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Marcia R. Saban  
*University of Oklahoma*

Helen L. Hellmich  
*University of Texas*

Mary Turner  
*Oklahoma Medical Research Foundation (OMRF)*

Ngoc-Bich Nguyen  
*University of Oklahoma and University of Texas*

Rajanikanth Vadigepalli  
*Thomas Jefferson University, raj@mail.dbi.tju.edu*

*See next page for additional authors*

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**Authors**

Marcia R. Saban, Helen L. Hellmich, Mary Turner, Ngoc-Bich Nguyen, Rajanikanth Vadigepalli, David W. Dyer, Robert E. Hurst, Michael Centola, and Ricardo Saban

Research article

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## The inflammatory and normal transcriptome of mouse bladder detrusor and mucosa

Marcia R Saban<sup>1</sup>, Helen L Hellmich<sup>2</sup>, Mary Turner<sup>3</sup>, Ngoc-Bich Nguyen<sup>1,7</sup>, Rajanikanth Vadigepalli<sup>4</sup>, David W Dyer<sup>5</sup>, Robert E Hurst<sup>6</sup>, Michael Centola<sup>3</sup> and Ricardo Saban\*<sup>1</sup>

Address: <sup>1</sup>Department of Physiology, The University Oklahoma Health Sciences Center, Oklahoma City, USA, <sup>2</sup>Department of Anesthesiology, University of Texas Medical Branch, Galveston, USA, <sup>3</sup>Oklahoma Medical Research Foundation (OMRF), Arthritis and Immunology Research Program, Microarray Core Facility, Oklahoma City, USA, <sup>4</sup>Daniel Baugh Institute for Functional Genomics and Computational Biology, Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, USA, <sup>5</sup>Department of Microbiology and Immunology, Laboratory for Genomics and Bioinformatics, Oklahoma University Health Sciences Center, Oklahoma City, USA, <sup>6</sup>Department of Urology, The University Oklahoma Health Sciences Center, Oklahoma City, USA and <sup>7</sup>Cellular & Structural Biology, The University of Texas Health Science Center at San Antonio, San Antonio, USA

Email: Marcia R Saban - marcia-saban@ouhsc.edu; Helen L Hellmich - hhellmic@utmb.edu; Mary Turner - Mary-turner@omrf.ouhsc.edu; Ngoc-Bich Nguyen - NGUYENN2@UTHSCSA.EDU; Rajanikanth Vadigepalli - raj@mail.dbi.tju.edu; David W Dyer - david-dyer@ouhsc.edu; Robert E Hurst - Robert-hurst@ouhsc.edu; Michael Centola - Mike-Centola@omrf.ouhsc.edu; Ricardo Saban\* - Ricardo-saban@ouhsc.edu

\* Corresponding author

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### Abstract

**Background:** An organ such as the bladder consists of complex, interacting set of tissues and cells. Inflammation has been implicated in every major disease of the bladder, including cancer, interstitial cystitis, and infection. However, scanty is the information about individual detrusor and urothelium transcriptomes in response to inflammation. Here, we used suppression subtractive hybridizations (SSH) to determine bladder tissue- and disease-specific genes and transcriptional regulatory elements (TRE)s. Unique TREs and genes were assembled into putative networks.

**Results:** It was found that the control bladder mucosa presented regulatory elements driving genes such as myosin light chain phosphatase and calponin I that influence the smooth muscle phenotype. In the control detrusor network the Pax-3 TRE was significantly over-represented. During development, the Pax-3 transcription factor (TF) maintains progenitor cells in an undifferentiated state whereas, during inflammation, Pax-3 was suppressed and genes involved in neuronal development (*synapsin I*) were up-regulated. Therefore, during inflammation, an increased maturation of neural progenitor cells in the muscle may underlie detrusor instability. NF- $\kappa$ B was specifically over-represented in the inflamed mucosa regulatory network. When the inflamed detrusor was compared to control, two major pathways were found, one encoding *synapsin I*, a neuron-specific phosphoprotein, and the other an important apoptotic protein, *siva*. In response to LPS-induced inflammation, the liver X receptor was over-represented in both mucosa and detrusor regulatory networks confirming a role for this nuclear receptor in LPS-induced gene expression.

**Conclusion:** A new approach for understanding bladder muscle-urothelium interaction was developed by assembling SSH, real time PCR, and TRE analysis results into regulatory networks. Interestingly, some of the TREs and their downstream transcripts originally involved in organogenesis and oncogenesis were also activated during inflammation. The latter represents an additional link between inflammation and cancer. The regulatory networks represent key targets for development of novel drugs targeting bladder diseases.

## Background

The lower urinary tract is subject to a number of functional disorders in which a cross-communication between urothelium and detrusor muscle is a factor. Bladder over-activity has been attributed to detrusor muscle dysfunction, and several *in vitro* and *in vivo* methodologies have been developed to better understand its pathophysiology [1]. Although the detrusor muscle participates intensively in the inflammatory response, practically every major disease of the urinary bladder, including cancer, interstitial cystitis, and infection [2], involves the urothelium [3].

The urinary bladder develops as a result of indispensable epithelial-mesenchymal interactions responsible for directing urothelial differentiation and for normal smooth muscle development [4,5]. However, in adulthood, the urinary bladder is a highly heterogeneous organ, consisting of a large variety of cell types, and this complexity presents challenges to the study of physiological and cellular processes in health and disease. This was evident in studies determining gene regulation [6-11] and target validation [12]. In the latter study, the expression of protease-activating receptors (PAR) was differentially distributed between bladder mucosa, detrusor smooth muscle, and nerve elements. Moreover, during inflammation, PAR expression was up-regulated in the mucosa contrasting with its down-regulation in the detrusor muscle [12]. These results suggested a possible differential distribution of proteins between bladder mucosa and detrusor muscle and indicated the need for reduction of the whole tissue into specific layers.

The present work was undertaken to elucidate the transcriptional complexity of inflammation, as modeled in the mouse urinary bladder. The first step towards the study of individual layers was the separation of the mucosa and submucosa layers away from the detrusor smooth muscle and adventitia (See Additional file 1). We went further to determine low abundant transcripts, using suppression subtractive hybridization (SSH) and selected ones were confirmed by real time PCR. Next, the SSH-originated transcripts were annotated and analyzed for significantly enriched transcriptional regulatory elements (TRE) using PAINT [Promoter Analysis and Interaction Network Toolset; [13]]. PAINT utilizes TRANSFAC database [14] containing eukaryotic cis-acting regulatory DNA elements and trans-acting factors. The pattern search tool MATCH in TRANSFAC suite is employed to identify the TREs on cognate 5' upstream regulatory sequences [15]. Putative regulatory networks were assembled using known interactions among genes and their coded proteins as well as information about TREs that were significantly over-represented in the genes comprising inflammatory and control transcriptomes. Together, these results constitute the first demonstration of the transcriptional complexity underlying the different layers of the urinary bladder and their contribution to the early phases of bladder inflammation.

## Results

SSH. From each library, at least 50 clones were further sequenced and annotated, for a total of 300 clones. The present work reports only tissue-specific transcripts

**Table 1: Primers for real time PCR**

Gene	GenBank ID	5' primer sequence	3' primer sequence	localization
Bcap3l	<a href="#">NM_009922</a>	AAAGAATATGACCGCCTGCTAGA	AAGCCTTTACTCCTCTTCTTGACT	761-847
Catenin	<a href="#">BC043108</a>	CCTCCCCACCCACATCTA	ACCCACACCCACCGA <sub>gaa</sub>	4957-5049
Cnn1	<a href="#">NM_009922</a>	AGTTGTTTGCTGCCAAGTCTGA	GGTGAAGGCAGTTTAAATGGAGT	2081-2168
Dlk1	<a href="#">D16847</a>	CTTTTTGTGGTGGAGTTTGCTCTAT	gCGTGGTAGCATGGCACACA	1859-1950
eiF-4E	<a href="#">NM_010124</a>	TGTGCTTGGCTGCTGAGAGA	ACGGACAGACGGACGATGA	1446-1531
Grn	<a href="#">X62321</a>	CTACCTAAAGGGTGTCTGCTGTAGA	AGGAATCTTCTTTCGCAAACACTT	1630-1729
Grp58	<a href="#">BC003285</a>	AAAACCCAGAGAGGACAGAATGGATAA	TGTATTTTCAAACAGTGCAGCTAAGAA	1627-1712
GSTM1	<a href="#">NM_010358</a>	TCTCCTTCCCGCTCCCTT	GAGAATGAAGGCTGTGTGGACTT	965-1047
GSTO1	<a href="#">NM_010362</a>	GGCAAGAGCCCTCAGCAA	TGAGAAAGGAGCCAGTGAGAATACT	826-912
IFIT3	<a href="#">BC003804</a>	TGTGGTGGATTCTTGGCAGTT	CTGCCTGTGCCCAAAGT	1279-1366
LAMP2	<a href="#">NM_010685</a>	TGGCACTGGCTTAATGCTGTT	GTGCTTTGGAGGTATCTCAATATGAA	3132-3218
Mafk	<a href="#">AF180338</a>	CCATCTTGAGAAGGTAGCAGCAA	AAAGTTGGGCTTGGTGGGTT	3731-3840
Mpp1	<a href="#">U38196</a>	AGATTGCCATCCTTGACATTGA	GTAGGTGCGATGAACACAATGAA	1301-1388
Pls3	<a href="#">NM_145629</a>	CACACCCAGGCTCAAAGGA	TTGTGATAAAGATTTCCAAACAACAA	2816-2902
Prss11	<a href="#">NM_019564</a>	AGTCAACATTTGCCCTTCCCTTA	GGCTGCGAGGACCTTCT	1752-1837
Sirt1	<a href="#">AF214646</a>	CCTGCATAGATCTTACCACA <sub>aat</sub>	ACACTCTCCCCAGTAGAAGTACCATT	3016-3108
Smoc2	<a href="#">NM_022315</a>	CCACTATGGGATGAAGGTTATGA	AGAAAGTGACAGCCAGCCATACA	2377-2465
SPRR2A	<a href="#">BC010818</a>	ATAGCAACACTTCCATCCTCCTTT	TGAGGAGCCATCATAAGCAT	379-471
SYN1	<a href="#">NM_013680</a>	GTTCTAAAGTCATCGTTCCGGTCTTAA	TTCCCAGCTCTGTGATCATCAA	3334-3424
TMPPRSS2	<a href="#">AF199362</a>	AATCACACCAGCCATGATCTGT	AATCAGCCACCAGATCCCAT	1364-1473
Upk1a	<a href="#">AF262335</a>	GGCAACTTCATCCCCATCAA	AGCAACCCTTGGTAAACAGGTAGT	580-652

**Table 2: Mouse bladder transcripts isolated by SSHs**

Abbrev	Name	Accession	Library	QPCR
2310015N07	RIKEN cDNA 2310015N07 gene	<a href="#">NM_025515</a>	MIDI	N
Actg1	actin, gamma 1, cytoplasmic	<a href="#">NM_009609</a>	MIC	N
Actg2	actin, gamma 2, smooth muscle, enteric	<a href="#">NM_009610</a>	MIDI	N
Actr3	ARP3 actin-related protein 3 homolog (yeast)	<a href="#">NM_023735</a>	MIC	N
Ambladder	adult male urinary bladder cDNA, RIKEN clone:9530014P05	<a href="#">AK020558</a>	MC	N
Amcq	adult male corpora quadrigemina cDNA, RIKEN clone:B230340L02	<a href="#">AK080832</a>	MIC	Y
Aplp2	amyloid beta (A4) precursor-like protein 2 isoform 751	<a href="#">U15571</a>	MIDI	N
Bcap31	B-cell receptor-associated protein 31	<a href="#">NM_012060</a>	MIDI	Y
Calm2	calmodulin 2	<a href="#">NM_007589</a>	MC	N
Catenin	similar to catenin src	<a href="#">BC043108</a>	MIC	N
Cnn1	calponin 1	<a href="#">NM_009922</a>	MC	N
Col3a1	collagen, type III, alpha 1, RIKEN 3200002K15	<a href="#">AK019448</a>	MIDI	N
Cox7b	cytochrome c oxidase subunit VIIb	<a href="#">NM_025379</a>	MIC	N
Coxl	mitochondrial gene for subunit I of cytochrome c oxidase	<a href="#">X57780</a>	MIC	N
Cpe	carboxypeptidase E (Cpe),	<a href="#">NM_013494</a>	MIC	N
Cts e	cathepsin E (Ctse)	<a href="#">NM_007799</a>	MIC	N
Cts h	cathepsin H	<a href="#">NM_007801</a>	DC	N
Cts l	cathepsin L	<a href="#">NM_009984</a>	MIC	Y
Ddx3	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3	<a href="#">NM_010028</a>	MIDI	Y
Dnaj	Hsp40 homolog, subfamily A, member 2	<a href="#">NM_019794</a>	MIDI	N
Eif4ebp2	eukaryotic translation initiation factor 4E binding protein 2	<a href="#">NM_010124</a>	DIC	N
Elp3	elongation protein 3 homolog, RIKEN clone:2610507P14	<a href="#">AK012072</a>	MIDI	Y
Endomucl	endomucin-1	<a href="#">BC003706</a>	MIC	N
Fth1	ferritin heavy chain 1	<a href="#">NM_010239</a>	MIC	N
Gpam/GPAT	glycerol-3-phosphate acyltransferase	<a href="#">NM_008149</a>	MIC	Y
Grn	epithelin 1 and 2 (granulin)	<a href="#">X62321</a>	MC	N
Grp58	glucose regulated protein	<a href="#">BC003285</a>	MIC	Y
Gst a4	glutathione S-transferase, alpha 4	<a href="#">NM_010357</a>	MIDI	N
Gst m1	glutathione S-transferase, mu 1	<a href="#">NM_010358</a>	DC	Y
Gst o1	glutathione S-transferase omega 1	<a href="#">NM_010362</a>	DC	Y
Gus	beta-glucuronidase	<a href="#">NM_010368</a>	MIDI	N
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	<a href="#">BC003804</a>	MIC	Y
Ifrg15	interferon alpha responsive gene	<a href="#">NM_022329</a>	DC	N
Lamp2	lysosomal membrane glycoprotein 2	<a href="#">NM_010685</a>	MIC	N
Lrrfip2	leucine rich repeat (in FLII) interacting protein 2	<a href="#">XM_284541</a>	MIDI	Y
Ly6d	mRNA for THB / lymphocyte antigen 6 complex, locus D	<a href="#">X63782</a>	MIDI	N
Mafb	transcription factor MAFB	<a href="#">AF180338</a>	MIC	N
Mgs1-182e11	clone mgs1-182e11 strain 129/Svj	<a href="#">AC096622</a>	MIDI	Y
MLZE	adult female vagina cDNA, RIKEN clone 9930109F21	<a href="#">AK037079</a>	MIDI	N
Mpp1	palmytoylated protein p55	<a href="#">U38196</a>	DIC	N
My h11	myosin heavy chain 11, smooth muscle	<a href="#">XM_147228</a>	MIDI	Y
My lc2b/MRLC2	myosin light chain, regulatory B	<a href="#">NM_023402</a>	MC	Y
My19	myosin, light polypeptide 9, regulatory	<a href="#">XM_283793</a>	MIDI	N
Pls3	similar to plastin 3 precursor (T-isoform)	<a href="#">NM_145629</a>	MIC	Y
Prdx1	peroxiredoxin 1	<a href="#">NM_011034</a>	MIDI	N
Prnp	prion protein	<a href="#">NM_011170</a>	DC	Y
Prss11	protease, serine, 11 (Igf binding) HtrA1	<a href="#">NM_019564</a>	MIC	N
RP23-452N23	clone RP23-452N23 on chromosome 4	<a href="#">AL928645</a>	MIC	N
RP23-70M6	chromosome 18 clone RP23-70M6	<a href="#">AC114820</a>	MIC	N
RPCI23	Strain C57BL6/J Chromosome 2, clone RP23-111A22	<a href="#">AC078911</a>	MIDI	Y
RPs21	ribosomal protein S21 / RIKEN cDNA 2410030A14 gene	<a href="#">BC027563</a>	MIC	N
RPs7	ribosomal protein S7	<a href="#">NM_011300</a>	MIDI	Y
SAC1	suppressor of actin mutations	<a href="#">AJ245720</a>	MC	Y
SC1	extracellular matrix protein precursor	<a href="#">U77330</a>	MC	N
SCP-1	SCP-1 mRNA for stromal cell derived protein-1	<a href="#">D16847</a>	MIC	Y
Sdfr1	stromal cell derived factor receptor 1	<a href="#">NM_009145</a>	DC	N
Sepp1/SePP	selenoprotein P, plasma, I glycoprotein	<a href="#">NM_009155</a>	MIC	Y
Serpina3n	serine (or cysteine) proteinase inhibitor (clade A, member 3N)	<a href="#">NM_009252</a>	DC	Y
Sf 3b2	splicing factor 3b, subunit 2, clone MGC:61326 IMAGE:6812422	<a href="#">BC049118</a>	MIDI	N

**Table 2: Mouse bladder transcripts isolated by SSHs (Continued)**

Sf rs6	splicing factor, arginine/serine-rich 6	<a href="#">NM_026499</a>	MIC	N
Sf sc35	splicing factor SC35	<a href="#">AF077858</a>	MIDI	N
Sirt1	Sir1 alpha protein	<a href="#">AF214646</a>	MIDI	N
Siva	Cd27 binding proapoptotic protein	<a href="#">NM_013929</a>	DIC	Y
Smoc2	SPARC related modular calcium binding 2	<a href="#">NM_022315</a>	MIC	N
Sparc	secreted acidic cysteine rich glycoprotein	<a href="#">NM_009242</a>	MIDI	N
Sprr 2A	small proline-rich protein 2A	<a href="#">BC010818</a>	MC	Y
Syn1	synapsin I or ribosomal protein S15a	<a href="#">NM_013680</a>	DIC	Y
Tcra-V13.1	T-cell receptor alpha/delta locus	<a href="#">AE008684</a>	MIC	N
Thsd6	thrombospondin, type I domain containing 6	<a href="#">NM_025629</a>	DC	Y
TMPRSS2	transmembrane protease, serine 2	<a href="#">NM_015775</a>	MIDI	N
Tnnt3	troponin T3, skeletal, fast	<a href="#">NM_011620</a>	MIDI	N
UDP-gluco	UDP-glucuronosyltransferase I family, member 2	<a href="#">BC019434</a>	MIC	N
Upk1a	uropod protein 1a	<a href="#">AF262335</a>	DC	N
Upk1b	uropod protein 1b	<a href="#">NM_178924</a>	DC	N
Wdr1	WD repeat domain 1	<a href="#">NM_011715</a>	MIDI	N
Zfp364	zinc finger protein 364/Rab7	<a href="#">NM_026406</a>	MIDI	N

(mucosa vs detrusor) and treatment-specific transcripts (saline vs LPS). 120 transcripts were considered to be unique. These indicate that each of these 120 transcript occurred in a specific subtraction. Transcripts that appeared in more than one subtraction were not considered unique and therefore, not included. Selection of clones ceased when a substantial number of additional sequences [approximately 180] did not reveal any additional unique transcript. Next, 120 clones were sequenced and analyzed for homology in the GenBank and EMBL databases.

Seventy six cDNA fragments (Table 2) were further annotated using the Mouse Genome Information [16] according to Gene Ontology and presented in Table 3. Among the 76 fragments selected for further annotation, 21 fragments had homology with expressed sequence tags (ESTs) and cDNA clones for which information about tissue specificity, biological or molecular function is not available. Interestingly, one of these clones (*ambladder*; RIKEN clone:9530014P05) was originally isolated from an adult male bladder cDNA library [17].

#### SSH-selected transcripts

Table 3 summarizes the isolated transcripts that in general are involved in: actin dynamics (*wdr1*); apoptosis (*sirt1*, *siva*, and *bcap31*); calcium ion binding (*pls3*, *sc1*, *scp-1*, *sparc*, and *smoc2*); cell adhesion (*col3a1* and *catenin*); cell cycle/ G-protein coupled receptor (*cam2*); cell growth (*prss11*); cell motility (*actr3*); cytoskeleton organization and biogenesis (*actg1*, *actg2*, and *mylc2b*); defense response (*ly6d*); electron transport (*cox7b*, *grp58*, and *cox1*); epithelial cell proliferation (*grn*); immune response (*ifit3*); insulin processing (*cpe*); iron ion transport (*fth1*); metabolism/ glutathione transferase activity (*gsto1*, *gsta4*, and *gstm1*); negative regulation of translational initiation (*eif4ebp2*); nuclear mRNA splicing (*sfsc35* and *sfrs6*); phos-

pholipid biosynthesis (*gpam*); positive regulation of transcription (*mafb*); post-embryonic development (*sepp1*); protein biosynthesis (*rps7* and *rps21*); protein folding (*dnaJ*); protein ubiquitination (*zfp364*); proteolysis (*ctse*, *ctsh*, *ctsl*, *Aplp2*, *serpina3n*, and *tmprss2*); regulation of cell shape (*sprr2A*); regulation of muscle contraction (*my19* and *cnn1*); response to oxidative stress (*prdx1* and *prnp*); synaptic transmission (*syn1*); and tRNA aminoacylation (*lamp2*).

#### Target validation by quantitative real-time polymerase chain reaction (QRT-PCR)

From the annotated transcripts (Table 2), twenty six were selected for further analysis by QRT-PCR. The results are summarized in Table 4. In tissues isolated from saline-treated mice the following transcripts were expressed preferentially in the detrusor muscle when compared to the mucosa layer: *ctsh*, *eif4ebp2*, *gstm1*, *gsto1*, *serpina3n*, *sprr2A*, *upk1a*, and *upk1b*. With the exception of *serpina3n*, all detrusor-specific transcripts were also preferentially up regulated in the inflamed detrusor. In contrast, *calm2*, *cnn1*, and *smoc2* were preferentially expressed in the bladder mucosa of control mice. With the exception of *calm2*, all other mucosa-specific transcripts were up-regulated during inflammation. Table 4 also segregates transcripts that were represented in both layers of control mice and that were up-regulated during the inflammatory process. The latter include: *bcap31*, *catenin*, *pls3*, *mafb*, *prss11*, *mpp1*, *syn1*, *lamp2*, and *sepp1*.

#### TREs

Figure 2 contains over-represented TREs and the downstream transcripts that were found primarily in the bladder mucosa when compared to detrusor muscle isolated from control mice (MC). In contrast, figure 5 contains over-represented TFs and transcripts that were found primarily in the control detrusor muscle (DC). TFs and

**Table 3: ANNOTATION (GENE ONTOLOGY) OF SSH-ISOLATED TRANSCRIPT FROM MOUSE URINARY BLADDER**

<i>Biological Process</i>	<i>Abbrev</i>	<i>Molecular Function</i>	<i>Cellular Component</i>	<i>Accession</i>
actin dynamics	Wdr1	actin binding	actin cytoskeleton	<a href="#">NM_011715</a>
apoptosis	Sirt1	NAD-dependent histone deacetylase	chromatin silencing complex	<a href="#">AF214646</a>
apoptosis	Bcap31	receptor binding	Golgi membrane	<a href="#">NM_012060</a>
apoptosis	Siva	CD27 receptor binding	cytoplasm	<a href="#">NM_013929</a>
calcium ion binding	SCP-1	calcium ion binding	integral to membrane	<a href="#">D16847</a>
calcium ion binding	Sparc	calcium ion binding	basement membrane	<a href="#">NM_009242</a>
calcium ion binding	Smoc2	calcium ion binding	extracellular space	<a href="#">NM_022315</a>
calcium ion binding	Pls3	calcium ion binding	unknown	<a href="#">NM_145629</a>
calcium ion binding	SC1	calcium ion binding	extracellular space	<a href="#">U77330</a>
cell adhesion	Col3a1	extracellular matrix structural constituent	collagen	<a href="#">AK019448</a>
cell adhesion	Catenin	protein binding	cytoskeleton	<a href="#">BC043108</a>
cell cycle/G-protein coupled receptor	Calm2	protein binding	plasma membrane	<a href="#">NM_007589</a>
cell growth (regulation)	Prss11	insulin-like growth factor binding	extracellular region	<a href="#">NM_019564</a>
cell motility	Actr3	structural molecule	actin cytoskeleton	<a href="#">NM_023735</a>
cytoskeleton organization and biogenesis	Actg1	motor activity	actin cytoskeleton	<a href="#">NM_009609</a>
cytoskeleton organization and biogenesis	Actg2	motor activity	actin cytoskeleton	<a href="#">NM_009610</a>
cytoskeleton organization and biogenesis	My1c2b	unknown	cytoskeleton	<a href="#">NM_023402</a>
defense response	Ly6d	unknown	plasma membrane	<a href="#">X63782</a>
electron transport	Grp58	electron transporter	endoplasmic reticulum	<a href="#">BC003285</a>
electron transport	Cox7b	oxidoreductase activity	mitochondrial electron transport chain	<a href="#">NM_025379</a>
electron transport	Cox1	ubiquinol-cytochrome-c reductase activity	mitochondrion	<a href="#">X57780</a>
epithelial cell proliferation	Grn	phospholipase A2	mitochondrion	<a href="#">X62321</a>
immune response	IFIT3	unknown	unknown	<a href="#">BC003804</a>
insulin processing	Cpe	carboxypeptidase A and E activity	extracellular space	<a href="#">NM_013494</a>
iron ion transport	Fth1	ferric iron binding	unknown	<a href="#">NM_010239</a>
metabolism	Gst a4	glutathione transferase	unknown	<a href="#">NM_010357</a>
metabolism	Gst m1	glutathione transferase	unknown	<a href="#">NM_010358</a>
metabolism	Gst o1	glutathione transferase	cytoplasm	<a href="#">NM_010362</a>
negative regulation of translational initiation	Eif4ebp2	insulin receptor signaling pathway	unknown	<a href="#">NM_010124</a>
nuclear mRNA splicing, via spliceosome	Sf sc35	DNA binding	spliceosome complex	<a href="#">AF077858</a>
nuclear mRNA splicing, via spliceosome	Sf 3b2	unknown	nucleus	<a href="#">BC049118</a>
nuclear mRNA splicing, via spliceosome	Sf rs6	pre-mRNA splicing factor	nucleus	<a href="#">NM_026499</a>
phospholipid biosynthesis	Gpam	acyltransferase	mitochondrion	<a href="#">NM_008149</a>
positive regulation of transcription	Mafb	DNA binding	transcription factor complex	<a href="#">AF180338</a>
post-embryonic development	Sepp1	selenium binding	extracellular space	<a href="#">NM_009155</a>
protein biosynthesis	RP521	structural constituent of ribosome	ribosome	<a href="#">BC027563</a>
protein biosynthesis	RP57	RNA binding	ribosome	<a href="#">NM_011300</a>
protein folding	Dnaj	unfolded protein binding	membrane	<a href="#">NM_019794</a>
protein ubiquitination	Zfp364	ubiquitin-protein ligase activity	ubiquitin ligase complex	<a href="#">NM_026406</a>
proteolysis and peptidolysis	Cts e	neutrophil collagenase activity	extracellular space	<a href="#">NM_007799</a>
proteolysis and peptidolysis	Cts h	cysteine-type endopeptidase activity	lysosome	<a href="#">NM_007801</a>
proteolysis and peptidolysis	Cts l	cysteine-type endopeptidase activity	lysosome	<a href="#">NM_009984</a>
proteolysis and peptidolysis	TMPRSS2	trypsin activity	integral to membrane	<a href="#">NM_015775</a>
regulation of cell shape	Sprr 2A	constituent of cytoskeleton	cornified envelope	<a href="#">BC010818</a>
regulation of muscle contraction	Cnn1	calmodulin binding	unknown	<a href="#">NM_009922</a>
regulation of muscle contraction	My119	calcium ion binding	myosin	<a href="#">XM_283793</a>
response to oxidative stress	Prdx1	antioxidant activity	unknown	<a href="#">NM_011034</a>
response to oxidative stress	Prnp	copper ion binding	Golgi apparatus	<a href="#">NM_011170</a>
synaptic transmission	Syn1	protein dimerization	synaptic vesicle membrane	<a href="#">NM_013680</a>
tRNA aminoacylation	Lamp2	tRNA ligase	platelet dense granule membrane	<a href="#">NM_010685</a>
unknown	RPC123	unknown	unknown	<a href="#">AC078911</a>
unknown	Mgs1-182e11	unknown	unknown	<a href="#">AC096622</a>
unknown	RP23-70M6	unknown	unknown	<a href="#">AC114820</a>

**Table 3: ANNOTATION (GENE ONTOLOGY) OF SSH-ISOLATED TRANSCRIPT FROM MOUSE URINARY BLADDER** (Continued)

unknown	Tcra-VI3.1	unknown	unknown	<a href="#">AF008684</a>
unknown	Upk1a	unknown	integral to membrane	<a href="#">AF262335</a>
unknown	SAC1	unknown	integral to membrane	<a href="#">AJ245720</a>
unknown	Elp3	N-acetyltransferase activity	mitochondrion	<a href="#">AK012072</a>
unknown	Ambladder	unknown	unknown	<a href="#">AK020558</a>
unknown	MLZE	unknown	unknown	<a href="#">AK037079</a>
unknown	Amcq	unknown	unknown	<a href="#">AK080832</a>
unknown	RP23-452N23	unknown	unknown	<a href="#">AL928645</a>
unknown	Endomuc1	unknown	integral to membrane	<a href="#">BC003706</a>
unknown	UDP-gluco	unknown	unknown	<a href="#">BC019434</a>
unknown	Sdfr1	receptor activity	integral to membrane	<a href="#">NM_009145</a>
unknown	Serpina3n	endopeptidase inhibitor	extracellular space	<a href="#">NM_009252</a>
unknown	Ddx3	ATP-dependent helicase activity	intracellular	<a href="#">NM_010028</a>
unknown	Gus	unknown	unknown	<a href="#">NM_010368</a>
unknown	Tnnt3	unknown	unknown	<a href="#">NM_011620</a>
unknown	lfrg15	unknown	unknown	<a href="#">NM_022329</a>
unknown	2310015N07	unknown	unknown	<a href="#">NM_025515</a>
unknown	Thsd6	unknown	extracellular space	<a href="#">NM_025629</a>
unknown	Upk1b	unknown	integral to membrane	<a href="#">NM_178924</a>
unknown	Aplp2	serine-type endopeptidase inhibitor activity	integral to membrane	<a href="#">U15571</a>
unknown	Mpp1	protein binding	membrane	<a href="#">U38196</a>
unknown	My h1 l	unknown	unknown	<a href="#">XM_147228</a>
unknown	Lrrfip2	unknown	unknown	<a href="#">XM_284541</a>

downstream transcripts specifically expressed in bladder mucosa during inflammation were compared to control mucosa (Figure 3) or to the inflamed detrusor (Figure 4). In contrast, the response of detrusor muscle to inflammation was determined in comparison to detrusor control (Figure 6) or inflamed mucosa (Figure 7). The results presented here demonstrate the concept that combining SSH methodology with PAINT-guided transcriptional regulatory element analysis permitted the generation of testable hypotheses regarding differences between mucosa and detrusor regulatory networks in health and disease states.

## Discussion

We used a highly effective method for differential gene analysis, termed suppression subtractive hybridization (SSH), which has been developed for the generation of subtracted cDNA libraries. It is based primarily on suppression PCR and combines normalization and subtraction in a single procedure [18]. The normalization step equalizes the abundance of cDNAs within the target population and the subtraction step excludes the common sequences between the target and driver populations. In a model system, the SSH technique enriched for rare sequences over 1,000-fold in one round of subtractive hybridization [18]. Unlike microarrays, which mainly identify moderate to high abundant genes, SSH identifies clones that are expressed at very low levels. It is possible that some of the extremely low-level gene expression is not biologically significant, as it might arise from 'random transcription' [19]. Therefore, we confirmed the differen-

tial expression of twenty six SSH-selected transcripts by QRT-PCR and the results were highly correlated. In addition, we introduced a redundancy factor by comparing the data generated from the analysis of multiple SSH libraries (MC, DC, MIC, MIDI, DID, and DIMI). Nevertheless, the question of validation certainly is an important one, and it is one we have considered. For one, we examined properties of the candidate genes that were independent of expression, such as the presence of promoter sequences, to increase the probability that mechanisms suggested by the expression data were not simply statistical anomalies. Moreover, the finding that mechanisms known to be operant emerged from the analysis also increases confidence that novel ones are valid as well.

This study was carried out at a single time point. The 24 hour time point was chosen because it coincides with the peak of acute inflammation [8,9]. This point characterizes the acute inflammatory responses to LPS and in addition to edema and vasodilation, the peak response of neutrophil infiltration occurred at 24 h in the mucosa and submucosa as well as in the detrusor smooth muscle [8,9]. The rationale for a single time point was to keep the number of variables within a reasonable limit. We understand that 24 h time point may not identify preceding or proceeding events as we indicated previously [7-9]. Therefore, the results of regulatory network here presented should be viewed as a snapshot of the inflammatory transcriptome at the point of maximal inflammatory response. Nevertheless, genes identified as important at



this time point can now be followed over time by other techniques.

By using cDNA subtractions between mucosa and detrusor smooth muscle layers isolated from control and inflamed bladders, several clones identified transcripts that were further annotated. It has to be taken in consideration that the mucosal layer contains the urothelial layer and the lamina propria which involves other cell types (fibroblasts, myofibroblasts, etc.) in addition to urothelial cells that may underlie the transcriptomes identified. In addition, during the inflammation, inflammatory cells are present in the detrusor as well as in the mucosa and may contribute to the inflammatory bladder transcriptome.

Some of the transcripts are RIKEN sequences and therefore, have no biological process attributed. Interestingly, some of these transcripts with unknown function, such as *ambladder*, have been reported in the adult male urinary bladder by others using SSH [17]. However, the present work indicates that the same transcript was isolated from female urinary bladder as well.

Central to elucidation of hypothetical cis-regulatory networks is the identification and classification of naturally occurring transcription factor-binding sites in subtracted libraries. The combination of SSH-derived transcripts with PAINT-guided query of the TRANSFAC database permitted the generation of regulatory networks containing upstream TREs connecting corresponding TFs to the respective transcripts. Interestingly, many of the genes identified by SSH are transcription factors which emphasize the use of SSH to identify low expression transcripts.

#### **Mucosa control [MC], (figure 2)**

The regulatory network for the control bladder mucosa (MC) was obtained in comparison to detrusor control and contained the following over-represented TREs: GA-binding protein (GABP), YY1 (yin yang 1), SF-1 (steroidogenic factor-1), EF2, CRE-BP1, and Sp-3 (trans-acting transcription factor 3).

GABP, also known as nuclear respiratory factor 2 (NRF-2), is a transcriptional coordinator of mitochondrial and nuclear-encoded subunits of cytochrome oxidase genes [20]. NRF-2 responds to increased neuronal activity by translocating from the cytoplasm to the nucleus, where it engages in transcriptional activation of target genes [21]. GABP is abundant in the kidney [22], however, the information about GABP is scanty in the rest lower urinary tract.

YY1 is a zinc finger TF which is thought to regulate cell growth and differentiation. YY1 normally antagonizes

serum response factor (SRF) [23]. Interestingly, in the inflamed mucosa YY1 was not expressed and SRF was considered over-represented (Figure 2). YY1 along with GAPB drives the expression of myosin light chain phosphatase (*mylc2b*) which can be developmentally regulated in mammalian urinary bladders [24] and it is involved in the bladder response to obstruction [25].

SF-1 is a zinc finger motif of nuclear receptors [26] essential for steroidogenesis as well as for the development of the reproductive axis [27]. CRE-BP1, which is an ubiquitous basic-leucine zipper, is required for normal skeletal development.

YY1, SF-1, and EF2 were found upstream of *ambladder* (RIKEN clone 9530014P05 or prothymosin alpha). Prothymosin alpha is an oxidative stress-protecting gene [28] and transgenic mice over-expressing this transcript develop polycystic kidney disease PKD [29]. Another gene downstream of SF-1 was calponin 1 (*cn1*). *Cnn1* encodes for a multifunctional protein whose expression is tightly restricted to differentiated smooth muscle cell lineages during embryonic and post-natal life [30].

SF-1 and CRE-BP1 were found upstream of *sac1* (a murine homolog of *S. cerevisiae* suppressor of actin mutations). Finally, an additional TRE, Sp-3 was found upstream of *sc1* which is a calcium binding protein and an extracellular matrix protein precursor.

Epithelial-stromal interactions in bladder development have been extensively studied [5]. However, the present results depicted in the MC regulatory network raises the hypothesis that the bladder mucosa exhibits TREs and genes whose proteins may regulate the smooth muscle phenotype (Figure 2).

#### **Detrusor control [DC] (figure 5)**

The regulatory network for the control detrusor smooth muscle suggests that Pax-3 plays a central role. In addition to Pax-3, other TREs such as: PITX2, CDP, and c-Myb were also found over-represented (figure 5).

Pax-3 was found upstream of the following transcripts: prion protein (*prnp*), cathepsin L (*ctsl*), stromal cell derived factor receptor 1 (*sdf1*) [31], thrombospondin, type I domain containing 6 (*thsd6*), and uroplakin 1b (*upk1b*). *Prnp* seems to be involved in cell-to-cell interaction and its expression has been observed in germ cell differentiation during spermatogenesis [32]. *Ctsl* is a lysosomal proteolytic enzyme and an imbalance between *ctsl* and its inhibitors is believed to correlate with bladder tumor progression [33].

**Table 4: Differential expression of selected SSH transcripts by quantitative real-time polymerase chain reaction (QRT-PCR) \*\*\***

Genes	Normalized CT values								log2 CT X1000000				Fold Change (Delta CT Values)*				
	Average (n = 3)				SEM				LD	LM	CD	CM	CD/CM	CM/CD	LD/LM	LD/CD	LM/CM
	LD	LM	CD	CM	LD	LM	CD	CM									
Cts h	26.5	24.2	26.1	24.1	0.03	0.01	0.05	0.01	96	19	70	18	<b>3.9</b>	0.3	<b>5.0</b>	1.4	1.1
Eif4ebp2	36.5	30.4	33.7	30.9	0.62	0.07	0.07	0.05	95252	1430	13833	1974	<b>7.0</b>	0.1	<b>66.6</b>	<b>6.9</b>	0.7
Gst ml	23.4	19.1	23.2	18.2	0.32	0.02	0.00	0.06	11	1	9	0	<b>29.9</b>	0.0	<b>19.7</b>	1.2	1.8
Gst ol	36.0	26.9	35.5	29.8	0.14	0.17	0.56	0.06	68660	125	47023	909	<b>51.7</b>	0.0	<b>550.9</b>	1.5	0.1
Serpina3n	21.8	21.1	26.1	23.5	0.03	0.02	0.09	0.05	4	2	72	12	<b>6.1</b>	0.2	1.6	0.1	0.2
Sprr 2A	29.4	21.8	25.4	19.8	0.06	0.14	0.03	0.00	697	4	45	1	<b>51.0</b>	0.0	<b>185.1</b>	<b>15.3</b>	<b>4.2</b>
Upk1a	32.5	24.4	30.0	23.7	0.18	0.01	0.02	0.13	6162	23	1050	14	<b>76.4</b>	0.0	<b>271.4</b>	<b>5.9</b>	1.7
Upk1b	26.5	22.2	26.7	21.4	0.04	0.02	0.12	0.05	98	5	112	3	<b>39.3</b>	0.0	<b>20.6</b>	0.9	1.7
Calm2	23.2	24.0	22.2	23.8	0.11	0.09	0.04	0.11	9	17	5	14	0.3	<b>3.0</b>	0.6	2.0	1.2
Cnn1	32.1	33.1	27.2	31.0	0.50	0.09	0.15	0.05	4547	9400	149	2177	0.1	<b>14.6</b>	0.5	<b>30.5</b>	<b>4.3</b>
Smoc2	33.9	34.7	26.0	28.4	0.06	0.27	0.04	0.06	15953	27402	68	352	0.2	<b>5.2</b>	0.6	<b>234.8</b>	<b>77.7</b>
Bcap3l	33.3	29.2	26.7	26.9	0.40	0.07	0.08	0.01	10331	627	106	127	0.8	1.2	<b>16.5</b>	<b>97.6</b>	<b>4.9</b>
Catenin	36.9	33.0	27.1	28.0	0.23	0.07	0.42	0.09	130085	8592	143	273	0.5	1.9	<b>15.1</b>	<b>912.8</b>	<b>31.5</b>
Mafb	32.1	30.1	31.8	30.5	0.65	0.10	0.34	0.44	4703	1154	3615	1570	2.3	0.4	<b>4.1</b>	1.3	0.7
Pls3	37.4	35.3	28.6	29.3	0.10	0.18	0.14	0.15	179012	41906	402	661	0.6	1.6	<b>4.3</b>	<b>444.9</b>	<b>63.4</b>
Prssl1	29.9	27.9	25.8	24.5	0.05	0.03	0.06	0.00	999	259	59	23	2.5	0.4	<b>3.9</b>	<b>17.1</b>	<b>11.0</b>
Syn1	30.1	25.3	24.1	22.8	0.22	0.04	0.21	0.13	1148	42	18	7	2.4	0.4	<b>27.3</b>	<b>65.6</b>	<b>5.9</b>
Lamp2	31.6	31.1	28.1	27.8	0.21	0.14	0.05	0.01	3342	2379	285	230	1.2	0.8	1.4	<b>11.7</b>	<b>10.3</b>
Mpp1	31.0	30.0	29.2	29.5	0.44	0.11	0.11	0.19	2114	1090	632	753	0.8	1.2	1.9	<b>3.3</b>	1.4
Sepp1	23.0	23.9	22.0	21.6	0.06	0.03	0.08	0.05	8	15	4	3	1.4	0.7	0.6	2.0	<b>4.8</b>
Thsd6	28.1	28.3	28.3	28.0	0.13	0.08	0.03	0.04	293	329	338	272	1.2	0.8	0.9	0.9	1.2
IFIT3	30.4	31.2	30.8	30.9	0.08	0.00	0.12	0.05	1455	2396	1885	1988	0.9	1.1	0.6	0.8	1.2
Ifrg15	27.9	27.9	28.2	27.6	0.03	0.05	0.09	0.07	254	245	307	210	1.5	0.7	1.0	0.8	1.2
Sdfr1	24.4	25.6	24.1	24.8	0.06	0.09	0.10	0.09	23	52	18	29	0.6	1.6	0.4	1.3	1.8
Siva	26.7	25.8	27.1	25.8	0.03	0.07	0.06	0.07	110	59	147	59	2.5	0.4	1.9	0.7	1.0
Prnp	24.1	25.7	23.3	24.6	0.02	0.07	0.02	0.00	18	53	11	26	0.4	2.5	0.3	1.7	2.0

\*(ratio of antilog2 of cycle threshold values)

\*\* (bolded cells indicate values greater than 3.0)

\*\*\*Female C57BL/6J mice were instilled with saline (n = 20) or LPS (n = 20). Twenty four hours after LPS instillation, mice were euthanized, the bladder was removed and placed in RNAlater™ (Ambion) for separation of the mucosa and submucosa from the detrusor smooth muscle, as described in Material and Methods. Four sample groups were obtained as follows: control mucosa (CM), control detrusor (CD), LPS-treated mucosa (LM), and LPS-treated detrusor (LD). The QRT-PCR amplifications were accomplished on an ABI PRISM 7700 using SYBRGreen I dye assay chemistry

All samples were run in triplicate with the appropriate single QRT-PCR controls (no reverse transcriptase and no template). From the QRT-PCR data, an average cycle threshold (Ct) value was calculated from the triplicate reactions. Averaged Ct values were then normalized (to adjust for different amounts of cDNA within each reaction) to the exogenous control gene, RCA. The relative expression level of each transcript within each sample group (CD, CM, LD, and LM) was determined by calculating the ratio of the antilog2 of the delta Ct values.

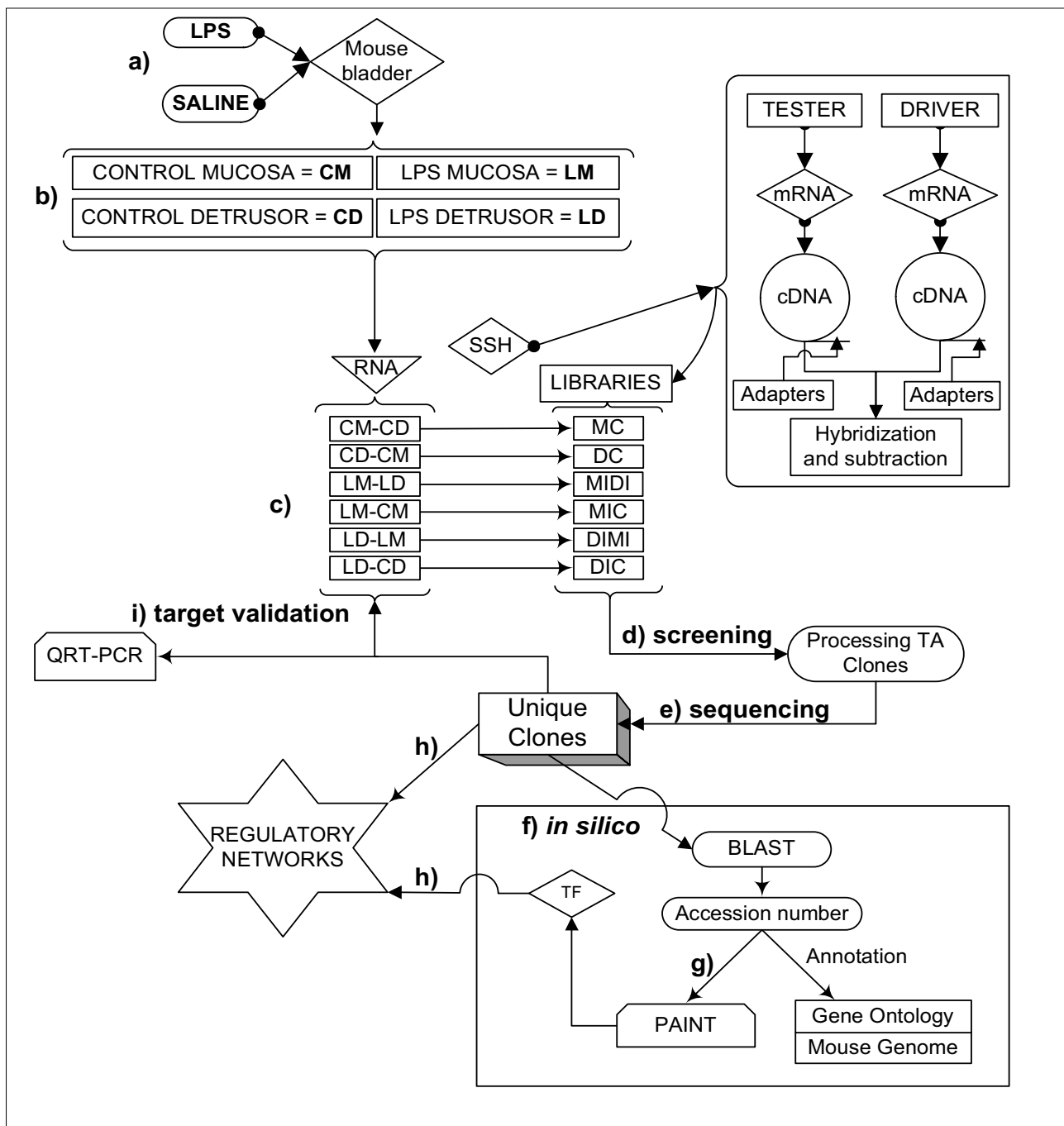
In addition to *ctsl*, the detrusor also presents *ctsh* under the control of c-Myb. Interestingly, *thsd* is involved in bladder cancer development [34] and is a target for methylation [35]. In addition, *thsd* has been described as inhibitor of angiogenesis. Finally, *upk1b* gene is highly expressed in normal human urothelium and its mRNA was undetectable or markedly reduced in bladder carcinoma [36].

Interestingly, the control detrusor normally expresses genes in the Pax-3 pathway that maintain neural progenitor cells [37] and myoblasts [38] undifferentiated. Therefore, our data suggests that Pax-3-regulated suppression of neural development in control detrusor changed substan-

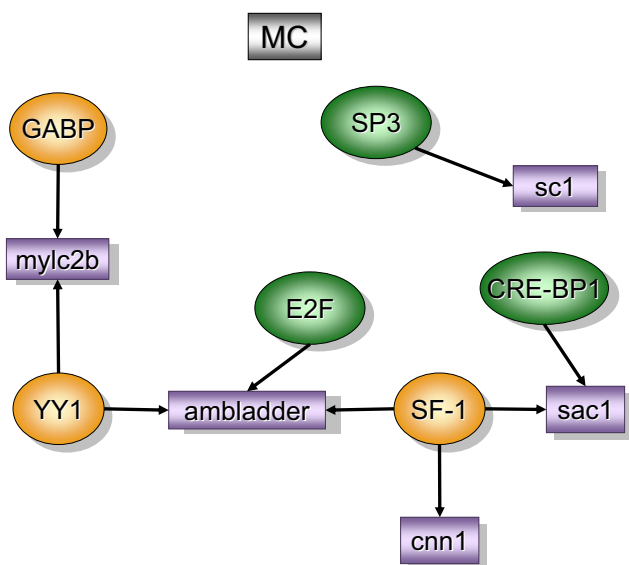
tially during inflammation and genes involved in neuronal development such as *syn* were found to be up-regulated. The later implies that detrusor instability may be a consequence of alterations in Pax-3 pathway leading to increased maturation of neural progenitor cells within the bladder smooth muscle.

**Mucosa inflamed versus control [MIC]. (figure 3)**

Of all regulatory networks (Figures 2, 3, 4, 5, 6, 7), MIC was the only one presenting NF-κB (p < 0.01), (Figure 3). These results confirmed our previous observation that it is the bladder mucosa and in particular, the urothelium, that responds to LPS with NF-κB translocation [39]. This



**Figure 1**  
**Overall experimental design.** a) A well established animal model of LPS- induced bladder inflammation was used. b) RNA was extracted from isolated detrusor muscle and mucosa layers. c) Extracted RNA was used to generate 6 different libraries by suppressive subtraction hybridization (SSH) in order to determine bladder tissue- and treatment- dependent transcripts. d) Transcripts were then screened and e) Sequenced f) All unique transcripts were fully annotated by querying public (PubMed, Gene Ontology, Mouse Genome [108]) and private (Transfac professional [109]) databases. g) The accession number of each SSH-selected transcript was uploaded into the PAINT 3.3 feasnet builder [110] to query the Transfac database [109]. h) A regulatory network for each library was originated by a combination of SSH-selected transcripts and over-represented TF ( $0 < p < 0.05$ ) in the matrix when compared to the PAINT database reference equivalent to the all the genes in the Ensembl annotated genome (Figures 2, 3, 4, 5, 6, 7). i) Unique clones were validated by QRT-PCR.



**Figure 2**  
**Transcripts and TREs over-represented in the control mucosa when compared to control detrusor (MC).** The regulatory network was determined by a combination of SSH-selected transcripts (green) and PAINT 3.3 query of transcription factor database (TRANSFAC). PAINT 3.3 was employed to examine 2000 base pairs of regulatory region upstream of the transcriptional start site of each differentially expressed transcript detected with the SSH. Genbank accession numbers were used as the gene identifiers in PAINT test files. Individual elements of the matrix are colored by the significance  $p$ -values. Over-representation in the matrix when compared to the reference (all TFs in the PAINT database) is indicated in orange ( $0 < p \leq 0.01$ ) or green ( $0.01 < p < 0.05$ ). For a detailed origin of each library please see Figure 1 and Material and methods.

independent identification of NF- $\kappa$ B [39], which is known to play a key role in bladder inflammation [40] strengthens confidence in the identification of novel pathways.

Three transcripts were found downstream of NF- $\kappa$ B: *catenin* (cadherin-associated protein, delta 1), *cox7b*, and *pls3* (platin 3 T-isoform). Regarding *catenin*, others have described its presence in the bladder mucosa and in particular in the urothelium [41]. Indeed, *alpha1-catenin* was reported to be reduced in bladder urothelial cells treated with anti-proliferative factor [42] which make this transcript a possible target in cystitis. Moreover, p120-catenin is frequently altered and/or lost in tumors of bladder [43]. Finally, bladder cancers harboring a *beta-catenin* mutation may represent aggressive biological behavior with enhanced proliferating activity [44]. *Cox7b* encodes cytochrome C oxidase, subunit VIIb which is the terminal component of the mitochondrial respiratory chain and catalyzes the electron transfer from reduced cytochrome C

to oxygen. Finally, *pls3* was found by differential display to have increased expression in cisplatin-resistant human cancer cells [45].

In addition to NF- $\kappa$ B, MIC also had SRF that along with several zinc finger TFs (SF-1 [Steroidogenic factor 1]; Gfi-1 (growth factor independent 1); ik-3 (Ikaros 3); and vMaf [basic region leucine zipper]), drive the activity of *actg1*. *Actg1* is a highly conserved protein involved in various types of cell motility, and maintenance of the cytoskeleton.

The MIC regulatory network also includes the following unique transcripts: *smoc2* (secreted modular calcium-binding protein 2), *amcq* (adult male corpora quadrigemina cDNA; RIKEN clone:B230340L02), *prss11* (Serine protease 11), and *ifit3* (Interferon-induced protein with tetratricopeptide repeats 3). *Smoc2* is a widespread glycoprotein with a calcium-dependent conformation [46]. *Prss11* encodes a secreted trypsin (HtrA1) that regulates the availability of insulin-like growth factors (IGFs) by cleaving IGF-binding proteins and therefore, may function as a regulator of cell growth [47]. HtrA is involved in stress response pathways [48]. Interestingly, down-regulation of *prss11* expression may be an indicator of melanoma progression [49].

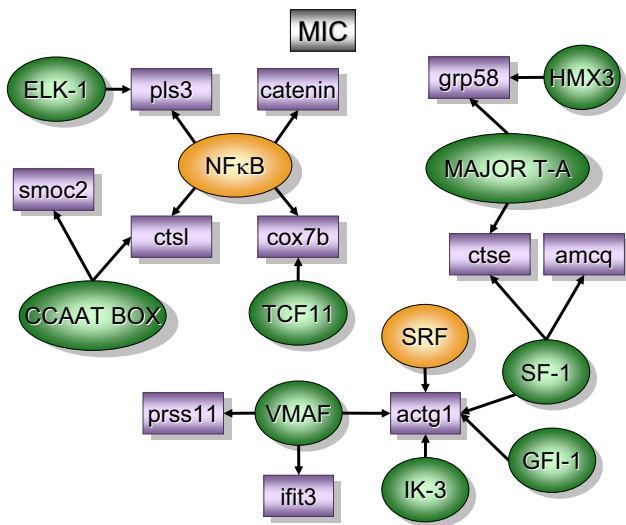
*Ifit3* was originally cloned from a cDNA library prepared from the murine cell line, RAW 264.7, after bacterial LPS stimulation [50]. Although it is a transcript induced by IFN [51], its function is still unknown.

*Grp58* encodes a ubiquitously expressed chaperone protein that resides in the endoplasmic reticulum and is part of the protein folding machinery [52]. It is likely that *grp58* is involved in the oncogenic transformation [53] since expression analysis revealed an up-regulation of *grp58* in breast, uterus, lung, and stomach tumors [54].

Together these results confirmed that the bladder mucosa expresses unique transcripts involved in cell growth, motility, and cytoskeleton, protein folding, and proteases. Some of these transcripts have been described to be altered in cystitis as well as in LPS-induced bladder inflammation. However, the most striking result was that of 6 different networks, the MIC library was the only one to have over-representation of NF- $\kappa$ B.

#### **Mucosa inflamed versus detrusor inflamed [MIDI], (figure 4)**

The question being answered by this experiment was whether or not the bladder mucosa sets the stage for LPS-induced inflammatory responses by up-regulating a unique set of genes and TFs distinct from the detrusor muscle.



**Figure 3**  
**Transcripts and TREs over-represented in the mucosa inflamed when compared to control mucosa (MIC).** The regulatory network was determined by a combination of SSH-selected transcripts (green) and PAINT 3.3 query of transcription factor database (TRANSFAC). PAINT 3.3 was employed to examine 2000 base pairs of regulatory region upstream of the transcriptional start site of each differentially expressed transcript detected with the SSH. Genbank accession numbers were used as the gene identifiers in PAINT test files. Individual elements of the matrix are colored by the significance *p*-values. Over-representation in the matrix when compared to the reference (all TFs in the PAINT database) is indicated in orange ( $0 < p \leq 0.01$ ) or green ( $0.01 < p < 0.05$ ). For a detailed origin of each library please see Figure 1 and Material and methods.

In terms of TFs, the upstream stimulatory factor (USF) was the most significantly over-represented ( $p < 0.001$ ) and driving unique transcripts (figure 4). USF dimerizes to regulate transcription through E-box motifs in target genes. Although widely expressed, they can mediate tissue-specific transcripts. USF is stimulated by glucose in murine mesangial cells, binds to TGF- $\beta$ 1 promoter, contributes to TGF- $\beta$ 1 expression, and may play a role in diabetes-related gene regulation in the kidney [55]. Others have shown that USF binding activity is enhanced in response to LPS [56].

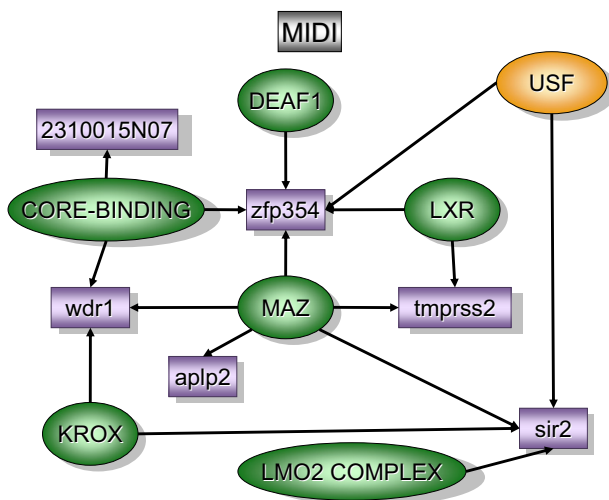
Another interesting TF was the liver X-activated receptors (LXR) found upstream of several transcripts bearing USF sequence. LXR is a member of the nuclear receptor superfamily [57] is a negative regulator of macrophage inflammatory gene expression [58], and a putative therapeutic

agent for the treatment of inflammation [58], diabetes [59], and neurodegenerative diseases [57,58,60].

LIM-only proteins (LMO), which consist of LMO1, LMO2, LMO3, and LMO4, are involved in cell fate determination and differentiation during embryonic development [61]. LMO2 was originally identified through its involvement in T-cell leukemia and subsequently shown to be critical for normal hematopoietic and endothelial development [61]. Accumulating evidence suggests that LMO1 and LMO2 act as oncogenic proteins in T-cell acute lymphoblastic leukemia, whereas LMO4 has recently been implicated in the genesis of breast cancer[61].

MAZ (Myc-associated zinc finger protein), also known as serum amyloid A-activating transcription factor-1 (SAF-1), plays a major role in regulating transcription of several inflammation-responsive genes, including matrix metalloproteinase-1 (81), the mouse mast cell protease (mMCP)-6 [62], and function as growth suppressor in fibroblasts [63]. SAF-1 transgenic mice are prone to develop a severe form of inflammation-induced arthritis [64]. MAZ is upstream of *Aplp2* which is a key regulator of structure and function of developing neuromuscular synapses [65].

In terms of unique transcripts isolated from the MIDI library, our results indicate that transcripts such as *zfp364* (zinc finger protein 364, also known as *rab7*) and *sir2* are downstream of series of TFs including: USF, LXR, DEAF1, MAZ, and Lmo2 complex (figure 4). *Sir2* gene encodes a member of the sirtuin family of proteins which are NAD-dependent histone/protein deacetylases [66]. *Zfp364* is a member of the Rab family of small G proteins, and regulates intracellular vesicle traffic to late endosomes [67]. Another unique transcript was the androgen-regulated *tmprss2* protease [68] known to be expressed in urogenital tissues [69]. *Tmprss2* has gained interest owing to its highly localized expression in the prostate and its over-expression in neoplastic prostate epithelium. Once activated, the serine protease domain of *tmprss2* is released from the cell surface into the extracellular space and activates PAR (protease-activated receptor)-2 that has a role in prostate cancer and tumor metastasis [70]. Among the proteins correlating with cytoskeleton dynamics, our SSH identified a transcript (*wdr1*) encoding a 67-kDa WD40 repeat protein 1 which is the vertebrate homologue of actin-interacting protein 1 [71]. *Wdr1* is involved in actin dynamics and seems to be required to induce cell morphologic changes, especially mitotic cell rounding [72]. Others have shown that *wdr1* was found upregulated in the lung [73] and cell lines [74] following exposure to nickel oxide-induced carcinogenesis. Finally, MIDI library also contained a RIKEN cDNA 2310015N07 gene that was



**Figure 4**  
**Transcripts and TREs over-represented in the mucosa inflamed when compared to detrusor inflamed (MIDI).** The regulatory network was determined by a combination of SSH-selected transcripts (green) and PAINT 3.3 query of transcription factor database (TRANS-FAC). PAINT 3.3 was employed to examine 2000 base pairs of regulatory region upstream of the transcriptional start site of each differentially expressed transcript detected with the SSH. Genbank accession numbers were used as the gene identifiers in PAINT test files. Individual elements of the matrix are colored by the significance  $p$ -values. Over-representation in the matrix when compared to the reference (all TFs in the PAINT database) is indicated in orange ( $0 < p <= 0.01$ ) or green ( $0.01 < p < 0.05$ ).

described to be isolated from developing mouse libraries but no function yet has been attributed [75].

Interestingly, a comparison between MIDI and DIMI libraries indicates that both share LXR as a TRE. The major difference between these two libraries was found downstream LXR activation. In the inflamed mucosa, LXR preferentially activates *zfp364* and *tmprss2* whereas in the inflamed detrusor LXR was found as a co-modulator of *actg2*.

In conclusion, the mucosa regulatory network presents USF in a central position raising the hypothesis that USF-target promoters such as the TGF- $\beta$ 1 promoter are involved in the mucosal response to inflammation and whether mucosa inflammation follows similar diabetes-related mucosal gene expression.

**Detrusor inflamed versus detrusor control [DIC], (figure 6)**  
 The regulatory network of the detrusor muscle, inflamed versus control, selected the following transcripts: *syn1*

(Synapsin I or ribosomal protein S15a), *siva* (CD27-binding protein), and *eif4ebp2* (negative regulation of translational initiation), (figure 6).

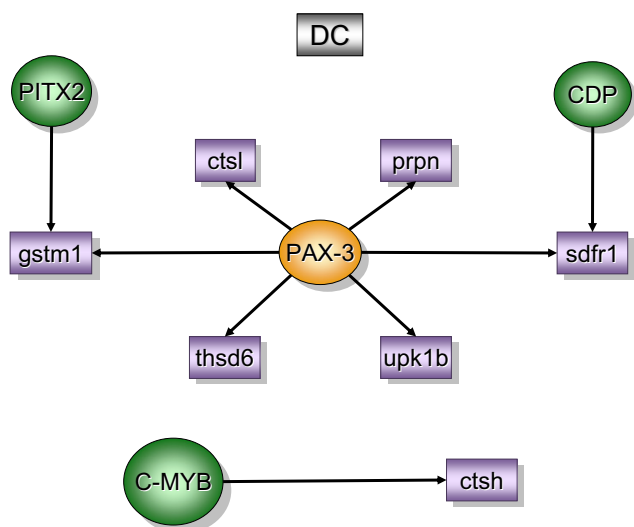
*Syn1* is a member of the synapsin gene family which is a neuron-specific phosphoprotein of small synaptic vesicles. *Syn1* has been mapped to an evolutionarily conserved linkage group composed of: *araf1*, *syn1*,  *timp*, and *properdin* located at human chromosome Xp11.2 [76] and mouse chromosome X [77]. Of interest, *araf1* is a proto-oncogene which is predominantly expressed in mouse urogenital tissues [77]. In contrast, *siva* has an important role in the apoptotic pathway induced by the CD27 antigen. Others have described that *siva* is a direct transcriptional target for both tumor suppressors, p53 and E2F1 [78]. Finally, the eukaryotic initiation factor *eIF4E* and *eIF4E*-binding proteins (4E-BPs) control the initiation of protein synthesis and are part of a translational signaling pathway sensitive to insulin [79] and rapamycin [80]. Changes in the state of phosphorylation of *eIF4E* and 4E-BPs occur at an early stage of apoptosis [81]. Interestingly, *eIF4E* selectively enhances the translation of powerful angiogenic factors such as FGF-2 and VEGF [82] and therefore may have a role in oncogenesis [82] as well as inflammation.

Over represented TFs in DIC regulatory network were NRSF (Kruppel-type zinc-finger transcriptional repressor RE1-silencing transcription factor [REST]; also known as the neuron-restrictive silencing factor), CREB/CRE-BP1, E2F-1, and Evi-1.

CREB/CRE-BP1, also called transcription factor ATF-2, binds to the cAMP response element and its activity is enhanced after phosphorylation by stress-activated protein kinases such as c-Jun N-terminal kinase and p38. ATF-2 plays a central role in TGF $\beta$  signaling by acting as a common nuclear target of both Smad and TAK1 pathways [83].

Nrf-1 (nuclear respiratory factor 1) regulates expression of nuclear-encoded mitochondrial genes and it was shown to be part of the response to LPS in rats [84].

FOXP3 belongs to the forkhead gene family which comprises a diverse group of "winged-helix" TFs with important roles in development, metabolism, cancer and aging [85]. Recently, several forkhead genes have been demonstrated to play critical roles in lymphocyte development and effector function [85]. FoxP3 is a potential target for treatment of experimental chronic inflammatory renal disease [86] and type I diabetes [87]. In addition, both FOXP3 and NRSF seems to be downstream of Wnt-Frizzled signaling [88] which was recently proposed to participate in the pathogenesis of interstitial cystitis [89].



**Figure 5**  
**Transcripts and TREs over-represented in the control detrusor when compared to control mucosa (DC).** The regulatory network was determined by a combination of SSH-selected transcripts (green) and PAINT 3.3 query of transcription factor database (TRANSFAC). PAINT 3.3 was employed to examine 2000 base pairs of regulatory region upstream of the transcriptional start site of each differentially expressed transcript detected with the SSH. Genbank accession numbers were used as the gene identifiers in PAINT test files. Individual elements of the matrix are colored by the significance *p*-values. Over-representation in the matrix when compared to the reference (all TFs in the PAINT database) is indicated in orange ( $0 < p \leq 0.01$ ) or green ( $0.01 < p < 0.05$ ). For a detailed origin of each library please see Figure 1 and Material and methods.

E2F, E2F-1, and Rb-E2F-1 belong to a family of TFs implicated in the regulation of cell proliferation and their binding sites are present in the promoters of several growth-regulating genes. E2F family members are functionally regulated, in part, by complex formation with one or more members of the nuclear pocket protein family such as the retinoblastoma protein (Rb) and play a role in neuronal development [90] by acting as negative regulator of cell proliferation. The interplay between Rb and E2F is critical for proper cell cycle progression [91]. Of interest, E2F-1 has a growth-promoting effect in bladder superficial TCC [92].

ATF-3 (activating transcription factor 3) is transcriptional repressor involved in survival and regeneration of sensory neurons [93,94] that responds to insulin [95]. ATF3 is also a novel stress-activated regulator of p53 protein stability/function providing the cell with a means of responding to a wide range of environmental insults [96]. In addition,

ATF3 represents a novel mechanism in which anti-inflammatory drugs exert their anti-invasive activity [97].

The proposed role of *nrsf/rest* is that of a transcriptional silencer that restricts neuronal gene expression to the nervous system by silencing their expression in non-neural tissues [98]. Interestingly, loss of *nrsf* function in human prostate carcinoma cells is associated with neuroendocrine phenotype, tumor progression, and androgen independence [99]. Others investigators indicated that *nrsf* also modulates the cholinergic gene locus [100,101] which may have some implication in detrusor instability. Recently, it was proposed that activation of the *rest/nrsf* target genes overrides muscle differentiation pathways and converted myoblasts to a physiologically active neuronal phenotype [102]. It remains to be determined whether *nrsf* promotes the same transformation in the inflamed detrusor muscle. The latter would explain the hyperactivity of detrusor muscle observed in over-active bladder disorders such as obstruction, incontinence, and inflammation.

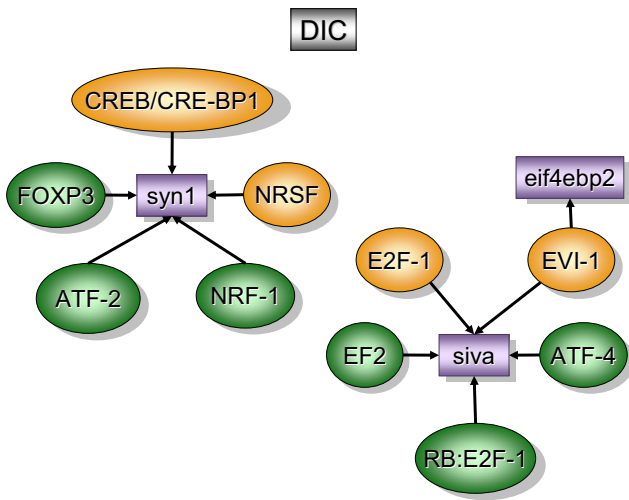
In conclusion two major networks are proposed to be active in the detrusor inflamed when compared to control. One containing a neuron-specific phosphoprotein of small synaptic vesicles (*syn*) and the other an important protein of apoptotic pathway (*siva*). In both cases, analysis of the intense upstream promoter network leads us to the hypothesis that both genes represent a common downstream target of several pro-inflammatory stimuli.

**Detrusor inflamed versus mucosa inflamed [DIMI], (figure 7)**

The question being answered by this experiment was whether or not the inflamed detrusor muscle expresses unique transcripts and TFs distinct from the bladder mucosa.

Two major pathways could be constructed with the combination of SSH and PAINT results. The first involves key smooth muscle proteins, a myosin light chain encoded by *my19* and gamma actin encoded by *actg2*. Gamma actins are highly conserved proteins that are involved in various types of cell motility, and maintenance of the cytoskeleton. In addition, a role for smooth muscle alpha actin in force generation by the urinary bladder has been suggested [103]. Several TREs upstream of *actg 2* and *my19* were over-represented in MIDI, including LXR which was described above, SRF, COUP, Pax-9, CP2, and RFX1.

A second pathway involved the transcripts *elp3*, *gus*, and *ddx3* and two TFs COMP1 and IRF. *Ddx3* is a putative RNA helicase and a member of a highly conserved DEAD box subclass. RNA helicases are highly conserved enzymes involved in transcription, splicing, and translation [104].



**Figure 6**  
**Transcripts and TREs over-represented in the detrusor inflamed when compared to detrusor control (DIC).** The regulatory network was determined by a combination of SSH-selected transcripts (green) and PAINIT 3.3 query of transcription factor database (TRANSFAC). PAINIT 3.3 was employed to examine 2000 base pairs of regulatory region upstream of the transcriptional start site of each differentially expressed transcript detected with the SSH. Genbank accession numbers were used as the gene identifiers in PAINIT test files. Individual elements of the matrix are colored by the significance *p*-values. Over-representation in the matrix when compared to the reference (all TFs in the PAINIT database) is indicated in orange ( $0 < p \leq 0.01$ ) or green ( $0.01 < p < 0.05$ ). For a detailed origin of each library please see Figure 1 and Material and methods.

There are several examples of the involvement of RNA helicases in differentiation of germ cells, particularly in spermatogenesis. Upstream of *ddx3*, PAINIT selected a TF that cooperates with myogenic proteins (COMP1) and Interferon Regulatory Factor (IRF). Transcription of IRF is synergistically activated by products of inflammation such as  $IFN\gamma$  and  $TNF\alpha$  [105].

Two other transcripts were found downstream of COMP1: *Gus* (beta-glucuronidase) and *elp3*. *Gus* is a sensitive indicator LPS activation of macrophages. *Elp3* is one of the sub units of the elongator complex, an acetyltransferase important for normal histone acetylation involved in elongation of RNA polymerase II transcription.

By comparing the inflamed detrusor and mucosa (DIM1), the fundamental difference observed was the up-regulation in the detrusor of genes and TFs related to smooth muscle function. It is fair to propose that this network could underlie detrusor instability during inflammation.

**Conclusion**

We here present a novel approach to understanding the bladder response to inflammation as a system. By using SSH, low abundance, differentially expressed transcripts could be detected that probably would have been lost in the background "noise" of a microarray study. That these genes were, in fact, key players was shown by the remarkable concordance in the transcriptional regulatory elements identified and by target validation with QRT-PCR. We suggest that the results identified key players governing the normal growth and differentiation of bladder mucosa and urothelium as well as the cross-communication of these layers during inflammation resulting from a number of pathologic processes.

As genes encoding DNA-binding TFs are the largest class of genes involved in human oncogenesis, it was obvious that in several instances the vast amount of information was related to cancer, in general and to bladder carcinoma, in particular. Interestingly, some of the TFs and their correlated downstream transcripts originally described to be involved in organogenesis were also activated during inflammation. The implications of these findings may represent one more link between inflammation and cancer.

The networks here described could well represent key targets for development of novel drugs for treatment of bladder diseases.

**Methods**

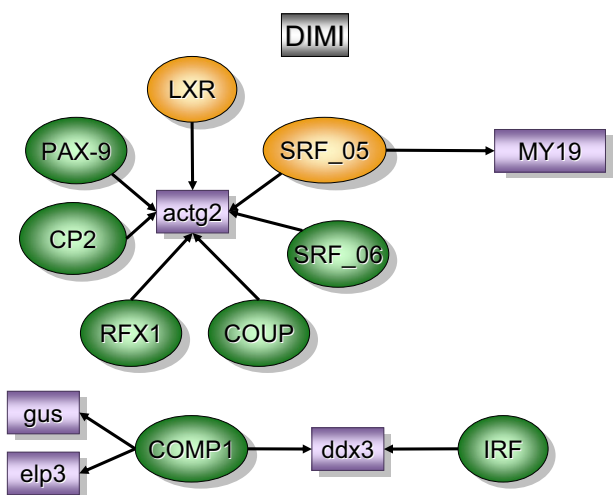
**Animals**

Ten to twelve-week old female C57BL/6J mice were used in these experiments that were performed in conformity with the "Guiding Principles for Research Involving Animals and Human Beings (OUHSC Animal Care & Use Committee protocol #002-109).

**Induction of inflammation**

Acute inflammation was induced by instillation of LPS into the mouse bladder, as described previously [8,9,106]. Female mice were anesthetized (ketamine 200 mg/kg and xylazine 2.5 mg/kg, i.p.), then transurethrally catheterized (24 Ga.; 3/4 in; Angiocath, Becton Dickson, Sandy, Utah), and the urine was drained by applying slight digital pressure to the lower abdomen. Because the bladder of 10-week old mice has an average capacity of 250  $\mu$ l, the urinary bladders were instilled with 200  $\mu$ l of one of the following substances: pyrogen-free saline (control) or *Escherichia coli* LPS strain 055:B5 (Sigma, St. Louis, MO; 100  $\mu$ g/ml,) (figure 1a). Substances were infused at a slow rate to avoid trauma and vesicoureteral reflux. To ensure consistent contact of substances with the bladder, infusion was repeated twice within a 1-hour interval and a 1-ml syringe was maintained in the catheter end during





**Figure 7**  
**Transcripts and TREs over-represented in the detrusor inflamed when compared to mucosa inflamed (DIMI).** The regulatory network was determined by a combination of SSH-selected transcripts (green) and PAINT 3.3 query of transcription factor database (TRANSFAC). PAINT 3.3 was employed to examine 2000 base pairs of regulatory region upstream of the transcriptional start site of each differentially expressed transcript detected with the SSH. Genbank accession numbers were used as the gene identifiers in PAINT test files. Individual elements of the matrix are colored by the significance  $p$ -values. Over-representation in the matrix when compared to the reference (all TFs in the PAINT database) is indicated in orange ( $0 < p \leq 0.01$ ) or green ( $0.01 < p < 0.05$ ). For a detailed origin of each library please see Figure 1 and Material and methods.

this period. The catheter was removed, and mice were allowed to void normally. Twenty-four hours after instillation, mice were euthanized with pentobarbital (100 mg/kg, i.p.) and bladders were removed rapidly.

#### **Tissue layers – separating the mucosa from detrusor**

Immediately after removal from the animal, bladders were placed in RNAlater™ (Ambion) and visualized under a dissecting microscope (Nikon SMZ 1500). The detrusor smooth muscle was separated by blunt dissection away from the mucosa which contained the epithelium and sub-epithelial layers, (see Additional file 1).

#### **Suppression subtractive hybridization (SSH) (9)**

A total of six libraries were obtained by SSH (figure 1c). In order to standardize the names of the groups and to correlate with delta CT values (see below), the SSH libraries were named after the *tester* minus *driver*. The first library (MC) was obtained by using the mucosa removed from control saline-treated mice (CM) as *tester* and the respective detrusor smooth muscle (CD) as *driver*. The resultant

subtraction MC was supposed to contain genes preferentially expressed in the control mucosa. The second library was the reverse of MC and therefore, CD was used as *tester* and CM as *driver* and will contain genes preferentially expressed in the detrusor smooth muscle of control mice (DC). The other 4 libraries were obtained to investigate genes whose expression was altered during LPS-induced inflammation (figure 1c). The samples used for each SSH were obtained by pooling RNA from 20 individual mice. The pooling was necessary in order to obtain enough RNA from each layer without amplification.

#### **Construction of subtractive cDNA libraries**

mRNA was isolated from total RNA using Poly(A) Quick mRNA Isolation Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. To compare the two populations of resulting cDNA the method of SSH was performed using PCR-Select cDNA Subtraction Kit (BD Biosciences – Clontech, Palo Alto, CA), as described by Diatchenko and colleagues [18]. This method selectively amplifies differentially expressed sequences, and the generation of high- and low-abundance sequences is equalized during the first hybridization. The PCR allows amplification of equalized differentially expressed sequences. Each step of the cDNA synthesis and subtractive hybridization procedure was monitored using the positive control samples provided by the manufacturer. We verified the efficiency of subtraction by PCR analysis by comparing GAPDH levels in subtracted and un-subtracted cDNA using the method and GAPDH primers provided by the manufacturer. For analysis of efficiency, please see Additional file 2. For analysis of ligation, please see Additional file 3. For the analysis of PCR products, please see Additional file 4. For PCR analysis and subtraction efficiency, please see Additional file 5.

Next, cDNAs from the testers and drivers were digested with *RsaI*. To select tissue- and treatment-specific transcripts, PCR adapters were ligated to the tester pool population. The tester cDNA pool was then hybridized with excess cDNAs from the driver pool. After hybridization suppression, PCR using primers specific for the tester PCR adapters selectively amplified differentially expressed transcripts.

#### **Screening the clones (plating out, growing up and analyzing the PCR clones), (figure 1d)**

After the PCR subtraction, the amplification products were cloned into the pCR 2.1 plasmid of the TA cloning kit (Invitrogen). Ligated DNA was transformed by heat shock in 100  $\mu$ l of INV $\alpha$ F competent *E. coli* cells. Colonies were grown overnight at 37 °C on Luria broth agar plates containing ampicillin, X-gal, and isopropyl-B-D-thiogalactopyranoside for blue/white colony selection. White colonies were isolated and grown individually in 2 ml of

LB medium containing ampicillin for 16 h. After plasmid DNA isolation (Wizard<sup>®</sup> Plus SV Minipreps DNA Purification System – Promega), digestion was performed using *Xba* I and *Bam*H I, and the products analyzed in 1% agarose gels. Positive clones (representing fragments larger than the original polylinker in the cloning vector) were sent to a sequencing service (figure 1e), and sequences were submitted for a BLAST analysis in GenBank for identification and annotation was done by searching the gene ontology [16], figure 1f.

#### **Tissue- and treatment-specific transcription factors**

After annotation, a bioinformatics approach to identify functionally relevant putative transcriptional regulatory elements (TREs) for all SSH-selected transcripts was used (figure 1g). We used the Promoter Analysis and Interaction Network Toolset [PAINT [13]], available online [107], to integrate functional genomics information from SSH-derived gene expression data with the genomic sequence and TRE data to derive hypotheses about relevant transcriptional regulatory networks. PAINT uses the TRANSFAC<sup>®</sup> database [14] of transcription factors and position weight matrix descriptions of cis-acting sequences and an associated pattern matching tool MATCH [15] to identify statistically over-represented regulatory sites in 5' upstream sequences of related genes. This information provides a substantially pruned list of TFs regulating tissue- and treatment-specific genes that were identified by SSH. Briefly, the accession numbers of all the SSH-selected transcripts were used as an input gene list in PAINT. Up to 2000 base pairs of 5' upstream sequences were analyzed for the presence of TREs using a MATCH/TRANSFAC setting to minimize false positives and filtering the results further in PAINT to consider only those hits with 100% match to the 5 bp core TRE sequence. The interaction matrix contained 79 genes and 162 TREs.

PAINT can analyze the interaction matrix for over-represented TREs in subsets/clusters of related genes, typically grouped based on gene expression data. The library for each transcript (MC, DC, MI, MIDI, DI and DIMI) was considered as the cluster label and provided as the GeneCluster information in the PAINT analysis and visualization step. The TRE over-representation in PAINT is calculated as the hyper-geometric probability of the observed number of TREs in a given cluster as compared to that in randomly selected gene clusters from a 'reference' list. In this study, all the genes in the genome as annotated in Ensembl were used to construct the interaction matrix for use as the reference. To construct the hypothesized regulatory networks, TFs were chosen based on the probability of over-representation ( $p < 0.05$ ) in any of the six gene groups as compared to all the genes in the genome (or equivalently in PAINT promoter database).

#### **Target validation by QRT-PCR (figure 1i)**

##### *RNA isolation and cDNA synthesis*

An additional 40 C57BL/6J female mice, ten to twelve-weeks old underwent the same intravesical treatment as described above [LPS, (n = 20 mice) and saline (n = 20 mice)]. Twenty four hours after LPS instillation, mice were euthanized, the bladder was removed and placed in RNAlater<sup>™</sup> (Ambion) for separation of the mucosa and submucosa from the detrusor smooth muscle, as described above. Four sample groups were obtained as follows: control mucosa (CM), control detrusor (CD), LPS-treated mucosa (LM), and LPS-treated detrusor (LD). Bladders were pooled and homogenized in Ultraspec RNA solution (Biotex Laboratories, Houston, TX) for isolation and purification of total RNA used for QRT-PCR. High RNA quality from each of these four groups was verified by capillary gel electrophoresis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc, Palo Alto, CA). RNA concentration was determined by spectrophotometry using a NanoDrop<sup>®</sup> ND-1000 UV-Vis Spectrophotometer (Nano Drop Technologies, Wilmington, DE). Subsequently, this total RNA was used as a template for each of the first-strand cDNA syntheses. Prior to cDNA synthesis, an exogenous standard of *A. thaliana* (RCA) mRNA (Stratagene, L Jolla, CA, USA) was added (0.1 ng) to each CM, CD, LM, and LD total RNA (2 µg) sample for normalization of succeeding gene expression data. RNA was reverse-transcribed according to the Omniscript RT<sup>™</sup> kit (Qiagen, Valencia, CA) instructions and subsequently purified using the Montage PCR 96-well cleanup plate (Millipore, Billerica, MA). Prior to the PCR, primer pairs were designed utilizing both Primer Express<sup>®</sup> (ABI, Foster City, CA) and NetPrimer (PREMIER Biosoft International, Palo Alto, CA) software. Primers were designed according to the general guidelines outlined in the Primer Express<sup>®</sup> User Bulletin. Details of the primers and the Genebank accession numbers are given in Table 1. The designed primers shared 100% homology with the target sequence but no significant homology with other sequences.

The QRT-PCR amplifications were accomplished on an ABI<sup>®</sup>PRISM 7700 using SYBR<sup>®</sup>Green I dye assay chemistry. A 15 µL PCR assay for each gene of interest consisted of 7.5 µL of 2X SYBR<sup>®</sup>Green PCR mix (Applied Biosystems Inc., Foster City, CA), 4.9 µL of H<sub>2</sub>O, 0.6 µL (30 pmoles) of gene-specific forward and reverse primers, and 2 µL (1 ng) of cDNA template. All samples were run in triplicate with the appropriate single QRT-PCR controls (no reverse transcriptase and no template). Cycling conditions used for all amplifications were one cycle of 95 °C for 10 minutes and 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Following the QRT-PCR, dissociation curve analysis was performed to confirm the desired single gene product.

From the QRT-PCR data, an average cycle threshold (Ct) value was calculated from the triplicate reactions. Averaged Ct values were then normalized (to adjust for different amounts of cDNA within each reaction) to the exogenous control gene, RCA. The relative expression level of each transcript within each sample group (CD, CM, LD, and LM) was determined by calculating the ratio of the antilog<sub>2</sub> of the delta Ct values. The resultant fold-change data is presented in Table 4.

### Authors' contributions

MRS participated in its design, carried out the animal experiments, removed the tissues, performed suppression subtractive hybridizations, and performed sequence alignments. NBN helped MRS on the suppression subtractive hybridizations experiments. HLH participated in the design of the study and trained both MRS and NBN to develop SSHs. MT and MC developed Q-PCR. RV developed the PAINT program and guided RS to perform TF analysis. DWD's laboratory sequenced all transcripts. REH participated in its design and helped to draft the manuscript. RS conceived of the study, developed annotation and TF analysis, and draft the manuscript.

### Additional material

#### Additional File 1

Separation of bladder layers (mucosa and detrusor).

Click here for file

[<http://www.biomedcentral.com/content/supplementary/1472-6793-6-1-S1.pdf>]

#### Additional File 2

Detailed methodology for SSH and analysis of the experimental procedures.

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[<http://www.biomedcentral.com/content/supplementary/1472-6793-6-1-S2.pdf>]

#### Additional File 3

Analysis of ligation.

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[<http://www.biomedcentral.com/content/supplementary/1472-6793-6-1-S3.pdf>]

#### Additional File 4

Analysis of PCR products.

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[<http://www.biomedcentral.com/content/supplementary/1472-6793-6-1-S4.pdf>]

#### Additional File 5

The reduced message of a known housekeeping gene between the subtracted and un-subtracted cDNA over the same number of PCR cycles.

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[<http://www.biomedcentral.com/content/supplementary/1472-6793-6-1-S5.pdf>]

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