

Supplemental Information

BPI Fold-Containing Family A Member 2/Parotid Secretory Protein Is an Early Biomarker of AKI

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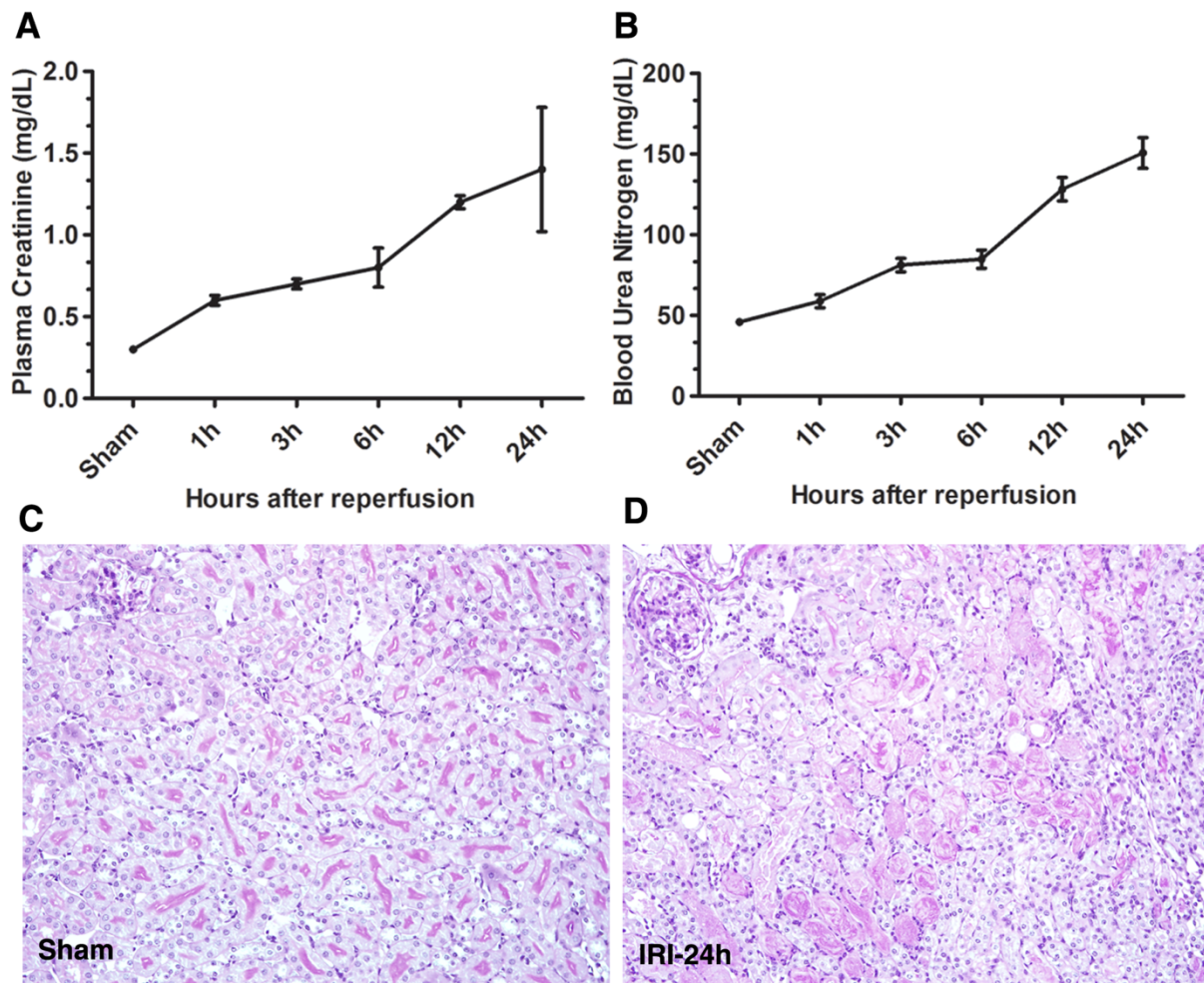
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Supplementary Figures

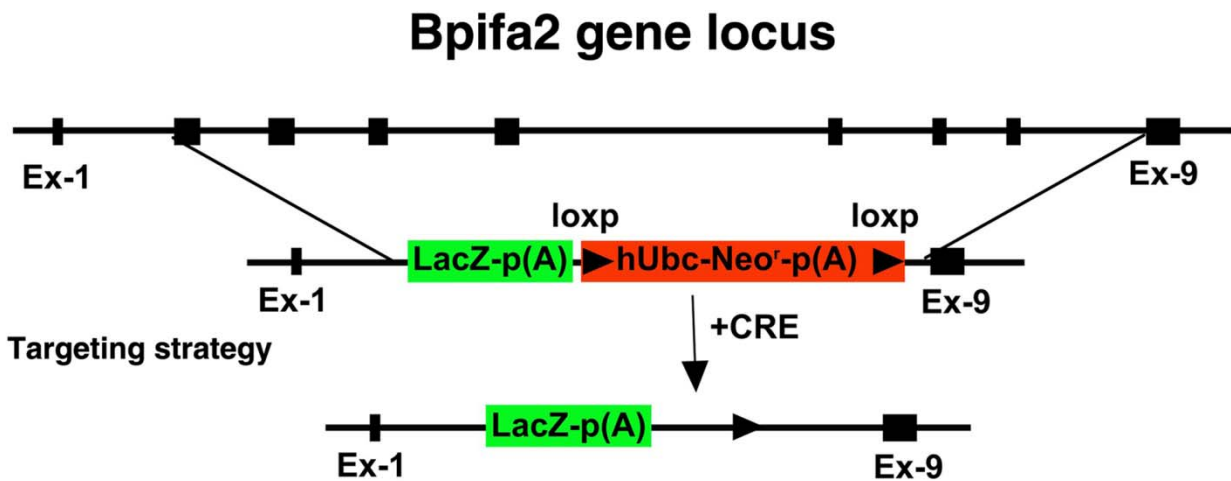
Supplementary Figure 1. Kidney injury parameters in renal ischemia reperfusion injury model.

Kidney injury was ascertained by colorimetric measurements of plasma (A) Creatinine and (B) Urea Nitrogen levels as well as following histological assessment of kidney injury following Periodic Acid-Schiff (PAS) staining.



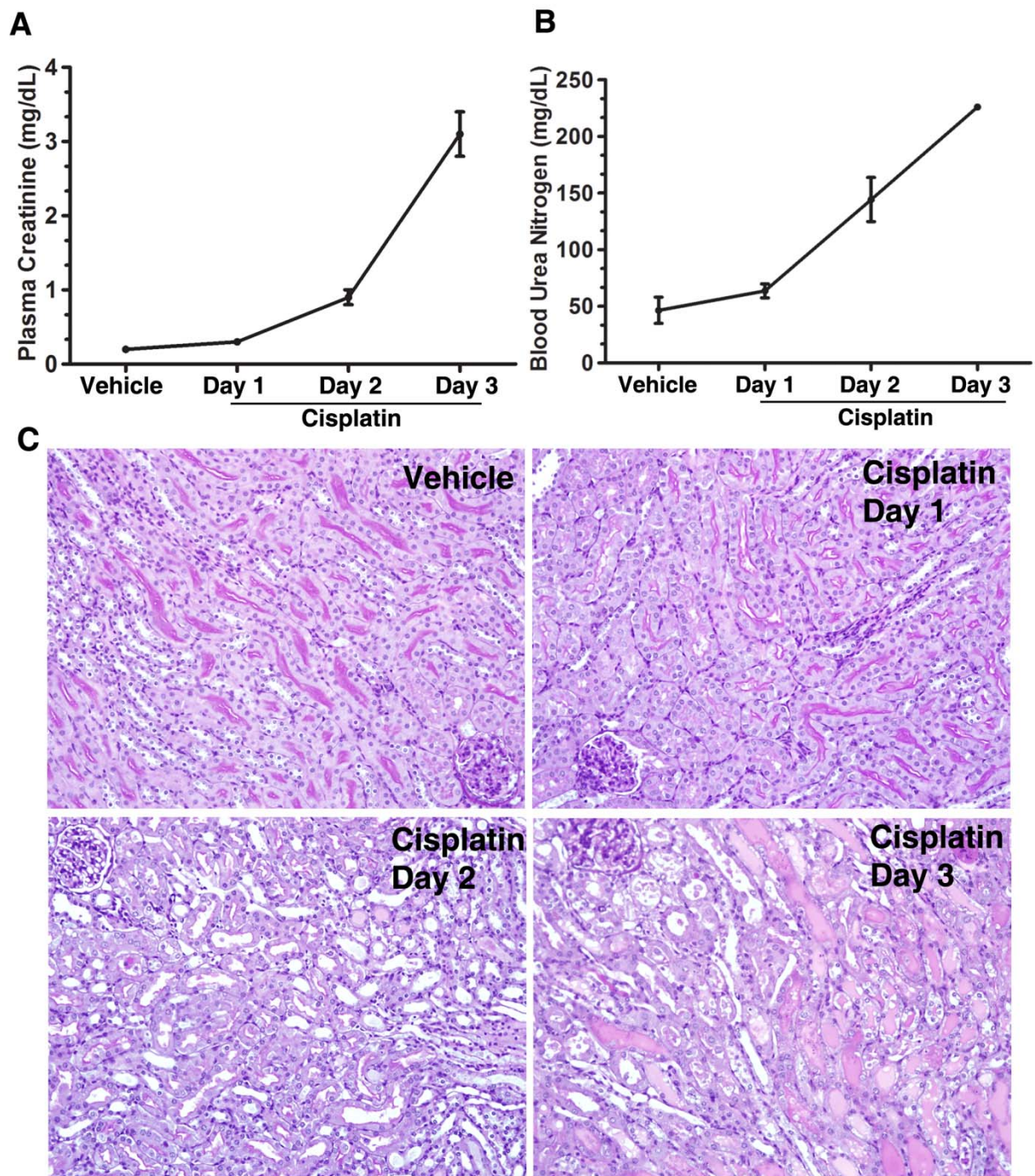
Supplementary Figure 2. *Bpifa2* mutant mouse strain carrying a *lacZ* reporter gene under the *Bpifa2* promoter at the endogenous locus.

LacZ tagged-*Bpifa2* null mouse strain was used in this study as a reporter of *Bpifa2* promoter activity. Neomycin cassette was removed by crossing with *Sox2* cre mouse strain.



Supplementary Figure 3. Kidney injury parameters in cisplatin induced AKI.

Kidney injury was ascertained by colorimetric measurements of plasma (A) Creatinine and (B) Urea Nitrogen levels as well as following histological assessment of kidney injury following Periodic Acid-Schiff (PAS) staining.



Supplementary Figure 4. Identification of Nur77 binding sites in the proximal promoter region of human and mouse *BPFA2* genes.

Consensus NBRE core binding site, AGGTCA was found in -2kb upstream region of TSS in both human and mouse *BPFA2* genes (highlighted in red).

Human *BPFA2*

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-840 TCCACTGGTT GCCCCACCAA ACTCGGCCCTT ACCAGGTCCT CCGTGATCAC CAGGTAGAGG AAGTCAATGG ATCATAGATG
-760 AGGTCAGCCT CATCTTGACC ATAGCACACA TACCACTTCC TCCTCCATGA ATCCAGTTCT TCCCTTGGCC TTA CTCCCT
-680 GGCTCTCCTC CTCTCCTCC TCTGACCATT CTACCTCTGC CTCTTTGCAG ATGTAATATT TTCAAATCGG CCAACCAAGT
-600 GCTTCCTTCA CCCTGGCCAC TTTGGTTGGT TCATTGCTCC GCATGTAACC CCAGCAGGAC CAATGGGAAT CAGTCCTAGA
-520 ACTTGTGAAG AGATATTAGG AAAGACATAC TTTCTTCTGC TAGACTATTA AGAGAGAATG TGGACCTGAA TCTGTTGGTG
-440 GCCATCTCTG CCACCTCTTT GGGGAAGTCA GTTTGAGGAG AAACCCCAAG CCAAGTCATG GAAAGAAACA AAGGCCTGGA
-360 GATTTCTTTT GAGCACCTAG ATTCAGCCAT GCCTGAAACC CGTTGGCCTG CTAGTTACAT GAGCCAATGA CTTTCTTTCA
-280 CATCTAAGTC ACTTTGGGTT GTGTTTTCAA TTACAAATAT AACCCTAAAGA ATCCTGACAA ATGCAACTGA CAAACATTTT
-200 AGTTTATTGG CAAGCCATGG TGGGAAACCA GAGATGAACC CCAGAGAGAA CTTGGCCTCC TGGAGTTGAC AATGGAGAAG
-120 AGACAGCTGT GGGTGTGTAG GCAAGGAAGA CACCTGTTCA AAAATGACAA GGCAGAACGT GAGAAAAGGT CAGCTGGGTG
-40 GCTCAATGAA GGCTTTATAT AAGAGGTGCT TGCAGGATGG GGAGAAATTG CAGGGGGGTG ACATCCTCAG ACCTGGCCCT
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Mouse *Bpifa2*

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-1680 TTGCAGCTAC AGCCCTTCC TGTCTGAACC TCCTGTCAGGT TCACTGGGAA ACAGATCAAG ATGGAACAGG ACAGCTCCTG
-1600 ATGGTAAATA AAAACAGTG GTCATGGCTA TTCATAGGGG TTTATGCTTC TTCAGTCCAC ACTGTGAAGA GCTGTGGGCA
-1520 TGAACCACAG TGTTTCGAGT AGAGTTGGGG TTCTGAAATT CACAGTGGGG TGAGCTCAGT AAATGTGAGC TGGAGGTCAC
-1440 TCGTGAGACA CACAGTCCTG CTGCTTCTGT TCCCAATATC CTGAGGAGAC GACACATCTA CTTTGTTCAG AGGCCACAGT
-1360 CTAGTTGACC TGAGAGTTAC CAGTTTCTTA TTTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TTGTTCTGTG
-1280 GTGAGTGCAG GTGCACATAT GATAGCGTAC ACGTTGAGGT CAGAGGATAA CTATCAGGCG TTGTCCCTC CTACTTTTCC
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Supplementary Figure 5. Potential N-glycosylation sites in Human and Mouse BPIFA2 protein.

N-X-S/T consensus sequences essential for N-glycosylation are found in both Human (2 sites) and mouse (1 site) proteins.

Human BPIFA2

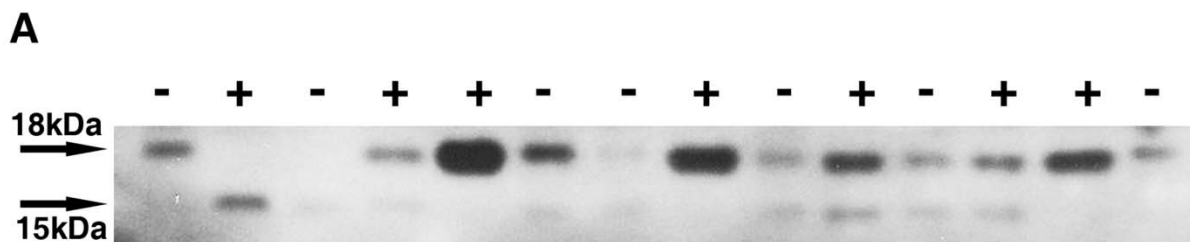
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1 MLQLWKLVLL CGVLTGTSES LLDNLGNDLS NVVDKLEPVL HEGLETVDNT LKGILEKLV DLGVLQKSSA WQLAKQKAQE
81 AEKLLNNVIS KLLPTNTDIF GLKISNSLIL DVKAEPIDDG KGLNLSFPVT ANVTVAGPII GQIINLKASL DLLTAVTIET
161 DPQTHQPVAV LGEASDPTS ISLSLLDKHS QIINKFVNSV INTLKSTVSS LLQKEICPLI RIFIHSLDVN VIQQVVDNPQ
241 HKTQLQTLI
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Mouse Bpifa2

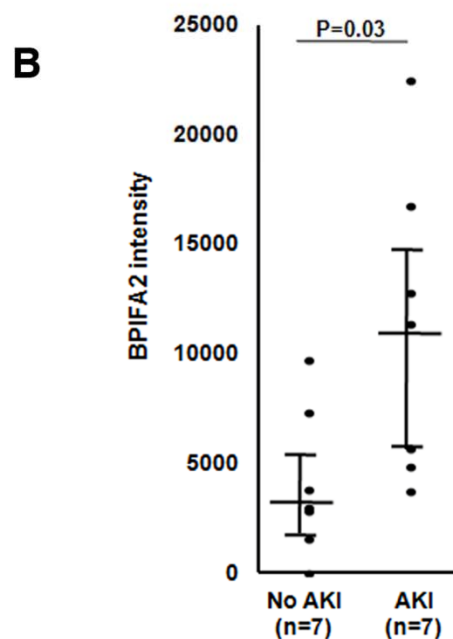
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1 MFQLGSLVVL CGLLIGNSES LLGELGSAVN NLKILNPPSE AVPQNLNLDV ELLQQATSWP LAKNSILETL NTADLGNLKS
81 FTSLNGLLLK INNKKVLDLQ AKLSSNGNGI DLTVPPLAGEA SLVLPFIGKT VDISVSLDLI NSLSIKTNAQ TGLPEVTIGK
161 CSSNTDKISI SLLGRRLPII NSILDGVSTL LTSTLSTVLQ NFLCPLLQYV LSTLNPSVLQ GLLSNLLAGQ VQLAL
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Supplementary Figure 6. Plasma levels of BPIFA2 in healthy volunteers and patients with established AKI.

Patients with established AKI had increased abundance of BPIFA2 as assessed by (A) Western blot analysis: 18 and 15 kDa bands reactive to anti-BPIFA2 antibody were detected in plasma; - denotes healthy individuals; + denotes AKI patients. (B) Quantification of BPIFA2 protein levels in plasma of AKI patients compared to healthy controls based on densitometric analysis of BPIFA2 bands observed in western blot. Bars indicate median (IQR). AKI was defined as an increase in serum creatinine ≥ 0.3 mg/dl within 48 hours or $\geq 50\%$ in 7 days.

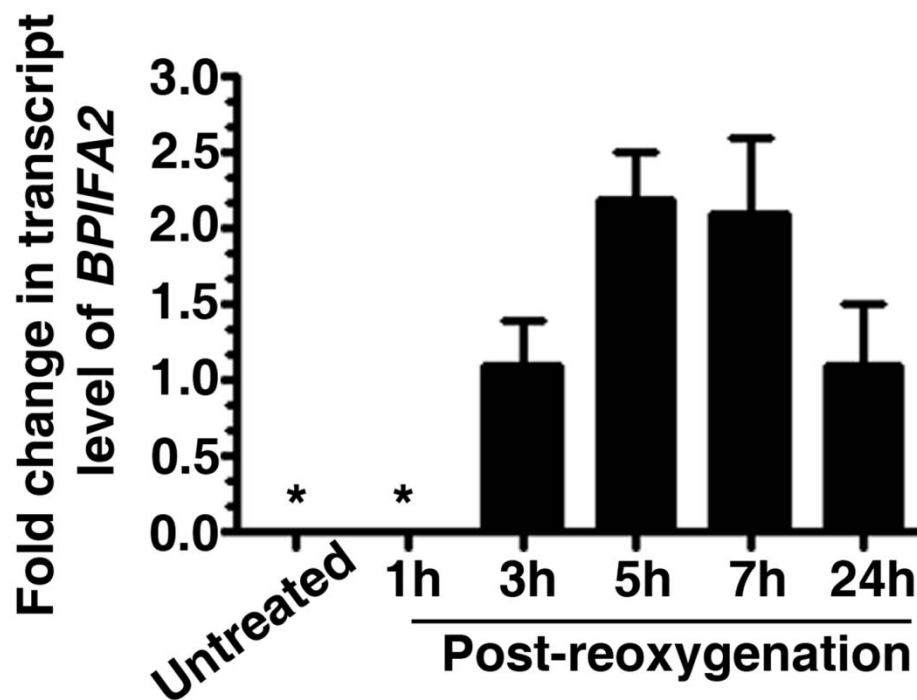


B: BPIFA2



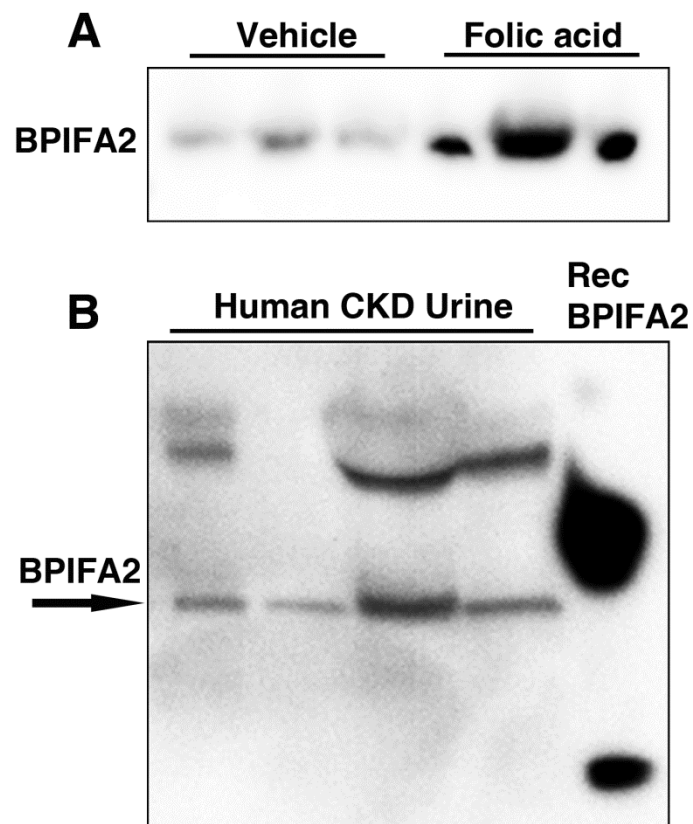
Supplementary Figure 7. *BPIFA2* gene expression was induced in HK2 cells in an *in vitro* ischemia/ reoxygenation model.

BPIFA2 transcripts were induced in the human immortalized proximal tubular epithelial cell line, HK2 upon treatment with a cocktail that depletes cellular ATP and glucose (Antimycin (10 μ M) and 2-Deoxy Glucose (10 mM)) with calcium overload (A23187 (1 μ M) for 1 hour and replaced with culture medium for indicated periods of time. * indicates transcript not detected.



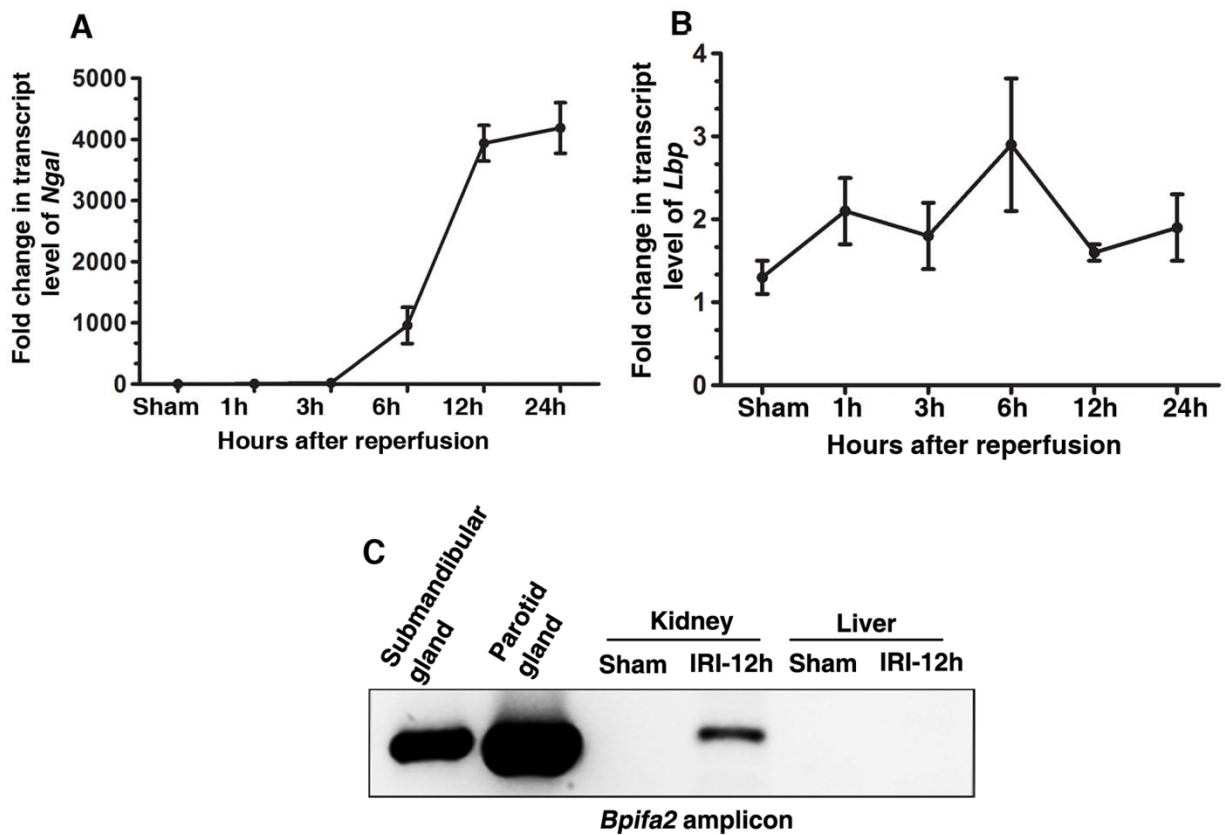
Supplementary Figure 8. BPIFA2 is detected during chronic kidney disease.

(A) Increased abundance of BPIFA2 was detected in the plasma of mice, 3 months post-administration of a single dose of folic acid (250 mg/kg body weight). (B) BPIFA2 was also detected in urine samples collected from CKD patients.



Supplementary Figure 9. Liver is not a source of *Bpifa2* during IRI mediated AKI.

Bpifa2 transcripts were undetectable in qRT-PCR mediated analysis of liver tissues obtained from mice that underwent bilateral renal IRI, whereas transcripts of (A) *Ngal* and (B) LPS binding protein (*Lbp*) were readily detected. (C) *Bpifa2* transcripts were amplified from kidney and not liver tissues following renal IRI in a semiquantitative RT-PCR analysis. Submandibular and parotid gland tissues served as positive controls.



Supplementary Table 1.

Top candidate proteins identified in the quantitative proteomics screen based on their fold change at 24h post reperfusion. Protein levels at sham operated mice kidneys served as baseline.

Protein Id	Gene Symbol	Sham	6 hr	24 hr	48 hr	Day 7
sp Q6GQT1 A2MP_MOUSE	<i>A2mp</i>	1	1.106571	8.006603	4.242642	1.367541
sp Q9DAU7 WFDC2_MOUSE	<i>Wfdc2</i>	1	1.432937	8.257983	9.167067	5.65521
sp P12032 TIMP1_MOUSE	<i>Timp1</i>	1	1.501873	6.971724	4.802022	2.233392
sp Q5QNS5 HAVR1_MOUSE	<i>Havcr1</i>	1	1.626663	28.65481	23.51179	15.61797
sp P02802 MT1_MOUSE	<i>Mt1</i>	1	1.801941	8.159717	7.597807	1.764346
sp Q9D312 K1C20_MOUSE	<i>Krt20</i>	1	1.823694	8.405963	9.314614	1.985935
sp P10923 OSTP_MOUSE	<i>Spp1</i>	1	1.995108	13.06103	7.784844	3.410112
sp P00688 AMYP_MOUSE	<i>Amy2</i>	1	2.220766	12.35956	2.816823	0.994746
sp Q9D975 SRXN1_MOUSE	<i>Srxn1</i>	1	2.452513	6.161515	4.508476	1.198853
sp P02798 MT2_MOUSE	<i>Mt2</i>	1	2.557624	7.884558	8.220662	2.086525
sp Q5FW60 MUP20_MOUSE	<i>Mup20</i>	1	3.639112	6.376291	6.385644	1.593279
sp P00687 AMY1_MOUSE	<i>Amy1</i>	1	3.946465	7.774237	2.216282	0.770808
tr G5E899 G5E899_MOUSE	<i>Serpine1</i>	1	4.080268	6.893168	3.508947	2.749006
sp P11247 PERM_MOUSE	<i>Mpo</i>	1	4.353757	6.506757	3.716976	1.30689
sp P27005 S10A8_MOUSE	<i>S100a8</i>	1	5.05806	6.484106	2.956633	1.14817
tr Q80YX8 Q80YX8_MOUSE	<i>Mup21</i>	1	5.123269	16.96224	11.60142	1.516608
sp Q9QXC1 FETUB_MOUSE	<i>Fetub</i>	1	5.852757	9.970386	6.599854	1.961896
sp Q8BNJ2 ATS4_MOUSE	<i>Adamts4</i>	1	5.994699	6.570403	6.421997	2.522707
tr Q58EV3 Q58EV3_MOUSE	<i>Mup1</i>	1	6.383092	16.66035	6.740625	1.377539
tr Q3KQQ2 Q3KQQ2_MOUSE	<i>Mup3</i>	1	8.403679	13.66151	10.89576	2.600822
sp P07743 BPIA2_MOUSE	<i>Bpifa2</i>	1	9.875035	7.678328	2.195403	0.889211
sp B5X0G2 MUP17_MOUSE	<i>Mup17</i>	1	10.25946	11.38849	8.672714	2.716147
tr A2BIN1 A2BIN1_MOUSE	<i>Mup10</i>	1	10.4255	11.43542	10.91587	3.654275
tr B8JI96 B8JI96_MOUSE	<i>Mup14</i>	1	11.18699	13.3182	9.968282	2.587072
tr L7MUC7 L7MUC7_MOUSE	<i>Mup7</i>	1	12.749	7.829818	5.531425	1.882403
tr H3BKP1 H3BKP1_MOUSE	<i>Hmox1</i>	1	13.9007	7.269489	1.980182	1.38771

Materials and methods

Mice

C57BL/6N and *Nur77* knockout (Stock # 006187) mice were purchased from Charles river and Jackson laboratories respectively. The *Bpifa2* mouse strain (*Bpifa2*^{tm1(KOMP)}*Vlcr*) carrying a *lacZ* reporter construct under the *Bpifa2* promoter (replacing ~6.2 kb region (Chr2:154,009,124-154,015,372) spanning exon2-exon8 at the *Bpifa2* gene locus) used in this study was created from ES cell clone Psp_AD12, generated by Regeneron Pharmaceuticals, Inc. and made into live mice by the KOMP Repository (www.komp.org) and the Mouse Biology Program (www.mousebiology.org) at the University of California Davis (1). Re-derivation of the strain was carried out the transgenic core facility at the Beth Israel Deaconess Medical Center (BIDMC). Neomycin selection cassette introduced in the above *Bpifa2* mutant mouse strain during gene targeting was removed by crossing with *Sox2* cre (strain 008454 / Jackson laboratory). Animals were housed in an AALAC-approved facility in a pathogen free environment. All the animal studies were approved by the Institutional Animal Care and Use Committee at BIDMC.

Renal ischemia reperfusion injury model

Male mice of age 8-10 weeks were used in this study. Animals were anesthetized using isoflurane administered via precision vaporizer or following administration of Ketamine / Xylazine and body temperature was maintained at 37 deg C throughout the procedure. The kidneys were exposed by flank incision and both the kidney pedicles were clamped for 30 min using nontraumatic microaneurysm clamps (Fine science tools). Reperfusion was confirmed visually, incision was sutured and 1 ml of warm sterile 0.9% saline was administered intraperitoneally. Sham operated controls underwent similar procedure as above minus kidney pedicle clamping.

Cisplatin mediated kidney injury

A single dose of Cisplatin (Sigma aldrich) (20 mg/ kg body weight) was administered intraperitoneally. Male mice of age 8-10 weeks were used.

LPS mediate kidney injury

Male mice of age 8-10 weeks were used. LPS from E. coli serotype O111:B4 (Sigma-Aldrich) was administered intraperitoneally at a dose of 10 mg/ kg body weight and mice were sacrificed at defined time points.

Folic acid mediated chronic kidney injury

A single dose of folic acid dissolved in 0.3 mM Sodium bicarbonate (Sigma aldrich) (250 mg/kg body weight) was administered intraperitoneally. Male mice of age 8-10 weeks were used. Plasma samples were collected on day 90 post administration.

Mouse saliva collection

Mice were anesthetized following administration of Ketamine / Xylazine. Salivary secretions were collected following intraperitoneal administration of Pilocarpine at a dose of 1 mg / kg body weight under complete anesthesia.

Analysis of kidney function

Blood plasma was collected at distinct time points viz., 1h, 3h, 6h, 12h, 24h post reperfusion (non-serial blood sampling) and stored at -80 deg C until further processing. Plasma and Urine Creatinine levels were quantified using QuantiChrom™ Creatinine Assay Kit (Bioassays systems). Blood Urea Nitrogen levels were measured using Infinity Urea liquid stable reagent (Thermo fisher).

Tandem tag mass spectrometry- LC-MS/MS

Samples for multiplexed quantitative mass spectrometry analysis were processed and analyzed through the Thermo Fisher Scientific Center for Multiplexed Proteomics at Harvard Medical School (2). Sample processing steps included cell lysis, tandem protein digestion using LysC and trypsin, peptide labeling with Tandem Mass Tag 10-plex reagents and peptide fractionation. Multiplexed quantitative mass spectrometry data were collected on an Orbitrap Fusion mass spectrometer operating in a MS3 mode using synchronous precursor selection for the MS2 to MS3 fragmentation (3). MS/MS data were searched against a Uniprot human database (February 2014) with both the forward and reverse sequences using the SEQUEST algorithm. Further data processing steps included controlling peptide and protein level false discovery rates, assembling proteins from peptides, and protein quantification from peptides.

Human urine and plasma samples

Urine samples were obtained from healthy volunteers (n=30) and patients with established AKI (n=24) resulting from diverse etiologies. Plasma samples were obtained from healthy volunteers (n=7) and patients with established AKI (n=7). AKI plasma samples were obtained from critically patients who enrolled in a prospective cohort study at Brigham and Women's Hospital (Boston, MA). AKI was defined as an increase in serum creatinine ≥ 0.3 mg/dl within 48 hours or $\geq 50\%$ in 7 days.

Renal histopathology, immunohistochemistry and immunofluorescence staining

Kidneys from animals subjected to IRI or sham operation were harvested at indicated time points. Transverse sections of the kidneys were prepared (by fixation in 4% paraformaldehyde, followed by incubation in 30% sucrose overnight) and frozen in OCT or fixed in 10% formalin and processed for paraffin embedded sections. Renal morphology was assessed by PAS staining (Periodic acid-Schiff reagent). *Bpifa2* expression on mouse kidneys was detected on frozen tissues following paraformaldehyde or acetone fixation. Formalin fixed paraffin embedded human kidney biopsies were processed following antigen retrieval (using sodium citrate pH 6.0). The following antibodies were used in the study. Goat anti-mouse BPIFA2 (Sigma aldrich, Cat # SAB2501987) (at a dilution of 1 ug/ml), Goat anti-human BPIFA2 (Santa cruz biotechnology, SPLUNC2 Antibody (C-20); Cat # sc-85932) (at a dilution of 1 ug/ml), Rabbit anti-beta galactosidase (Invitrogen, Cat #: A-11132) (at a dilution of 2 ug/ml). Biotinylated LTL (Vector labs, Cat # B-1325) was used to identify renal proximal tubules.

Real time PCR quantification

Total RNA was prepared from cultured cells or kidney tissues using RNeasy kit (Qiagen). DNA contamination was eliminated by DNaseI digestion. For the detection of *Bpifa2* transcripts, 1000 ng of RNA were reverse transcribed in a 10 ul of reaction using multiscript reverse transcription system (Applied biosystems). The reverse transcribed cDNA was subjected to qPCR using a Sybr green based detection system (Qiagen). The following cDNA equivalents of RNA were used for the detection of transcripts: *Bpifa1*, *Bpifa2*, *Bpifa3*: 100 ng; *Ngal*, *Kim-1*: 50ng; *Gapdh*: 5ng. Relative levels of transcripts were normalized to *Gapdh* levels and quantified based on 2^{-deltaCT} method.

Western blotting analysis

For the detection of kidney levels of BPIFA2, kidney tissue lysates were prepared by homogenizing the tissue in lysis buffer (50mM Tris pH7.5, 150mM NaCl, 5mM EDTA, 10% Glycerol, 0.5% Triton X-100, 1% NP-40 and protease inhibitor cocktail) at 4 deg C. Protein concentrations were quantified using BCA protein assay kit (Pierce-Thermo fisher) and 15 ug of protein samples were separated on 4-15% polyacrylamide gel (Bio-rad laboratories) and blotted onto PVDF membrane (Millipore). In the case of parotid gland that express a large amount of BPIFA2, only 100 ng of lysate were loaded per lane to obtain optimal signal. The membrane was probed with a goat anti-mouse BPIFA2 antibody at a concentration of 0.67ug/ml (Sigma aldrich, Cat # SAB2501987), followed by stripping and probing with anti-GAPDH antibody (Santa Cruz, Cat # sc-25778).

For the detection of plasma levels of BPIFA2, equal volumes of plasma either 2.5 ul (for cisplatin experiment) or 7.5 ul (for renal IRI experiment) were resolved on a 4-12% polyacrylamide gel, transferred to a PVDF membrane. Plasma BPIFA2 was detected using a goat anti-mouse BPIFA2 antibody at a concentration of 0.67 ug/ml (Sigma aldrich, Cat # SAB2501987).

For the detection of BPIFA2 in mice urine samples, 125 ng -1000 ng creatinine equivalents of urine samples were separated on a 4-15% non-reducing polyacrylamide gel, transferred to PVDF membrane. Urine BPIFA2 was detected using a goat anti-mouse BPIFA2 antibody at a concentration of 0.67 ug/ml (Sigma aldrich, Cat # SAB2501987).

For the detection of BPIFA2 in human urine samples, 10 ul of urine samples were resolved in a 4-15% reducing polyacrylamide gel, transferred to PVDF membrane. Urinary BPIFA2 was detected using a goat anti-human BPIFA2 antibody at a concentration of 0.4 ug/ml (Santa cruz, SPLUNC2 Antibody (C-20); Cat # sc-85932). Urinary NGAL were detected using a goat anti-human NGAL antibody at a concentration of 1.0 ug/ml (R&D systems, Cat # AF1757).

For the detection of BPIFA2 in human plasma samples, 5 ul of 1:5 diluted plasma samples were resolved in a 4-15% reducing polyacrylamide gel, transferred to PVDF membrane. Plasma BPIFA2 was detected using a goat anti-human BPIFA2 antibody at a concentration of 0.4 ug/ml (Santa cruz, SPLUNC2 Antibody (C-20); Cat # sc-85932) or a mouse monoclonal antibody that is reactive against human BPIFA2 at a concentration of 2 ug/ml (Novus Biologicals, PSP/BPIFA2 (1F12); Cat # NBP2-37493).

Protein deglycosylation

Human saliva and Human recombinant BPIFA2 protein (R&D systems) were incubated with PNGaseF (New England Biolabs) for 1h at 37 deg C. Mock treated samples were treated same in the absence of PNGaseF. Complete deglycosylation was ascertained by mobility shift by western blot analysis.

RNA In Situ Hybridization

BPIFA2 RNA expression was localized in formalin fixed paraffin embedded (FFPE) human kidney biopsies by RNA *in situ* hybridization by adapting the protocol for low signal detection based on the method as described elsewhere (4). Digoxigenin-labeled anti-sense riboprobe against human *BPIFA2* was generated by gene-specific PCR followed by *in vitro* transcription using MAXIscript®Kit (Ambion / Thermo Fisher Scientific). *BPIFA2* riboprobe corresponds to 300 nucleotides complementary to 245-544 bp of the coding sequence of human *BPIFA2*.

Briefly, FFPE human kidney biopsies were prepared for hybridization by baking at 60 deg C for 1h followed by rewaxing in xylene and subsequent rehydration steps. Sections were fixed in 4% paraformaldehyde followed by three washes in PBS. Sections were then incubated in Proteinase K (10 ug/ml) for 10 min followed by three washes in PBS, refixed in paraformaldehyde. After three washes in PBS, the sections were acetylated, sequentially dehydrated and prehybridized at 50 deg C for 30 min. Hybridization was performed with 750 ng/ml digoxigenin-labeled anti-sense riboprobe in a buffer comprised of 50% Formamide, 5X SSC, 5X Denhardt's solution, 250 ug/ml Baker's Yeast RNA, 500 ug/ml Herring sperm DNA at 50 deg C overnight. After RNase A (2 ug/ ml) mediated digestion of unhybridized probe by incubating at 37 deg C for 15 min, sections were washed in a series of low and high stringency wash buffers at 50 deg C for 1 h. Sections were then blocked and incubated with anti-digoxigenin antibody (1:4000 dilution, Roche diagnostics) overnight at 4 deg C, washed and color was developed using BM purple (Roche diagnostics).

***In vitro* ischemia reoxygenation injury**

To induce ischemia / reoxygenation injury *in vitro* in HK-2 cells, the cells were washed with twice with Hank's Buffered Salt Solution (HBSS) and incubated with HBSS containing 10 uM Antimycin, 10mM 2-Deoxy Glucose and 1 uM A23187 (all Sigma aldrich chemicals) for 1 hour at 37° C, 5% CO₂. Subsequently the cells were washed with HBSS and cultured in complete culture medium (DMEM:F12) for defined periods of time.

Realtime PCR primers

The following oligonucleotides were used to quantify transcripts in Sybr green based qRT-PCR reactions: Mouse Bpifa2: *forward*: 5'-CTCCATTTCTTGTGGGAA-3'; and *reverse*: 5'-AGTTTTGCAGGACGGTTGAC-3'. Human BPIFA2: *forward*: 5'-AACCTGTTCTTCACGAGGGA-3'; and *reverse*: 5'-GCAATTTCTCAGCTTCCTGG-3'.

Supplementary references

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