Supplementary information:

1- Results and Discussion

c-Cbl Androgen dependency

1 c-Cbl expression is androgen-dependent in rat ventral prostate

Before investigating the role of c-Cbl in cancer survival, we explored its relationship with apoptosis \textit{in vivo} then \textit{in vitro}. Considering the ability of androgens to affect the survival/apoptosis cellular balance of ventral prostate (VP) but not cranial and dorsal prostate [56], we assessed the dependency of c-Cbl to androgens and its effect on rodent VP cell apoptosis (Fig. S1 and S2). Rats were treated by the anti-androgen flutamide responsible for cell apoptosis in a dose-dependent manner [57]. Weak doses of flutamide were used with minimal impact on the number of living cells as reported [58] and without affecting the integrity of the epithelium, as observed in \textit{in-situ} experiments (Fig. S2A, rows b and c).

RNA and protein c-Cbl expression decreased in VP after 24 hours of treatment (Fig. S1A) but did not in the other lobes (Fig. S1B and C). These results reinforce the role of c-Cbl in androgen receptor (AR)-related apoptosis/survival balance in androgen-dependent cells. Dose-effect experiments confirmed the c-Cbl-decreased expression with increased flutamide doses in VP (Fig. S2B). Castration performed on rats led to a drop of c-Cbl expression as soon as one day after surgery, quickly followed by its reinstatement after substitutive testosterone treatment (Fig. S2C). Epithelial-cell repopulation is unlikely for such a short time after surgery. These results strongly suggest androgen-dependency of c-Cbl in VP.

\textit{In-situ} experiments showed that only VP differentiated, non-basal luminal cells [59] were c-Cbl stained (Fig. S2A, column 2, row a). Flutamide led to a noticeable decrease of c-Cbl staining (Fig. S3A, column 2, rows b and c), compatible with the decrease observed in Western blot (Fig. S2B). The c-Cbl down-regulation was accompanied by a reduction of AR
staining co-localizing with c-Cbl (Fig. S2A, column 1). Androgen antagonists are known to weaken AR expression [60] and our findings underline the tight functional relationship between c-Cbl and AR in rat VP.

2 Appearance of c-Cbl androgen-dependency during maturation of mouse ventral prostate

In order to relate c-Cbl expression to AR activation in mouse VP, we analyzed c-Cbl expression days 16-20 after birth, when the first wave of testosterone is released (Fig. S2D). We observed that c-Cbl expression in mouse VP compared with the epithelium marker CK-18, increased during the development of androgen-dependent tissues. We can conclude there is a strong, positive correlation between AR activation and c-Cbl expression in mice as in rats.

3 Flutamide-induced apoptosis in rat ventral prostate is associated to c-Cbl down-regulation

AR activation is closely related to the survival of VP epithelial cells and anti-androgen treatment promotes their mitochondrial apoptotic pathway [28, 58, 59]. Accordingly, we show here that pro-apoptotic factor BimEL expression was up-regulated upon flutamide treatment (Fig. S2A, column 3; Fig. S2E), whereas the inhibitor of apoptosis (IAP) c-IAP2 expression was down-regulated (Fig. S2F).

c-Cbl behaves as an anti-apoptotic factor in LNCaP cell line

In order to link the apoptotic resistance against oxidative stress led by c-Cbl in MEFs and the effect of c-Cbl in human malignant cells, c-Cbl’s effect in a human cancer cell line was analyzed. We asked how c-Cbl behaves in a range of growing or apoptotic conditions, using the androgen-dependent human cancer prostatic cell line LNCaP. A first set of experiments was
done in de-steroided foetal calf serum with gradual addition of testosterone (R1881®). It has already been described that beyond an optimal concentration—in our hands $10^{-10}$ M of R1881-LNCaP cell proliferation decreases. We confirm this observation (Fig. S5A), which is either due to apoptotic onset [28] or to G1 arrest, as reported by Kokontis and al. [55]. It is possible that G1 arrest allows cells to escape apoptosis. Beyond this point, we observed a significant post-transcriptional decrease of c-Cbl protein expression (Fig. S5B), accompanied by a mitochondrial apoptotic pathway activation. Indeed, we observed a significant increase of the pro-apoptotic factors BimEL and Smac/DIABLO expression (Fig. S5C and D) and a significant decrease of the anti-apoptotic factors XIAP and c-IAP1 (Fig. S5E and F). These results exactly mirror what we observed in rat and mouse VP in apoptotic conditions, associating enhancement of pro-apoptotic regulation and c-Cbl expression decrease (Fig. 1 and 2).

Other sets of experiments have been showed and explained in the main article (Fig. 5), using LNCaP cells untreated or treated with hydrogen peroxide or etoposide or using LNCaP cells submitted to c-Cbl interference.

2- Methods

RT-PCR analysis

Total RNAs were extracted from rat prostatic tissues with TRIzol reagent. The amount of RNA was estimated by spectrophotometry at 260 nm. The cDNAs were obtained from RT of 5 mg of total RNAs using random hexanucleotides as primers (5 mM) in the presence of dNTP (0.2 mM), dithiothreitol (10 mM), Moloney murine leukemia virus reverse transcriptase (10 U/ml) for 1 h at 37°C. For PCR analysis, the target gene (c-cbl) was coamplified with an endogenous standard gene (Ribosomal S-20 RNA). The stock reactions (20 ml) were prepared on ice and contained 0.02 U/ml of Taq polymerase, 1.5 mM MgCl2, 200 mM dNTPs, 1 mM caspase primers, 10 nM S-20 primers, and 2 ml of RT mixture (cDNA). The PCR conditions were 94°C
for 5 min; 25 cycles of 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min; and then 72°C for 7 min. After amplification, the PCR products were separated by electrophoresis on 2% agarose gels containing 0.005% ethidium bromide visualized by ultraviolet light. Band intensities were estimated by densitometric scanning using a GelDoc scanner (Bio-Rad Life Science, Marnes la Coquette, France). Data were expressed as c-cbl / S-20 mRNA ratios. Primers used for c-cbl were:

(forward) ATGGACAAGGTTGGTGCGGTTGTGGT

and (reverse) GAAGAGGCTGATAGTCTGCTTAGT producing a 213 bp product.

The PCR analysis for PCR products was carried out from the logarithmic phase of amplification. The PCR-amplified products were checked by direct sequencing. The RT-PCR primers were designed inside separate exons to avoid any bias caused by residual genomic contamination. Moreover, no amplification was observed when PCR was performed on RNA preparations.

References


3- Figure Legends

SI Figure 1. Androgen-dependency of c-Cbl expression in rat prostatic lobes. c-Cbl expression in prostate lobes of rats treated by the anti-androgen flutamide at a dose of 10mg/kg/day from one to four days, compared to untreated rats. Each row corresponds to a lobe, respectively ventral prostate (A), caudal prostate (B) and dorso-lateral prostate (C). The left column reports co-amplification c-Cbl RT-PCR expression whereas the right part of the figure reports c-Cbl western-blotting. Prostate lobes were dissected either for western-blotting or RT-PCR assays from adult rats (90 post-natal days, pnd) exposed or non-exposed (CT) to the anti-androgen flutamide for different times (24, 48, 72 and 96 hours). The histograms represent the % of the diverse c-cbl/S-20 (RT-PCR) and c-Cbl/Actin (Western-blotting) expression level ratio compare to the control ratio (considered as 100%). Three independent experiments were done for each lobe and one of them is reported. A) Considering Ventral Prostate lobe, the RT-PCR shows that c-Cbl expression is significantly lower than untreated control (CT) after 24 hours of flutamide exposure (p = 0.0012, p < 0.0001 and p < 0.001 respectively). For western-blotting, c-Cbl expression was significantly lower than untreated control after 48 hours of flutamide exposure (p = 0.043 and p < 0.0001).
SI Figure 2. c-Cbl expression is androgen-dependent in rodent ventral prostate and associated to apoptosis. A) Immuno-staining was performed with anti-androgen receptor (AR, column 1), anti-c-Cbl (column 2) or anti-Bim (column 3) antibody. VPs came from untreated animals (row a), or 10mg/kg/day flutamide treated for 24H (row b) or 96H (row c). The magnification bar represents 50 μm. B) c-Cbl western-blotting of rat ventral prostate (VP) after different doses of three day-exposure flutamide. The results of 1 experiment among 3 independent experiments with at least 6 rats (n = 6) giving equivalent results is reported. The c-Cbl expression upon 10 mg/kg/day doses were significantly inferior to the control group (0) (p = 0.0288). C) c-Cbl western blotting of rat VP one day after castration (Cast) or not (C). Another rat group received 1.6mg/kg/day testosterone during 4 days (Cast.+Testo.). c-Cbl in castrated rats is significantly lower than control (p = 0.0137). D) c-Cbl western blotting of mouse VP 16th to 20th days after birth. Cytokerasin-18 (CK-18) solely expressed in epithelial cells was used as the control-marker. c-Cbl expression is significantly higher from the 18th day (p = 0.035). E) c-IAP2 expression in rat VP after 24, 48, 72 and 96H of 10mg/kg/day flutamide. c-IAP2 expression is significantly lower after 24H of flutamide exposure (p = 0.011). F) BH3-only BimEL expression in rat VP after 24, 48, 72 or 96H of 10mg/kg/d flutamide. After 4 days exposure, BimEL is significantly higher than control (0h) (p = 0.0303).

SI Figure 3. c-Cbl immuno-staining considered as negative compared to the corresponding healthy tissues of diverse human malignancies. Panels are showed associated in two. Several healthy tissues (left pictures) and tumour tissues of the same origin (right pictures) stained with the anti-c-Cbl polyclonal antibody C15 through immunohistochemistry experiments, are associated and displayed from A to H. Spots of at least 6 different patients on tissue microarray (TMA) slides were independently compared several times by two investigators, showing equivalent results. A): liver versus hepato-cellular carcinoma; B): skin

SI Figure 4. Correlation between c-Cbl expression intensity in human prostatic adenocarcinoma and the aggressiveness of the tumor. The reported cases are representative of the the c-Cbl immuno-stained sections that were selected from 26 patients A): benign hypertrophy of the prostate (BHP); B and C): adenocarcinoma of low grade, infiltrating and well differentiated; D and E): adenocarcinoma of high grade with tumor cell clusters weakly differentiated; F): undifferentiated adenocarcinoma of high grade. Magnification bar = 50µm.

SI Figure 5. Androgen-dependency of c-Cbl expression in the androgen dependent prostatic adenocarcinoma LNCaP cell line. The graphs report the % of the diverse c-cbl/S-20 (RT-PCR) and c-Cbl/Actin (Western-blotting) expression level ratio compare to the control ratio considered as 100% (CT). Each experiment was done at least three times with concordant results. A) isotopic thymidine incorporation in LNCaP cell line upon increased culture concentration of R1881® with significant differences relative to the ethanol control (lower proliferation) for thymidine incorporation at 5.10^{-11} M R1881® (p < 0.001) and 10^{-10} M (p < 0.001) (maximal incorporation). Relative to the highest thymidine incorporation at 10^{-10} M R1881®, increasing R1881® concentrations lead to a drop of thymidine incorporation with p values of p = 0.0097 for 10^{-9} M R1881® and p = 0.0019 for 10^{-8} M R1881®. B) c-Cbl mRNA expression of LNCaP cell line upon increasing R1881® concentrations (CT, 10^{-12} M, 10^{-11} M, 10^{-10} M, 10^{-9} M and 10^{-8} M) is showed on the left graph. c-Cbl protein expression under the same culture conditions is displayed on the right graph. The c-Cbl protein levels of LNCaP cell
line showed a low significant expression compared to untreated control cells for 10^{-9} and 10^{-8} M R1881® treatment (respectively p = 0.0321 and p = 0.0228). C) and D) western blotting of the cellular proapoptotic proteins, respectively Bim EL and Smac/DIABLO of LNCaP cell line cultured in the presence of R1881® at the previous indicated concentrations. Bim and Smac expression are compared to untreated control cells (Ethanol). For 10^{-9} and 10^{-8} M R1881®, Bim EL is significantly higher with p = 0.002 and p = 0.0163 respectively. For 10^{-8} M R1881®, Smac/DIABLO significantly increased with p = 0.0342. E) and F) western blotting of the inhibitors of apoptosis, respectively XIAP and c-IAP1 of the same cells cultured in the previous conditions. XIAP and c-IAP1 expression are compared to untreated controls (Ethanol). 10^{-9} M and 10^{-8} M R1881® treatment significantly down-regulate XIAP (p < 0.0001 and p = 0.0175 respectively) when c-IAP1 is downregulated at concentrations of R1881® higher than 10^{-12} M ( p = 0.0219, p = 0.0367, p = 0.039 and p = 0.0102 respectively).
SI Figure 1
SI Figure 2
SI Figure 5