REVIEW ARTICLE

The urothelial gene regulatory network: understanding biology to improve bladder cancer management

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The urothelium is a stratified epithelium composed of basal cells, one or more layers of intermediate cells, and an upper layer of differentiated umbrella cells. Most bladder cancers (BLCA) are urothelial carcinomas. Loss of urothelial lineage fidelity results in altered differentiation, highlighted by the taxonomic classification into basal and luminal tumors. There is a need to better understand the urothelial transcriptional networks. To systematically identify transcription factors (TFs) relevant for urothelial identity, we defined highly expressed TFs in normal human bladder using RNA-Seq data and inferred their genomic binding using ATAC-Seq data. To focus on epithelial TFs, we analyzed RNA-Seq data from patient-derived organoids recapitulating features of basal/luminal tumors. We classified TFs as "luminal-enriched", "basal-enriched" or "common" according to expression in organoids. We validated our classification by differential gene expression analysis in Luminal Papillary vs. Basal/Squamous tumors. Genomic analyses revealed well-known TFs associated with luminal (e.g., PPARG, GATA3, FOXA1) and basal (e.g., TP63, TFAP2) phenotypes and novel candidates to play a role in urothelial differentiation or BLCA (e.g., MECOM, TBX3). We also identified TF families (e.g., KLFs, AP1, circadian clock, sex hormone receptors) for which there is suggestive evidence of their involvement in urothelial differentiation and/or BLCA. Genomic alterations in these TFs are associated with BLCA. We uncover a TF network involved in urothelial cell identity and BLCA. We identify novel candidate TFs involved in differentiation and cancer that provide opportunities for a better understanding of the underlying biology and therapeutic intervention.

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INTRODUCTION

The urothelium is a highly specialized epithelium lining the lower urinary tract, from the renal pelvis to the proximal urethra. It is composed of 3–8 cell layers [1]. Cells in the basal layer are small and lack expression of genes related to urothelial identity. Urothelial stem cells are thought to reside in this layer. Cells in the intermediate layers engage in differentiation and display increasing levels of uroplakins (UPKs), transmembrane proteins involved in the formation of urothelial plaques. The superficial layer is composed of umbrella cells, which are large [2], multinucleated, long-lived [3], differentiated cells generated by endoreplication [4]. Umbrella cells contain discoidal-shaped/fusiform vesicles that allow apical membrane expansion in response to bladder filling [5] and provide barrier impermeability [6]. Acquisition of urothelial lineage identity involves the extinction of the basal program-which is reminiscent of the epidermis and is characterized by expression of KRT5 - and the expression of urothelial markers, most notably UPKs and KRT20 [7, 8]. KRT14 is expressed by a small fraction of basal cells thought to have stem cell properties [9] (Fig. 1).

The urothelium is a slow-renewal epithelium: in homeostatic conditions, <1% of cells express Ki67 or are labeled with BrdU [10]. The lifespan of a rodent urothelial cell is estimated to be ~200 days [11]. However, upon injury, rapid regeneration ensues: basal cells secrete Sonic hedgehog (SHH) which stimulates stromal expression of Wnt agonists [12], promoting cell proliferation. Little is known about the spatial arrangements of the cellular neighborhood(s) in which these signaling loops take place. KRT14$^+$ basal cells are involved in regeneration upon cyclophosphamide injury [9]. The Mendelsohn lab has proposed that cells other than basal are also important in urothelial regeneration [8]. SHH-expressing cells include both KRT5$^+$ basal cells, as described by Shin et al., and two KRT5$^-$ UPK$^+$ cell types: P-cells, present only in the embryo and I-cells, also present in the adult. Lineage tracing shows that transient intermediate urothelial cells act as progenitors both in the embryo and in the adult [8]. The emerging scenario is one where the urothelial lineage may not simply progress in a linear fashion, from basal to intermediate to umbrella cells; urothelial progenitors may instead display a wider range of phenotypes (or be in a wider range of states), depending on the extent/type of damage.

A deeper understanding of these aspects of urothelial biology is required to dissect the regulatory mechanisms involved in diseases of the bladder. Here, we combine a comprehensive review of the literature with new analyses of public data to provide a better understanding of the landscape of alterations in the regulation of urothelial differentiation associated with bladder cancer (BLCA) through the identification of well-established and novel candidate transcription factors (TFs) involved therein.

AN OVERVIEW OF BLADDER CANCER GENETICS

The majority of BLCA arise in the urothelium and >90% are urothelial carcinomas. Conventionally, BLCA are categorized into

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non-muscle invasive BLCA (NMIBC) (ca. 75%) and muscle-invasive BLCA (MIBC) (ca. 25%). NMIBC are often indolent, papillary tumors. Most of them are genomically stable, unlike MIBC tumors which are life-threatening and genomically unstable [13, 14]. It is thought that BLCA arises through two pathways: papillary tumors constitute the majority of NMIBC and develop from papillary hyperplasia displaying lineage fidelity, whereas the majority of MIBC arise from dysplasia/carcinoma in situ. Recent work suggests that MIBC develops through preneoplastic lesions (carcinoma in situ) that can either maintain or lose urothelial lineage fidelity [15, 16], the latter resulting from epigenetic changes [17] (Fig. 2).

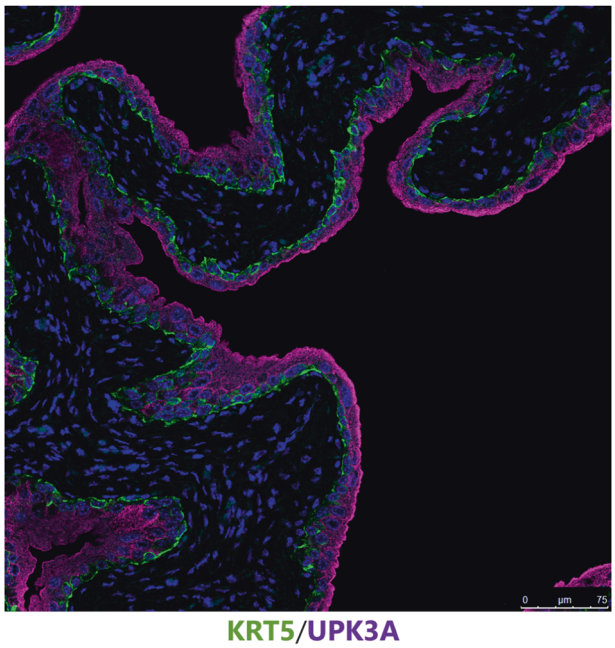

Fig. 1 The urothelium: morphological and molecular stratification during differentiation. Immunofluorescence analysis of the expression of urothelial differentiation markers in normal mouse urothelium. KRT5 (green) is expressed in basal/intermediate cells; UPK3A (purple) is expressed in luminal cells.

Mutations in some BLCA-relevant genes—such as *STAG2*, *RBM10*, and *KDM6A*—can already be detected in normal urothelial cells from subjects without BLCA and undergo positive selection [18]. *TERT* hotspot promoter mutations and deletions in chr. 9 are shared by tumors evolving through both BLCA progression pathways, suggesting that they are early events in tumorigenesis. Papillary NMIBC commonly harbor activating mutations in *FGFR3* and *PIK3CA*, and loss-of-function mutations in the tumor suppressors *STAG2* and *KDM6A* [13, 19, 20]. Alterations in the RB (e.g., through inactivation of *CDKN2A*) and p53 pathways drive progression to MIBC [21]. Non-papillary tumors often invade muscle and are characterized by inactivation of tumor suppressors (e.g., *TP53*, *RB1*, *ARID1A*): *TP53* mutations are highly frequent in urothelial dysplasia [22] and the RB pathway is altered in the majority of MIBC through a variety of genetic mechanisms [23]. Mutations in genes involved in chromatin regulation (e.g., *MLL2*, *ARID1A*, *KDM6A*, *EP300)* are enriched in MIBC more than in any other malignancy [23], 76% of the samples being mutated for at least one epigenetic regulator. Figure 2 summarizes current knowledge on the morpho-genetic pathways leading to BLCA.

IDENTIFICATION OF CANDIDATE TFs RELEVANT FOR UROTHELIAL LINEAGE IDENTITY AND CARCINOGENESIS

The disruption of cell differentiation during BLCA development and progression is reflected by the loss of lineage fidelity, underlined by the transcriptional taxonomic classification of tumors. Consequently, an improved understanding of the gene regulatory networks (GRN) and epigenomic mechanisms involved therein is necessary.

To systematically identify candidate **TFs relevant for urothelial differentiation and carcinogenesis**, we assessed the expression of all TFs from the consensus list of Lambert et al. [24] in patient-derived tumor organoids [25], recapitulating features of luminal and basal BLCA subtypes (Supplementary Fig. 1). We classified TFs as "luminal-enriched" or "basal-enriched" if they were: 1) present

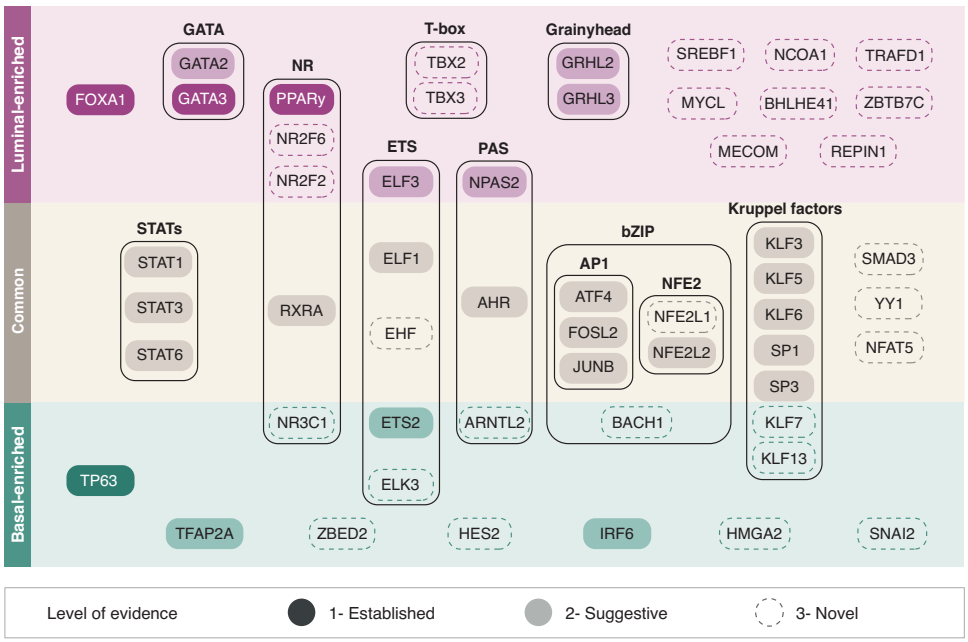

Fig. 2 Established and novel candidate TF involved in urothelial differentiation and BLCA. Topological scheme depicting the involvement of well-established (highlighted in dark background) and novel candidate (highlighted in discontinuous outline) TFs participating in the luminal vs. basal differentiation programs. Color code: purple for "Luminal-enriched", beige for "Common" and teal for "Basal-enriched" TFs.

among the top 100 highest-expressed TFs only in luminal or basal BLCA organoids, respectively, 2) significantly differentially expressed in luminal vs. basal organoids (log2 fold change cutoff of ±1 and FDR < 0.05), and 3) significantly differentially expressed in Luminal Papillary vs. Basal/Squamous tumors from the TCGA series (FDR < 0.05). We also defined a category of "Common TFs" including those that were among the top 100 highest-expressed TFs in both basal and luminal organoids. Common TFs were analyzed further if a significant enrichment (*P* values < 0.05) of their associated motif was found in ATAC-Seq peaks in normal human bladder [26] (Table 1 and Fig. 3). This strategy unveiled known, as well as novel, TFs involved in urothelial lineage specification. See Box 1 for more details. To further validate the role of these TFs, we assessed their activity according to target expression and transcription factor enrichment analysis with differentially expressed genes from luminal vs. basal BLCA organoids, with the ChEA 2022 database through the Enrichr website and filtering by TFs from the consensus list of Lambert et al. [24]. The results show significant enrichment of selected well-established urothelial TFs, thus validating the overall strategy used (Supplementary Fig. 2). Tables 2 and 3 provide detailed information on the molecular features of the TFs selected and their relevance to cancer.

Additionally, we assessed the expression of all consensus TFs in normal human bladder samples from GTEx, investigated genomic TF binding motifs in open chromatin regions identified from ATAC-Seq data of normal human urinary bladder mucosa [26], and confirmed their expression in normal urothelial cells using single cell RNA-Seq [27]. Highly expressed TFs (> 4000 DESeq2-normalized counts) with significant enrichment of their associated motif (*P* values < 0.05) were categorized as "normal-enriched TFs" and selected for further analysis (Supplementary Table 1).

TFs associated with luminal differentiation ("luminal-enriched")

PPARG and RXRA. PPARG is a member of the peroxisome proliferator-activated receptor subfamily of nuclear receptors, together with PPARA and PPARD. Its N-terminus contains the ligand-independent activation function domain (AF-1), which is poorly conserved between the 3 family members. The highly conserved central region contains the DNA-binding domain (DBD) with two zinc fingers that bind to PPAR response elements (PPREs) in target genes and a flexible hinge region that separates the DBD from the highly conserved ligand-binding domain (LBD). The C-terminus contains a ligand-dependent activation domain, AF-2.

PPARG heterodimerizes with retinoid X receptor alpha (RXRA) and regulates transcription by binding to PPREs present in regulatory regions of target genes. RXRA homo- or hetero-dimerizes with 14 nuclear receptors, including PPARG, PPARA, and PPARD [28]. In the absence of ligand, PPARG/RXRA dimers bind to co-repressors such as NCoR1 and SMRT, which recruit histone deacetylases. Upon ligand binding, co-repressors are replaced by co-activators such as mediator complex subunit 1 (MED1) and PPARG coactivator 1 alpha. Natural PPARG ligands include fatty acids, arachidonic acid and its metabolites. PPARG is expressed in adipose tissue and several epithelia and it plays important roles in adipogenesis and glucose homeostasis. Synthetic agonists (e.g., thiazolidinediones) acting as insulin sensitizers are used to treat type 2 diabetes [29]. There is some evidence that they can increase BLCA risk [30] although the results are controversial.

In the urothelium, PPARG is expressed in all cell layers and is a key regulator of differentiation. PPARG activation, together with EGFR inhibition, promotes the differentiation of normal human urothelial cells [31], mouse urothelial organoids [32], and human pluripotent stem cells induced to acquire an urothelial fate [33]. In vivo, it is required for terminal differentiation of umbrella cells in the developing ureter [34]. *Pparg* deletion throughout the urothelium (using the *Shh-Cre* driver allele), or in basal (*Krt5-CreERT2*) or suprabasal (*Upk2-CreERT2*) cells, reveals its critical role in mitochondrial biogenesis, fatty acid transport, and urothelial identity. In the absence of PPARG, basal cells undergo squamous-like differentiation and umbrella cells fail to mature [35]. This is reminiscent of the squamous metaplasia reported to occur upon retinoid deficiency [36]. PPARG is also required for homeostatic regeneration: upon injury/uropathogenic *E. coli* infection, NF-kB signaling is transiently activated and PPARG is required to dampen NF-kB signaling, which otherwise results in chronic inflammation [35, 37]. Ectopic expression of an activated form of PPARG in basal urothelial cells in mice induces enhanced differentiation but not tumor formation. However, upon N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN) administration, luminal, immune-cold tumors develop, likely through reduced NF-kB signaling [38]. A subset of luminal tumors loses canonical identity and acquires basal features over time, in association with PPARG down-regulation [38].

PPARG focal amplifications occur in ~10% of MIBC [39] leading to overexpression of the protein and its target genes. In addition, seven recurrent *PPARG* point mutations have been identified, five of which confer gain-of-function. Those affecting the LBD (M280I, I290M and T475M) endow the protein with ligand-independent activity and favor its interaction with coregulators [40]. *PPARG* mutations and amplifications are associated with luminal tumors, which display an active PPARG regulon. In the consensus publication of BLCA subtypes, *PPARG* gains/amplifications or fusions were found in 76% of LumNS tumors and in 89% of LumU tumors [23]. *RXRA* hotspot mutations (S242F/Y) have also been identified in 5% of MIBCs, mostly in the luminal subgroup [39]. These gain-of-function mutations confer enhanced binding affinity for PPARG and promote ligand-independent activation [41, 42]. Despite these important data, and work supporting that retinoic signaling promotes luminal differentiation [8], there is no direct evidence showing a role for RAR-RXR heterodimers in the regulation of luminal cell identity.

The role of the PPARG/RXRA axis in BLCA is context-dependent: luminal tumors are addicted to PPARG/RXRA signaling whereas Basal-Squamous (Ba/Sq)-like tumors display down-regulation of this pathway. PPARG loss-of-function mutations, hemizygous deletions, and DNA hypermethylation - associated with decreased PPARG activity - have been described in Ba/Sq-type MIBC [43]. Overexpression of PPARG in low-expressing BLCA cells activated PPARG signaling and inhibited growth, partly by down-regulating EGFR expression, while no effect was seen in PPARG high-expressing cells. Transcriptomic analysis revealed increased activity of lipid metabolism and urothelial differentiation pathways, and decreased activity of immunity/inflammation pathways [43]. These effects may require the cooperation of GATA3 and/or FOXA1 [44]. Conversely, a recent genome-wide CRISPR knockout screening identified GATA3, SPT6, and cohesin complex components as upstream positive regulators of PPARG expression in luminal BLCA cells [45].

In NMIBC, PPARG activity and immune cell infiltration are anticorrelated. Consistently, PPARG/RXRA activation in cancer cells inhibits secretion of pro-inflammatory cytokines, such as IL6 and IL8, that function as chemoattractants of effector T cells. Tumors from MBT2 murine cells overexpressing RXRA (S242F/Y) have substantially fewer $CD3^+$ and $CD8^+$ T cells and are partially resistant to immunotherapy. Down-modulating PPARG or RXRA significantly increases cytokine expression, suggesting therapeutic strategies that could sensitize patients to immunotherapies [42]. Pharmacological or genetic PPARG inhibition reduces proliferation, migration, and invasion of BLCA cells, especially those harboring *PPARG* amplification or *RXRA* activating mutations, highlighting the potential of PPARG as a therapeutic target in luminal BLCA [46, 47].

GATA proteins. GATA2 and GATA3 are Zn finger TFs that bind the GATA consensus sequence [48]. GATA proteins play fundamental

Table 1. BLCA-associated candidate TFs classified by cellular identity.

	TF	Average expression in **luminal**-like organoids	Average expression in **basal**-like organoids	**Log2FC expression** luminal vs basal organoids	**Log2FC expression** LumP vs BASQ TCGA	**Rank by expression in normal bladder**
Luminal-enriched	TBX2	178	5	4.81	2.8	70
	GATA2	219	10	4.22	1.72	124
	FOXA1	198	10	4.15	2.2	469
	ELF3	418	27	3.82	1.59	18
	MYCL	252	17	3.77	2.36	431
	BHLHE41	202	16	3.6	1.58	331
	ZBTB7C	124	11	3.35	1.88	571
	TBX3	437	60	2.77	2.89	29
	PPARG	210	32	2.61	2.08	351
	GRHL3	312	48	2.58	2.08	615
	REPIN1	167	37	2.1	0.74	125
	GATA3	255	53	2.1	2.46	115
	SREBF1	372	93	1.89	0.3	59
	NR2F6	112	34	1.65	1.03	293
	NCOA1	108	53	1.62	0.18	138
	NR2F2	161	51	1.53	0.53	61
	MECOM	142	44	1.45	1.6	214
	TRAFD1	109	41	1.36	0.24	239
	GRHL2	109	44	1.11	0.56	600
	NPAS2	135	58	1.1	1.59	271
Common	AHR	245	123	0.8	0.82	69
	JUNB	176	118	0.35	0.44	5
	EHF	570	389	0.31	0.49	405
	SP1	195	160	0.24	0.31	67
	STAT6	303	251	0.21	0.58	11
	SMAD3	195	158	0.18	0.74	54
	RXRA	228	195	0.17	0.51	43
	STAT1	192	164	0.16	-1.52	137
	NFAT5	165	139	0.15	0.57	156
	FOSL2	547	479	0.12	0.3	7
	KLF5	573	506	0.11	0.68	134
	ELF1	120	114	0.05	0.24	172
	STAT3	197	200	-0.02	-0.7	17
	YY1	168	182	-0.12	0	79
	NFE2L2	236	279	-0.2	-0.05	27
	KLF3	187	225	-0.2	0.47	136
	SP3	113	139	-0.26	-0.32	147
	NFE2L1	307	433	-0.4	-0.56	20
	ATF4	252	372	-0.46	0.18	2
	KLF6	356	607	-0.56	-0.58	12
Basal-enriched	ETS2	157	377	-1.11	-0.08	16
	BACH1	72	194	-1.38	-0.89	347
	ARNTL2	41	123	-1.39	-1.84	900
	TFAP2A	38	112	-1.41	-0.88	1108
	IRF6	213	612	-1.46	-0.42	304
	TP63	115	369	-1.61	-0.1	288
	KLF7	34	178	-2.28	-1.08	267
	NR3C1	28	159	-2.44	-1.54	189
	KLF13	55	327	-2.53	-1.23	129
	ELK3	34	215	-2.61	-0.96	290
	HES2	7	135	-4.15	-2.31	1428
	SNAI2	9	201	-4.31	-1.61	238
	ZBED2	1	103	-6.47	-2.34	1444
	HMGA2	1	402	-7.86	-4.51	1384

TFs were classified into "Luminal-enriched", "Basal-enriched", or "Common" according to their expression levels in luminal- vs. basal-like patient-derived organoids and in LumP vs. Ba/Sq bladder tumors from the TCGA cohort. TFs within each category are prioritized by Log2FC expression in luminal vs. basal organoids. Colors range from blue to red according to Log2FC expression. In the companion column, rank by expression in normal bladder GTEx samples is noted (among 1781 TFs). "Novel" candidate TFs are highlighted in orange.

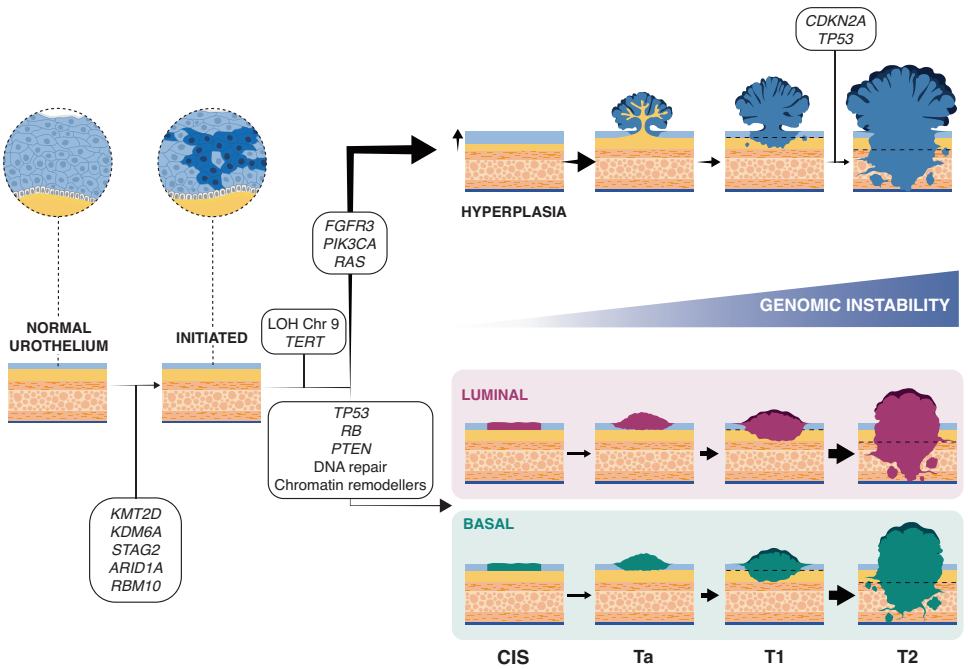

Fig. 3 An updated morphogenetic model of BLCA progression. Normal urothelial cells acquire somatic mutations during aging, some of which endow them with clonal selection capacity. [18] A subset of the progeny acquires additional mutations that promote tumor development, associated with specific phenotypes. Non-invasive preneoplastic lesions arising through the papillary pathway (upper track) have a lower propensity to invade muscle, unlike those arising through the non-papillary pathway (lower tracks). Carcinoma in situ, a precursor participating in the non-papillary pathway, can display either luminal or basal features, [15, 16] possibly accounting for the existence of luminal as well as basal tumors along this track. However, this model does not rule out that—in some cases—luminal tumors arising through the papillary pathway can evolve to acquire basal features. It is also possible that some of the CIS-like lesions represent the intraluminal expansion of tumor cells along the urothelial lining (surface "cancerization").

Box 1. Analytical approach to identify TFs relevant to urothelial differentiation and BLCA

To obtain a consensus list of **TFs relevant to urothelial carcinogenesis**, we used normalized expression data from patient-derived organoids (GEO accession GSE103990) that recapitulate luminal and basal BLCA subtypes. We grouped organoid lines according to their molecular subtype, determined average gene expression in each subtype, and filtered by genes included the consensus list of human TFs from Lambert et al [24]. Zinc finger proteins and chromatin remodelers were not considered in the analyses. The overlap between the top 100 highest-expressed TFs in luminal and basal organoids was further analyzed to identify three classes of TFs: TFs expressed among the top 100 in both luminal and basal organoid lines were defined as "common"; TFs from the top 100 highest-expressed TFs that were selectively present in luminal and basal lines were assigned as "luminal-enriched" and "basal-enriched", respectively. Next, DESeq2 [216] was used to identify TFs differentially expressed in luminal vs. basal organoid samples.

Luminal- and basal-enriched TFs were selected for further study according to the following criteria: 1) present among the top 100 highest-expressed TFs in luminal and basal organoids and showing a normalized expression value higher than 100, 2) significantly differentially expressed in luminal or basal organoid lines (Log2 fold change cutoff of ±1 and FDR of 5%), and 3) significant and consistently differentially expressed in luminal papillary vs. Ba/Sq tumors from the TCGA database (Log2 fold change cutoff of ±1 and FDR of 5%), respectively.

To validate "common" TFs, we analyzed ATAC-Seq data from normal human bladder (GEO accession GSE170508) to check for TF binding enrichment in open chromatin regions assigned to genes expressed in normal urothelium (Log2 average normalized expression). Reads were aligned to human assembly hg38 with bowtie2 using the default parameters. Reads mapped to mitochondria were filtered out using Samtools and duplicated reads were removed using the Picard tool MarkDuplicates. Reads were filtered against genomic "blacklisted" regions (http://mitra.stanford.edu/kundaje/akundaje/release/blacklists/hg38-human/hg38.blacklist.bed.gz). Enriched regions were identified using MACS2. High confidence peaks (q. val <0.05) were combined with the Irreproducible Discovery Rate (IDR) tool. Peaks were annotated with annotatePeaks.pl from HOMER [217]. Motif signatures were obtained using the 'de novo' HOMER approach. To identify putative functional TF binding sites, motif analysis was restricted to open chromatin regions of expressed genes. "Common" TFs were considered eligible for further study when they met the criteria: significant enrichment (Benjamini q-value < 0.05) of their associated motif and fold change between % of target/background sequences with motif >1. To further validate our findings, we assessed the expression of our candidate TFs on scRNA-Seq data from BLCA tumor samples (available in GSA-Human under the accession code HRA000212, [27] by restricting the analysis to the epithelial cancer cells.

To obtain a consensus list of **TFs relevant to normal urothelium** (Supplementary Table 1), we used expression data from normal bladders from the GTEx database. The threshold to define highest expressed genes was set at 4000 DESeq2-normalized counts based on gene expression distribution. Data were filtered by TFs according to the consensus list of human TFs [24]. To identify functional TFs, highly expressed TFs with significant enrichment (q.val <0.05) of their associated motif and fold change between % of target/background sequences with motif > 1 were considered. To overcome the limitations of analyzing bulk transcriptional data from normal bladders, TFs were eligible for further analysis when they showed an average SCT expression >0.4 in scRNA-Seq data from normal urothelium [27].

roles in development and differentiation, contributing to cell lineage specification and morphogenesis in a wide variety of tissues. In the adult, GATA2 is expressed broadly while GATA3 displays a more tissue-restricted expression pattern including skin, breast, and urothelium. They can act as "pioneer factors", being able to access silenced chromatin and facilitating the binding of other TFs. *GATA3* germline mutations cause Congenital Anomalies of the Kidney and Urinary Tract (CAKUT) syndrome, characterized by urogenital morphogenesis defects [49].

Gata3 ablation in the adult murine mammary gland results in luminal cell death, disruption of epithelial architecture, and expansion of an undifferentiated luminal cell population [50]. In the bladder, GATA3 is expressed in all urothelial layers and it is a well-established urothelial lineage marker [51]. In mice, inactivation of *Gata2* and *Gata3* in the urogenital system leads to morphological alterations that recapitulate the human CAKUT phenotype [52, 53]. GATA3, together with FOXA1, plays a central role in differentiation through PPARG signaling. *GATA3* silencing prevents the differentiation-associated down-regulation of TP63 and the expression of intermediate/late urothelial markers [54].

Ectopic GATA3 expression in T24 and mesenchymal-like UMUC3 BLCA cells reduces proliferation [55] and the combined overexpression of GATA3 and FOXA1 in cells with a basal phenotype leads to the expression of a luminal-specific program [44]. GATA3 binding motifs are enriched in chromatin regions that become accessible upon differentiation of normal human urothelial [54] and BLCA cells [56].

GATA3 has been proposed as one of the best markers of urothelial cell identity [57]. It is amplified in 9.8% of MIBC [14]. Expression is associated with luminal differentiation and down-regulation is common in high-grade tumors [58, 59], most notably in the Ba/Sq subtype. *GATA2* is also one of the most down-regulated genes in the Ba/Sq subtype [60] and promoter hypermethylation is significantly associated with MIBC progression [61].

FOXA1. FOXA1 (also known as HNF3α) is the founding member of the Forkhead box (Fox) superfamily. It contains a winged-helix DNA binding domain, with a helix-turn-helix core flanked by two loops that stabilize chromatin binding, and is structurally similar to linker histones H1 and H5 [62, 63]. FOXA1 typically binds as a monomer and acts as a pioneer factor [64]. FOXA1 cooperates with nuclear hormone receptors, most notably with the estrogen receptor (ER) and androgen receptor (AR) in breast and prostate epithelium, respectively, and displays sexual dimorphism [65]. It also interacts with the repressor NR0B2 [66] and with the glucocorticoid receptor (GR or NR3C1) [67].

FOXA1 is required for the development/differentiation of endoderm-derived organs such as liver, lung, pancreas, or bladder and its expression is maintained throughout adulthood [68, 69]. In the bladder, FOXA1 is expressed in all urothelial layers [54] and it cooperates with PPARG to drive differentiation [70]. In normal human urothelial (NHU) cells induced to differentiate with troglitazone and EGFR inhibition, FOXA1 is up-regulated, binds to the promoters of UPKs, and activates their expression [70].

FOXA1 is expressed in luminal BLCA and its knockdown in RT4 cells enhances proliferation while its overexpression in T24 cells decreases cell proliferation and invasion, and increases E-cadherin expression [71]. GABPA has recently been proposed as an upstream activator of FOXA1 transcription in BLCA, dictating luminal differentiation and suppressing stem cell traits and invasion. GABPA expression correlates positively with luminal signatures and with better patient survival [72]. *Foxa1* knockout mice develop preneoplastic urothelial lesions in adulthood: males develop urothelial hyperplasia while females show keratinizing squamous metaplasia [73]. The combined deletion of *Foxa1* and *Pten* in superficial and intermediate cells leads to squamous tumors [74].

FOXA1 has been proposed to have mainly a tumor suppressor role but it can also act as an oncogene in acute myeloid leukemia, esophageal, or lung adenocarcinoma [75]. It is a significant cancer driver in prostate and breast tumors [76, 77] and it is mutated in 5% of BLCA-TCGA cases [14]. FOXA1 is detected in the majority of early-stage BLCA and in luminal-type MIBC and its expression is reduced/lost in a subset of MIBC with Ba/Sq or neuroendocrine features [14, 23, 57, 59, 71, 78].

ELF3. ELF3, a member of the ETS family of TFs, is highly expressed in the bladder as well as in other endodermal tissues. ELF3 is induced during epidermal differentiation, where it regulates the expression of SPRR2A, a marker of terminal maturation [79]. Constitutive *Elf3* inactivation leads to embryonic or early postnatal lethality, associated with intestinal alterations [80]. In the urothelium, ELF3 expression increases with differentiation; in NHU cells, its up-regulation is driven by PPARG and is required for the induction of FOXA1 and GRHL3 and the expression of UPK3A [81].

ELF3 is a downstream component of WNT7B signaling, which is down-regulated in high-grade BLCA and associated with poor prognosis. Loss- and gain-of-function studies indicate that the WNT7B/FZD5-ELF3-NOTCH1 axis suppresses the epithelial-mesenchymal transition (EMT) and stem-like properties in BLCA cells [82]. ELF3 overexpression in UMUC3 mesenchymal-like cells induces an epithelial phenotype and reduces invasiveness [83].

ELF3 is a mutational driver in BLCA and cholangiocarcinoma: truncating, splice site and missense mutations, and deletions, occur in up to 14% of BLCA consistent with a tumor suppressor role. *ELF3* alterations are enriched in the LumNS consensus subtype, as are *PPARG* amplifications [23]. ELF3 is expressed in low-grade human BLCA and, together with PPARG, FOXA1, GATA3, and GATA2, it is among the most down-regulated TFs in basal tumors [60].

GRHL3. GRHL3 (or GET1**)** is a Grainyhead family member that can act both as an activator or a repressor [84, 85]. GRHL3 is highly expressed in the esophagus, skin, vagina, and bladder. In the mouse bladder, GRHL3 is mainly expressed in luminal and umbrella cells; at E16.5 and E18.5, *Grhl3*$^{-/-}$ mice show defective urothelial barrier formation and down-regulation of UPKs, which are direct target genes [86]. The Southgate laboratory showed that, together with FOXA1, it is a downstream effector of PPARG and ELF3 in NHU cells [81]. Up-regulation of GRHL3 has also been described during the urothelial differentiation of human embryonic stem cells and induced pluripotent cells [87]. It is expressed at high levels in differentiated human BLCA cells. *GRHL3* knockdown in luminal RT4 cells promotes a more invasive phenotype while overexpression in T24 cells reduces migration and invasiveness but it does not impact on proliferation/survival. These results suggest a tumor suppressor role [88].

Our analyses additionally identified **BHLHE41, MECOM, MYCL, NCOA1, NR2F2, NR2F6, REPIN1, SREBF1, TBX2, TBX3, TRAFD1**, and **ZBTB7C** as novel putative TFs that are enriched in the luminal transcriptional program and for which there is suggestive evidence for having a role in urothelial differentiation and/or BLCA (Box 2). Therefore, they merit additional study in BLCA.

TFs associated with basal/squamous differentiation ("basal-enriched")

TP63. TP63 is a member of the P53 family that contains an extended C-terminal region thought to have repressor activity [89]. At least six isoforms (TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, and ΔNp63γ) are generated through alternative splicing and alternative promoter usage [90–92]. The latter leads to TAp63 or ΔNp63, containing—or lacking—a N-terminal transactivation domain, respectively. TAp63 can transactivate P53 target genes (e.g., *CDKN1A*, *BAX* and *MDM2*), whereas ΔNp63 has both activator and repressor functions [92–94]. Both isoforms regulate transcriptional programs related to cell cycle [93], differentiation [95], apoptosis [92], and senescence [96]. Cell type-specific cofactors may contribute to determine transcriptional outputs of ΔNp63 [97]. Using epigenomic profiling, most ΔNp63 binding sites were found in enhancers, where ΔNp63 has been proposed to bookmark genes in stratified epithelia and in the squamous lineage in pancreatic cancer [98, 99]. TP63 levels are also controlled post-translationally, DNA damage being a major regulator.

TP63 proteins are expressed in the basal layer of stratified epithelia [100] and are required for epidermal development and differentiation [95]. In mice, TAp63 isoforms are detected in the urothelium at E16.5, where ΔNp63 is first detected at postnatal day 1. In the adult murine and human urothelium, TP63 isoforms are expressed in the basal/intermediate layers [101]. The diversity of TP63 isoforms has hampered acquiring a precise understanding of their spatiotemporal expression and caution is suggested when making cross-species extrapolations. *Trp63* knockout mice have severe ectodermal and heart defects and die shortly after birth

Box 2. Novel candidate TFs involved in urothelial differentiation and BLCA. TFs for which we found novel suggestive evidence favoring a role in urothelial differentiation or BLCA are listed below and basic information on their features and biology is summarized. Functional work is needed to confirm their relevance in urothelial differentiation and cancer

Luminal-enriched	
MECOM	Locus encoding for several proteins involved in transcriptional regulation and cancer, which interact with chromatin regulatory proteins (e.g., KAT2B, CTBP1, CREBBP, SMAD3) that are often deregulated in BLCA [218].
MYCL	Oncogene for which there is essentially no information on a role in urothelium. A recent study showed that transduction of human adult dermal fibroblasts with *MYCL* together with *FOXA1*, *TP63* and *KLF4* induces their conversion into urothelial cells [219]. MYCL amplifications occur in 6% of tumors in the TCGA-BLCA cohort, although deletions at the *MYCL* locus have also been reported in BLCA [220].
NCOA1	Transcriptional coactivator of steroid and nuclear hormone receptors. It is a Histone H3 and H4 acetyltransferase that cooperates with nuclear hormone receptors including RXR, PPAR, and sex hormone receptors. Its expression in BLCA is lower than in adjacent urothelium [221].
NR2F2	Ligand inducible nuclear receptor which expression is down-regulated in the bladder postnatally and a direct relationship between NR2F2 and PPARG has been suggested [222].
NR2F6	Transcriptional repressor for which no endogenous ligands have been identified [223, 224].
SREBF1	Transcriptional activator involved in cholesterol and lipid metabolism. In BLCA cells, FGFR3 signaling controls lipogenic metabolism through SREBF1 which, in turn, regulates the expression of stearoyl coA desaturase 1 which is required for cell proliferation [225]. An association with immune tumor features has been proposed [226, 227].
TBX2 and TBX3	Widely expressed T-box family repressors involved in a wide range of processes [228–230]. TBX3 is important for epidermal stem cell homeostasis [231], it is gradually down-regulated in the rat urothelium as cells differentiate [232], and it participates in the WNT-mediated mesenchymal-epithelial cell cross-talk in the murine ureter [233]. TBX2 and TBX3 are strongly down-regulated in BLCA with SCC-like features [60].
TRAFD1	Negative regulator of innate immune responses that attenuates NF-KB activation. There is essentially no information on a role in BLCA, but high expression is associated with significantly improved patient survival (HR = 0.53; $p = 0.005$, Gepia).
BHLHE41, ZBTB7C, REPIN1	No relevant publications found in Pubmed as of February 10, 2023 using the search terms "each TF identified AND bladder cancer" or "each TF identified AND urothelium"
Basal-enriched	
HMGA2	High mobility group protein family member with architectural chromatin functions. It is widely up-regulated in tumors though little is known about its function. HMGA knockdown in BLCA cells inhibits cell proliferation, migration, and invasion through TGFβ signaling [234, 235]. *HMGA2* expression is associated with an EMT phenotype [236] and mediated by several miRNA (e.g., miR-let-7c-5p and miR15a-5p) [235, 237, 238].
NR3C1	NR3C1, also known as glucocorticoid receptor (GR), is increasingly recognized as being involved in cell proliferation, invasion, and BLCA prognosis [239, 240]. Epigenomic profiling of BLCA tumors and cell lines recently identified a higher enhancer activity associated with NR3C1 in the basal subtype [176].
BACH1	Basic leucine zipper transcription factor that cooperates with MAFK in gene repression and it modulates the expression of genes involved in the oxidative stress pathway regulated by NFE2L2 [241, 242]. BACH1 is expressed broadly with highest levels in the skin and moderate levels in the bladder. In BLCA, high expression is associated with worse patient outcome.
ZBED2	Transcriptional repressor that competes with IRF1 [243]. ZBED2 is expressed at high levels in the basal layer of the epidermis, where it plays a role in keratinocyte differentiation [244]. Consistent with the enrichment for ZBED2 in basal-like organoids found in our analysis, it has recently been proposed to be a master TF for the Ba/Sq BLCA subtype [176].
SNAI2	Transcriptional repressor known for its role in epithelial-to-mesenchymal transition.
ARNTL2, ELK3, HES2, KLF7, KLF13	No relevant publications found in Pubmed as of 10th February 2022 using the search terms "each TF identified AND bladder cancer" or "each TF identified AND urothelium"
Common	
EHF	Epithelial-specific TF that acts as a transcriptional repressor and is involved in epithelial differentiation in the intestine, airways, and skin [245–247]. A tumor promoting role has been proposed in gastric, colorectal, thyroid, and ovarian cancers [248–251]. A tumor suppressive role has been described in prostate, pancreatic, and esophageal cancers [252–254]. Both amplifications and deep deletions of *EHF* have been reported at low frequency (<2%) in the BLCA-TCGA study.
NFAT5	Atypical member of the NFAT family sharing features with NF-KB [255] which is involved in the regulation of osmotic stress [256], an important process in the urothelium. *NFAT5* amplifications and missense mutations are found in 7% of cases from the TCGA cohort but little is known about their role in urothelial homeostasis and carcinogenesis.
NFE2L1	Poorly characterized TF that activates or represses expression of target genes in response to stress such as cholesterol excess, oxidative stress or proteasome inhibition [257–259]. NFE2L1 is also involved in heart regeneration [260] and its deletion in mice results in liver nonalcoholic steatohepatitis and liver cancer [261]. A TF-independent tumor suppressive effect of NFE2L1 has been described in human cancer cells through repression of Wnt/β-catenin signaling [262].
SMAD3	YY1 and SMAD3, a downstream effector of TGFβ, were identified as enriched in the luminal and basal BLCA subtypes. Both of them have been shown to participate in EMT [263–265].
YY1	

[102–104]. They develop a single-layered, umbrella cell-like, urothelium [105–107]. Genetic defects in human *TP63* have similar, but milder, phenotypic consequences [108–111].

There is little functional evidence on how TP63 proteins regulate differentiation in BLCA at the genomic level. Studies in pancreatic cancer show that, for the basal program to emerge, a concomitant down-regulation of lineage identity programs (i.e., GATA/FOXA) and an up-regulation of ΔNp63 is required [112].

TP63 is a mutational driver in BLCA, largely through missense mutations. In MIBC, *ΔNp63α* is the most abundant transcript, present at highest levels in Ba/Sq tumors and at low/undetectable levels in luminal and neuronal subtypes [113]. *ΔNp63* is up-regulated in NMIBC and MIBC compared to normal urothelium while *TAp63* is undetectable [114]. Similar findings were made when analyzing total *TP63* in the UROMOL and TCGA-BLCA cohorts [14, 21, 114]. Expression of TP63 isoforms has been associated with tumor stage and molecular subtype: $\Delta Np63^{-}$ tumors are $KRT20^{+}$ while $\Delta Np63^{+}$ tumors are $KRT5^{+}$ [101]. The association of TP63 expression with patient outcome is controversial [101, 113]. Sarcomatoid MIBC lack ΔNp63 expression in association with the worst outcome [115].

TFAP2s. TFAP2 family members (A-E) contain a N-terminal transactivation domain, a C-terminal helix-span-helix domain, and a central basic region. They are expressed broadly and play important roles in mouse development [116]. TFAP2s have been implicated in epidermal differentiation in mice [117] and humans [118]: TAp63α directly induces *Tfap2c* during differentiation and its knock-down blunts *Krt14* expression [116]. In keratinocytes, functional TFAP2 binding sites have been identified in the promoter of *Krt5* and other differentiation genes [117–119] and TFAP2A and TFAP2C co-regulate a subset of ΔNp63 targets [120]. In the murine urothelium, expression of *Tfap2a*, *Krt6a*, *Krt14*, and *Krt15* is repressed by PPARG, further suggesting that TFAP2A is part of the basal program [35].

TFAP2A and TFAP2C are up-regulated in Ba/Sq BLCA cells and tumors and in areas of squamous metaplasia [121]. In BLCA patients treated with cisplatin, TFAP2A expression is an independent predictive marker of response and survival. Neither *TPAP2A* nor *TFAP2C* are identified as mutational driver genes in BLCA.

MYC. MYC is a basic helix-loop-helix (bHLH) and leucine zipper (LZ) protein that is ubiquitously expressed and plays a central role as an oncogene in almost all tumor types as it is crucially required for cell proliferation [122]. It regulates the expression of ~10% of the protein-coding genes.

Aberrant MYC activity in BLCA occurs through a wide variety of mechanisms such as genetic alterations [123], transcriptional and post-transcriptional regulation [124–126], or altered protein stability [127]. *MYC* amplifications occur in ~9% of MIBC and it is overexpressed—and its program is enriched—in a subset of T1 BLCA [128]. A recent study identified MYC as a key effector of mutant FGFR3 signaling and *FGFR3* as a direct transcriptional MYC target. Consistently, MYC and FGFR3 levels were directly correlated in *FGFR3*-mutant tumors [125]. However, other work has shown an enrichment of *MYC* expression and activity in basal tumors [59, 129] and functional studies in human BLCA cells revealed that MYC expression is controlled by TP63 [130]. These findings highlight complex, context-dependent, roles of MYC in BLCA biology.

NOTCH. NOTCH is involved in a wide range of biological processes and mechanistically links cell-cell interactions to transcriptional responses. In our analyses, its critical effector RBPJ is among the top expressed TFs in normal urothelium and BLCA organoids and its target HES2 is significantly enriched in basal BLCA organoids. Notch signaling is required at multiple levels during development.

In normal urothelium, *NOTCH1* is up-regulated during differentiation [131] and, in normal murine urothelial organoids, Notch pathway inhibition results in up-regulation of *Tp63* and reduced expression of luminal markers [32]. Tissue-specific inducible inactivation of Notch in the mouse urothelium led to hyperplasia, inflammation, and mucosal sloughing [132].

Both oncogenic and tumor-suppressive roles have been described for the Notch pathway in a context-dependent manner in several tumor types. In BLCA, *NOTCH1* inactivation is more common in Ba/Sq tumors and results in increased MAPK signaling [131, 133, 134]. In contrast, *NOTCH2* appears to act as an oncogene [135]. Both tumor-suppressive and oncogenic roles have been attributed to NOTCH3 [135, 136]. Mutations in *NOTCH1*, *NOTCH2,* and *NOTCH3* occur in 6%, 12% and 4% of samples in the TCGA-BLCA cohort [14], respectively. Missense mutations are the most frequent alteration in *NOTCH1* and *NOTCH3* though truncating mutations and amplifications are also reported. *NOTCH2* copy number gains/amplifications have been reported in Ba/Sq tumors, associated with lower survival in the TCGA cohort [135]. Other NOTCH pathway genes such as *MAML1*, *NCSTN*, *PSEN1* are also significantly mutated in BLCA, regardless of stage or grade [133].

ETS family. ETS1 and ETS2 are characterized by the presence of a conserved ETS DNA-binding domain that recognizes the core consensus DNA sequence GGAA/T in target genes. They can act as transcriptional activators or repressors and play a key role in the regulation of cell proliferation, differentiation, survival, invasion, and angiogenesis [137–139].

ETS1 is expressed in the normal urothelium and its down-regulation is associated with high-grade/stage BLCA [140]. However, another study reported high levels of *ETS1* mRNA in BLCA, compared to paired normal tissue. *ETS1* silencing in BLCA cells inhibits cell migration and invasion, suggesting an oncogenic role [141]. Two microRNAs (miR-193b-3p and mir-106a) whose down-regulation is associated to tumor progression suppress ETS1 expression and, as a consequence, proliferation, migration and invasion of BLCA cells [142, 143]. Deep deletions of *ETS1* and *ETS2*, as well as *ETS2* amplification, have been reported in <1% tumors from the TCGA.

As shown in Box 2 and Table 1, our analyses additionally identified **ARNTL2, BACH1, ELK3, HES2, HMGA2, KLF7, KLF13, NR3C1/GR, SNAI2** and **ZBED2** as significantly associated with the basal transcriptional program and there is suggestive evidence of their role in urothelial differentiation and/or BLCA. Therefore, these TFs merit further investigation in BLCA.

TFs common to luminal and basal subtypes

KLF4 and KLF5. Krüppel-like factors (KLFs) act either as activators or repressors and participate in a wide range of cellular processes, including epithelial differentiation, in multiple tissues.

Klf5 deletion in the developing murine bladder impaired urothelial stratification and differentiation [144], accompanied by reduced expression of *Pparg*, *Elf3*, and *Grhl3*, supporting its participation in a urothelial GRN [144]. Similarly, KLF5 is required for basal-to-luminal differentiation in the prostate [145]. In BLCA cells, KLF5 overexpression increases proliferation, lamellipodia formation, and cell migration by direct promoter binding and up-regulation of the *FYN* kinase [146]. KLF5 may also promote angiogenesis by directly regulating *VEGFA* [147].

Genetic alterations in *KLF4* (3%; amplifications and missense mutations), *KLF5* (8%; amplifications, deletions, missense mutations), and *KLF6* (9%; amplifications) are most frequent in the TCGA-BLCA cohort. KLF4 and KLF5 are abundant in normal urothelium [148]. *KLF4* is frequently down-regulated in BLCA cells and tissues through promoter methylation [149]. Its overexpression triggers apoptosis in vitro [148, 150] and inhibits tumor growth in vivo [151], consistent with a tumor suppressor role.

SP1 and SP3. The specificity protein (Sp) TFs belong to the Sp/KLF family and consist of four members in humans: Sp1, Sp2, Sp3,

and Sp4 [152]. Sp1 and Sp3, which emerge as "common" TFs in our analyses, are ubiquitous TFs implicated in the control of a wide variety of cellular processes [153]. In BLCA, they have been reported to play a role in cell invasion through regulating the expression of the metalloproteinase MMP2 [154, 155]. In tumors, SP1 expression is associated with poor prognosis and progression [156].

BIOLOGICAL PROCESSES/PATHWAYS INVOLVED IN BLCA UROTHELIAL LINEAGE FIDELITY

Our analyses have identified other TFs associated with biological processes for which there is evidence of their involvement in urothelial differentiation and/or BLCA. The majority of them were highly expressed in both luminal and basal organoids—hence classified as "common TFs" (Table 1).

Xenobiotic metabolism and oxidative stress. The P450 cytochromes, epoxide hydrolase, and glutathione S-transferases (GST) are involved in xenobiotic metabolism, protecting urothelial cells from damage impinged by the continued contact of urothelial cells with urine and exposure to carcinogens. Consequently, polymorphisms in genes involved in xenobiotic metabolism are associated with an increased BLCA risk (reviewed in [157]). Tobacco and urinary infections, which are known to cause oxidative stress, are also associated with increased BLCA risk [158].

AHR encodes a bHLH protein that acts as a "sensor" through binding to a wide variety of xenobiotics (e.g., halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons) as well as endogenous molecules (e.g., flavonoids and metabolites of tryptophan) (reviewed in [159]). Upon ligand binding and nuclear translocation, AHR dimerizes with **ARNT** and regulates expression of multiple phase I (e.g., *CYP1A1*, *CYP1A2*, and *CYP1B1*) and phase II genes (e.g., NAD(P)H Quinone Dehydrogenase 1, *NQO1* and *GSTs*) [160–162].

AHR is expressed at high levels in the urothelium (GTEx), in NMIBC [163], and in luminal MIBC [164] and the gene is mutated in 12% of MIBC in the TCGA cohort, 52% of which are amplifications. Recurrent in-frame deletions of *AHR* exons 8 and 9 occur in primary and metastatic BLCA [165]. Mutant AHR is constitutively active, down-regulates differentiation markers, up-regulates stem cell markers, and confers anchorage independent growth to bladder organoids [166]. Upon BBN administration, *Ahr*-null mice develop MIBC at a much higher frequency than *Ahr* wild-type mice suggesting a tumor-suppressive role in normal urothelium [163].

NFE2L2 (Nuclear factor, erythroid 2 like 2 or NRF2) is a master regulator of the response to oxidative stress. NRF2 regulates the expression of AHR as well as genes coding for proteins with antioxidant activity (e.g., *NQO1, SQSTM1, GSTA1, GSTM1, and GSTP1*) that may be important in bladder carcinogenesis [166]. *NRF2* amplifications or hotspot missense mutations in 7% of MIBC in the TCGA cohort suggest an oncogenic role in BLCA, but little is known at the mechanistic level. Additional processes may lead to NRF2 hyperactivation including down-regulation of the KEAP1-binding protein GULP1 as a result of promoter methylation, especially in MIBC [167]. **BACH1**, a basic LZ TF that regulates the expression of genes involved in the oxidative stress pathway mediated by NFE2L2, is enriched in basal-like organoids and tumors (Table 1). Our analysis also found **NFE2L1** (or NRF1) among the highest expressed TF in both BLCA organoids and normal bladder, although there is virtually no information on its role in the urothelium or in BLCA (Box 2).

The circadian clock machinery harmonizes physiological processes to provide homeostasis within the 24h cycle and is modulated by clock genes. The diurnal variation in bladder function and the time-dependent expression of these genes support a role in urothelial function [168], consistent with the robust expression of **NPAS2, ARNTL2/BMAL2**, and **BHLHE41/DEC2** in BLCA organoids (Table 1). Altered expression of clock-related genes has been reported in BLCA [169]. Iyyanki et al. have recently described NPAS2 as a novel luminal TF that binds and regulates the *FOXA1* promoter and is, in turn, up-regulated upon overexpression of FOXA1 or GATA3. This regulatory loop may contribute to BLCA cell proliferation and migration [56]. **BHLHE41/DEC2** is also luminally-enriched, interacts with ARNTL and other nuclear receptors (e.g., RXRA, LXR, and VDR), and may contribute to repress the expression of genes controlled through the circadian rhythm pathway [170].

Inflammatory pathways are critically involved in cancer and a transcriptional link between cell differentiation and inflammation is increasingly recognized [171]. Several families of TFs involved in inflammation appeared in our analysis, including Activator protein 1 (AP-1), Interferon response factors (IRFs), Nuclear Factors of Activated T-cells (NFATs), and Signal Transducers and Activators of Transcription (STATs) (Fig. 2, Table 2).

AP-1 includes a ubiquitous type of homo- or heterodimeric complexes composed of members of four gene families: *JUN*, *FOS*, *ATF*, and *MAF*, all of which were represented in our analysis both in BLCA organoids and normal bladder (Table 1 and Supplementary Table 1). AP-1 signaling is activated in response to multiple stimuli (e.g., inflammation, stress, pathogens) and participates in proliferation, differentiation, and apoptosis [172]. The effects of AP-1 are highly context-dependent. In the urothelium, its activation by tobacco smoke results in abnormal differentiation [173]. AP-1 cooperates with several of the major BLCA TFs. Genomic regions bound by FOXA1, GATA3, and KDM6A are enriched in AP-1 motifs in BLCA cells [44, 56, 174, 175]. A recent epigenomic map of co-regulated enhancers and associated transcription factors identified AP-1, as well as SMAD2/3, NF-kB, and STAT3, as potential regulators of basal enhancers [176]. This is consistent with our findings of a significant up-regulation of AP-1 target genes in basal organoids (Supplementary Fig. 2).

Amplifications are the most common alterations in AP-1 genes in tumors. Highly frequent copy number gains of *FOSL2/FRA2* significantly associate with advanced stage, high grade, and low disease-free survival in both MIBC and NMIBC patients [177].

IRFs activate or repress gene expression by binding to the interferon-stimulated response element. *IRF6* and *IRF3* are among the highest expressed TFs in basal-like BLCA organoids and normal bladder, respectively. IRF6 is involved in epithelial differentiation and cell cycle regulation, partly synergizing with SMAD4 in response to TGF-β [178, 179] and NOTCH signaling [180]. In NHU cells, IRF1 binds the promoter of differentiation-associated genes (e.g., *UPK*) and enables their expression upon PPARG activation [70].

STATs are expressed broadly in the bladder, in epithelial and non-epithelial cells, and mediate cellular immunity, proliferation, apoptosis, and differentiation. They activate gene expression upon phosphorylation by Janus kinases, emphasizing that post-translational modifications add an important layer of regulation that cannot be fully captured through the sole analysis of genomic data. In our analyses, *STAT1*, *STAT3*, and *STAT6* appeared as relevant TFs for normal bladder and both basal and luminal tumors (Table 1). They are altered in 4%, 1.7%, and 2.9% of the TCGA-BLCA samples, respectively, mainly through missense mutations and amplifications. In this cohort, STAT1 was identified as a key regulator of an immune GRN [181, 182]. STAT1 expression positively correlates with levels of PD-L1 [183].

Several studies point to STAT3 as tumor-promoting. p-STAT3 levels are significantly higher in basal BLCA, particularly in SCC-like tumors [60, 184, 185]. Consistently, our differential gene expression analysis in luminal vs. basal organoids reveals a significant up-regulation of STAT targets in basal urothelial cells (data not shown). STAT3 is critical for the regulation of basal subtype-specific genes [56] and STAT3 regulon activity is significantly enriched in Ba/Sq tumors [23]. Increased expression of STAT3

Table 2. Molecular features of candidate TFs.

TF	Structural family	Activator/ Repressor	Tissue distribution	Major roles	Mouse phenotype	Refs
AHR	Nuclear receptor	Activator	Enhanced in liver and in barrier tissues such as skin, lung, gut and urothelium. Important role in immune cells	Xenobiotic metabolism and oxidative stress; endogenous ligands	Null mutants have liver defects, impaired female fertility, neonatal or postnatal lethality, and spleen abnormalities. Conditional KO in the retina leads to retinal degeneration. Conditional KO in neural progenitor cells leads to decreased susceptibility to ischemic brain injury	(1,2)
AR	Nuclear receptor	Both	Enhanced in male tissues, liver and bladder	Male sexual phenotype	Conditional activation of transgenic AR expression increases BBN-induced BLCA. AR knockout reduces BLCA incidence	(3–5)
ARNTL2	bHLH-PAS	Activator	Tissue enhanced (esophagus, lymphoid tissue, bladder, female tissues)	Circadian clock	Circadian clock, metabolic phenotypes, and decrease thymocyte apoptosis and proliferation	(6,7)
BACH1	bZIP	Both	Broad (skin)	Oxidative stress pathway; antagonizes NRF2	No obvious phenotype	(8,9)
BHLHE41	bHLH	Both	Tissue enhanced (retina, skeletal muscle, brain)	Circadian clock	Homozygous *Bhlhe41/Sharp-1* KO mice display altered circadian rhythmicity (jet lag phenotype), sleep length, and immune cell development	(10)
EHF	ETS family	Repressor	EHF is expressed mainly in the salivary gland, esophagus, vagina and - to a lower extent - in prostate, colon, skin, bladder, and breast	Epithelial differentiation in the intestine, airways and skin	No global KO; *Ehf*-KO intestinal crypts exhibit reduced stem cells with impaired organoid formation capacity	(11)
ELF1	ETS family	Both	Tissue enhanced (bone marrow)	Haematopoiesis and angiogenesis	No obvious phenotype	(12)
ELF3	ETS family	Both	Epithelia (bladder, other endodermal, epidermis,)	Cell differentiation; interacts with the Wnt and Notch pathways; suppresses EMT	Embryo or early postnatal lethality, associated with altered intestinal differentiation	(13)
ELK3	ETS family	Both	Low tissue specificity	May be a negative regulator of transcription, but can activate transcription when coexpressed with Ras, Src, or Mos	Elk3 deficiency causes transient impairment in post-natal retinal vascular development and formation of tortuous arteries in adult murine retinae	(14)
ER	Nuclear receptor	Both	Enhanced in female tissues	Female sexual phenotype	ERα KO increases BBN-induced BLCA incidence; ERβ KO reduces BBN-induced BLCA incidence	(15,16)
ETS2	ETS family	Both	Low tissue specificity	Development and apoptosis	Embryonic lethal by E8.5 (defective trophoblast formation). Rescued Ets2-deficient mice are viable and fertile but have wavy hair, curly whiskers, and abnormal hair follicle	(17)
FOSL2	bZIP	Both	Broad	proliferation, differentiation, apoptosis	Global *Fosl2* KO mice die shortly after birth	(18)
FOXA1	Forkhead	Activator	Broad epithelial	Pioneering functions	*Foxa1* KO present preneoplastic lesions in the urothelium	(19)
GATA2	GATA	Activator	Broad	Pioneer factor (master hematopoietic regulator)	*Gata2* KO in development leads to severe anemia and subsequent lethality	(20)
GATA3	GATA	Activator	Moderate levels are detected in bladder, kidney, and breast and to a lesser extent in lymphoid tissues and both male and genital system	Pioneer factor	*Gata3* null embryos die due to severe defects in the nervous system at E11-12	(21)
GRHL2	Grainyhead	Both	Tissue enhanced (skin)	Primary neurulation and epithelial development	Embryonic lethal by E11.5 (failed neural tube closure resulting in thoraco-lumbo-sacral spina bifida)	(22)
GRHL3	Grainyhead	Both	Restricted (esophagus, skin, vagina, bladder)	key role in the differentiation of stratified epithelia of both ectodermal and endodermal origin	*Grhl3* KO mice die at birth, showing neural tube defects, defective skin barrier formation, wound repair, and eyelid fusion	(23,24)
HES2	bHLH	Repressor	Restricted (skin, esophagus, placenta)	Notch effector	Lethal	(25)
HMGA2	High mobility group	Both	Broad (low)	Chromatin architectural function	Impaired muscle development and reduced myoblast proliferation; impaired lung development	(26,27)
IRF6	IRF family	Activator	Broad (esophagus, skin)	Epithelial homeostasis	Perinatal lethality due to esophageal adhesions, epidermal hyperproliferation and soft-tissue fusions	(28)
JUNB	bZIP	Mainly activator	Broad	Proliferation, differentiation, apoptosis	Global KO is embryo lethal due to impaired placentation and vascular defects	(29)
KLF13	C2H2 zinc finger	Repressor	Low tissue specificity	Suppress proliferation and cholesterol biosynthesis, regulates T Lymphocyte survival	*Klf13* KO mice show a defect in lymphocyte survival	(30)
KLF3	C2H2 zinc finger	Repressor	Broad (high)	Differentiation, proliferation, haematopoiesis	Homozygous: perinatal lethality + abnormal adiposity, B cell development, erythroid maturation + heart defects// Heterozygous: cardiovascular abnormalities	(31–33)
KLF5	C2H2 zinc finger	Activator	Broad epithelial (skin, esophagus, bladder, intestine)	Epithelial differentiation	*Klf5* KO embryos are lethal due to implantation defects. Deletion of *Klf5* in the developing murine bladder impaired urothelial stratification and differentiation	(34–36)
KLF6	C2H2 zinc finger	Activator	Broad (enhanced in bone marrow)	Tumor suppressor, role in proliferation, differentiation, immunity	*Klf6* KO mouse is embryonic lethal, with reduced hematopoiesis and disorganized vascularization	(37,38)
KLF7	C2H2 zinc finger	Repressor	Low tissue specificity	Proliferation, differentiation, adipogenesis, metabolism	Neonatal lethality, impaired neurogenesis	(39)
MECOM	C2H2 zinc finger	Activator	Broad (bladder, kidney, lung and stomach)	Oncogenic activity; complex locus	Homozygous disruption of the *Evi1* leads to embryonic lethality	(40)
MYCL	bHLH	Activator	Broad epithelial (pancreas, skin, bladder, oesophagus and vagina)	Cell proliferation; proto-oncogene	Lack of apparent phenotype pointing to a dispensable role for MYCL for gross morphological development	(41)
NCOA1	bHLH	Activator	Broad	Nuclear receptor family coactivator	*Ncoa1* KO mice are viable but display hormone resistance	(42,43)
NFAT5	NFAT family	Activator	Broad (kidneys, skin, eyes)	Regulates osmotic stress	Embryonic lethality due to cardiac failure	(44)
NFE2L1	CNC-bZIP	Both	Tissue enhanced (skeletal muscle, tongue, kidney, bladder)	Redox homeostasis and metabolism	Impaired metabolism homeostasis, liver steatohepatitis and neoplasia	(45–47)
NFE2L2	bZip-CNC	Both	Broad (enhanced in esophagus)	Response to oxidative stress	Increased incidence of BLCA induced by BBN in *Nrf1* KO mice	(48)
NPAS2	bHLH-PAS	Activator	Broad (bladder, esophagus, and skin)	Circadian clock	-	-
NR2F2	Nuclear receptor	Activator	Tissue enhanced (ovary)	Heart development and stomach patterning	KO mice show congenital diaphragmatic hernia	(49)
NR2F6	Nuclear receptor	Repressor	Tissue enhanced (liver)	Development of forebrain circadian clock	KO mice show abnormal development of the locus coeruleus impairing functionality of the forebrain clock and affects nociception	(50)
NR3C1	Nuclear receptor	Activator	Low tissue specificity	Binds to glucocorticoid response elements in the promoters of glucocorticoid responsive genes to activate their transcription. Involved in inflammatory responses, cellular proliferation, and differentiation in target tissues	KO mice show variable phenotype depending on background	-
PPARG	Nuclear receptor	Both	Expressed at high levels in white and brown adipose tissue and in breast and to a lesser extent in bladder, colon, and lung (GTEx portal)	Metabolism, glucose homeostasis, adipogenesis; Heterodimerizes with RXRA	Lethal at E10.5–11.5 due to placental dysfunction	(51)
REPIN1	C2H2 zinc finger	-	Broad	Metabolism modulation in liver and adipose tissue	KO mice show altered glucose and lipid metabolism	(52,53)
RXRA	Nuclear receptor	Both	Broad	Metabolism, glucose homeostasis, adipogenesis. Broader role than PPARG; Heterodimerizes with PPARG and other nuclear receptors	KO mice die at E14.5 and show myocardial and ocular malformations	(54)
SMAD3	SMAD family	Both	Broad	Cell proliferation (tumor suppressor)	*Smad3* KO mice at 4 to 6 months develop colorectal carcinomas	(55)
SNAI2	C2H2 zinc finger	Repressor	Low tissue specificity	Binds to E-box motifs and is likely to repress E-cadherin expression. Involved in epithelial to mesenchymal transition.	KO mice show postnatal growth retardation and hematopoietic progenitor cells have increased sensitivity to radiation-induced apoptosis in vivo	(56)
SP1	Sp/XKLF family	Both	Low tissue specificity	Cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodelling	KO mice die during organogenesis (E11) with a broad range of developmental defects	(57)
SP3	Sp/XKLF family	Both	Low tissue specificity	Cell-cycle regulation, hormone-induction and house-keeping.	KO mice die immediately after birth due to respiratory failure	(58)

Table 2. continued

SREBF1	bHLH-Zip	Activator	Broad	Cholesterol and lipid metabolism	*Srebf1* KO mice exhibit alterations in fatty acid and triglyceride biosynthesis	(59)
STAT1	STAT family	Mainly activator	Low tissue specificity (low)	IFN response	Failed biological responses to IFNα or IFNγ; affected lymphocyte survival and proliferation	(60)
STAT3	STAT family	Activator	Broad (medium)	Proliferation, apoptosis, immunity, and cell motility	Early embryonic lethality; Stat3-transgenic mice (K5.Stat3C) develop a skin phenotype and invasive bladder cancer directly from carcinoma in situ (CIS) bypassing the non-invasive papillary tumor stage	(61–63)
STAT6	STAT family	Activator	Broad (enhanced in bladder, female tissues, bone marrow)	Central role in IL4 and IL3 mediated responses	Impaired Th2 immunity	(64)
TBX2	T-box family	Repressor	Broad	Processes involved in development, including proliferation, cell fate determination and differentiation	*Tbx2* homozygous loss result in lethal cardiovascular defects by day 14.5	(65)
TBX3	T-box family	Repressor	Broad but enriched in adrenal gland, seminal vesicle and prostrate	Linked to stem-cell properties and is a pluripotency-related transcription factor	Homozygous deletion of *Tbx3* results in abnormal heart, limbs, mammary glands and yolk sac and the mutants die in utero by day E12.5. In adult mice, deletion of TBX3 in the epidermis impairs regeneration after injury	(66–68)
TFAP2A	AP-2	Both	Broad but enriched in retina, trophoblastic cells and squamous epithelia (breast ad skin).	Epidermal differentiation; target of TP63; regulates epidermal KRT expression	Homozygous null mutants die perinatally with anencephaly, craniofacial and neural tube defects, thoraco-abdominoschisis and defects in sensory organs, cranial ganglia, skeleton, and heart. On some genetic backgrounds, heterozygotes may exhibit exencephaly	(69–71)
TP63	p53 family; many isoforms	Both	Stratified epithelia, bladder	Skin development and maintenance	All squamous epithelia and its descendants, including the mammary, lachrymal, salivary, and prostate glands, are absent in mice with homozygous *Tp63* null mutation. Mutants have tiny genitalia, missing or amputated limbs, craniofacial deformities, and neonatal death	(72–74)
TRAFD1	-	-	Broad (bladder, skin, esophagus, and intestine)	Innate immune response; attenuates NF-KB	Reduced body weight	(75)
YY1	GLI-Kruppel family of Zn finger	Both	Broad (bladder, esophagus, and thyroid)	Regulates histone acetylation; recruits PRC2	Preimplantation lethality	(76)
ZBED2	Zinc Finger BED-Type Containing 2	Repressor	Tissue enhanced (lymphoid tissue, thyroid gland)	Keratinocyte differentiation	-	(77)
ZBTB7C	BTB domain containing, Zinc fingers C2H2-type	Repressor	Enhanced in esophagus	Cell cycle and metabolism	KO mice show altered adipogenesis and lipid metabolism	(78)

Main features of candidate TFs summarized from the GeneCards human gene database and Uniprot. Tissue distribution information was retrieved from The Human Protein Atlas. Note: AR and ER do not directly come up in our analysis but are included in the Table based on the existing evidence of their participation in BLCA and for the sake of completeness. A list of detailed references can be found in Supplementary Material. Color code: luminal-enriched (purple), basal-enriched (teal), and common (beige).

targets predicts basal-type phenotype and associates with worse prognosis [186]. Furthermore, STAT3 signaling is activated in response to urological infections, which may increase the risk of BLCA, and is required for their resolution [187, 188]. Collectively, these data suggest that STAT signaling may play a pro-tumorigenic role in BLCA at early stages of tumor development.

Two transcriptional pathways that did not stand out in our analyses but that are relevant to differentiation and BLCA are sex hormones and the HOX developmental gene family.

Sex hormones. The gender bias associated with BLCA has long raised interest in the role of sex hormones. In MIBC, males are enriched in the LumP and neuroendocrine-like subtypes and females are enriched in the Ba/Sq-like subtype. Sex-specific differences in AR activity have been reported among the luminal-like subtypes, suggesting that luminal differentiation could be partly driven by AR signaling. The activity of the ER pathway has been associated with the luminal MIBC subtype [59].

Several studies have reported an inverse association between AR expression and tumor stage [189–191]. Moreover, AR co-regulators (e.g., NCOA1, NCOA2, NCOA3, and CREBBP) are enriched in BLCA [189]. Functional analysis suggests that high AR activity could increase the susceptibility of BLCA cells to carcinogens, as well as exert a direct pro-tumoral effect [192, 193]. *Ar* knockout mice of both sexes were completely protected from BBN-induced BLCA and androgen deprivation suppressed tumor growth in vivo [192].

The involvement of ER in BLCA is more complex. ERβ expression has been positively associated with stage and grade [194]. Regarding ERα, there is controversy on its association with tumor grade, stage, and outcome. Studies in knockout mice have reported opposing roles for ERα and ERβ, the former having a protective role and the latter promoting carcinogenesis [195, 196].

Developmental HOX genes. HOX genes are expressed upon urothelial differentiation and their levels are higher in the urethra than in the bladder urothelium. In our analyses, *HOXA13* was found to be expressed at highest levels in normal bladder. In mice, *Hoxa13* is expressed in the embryonic urinary tract and postnatally, and it is essential for the morphogenesis of the urogenital and digestive tracts [197]. Mutations in *HOXA13* are associated with hand-foot-genital syndrome and urinary tract malformations [198].

In NMIBC, a transcriptional network of cell cycle dysregulation pointed to *HOX* gene activity [199]. Marzouka et al. reported an inverse association between *HOXB* and late cell-cycle gene expression in BLCA [200]. Approximately 20% of MIBC from the TCGA cohort harbor mutations and/or copy number changes in *HOX* genes, amplification being the most frequent alteration. Genome-wide methylation analyses revealed an association between gene copy number gains, high methylation, and low expression for *HOX* genes in MIBC [201]. This highlights the importance of DNA methylation for *HOX* gene regulation and points to a tumor suppressor-like activity of the *HOX* family. Consistently, Aine et al. reported a correlation between epigenetic inactivation of *HOX* genes and tumor differentiation [202]. In the TCGA cohort, expression of 24 *HOX* genes (e.g., HOXA13, HOXB1-8) was significantly higher in LumP than in Ba/Sq tumors (FDR < 0.05), whereas eight genes were significantly down-regulated (e.g., *SHOX2*, *PHOX2A*, *HOXD10-13*). This is consistent with the fact that luminal BLCA organoids express significantly higher levels of *HOXB* genes, the expression of which is activated upon retinoid-induced differentiation [203]. In contrast, expression of *HOXD* genes was reduced. Altogether, these findings provide support to the role of this gene family in urothelial differentiation and BLCA.

BLCA GENE REGULATORY NETWORKS

Few studies have aimed at building BLCA GRNs, possibly due to the scarcity of functional genomics data. The CoRegNet (Co-regulatory network) tool integrates transcriptomic, gene copy number, and ChIP data [204, 205]. When applied to BLCA, CoRegNet confirmed

the driver role of PPARG, FOXA1, and GATA3. This is consistent with another study combining chromatin accessibility and gene expression data to build a network of TFs modulating urothelial differentiation in vitro upon PPARG activation. This work also found that GATA3 and FOXA1 cooperate, driving expression of luminal genes, which were repressed by TP63 [54].

Champion et al. have built a reference GRN from NHU cells and analyzed perturbations thereof in BLCA [206]. They inferred GRNs for the TCGA molecular subtypes, computed a deregulation score for each target gene in each subtype, and identified deregulated TFs. This approach highlighted 108 and 137 deregulated TFs in Ba/Sq and LumP samples, respectively. PPARG and NOTCH4 were among the 10 most important TFs accounting for transcriptomic deregulation in LumP and Ba/Sq BLCA, consistent with previous knowledge. Liang et al. have used H3K27ac ChIP-Seq data to identify super-enhancer (SE)-regulated genes and reconstruct a network including SMAD3, ETS1, and HOXB2 as core TFs in BLCA cells [207].

More recently, Neyret-Kahn et al. integrated RNA-Seq with ChIP-Seq data of active (H3K27ac) and repressive (H3K27me3, H3K9me3) histone marks in human primary tumors and cultures of BLCA and primary NHU cells [176]. They found chromatin states distinguishing basal and luminal samples that were associated with molecular BLCA subtypes. By analyzing H3K27ac, they inferred subsets of cancer-specific and subtype-specific SE and created SE-regulated networks. They confirmed FOXA1 as a major driver, provided evidence indicating that it also suppresses inflammatory programs, and discovered ZBED2 as a novel basal-specific TF that dampens IFN responses through STAT2. This is consistent with the enrichment we find for ZBED2 in basal-like organoids. These findings support that cell identity and inflammatory programs are part of the same TF networks, as previously described in the pancreas [171].

To expand this knowledge, we have used PPARG, RXRA, FOXA1, and GATA3 ChIP-Seq data to assess their binding to the promoter, gene body, and intergenic regions of the genes coding for the BLCA TFs identified in our analysis (Fig. 4). RXRA and PPARG show the highest and the lowest number of targets in our network, respectively. However, ChIP-Seq for them was performed in basal but not in luminal BLCA cells, which could affect these findings. In RT4 cells, FOXA1 and GATA3 binding was enriched in gene bodies and intergenic regions, compared to promoters, consistent with their reported role at distal enhancers [56]. The paucity of available data calls for expanding functional genomics analyses to establish the landscape of transcriptional regulation mechanisms in BLCA.

TRANSCRIPTIONAL REGULATION OF BLCA PLASTICITY AND TUMOR HETEROGENEITY

Histological and genomic analyses of BLCA have provided insight into cellular heterogeneity and plasticity, two prominent contributors to tumor progression and therapy resistance. The underlying evidences include: (1) histological heterogeneity and morphological changes occur during the disease course, (2) subclonal mutations undergo clonal selection over time, and (3) tumors with distinct gene expression signatures cluster into molecular subtypes. However, bulk tissue analyses fail to capture the diversity of individual cells and subclones, cannot fully explain the molecular underpinnings of heterogeneity and plasticity, and fail to provide information on the spatial configuration of cell-cell interactions. Single-cell technologies and multiplex analyses - at the RNA or protein level - provide resolution at the individual or quasi-individual cell level, allowing discovery of novel cell populations or states following gene ontologies. Single cell RNA-Seq (scRNA-Seq) has been used to infer cell lineage plasticity, hierarchies, and developmental trajectories. Understanding intermediate states in tumor evolution is of particular interest as this can lead to discovering key TFs acting as drivers of this process.

To delineate BLCA plasticity and tumor heterogeneity, Sfakianos et al. performed scRNA-Seq in human MIBC and in murine BBN-induced tumors [208]. Immune cell and non-immune cells were sorted from BBN-treated urothelium and scRNA-Seq revealed the existence of multiple cell populations within the tumor compartment. Differential expression analysis between the clusters showed their correspondence with well-known transcriptomic subtypes. For example, distinct populations expressed high levels of luminal-type TFs (*Pparg*, *Foxa1*, and *Gata3*) while others expressed basal-type (*Trp63*) or EMT-like (*Zeb1*, *Zeb2*, *Snai2*, and *Twist1*) TFs. These tumors are, thus, composed by a mosaic of heterogeneous cell populations. When analyzing human samples, they further identified individual epithelial cells with gene expression patterns characteristic of luminal, basal, and EMT-like transcriptomic subtypes. Using patient-derived xenografts, human BLCA cell populations with basal and EMT-like subtypes that can undergo lineage plasticity and subtype switch were identified [208].

In another study, scRNA-Seq combined with scATAC-Seq were used to resolve cell types, cell states, and tumor heterogeneity in BLCA [209]. Cancer stem cell markers were enriched in basal cell clusters. Regulon analysis revealed two groups of BLCA stem cells: one expressing *CHD2*, *SIN3A*, *YY1*, and *KDM5B* and another group expressing *EZH2, SOX15*, *ATF3*, and *KLF10*. EZH2 is a component of the Polycomb Repressive Complex 2 responsible for the H3K27me3 mark associated with transcriptional repression. Depletion of EZH2 in BLCA cells compromised cell proliferation, colony formation, and migration. *EZH2*-null BLCA xenografts in nude mice had decreased size and reduced proliferation. Loss of *EZH2* was associated with enhanced chromatin accessibility near up-regulated genes, including those involved in stemness, differentiation, and cell-adhesion. Conversely, *CD44*, *NCAM1*, *CDH2*, and *VIM* genes showed reduced accessibility. DNA footprinting analysis of open chromatin regions specific for EZH2 wild-type cells further identified the TCFL5, TCF4, and CEBPB motifs, suggesting that EZH2 regulates BLCA stemness through these TFs. Our analyses revealed that *CEBPB* expression was highly enriched in normal and cancer urothelial cells according to bulk and scRNA-Seq data. Altogether, these data suggest that EZH2 is important for the stemness phenotype of certain BLCA populations and is relevant to patient outcome [210].

We have also analyzed public scRNA-Seq data on eight BLCA tumor samples [27]. We identified three clusters of urothelial cells defined as "Luminal_FABP4^{+}", "Luminal_UPK^{+}" and "Basal_KRT5^{+}" (Fig. 5A, B). The basal cluster shows high expression of genes enriched in the Ba/Sq subtype signature [23], whereas the luminal clusters are enriched in the LumP gene signature [23]. TFs identified by our analysis (see Box 1) as "Basal-enriched" and "Luminal-enriched" are highly expressed in the basal and the luminal clusters, respectively (Fig. 5C–F). In contrast, "Common" TFs are expressed widely (Fig. 5G). The candidate TFs identified here display distinct expression patterns. For example, *ELF3* is highly expressed in the "Luminal_UPK^{+}" cluster but it is undetectable in the "Luminal_FABP4^{+}" cluster, which shows higher levels of *GATA3*, *PPARG*, and *TBX3* (Fig. 5E, F; Supplementary Fig. 3). These findings highlight the existing heterogeneity even within molecular subtypes, which may be governed by different transcriptional programs. Moreover, we find an enrichment of novel candidate TFs, such as *HMGA2* or *MYCL*, in basal and luminal cells, respectively (Fig. 5E, F), further supporting the relevance of the proposed novel candidate TFs in urothelial differentiation.

TF-BASED OPPORTUNITIES FOR IMPROVED BLCA MANAGEMENT

As discussed above, TFs can display both oncogenic or tumor suppressor function. Rescue of loss-of-function defects is more challenging than pharmacological inhibition and, with the exception of nuclear receptors, most oncogenic TFs have classically been

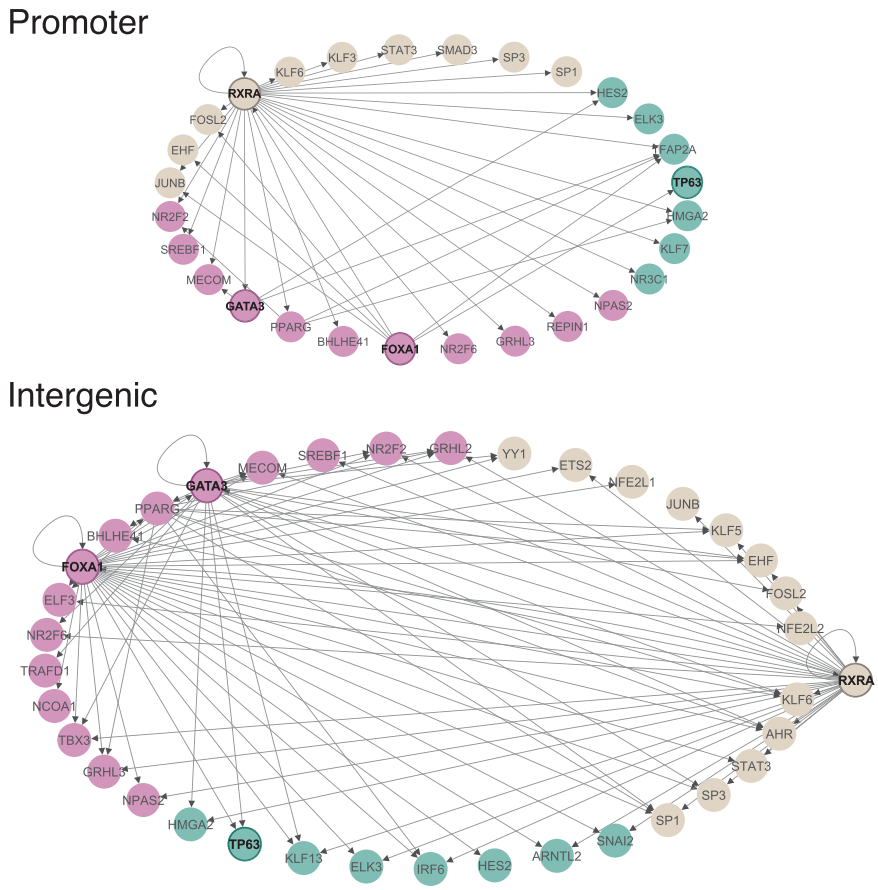

Fig. 4 Building a BLCA TF gene regulatory network. Regulatory network of TFs involved in luminal BLCA. ChIP-Seq data for the core nodes of the BLCA and RXRA (in bold) unveil genomic positions in promoters and intergenic regions of luminal-enriched (purple), basal-enriched (teal) and common (beige) BLCA TF nodes. The network displayed was constructed using Cytoscape.

considered undruggable. MYC is a prototypic TF the inhibition of which has long been sought because of its central role in oncogenesis. A variety of strategies to inhibit MYC have been applied [211] and a cell-penetrating miniprotein that acts as a dominant negative for MYC has antitumor activity in vitro and in vivo preclinical models [212]. A clinical trial in patients with advanced solid tumors is ongoing (https://clinicaltrials.gov/ct2/show/NCT04808362).

Several of the TFs discussed here are attractive pharmacological targets in BLCA, including PPARG and RXRA. The oncogenic activity of the PPARG/RXRA heterodimer in BLCA resembles that of ER/PR in breast cancer and AR in prostate cancer. In homeostatic conditions, these TF play an important role in the respective tissues, including the promotion of cell differentiation. As discussed above, PPARG can also promote tumorigenesis with both a direct role at the tumor cell level by controlling transcription and, indirectly, through effects on the immune TME [38]. We are not aware of any studies using PPARG antagonists in BLCA, possibly because of the broad metabolic functions of this target. On the other hand, the activating point mutations in *RXRA* and the recently described *AHR* deletion mutations, both present in a small proportion of BLCA, may provide therapeutic selectivity.

Regulon analyses of RNA-Seq data from NMIBC and MIBC suggest that sex hormone receptors may play a significant role in BLCA. In NMIBC, the ERβ regulon is up-regulated in Class 2a tumors whereas the ERα and PR regulons are up-regulated in a subset of Class 2b tumors, and the AR regulon is broadly up-regulated in class 3, *FGFR3*-mutant, tumors [213]. Consistent with these findings, increased activity of the AR regulon has been found in the three MIBC consensus papillary subtypes. The ERβ regulon is also activated in luminal tumors whereas the PR and ERα regulons are up-regulated in the stromal-rich and Ba/Sq-like subtypes [23]. However, these bioinformatics analyses need to be followed by rigorous experimental validation.

In the last few 7s, there has been renewed interest in TFs as drug targets, most notably thanks to the development of "degraders", small molecules that induce target protein degradation by the proteasome [214]. Proteolysis targeting chimerics (PROTAC) are small heterobifunctional molecules that can bind a protein of interest and an E3 ubiquitin ligase, leading to target degradation. PROTACs may, but do not need to, involve the active or ligand-binding site of a protein but they must have target protein binding selectivity (target must be ligandable). PROTAC-type degraders have been developed to a variety of oncogenic molecules including fusion proteins (e.g., BCR-ABL), kinases (e.g., BTK, CDK6), and TFs (e.g., AR and ER) [215]. Molecular glues are degraders that act as protein-protein scaffolds, involve highly cooperative E3-neosubstrate interactions, and are discovered agnostically through molecular screening. Natural examples of such molecules are thalidomide and the plant signaling hormones auxin and jasmonate. Several degrader molecules targeting TF, namely the AR for prostate cancer and the ER for breast cancer, have entered clinical trials, most of them being of the molecular glue type. The degrader strategy may thus be more broadly applied to TFs acting through gain-of-function.

CONCLUDING REMARKS

The evidence presented here underlies the notion that a profound dysregulation of transcriptional programs involved in cell

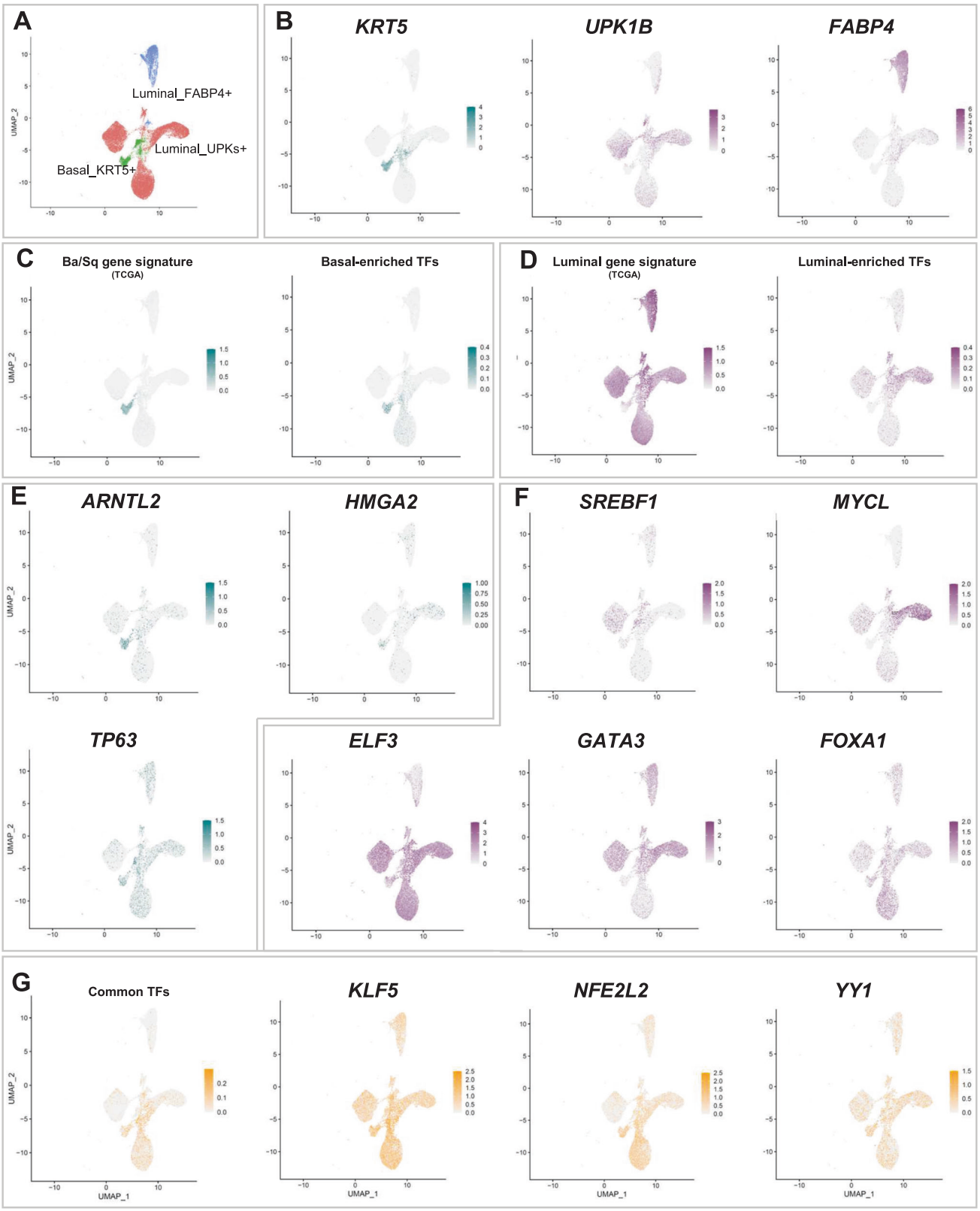

Fig. 5 The single-cell transcriptomic landscape of urothelial BLCA. A, B UMAP plots of urothelial cells disclose a cluster of basal cells enriched in *KRT5* expression, a cluster of luminal cells enriched in UPK expression, and a cluster of luminal cells enriched in *FABP4* expression **(B)**. **C** UMAP plots depicting the activity of the Ba/Sq signature from Kamoun et al. [23] (left) and the "basal-enriched TF" gene set (right) from our analysis. **D**. UMAP plots depicting the activity of the Luminal papillary signature from Kamoun et al. [23] (left) and the "luminal-enriched TF" gene set (right) from our analysis. **E** UMAP plots showing the expression of *TP63* and selected basal-enriched novel candidate TFs (*ARNTL2* and *HMGA2*). **F** UMAP plots showing the expression of *ELF3*, *GATA3*, *FOXA1* and selected luminal-enriched novel candidate TFs (*SREBF1* and *MYCL*): differential expression among the luminal clusters. **G** UMAP plots depicting expression activity of the "Common TFs" gene set and expression of *KLF5* and *NFE2L2* and novel candidate TF *YY1*.

Table 3. Cancer-related features of BLCA TFs.

TF	Expression in normal urothelium (protein, HPA)	Mutational driver in BLCA (Intogen)	Copy number alterations (% total)	Amplification (cases out of 408)	Amplification (%)	Deletion (cases out of 408)	Deletion (%)	Association with OS in TCGA-BLCA (GEPIA2; High n=101 vs Low Quartiles n=101)	Association with DFS in TCGA-BLCA (GEPIA2; High n=101 vs Low Quartiles n=101)
AHR	Medium	No	6.4	25	6.1	1	0.2	P=0.9; HR=0.95	P=0.74; HR=1.1
AR	Not detected	No	1.5	1	0.2	5	1.2	P=0.89; HR=1	P=0.27; HR=1.3
ARNTL2	Not detected	No	2.7	10	2.5	1	0.2	P=0.41; HR=1.2	P=0.45; HR=1.2
BACH1	Medium	No	2.0	6	1.5	2	0.5	P=0.056; HR=1.5	P=0.036; HR=1.7
BHLHE41	No data	No	2.5	9	2.2	1	0.2	P=0.009; HR=0.56	P=0.3; HR=0.78
EHF	Medium	No	3.9	11	2.7	5	1.2	P=0.33; HR=0.81	P=0.15; HR=1.4
ELF1	High	No	5.4	4	1.0	18	4.4	P=0.87; HR=1	P=0.073; HR=1.5
ELF3	High	Yes	1.2	1	0.2	4	1.0	P=0.077; HR=0.68	P=0.1; HR=0.68
ELK3	Low	No	1.2	5	1.2	0	0	P=0.62; HR =1.1	P=0.22; HR=1.3
ER	Not detected	No	1.2	1	0.2	4	1.0	P=0.2; HR=1.3	P=0.6; HR=0.88
ETS2	No detected	No	1.7	3	0.7	4	1.0	P=0.71; HR=1.1	P=0.15; HR=1.4
FOSL2	High	No	2.2	9	2.2	0	0	P=0.54; HR=0.88	P=026; HR=1.8
FOXA1	Medium	Yes	2.5	1	0.2	9	2.2	P=036; HR=0.62	P=0.34; HR=0.79
GATA2	Medium	Yes	1.7	6	1.5	1	0.2	P=062; HR=0.67	P=038; HR=0.59
GATA3	High	Yes	10	40	9.8	1	0.2	P=0.13; HR=0.72	P=038; HR=0.59
GRHL2	Medium	No	16.7	68	16.7	0	0	P=0.53; HR=0.88	P=0.13; HR=1.4
GRHL3	Medium	No	1.7	7	1.7	0	0	P=0.24; HR=0.78	P=0.31; HR=0.78
HES2	Not detected	No	0.5	2	0.5	0	0	P=011; HR=1.7	P=0.13; HR=1.4
HMGA2	Medium	No	3.7	15	3.7	0	0	P=079; HR=1.5	P=0.33; HR=1.3
IRF6	Medium	No	1.5	4	1.0	2	0.5	P=0.57; HR=1.1	P=0.22; HR=1.3
JUNB	High	No	0.2	1	0.2	0	0	P=0.58; HR=0.89	P=0.42; HR=0.82
KLF13	No data	No	1.5	1	0.2	5	1.2	P=0.19; HR=1.3	P=071; HR=1.6
KLF3	High	No	1.0	3	0.7	1	0.2	P=0.7; HR=0.95	P=0.66; HR=1.1
KLF5	Medium	Yes	6.9	20	4.9	8	2.0	P=0.3; HR=0.79	P=0.48; HR=1.2
KLF6	Low	No	7.6	30	7.4	1	0.2	P=026; HR=1.6	P=0.23; HR=1.3
KLF7	Low	No	2.2	8	2.0	1	0.2	P=0.95; HR=1	P=0.15; HR=1.4
MECOM	High	Yes	5.6	20	4.9	3	0.7	P=041; HR=0.64	P=0.91; HR=1
MYCL	No data	No	6.9	27	6.6	1	0.2	P=0.44; HR=0.85	P=0.9; HR=1
NCOA1	High	No	2.7	9	2.2	2	0.5	P=0.11; HR=1.5	P=0.19; HR=1.4
NFAT5	Medium	No	2.2	9	2.2	0	0	P=0.73; HR=0.93	P=023; HR=1.8
NFE2L1	High	No	1.2	5	1.2	0	0	P=065; HR=1.5	P=044; HR=1.6
NFE2L2/	Medium	Yes	1.5	5	1.2	1	0.2	P=0.63; HR=0.9	P=0.49; HR=1.2
NPAS2	Medium	No	1.7	7	1.7	0	0	P=0.1; HR=0.71	P=0.27; HR=0.77
NR2F2	No data	No	1.2	1	0.2	4	1.0	P=0.51; HR=1.1	P=0.58; HR=1.1
NR2F6	No data	No	0	0	0	0	0	P=088; HR=0.69	P=0.27; HR=0.75
NR3C1	Medium	No	0.7	2	0.5	1	0.2	P=0.33; HR=1.2	P=077; HR=1.5
PPARG	High	No	11.8	48	11.8	0	0	P=0046; HR=0.54	P=0.17; HR=0.73
REPIN1	High	No	1.2	3	0.7	2	0.5	P=0.54; HR=0.88	P=0.89; HR=1
RXRA	High	Yes	1.0	1	0.2	3	0.7	P=0.24; HR=1.3	P=0.45; HR=1.2
SMAD3	High	Yes	0.7	0	0	3	0.7	P=0.42; HR=0.85	P=0.68; HR=1.1
SNAI2	High	No	5.4	20	4.9	2	0.5	P=0.12; HR=1.4	P=079; HR=1.5
SP1	Medium	No	0.2	1	0.2	0	0	P=0.76; HR=0.93	P=0.33; HR=1.3
SP3	Not detected	No	0.5	2	0.5	0	0	P=0.69; HR=1.1	P=082; HR=1.6
SREBF1	Not detected	No	3.2	3	0.7	10	2.5	P=0.13; HR=1.4	P=0.96; HR=0.98
STAT1	Medium	No	2.0	5	1.2	3	0.7	P=0.9; HR=0.97	P=0.61; HR=1.1
STAT3	Medium	No	1.2	5	1.2	0	0	P=0.2; HR=1.3	P=0.2; HR=1.4
STAT6	High	No	1.0	4	1.0	0	0	P=055; HR=0.66	P=0.28; HR=0.78
TBX2	High	No	4.2	17	4.2	0	0	P=0.34; HR=0.81	P=092; HR=0.67
TBX3	No data	Yes	2.2	9	2.2	0	0	P=0093; HR=0.58	P=0.68; HR=0.91
TFAP2A	Not detected	No	2.9	9	2.2	3	0.7	P=062; HR=1.5	P=0.1; HR=1.5
TP63	High	Yes	4.4	18	4.4	0	0	P=0.18; HR=0.76	P=0.64;HR=1.1
TRAFD1	Low	No	2.0	8	2.0		0	P=0046; HR=0.53	P=0.44; HR=0.83
YY1	High	No	2.0	3	0.7	5	1.2	P=0.81; HR=1.1	P=014; HR=1.8
ZBED2	No data	No	1.5	6	1.5	0	0	P=0.18; HR=1.4	P=0.32; HR=0.78
ZBTB7C	No data	No	1.7	4	1.0	3	0.7	P=0.31; HR=0.8	P=0.77; HR=1.1

For all selected TFs, evidence on a mutational driver role was acquired from the Intogene database. Values for copy number alterations, amplifications, and deletions in the Bladder Urothelial Carcinoma (TCGA, Cell 2017) dataset were obtained from the cBioPortal site. Association with disease-free survival (DFS) and overall survival (OS) in the TCGA-BLCA dataset was retrieved from Gepia2, comparing the upper ($n = 101$) vs. the lower ($n = 101$) expression quartile. Color code: luminal-enriched (purple), basal-enriched (teal), and common (beige).

differentiation contributes to BLCA. Furthermore, the GRNs involved play a key role in tumor cell heterogeneity and in the plasticity associated with tumor evolution and therapeutic resistance. Several of the key TFs participating in these processes display features that are amenable to therapeutic targeting. The high frequency of alterations in genes coding for proteins involved in chromatin function suggests that an in-depth analysis of epigenetic changes is mandatory. Acquiring a more profound mechanistic knowledge of both GRN and epigenetic regulation should provide improved opportunities to understand heterogeneity and leverage cell plasticity in the stratified management of BLCA.

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AUTHOR CONTRIBUTIONS

All authors reviewed and summarized literature on the topic. MR also performed new bioinformatics analyses included in the manuscript. SC took the main responsibility for the illustrations.

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COMPETING INTERESTS

The authors declare no competing interests.

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