SUPPLEMENTARY MATERIALS - Hoorn et al.

Supplementary Methods

IMCD sample preparation: After harvesting the kidneys from the animals, inner medullas were dissected, finely minced with a razor blade and transferred to a 12 x 75-mm glass tube, containing tubule suspension solution (118 mM NaCl, 5 mM KCl, 4 mM Na_2HPO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.5 mM glucose, 5 mM sodium acetate) supplemented with 2 mg/ml collagenase B (Boehringer Mannheim, Indianapolis, IN) and 540 U/ml hyaluronidase (Worthington Biochemical, Freehold, NJ). Suspensions were incubated at 37ºC with 95% air - 5% CO_2 superfusion. Every 15 minutes, suspensions were aspirated with a large-bore Pasteur pipette until all large tissue clumps were digested (75-90 minutes). Then, suspensions were centrifuged at 50 x g, after which the supernatant was discarded and the pellet was resuspended in tubule suspension solution. The procedure was repeated twice, and after the third centrifugation, the resuspended pellet was centrifuged at 1,000 x g for 5 min. Previously, we have demonstrated that this procedure gives an approximately 10-fold enrichment of the collecting duct marker AQP2 and that the resulting pellet is contaminated with only very small amounts of non-IMCD cells (17).

Difference gel electrophoresis: For sample labeling, 50 µg of protein were mixed with 400 pmol of NHS-Cy dye and incubated on ice in the dark for 30 min. 50 µg of each Cy3 and Cy5-labeled protein as well as 50 µg of pooled internal standard (Cy2) were mixed with 50-900 µg of unlabeled protein. Rehydration buffer (7 M urea, 2 M thiourea, and 4 % CHAPS, 2 % Dithiothreitol (DTT), 2 % pH 3-10 Pharmalyte^{TM}) was added to give a total volume of 450 µl. This

was loaded on a 24 cm Immobiline DryStrip (pH 3-10 linear) by active rehydration for 10 h (Ettan IPGphor, Amersham). Isoelectric focusing was accomplished by subsequently applying 500 V for 500 V-hrs, 1000 V for 1000 V-hrs and 8000 V for 62533 V-hrs. After isoelectric focusing, strips were equilibrated in SDS equilibration buffer (50 mM TrisHCl pH 8.8, 6 M Urea, 30 % glycerol, 2 % SDS, 0.025 % bromophenol blue) containing 0.5 % DTT for 10 min at room temperature followed by incubation in SDS equilibration buffer containing 2.5 % iodoacetemide for 10 min at room temperature.

Semi-quantitative immunoblotting: The following affinity-purified primary antibodies were used, AQP2, α-ENaC, UT-A1, and UT-A3, which were all characterized previously. Other primary antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA): annexin A4 [sc-1930], HSP70 [sc-1060]), c-fos [sc-253], c-jun [sc-45], RACK1 [sc-17754], GRP78 [sc-1050], phosphorylated c-myc (Thr 58/ Ser 62)-R [sc-8000-R], c-myc [sc-42], c-src [sc-19], β-tubulin [sc-5274]; Upstate (Lake Placid, NY): caspase 3 [06-735], SRC-1 [05-522]; Sigma (St. Louis, MO): protein disulfide isomerase [P7496]. Additional antibodies were supplied by independent investigators: p53 (Dr. N.I. Dmitrieva), calreticulin (Dr. M. Michalak). Antibodies were diluted in a diluent containing 50 mM $NaPO_4$, 150 mM NaCl, 0.05% Tween-20, and 0.1% BSA. The peroxidase-conjugated secondary antibodies were diluted at 1:5000 in blot wash buffer (50 mM $NaPO_4$, 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat dried milk. Band visualization was achieved using an enhanced chemiluminescence substrate (LumiGLO for Western blotting, Kirkegaard and Perry no. VC110) before exposure to X-ray film (Kodak 165-1579). The band densities were quantitated by laser densitometry (model PDS1-P90, Molecular Dynamics). To facilitate comparisons, the densitometry values were normalized to control,

defining the mean for the control group as 100%.

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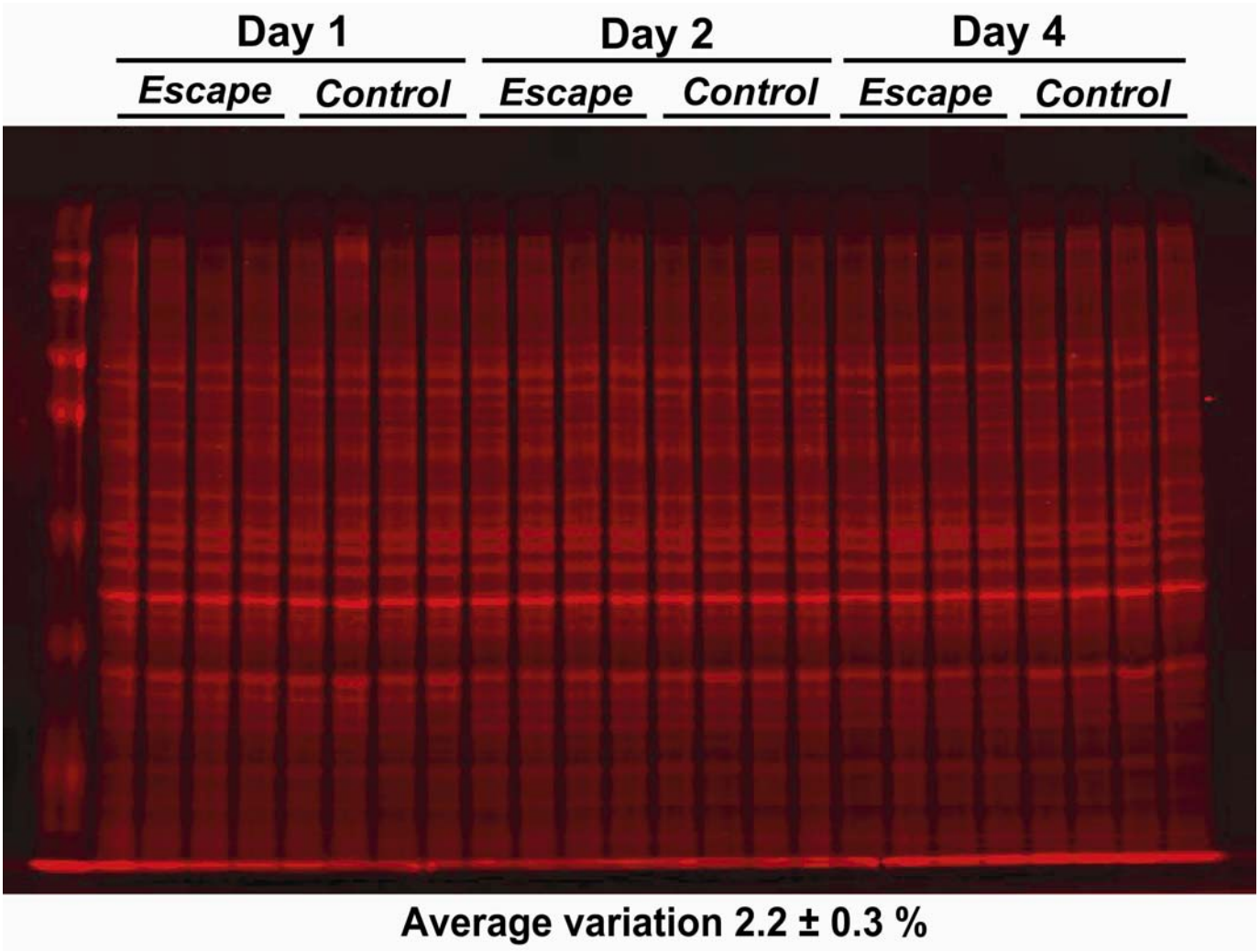
Supplementary Materials Figure 1: Loading gel confirming equal loading of all three time-points



Legend Supplemental Materials Figure 1: Loading gel confirming equal loading of all three time-points

Immunoblot of inner medullary collecting duct suspensions, prepared at the early (Days 1 and 2) and later (Day 4) stages of vasopressin escape. Equal loading was confirmed by staining identically loaded gels with Coomassie blue and scanning these with a linear fluorescence scanner (Odyssey, Li-Cor Biosciences) at an excitation wavelength of 700 nm. Densitometry was performed with Odyssey software (v. 1.1), yielding an overall average variation of 2.2 ± 0.3 %.

Supplemental Materials Table 1: Detailed description of the protein-protein interactions in the "vasopressin escape pathway" represented by edges in network diagram (Figure 4)

Central node	Interaction with	Nature of interaction	Reference(s)
c-Myc	p53	p53 decreases expression of c-Myc	A.o. Soengas, Science 1999
	Caspase 3	c-Myc decreases expression of of caspase 3 mRNA	-Louro, Canc Res 2002 -Grassili, JBC 2004
	Annexin A4	c-Myc decreases expression of Annexin A4 mRNA	Louro, Canc Res 2002
	Calnexin	c-Myc protein decreases expression of rat Calnexin mRNA	Louro, Canc Res 2002
	Elongation Factor 2	c-Myc protein increases expression of rat Elongation Factor 2 mRNA	Louro, Canc Res 2002
	Alpha B crystallin	c-Myc protein increases expression of rat Alpha B crystallin mRNA	Louro, Canc Res 2002
	F-actin capping protein β	In cells, human Myc protein decreases expression of rat F-actin capping protein β protein.	Shiio, EMBO J 2002
	Glucose phosphate isomerase	In Rat1a cells, c-Myc protein increases expression of rat Gpi mRNA.	Osthus, JBC 2000
	Lysosomal associated membrane protein 2	c-Myc protein decreases expression of rat Lamp2 mRNA	Watson, JBC 2002
	Transgelin 2	In cells, human c-Myc protein decreases expression of rat Transgelin2 protein.	Shiio Y et al, EMBO J 2002
	Retinoic acid receptor α	In mutant cells with a homozygous knockout of rat c-Myc, c-Myc protein decreases expression of rat retinoic acid receptor α mRNA.	Guo QM et al , Canc Res 2000
	Ubiquitin-like protein SMT3B	In mutant cells with a homozygous knockout of rat c-Myc, c-Myc protein increases expression of rat Smt3b mRNA.	Guo QM et al, Canc Res 2000
	Immunoglobulin kappa locus	c-Myc protein decreases transcription of immunoglobulin kappa locus gene	Oster SK et al, Adv Canc Res 2002
Glucocorticoid receptor (GR)	c-Jun	-GR protein decreases activation of a protein-protein complex consisting of c-Fos and of c-Jun -Heterodimerization of 39 kd c-Jun protein and GR protein occurs. -Binding of AP1 binding site and dimer consisting of c-Jun and GR protein occurs. -GR interacts with c-Jun.	-Touray M et al, Oncogene 1991 -Hsu TC et al, Biochem Biophys Res Commun 1993 -Ribeiro RC, Annu Rev Med 1995 -Chen H et al, J Biol Chem 1997 - Herdegen T et al, Brain Res Brain Res Rev 1998
	SRC-1	GR interacts with SRC-1	-Makishima M et al, Science 2002 -Kucera T et al, J Biol Chem 2002
	c-Myc	GR binds c-Myc promoter	Ma T et al, Mol Endocrinol 2000
	p53	-In cytoplasm, dexamethasone increases binding of human HDM2 protein and human GR protein and human p53 protein. -Binding of GR protein and P53 [TP53] protein occurs in HSC-2 cells. -Wild-type p53 interacts with GR.	-Yu C et al, Cancer Lett 1997 -Sengupta S et al, EMBO J 2000 -Sengupta S et al, Genes Dev 2001
	Calreticulin	-GR binds calreticulin. -GR binds N-domain of calreticulin.	-Burns K et al, Nature 1994 -Roderick HL et al, FEBS Lett 1997
	GRP78	GR binds GRP78.	Hutchison KA et al, J Steroid Biochem Mol Biol 1996
	c-Src	Binding of mouse Hsp90 protein(s) and mouse GR protein and mouse Src protein occurs in mouse thymocytes.	Marchetti MC, Blood 2003
c-Fos	c-Jun	c-Fos and c-Jun form a dimer representing the AP1 transcription factor.	
	SRC-1	-Binding of c-Jun protein and SRC-1 protein occurs in a cell-free system. - Binding of c-Jun protein and human SRC-1 protein occurs in a system of purified components	Lee SK et al, Mol Endocrinol 2000 Lee SK et al, J Biol Chem 1998
	CREB	-In mouse CA1 neuron, transgenic CREB protein increases expression of mouse c-Fos protein.	Barco A et al, Cell 2002

		-CREB protein increases expression of c-Fos mRNA. -A protein-protein complex has members mouse CREB protein and mouse Crem alpha protein and mouse c-Fos protein and mouse c-Jun protein and mouse Junb protein and mouse Jund protein(s).	
	Protein kinase A	PKA protein increases phosphorylation of c-Fos protein	Herdegen T et al, Brain Res Brain Res Rev 1998
	Annexin A4	In 208F cells, v-Fos protein increases expression of rat Annexin 4 mRNA.	Johnston IM et al, Oncogene 2000
	Keratin 8	In 208F cells, v-Fos protein increases expression of rat Cytokeratin-8 mRNA	Johnston IM et al, Oncogene 2000
	Sec23B	In 208F cells, v-Fos protein decreases expression of rat Sec23b mRNA.	Johnston IM et al, Oncogene 2000
	p53	p53 protein increases transcriptional activation of c-Fos gene.	Kohn KW et al, Mol Biol Cell 1999
	X-box binding protein 1	Binding of c-Fos protein and human XBP1 protein occurs in vitro	Ono SJ et al, Proc Natl Acad Sci U S A 1991
	Lysosomal associated membrane protein 2	In 208F cells, v-Fos protein decreases expression of rat Lamp2 mRNA.	Johnston IM et al, Oncogene 2000
	Ubiquitin-like protein SMT3B	In 208F cells, v-Fos protein increases expression of rat Smt3b mRNA.	Johnston IM et al, Oncogene 2000
c-Jun	CREB	-PD98059 in cell culture decreases binding of human CREB protein in a nuclear fraction of serum-deprived JEG3 cells treated with EGF protein and cAMP response element in a system of purified components and human c-Jun protein in a nuclear fraction of serum-deprived JEG3 cells treated with EGF protein. -EGF protein in cell culture increases binding of human CREB protein in a nuclear fraction of serum-deprived JEG3 cells and CRE in a system of purified components and human c-Jun protein in a nuclear fraction of serum-deprived JEG3 cells. -A protein-protein complex has members mouse Atf2 protein and mouse CREB protein and mouse c-Jun protein and mouse Junb protein. -A protein-protein complex has members mouse CREB protein and mouse Crem alpha protein and mouse Fos protein and mouse C-jun [Jun] protein and mouse Junb protein and mouse Jund protein(s).	Rutberg SE et al, Oncogene 1999 Roberson MS et al, Mol Cell Biol 2000 Andrecht S et al, J Biol Chem 2002
	p53	-c-Jun protein increases expression of mouse p53 protein. -Mouse p53 protein is necessary for the expression of mouse c-Jun mRNA that involves gamma radiation.	Fuchs SY et al, Genes Dev 1998
	Src	In Rat-1 cells, v-Src protein increases (S73) serine phosphorylation of rat c-Jun protein	Paasinen-Sohns A et al, Oncogene 1997
p53	HSP70	In Hct 116 cells, human p53 protein decreases expression of human HSP70 mRNA	Daoud SS et al, Cancer Res 2003
	Transgelin 2	In Hct 116 cells, human p53 protein is necessary for the Topotecan-independent expression of human transgelin 2 mRNA	Daoud SS et al, Cancer Res 2003
	Glucose phosphate isomerase	Mouse p53 protein is necessary for the expression of mouse glucose phosphate isomerase mRNA that involves gamma radiation.	Komarova EA et al, Oncogene 1998
c-Src	Annexin A2	pp60src phosphorylates annexin A2	Ohnishi M et al, J Cell Sci 1994
	RACK1	Binding of human RACK1 protein and mouse c-Src protein occurs in rabbit reticulocyte lysates and NIH/3T3 cells.	Chang BY et al, Mol Cell Biol 1998
Protein kinase A (PKA)	CREB	-PKA protein increases (S133) phosphorylation of CREB protein. -BMP2 protein increases the mouse PKA protein-dependent phosphorylation of mouse Creb [Creb1] protein. -PKA activates CREB.	Rao A et al, Annu Rev Immunol 1997 Gupta IR et al, J Biol Chem 1999 Richards JP et al, J Biol Chem 1996
	AQP2	PKA phosphorylates AQP2.	Bouley R et al, J Clin Invest 2000
GRP78/BiP	Translation initiation factor 2	-GRP78/BiP protein decreases activation of PERK protein. -Thapsigargin decreases binding of human PERK protein and human GRP78/BiP protein. - Binding of human PERK protein and human GRP78/BiP protein occurs in 293t cells.	Bertolotti A et al, Nat Cell Biol 2000
	Immunoblogin	A protein-protein complex has members mouse GRP78/BiP	Chillarón J, Mol Biol Cell 2000

	kappa locus	protein and Immunoblogin kappa locus protein.	
Activating TF 6	GRP78/BiP	-In a microsomal fraction of 293t cells, binding of human ATF6 protein and Grp78/BiP protein is the same as binding of mutant human ATF6 protein (T645I with its N-linked glycosylation site mutated) and Grp78/BiP protein. -Binding of human ATF6 protein and Grp78/BiP protein occurs in a microsomal fraction of 293t cells.	Yoshida H et al, Mol Cell Biol 2000 Hong M et al, J Biol Chem 2004
	X-Box binding protein 1	Soluble ATF6 protein increases expression of human XBP1 mRNA.	Yoshida H et al, Mol Cell Biol 2000
Calreticulin	Retinoic acid receptor α	Retinoic acid receptor α interacts with calreticulin	Desai D et al, J Biol Chem 1996