

# Androgen-dependent apoptosis in male germ cells is regulated through the proto-oncoprotein Cbl

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The proto-oncoprotein Cbl is known to control several signaling processes. It is highly expressed in the testis, and because spermatogenesis is androgen dependent, we investigated the androgen dependency expression of Cbl through its testicular sublocalization and its expression levels in rats that were exposed to the antiandrogen flutamide or were hypophysectomized. We report the androgen dependency of Cbl as it localizes in pachytene spermatocytes during androgen-dependent stages, is down-regulated upon flutamide exposure, and is up-regulated with testosterone in hypophysectomized rats. Coculture experiments showed the key control ex-

erted by the Sertoli cell on Cbl activity. As flutamide induces germ cell apoptosis, we investigate members of the Bcl-2 family upon flutamide exposure. We show that the proapoptotic Bcl-2 family member Bim mirrored Cbl expression through a posttranscriptional process. We also show that in Cbl knockout mouse testes, the imbalance between the high expression of Bim and Smac/Diablo and antiapoptotic factors such as cellular inhibitor of apoptosis 2 favors a survival process, which makes these mice unresponsive to androgen withdrawal and could explain their hypofertility.

## Introduction

Investigations concerning the proto-oncogene *c-cbl* have significantly increased over the past few years. Cbl performs a well-described E3 ligase function, and its role as a multidomain adaptor protein has expanded, which allows the cytoplasmic p120<sup>cbl</sup> (Cbl) to interfere with numerous metabolic pathways (Schmidt and Dikic, 2005; Thien and Langdon, 2005).

Recently, its involvement has been reported in the regulation of apoptosis through the suppression of this process in cell lines overexpressing Cbl oncogenic forms (Hamilton et al., 2001; Sinha et al., 2001) or through the scaffolding of the antiapoptotic 14-3-3 proteins (Melander et al., 2004). It is also associated with thymic apoptosis (Denis et al., 1999; Corsois et al., 2002). It is responsible for the constitutive degradation through ubiquitylation of the proapoptotic Bim factor in primary cultures of osteoclasts (Akiyama et al., 2003). Much of our knowledge about *c-cbl* is based on in vitro studies on cell

lines, and despite its high, specific physiological expression in the thymus and testis (Langdon et al., 1989), the role of Cbl, particularly in the testis, has not been well explored yet. Knowing that apoptosis plays a key role in germ cell development (Rodriguez et al., 1997), we have focused our efforts on correlating in vivo Cbl expression with the onset of apoptosis in this tissue. As far as we know, Cbl has never been analyzed in the testis upon apoptotic conditions, which prompted us to investigate its expression in vivo after the induction of testicular germ cell (TGC) apoptosis according to the importance of this process in the development of spermatogenesis.

Androgen receptor (AR) inhibition models (Brinkworth et al., 1995; Kim et al., 2001) by means of the antiandrogen compound flutamide lead to TGC apoptosis. This nonsteroidal synthetic chemical and its active metabolite hydroxyflutamide inhibits the action of androgen at the receptor level by competing with the physiological ligand (Sharpe et al., 1998; Omezine et al., 2003). In utero exposure to this antiandrogen leads to different types of testis alterations, from histological effects to only a faint decrease in the number of TGCs that correlates to the flutamide impregnation (Kassim et al., 1997; McIntyre et al., 2001). We already showed that in utero exposure to flutamide led to a long-term apoptotic cell death process in TGCs, whereas adulthood exposure led to a transient apoptosis

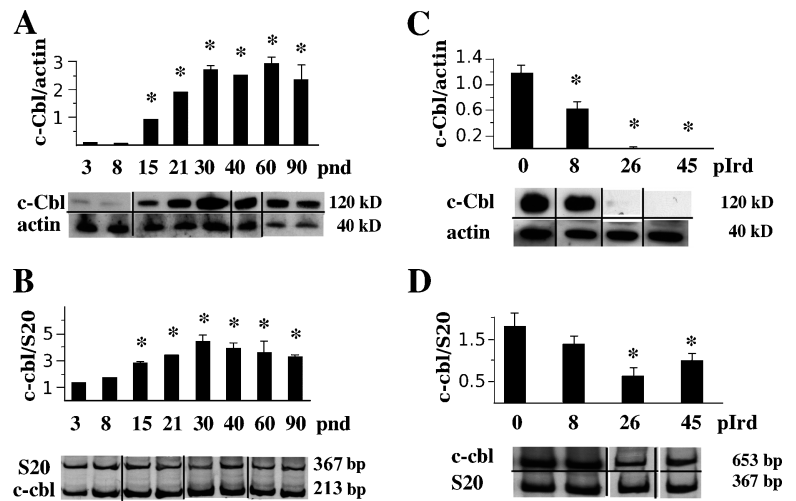
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Abbreviations used in this paper: AR, androgen receptor; HC, hydrocortancyl; HX, hypophysectomy; IAP, inhibitor of apoptosis; IHC, immunohistochemistry; IP, immunoprecipitation; KO, knockout; PMC, peritubular myoid cell; pnd, postnatal day; PS, pachytene spermatocyte; SC, Sertoli cell; TGC, testicular germ cell; WB, Western blotting; WT, wild type.

Figure 1. **Spermatocytes expressed Cbl.** (A–D) WB and co-amplification RT-PCR were performed on 3–90 postnatal day (pnd) rat testes (A and B) and on 9 Gy-irradiated testes of 90 pnd rats 8, 26, and 45 d after irradiation (pIrd) or on untreated rats (0; C and D). WB data are given as the Cbl/actin ratio, and RT-PCR is given as the Cbl/S20 ratio. (A and B) Significant data refer to day 3 for the ontogeny study: (A) \*,  $P < 0.0001$ ; (B) \*,  $P < 0.003$ . (C and D) Significant data refer to nonirradiated animals for irradiation experiments: (C) \*,  $P < 0.0001$  for postirradiation days 26 and 45; (D) \*,  $P < 0.005$ . Values represent the means  $\pm$  SD determined from five different animals for ontogeny experiments and 12 for irradiation experiments.



(Omezzine et al., 2003). We also reported that this TGC alteration upon fetal androgen disruption is related to the mitochondria-dependent pathway (Bozec et al., 2004), showing androgen-dependent alterations of Bcl-2 family member expression. It has also been recently reported that a selective AR knockout (KO) of the nursing Sertoli cells (SCs) leads to spermatid and, in part, spermatocyte apoptosis (Chang et al., 2004). In addition, it alters the expression of several androgen-dependent testicular proteins, which could directly or indirectly be involved in the regulation of spermatogenesis, particularly in the apoptotic process (Tan et al., 2005).

We first report that Cbl activity in the testis is androgen dependent. Indeed, its expression was localized in pachytene spermatocytes (PSs) inside the androgen-dependent seminiferous tubules, decreasing upon flutamide exposure or hypophysectomized rats and reexpressing after testosterone treatment. Coculture experiments demonstrated that AR-expressing SCs control Cbl activity. The relationship between Cbl and apoptosis was then proven by the study of flutamide-treated or untreated Cbl KO mice compared with their wild-type (WT) counterparts. The imbalance we observed (Liston et al., 2003) between proapoptotic (Bim EL and Smac/Diablo) and survival factors (cellular inhibitor of apoptosis [c-IAP] 2) might explain the noticeable reduction of the number of apoptotic cells in the Cbl KO testis and the deeply impaired androgen dependency of testicular apoptosis. Those aspects could also lead the Cbl KO mouse hypofertility that is analyzed in this study.

## Results

### Cbl is predominantly localized in PSs at an androgen-dependent stage

The high Cbl expression in the testis (Langdon et al., 1989) prompted us to investigate this aspect. Cbl protein levels drastically increased from the 15th to the 21st postnatal day (pnd), which corresponds to the growth of spermatocytes around pnd 18 (Fig. 1 A). Its protein expression level compared with its expression at pnd 15 doubled in a few days and reached a plateau at pnd 30. The Cbl transcripts followed a similar pattern (Fig. 1 B), although their expression was already detectable at pnd 3

and 8, unlike Cbl protein. These data could be attributed to noncoding Cbl mRNA at pnd 3 and 8, likely in precursor TGCs and/or possibly in somatic cells as SCs.

To assess the testicular cell population supporting Cbl transcript and protein expression, we used rats that were locally 9 Gy irradiated on testes. It has been demonstrated that only spermatogonia in the testes are radiosensitive (Kangasniemi et al., 1990). According to the spermatogenesis cycle, a delayed and progressive disappearance of the downstream daughter cells occurs after  $\gamma$  irradiation (Russell et al., 1990). Then, 26 d after irradiation, spermatocytes were totally absent, and 45 d after irradiation, both spermatocyte and spermatid populations were discarded (Table I, first three rows). Cbl was not detected at these two last periods (Fig. 1 C), suggesting that the disappearing cells for the two periods, including spermatocytes in either case, were mainly supporting Cbl expression (Table I, last two rows). The Cbl RNA expression at 26 and 45 d after irradiation was low but still present, suggesting the presence of noncoding Cbl transcripts in cells other than spermatocytes (Fig. 1 D). 8 d after irradiation, only the radiosensitive spermatogonia were removed, whereas spermatocytes and spermatids were still present. At this time, we observed that Cbl expression was still high but was decreasing at half of the level of the intact testes (Fig. 1 C). This decrease could be attributed to the disappearance of radiosensitive spermatogonia or already some spermatocytes and/or to a lower Cbl expression under irradiation (Corsois et al., 2002).

Immunohistochemistry (IHC) experiments performed on rat testis sections revealed that PSs, which are long-lasting cells situated at the meiotic prophase, are highly stained within the cytoplasm in a finely and uniformly dispersed manner (Fig. 2 A). The seminiferous tubules were not all stained, giving a heterogeneous Cbl staining likely including the androgen-dependent stages VII, VIII, and IX of the cycle of the seminiferous epithelium (Fig. 2 B; Russell et al., 1990). A few spermatogonia (Fig. 2 A, inset), which localize to androgen-insensitive stages, were also stained to a lesser extent.

Altogether, these data strengthen the PS population as supporting the main Cbl expression in the testis. As Cbl expression was mainly observed in androgen-dependent stages of the

**Table 1. Cbl expression is reduced or absent when spermatocytes are removed from germ cells after testis irradiation**

9 Gy $\gamma$ irradiated germ cells (or TGCs)	8 plrd	26 plrd	45 plrd
Spermatogonia	–	–	+
Spermatocytes	+	–	–
Spermatids	+	+	–
Cbl transcripts	78%	35%	53%
Cbl protein (p120 <sup>cbl</sup> )	50%	NF	NF

The two last rows represent the percentage of Cbl expression in terms of p120<sup>cbl</sup> protein (fourth row) or Cbl mRNA (fifth row) at different days after local testis 9 Gy irradiation. These calculations are based on the experiments reported in Fig. 1 (C and D). The three first rows report the absence or presence of the different germ cell populations after irradiation (Kangasniemi et al., 1990). plrd, days after irradiation; NF, not found.

seminiferous epithelium, the question arose of the regulation of Cbl expression levels upon inhibition of testosterone activity. Moreover, because TGC apoptosis occurs upon hormonal changes (Omezzine et al., 2003), the relationship between Cbl expression and this process could also be questioned.

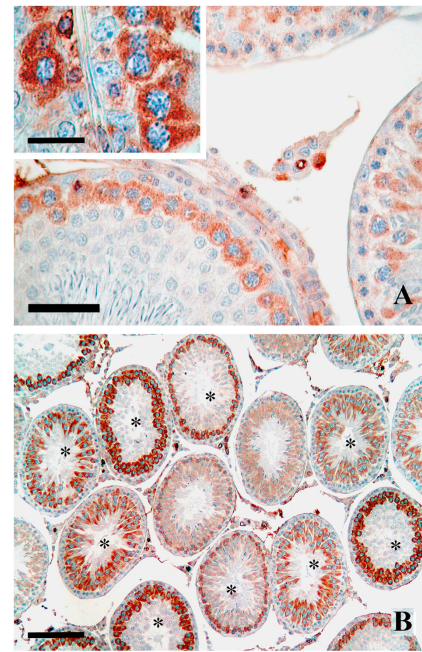
#### **Cbl but not Cbl-b expression is androgen dependent in the testis**

Androgen action in the testis was altered according to two different protocols: (1) through treatments of adult rats that consist of either exposure to the antiandrogen flutamide competing with testosterone at the AR level or hypophysectomy (HX) leading to luteinizing hormone and testosterone suppression and (2) through in utero flutamide exposure.

To first appreciate the optimal time of flutamide exposure in adult rats that is needed to interfere with the level of testicular Cbl expression, various lengths of treatment were assessed. 3 d of treatment were needed for the protein and the transcripts as well to significantly reach about half of the control (Fig. 3, A and B). Thus, a 3-d treatment is needed to assure the androgen dependency of Cbl.

Because the Cbl family member Cbl-b is structurally very similar to Cbl and it has been demonstrated that the Cbl-b effect on ubiquitination is Cbl-like (Ettenberg et al., 1999), we investigated the androgen dependency of Cbl-b in the testis. The level of Cbl-b expression, which is quite low in the testis compared with Cbl, did not change after 3 d of flutamide exposure (Fig. 3 C), showing the unresponsiveness of Cbl-b expression to androgen deficiency, unlike Cbl in the same conditions.

Cbl fulfills a variety of functions that are essentially sustained through extensive phosphorylation and cytoplasmic translocations (Dikic et al., 2003). We assessed the effect of flutamide exposure on Cbl tyrosine phosphorylation in the testes of an adult pnd 90 rat treated for 3 d with flutamide. Immunoprecipitation (IP) experiments with the anti-Cbl monoclonal antibody showed a decrease of Cbl expression around one third of the control (Fig. 3 D, right), confirming the aforementioned data. The tyrosine phosphorylation of Cbl was low after flutamide exposure around half of the control (Fig. 3 D, left). Thus, the alteration of androgen activity had an effect on Cbl phosphorylation. However, the real degree of Cbl phosphorylation



**Figure 2. PSs expressed Cbl in an androgen-dependent manner.** (A) IHC experiments were performed on testis sections of 90 pnd untreated rats. (Inset) Staining of spermatogonia is observed. (B) Strong Cbl staining (\*) in androgen-dependent stages of the seminiferous epithelium is observed. Bars (A), 50  $\mu$ m; (A, inset) 20  $\mu$ m; (B) 200  $\mu$ m.

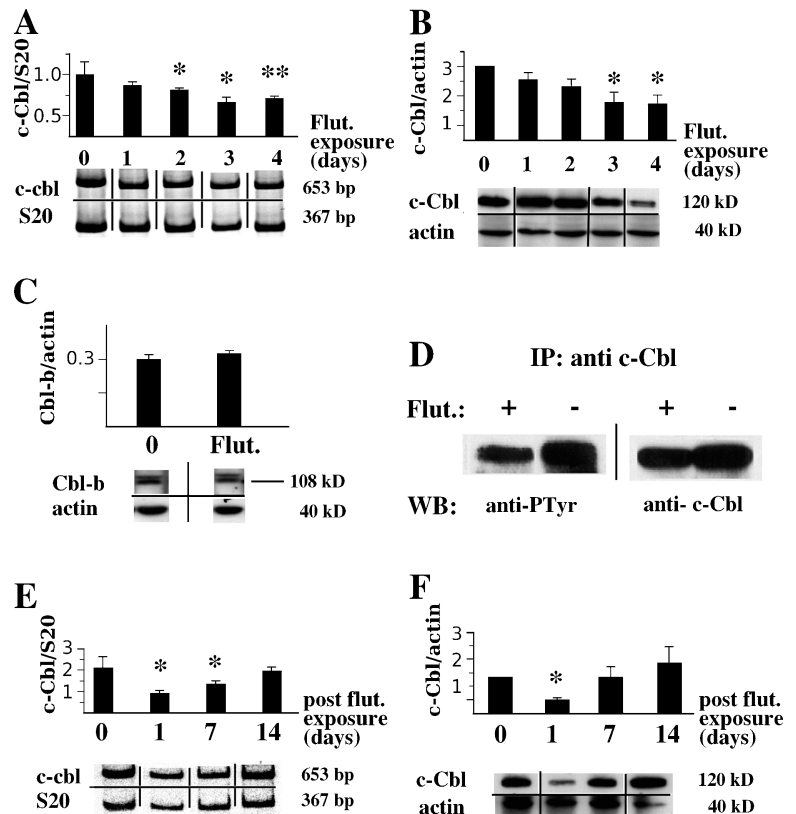
decrease after flutamide exposure has to be indirectly evaluated as the level of both signals decrease, and we chose to directly analyze Cbl expression level for further investigation.

The length of decay of testicular Cbl expression was then assessed. The lower expression of Cbl mRNA was significantly reached the day after the arrest of flutamide administration up to around half that of the control and was increased after this point (Fig. 3 E, 1 d after flutamide exposure arrest). A similar pattern could be seen for the Cbl protein level, which was still low a week after cessation of the flutamide exposure and reached the control level between 7 and 14 d postflutamide treatment arrest (Fig. 3 F). These results confirmed the androgen dependency of Cbl expression in the testis and pointed out its transient down-regulation, which lasted  $\sim$ 10 d after 3 d of flutamide adult rat exposure.

The reverse experiment consisted of measuring the Cbl testis expression of surgical hypophysectomized rats that were complemented or left uncomplemented for 4 d after surgery with testosterone (El Shennawy et al., 1998). Cbl mRNA expression was significantly low 3 d after HX and increased to control levels after testosterone treatment (Fig. 4 A). Cbl protein dropped to an undetectable expression and reached the control level after androgen administration (Fig. 4 B).

We know the essential nursing role of SCs for spermatogenesis (TGCs cannot be cultured without SCs) and that SCs express ARs, whereas TGCs do not, so spermatocyte-enriched populations from control 90 pnd rats were cocultured with SCs obtained from 15 pnd untreated rats (no contaminating TGCs). Testosterone analogue R1881 was added to the coculture. The SC controls were Cbl unresponsive to this in vitro treatment,

**Figure 3. Testicular Cbl expression is transiently down-regulated after flutamide exposure of adult rats.** (A and B) Coamplification RT-PCR (A) and WB (B) performed the day after treatment arrest on testis extracts of 90 pnd rats subjected or not subjected to 10 mg/kg/d flutamide exposure are shown for 1–4-d treatment. Significant data refer to the untreated animal group (0): (A) \*,  $P < 0.001$ ; \*\*,  $P < 0.004$ ; (B) \*,  $P < 0.005$  for each 3 and 4 flutamide exposure days. (C) Testicular Cbl-b protein expression in rats with or without 3-d flutamide exposure (means  $\pm$  SD from four animals). (D) Anti-Cbl IP was followed by Cbl immunoblotting (right). Another blot was revealed by the antiphosphotyrosine antibody (left). (E and F) Testicular Cbl expression in terms of mRNA (E) or protein (F) is shown 1–14 d after the arrest of flutamide exposure (postflut expo) of 90 pnd rats. Significant data refer to the untreated group (0): (E) \*,  $P < 0.01$ ; (F) \*,  $P < 0.0005$ . The values shown for A, B, E, and F represent the means  $\pm$  SD determined from six different animals.



but R1881 did induce a significant increase of Cbl transcript expression when spermatocyte cocultures were performed (Fig. 4, C and D). These results are in accordance with the aforementioned HX experiment data and show that spermatocyte Cbl transcript expression is up-regulated upon testosterone exposure of SCs.

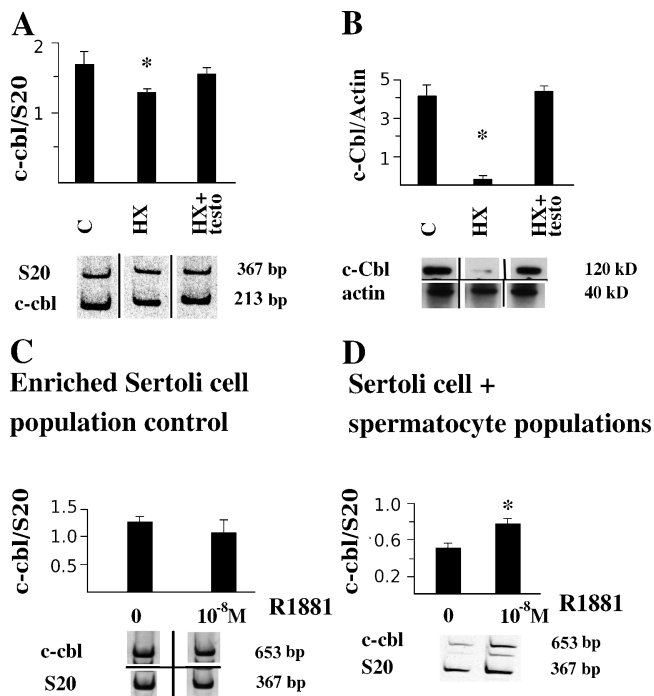
On the other hand, spermatocytes cocultured with primary SCs that were extracted from in utero flutamide-exposed animals showed a slight but still significant decrease of the Cbl transcript expression compared with coculture performed with untreated SCs (Fig. 5 B). These data show that expressing AR SCs that had been flutamide exposed long before experiments (in utero) are able to lead a Cbl transcript down-regulation in spermatocytes and a delayed effect over Cbl expression at the adult age. Surprisingly, the primary SC population cultured alone as a control showed a Cbl mRNA expression that was significantly up-regulated in flutamide-exposed cells compared with untreated ones (Fig. 5 A). This could be ascribed to the SCs themselves and/or to the contaminant cells (peritubular myoid cells [PMCs] and spermatogonia). Nevertheless, this down-regulation of Cbl mRNA in coculture conditions prompted us to confirm these data in vivo through in utero flutamide-exposed rat testes. We observed a significant decrease of about three times the mRNA level at the dose 0.4 mg/kg/d, reaching its lower expression at 2 mg/kg/d (Fig. 5 C). The Cbl protein also decreased significantly by about three times at the dose of 10 mg/kg/d, with a slight decrease at 2 mg/kg/d (Fig. 5 D).

Altogether, these data sustain an androgen dependency of Cbl expression either after the inhibition of testosterone activity during a short time in adult rats (transient Cbl down-regulation) or after its in utero inhibition (long-term Cbl down-regulation).

As the mechanisms leading to transient or long-term germ cell apoptosis, respectively, are different (Omezzine et al., 2003) and Cbl activity matches the processes, these results tightly associate Cbl with TGC apoptosis.

#### Cbl expression is androgen dependent in the thymus

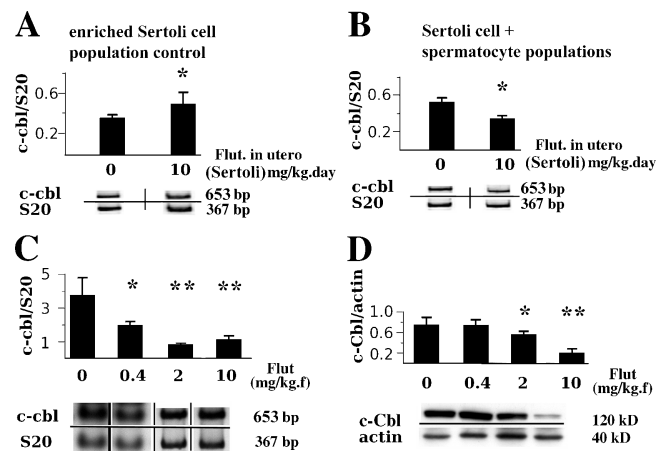
Testosterone exerts an important effect on the size of the thymus. Removal of androgens by castration results in thymus enlargement, whereas androgen replacement accelerates CD4<sup>+</sup>CD8<sup>+</sup> thymocyte apoptosis (Olsen et al., 1998; Dulos and Bagchus, 2001). This effect is opposite to what we observe in testes. Exploration of Cbl expression in this model showed that 3-d flutamide exposure of a rat killed at 30 pnd led to a slight (around one fourth) but significant increase of thymic Cbl expression in terms of mRNA (Fig. 6 A) and protein (Fig. 6 B). Because Cbl expression increases in cells considered to be protecting themselves from apoptosis, it was of interest to test Cbl expression in the same cells (e.g., thymocytes) undergoing apoptosis. Glucocorticoids are potent initiators of apoptosis in the thymus (Cohen, 1992). After a 1-h treatment with an intraperitoneal injection of 20 mg hydrocortancyl (HC) in 30 pnd rats, the Cbl protein expression level in the thymus was significantly decreased by half compared with control (Fig. 6 B). Thus, HC induced a down-regulation of Cbl (p120<sup>cbl</sup>) in the thymus, paralleling the induction of apoptosis before the outset of caspase processing as was previously reported (Denis et al., 1999), whereas Cbl up-regulation is associated with the protective effect against flutamide-induced apoptosis.



**Figure 4. Testicular Cbl is up-regulated upon testosterone treatment given during rat adulthood.** (A and B) Coamplification RT-PCR (A) and WB (B) were performed on hypophysectomized rat testes (HX; five animals per group). Testosterone treatment is indicated (HX + testo). C, control mice of the same age. Significant data refer to normal untreated animals (0): (A) \*,  $P < 0.0004$ ; (B) \*,  $P < 0.0001$ . (C and D) Cbl RT-PCRs were performed from cocultures of 15 pnd SCs without (C) or with (D) spermatocytes and with or without R1881 as indicated. Values represent the means  $\pm$  SD determined from five different cultures from three unrelated experiments. (D) Significant data refer to untreated cocultures (0): \*,  $P < 0.0005$ .

### The BH3-only proapoptotic protein Bim and mitochondrial proapoptotic Smac/Diablo expression are indirectly regulated through Cbl

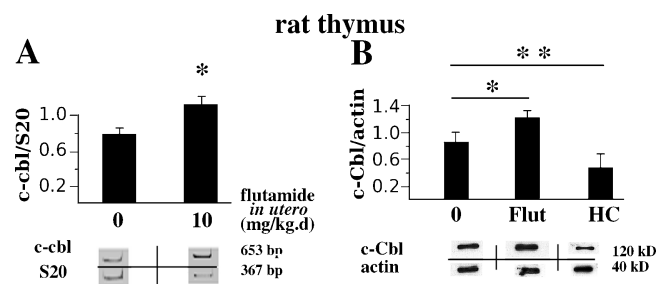
It has been recently described that the regulation of osteoclast apoptosis occurs through ubiquitination and subsequent degradation of the proapoptotic Bcl-2 family member BH3-only protein Bim EL through Cbl (Akiyama et al., 2003). This study prompted us to ask whether Bim follows the same Cbl regulation after testosterone withdrawal in the testis. Indeed, Bim EL is significantly up-regulated in rat (five times the control) as well as in mouse testes (1.5 times the control; Fig. 7, A and D, compare WT with WT + F) when Cbl is significantly down-regulated in the same species (four times the decrease in mice compared with control) upon flutamide exposure (Fig. 7 B). To directly link Bim expression to Cbl in the testis, anti-Bim EL Western blotting (WB) was performed in Cbl KO adult mouse testes. The result showed a significant doubling of Bim EL expression in the testes of Cbl KO mice compared with littermate WT controls (Fig. 7 D, KO vs. WT). These data showed the causal effect of the regulation of Cbl expression upon Bim expression levels in the testis. Flutamide-exposed Cbl KO mice showed a significant increased Bim expression (1.5 times) compared with untreated KO mice (Fig. 7 D, KO + F vs. KO), suggesting a way of andro-



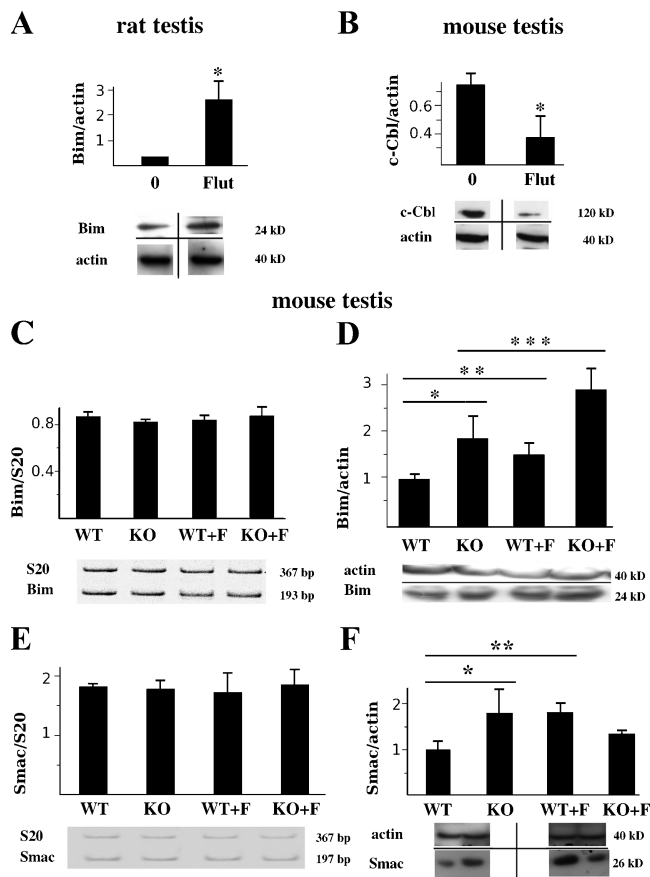
**Figure 5. Testicular Cbl expression undergoes a long-term down-regulation after in utero flutamide exposure.** (A) RT-PCR experiments were performed with isolated 15 pnd SCs from untreated animals (0) or from in utero flutamide-exposed animals (10 mg/kg/d; 10). (B) These SCs were also cocultured with naive elutriated spermatocytes. Values represent the means  $\pm$  SD determined from five different cultures from three unrelated experiments. Significant data refer to untreated cocultures (0): (A) \*,  $P < 0.03$ ; (B) \*,  $P < 0.04$ . (C and D) Rats of 90 pnd (10 rats per group) were exposed during fetal time to 0.4–10 mg/kg/d flutamide, and coamplification RT-PCR (C) and WB (D) were performed on testes. Significant data refer to untreated rats: (C) \*,  $P < 0.002$ ; \*\*,  $P < 0.005$ ; (D) \*,  $P < 0.01$ ; \*\*,  $P < 0.004$ .

gen regulation of Bim in the testis other than that exerted through Cbl. Regulation of Bim expression is posttranscriptional, as no change of Bim transcripts were detected between WT and KO mice, whether they were treated or untreated (Fig. 7 C).

Next, the expression level of the proapoptotic mitochondrial negative regulator of IAPs, Smac/Diablo, was explored. We found a significant increase expression of 1.8 times the Smac in KO testes compared with WT (Fig. 7 F), which is associated with a posttranscriptional control as with Bim (Fig. 7 E). Flutamide treatment did lead to a significant increase in Smac expression in WT mouse testes (1.8 times), but, in contrast to Bim, this did not occur in KO testes. This stressed the loss of Smac androgen dependency when Cbl was inactive. Immunolocalization of Bim was positive in all TGCs except in sper-



**Figure 6. Cbl is up-regulated in protective conditions against apoptosis in thymus.** (A and B) Thymic Cbl protein (A) and transcript (B) expression of 3-d flutamide-exposed (10 mg/kg/d) or untreated (0) 30 pnd rats are shown. (B) Normal 30 pnd rats were also treated with 20 mg intraperitoneal hydrocortancyl (HC) and tested for thymic Cbl expression through WB. Values represent the means  $\pm$  SD determined from three different animals. Significant data refer to untreated animals (0): (A) \*,  $P < 0.03$ ; (B) \*,  $P < 0.04$ ; \*\*,  $P < 0.006$ .

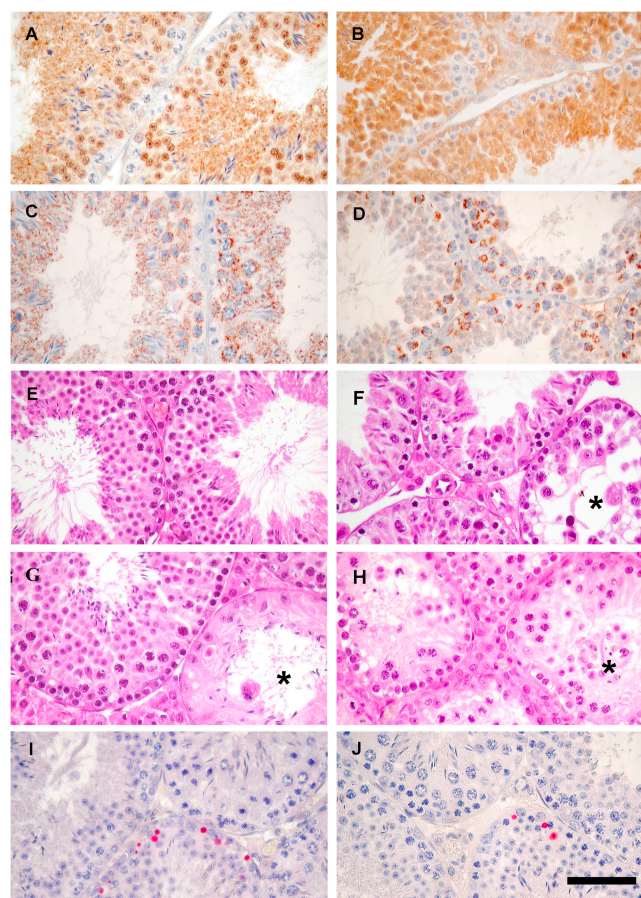


**Figure 7. Bim EL and Smac/Diablo are up-regulated in the Cbl KO mouse testis.** Cbl (B), Bim (D), and Smac/Diablo (F) WB was performed with testes of adult sv129 WT or Cbl KO mice with or without 3-d flutamide exposure. (A) Bim WB was performed with treated or untreated 90 pnd rat testes. (C and E) Coamplification RT-PCR was performed for Bim and Smac from the same mouse testis. Values represent the means  $\pm$  SD determined from eight different animals. Significant data refer to untreated animals (0; WT or KO) for the flutamide exposure experiments: (A) \*,  $P < 0.001$ ; (B) \*,  $P < 0.033$ ; (D) \*\*,  $P < 0.008$ ; \*\*\*,  $P < 0.0005$ ; (F) \*\*,  $P < 0.008$ . Significant data also refer to WT for untreated (KO/WT) mice experiments: (D) \*,  $P < 0.0003$ ; (F) \*,  $P < 0.0085$ .

matogonia (Fig. 8 A), which, interestingly, had a markedly more homogenous staining in Cbl KO testes (Fig. 8 B). Smac is principally immunolocalized in the PS (Fig. 8 C), and, as for Bim, it is also more markedly stained in KO testes (Fig. 8 D). Thus, both proteins colocalized with Cbl.

### Androgen-dependent apoptosis is drastically affected in Cbl KO mouse testes

The alteration of the apoptosis pathway prompted us to look for any change in the testicular apoptosis pattern of Cbl KO mice and particular morphological aspects on testis sections. Compared with WT mice, we observed in some Cbl KO seminiferous tubules important abnormalities that consist of a complete disruption of germ cell organization and disappearance of postmeiotic cells (Fig. 8, E vs. F). The flutamide exposure did not obviously modify this aspect (Fig. 8 H), whereas in the WT mouse testis, a few tubules were devoid of postmeiotic and part of premeiotic cells as a result of apoptosis (Fig. 8 G). Surprisingly, no evident

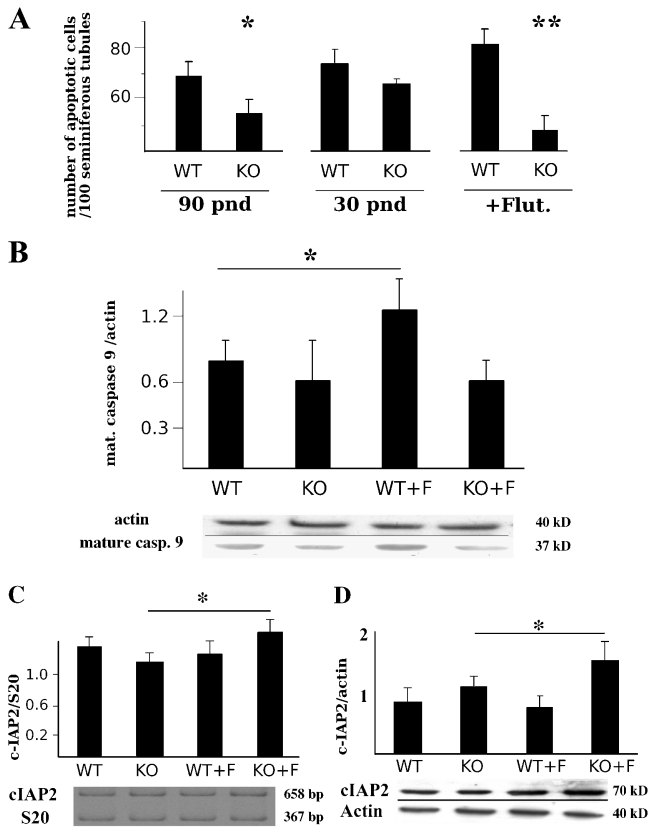


**Figure 8. Colocalization of Cbl with Bim EL and Smac/Diablo and alteration of spermatogenesis in Cbl KO mouse testes.** (A–D) IHC experiments were performed on testis sections of untreated WT (A and C) or Cbl KO (B and D) 90 pnd mice. Staining was performed with anti-Bim (A and B) or with anti-Smac/Diablo (C and D). (E–H) Classic HE (hematoxylin–eosin) staining of testis sections is outlined in E and F, respectively, for untreated WT and KO mice and in G and H, respectively, for 3-d flutamide-exposed WT and KO mice. (F and H) Some tubules have a disrupted cell organization completely devoid of postmeiotic cells in treated as well as in flutamide-exposed Cbl KO mice. (G) Only in flutamide-treated WT animals, some tubules show a classic aspect of spermatid  $\pm$  spermatocyte removal, resulting from androgen-dependent apoptosis (\*). (I and J) TUNEL-positive (apoptotic) cells from an untreated WT mouse testis (I) or Cbl KO mouse testis (J). Bar, 50  $\mu$ m.

sign of apoptosis was observed in Cbl KO testes, but rather an alteration of spermatogenesis and the obvious inefficacy of androgen withdrawal to initiate apoptosis was observed (Fig. 8 H).

Testicular TUNEL experiments confirmed this aspect and clearly showed that the apoptotic level is significantly lower in KO than in WT mice, targeting spermatogonia and spermatocytes as well (Fig. 8, I and J). Differences between the number of WT and KO apoptotic cells seems to depend on the age of the animals: young KO mice did not present any significant variation with WT counterparts (Fig. 9 A, 30 and 90 pnd). Flutamide exposure had no effect in KO mouse testes, whereas it increased the WT apoptotic TGC number as expected (Fig. 9 A, 90 pnd and +Flut).

The processed mitochondrial initiator caspase 9 was then assessed (Fig. 9 A). We could not observe any alteration of mature caspase 9 in the KO mouse testis compared with its WT



**Figure 9. Low expression of spontaneous and flutamide-induced germ cell apoptosis in Cbl KO mice.** (A) The number of TUNEL-positive (apoptotic) germ cells per 100 seminiferous tubules was counted from WT or Cbl KO mouse testes at 90 pnd (2 histograms on the left), 30 pnd (2 histograms in the middle), and flutamide-exposed WT and Cbl KO (2 histograms on the right). (B) WB with antiprocessed caspase 9 was performed on testes of flutamide-unexposed or -exposed (25 mg/kg/d  $\times$  3 d) WT or Cbl KO mice. (C and D) Testicular c-IAP2 RT-PCR (C) and WB (D) from flutamide-exposed or -unexposed 60 pnd (adult) WT and Cbl KO mice are shown. Eight treated or untreated WT or KO mice are considered. Significant data refer to WT mice: (A) \*,  $P < 0.004$ ; \*\*,  $P < 0.002$ . Significant data also refers to untreated mice: (B) \*,  $P < 0.033$ ; (C)  $P < 0.01$ ; (D)  $P < 0.008$ .

counterpart. However, upon flutamide exposure, we did observe a significant increase (1.5 times) of this caspase in WT mouse testes but not in Cbl KO mice, which is reminiscent of the Smac/Diablo expression pattern. Thus, caspase 9 is not processed in androgen-dependent apoptosis in testicular Cbl KO mice.

These results prompted us to assess the testicular IAPs. c-IAP2 in KO mouse testes had a pattern of activity different to what we observed in WT mice. There was a slightly higher expression of testicular protein c-IAP2 in Cbl KO mice than in WT, but, noticeably, the flutamide exposure led first, as expected, to a decreased expression in WT mouse testes, whereas it significantly increased in flutamide-exposed KO mice (Fig. 9 D). Testicular c-IAP2 transcripts of flutamide-exposed KO mice were also significantly higher than in WT (Fig. 9 C). Thus, despite the highest expression of Smac/Diablo, increased c-IAP2 expression could reflect the transcriptional activation of this prosurvival factor and explain, in part, the diminished apoptosis pattern observed in Cbl KO TGCs, as we know that the apoptosis process reflects the balance between proapoptotic and prosurvival factors (Liston et al., 2003).

**Table II. Cbl KO mice are hypofertile**

Offspring	Cbl KO	WT	M Cbl KO $\times$ F WT	M WT $\times$ F Cbl KO
Cages with litter	40 $\pm$ 6% <sup>a</sup>	80 $\pm$ 4.5%	33 $\pm$ 8% <sup>c</sup>	66 $\pm$ 6%
Newborn/cages	1.2	5.4	2	4
Newborn/pregnant F	3 $\pm$ 1.5 <sup>b</sup>	6.8 $\pm$ 2	NR	NR
Cbl WT/KO birth	NR	$\times$ 4.6		

Six males and six females of the same strain (WT or Cbl KO) were separately mated. We considered the percentage of cages with newborns (first row), the mean number of newborns by cage (second row), or the mean number of newborns by pregnant female (third row). The ratio of Cbl KO versus WT newborn number or the inverse has also been calculated (fourth row). All of these results are indicated in the first and second columns. Mating of one strain to another was also performed as Cbl<sup>-/-</sup>  $\times$  Cbl<sup>+/+</sup> of male and female of each or of another strain. The results are reported in the first and second rows (third and fourth columns). The values represent the means  $\pm$  SD determined from three unrelated experiments. Significant data refer to the WT strain. M, male; F, female; NR, not reported.

<sup>a</sup> $P < 0.01$ .  
<sup>b</sup> $P < 0.005$ .  
<sup>c</sup> $P < 0.008$ .

### Cbl-deficient mice are hypofertile

This altered mitochondrial pathway of apoptosis as well as the morphological disruption of TGCs in the Cbl KO mouse urged us to assess the reproductive function of these animals. We already observed that Cbl KO mice are seemingly hypofertile even if they are healthy and do not display any obvious developmental abnormalities (Naramura et al., 1998). Each strain was respectively mated as indicated in Table II. 80% of couples gave birth to newborns when the mice were of the WT strain, and 40% gave birth when the mice were deficient for Cbl ( $P < 0.003$ ). On average, Cbl KO mouse couples gave birth to 1.2 newborns, and WT couples gave birth to 5.4, indicating that Cbl KO mice are 4.5 times less fertile than their WT counterparts.

Moreover, we observed 3 newborns per KO pregnant female and 6.8 newborns per WT pregnant female, meaning there were 2.3 more newborns in a WT mouse litter compared with a Cbl KO mouse ( $P < 0.001$ ). To assess what gender bears the hypofertility, we performed mating with each gender (c-Cbl<sup>-/-</sup>  $\times$  c-Cbl<sup>+/+</sup>). Six males were mated with six females of the opposite strain. We then found that the WT male was two times more fertile than its Cbl KO counterpart when each of them were respectively mated with the female of the other strain ( $P < 0.03$ ). Thus, Cbl KO male mice are significantly responsible for the decrease of Cbl mouse fertility, corresponding, after calculations, to  $>80\%$  of the fertility loss carried by the KO strain. This could be caused, at least in part, to the imbalance of the aforementioned testicular apoptotic pathway.

## Discussion

Numerous studies have shown that Cbl could perform several types of regulation illustrating the functional complexity of this protein. The E3 ligase function of Cbl has been well established, and the potential of Cbl for apoptotic regulation has also been recently stressed.

The high Cbl expression in testes justified our investigations. We found that it was predominantly expressed in the PS,

with a lower expression in spermatogonia. Testis irradiation, *in situ*, and postnatal ontogeny experiments confirmed these points. They also suggest that somatic cells (mainly SCs, PMCs, and Leydig cells) display very low, if any, Cbl protein expression. Cbl mRNA expression was reduced but was clearly present either between birth and pnd 15, when spermatocytes did not yet appear, or after postirradiation day 26, when they were discarded. These results could mean that testicular cells other than spermatocytes might express Cbl transcripts without any protein translation, an aspect that has been largely documented (Mattick, 2004). Indeed, the increased mRNA signal at postirradiation day 45 without any Cbl protein signal likely suggests a delayed reoccurrence of protein Cbl expression in spermatogonia. It cannot be ruled out that testicular somatic cells expressed noncoding Cbl transcripts, as we found it in SC primary cultures or SERW3 cell lines (unpublished data).

Only the androgen-dependent stages were Cbl stained, particularly stage IX (Russell et al., 1990). Other latter androgen-independent stages, particularly where spermatogonia are detected, are slightly Cbl stained. According to these data, we might then expect a steady-state level of Cbl expression with some alteration upon antiandrogen exposure.

We then explored the expression level of Cbl in TGCs of rats that had been exposed to flutamide during either adulthood or the fetal stage. We showed that flutamide exposure during adulthood or *in utero* led to a significantly lower Cbl mRNA as well as protein expression in the testis. These data clearly showed that the expression level of Cbl is androgen dependent in the TGC. Cbl expression would hardly have been influenced by the vanishing of germ cells upon flutamide exposure for the following reasons: (1) the flutamide doses used are not deleterious (Kassim et al., 1997; Kim et al., 2001) and barely induced histological alterations (Omezzine et al., 2003; Bozec et al., 2004); (2) flutamide-induced apoptosis predominately targets spermatids, where Cbl is weakly expressed; (3) the number of apoptotic cells is low and could hardly account for alteration in Cbl expression; and (4) flutamide exposure during adulthood led to a transient apoptosis in which Cbl expression reversion to control level could not be a result of TGC renewal. Moreover, the hypophysectomized rat testes showed a quick Cbl expression reversion to normal values upon testosterone treatment, which could not be ascribed to cell turnover.

The change of Cbl phosphorylation status that we report is observed in several models (Dikic et al., 2003), but as far as we know, the concomitant alteration of Cbl expression has never been reported, particularly as an androgen effect. HX experiments entirely support the results given by the adulthood flutamide exposure, as a dramatic decrease of testosterone led to a drop of Cbl protein expression, and supplemental testosterone treatment rapidly reinstates Cbl to control level. These results also confirmed that the testosterone antagonist effect of flutamide is mainly responsible for the alteration of Cbl expression in testes.

Coculture experiments supported the *in vivo* Cbl expression data and highlighted the key role of SCs/PMCs as controlling Cbl androgen-dependent regulation in TGCs either through direct, specific interaction of androgen with SCs or through DNA imprinting that is acquired *in utero*. Surprisingly,

Cbl mRNA expression was significantly up-regulated in flutamide-exposed SCs, masking, in part, the Cbl down-regulation observed in cocultured spermatocytes.

However, it is worth noting that in cells considered resistant to apoptosis (e.g., SCs; Brinkworth et al., 1995; Tan et al., 2005), Cbl mRNA expression could possibly be up-regulated upon flutamide exposure, whereas it is down-regulated in cells susceptible to apoptosis (e.g., TGCs). This last assumption was supported by the thymic model: flutamide exposure has an opposing effect in the thymus as compared with the testis (Olsen et al., 1998; Dulos and Bagchus, 2001). The protective effect against thymic apoptosis driven by flutamide exposure was concomitant to a significant increased expression of Cbl, which could be compared with the high mRNA Cbl expression in apoptosis-resistant SCs. On the contrary, HC treatment initiating a broad thymic apoptosis was accompanied by an early significant decrease of Cbl. These data stressed the fact that Cbl expression is likely to be regulated in direct relationship with the onset of apoptosis and independently of the treatment necessary to achieve it (e.g., flutamide, HC, and R1881) or of the tissue where apoptosis is initiated (testis and thymus). Importantly, the testosterone effect in the thymus is indirect because it targets thymic reticuloepithelial AR-presenting cells exactly as it does with AR-presenting SCs and/or PMCs in the testis.

Moreover, the expression pattern of Cbl shown in this study entirely follows the flutamide-initiated pattern of apoptosis reported by Omezzine et al. (2003): the Cbl down-regulation paralleled the course of activated caspases reflecting the transient or established apoptosis, depending on the period of flutamide administration (adult age or *in utero*). The mechanisms of Cbl alteration are different in those two processes. They are correlated to a short time of testosterone deficiency upon adult flutamide exposure, whereas the long-lasting Cbl decrease finds its cause in a reprogramming of the testicular line during embryonic development, where SCs/PMCs play a key role (Anway et al., 2005). In support of this last statement, studies (for review see Sinha Hikim and Swerdloff, 1999; Kim et al., 2001) have confirmed that the testosterone intratesticular level at the adult age was normal after *in utero* flutamide exposure. Cbl could be involved at the very first step of apoptosis, as we report that HC treatment led to a significant decrease of thymic Cbl <1 h after treatment, which was unrelated to caspase processing as mentioned previously (Denis et al., 1999).

Because germ cells do not require a functional AR, androgen must affect spermatogenesis indirectly via the SC and/or PMC. Despite extensive investigations, limited changes in protein expression in the SC in response to androgen have been shown. It is only recently that selective SC AR KO mice have been constructed (Chang et al., 2004; Tan et al., 2005), allowing us to point out some SC key factors that could target spermatogenesis through poorly known paracrine factors. There is also growing evidence that nongenomic actions of androgen could play a role in spermatogenesis (Fix et al., 2004). As a consequence, the SC AR KO mice get rid of the majority of postmeiotic spermatids and a part of premeiotic spermatocytes exactly as does flutamide exposure at a lower extent, depending on the dose of flutamide given.



Recently, the proapoptotic Bcl-2 family member BH3-only protein Bim has been involved in the apoptosis of diverse tissues. It localizes in germ cells (Bouillet et al., 2002) and belongs to the essential initiators of the mitochondrial apoptotic cell death (Huang and Strasser, 2000). Recently, it has been associated directly with Cbl in the control of osteoclast apoptosis (Akiyama et al., 2003). However, Bim is dispensable for TGC development, suggesting that other proapoptotic Bcl-2 family members overlap Bim in testicular apoptosis (O'Reilly et al., 2000). Some studies along with ours show that the apoptotic mitochondrial/Bcl-2 family members pathway is implicated in spermatogenesis (Yan et al., 2000; Bozec et al., 2004) and plays a role at the late androgen-dependent spermatocyte/post-meiotic stages of TGC development. Thus, we extensively investigated the caspase cascade regulation at work in Cbl KO mouse testes and assessed the testicular apoptosis regulation of those mice. Flutamide exposure during adulthood allowed us to explore the androgen dependency of the process. Table III summarizes the significant testicular alterations observed in comparing WT/Cbl KO mice.

In addition, the proapoptotic factors Bim EL and Smac/Diablo were spontaneously highly expressed in KO mouse testes (Table III, third column) as a result of posttranscriptional regulation. So far, pull-down experiments with the GST-Bim EL fusion protein and/or co-IP performed with rat spermatocytes that were shortly cultured with the antiproteasome MG132 did not allow us to detect any direct Cbl association with Bim or Smac nor any specific ubiquitination of these proteins. We concluded that the Cbl effect on testicular Bim or Smac is indirect, unlike what was described in osteoclasts for Bim and possibly close to what was reported concerning the negative selection in the thymus (Villunger et al., 2004).

The protein Smac/Diablo is released from mitochondria upon apoptotic stress from permeability transition pores, which would require primary caspase activation (Morizane et al., 2005). Bim is thought to inactivate pro-survival Bcl-2 protein members, leading to increased mitochondria permeability (Strasser, 2005). It could be the indirect cause of Smac overexpression. Smac binds all IAPs and is able to interfere with the caspase-inhibiting properties of these proteins. In a so-called "proximity model," Smac is processed and released to link and allow autoubiquitination of IAPs, allowing unrestricted caspase activation.

Surprisingly, the activation of these two proapoptotic factors through Cbl disruption did not induce testicular apoptosis in adult mice. In this Cbl KO model, the initiator-processed caspase 9 is weakly activated even after flutamide exposure, accounting for the weak number of apoptotic cells observed in Cbl mouse testes. Thus, Smac/Diablo seems ineffective, perhaps because it does not escape mitochondria, is not expressed enough, or a blockade of caspases occurs that is unrelated to Smac.

Major factors that have been shown to control caspase activity are the IAPs. Among IAPs, we found that only c-IAP2 was more weakly expressed in KO mice. But in complete contrast to what happened in WT mice, the flutamide exposure of these mice induced a significant increase of this apoptotic inhibitor. This result could then entirely explain why the flutamide exposure of Cbl KO mice did not initiate any caspase

Table III. **Altered androgen-dependent apoptosis in the Cbl KO mouse testis**

Apoptosis labeling	WT	WT + F	KO	KO + F
Bim EL	+	++	++	+++
Smac/Diablo	+	++	++	+
Procaspase 9	+	+++	+/-	+/-
c-IAP2	+	+/-	+	++
TUNEL	+	++	+/-	-

Summary of the WB and TUNEL significant data obtained through experiments shown in Figs. 6–9. Only the alteration of expression of c-IAP2 in Cbl KO mice showed a transcriptional regulation. F, flutamide.

activation, contrary to WT mice. It is striking that flutamide treatment had an opposite effect in Cbl KO compared with WT mice, including Smac (reduced), processed caspase 9 (reduced), and apoptotic cell number (reduced). Only Bim is significantly overexpressed in flutamide-treated Cbl KO mice, clearly showing that Bim is, at least in part, down-regulated through a pathway other than that used by Cbl. At this point, it is difficult to claim whether Bim and Smac posttranslational overexpression are led indirectly by Cbl activity and/or through an apoptosis deficiency feedback regulation, as could be the case with c-IAP2. However, c-IAP2 is activated at an upstream level, which favors an indirect effect of Cbl on it and could also explain how it escapes from Smac degradation.

The morphology of KO mouse testes as well as TUNEL experiments performed at different ages of these mice suggest that a possible strong apoptosis occurred when spermatocytes/spermatids appeared in the testis (15–20 pnd), which then could be relayed by a blockade of this process. During this period, the normal mouse testis also undergoes a peak of apoptosis related to androgens. Experiments are in progress to elucidate this aspect. However, it appears clear from all of these data that Cbl controls the androgen dependency of testicular apoptosis and the balance between proapoptotic and pro-survival factors.

In light of our recent works (Omezzine et al., 2003; Bozec et al., 2004) and others (Anway et al., 2005; Tan et al., 2005), the transgenerational effect of an external agent requires stable chromosomal alterations or an epigenetic phenomenon such as DNA methylation. It is then very likely that the antiandrogen given during fetal development induced a permanent DNA reprogramming of the germ line affecting *c-cbl*, which was strongly suggested through our in vivo and coculture experiments. Considering our data and the particular sensitivity of Cbl transcripts to low doses of flutamide in the embryo, there is also a possible compensatory translational regulation allowing Cbl to be more actively expressed. All of these aspects open new interesting fields.

In summary, we have shown that the intrinsic apoptotic pathway in Cbl KO mouse germ cells is altered, leading to a defect of testicular apoptosis. Despite overexpression of the proapoptotic factors Bim and Smac/Diablo, the blockade of caspase activation could be explained, in part, by translational overexpression of c-IAP2, allowing it to escape Smac inhibition. Relevant to this aspect, we report that Cbl expression is mandatory to activate androgen-dependent TGC apoptosis.

## Materials and methods

### Animals, chemicals, and antibodies

All animals and chemicals used were previously described in Omezzine et al. (2003) and Bozec et al. (2004). The Cbl KO mice constructed from the sv129 WT mice used in our experiments were gifts from H. Gu and M. Naramura (National Institutes of Health, Bethesda, MD). Human Cbl cDNA was provided by W.Y. Langdon (University of Western Australia, Western Australia, Australia); GST fusion protein Bim EL was provided by S. Tanaka (Kyoto University, Kyoto, Japan); and Flag-Cbl and HA-Bim EL was provided by D.C.S. Huang (The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia). A. Bègue (Institut de Biologie de Lille, Lille, France) engineered the mouse Cbl cDNA.

The anti-Cbl polyclonal antibody directed to the 15 COOH-terminal amino acids of Cbl (C-15) was obtained from Santa Cruz Biotechnology, Inc. (sc-170), as were the anti-Cbl-b (C-20), anti-Bim EL, L and S (H-191), anti-Smac (V-17), anticaspase-9 p10 (H-83), and anti-cIAP2 (H-85) polyclonal antibodies. The monoclonal anti-Cbl antibody (clone 7G10) and antiphosphotyrosine (clone 4G10) were purchased from Upstate Biotechnology. Protein A/G-agarose was obtained from Santa Cruz Biotechnology, Inc., and testosterone-agonist methyltrienolone (R188) was purchased from NEN Life Science Products.

### Animal treatments

Rat experiments were performed with Sprague Dawley strain or with mice mentioned in the previous paragraph. Adulthood as well as in utero flutamide exposures have been described previously (Omezzine et al., 2003; Bozec et al., 2004). Experiments with locally irradiated rat testes were reported in Florin et al. (2005). Experiments performed with hypophysectomized rats are described in Boussouar et al. (2003). 1.6 mg/kg testosterone treatment was performed for 4 d consecutively. Mouse treatments consisted of 25 mg/kg/d flutamide exposure during adulthood (10 wk old), whereas rat treatment consisted of 10 mg/kg/d.

Only bilateral descended testes were studied. Studies on animals were conducted in accordance with current regulations approved by the Institut National de la Santé et la Recherche Médicale animal care committee.

WB, IHC, and RT-PCR coamplifications were performed as previously reported (Omezzine et al., 2003; Bozec et al., 2004). For WB, the primary antibodies were used at 1:3,000 (C-15 Cbl), 1:500 (Cbl-b), 1:200 (Bim, Smac, and caspase-9), and 1:500 (c-IAP2) dilutions. For IHC, Cbl (C-15), Bim, and Smac, antibodies were used at 1:200 dilutions on paraffin sections of 5  $\mu$ m.

For PCR analysis, the target genes (*c-cbl*, *Smac/Diablo*, and *c-IAP2*) were coamplified with an endogenous standard gene (Ribosomal S-20 RNA).

### Image acquisition and manipulation

We used a microscope (Axioskop; Carl Zeiss Microimaging, Inc.) with plan-Neofluar objective lenses (Carl Zeiss Microimaging, Inc.) at 40 $\times$ /NA 0.75. Observation was performed with a 3,200-K halogen light plus a daylight blue filter using digital imaging medium. We used DAB as chromogen. The camera (Coolpix 990; Nikon) used Nikon acquisition software. All manipulations were performed at room temperature. Image processing was performed with Adobe Photoshop, and only the whole images were processed with brightness, contrast, and color balance adjustments.

### IP experiments

IP was performed as described previously (Corsois et al., 2002) using the monoclonal anti-Cbl antibody (7G10) for IP and WB on testes of 3-d flutamide-exposed and unexposed (10 mg/kg/d) 90 pnd rats.

### Coculture experiments

Isolation and culture of SCs were performed as described previously (Florin et al., 2005). Spermatogenic cells were isolated and submitted to a centrifugal elutriation as described previously (Le Magueresse-Battistoni et al., 1997). Enriched spermatocytes (80%) were collected. 10<sup>6</sup> spermatocytes were added to SC cultures, and cells were harvested after 3 d of coculture.

### Data analysis

Data are expressed as means  $\pm$  SD. The number of animals used is indicated, and experiments were repeated three times. For statistical analysis, one-way analysis of variance between groups and Bonferroni/Dunn tests were performed.  $P < 0.05$  was considered to be significant.

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**Note added in proof.** While this paper was in production, a paper on a similar topic was published by Thien et al. (Thien, C.B., F.D. Blystad, Y. Zhan, A.M. Lew, V. Voigt, C.E. Andoniou, and W.Y. Langdon. 2005. *EMBO J.* 24:3807–3819). These authors engineered a Cbl knock-in mouse with a loss-of-function mutation in the Cbl ring finger domain and reported original results that contrast with those obtained through the Cbl KO mice. It would be of great interest to explore the impacts of such a mutation in TGCs, which could allow us to more accurately analyze the role of Cbl in spermatogenesis.

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