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In vivo disruption of an Rb-E2F-Ezh2 signaling loop causes bladder cancer

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Abstract

Bladder cancer (BC) is a highly prevalent human disease in which retinoblastoma (Rb) pathway inactivation and epigenetic alterations are common events. However, the connection between these two processes is still poorly understood. Here we show that the *in vivo* inactivation of all Retinoblastoma (Rb) family genes in the mouse urothelium is sufficient to initiate BC development. The characterization of the mouse tumors revealed multiple molecular features of human BC, including the activation of E2F transcription factor and subsequent Ezh2 expression, and the activation of several signaling pathways previously identified as highly relevant in urothelial tumors. These mice represent a genetically defined model for human high-grade superficial BC. Whole transcriptional characterizations of mouse and human bladder tumors

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CONFLICTS OF INTEREST

The authors declare no conflict of interest

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revealed a significant overlap and confirm the predominant role for Ezh2 in the downregulation of gene expression programs. Importantly, the increased tumor recurrence and progression in human superficial BC patients is associated with increased E2F and Ezh2 expression and Ezh2-mediated gene expression repression. Collectively, our studies provide a genetically defined model for human high-grade superficial BC and demonstrate the existence of an Rb-E2F-Ezh2 axis in bladder whose disruption can promote tumor development.

INTRODUCTION

Bladder cancer (BC) is a complex and heterogeneous disease caused by both genetic and environmental factors (1). Most tumors arise from the inner lining epithelial cells of the bladder wall, being over 90% transitional cell carcinomas. At diagnosis, two major types of transitional cell carcinomas can be identified according to the pathological characteristics: two thirds of patients present with superficial non-muscle invasive tumors (NMIBC), the remaining third of patients presents (or develops) a highly aggressive, muscle invasive disease (MIBC) that leads to the death of 50% of patients. In general, NMIBC have a favorable prognosis and are treated by transurethral resections and intravesical therapy. However, these tumors show a high rate of recurrence, which in some cases can progress into MIBC. This makes necessary a regular surveillance with cystoscopy and urine cytology indefinitely (EAU guidelines) (2). Therefore, NMIBC represents one of the most costly malignancies to health care systems in developed countries (3). In MIBC, surgery, radiotherapy, and chemotherapy are effective treatments for the disease but there has been little progress in survival in the last 20 years.

The interpretation of the genetic landscape of BC has largely been influenced by the current clinical (NMIBC vs. MIBC) and pathological (stage and grade; papillary vs. solid-invasive) classifications (4). Molecular taxonomy provides a distinct view of BC subphenotypes and their relationship with the disease progression (5). *TP53* mutations, and *RBI* inactivation are more prevalent in MIBC and may favour tumor progression and muscle invasion (6). However, there is insufficient evidence to use them as independent predictors of poor outcome in the clinical practice of BC patients (7). Low grade NMIBCs are genomically stable whereas high-grade NMIBC and MIBC display genomic instability (8). *FGFR3* and *PIK3CA* mutations are more prevalent in NMIBC and can also be predictive of local recurrences in NMIBC (9). Bladder cancer is also characterized by significant alterations in genes involved in chromatin regulation, affecting in particular to those genes implicated in histone modification (10).

In an attempt to reproduce human tumors, multiple genetically engineered mouse models have been generated (11). In these, the expression of large TAg from SV40 (leading to the elimination of all Rb family members and p53) can induce bladder aggressive tumors in transgenic mice (11, 12). This is in contrast with the absence of bladder tumors upon *Rb1* ablation in the urothelium (13). In this regard, we did not observe bladder tumor development in large cohorts of mice bearing the specific elimination of *Rb* gene directed by keratin *K14cre* expression (14), which is active in the adult mouse urothelium (15), even in the absence of p107 (16), E2F1 (17) or p130 (18). These data indicate the existence of large

overlapping roles for the Rb family members in bladder urothelial cells, similar to the reported for other mouse epithelial cells (19). To circumvent this possible problem and to specifically delete Rb family function in the urothelium of adult mice, we took advantage of a mouse strain bearing floxed alleles of *Rb1* and *Rbl2* genes and whole deficiency in *Rbl1* gene (*Rb^{F/F};p130^{F/F};p107^{-/-}*) (20). Conditional gene deletion in the urothelium was induced by delivering an Adeno-Cre into the bladder lumen of adult male mice (13). Here we show that the ablation of all three Rb family members in the mouse bladder urothelium leads to tumor development. The molecular characteristics of these triple knock out (TKO) mouse tumors combined with their genomic characterization provide new possible molecular mechanisms of BC development. Collectively, the present data support a molecular connection between Rb-E2F and Ezh2 that may explain human NMIBC recurrence and progression.

MATERIAL AND METHODS

Patients

Tumor samples and medical records were analyzed from 77 patients who had been consecutively evaluated at the Urology Department of the University Hospital “12 de Octubre” between October 2009 and March 2011, and diagnosed with a Ta or T1 bladder cancer. Informed consent was obtained from all patients and the study was approved by the Ethical Committee for Clinical Research of University Hospital “12 de Octubre”. The pathologic and clinical data, as well as the sample recollection and preservation procedures have been reported elsewhere (9). Samples and united data from patients included in this study were provided by the Biobanco i+12 in the Hospital 12 de Octubre integrated in the Spanish Hospital Biobanks Network (RetBioH; www.redbiobancos.es) following standard operation procedures with appropriate approval of the RETHICAL AN Scientific Committees.

Retinoblastoma family-deficient bladder tumor mouse model

All the animal experiments were approved by the Animal Ethical Committee and conducted in compliance with Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) Guidelines. *Rb^{F/F};p130^{F/F};p107^{-/-}* mice were generated by breeding *Rb^{F/F};p107^{-/-}* (16) and *p130^{F/F}* (20) mice. Adenovirus expressing Cre recombinase was obtained from University of Iowa's Vector Core Facility (www.uiowa.edu) and surgically delivered to the bladder lumen as previously described (13). At the time of sacrifice, tissues were collected and processed as previously reported (16, 17, 21).

Tissue microarray (TMA)

The construction and analysis of tissue microarray containing the human samples has been reported elsewhere (9). At least two representative duplicate cores for each case were scored.

Immunohistochemistry

Immunohistochemistry analyses of human and mouse were performed essentially as previously described (9). Antibodies used were anti Ezh2 (Abnova mAb9542 diluted 1/200),

anti K5 (Covance, Princeton, NJ, USA diluted 1/500), anti K8 (TROMA, Univ of Iowa rat mAb diluted 1/10), anti laminin (Sigma L9393, diluted 1/100), anti p63 (Sta Cruz Biotech. mAb 4A4), anti pRb (Sta Cruz Biotech. sc50, diluted 1/50) and anti p130 (Sta Cruz Biotech. sc317, diluted 1/50). Signal was amplified using avidinperoxidase (ABC elite kit Vector) and peroxidase was visualized using diaminobenzidine as a substrate (DAB kit Vector). Negative control slides were obtained by replacing primary antibodies with PBS (data not shown). Scoring of the results and selection of the thresholds, internal controls for reactivity of each antibody, and tissue controls for the series were done according previously published methods (9). Mice were injected intraperitoneally with BrdU (0.1mg/g weight in 0.9% NaCl; Roche, Basel, Switzerland) 1h before sacrifice. BrdU incorporation was monitored in formalin-fixed sections using an anti-BrdU antibody (Roche) as described (16, 17, 21).

Determination of FGFR3 and PIK3CA gene mutation

The presence of mutations in the PIK3CA and FGFR3 genes was assessed in tumor genomic DNA by PCR test (QIAGEN) and/or by snapshot technique has been reported elsewhere (9).

RTqPCR

Total RNA was isolated from mouse and human samples and analyzed by RTqPCR as previously described (9, 16, 17, 21, 22). Briefly, total RNA was isolated using miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions and DNA was eliminated (Rnase-Free Dnase Set Qiagen). Reverse transcription was performed using the Omniscript RT Kit (Qiagen) and a primer mix specific for all genes of interest, in the case of human samples, and oligo dT primer for the mouse samples, using 10ng and 1µg of total RNA, respectively. PCR was performed in a 7500 Fast Real Time PCR System using Go Taq PCR master mix (Promega) and 1 µl of cDNA as a template. Melting curves were performed to verify specificity and absence of primer dimerization. Reaction efficiency was calculated for each primer combination, and TBP gene was used as reference gene for normalization (23). The sequences of the specific oligonucleotides used are listed in Supp Table 1. Discrimination between samples showing increased or decreased relative expression was made using the median.

Whole transcriptome analysis

Genome-wide transcriptome experiments were performed using the Affymetrix HuGene-1_0-st-v1 microarray or Mo Gene-1_0-st-v1 at the Genomics Facility of the Cancer Research Center (Salamanca, Spain) using standard procedures (see Supp Information). Datasets have been deposited in GEO (GSE38264). Mouse to human comparison was performed essentially as described elsewhere (22).

Immunoblot

Immunoblot was performed as described previously (16, 21). Briefly, dissected bladder tumors were disrupted by freeze-thawing cycles in lysis buffer (200mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.9, 25% glycerol, 400mM NaCl, 1mM ethylenediaminetetraacetic acid, 1mM ethylene glycol tetraacetic acid, 1mg/ml aprotinin,

1mg/ml leupeptin, 1mM phenylmethanesulfonyl fluoride, 20mM NaF, 1mM NaPPi, 1mM Na₃VO₄, 2.5mM dithiothreitol), and centrifuged to obtain supernatant containing total protein. 35 µg protein per sample were resolved in sodium dodecyl sulfate—polyacrylamide gel electrophoresis, gels and transferred to nitrocellulose membranes (Amersham, Little Chalfont, UK). Membranes were blocked with 5% non-fat milk diluted in Tris-buffered saline (TBS) and incubated with the appropriate antibodies diluted in TBS-T 0.5% bovine serum albumin. Secondary antibodies were purchased from Jackson ImmunoResearch. Super Signal West Pico Chemiluminescence Substrate (Pierce) was used according to the manufacturer's recommendations to visualize the bands. Antibodies used are against E2F1, E2F2, E2F3, pRb, p130 (Santa Cruz Biotechnology), Ezh2 (Abnova), phosphoSer473-Akt (Epitomics or Cell Signaling), Phospho-Erk1/2 (Thr202/Tyr204-P), p19Arf (AbCam), p53 (Novocastra) Stat3-Tyr805-P and S6-P (Cell signaling). Loading was controlled by using an anti-Actin antibody (Santa Cruz Biotechnology).

Statistical analysis

Comparisons were performed using the Wilcoxon–Mann–Whitney test (for two groups) and Student's t Test for paired samples showing normal distribution. Survival analyses (recurrence free or tumor progression in recurrence) according to various variables were performed using the Kaplan–Meyer method and differences between the patient groups were tested by the log-rank test. Discrimination between samples showing increased or decreased gene expression was made using the median. Overlapping significance was monitored by exact F Fisher test. SPSS 17.0 and Graph prism 5.0 software were used.

RESULTS

Ablation of all Rb family members causes bladder tumor development

To study the functional consequences of Rb family loss in the urothelium of adult mice, conditional gene deletion mediated by delivering an Adeno-Cre into the bladder lumen (13) of adult male *Rb^{F/F};p130^{F/F};p107^{-/-}* mice (20) was used. We observed the development of bladder lesions in all the Adeno Cre infected mice (n=18) (Fig 1A B), but not in control mice (non-infected littermates or infected with Adeno GFP n=30). The presence of these lesions was easily monitored by computerized tomography (Supp Fig 1), and in some cases was accompanied by hematuria. The histological analysis of these lesions revealed papillary masses growing inward to the bladder lumen (Fig 1A, Supp Fig 1), displaying histological features of high-grade non-muscle invasive carcinoma. The mouse bladder tumors were keratin K5-positive, keratin K8-negative (Fig 1C), displayed high proliferation (analyzed by BrdU incorporation, Fig 1C), and invaded the basal lamina (as determined by laminin staining, Fig 1C). When mice were followed up to one year after infection (Fig 1B) no visible metastases were detected.

We observed the ablation of all Rb family members (Supp Fig 2), and the increased expression of E2F1, 2 and 3a (Fig 1D) in these tumors, by both immunoblot and RTqPCR analyses (Supp Fig 3). In addition, tumors were characterized by the upregulation of p19^{Arf} and p53 and the activation of Erk, S6 and Stat3 signaling pathways, as demonstrated by

immunoblot analysis using phospho-specific antibodies (Fig 1D). In contrast, tumors did not show increased Akt activity (Fig 1D).

Tumors in retinoblastoma-deficient mouse resemble human BC

To further characterize the Rb-family mutant mouse bladder tumors and to compare with human BC, a whole transcriptome characterization of the mouse samples was performed. This analysis revealed a differential expression of 3053 transcripts (1849 downregulated and 1204 upregulated) in tumors compared to normal bladder (Fig 2A and Supp Table S2). Gene ontology characterization of these deregulated transcripts indicated that downregulated genes were involved in oxido-reduction, small GTPases transduction signaling, cell adhesion and translation processes (Fig 2B and Supp Table S3), whereas the upregulated genes were primarily involved in cell cycle and DNA repair processes, as would be expected in tumors with high E2F activity (Fig 2B and Supp Table S4). To characterize the putative transcription factors controlling these differentially expressed genes, we performed ChIP enrichment analyses (ChEA) (24). These revealed the possible involvement of Polycomb repressive group 2 (PRC2) in downregulated genes (Fig 2C and Supp Table S5), whereas the upregulated genes showed a primary involvement of E2F and Myc, together with histone demethylases (Fig 2C and Sup Table S6). Gene set enrichment analysis (GSEA) showed significant overlap of the upregulated and downregulated genes in mouse bladder tumors with various human bladder datasets and also with stem cell signatures, including genes silenced in ES cells by H3K27me3 (Supp Table S7), thus reinforcing the possible role of PRC2 in gene expression deregulation. The comparative analyses between the downregulated (Fig 2D upper panel and Supp Table S8) or upregulated (Fig 2D lower panel and Supp Table S9) mouse genes and human bladder tumors available in Oncomine database (25) showed a very significant overlapping with multiple external bladder cancer datasets comparing normal vs. tumor samples, tumors with poor clinical outcome, and the presence of specific gene mutations in human tumors, including *KDM6A* and *RB* mutations. Remarkably, this comparison (Fig 2D and Supp Table S9) also showed a statistically significant overlap between the upregulated mouse genes and upregulated genes in human recurrent bladder tumors, and also between the downregulated genes in mouse tumors and multiple datasets identifying genes silenced by PRC2 in stem cells (Supp Table S8).

Increased Ezh2 in retinoblastoma family-deficient mouse bladder tumors

The observed similarities between downregulated genes in mouse bladder tumors and those being silenced by PRC2 prompted us to analyze possible changes in the expression of PRC2 elements, *Ezh2*, *Suz12* and *Eed*, in the mouse tumors. RTqPCR analysis (Fig 3A) showed significant increased expression of *Ezh2* and *Suz12* genes, but not *Eed* in mouse tumors compared with control samples, being more relevant the increased expression of *Ezh2* gene. Immunoblot analysis confirmed the increased expression of Ezh2 protein (Fig 3B), the catalytically active member of the PRC2 complex and responsible of trimethylation of H3K27. Finally, immunohistochemistry analysis of Ezh2 expression (Fig 3D) compared to that of urothelium basal layer marker p63 (Fig 3C), revealed the increased expression of Ezh2 in mouse tumors, but not in the adjacent normal urothelium.

Genome-wide transcriptome analysis of human recurrent bladder tumor

These observations indicate that the mouse Rb family mutant tumors represent a *bona fide* model of human BC. The pathological characteristics resemble those of human high grade NMIBC, a possibility also reinforced by the genomic comparisons. To confirm this, a whole transcriptome study was performed using a series of human NMIBC samples focusing in differential gene expression between recurrent and non-recurrent tumors. The analysis identified 351 transcripts (162 overexpressed and 299 underexpressed) differentially expressed in recurrent tumors (Fig 4A, and Supp Table S10), which also discriminated normal tissue. Remarkably, this classification did not discriminate between Ta and T1 stages, high and low grade tumors, or tumors bearing *FGFR3* mutations (Fig 4A). On the contrary, tumors bearing *PIK3CA* gene mutation were predominantly associated with non-recurrent samples (Fig 4A). These findings may help to explain our previously reported association between *PIK3CA* gene alterations and reduced recurrence in superficial bladder tumors (9). Gene Ontology (GOBP Supp Table S11 and Supp Table S12) and GSEA (Supp Table S13) revealed that the upregulated genes in recurrent tumors played a major role in cell cycle control, proliferation and proteosomal protein degradation, whereas the downregulated genes displayed an association with ribosome, 3'UTR mediated translational regulation and protein translation. The GSEA also indicated similarities with stem cell transcription and PRC2 silencing (Supp table S13). The comparison of these differentially expressed genes with other human bladder cancer datasets in Oncomine database revealed a significant overlap and trend with previously reported bladder cancer datasets (Supp. Table S14). Of note, we found that the downregulated genes in recurrent samples of our study were able to identify early recurrence of NMIBC in an external dataset (26) (Fig 4B). ChEA of these deregulated genes revealed, besides the common involvement of multiple transcription factors in all datasets (Supp Table S15 and S16), a statistically significant involvement of histone modifying enzymes, affecting histone methyl transferases and demethylases, and including the PRC2 members: Ezh2, Suz12 and Eed (Fig 4C). In agreement, we also observed a significant overlap with H3K27me3 in downregulated genes in recurrent tumors by GSEA analysis (Supp Table S13).

Finally, we integrated our transcriptome data from both mouse and human genes in a common dataset. The unsupervised classification of this common dataset according i) the expression of genes discriminating mouse normal and tumor bladder samples (Fig 4D upper panel), or ii) human genes discriminating human recurrent and non-recurrent NMIBC samples (Fig 4D Lower panel), invariably showed that mouse tumors were clustered with human recurrent tumors. Thus, the mouse bladder tumors initiated by the ablation of all retinoblastoma family members represent a putative model of human recurrent NMIBC.

Increased EZH2 expression in human recurrent bladder tumors

Genomic data in mouse and human bladder tumors analyzed pointed to a major involvement of PRC2, and in particular Ezh2, in the development and recurrence of NMIBC. To further support this, those genes identified by ChEA to be bound by any PRC2 element or specifically by Ezh2 (n= 38 and n=12, respectively) were loaded into Oncomine database. A statistically significant overlap was found with the Lee-bladder dataset (26) in the case of PRC2-bound genes (overlapping genes n= 4 of 38, *p*-value= 0.00044; Odds ratio 12.7).

Moreover, we also observed a significant overlap with both PRC2- and Ezh2-bound genes in another external dataset (27) (overlapping genes n=5 of 38; p-value=0.002; Odds ratio 9.1, and n=3 of 12, p-value= 0.008; Odds ratio= 13.6, respectively). Importantly, even these limited numbers of overlapping genes were able to discriminate patients of NMIBC with high likelihood of recurrence (Supp Fig S4A, B, C). These observations point to a primordial role of Ezh2-mediated gene silencing in NMIBC early recurrence

We next analyzed expression changes of PRC2 members in a series of human NMIBC samples previously described (9). RTqPCR analysis revealed a significant increase of *EZH2* expression (Fig 5A), but not of *SUZ12* and *EED* (Supp Fig S5A, B), in tumors compared with paired normal bladder samples. The *EZH2* gene expression was also higher in recurrent tumors compared to non-recurrent (Fig 5A). When the patients were stratified according Ezh2 expression in a tissue microarray (Fig 5C, C'' and data not shown), we found that Ezh2 increased expression correlated with tumor recurrence (Fig 5D; $p=0.000021$).

A possible involvement of E2F in Ezh2 deregulated expression

The expression of *EZH2* gene is regulated by E2F activity (28). Accordingly, we monitored whether *EZH2* expression correlated with the expression of different E2Fs in our series of human bladder tumor samples. This analysis revealed a striking correlation between *EZH2* and *E2F3a* gene expression (Fig 6A). We also observed that *E2F3a* expression was significantly higher in tumor than in normal samples, and in recurrent than in non-recurrent tumors (Fig 6B). Importantly, increased *E2F3a* expression levels stratified recurrent NMIBC patients (Fig 6C). On the other hand, *E2F1*, 2 and *3b* gene expression did not discriminate between normal and tumor samples (Supp Fig S6A), or between recurrent and non-recurrent human NMIBC (Supp Fig S6B). Further the expression of *E2F1*, 2 and *3b* genes did not show significant correlation with *EZH2* gene expression (Supp Fig S6C, E, G), and did not allow statistical stratification of recurrence in our series of NMIBC patients (Supp Fig S6D, F, H). These results indicate that the early recurrence mediated by increased *EZH2* expression could be facilitated primarily by increased *E2F3a* expression. However, a concerted action of other E2F family members in this process could not be discarded at this point.

EZH2 affects tumor progression in recurrence

Besides the high recurrence rate, an important problem in NMIBC is the relatively high rate of recurrent tumors appearing with increased stage and/or grade compared with the primary tumors indicative that tumor progression upon recurrence occurred. During this study, 11 out of 33 patients suffering recurrence in our series also displayed tumor progression. To study if similar events to those described for recurrence were also affecting tumor progression, the differential gene expression between tumors showing or not tumor progression upon recurrence was studied. This analysis revealed a very limited number of transcripts (Supp Table S17). ChEA analysis of these transcripts showed that a significant number of under-expressed genes in tumors showing progression were bound by PRC2 complex elements (Fig 7A). In addition, these transcripts displayed a significant overlap with external datasets of gene lists regulated by PRC2, including genes with H3K27me3 marks (Fig 7B). Accordingly, samples of tumors showing progression in recurrence showed

also a significant increase in *EZH2* and *E2F3a* expression (Fig 7C). Moreover, the increased expression of EZH2 protein was an independent predictor of tumor progression in the recurrences of our series of NMIBC samples (Fig 7D).

These data indicated that increased EZH2 expression mediates not only early recurrence, but also increased probability of tumor progression in human NMIBC.

DISCUSSION

Bladder cancer is one of the most common cancers in men worldwide making it a current problem in terms of social and medical relevance. The genomic landscape of these tumors may contribute to identify novel targets of possible therapeutic interest. These studies have revealed multiple alterations and mutations in BC, including chromatin remodeling, cell cycle and specific signal transduction pathways (5, 10, 29-33). Regarding cell cycle, *RBI* mutations are relevant. However, these studies are biased by the analysis of mainly aggressive, MIBC tumors and few examples of NMIBC are included. Nonetheless, even in these few examples, *RBI* alterations are present (29, 32). This, together with other possible alterations leading to functional pRb inactivation, such as CycD or E2F amplification (26, 30, 32), may suggest that Rb-dependent pathway is also of relevance in NMIBC. Our present data, showing that the complete ablation of Rb family *in vivo* leads to urothelial tumors supports this possibility.

It has been previously shown that the inactivation of *Rb1* gene in mouse urothelium is insufficient to allow spontaneous tumor development (13). Regarding our observations of complete penetrance of BC development upon Rb-family ablation, this apparent discrepancy could be attributed to functional compensation by the other family members, as previously reported in mouse epithelia (19). Our findings are in agreement with the reported results of urothelial expression of T antigen in transgenic mice (12, 34). However, T antigen transgenic mice usually develop muscle invasive tumors; this could be attributed to impaired p53 tumor suppressive functions. Such possibility, which is also reinforced by our previous findings indicating that p53 loss in *Rb*-deficient stratified epithelia facilitate the development of invasive metastatic disease (21, 35), remains to be determined. The pathological characteristics of the mouse bladder tumors suggest that these mice could represent a bona fide NMIBC model. Also, the mouse tumors display activation of MAPK/Erk, Stat3 and S6 pathways, which have been previously involved in human BC (36-38).

In view of the findings in the mouse model, we aimed to use it as a tool to gain a better understanding of the molecular mechanisms responsible for the initiation, recurrence and progression of BC. This would also provide potential biomarkers. In a first approach, we performed a whole transcriptome analysis. This reinforced the similarities between mouse and human tumors, and provided a possible mechanism for the upregulation and downregulation of genes in the mouse tumors involving E2F and PRC2. We found that the removal of Rb family led to overexpression of E2F family members, and these mouse tumors invariably showed increased *Ezh2* expression. Further, our GSEA and ChEA data indicated a predominant role for E2Fs, in particular *E2F3a*, in gene upregulation in mouse tumors. Given that i) *E2F3* is frequently amplified and overexpressed in human BC (39-42),

ii) the inactivation of the Rb pathway is required in addition to *E2F3* overexpression in human bladder carcinogenesis (40), iii) *E2F3* is a primary regulator of *EZH2* expression (28), and iv) there is emerging evidence supporting the RB-*E2F3*-*EZH2* pathway as a key oncogenic axis in cancer development and aggressiveness (43), our findings provide a new molecular mechanism for bladder tumor development. Importantly, various *Ezh2* inhibitors have been developed and are being tested in various preclinical models (44-46). The possibility that these inhibitors may impair bladder tumor growth in our Rb family-deficient model will be the subject of future investigations. In addition the identification of possible overlapping mutations between human NMIBC and our mouse model will provide further evidence of the relevance of this model for preclinical studies.

In order to confirm these findings and to validate the mouse model as a suitable tool for understanding human NMIBC, we carried out a whole transcriptome analysis in human bladder cancer samples. Furthermore, as recurrence and progression are common in this type of human tumors and constitute a severe clinical problem, this study was designed to determine possible molecular factors affecting recurrence in NMIBC. The metagenomic analyses also reinforced the extreme similarities between mouse and human recurrent tumors. We also observed that recurrence is coupled to gene upregulation mediated by *E2F* activity, and downregulation associated with increased *PRC2* activity. Importantly, we could also validate the role of *PRC2*- and *Ezh2*-mediated gene silencing in NMIBC recurrence in external datasets, indicating that increased *Ezh2* expression and activity could act as a predictor of early recurrence. Further, we could also associate the increased *Ezh2* expression and activity with progression upon recurrence in human NMIBC. In this regard, the increased expression of *Ezh2* had been previously reported associated with increased malignancy in bladder cancer (47, 48). In agreement, *KDM6A*, (which catalyzes the *H3K27* demethylation and thus acting in opposite manner to *EZH2*), had also been described as frequently mutated in bladder cancer, in some cases in association with *RBI* gene mutation in high stage or grade human bladder tumors (29, 30, 33). Future research, aimed to detect possible overlapping mutations in these genes in our mouse model, will provide further data of the relevance of this model for preclinical studies. Nonetheless, to the best of our knowledge, this is the first report associating *EZH2* activity with NMIBC recurrence and progression.

Collectively, these findings could be highly relevant in the clinical management and therapy of human bladder cancers. The possible therapeutic use of *Ezh2* inhibitors in the management of BC is a crucial aspect that merits future research. In this regard, the mouse model here described arises as an essential and invaluable tool for such analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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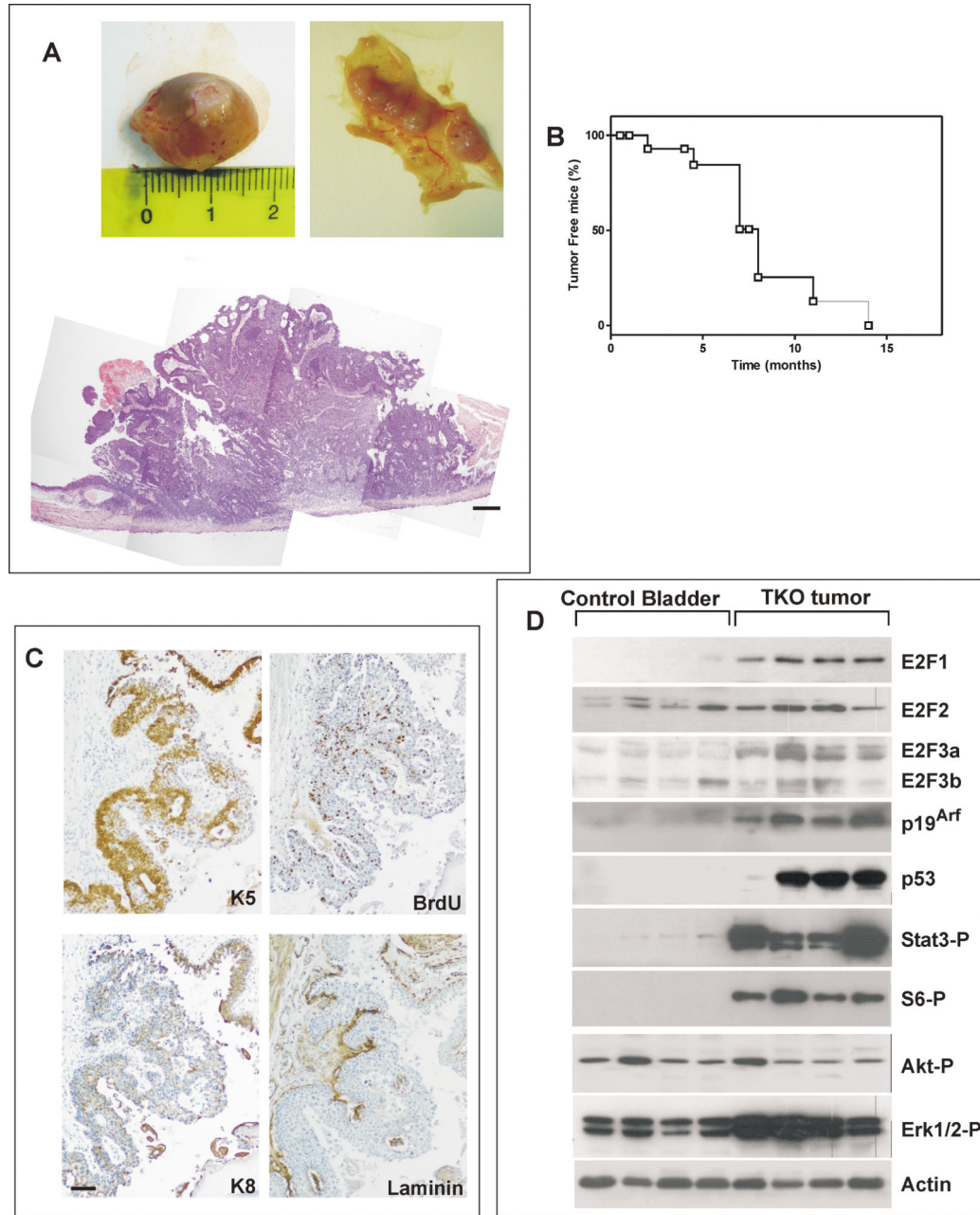


Fig. 1. In vivo Rb family ablation causes bladder cancer development in mice

A) Example of external (upper left panel) or internal (upper right panel) view of a bladder from an $Rb^{F/F};p130^{F/F};p107^{-/-}$ mouse 11 months after inducing the gene recombination by AdenoCre infection. Lower panel depicts an example of H&E stained section of the lesions shown in upper panels. Bar =500 μ m

B) Kaplan-Meier curve showing the kinetics of tumor development as assessed by CT scan in the adenoCre injected mice (n=18).

C). Representative immunohistochemistry of a mouse tumor stained for Keratin K5, BrdU, Keratin K8 and Laminin as indicated. Bar=150 μ m

D) Immunoblot analyses of control (uninfected littermates bladder samples) and mouse bladder tumors (denoted TKO: triple knock out) showing the expression of the quoted proteins, Actin was used to normalize protein loading.

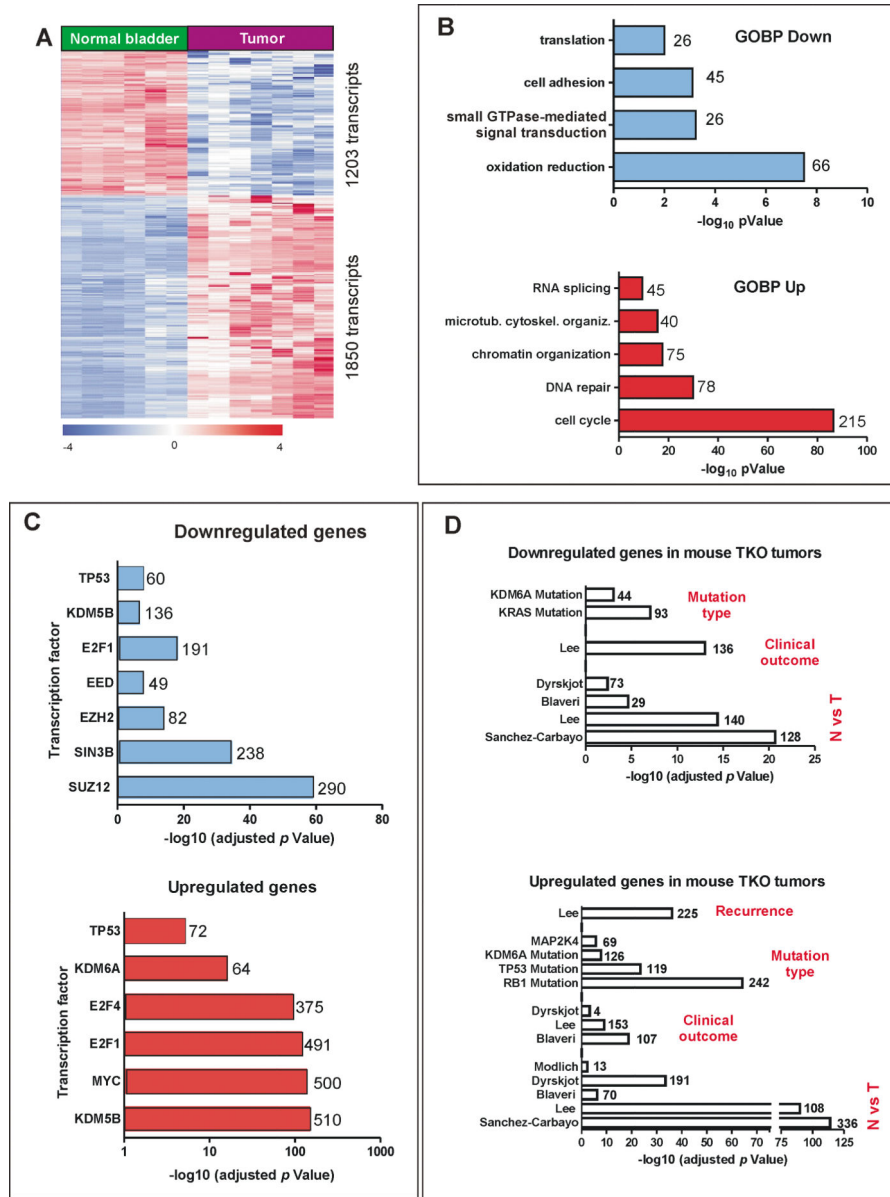


Fig. 2. Transcriptome analysis of mouse bladder tumors

A) Heatmap showing the distribution of genes (rows) identifying tumors and normal control uninfected mouse bladder samples. Each column represents a sample. A red (overexpressed) to blue (downregulated) scheme following the above scale limits (in log₂ scale) is shown. Numbers on the right denote the number of transcripts of each group (upregulated or downregulated).

B) Representative Gene Ontology biological processes affecting the functions of the downregulated (upper panel) or upregulated (lower panel) genes in mouse bladder tumors. Numbers on the right of each bar indicate the genes on COBP category:

C) Relative relevance of transcription factors and histone modifying enzymes in the genes downregulated (upper panel) or upregulated (lower panel) in tumors as obtained by ChEA.

The relevance of each factor is provided by the p -value (in $-\log_{10}$ scale). Numbers on the right of each bar represent genes bound by each transcription factor from the database.

D) Summary of relevant overlap between downregulated (upper panel) or upregulated (lower panel) genes in mouse bladder TKO (triple knock out) tumors with human bladder tumors from the Oncomine database. The different comparison concepts (Tumor vs Normal, Mutation Type, Clinical Outcome and Recurrence) are provided for each group. p -values were obtained by exact F Fisher test. The number of overlapping genes is shown for each dataset.

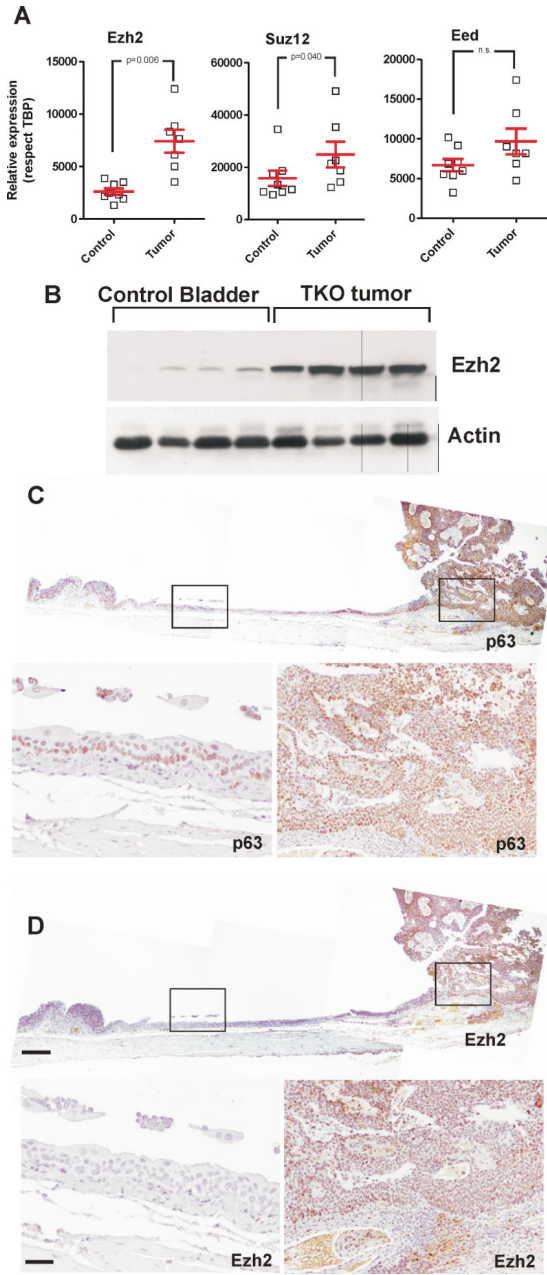


Fig 3. Mouse bladder tumors display increased Ezh2 expression

A) Expression of *Ezh2*, *Suz12* and *Eed* genes in control (uninfected bladder samples) and mouse bladder tumors as assessed by RTqPCR (respect to *Tbp*). *p*-value was obtained by the Mann Whitney t-test, mean and SEM are denoted in red.

B) Immunoblot analyses of control (uninfected bladder samples) and mouse bladder TKO (triple knock out) tumors showing the expression of Ezh2 protein. Actin was used to normalize protein loading.

C) Representative examples of the immunohistochemistry analysis of mouse bladder tumor showing the expression of p63. Lower panels are higher magnification of the corresponding areas denoted in upper panel.

D) Representative examples of the immunohistochemistry analysis of mouse bladder tumor showing the expression of Ezh2. Lower panels are higher magnification of the corresponding areas denoted in upper panel. Bar in upper panel=500 μ m, in lower panel =150 μ m

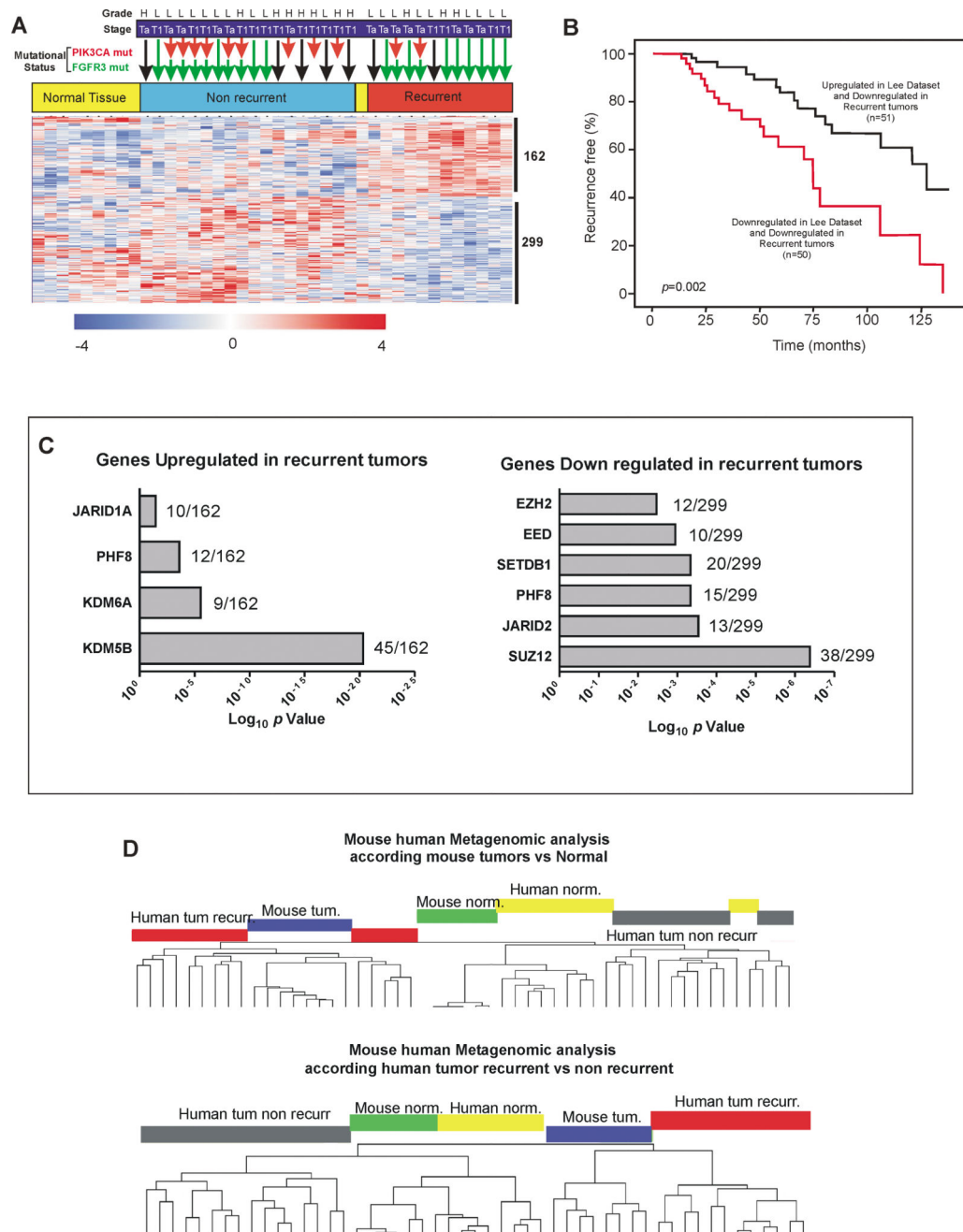


Fig. 4. Transcriptome studies of human NMIBC (non-muscle invasive bladder cancer) recurrence

A) Heatmap showing the distribution of genes (rows) identifying recurrent tumor samples. Each column represents a sample, the mutation of *PIK3CA* or *FGFR3* genes is denoted by red and green arrows, respectively. The stage (Ta and T1) and grade (high: H, low: L) for each tumor sample is also provided. A red (upregulated) to blue (downregulated) scheme following the scale limits (in \log_2 scale) is shown. Numbers on right side denote the number of transcripts characterizing each class.

B) Kaplan-Meier distribution of patients in Lee Dataset (26) according the expression levels of the downregulated genes identified in our study. p -values were obtained by the Log rank test. n denotes the number of samples scored of each group.

C) Relative relevance of the different histone modifying enzymes in the genes of each quoted group obtained by ChEA. The relevance of each enzyme is provided by the p -value. The number on the right of each bar represents the number of specific genes bearing this type of modification and the total number of transcripts on each group: upregulated (left panel) or downregulated (right panel) in recurrent tumors.

D) Dendrograms of mouse-human transcriptome comparison upon unsupervised clustering (Pearson correlation and average linkage method) according the expression of genes differentiating mouse control and bladder tumor samples (upper panel) or genes discriminating human recurrent tumors (lower panel).

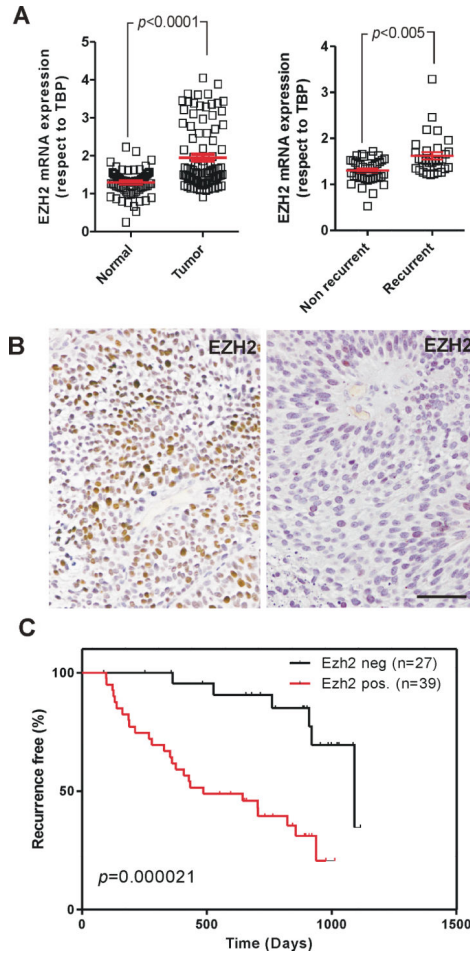


Fig. 5. EZH2 Expression in human NMIBC (non-muscle invasive bladder cancer)

A) Expression of *EZH2* gene as assessed by RTqPCR (respect to *TBP*) in normal vs. tumor samples, and in recurrent vs non-recurrent tumors. *p*-values were obtained by the Mann Whitney t-test, mean and SEM are shown.

B) Representative examples of human tumors showing positive (left panel), and negative (right panel) staining for EZH2 protein. Bar= 100 μ m.

C) Kaplan-Meier distribution of recurrence according the expression of EZH2 protein form tissue microarrays. *p*-values were obtained by the Log rank test (n denotes the number of samples in each group).

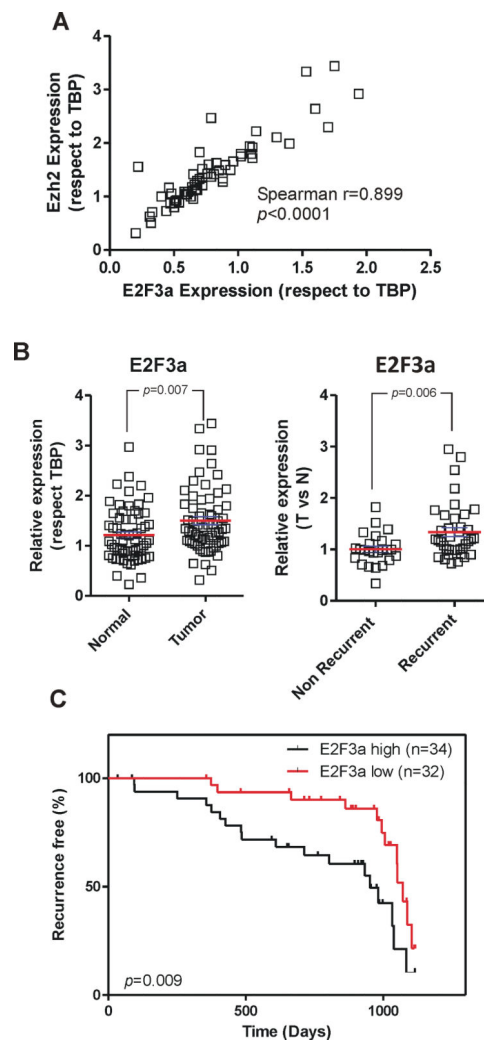


Fig. 6. *E2F3a* expression in human NMIBC (non-muscle invasive bladder cancer)

A) Expression of *EZH2* gene as a function of *E2F3a* gene expression as assessed by RTqPCR in NMIBC samples. The r and p values are provided according the Spearmann correlation method

B) Expression of *E2F3a* gene in normal and tumor bladder samples (left panel) and in recurrent and non-recurrent bladder tumors (right panel). p -values were obtained by the Mann Whitney t-test, mean and SEM are denoted in red.

C) Kaplan-Meier distribution of NMIBC recurrence according increased expression of *E2F3a* gene p -value was obtained by the Log rank test. n denotes the number of samples scored of each group.

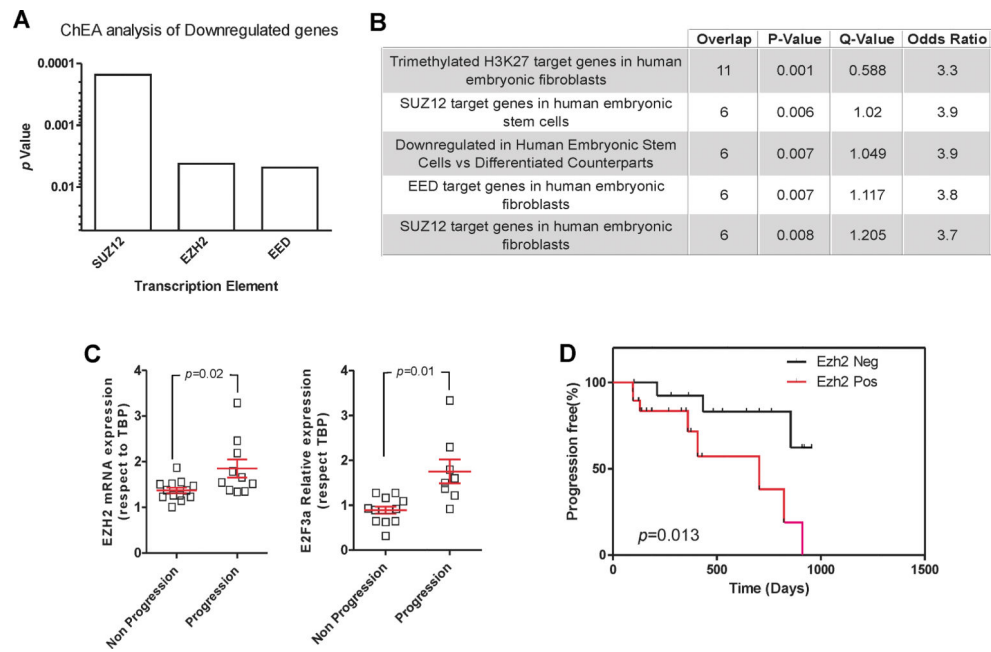


Fig. 7. EZH2-dependent signaling in tumor recurrence and progression

A) Relative relevance of the binding of the different PRC2 elements to the downregulated genes in tumors showing progression upon recurrence as assessed by ChEA. The relevance of each enzyme is provided by the p -value.

B) Summary of relevant overlap of the downregulated genes in tumors showing progression upon recurrence and Oncomine database in the concept “literature defined” showing the relevance of PRC2 components.

C) Expression of *EZH2* and *E2F3a* genes as assessed by RTqPCR (respect to *TBP*) in tumors displaying or not progression upon recurrence. p -values were obtained by the Mann Whitney t-test, mean and SEM are denoted in red.

D) Kaplan-Meier distribution of tumor progression in recurrences according increased expression of *EZH2*. p -values were obtained by the Log rank test.