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Consequences of the loss of the Grainyhead-like 1 gene for renal gene expression, regulation of blood pressure and heart rate in a mouse model*

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Aim: **The Grainyhead-like 1 (GRHL1) transcription factor is tissue-specific and is very highly expressed in the kidney. In humans the *GRHL1* gene is located at the chromosomal position 2p25. A locus conferring increased susceptibility to essential hypertension has been mapped to 2p25 in two independent studies, but the causative gene has never been identified. Furthermore, a statistically significant association has been found between a polymorphism in the *GRHL1* gene and heart rate regulation. The aim of our study was to investigate the physiological consequences of *Grhl1* loss in a mouse model and ascertain whether *Grhl1* may be involved in the regulation of blood pressure and heart rate. Experimental approach**: **In our research we employed the *Grhl1* "knock-out" mouse strain. We analyzed renal gene expression, blood pressure and heart rate in the *Grhl1*-null mice in comparison with their "wild-type" littermate controls. Most important results**: **The expression of many genes is altered in the *Grhl1*$^{-/-}$ kidneys. Some of these genes have previously been linked to blood pressure regulation. Despite this, the *Grhl1*-null mice have normal blood pressure and interestingly, increased heart rate. Conclusions**: **Our work did not discover any new evidence to suggest any involvement of *Grhl1* in blood pressure regulation. However, we determined that the loss of *Grhl1* influences the regulation of heart rate in a mouse model.**

Key words: blood pressure; genetics; grainy head; heart rate; kidney; transcription factor.

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INTRODUCTION

The Grainyhead-like 1 (GRHL1) transcription factor, previously known as MGR (Mammalian Grainyhead), belongs to the Grainyhead-like family of proteins (Wilanowski *et al.*, 2002). The *Grhl1*-null mice are viable and fertile, but they display symptoms reminiscent of palmoplantar keratoderma, as well as hair loss due to the poor anchoring of the hair shaft in the follicle (Wilanowski *et al.*, 2008). It is also known that *Grhl1* acts as a tumor suppressor in squamous cell carcinoma of the skin (Mlacki *et al.*, 2014) and in neuroblastoma (Fabian *et al.*, 2014).

In humans, the *GRHL1* gene is located on chromosome 2, in the 2p25 region. In two independent studies, a locus conferring increased susceptibility to essential hypertension was mapped to this chromosomal region, with the causative gene remaining unknown until this day (Angius *et al.*, 2002; Zhu *et al.*, 2001). This locus has later been named *HYT3* (Hypertension, essential, susceptibility to, 3) [Online Mendelian Inheritance in Man (OMIM) ID 607329]. There are literature reports suggesting that other Grainyhead-like factors are involved in the regulation of blood pressure: upstream binding protein 1 (UBP1), a transcription factor closely related to GRHL1, is crucial for blood pressure regulation in humans (Koutnikova *et al.*, 2009); and transcription factor CP2-like 1 (TFCP2L1), another transcription factor closely related to GRHL1, is required for the proper electrolyte excretion in the kidney in a mouse model, and this function is essential for the regulation of blood pressure (Yamaguchi *et al.*, 2006). The expression of *GRHL1* is tissue-specific, and is very high in the kidney (Auden *et al.*, 2006; Wilanowski *et al.*, 2008). It is well established that kidney malfunctions often cause hypertension (Messerli *et al.*, 2007). On the basis of the above observations we propose a hypothesis that GRHL1 may be involved in the regulation of blood pressure.

In our preliminary analyses we identified four potential target genes of GRHL1 regulation that are expressed in the kidney and may be involved in the regulation of blood pressure. Two such potential target genes were se-

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Abbreviations: CACNA1D, calcium channel, voltage-dependent, L type, alpha 1D subunit; DBP, diastolic blood pressure; DDC, DOPA decarboxylase; FLOT2, flotillin 2; GRHL, Grainyhead-like; HPRT, hypoxanthine phosphoribosyltransferase; HYT3, hypertension, essential, susceptibility to, 3; MBP, mean blood pressure; Q-RT-PCR, quantitative real time polymerase chain reaction; SBP, systolic blood pressure; TSLP, thymic stromal lymphopoietin; XPO1, exportin 1

lected on the basis of their homology to known targets of Grainyhead (GRH) regulation in the fruit fly *Drosophila melanogaster*. Such regulation events may be conserved in at least some cases, because the Grainyhead-like family of proteins displays a very high level of conservation of their functions throughout Metazoa, with some of the functions being conserved even in the fungi (Pare *et al.*, 2012). In *D. melanogaster*, GRH directly regulates the expression of DOPA (L-3,4-dihydroxyphenylalanine) decarboxylase (DDC) [name approved by the Human Genome Organization Gene Nomenclature Committee (HGNC ID 2719)], also known as aromatic-L-amino-acid decarboxylase (AADC) [name approved by Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (EC 4.1.1.28)] (Dynlacht *et al.*, 1989). DDC is highly expressed in the mammalian kidney, where it is important for sodium transport and perfusion, and for the regulation of systemic blood pressure (Harris & Zhang, 2012). In a mouse model, conditional deletion of *Ddc* in the renal proximal tubules leads to hypertension (Zhang *et al.*, 2011). It has also been experimentally proven in the fruit fly that GRH directly regulates the expression of flotillin 2 (*Flot2*) (Juarez *et al.*, 2011). FLOT2 is an important component of lipid rafts involved in dopamine receptor signaling (Yu *et al.*, 2004) and this signaling pathway is crucial for the regulation of renal function and blood pressure (Harris & Zhang, 2012).

Next, two potential target genes of GRHL1 regulation were identified using bioinformatic analyses. Previously, we defined the GRHL1-binding consensus DNA sequence to be a palindrome AACCGGTT (Wilanowski *et al.*, 2008). With this sequence we interrogated a customized dataset of genomic regions located in the vicinity of gene transcriptional start sites that are highly conserved in placental mammals (Caddy *et al.*, 2010). We found one well conserved potential GRHL1 binding site 41866 bp upstream of the transcriptional start site of the calcium channel, voltage-dependent, L type, alpha 1D subunit (*Cacna1d*) gene, and two conserved tandem GRHL1 binding sites 9 bp downstream of the transcriptional start site of exportin 1 (*Xpo1*), also known as CRM1 homolog (Fig. 1). Genetic polymorphisms in the *CACNA1D* gene confer sensitivity to certain antihypertensive drugs in human patients (Kamide *et al.*, 2009). The link between XPO1 and the regulation of blood pressure is less direct. XPO1 is necessary for nuclear export of tonicity enhancer-binding protein (TonEBP) under isotonic conditions, but not under hypertonic stress (Andres-Hernando *et al.*, 2008). In turn, TonEBP enhances DDC expression in the epithelial cells of renal proximal tubule upon hypertonic stress (Hsin *et al.*, 2011) and the link between DDC and the regulation of kidney function and blood pressure is well established (Harris & Zhang, 2012).

Recently, a genome-wide association study was conducted to search for loci contributing to variance in heart rate responses to submaximal exercise training in human subjects (Rankinen *et al.*, 2012). This study discovered a statistically significant association between these responses and a single nucleotide polymorphism in the *GRHL1* gene, which indicates that *GRHL1* may be connected to the regulation of heart rate in response to exercise. Therefore, one of the aims of our study was to investigate the possibility of *GRHL1* involvement in the regulation of heart rate.

In summary, we hypothesize that the *GRHL1* gene may be linked to the regulation of blood pressure and heart rate. We propose that the underlying mechanism is connected to the role of *GRHL1* in the functioning of the kidney. The expression of *GRHL1* is tissue-specific; this gene is not expressed in many tissues and organs, but it is very highly expressed in the kidneys, and kidney malfunctions often cause hypertension. Since the *GRHL1* gene codes for a transcription factor, the molecular mechanism is very likely to involve regulation of gene expression. The GRHL1 transcription factor may regulate, directly or indirectly, the expression of genes whose products are necessary for the correct regulation of blood pressure and heart rate.

To test these hypotheses, we employed the *Grhl1* "knock-out" mouse strain, which we previously used in the analyses of *Grhl1* function in the skin (Mlacki *et al.*, 2014; Wilanowski *et al.*, 2008). If *Grhl1* is important for the regulation of blood pressure and heart rate, the *Grhl1*-null mice are likely to have abnormal blood pressure and heart rate. In our study, we assayed blood pressure and heart rate in this mouse model. We also analyzed changes in gene expression in the kidneys of *Grhl1*$^{-/-}$ mice.

MATERIALS AND METHODS

Mice. In our experiments we used the *Grhl1* "knock-out" mouse strain. The making of this strain, as well as breeding conditions, are described elsewhere (Wilanowski *et al.*, 2008). The genetic background is C57BL/6 (Black 6). The animals were fed ssniff® R/M-H Ered I chow (ssniff, Soest, Germany) containing 0.24% sodium. This study was carried out in strict accordance with the regulations of the Experiments on Animals Act (Act of 21 January 2005 on experiments on live animals, the Parliament of the Republic of Poland, Dz. U. Nr 33, poz. 289); as well as with the Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes. All animal experiments were approved by the First Warsaw Local Ethics Committee for Animal Experimentation (permit number 28/2010) and by the Fourth Warsaw Local Ethics Committee for Animal Experimentation (permit number 66/2012). All efforts were made to minimize suffering. In all our experiments we used only male mice, in order to avoid blood pressure variations caused by the menstrual cycle in females.

Kidney microscopic sample preparation. According to a protocol described in (Mlacki *et al.*, 2014), with modifications. Briefly, mice of age about 6 months were sacrificed and kidneys were dissected, fixed in 4% paraformaldehyde (Acros Chemicals, Geel, Belgium) in phosphate-buffered saline (PBS) and embedded in paraffin (POCH, Gliwice, Poland). Samples were cut into 7 μm sections using microtome Hyrax M55 (Zeiss, Jena, Germany) and placed on Superfrost Ultra Plus microscope slides (Thermo Scientific, Waltham, MA, USA). Sections were then deparaffinized with xylene and decreasing concentrations of alcohols.

Immunohistochemistry. According to a protocol described in (Mlacki *et al.*, 2014), with modifications. Briefly, prepared 7 μm kidney sections were incubated in citrate buffer at 60°C overnight (antigen retrieval). The endogenous peroxidase activity was blocked by incubation in 1% hydrogen peroxide in PBS for 15 minutes. The following rabbit polyclonal anti-mouse antibodies were used: anti-flotillin 2 (sc-25507 H-90), anti-CRM1 (sc-5595 H-300), anti-L-type Ca^{++} CP α1D (CACNA1D) (sc-25687 H-240) (all from Santa Cruz Biotechnol-

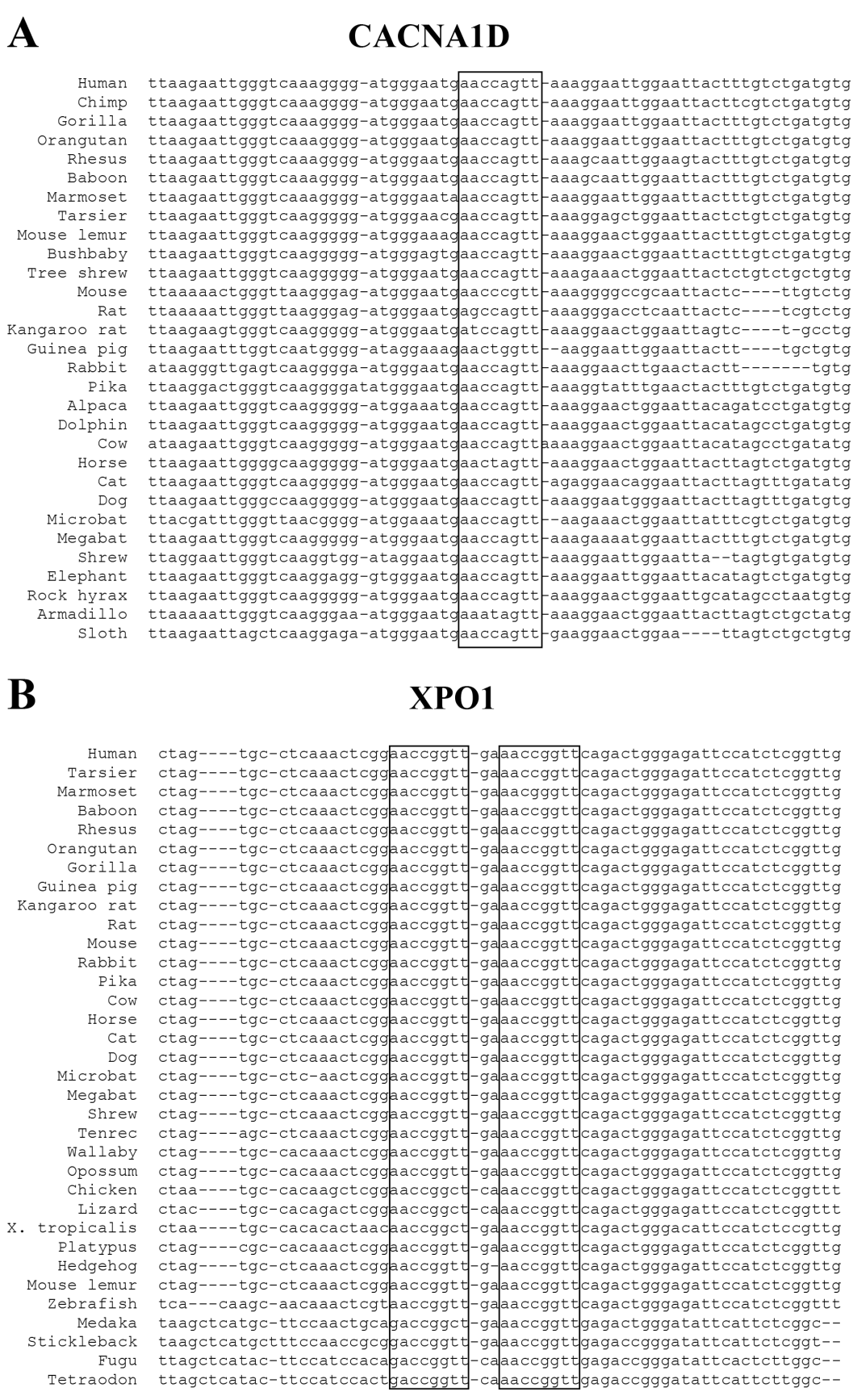

Figure 1. Alignment of the promoter regions of genes coding for CACNA1D (A) and XPO1 (B) from the indicated species.
The GRHL1 DNA consensus sequence is boxed in red. Regions shown correspond to chr3:53,521,200-53,521,267 (**A**) and chr2:61,765,392-61,765,453 (**B**). According to UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly, http://genome.ucsc.edu/ (Kent *et al.*, 2002).

ogy, Dallas, TX, USA), anti-N-terminal region of DDC (ARP41425_T100 from Aviva Systems Biology, San Diego, CA, USA). The rabbit polyclonal anti-beta-galactosidase antibody (ab4761) was purchased from Abcam (Cambridge, UK). For immunodetection the 3,3'-diaminobenzidine (DAB) Detection Kit (USA™ Ultra Streptavidin Detection System, Covance, Princeton, NJ, USA SIG-32232) was used according to the manufacturer's instructions. The results were documented using microscope (Eclipse 80i, Nikon, Tokyo, Japan) with digital camera.

RNA and cDNA preparation, Quantitative Real Time Polymerase Chain Reaction (Q-RT-PCR). According to a protocol described in (Mlacki *et al.*, 2014), with modifications. Briefly, mice of age about 6 months were sacrificed, their kidneys dissected and immediately frozen in liquid nitrogen. The samples were ground in mortar in liquid nitrogen, the Ron's FastTRI Extraction Reagent (Bioron, Ludwigshafen, Germany) was added and the solution was homogenized using Polytron (PRO2000, PRO Scientific, Oxford, CT, USA). The RNA was isolated according to the manufacturer's instructions. The RNA was then reverse transcribed into cDNA using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (Bioron) according to the producer's protocol. The Q-RT-PCR reactions were carried out using SYBR Green PCR Mastermix on 7500 Real Time PCR System (Applied BioSystems, Grand Island, NY, USA). Relative expression levels were standardized to hypoxanthine phosphoribosyltransferase (*Hprt*) expression, and statistical differences were determined by Student's t-test. In each assay, we used three *Grhl1*$^{-/-}$ and three *Grhl1*$^{+/+}$ animals. Primer sequences were obtained from PrimerBank (Wang & Seed, 2003) with the exception of primers specific for *Hprt*, where we employed primer sequences published in literature (Darido *et al.*, 2011). Used primers are listed in Table 1.

Expressional microarray analysis of gene expression. We used 8 *Grhl1*$^{-/-}$ and 7 *Grhl1*$^{+/+}$ animals in this experiment. RNA was extracted as described in the previous section. Mouse RNA samples (500 ng) were processed and labeled for array hybridization using the Ambion WT Expression Kit (Life Technologies, Carlsbad, CA, USA; catalogue number 4411974). Labeled, fragmented cDNA (Affymetrix GeneChip WT Terminal Labeling and Controls Kit; catalogue number 901524) was hybridized to Mouse Gene 2.0 arrays for 16 hours at 45°C (at 60 rpm) (Affymetrix GeneChip Hybridization, Wash, and Stain Kit; catalogue number 900720). Arrays were washed and stained using the Affymetrix Fluidics Station 450, and scanned using the Hewlett-Packard GeneArray Scanner 3000 7G.

Eight Mouse Gene 2.0 ST microarrays (Affymetrix, Santa Clara, CA, USA) containing RNA from the kidneys of *Grhl1*$^{-/-}$ mice and seven microarrays containing RNA from the kidneys of *Grhl1*$^{+/+}$ mice were analyzed using R/Bioconductor environment (Gentleman *et al.*, 2004). Firstly, all CEL files comprising intensities for all probes in the microarray were loaded using *oligo* package (Carvalho & Irizarry, 2010). After quality assessment (histograms, boxplots, MA plots, principal component analysis) five chips (two with wild-type samples and three with knock-out samples) were removed from further analyses. Probe intensities of ten microarrays were $\log_2$-transformed and normalized by Robust Multi-array Average (RMA) method (Irizarry *et al.*, 2003). The comparison between tested conditions of each transcript on microarray was performed by linear modelling using *limma* package (Smyth *et al.*, 2005). Gene expression p values were corrected for multiple testing using Benjamini-Hochberg threshold of 0.05 (Benjamini & Hochberg, 1995).

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE62252 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62252).

Assay of dopamine levels. Dopamine levels in mouse blood serum were assayed using the mouse dopamine ELISA (enzyme-linked immunosorbent assay) kit from MyBioSource (San Diego, CA, USA); catalogue number MBS162171. We followed the manufacturer's protocol exactly.

Measuring blood pressure and heart rate in mice using tail-cuff method. Systolic, mean and diastolic blood pressure (SBP, MBP, DBP) and heart rate were measured during three to four consecutive days using tail-cuff method (CODA System, Kent Scientific Corporation, Torrington, CT, USA). During the three days before the start of experimental measurements animals were accustomed to the restrainment needed for measurement.

Blood pressure and heart rate monitoring in mice by telemetry. The telemetry experiment was carried out according to a published protocol (Singh *et al.*, 2013). Briefly, following acclimatization, radio transmitters (TA11PA-C10, Data Sciences International, St. Paul, MN, USA) were implanted. The catherer was placed into the common carotid artery, and the transmitter — under the skin in the abdominal region. Mice were kept in a 12 hour light — 12 hour dark cycle, and received food and water *ad libitum*. After 8–10 days of recovery, we began monitoring blood pressure and heart rate continuously using the telemetry data acquisition system (Data Sciences International, St. Paul, MN, USA).

Statistical analysis. The statistical analysis was performed using Student's t-test incorporated into Microsoft Office Excel 2003 package. For some

Table 1. List of primers used in Q-RT-PCR.

Primer name	Primer sequence	PrimerBank ID
Cacna1d-F	5′-GCTTACGTTAGGAATGGATGGAA-3′	134288874c2
Cacna1d-R	5′-GAAGTGGTCTTAACACTCGGAAG-3′	
Ddc-F	5′-TAGCTGACTATCTGGATGGCAT-3′	22094149a1
Ddc-R	5′-GTCCTCGTATGTTTCTGGCTC-3′	
Flot2-F	5′-AGGCTGTTGTGGTTCTGACTA-3′	12835861a1
Flot2-R	5′-TGCAACGTCATAATCTCTAGGGA-3′	
Hprt-F	5′-GCTGGTGAAAAGGACCTCT-3′	none (Darido *et al.*, 2011)
Hprt-R	5′-CACAGGACTAGAACACCTGC-3′	
Xpo1-F	5′-TGGAGAAGTAATGCCGTTCATTG-3′	19527232a1
Xpo1-R	5′-CCCACACTTGATTAGGGAGTAGC-3′	

The abbreviations are: F — forward, R — reverse.

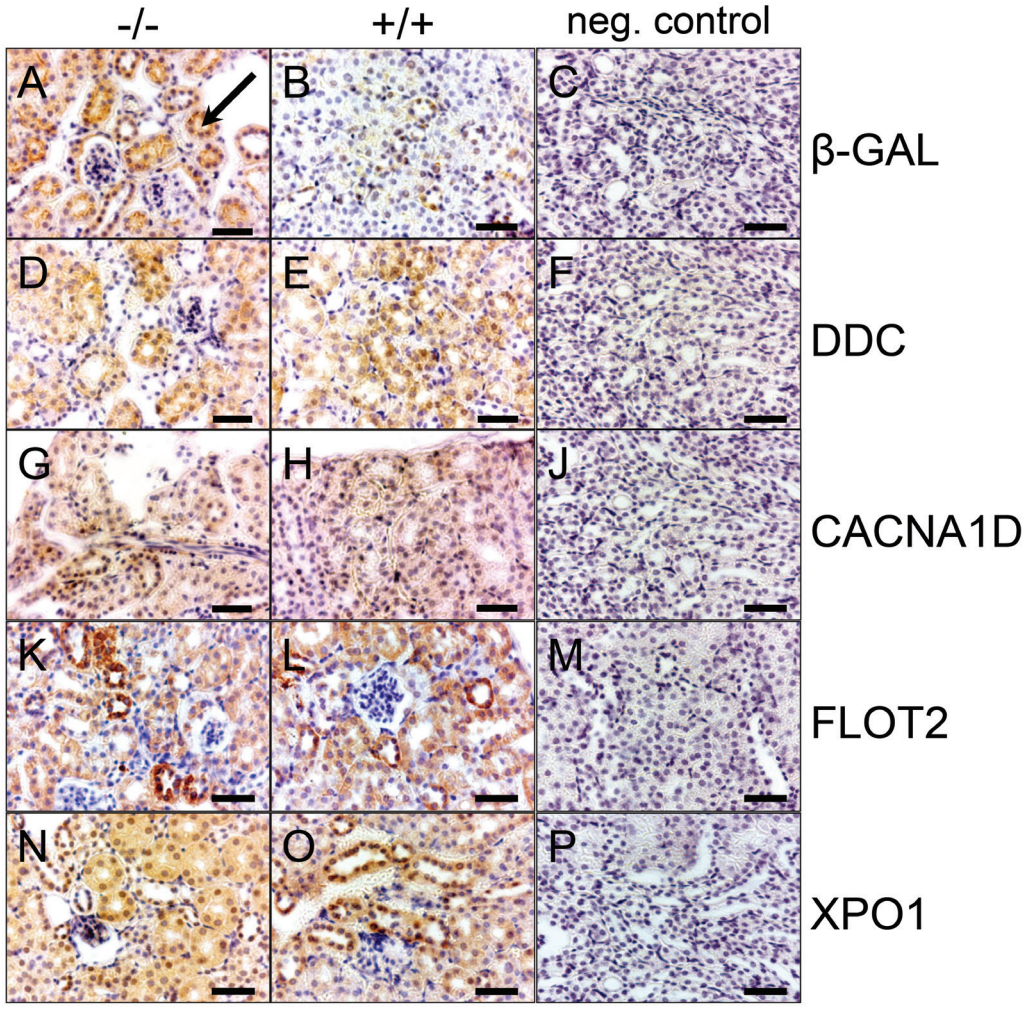

Figure 2. Immunohistochemical analysis of the kidneys of *Grhl1*$^{-/-}$ mice (left panels) in comparison to their wild-type littermates *Grhl1*$^{+/+}$ (middle and right panels).
Kidney sections obtained from mice were cut and placed on microscope slides, as described in Materials and Methods. Subsequently, these sections were stained using specific antibodies. Antibodies specific for the following proteins were used: beta-galactosidase (**A**, **B**, black arrow indicates a proximal tubule), DDC (**D**, **E**), CACNA1D (**G**, **H**), FLOT2 (**K**, **L**) and XPO1 (**N**, **O**). Panels in the right column (**C**, **F**, **J**, **M**, **P**) show kidney sections from wild type mice stained without the use of primary antibodies, but following the staining protocol starting from the application of secondary antibody (negative controls). Scale bars represent 50 μm.

tests we also used software available from Kirkman, T.W. (1996) Statistics to Use. http://www.physics.csbsju.edu/stats/ For statistical analysis of blood pressure and heart rate measurements, differences in mean values between groups were first analyzed by one-way ANOVA followed by modified Student's t-test for independent variables (STATISTICA, version 10.0, StatSoft Inc.). The standard error of mean (SEM) was used as the measure of data dispersion. $P < 0.05$ was taken to indicate the significance.

RESULTS

The expression patterns of four potential targets of GRHL1 regulation overlap with the expression of GRHL1 in the kidney

In the *Grhl1* "knock-out" mouse strain, the *lacZ* reporter gene is inserted into the *Grhl1* locus (Wilanowski *et al.*, 2008), so we carried out immunohistochemistry with anti-beta-galactosidase antibodies, using kidney sections from this strain of mice. As shown in Fig. 2A,

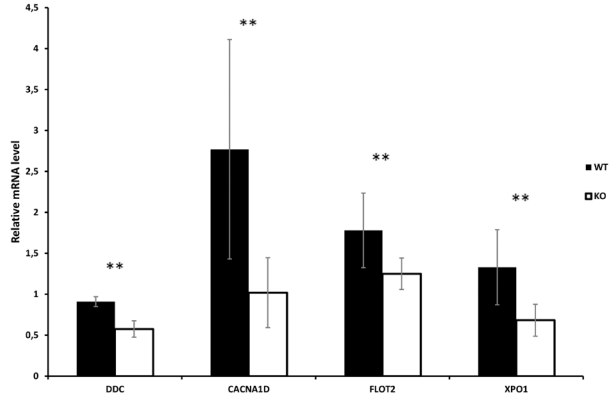

Figure 3. Levels of expression of potential target genes of GRHL1 regulation in the kidneys of *Grhl1*$^{+/+}$ (solid bars) and *Grhl1*$^{-/-}$ mice (open bars).
Total RNA samples were isolated from whole kidneys extracted from mice. RNA was reverse transcribed into cDNA and levels of cDNA corresponding to different genes were measured using Q-RT-PCR.

Table 2. Results of the microarray experiment.
List of ten protein-coding genes with the greatest fold change decrease in expression levels, and six protein-coding genes with an increase in expression levels in the *Grhl1*$^{-/-}$ kidneys, as determined by microarray analysis. Only genes with statistically significant changes in expression are shown (adjusted p-value<0.1).

Gene name	Accession No.	Fold change	*p*-value	Adjusted *p*-value	Description
Ighg	BC092269	0.0814	2.3E-05	2.3E-02	immunoglobulin heavy chain (gamma polypeptide)
Zfp125	AJ005350	0.0937	2.9E-10	1.0E-05	zinc finger protein 125
Igkv10-96	BC128281	0.171	1.1E-04	6.1E-02	immunoglobulin kappa variable 10–96
Ighm	GENSCAN00000028100	0.185	4.7E-05	3.8E-02	immunoglobulin heavy constant mu
Igkv4-72	AY835663	0.199	2.4E-05	2.4E-02	immunoglobulin kappa chain variable 4–72
Igkv4-70	AJ416331	0.248	1.6E-04	7.7E-02	immunoglobulin kappa chain variable 4–70
Igh-VJ558	GENSCAN00000028118	0.261	1.6E-05	2.1E-02	immunoglobulin heavy chain (J558 family)
Igkv6-23	GENSCAN00000031267	0.264	1.8E-04	8.4E-02	immunoglobulin kappa variable 6–23
Igkc	ENSMUST00000103349	0.292	1.3E-05	2.1E-02	immunoglobulin kappa constant
Igk-V28	V00810	0.302	7.2E-05	5.4E-02	immunoglobulin kappa chain variable 28
B930095G15Rik	AK047587	1.205	1.2E-04	6.2E-02	RIKEN cDNA B930095G15 gene
Wdr27	NM_175173	1.245	7.8E-05	5.5E-02	WD repeat domain 27
Dusp9	NM_029352	1.358	1.6E-05	2.1E-02	dual specificity phosphatase 9
Taf1b	NM_020614	1.390	7.7E-05	5.5E-02	TATA box binding protein (TBP)-associated factor, RNA polymerase I, B
Nepn	NM_025684	1.394	2.0E-04	8.7E-02	nephrocan
Greb1	NM_015764	2.498	3.3E-06	9.6E-03	growth regulation by estrogen in breast cancer 1

GRHL1 is expressed primarily in the lining of proximal tubules, and is either absent or expressed at much lower levels in the other kidney structures. Lack of staining in Fig. 2B served as a negative control in this experiment, as the "wild-type" mice do not express bacterial beta-galactosidase.

We employed immunohistochemistry with commercially available antibodies to determine patterns of renal expression of potential targets of GRHL1 regulation in the kidneys of *Grhl1*$^{-/-}$ and *Grhl1*$^{+/+}$ mice (Fig. 2). There is clearly at least partial overlap between the expression patterns of CACNA1D, DDC, FLOT2, XPO1 and GRHL1, as all these proteins are found in the lining of proximal tubules, although most of them have broader patterns of expression than GRHL1.

The expression of four potential targets of GRHL1 regulation is reduced in the *Grhl1*-null mice

We employed Q-RT-PCR to determine whether the expression of *Cacna1d*, *Ddc*, *Flot2* and *Xpo1* is altered in the kidneys of *Grhl1*$^{-/-}$ mice, as compared to their *Grhl1*$^{+/+}$ control littermates. All four genes were significantly downregulated in *Grhl1*$^{-/-}$ kidneys (Fig. 3).

The levels of expression of many other genes, including those involved in susceptibility to hypertension or in the regulation of blood pressure, are significantly altered in the kidneys of *Grhl1*$^{-/-}$ mice

We used the microarray technology to investigate changes in gene expression in the kidneys of *Grhl1*-null mice. The results are summarized in Table S1 (Supplementary Materials on web site www.actabp.pl). We then assembled a list of ten protein-coding genes with the greatest fold change decrease in expression levels in the *Grhl1*$^{-/-}$ kidneys, as well as six protein-coding genes with an increase in expression levels (Table 2). Only six protein-coding genes displayed a statistically significant increase in expression under our criteria (adjusted *p*-value<0.1), consequently only those genes are included in Table 2. Nine of the ten most downregulated genes code for various immunoglobulin polypeptides. On the other hand, the group of six upregulated genes is diverse, and their products perform different functions. The

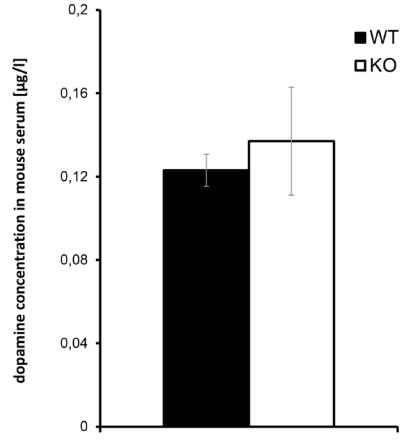

Figure 4. Dopamine levels in the blood serum of *Grhl1*$^{+/+}$ (solid bar) and *Grhl1*$^{-/-}$ mice (open bar).

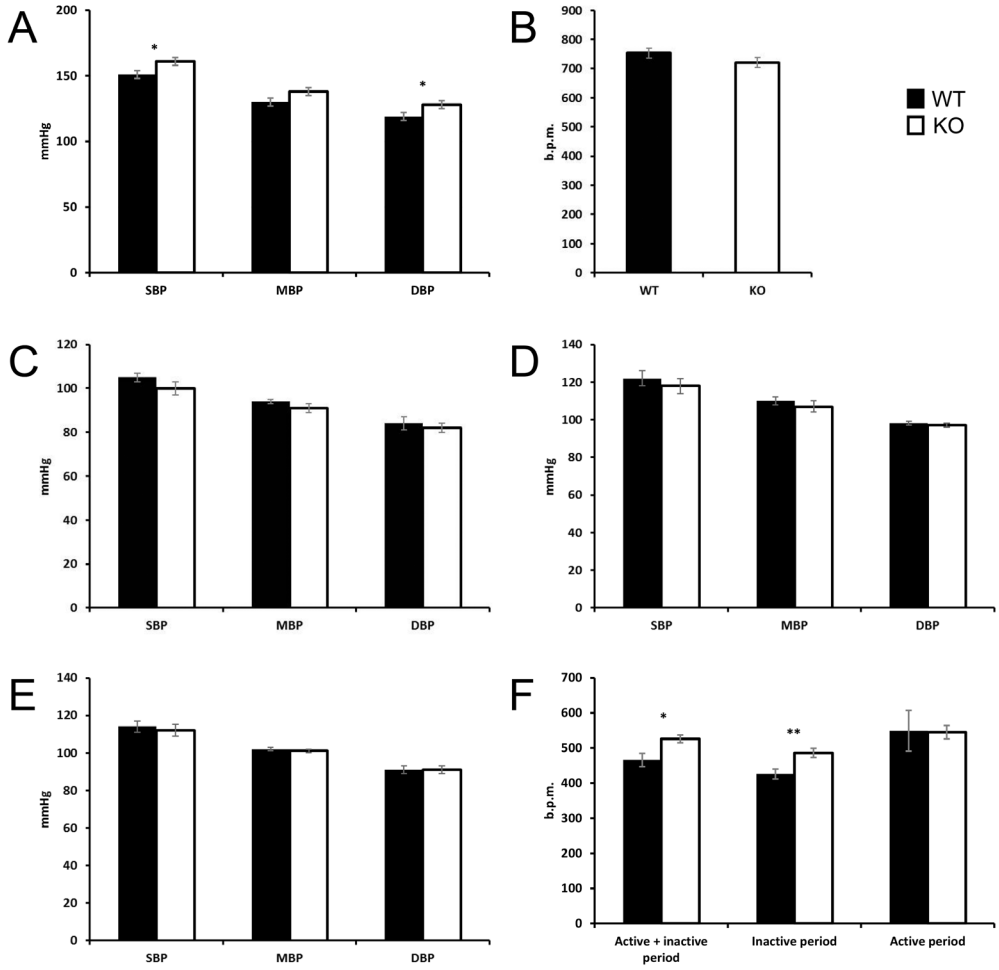

Figure 5. The results of blood pressure and heart rate measurements.
Blood pressure and heart rate were measured in conscious mice using the tail cuff method (**A**, **B**) and by telemetry (**C**–**F**). (**A**) Blood pressure. (**B**) Heart rate. (**C**) Blood pressure during animal's inactivity. (**D**) Blood pressure during animal's activity. (**E**) Average blood pressure. (**F**) Heart rate. In all diagrams, measurements from knockout mice are indicated by open bars, and results of control wild-type littermates — by solid bars. Asterisks indicate $p<0.05$, double asterisks $p<0.01$.

gene with the highest fold change increase (*Greb1*) has previously been linked to susceptibility to hypertension in human subjects (Kamide *et al.*, 2005). Consequently, we investigated whether expression of other genes with known links to hypertension or the regulation of blood pressure, is changed in the kidneys of *Grhl1*-null mice. The summary of this analysis is shown in Table 3. Overall we identified four such genes using gene expression microarrays, which provided further support to the notion that the *Grhl1*$^{-/-}$ mice display aberrant kidney function.

Subsequently, we conducted detailed bioinformatic analyses of 250 genes with the lowest adjusted p-values associated with differences in their expression in the kidneys of *Grhl1*$^{-/-}$ mice, as compared to the kidneys of *Grhl1*$^{+/+}$ control littermates. For this purpose we employed g:Profiler algorithm (http://biit.cs.ut.ee/gprofiler/). The results are summarized in Table S2 (Supplementary Materials on web site www.actabp.pl). In general, these genes can be divided into two major groups: those associated with response to misfolded protein and those associated with the immunological response.

Independently, we carried out enrichment analysis for Gene Ontology terms using web-based application GOrilla (http://cbl-gorilla.cs.technion.ac.il/). The results are shown in Tables S3-S5 (Supplementary Materials on web site www.actabp.pl), separately for terms categorized as "Biological Process", "Molecular Function" and "Cellular Component". Overall, these results are consistent with those obtained using g:Profiler described in the previous section.

Grhl1-null mice have normal dopamine levels in the blood serum

We assayed the levels of dopamine in the blood serum of 7 *Grhl1*$^{-/-}$ mice and 5 *Grhl1*$^{+/+}$ control mice. The results are shown in Fig. 4. The difference between the knockout and wild type mice was not statistically significant (p=0.29).

Grhl1$^{-/-}$ mice have normal blood pressure

We measured blood pressure in 14 *Grhl1*$^{-/-}$ males and 20 *Grhl1*$^{+/+}$ males using the tail-cuff method. As shown in Fig. 5A, there were statistically significant differences between the two groups regarding their SBP (p=0.031) and DBP (p=0.0495). However, in this experiment we failed to observe statistically significant differences in MBP (p=0.096). Subsequently, we measured blood pressure in 7 *Grhl1*$^{-/-}$ males and 7 *Grhl1*$^{+/+}$ males using telemetry. We did not detect statistically significant differences between *Grhl1*$^{-/-}$ males and their *Grhl1*$^{+/+}$ male littermates using this method (Fig. 5C–E).

Grhl1$^{-/-}$ mice have increased heart rate in comparison with their *Grhl1*$^{+/+}$ littermates

Simultaneously we measured the heart rates in the analyzed animals as in the previous section. With the tail cuff method we did not detect statistically significant differences between *Grhl1*$^{-/-}$ and *Grhl1*$^{+/+}$ mice (Fig. 5B). Using telemetry we discovered that the heart rates of *Grhl1*$^{-/-}$ males are significantly higher than in the *Grhl1*$^{+/+}$ littermate control males on average and during periods of inactivity. Surprisingly, the differences during active periods were not statistically significant (Fig. 5F).

DISCUSSION

Using the *Grhl1* "knock-out" mouse model we determined that the expression of four potential GRHL1 target genes is altered in the kidneys of *Grhl1*$^{-/-}$ mice, and all these four potential targets can be linked to the kidney function and regulation of blood pressure. This observation provided the first clear evidence that, despite the apparently normal kidney structure in the *Grhl1*-null mice (both at the macroscopic and microscopic levels – see Fig. 2), there are changes in their functioning at the molecular level. Subsequently, we employed microarray analysis to investigate gene expression in the kidneys of *Grhl1*-null mice. With this method we identified four additional genes with significantly altered expression which have previously been linked to susceptibility to hypertension or to the regulation of blood pressure (Table 3). We are cognizant that these genes do not have to be direct targets of GRHL1 regulation; their expression may have been altered as an indirect effect of *Grhl1* loss on kidney function.

Our more detailed analyses of results of the microarray experiment have shown that there are two major categories of genes whose expression is altered in the kidneys of *Grhl1*-null mice. One category consists of genes whose products are either chaperones or other proteins associated with response to wrongly folded protein. The other category includes genes involved in the immunological response. At the present moment we can offer no obvious explanation for the former finding, but the latter may be linked to the mild chronic inflammatory state observed in the *Grhl1*$^{-/-}$ mice. In our previous publication we reported that these mice display symptoms of skin inflammation; moreover, the levels of cytokine thymic stromal lymphopoietin (TSLP) are increased in the blood serum of *Grhl1*-null mice (Mlacki *et al.*, 2014). TSLP signaling is involved in the regulation of various immunological processes, hence it is possible that altered expression of this group of genes is caused by a response to elevated levels of TSLP in the peripheral blood (He & Geha, 2010).

Our blood pressure measurements using the tail cuff method revealed that SBP and DBP were significantly increased in the *Grhl1*-null mice, in comparison with their control wild-type littermates, but the change in MBP was not statistically significant (Fig. 5A). MBP is defined as the average arterial blood pressure during a single cardiac cycle, and is usually calculated as MBP=DBP+((SBP-DBP)/3) (Chaignon *et al.*, 1986). Thus MBP is a function of DBP and SBP, which is why we were surprised that, despite statistically significant increases in SBP and DBP, the increase in MBP was not significant. As seen from the above equation, the DBP component has greater influence on MBP than SBP, and in our mice the difference in DBP was on the border of significance (p=0.0495). We wanted to verify whether the greater difference in SBP than in DBP was caused by the use of tail cuff method, so we decided to employ a different method to measure blood pressure. Telemetry is much more expensive and more technically difficult than the tail cuff method, but it provides much more accurate results (Malkoff, 2005). With this method we established that the blood pressure of *Grhl1*$^{-/-}$ mice is not significantly different from their *Grhl1*$^{+/+}$ littermates (Fig. 5C–E). Therefore, since the *Grhl1*-null mice do not express the *Grhl1* transcript in any organ or tissue including the kidney (Wilanowski *et al.*, 2008), and in spite of that they have normal blood pressure, it is unlikely that a mutation in the *GRHL1* gene might be responsible for increased susceptibility to essential hypertension in humans.

The reason for the lack of change in blood pressure might be the relatively minor, albeit statistically significant, decrease in the expression of potential target genes of GRHL1 regulation *Cacna1d*, *Ddc*, *Flot2* and *Xpo1* in the kidneys of *Grhl1*$^{-/-}$ mice. This decrease ranged from 1.4-fold (*Flot2*) to 2.7-fold (*Cacna1d*) (Fig. 3) which might be insufficient to trigger significant physiological consequences in terms of renal pathology and blood pressure. The same can be said about genes identified in the microarray experiment, whose changes in expression ranged from 1.6-fold decrease (*Cyp4a12b*) to 2.5-fold increase (*Greb1*) (Table 3). This possibility is further supported by the fact that, despite reduced *Ddc* expression, the *Grhl1*-null mice have normal dopamine levels in the blood serum (Fig. 4).

We can envisage a number of reasons for the discrepancies between the results obtained using the tail cuff method and telemetry. In the tail cuff method animals are always subjected to certain levels of stress. It is possible that the *Grhl1*$^{-/-}$ mice display an excessive response to stress – for example, due to a neurogenic malfunction – and this may account for the increased blood pressure observed in tail cuff measurements (Currie *et al.*, 2012).

Despite normal blood pressure, the *Grhl1*-null mice display significantly increased heart rate in comparison with their wild-type littermates, on average and during the periods of inactivity when measured by telemetry. However, their heart rate is normal when measured using tail cuff method and during periods of activity (Fig. 5B, 5F). One possible way to explain these results is that

Table 3. Results of the microarray experiment – continued.
List of genes with altered expression in the *Grhl1*$^{-/-}$ kidneys that have been linked to hypertension or regulation of blood pressure. Only genes with statistically significant changes in expression are shown (adjusted p-value<0.1).

Gene name	Accession no.	Fold change	p-value	Adjusted p-value	References
Cyp4a12b	NM_172306	0.637	1.4E-04	0.069	(Zhou *et al.*, 2008)
Cys1	NM_138686	0.658	1.6E-04	0.077	(Wu *et al.*, 2013)
Srebf1	NM_011480	0.721	9.6E-06	2.0E-02	(Pravenec *et al.*, 2008)
Greb1	NM_015764	2.498	3.3E-06	9.6E-03	(Kamide *et al.*, 2005)

the loss of *Grhl1* leads to intravascular volume depletion and subsequent increase in heart rate which is a compensatory mechanism allowing to maintain the correct blood pressure (Arikan & Citak, 2008). This volume depletion may have various causes: polyuria; a concentrating defect; or impaired thirst. If during periods of inactivity the *Grhl1*$^{-/-}$ mice are polyuric, this may result in intravascular volume depletion and increased heart rate. Then, if during periods of activity the same mice increase their water intake and thus replenish their intravascular volume, their heart rate returns to normal values. This would explain the differences in heart rates while maintaining normal blood pressure.

We examined an alternative mechanism which could involve dopamine signaling. Dopamine is a known regulator of blood pressure, and intravenous dopamine administration is recommended for treatment of slow heart rate (Neumar *et al.*, 2010). However, the *Grhl1*-null mice have normal serum levels of dopamine (Fig. 4), so this hypothesis proved to be false. Yet another possibility involves a degree of sympathetic activation in the *Grhl1*$^{-/-}$ mice. This hypothesis would require a further study.

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