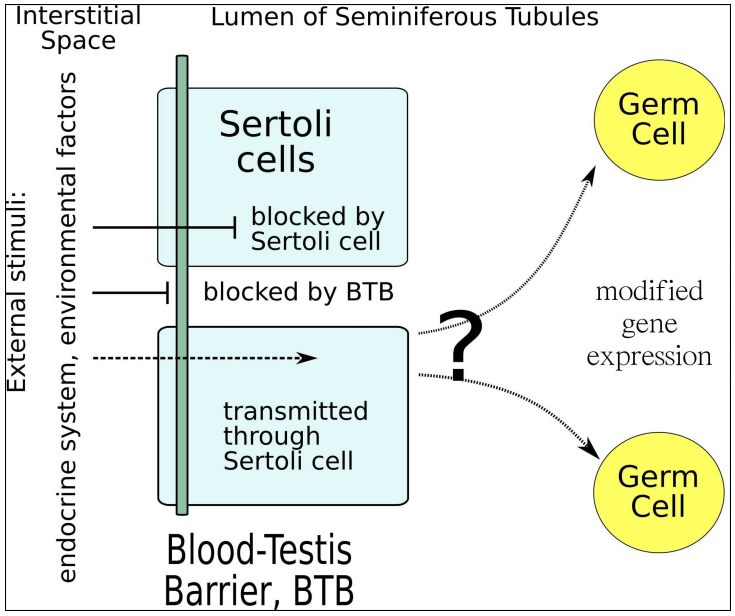


Figure 10.1: modulation of external stimuli by Sertoli cells

The exact mechanism of apoptosis induction by androgen deprivation remains a mystery however. In developing germ cells - to our current knowledge - there is no androgen receptor present [182]. Germ cells beyond the pre-leptotene stage are also behind the blood-testis barrier and testosterone-induced physiological events can only be the results of interactions between Sertoli and germ cells[2] (*see Figure* 10.1). It is highly likely that Sertoli cells are capable of influencing spermatogenesis by both directly regulating transcription of target genes and activating **or modulating** signal transduction events in maturating GCs.

Our results suggest that *c-Cbl* expression is restricted to a well precised stage of germ cell development where cells are susceptible to androgen-regulated apoptosis. *c-Cbl* is down-regulated in the thymus where androgens accelerate apoptosis[128] and it is up-regulated in the testis where they protect from

[2]There are many examples of such interactions already well documented, like the secretion of lactate - it seems to be the energy source of choice for spermatocytes and round spermatids [74][16] - by Sertoli cells, or receptor-ligand interactions between the adjacent Sertoli and germ cells like Stem Cell Factor (produced by Sertoli cells) and c-kit (expressed by germ cells) interaction [45] [29].

apoptosis[129]. In both cases the relation between androgens and apoptosis is indirect, as in the testis the cells possessing functional AR are not the cells dying with apoptosis while in the thymus it was also shown that the regulation by androgens is indirect [37].

Although the above results suggest that *c-Cbl* plays an anti-apoptotic role in the testis, a role already described in connection with *v-Cbl* [152]. Unfortunately the picture is not so clear as KO$^{\text{c-Cbl}}$mice (showing signs of hypofertility and irregularities of spermatogenesis[39]) have decreased levels of baseline apoptosis in pachytene spermatocytes and they are no longer prone to increased apoptosis upon flutamide treatment[39]. This is explained by the slight over-expression of cIAP-2 in normal and strong over-expression in flutamide treated KO$^{\text{c-Cbl}}$animals, compared to wild-type counterparts [39].

The above results, once summarised with other, already published results, indicate that the expression of *c-Cbl* in pachytene spermatocytes is not purely coincidental with but necessary for the correct regulation of androgen-dependent apoptosis and it is one of those proteins through which Sertoli-cell initiated pro-apoptotic signals are passed on to their targets in germ cells.

10.2 Isoforms *in vivo*

We managed to find three new isoforms of *c-Cbl*, all of them the results of alternative splicing and transcribed at the same time in pachytene spermatocytes (*Figure* 7.3). These isoforms are due to parts of them missing, corresponding what we named "deletions" $\Delta 1$ and $\Delta 2$. $\Delta 1$ spans sequences corresponding to exon10 of *c-Cbl,* presenting a simple case of alternative splicing where a whole exon is excluded cassette-like, while $\Delta 2$ starts within exon 11 and ends in the middle of exon 15, generated by splicing using alternative splice sites[3] [40]. The three novel isoforms were named p115$^{\text{c-Cbl}}$, p90$^{\text{c-Cbl}}$ and p85$^{\text{c-Cbl}}$, lacking sequences corresponding to $\Delta 1$, $\Delta 2$ or both

[3] our un-proven hypothesis, as we were yet unable to locate the sequences corresponding to these "alternative" splice sites

deletions. For brevity, the four isoforms will be referred to as p120 (wt), p115, p90 or p85, respectively.

The presence of p115 is almost ubiquitous at transcript level, as it can be easily detected using RT-PCR in many different tissues apart the testis or the thymus and it is present both in samples of rat or mouse origins (*Figure* 7.4/ *b, a*). Interestingly enough, it was not amplified from any of the human carcinoma or the human seminoma cell lines we examined (*Figure 7.4/c*), neither were we capable of amplifying it from the fish *Danio rerio* even though we had not problems detecting the p120 present in fish[4]. These results suggest that these alternatively spliced forms of *c-Cbl* are restricted to rodents although we cannot conclude this definitively without checking p120 and p115 expression in bird, reptile and amphibian species.

The amount of p115 mRNA present shows much greater variation. In mouse tissues - with cyclophilin as an internal reference - p115 is barely amplified anywhere else than in testis where it is present in amounts comparable to p120 (*Figure* 7.4/*a*). Although without internal control, results obtained with rat tissues gave similar results : with p120 amplified to saturation, only in enriched germ cell fractions or in testis do we have a considerable amplification of p115 mRNA (*Figure* 7.4/*b*).

The expression of p90 and p85 was not examined at such a depth neither at mRNA nor at protein level, the reasons behind this are the rarity of these smaller isoforms and the great difference in transcript length, making their co-amplification with p120 or p115 painstaking. As we were only able to amplify them from mRNA purified from enriched germ cell fractions (*Figure* 7.3/*a*) or from whole testis, we concentrated our efforts examining the *in vivo* presence of isoforms on p120 and p115[5].

When comparing the transcription of p120 and p115 in developing rat testis we can see that p120 mRNA is already well present at the age 20dpn and reaches its peak (plateau) between the ages of 30 and 60dpn (*Figure* 7.5/*a*)

[4]results not shown

[5]Results of unsuccessful experiments trying to amplify p90 and p85 apart of testis or germ cells are not presented

, corresponding well to already published results [39]. p115 transcription shows a similar profile with considerably lower levels at 20dpn, followed by rapid increase of its transcription between ages 20 and 30dpn (*Figure* 7.5/*b*), doubling the ratio of p120/p115 transcripts within this period (*Figure* 7.5/*c*). Although we were unable to do IHC studies of p115 separately, this delay in the appearance of p115 transcript suggests that it is practically missing from spermatogonia and all primary spermatocyte stages that appear before the age of 20dpn.

We could also observe a *c-Cbl* immun-reactive band of approximately 115kDa that is barely visible in 20dpn testis but becomes almost as pronounced as p120 in 30, 60 or 90 dpn testis (*Figure* 7.5/*d*). As this smaller band was detected with both polyclonal and monoclonal antisera raised against the C-terminal of *c-Cbl* and the appearance of this band corresponds well with the expression profile of p115 mRNA in the developing testis, we are convinced that this Western Blot is the first to show the in vivo expression of the 115kDa *c-Cbl* isoform. Its inferior size compared to p120 excludes the possibility that it is indeed p120 undergone post-translational modifications.

The smaller band could still be the result of proteolitic degradation but the band is well-defined and sharp while degradation produces a smear. As the epitope of the antibody used[6] is within the last 30 amino acids of the C-terminal, the degradation - usually - starting at the C-terminal would render the protein unrecognisable to the antibody before the observed 5-8 kDa size difference could appear.

It is important to point out that in all of the experiments presented in *Figure* 7.5, p120 and p115 are present in spermatocytes (marked SCY) and in spermatids (marked STD) both as transcripts and as proteins (*Figure* 6.2/*e*) while IHC studies did not show marking in spermatids (*Figure* 6.1). This can be due to inefficient cell separation during the elutriation process resulting in a considerable contamination of the round spermatid fraction by pachytene spermatocytes.

Although the rarity of the two smaller isoforms could suggest that they are

[6] αc-Cbl mouse monoclonal antibody, clone 7G10, Upstate

	Fetal Calf Serum		
MEF genotype	1%	5%	10%
$KO^{c\text{-}Cbl}$	72h	18h	17h
$WT^{c\text{-}Cbl}$	36h	24h	18h

Table 10.1: cell-cycle duration in $KO^{c\text{-}Cbl}$ and $WT^{c\text{-}Cbl}$ MEFs in function of FCS concentrations

splice artifacts, their recognition by C-terminal specific α c-Cbl antibody indicates that both deletions preserve the ORF of C-terminal sequences, suggesting the opposite.

10.3 Isoforms *in vitro*

The transfection of *c-Cbl* isoforms into $KO^{c\text{-}Cbl}$ MEFs did not reveal any differences, all of them were found in the cytoplasm, well-excluded from the nucleus (*Figure* 9.4). The filament-like and finely dotted appearance suggest that they show similar propertied as $wt^{c\text{-}Cbl}$, associating both with endosomes and with certain elements of the cytoskeleton[33]. These results are also the first clues that probably neither p90 nor p85 corresponds to CARP-90, the protein we initially started looking for - even though of the similar molecular weight they don't show nuclear localisation [30].

We decided to use transfection and a cell line devoid of endogenous *c-Cbl* to study the possible functional differences between the newly described isoforms. Isolating MEFs from $KO^{c\text{-}Cbl}$mice seemed the obvious choice for this, creating also immortalised versions for experiments of longer duration. Before starting the transfection of isoforms, we examined if there are differences between these cell lines regarding proliferation and apoptosis, two qualities likely to be affected by the absence of *c-Cbl* in $KO^{c\text{-}Cbl}$ cells.

In long term proliferation assays $KO^{c\text{-}Cbl}$ MEFs were found more sensitive to changes in growth-factor concentration added to their medium with FCS. Their response to increasing concentrations of FCS was all-or-nothing like, with a 72 hour long cell cycle in the presence of 1% FCS reduced to 18

hours and 17 hours in the presence of 5% and 10% FCS, respectively (*Table* 10.1). The acceleration of proliferation was more gradual in $WT^{c\text{-}Cbl}$ MEFs (*Table* 10.1), suggesting that in the absence of *c-Cbl* growth factor receptors reached maximal activation in the presence of lower ligand concentrations. These findings correspond well with the known inhibitory role of *c-Cbl* in RTK/TK signalling [159].

$KO^{c\text{-}Cbl}$ and $WT^{c\text{-}Cbl}$ MEFs reacted in a similar fashion to etoposide induced apoptosis but $KO^{c\text{-}Cbl}$ cells were more sensitive to oxidative stress as they exhibited higher percentage of apoptotic nuclei, determined by DAPI staining (*Figure 9.2/a*). This was accompanied by a strong increase of cleaved caspase-3 levels (*Figure* 9.3) which was not simply due to differences in procaspase 3 expression between untreated $KO^{c\text{-}Cbl}$ and $WT^{c\text{-}Cbl}$ MEFs (*Figure 9.2/b*).

Interestingly enough, xIAP and especially cIAP-2 levels were reduced in apoptotic $KO^{c\text{-}Cbl}$ MEFs (*Figure* 9.3). This indicates that *c-Cbl* is certainly not promoting the degradation of these IAPs but rather "protects" them from degradation. The mechanism of this "protection" is certainly indirect and is probably achieved through the negative regulation of the ceramide generation, leading to the liberation of *CytC* from mitochondria [97]. The indirect nature of *c-Cbl*s influence on cIAP2 expression is further supported by results showing just the opposite happening in the testis of flutamide treated $KO^{c\text{-}Cbl}$ mice, where apoptosis induction is reduced with elevated levels of cIAP2 [39].

GST pull-down experiments have confirmed our idea that isoforms will show differences in their ability to bind key interacting partners, as p85 was not pulled down by GST tagged Grb2 (*Figure 9.7*). The differences in the quantity of each isoform pulled down in function of serum starvation and EGF stimulation suggest that although the $KO^{c\text{-}Cbl}$ MEFs used for the pull-down do not display EGF induced tyrosine-phosphorylation changes, the EGF-EGFR signalling pathway is well present and working in these cells, probably in a state of elevated baseline activity due to the absence of *c-Cbl.*

Knowing the results of the Grb2-GST pull-down assay we noticed with sur-

prise that when transfecting isoforms into $KO^{c\text{-}Cbl}$ cells it is p90 expressing MEFs that proliferated the fastest. We suspect that in reality these results show the relative inability of p90 to down-regulate extracellular signals rather than promoting proliferation, but why is it p90 and not p85 producing these results is not clear yet.

10.4 Summary

We were first to examine, after 16 years of neglect, the expression of the *c-Cbl* proto-oncogene in the testis and found that *c-Cbl* expression is restricted to a small subset of pre-meiotic germ cells, susceptible to androgen regulated apoptosis and that the expression of *c-Cbl* is indeed regulated by testosterone.

We also discovered the existence of multiple new *c-Cbl* splice variants differing from wild-type *-c-Cbl* in their C-terminal sequences, created by alternative splicing of *c-Cbl* mRNA. All three isoforms are expressed alongside $p120^{c\text{-}Cbl}$ in pachytene spermatocytes with $p115^{c\text{-}Cbl}$ showing expression levels approaching that of $p120^{c\text{-}Cbl}$s , while in other tissues we detected only trace amounts of isoforms other than $p120^{c\text{-}Cbl}$. The difference in the prevalence of these isoforms can be explained by the fact that $p115^{c\text{-}Cbl}$is created by the exclusion of an entire exon (exon 10, excluded cassette-like) while the mRNA of $p90^{c\text{-}Cbl}$ and $p85^{c\text{-}Cbl}$demands splicing along alternative splice sites, situated inside coding sequences.

We had shown that the sequences corresponding to deletions $\Delta 1$ and $\Delta 2$ are important for the correct interaction of *c-Cbl* with the adaptor protein Grb2 as $p85^{c\text{-}Cbl}$ fails to bind to Grb2 in GST pull-down experiments. Interaction with other binding partners are likely to be compromised, too, as Crk, Abl, Src, PI3K (list not exhaustive) are all predicted to have docking sites within the same sequences.

By exposing $WT^{c\text{-}Cbl}$, $KO^{c\text{-}Cbl}$ and $KO^{c\text{-}Cbl}$ MEFs expressing *c-Cbl* isoforms to apoptosis-inducing agents etoposide and H_2O_2 we had shown that *c-Cbl* is involved in the regulation apoptosis, affecting the mitochondrial or intrinsic

pathway. The absence of *c-Cbl* increased oxidative-stress induced caspase-3 activation, while in presence of different isoforms caspase-3 activation varied with etoposide treatment. Our conclusion is that *c-Cbl* must be capable of regulating apoptosis at points upstream of the first common step between etoposide and H_2O_2 induced apoptosis, the release of cytochrome c from mitochondria; and it can possibly interfere both with ceramide production (oxidative stress [97]) or events leading to the activation of Bax (etoposide [76]).

$p115^{c\text{-}Cbl}$ expressing MEFs were especially susceptible to etoposide induced caspase-3 activation. It is also the *c-Cbl* isoform the most abundantly expressed in pachytene spermatocytes, subject to apoptosis induction either by androgen withdrawal or by the failure of these cells to complete meiotic recombination[139]. Theses results suggest that expression of $p115^{c\text{-}Cbl}$might play a role in determining spermatocyte fate at this key regulatory stage of spermatogenesis.

10.5 Perspectives

The study of *c-Cbl* - judging simply by the number of related publications in prestigious journals - has always been rewarding. By studying its expression in testis we made an important contribution to what is known about *c-Cbl* but - when describing three new isoforms and their possible role in the regulation of cell proliferation and apoptosis - we have also opened up multiple new directions for further research, with the number of questions arising outnumbering the number of questions we've just answered.

It would be worthwhile examining what growth factor(s) or cytokine(s) exactly cause the differences in the proliferation of $KO^{c\text{-}Cbl}$ and $WT^{c\text{-}Cbl}$ MEFs and if the expression of the different isoforms could restore the regulation of proliferation in $KO^{c\text{-}Cbl}$ to what is observed in $WT^{c\text{-}Cbl}$ MEFs. The expression of candidate growth factor/cytokine receptors should also be checked in the testis, notably in pachytene spermatocytes, to see if these interactions might be relevant *in vivo*.

Examining the capacity of Crk, Abl, Src, p85 subunit of PI3K and PLCγ to bind the different isoforms in GST-pull down or co-immunoprecipitation experiments could provide more answers to the question posed in the previous paragraph and further elucidate the functional role of the Δ1 and Δ2 deletions.

Exploring the levels of ceramide and Bcl-2 in $KO^{c\text{-}Cbl}$/ $WT^{c\text{-}Cbl}$ MEFs could give an answer why $KO^{c\text{-}Cbl}$ MEFs are so sensitive to oxidative stress and could give further proof that Bcl-2 expression can be affected by *c-Cbl* [152].

Cells expressing either isoform had shown a reduced sensibility to H_2O_2 provoked apoptosis, but stating that they restore wild-type phenotype in this regard would be irresponsible. A decisive experiment should be done with $KO^{c\text{-}Cbl}$ MEFs transfected with a control plasmid and treated with etoposide and H_2O_2. The experiment seen in *Figure* 9.6 should be also done with DAPI staining of cells in paralell to asses if the elevated caspase-3 levels result in apoptosis or it is blocked by IAP family proteins.

Examining how the EGFR - Grb2 - Ras - MAPK signalling pathway is modulated in the presence of the different isoforms and if there are differences in EGFR down-modulation and in its endosomal trafficking using fluorescence-tagged isoforms could show if results obtained with the Grb-GST pull-down experiment are relevant *in vivo* and if the presence of competing isoforms lacking one or two Grb2 docking sites indeed reduces the efficacy of EGFR down-regulation by $p120^{c\text{-}Cbl}$. These results should be applicable to numerous other situations where Grb2 plays the role of adaptor protein between *c-Cbl* and a RTK.

Transgenic mice expressing only $p115^{c\text{-}Cbl}$ could be an extremely useful model to study the relation between apoptosis and this isoform. We would expect $p115^{c\text{-}Cbl}$/ $p115^{c\text{-}Cbl}$mice to either have high occurrence of apoptosis in pachytene spermatocytes already in control animals (and/)or to have an increased susceptibility to apoptosis induced upon androgen deprivation (flutamide treatment).

Finally, we have collected enough evidence of *c-Cbl* being involved in apoptosis to grant further research into this subject. It would be rewarding to

find out how exactly p115$^{\text{c-Cbl}}$ interferes with DNA-damage induced apoptosis and why KO, but not cells expressing any of the *c-Cbl* isoforms, show increased sensitivity to oxidative stress. The possible involvement of a short, conserved PR or the UBA domain is highly likely as these are the only C-terminal domains preserved between all isoforms (*Figure* 10.5).

Part V

Annexes

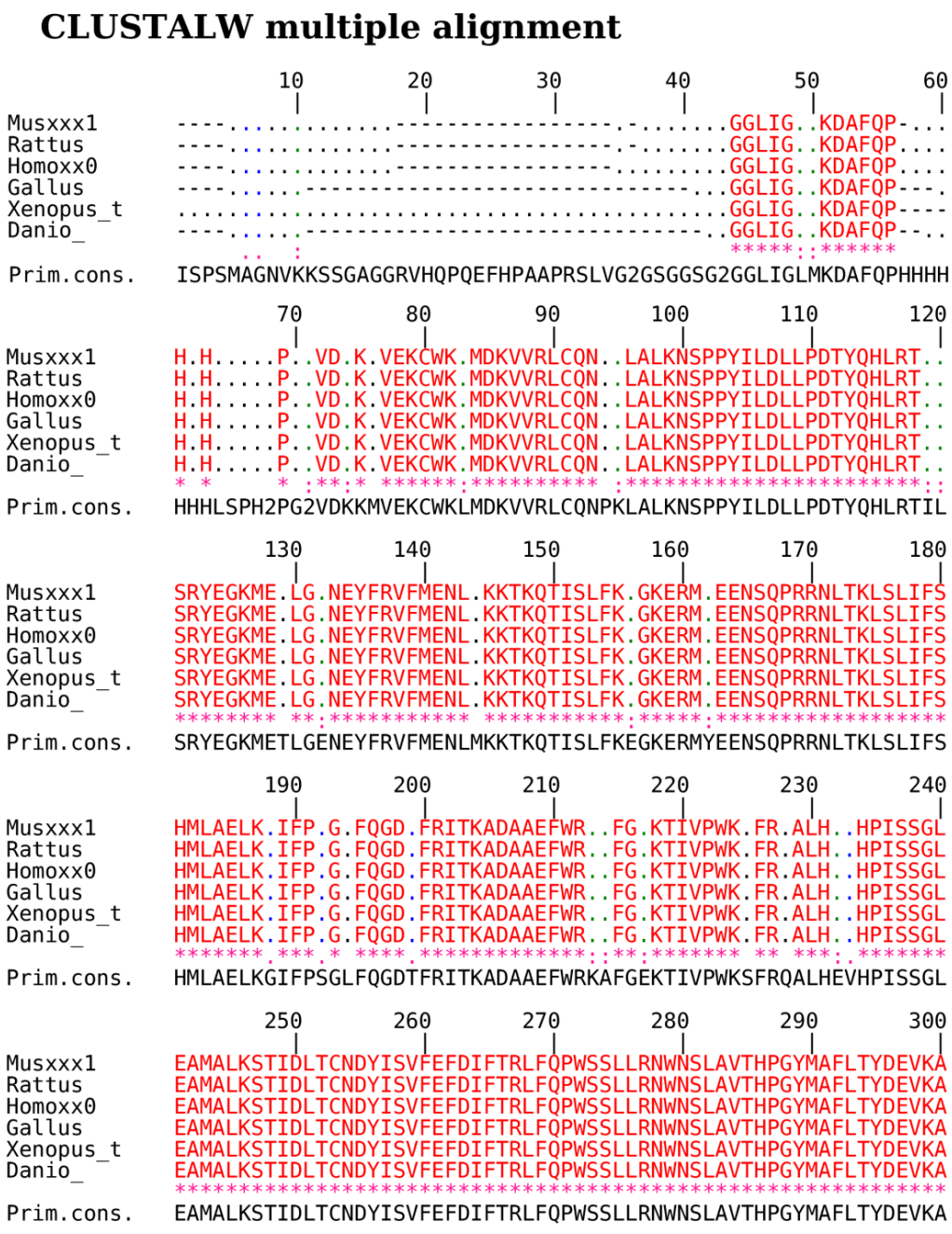


Figure 10.2: N-terminal protein sequences aligned - page 1.

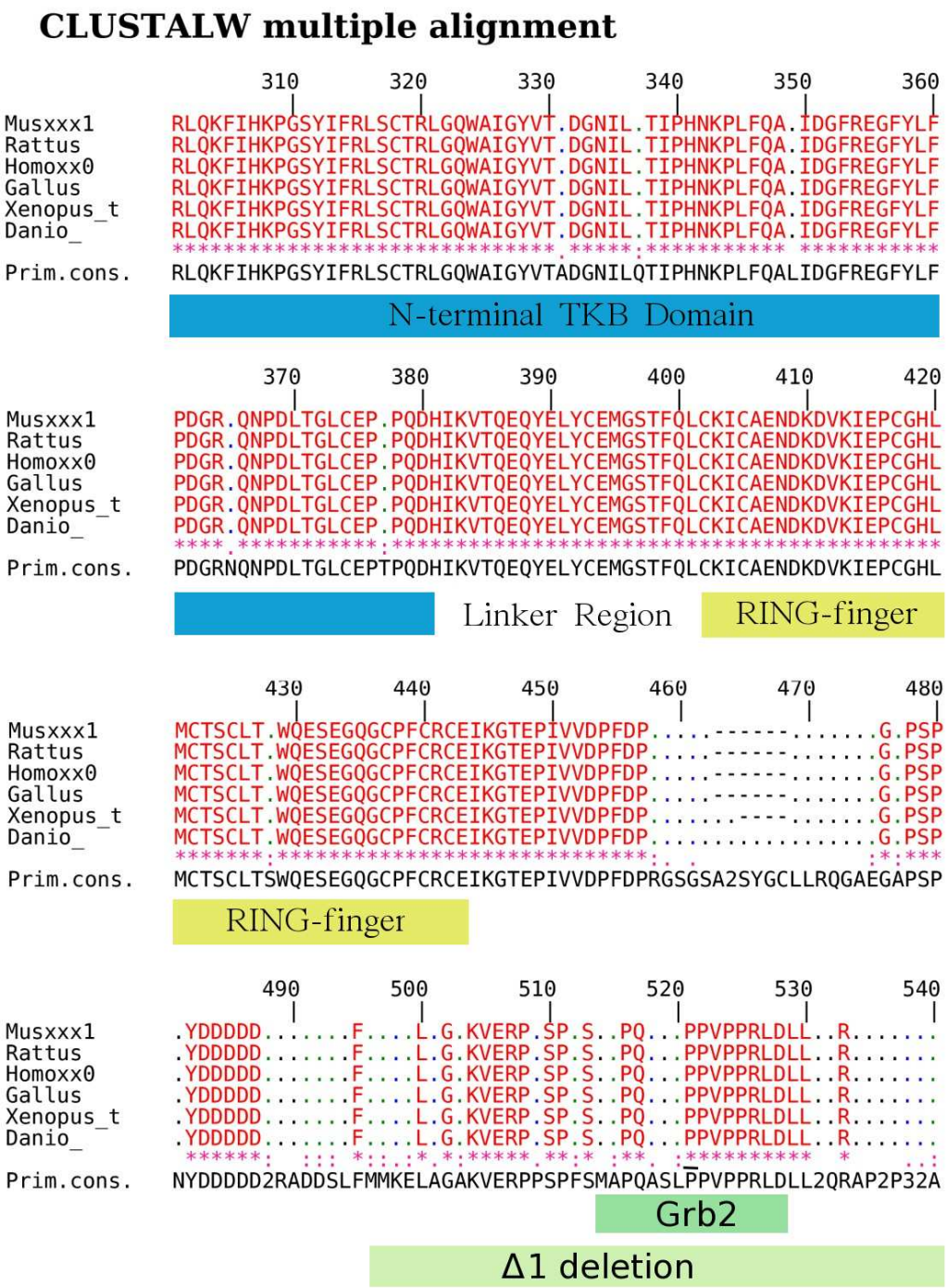


Figure 10.3: proximal C-terminal sequences - page 2.

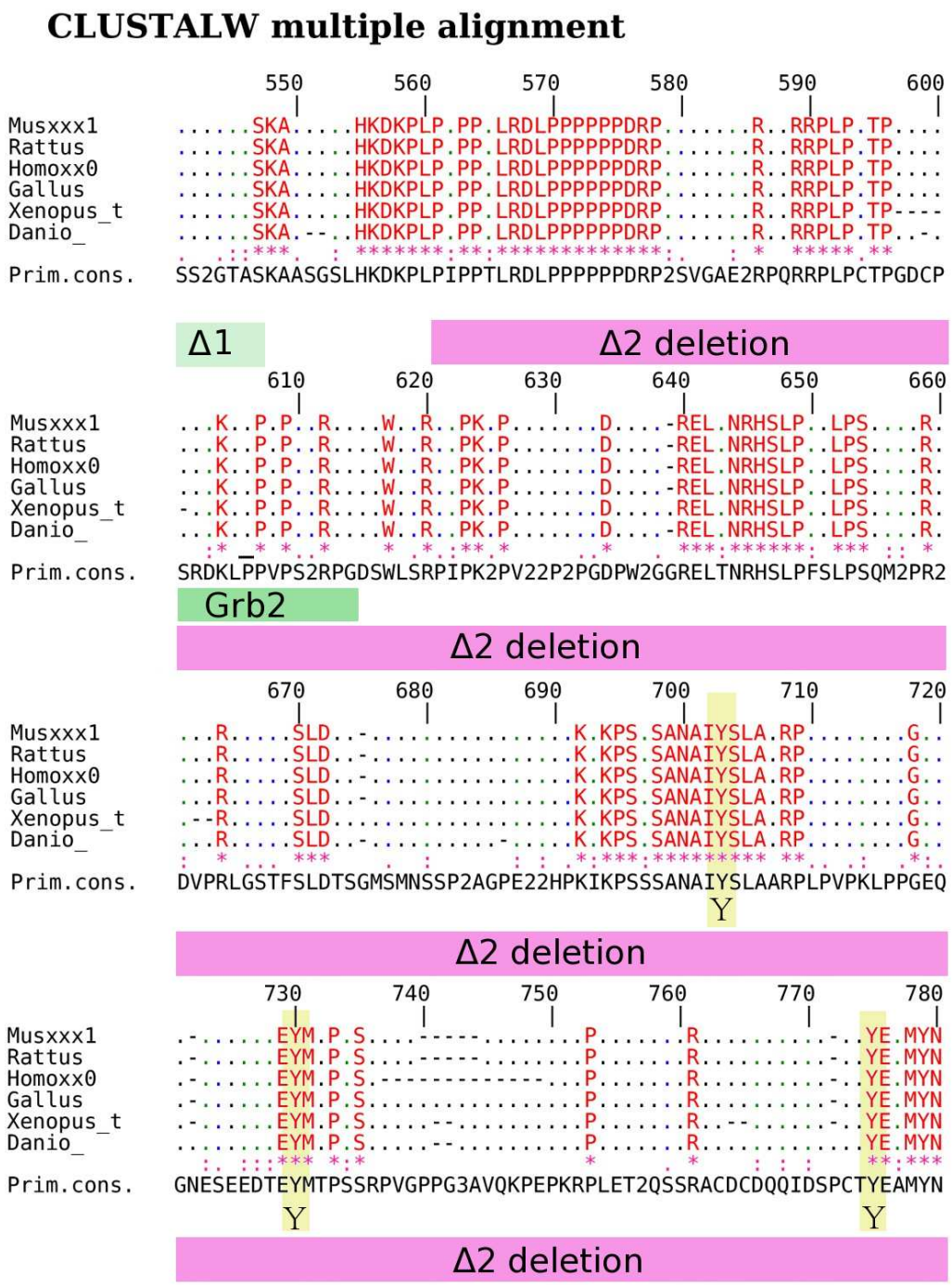


Figure 10.4: PR-rich and Tyrosine containing sequences - page 3.

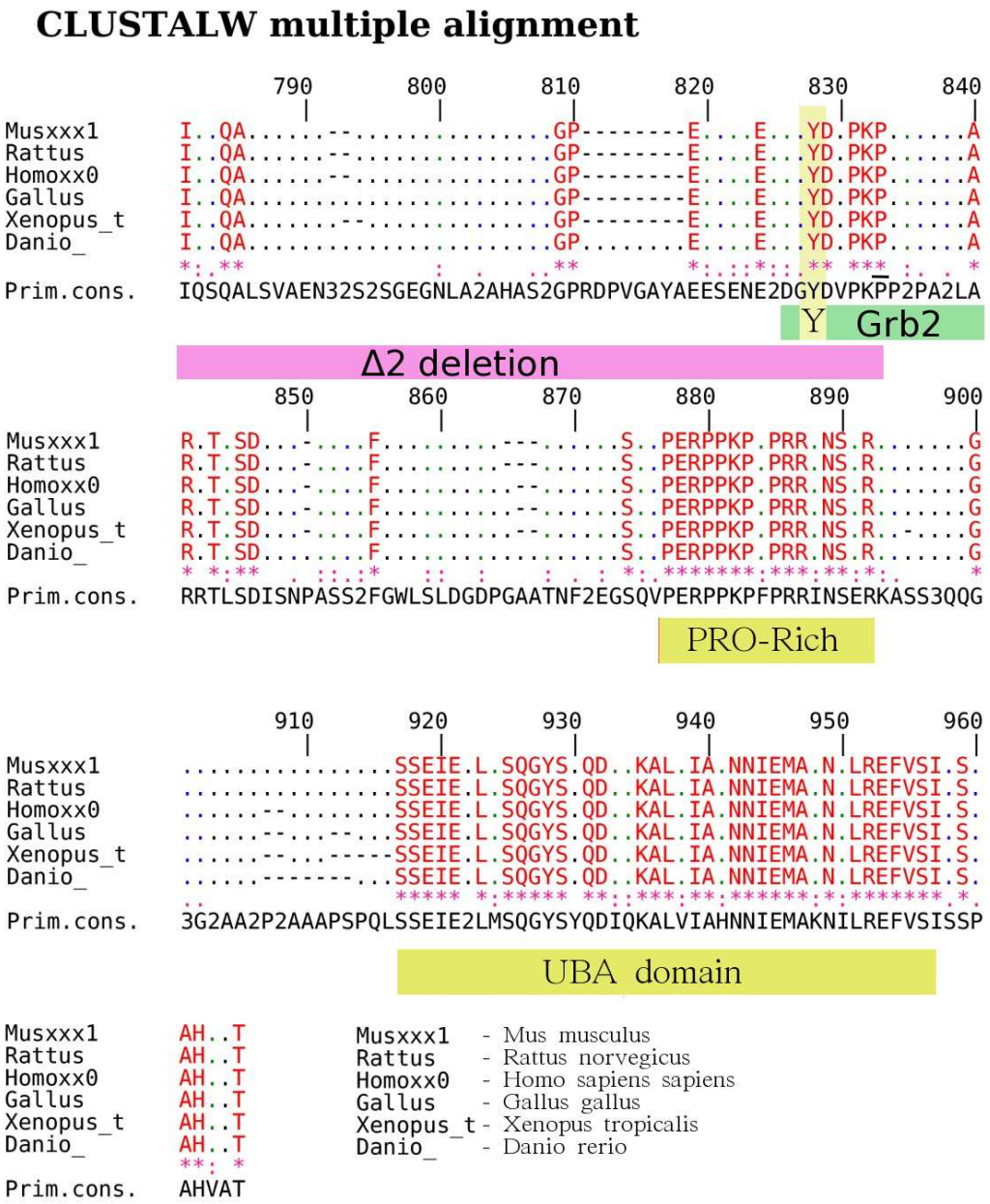


Figure 10.5: distal C-terminal sequences aligned - page 4.

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RESUME : Le proto-oncogène c-Cbl code pour une protéine cytoplasmique abondamment exprimé dans les tissus hématopoïétiques et testiculaires. Elle possède un rôle de protéine multi-adaptatrice et/ou de régulateur négatif des RTKs. Nous rapportons ici que l'expression majoritaire de c-Cbl dans le testicule de rat et de souris est limité aux cellules germinales et son expression augmente en présence de testostérone et diminue après traitement *in vivo* par l'anti-androgène flutamide. L'apoptose dépendante des androgènes survenant à la fin de la méïose 1 est notablement moins importante dans le testicule de souris KO pour c-Cbl (c-Cbl^{KO}). Inversement, le nombre de Fibroblastes Embryonnaires de souris c-Cbl^{KO} entrant en apoptose sous l'effet du stress oxydatif est bien plus élevé que celui des cellules provenant d'animaux sauvages. Ces résultats indiquent le rôle sur la survenue du processus apoptotique de c-Cbl. Nous avons aussi cloné trois autres isoformes de p120$^{c\text{-}Cbl}$ à partir d'extraits de spermatocytes pachytènes, appelées p115$^{c\text{-}Cbl}$, p90$^{c\text{-}Cbl}$ et p85$^{c\text{-}Cbl}$, en considération de leur taille déduite. Ces isoformes présentent, par rapport à la p120$^{c\text{-}Cbl}$, soit une délétion (Δ1 pour la p115$^{c\text{-}Cbl}$; Δ2 pour la p90$^{c\text{-}Cbl}$) soit les deux délétions combinées (Δ1 + Δ2, p85$^{c\text{-}Cbl}$). L'expression de la p120$^{c\text{-}Cbl}$ et de la p115$^{c\text{-}Cbl}$ a été détectée dans le testicule, le poumon, la rate, le thymus et le cerveau alors que celle de la p90$^{c\text{-}Cbl}$ et p85$^{c\text{-}Cbl}$ semble être restreinte aux cellules germinales. La délétion Δ1 correspond à l'exon 10 (acides aminés 459-502) et la Δ2 s'étend des exons 11 à 15 (acides aminés 514-768), provenant d'épissages alternatifs. Les deux délétions contiennent des régions riches en prolines (PR) possédant pour chacun d'eux un site de liaison à la protéine adaptatrice Grb2. Seule la p85 $^{c\text{-}Cbl}$ ne peut pas se lier à Grb2, démontrant que chacun de ces sites de liaison sont suffisants pour que la liaison Grb2-c-Cbl puisse avoir lieu. D'autre part, les cellules transfectées par la p90 c-Cbl prolifèrent plus rapidement que celles transfectées avec les autres isoformes de c-Cbl. L'ensemble de ces résultats montrent que c-Cbl possède un rôle finement régulé dans la spermatogenèse, comme l'atteste l'hypofertilité des animaux c-Cbl KO, et que cette régulation pourrait en parti provenir d'une balance entre les différentes isoformes de c-Cbl.

Mots-Clés: c-Cbl, apoptose, spermatocyte, flutamide, épissage alternatif, Grb2, domaine SH3

Identification and characterisation of new isoforms of the proto-oncogene c-Cbl and its new roles in apoptosis and cell proliferation

ABSTRACT: The proto-oncogene *c-cbl* is cytoplasmic protein expressed in heamatopoetic tissues and testis, implicated in cellular signalling either as a scaffolding protein and/or as a negative modulator of cellular signalling. Although it has been studied extensively, not much attention was granted to examine it's role in the testis, one of the principal organs of it's expression. We report here that c-Cbl expression in rat and mouse testis is limited to developing germ cells with strongest expression in pachytene spermatocytes and round spermatids and its expression is upregulated by testosterone that can be inhibited by the anti-androgen flutamide. Androgen-regulated apoptosis occurring at the end of meiosis I is markedly lower in the testis of c-Cbl ^{KO} mice, while KO$^{c\text{-}Cbl}$ Mouse Embryo Fibroblasts (MEFs) show an increased sensitivity to apoptosis provoked by oxidative stress. We also cloned three other isoforms of p120$^{c\text{-}Cbl}$ from pachytene spermatocytes, named p115$^{c\text{-}Cbl}$, p90$^{c\text{-}Cbl}$ and p85$^{c\text{-}Cbl}$ after their expected size, harbouring either one (Δ1 – p115; Δ2 - p90) or two (Δ1 & Δ2 - p85) deletions. The expression of p120$^{c\text{-}Cbl}$ and p115$^{c\text{-}Cbl}$ can be detected in testis, lung, spleen, thymus and brain while p90$^{c\text{-}Cbl}$ and p85$^{c\text{-}Cbl}$ expression seems to be restricted to germ cells. The deletion Δ1 corresponds to exon 10 (amino acids 459-502), deletion Δ2 originates from alternative splicing, stretching over exons 11 to 15 (amino acids 514-768). Both deletions contain PR regions with a Grb2 binding site but only p85$^{c\text{-}Cbl}$ failed to bind to Grb2, showing that the presence of any of these two sites is sufficient for Grb2 – c-Cbl binding. p90$^{c\text{-}Cbl}$ transfected cells had shown increased proliferation compared to the other isoforms. The above results suggest that c-Cbl has a finely regulated role in the regulation of spermatogenesis and some of this regulation is realised through a balance between alternative isoforms.

Keywords: c-Cbl, apoptosis, spermatocyte, flutamide, alternative splicing, Grb2, SH3 domain

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