Charles University

First Faculty of Medicine

Summary of the PhD Thesis

FIRST FACULTY
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GENETIC FACTORS RESPONSIBLE FOR DEVELOPMENT OF METABOLIC SYNDROME

Genetické faktory podílející se na vzniku metabolického syndromu

MUDr.Lukáš Farhád Mirchi

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Study Program: Molecular and Cell Biology, Genetics and Virology

Chair: doc. RNDr. Dana Holá, PhD.

Research center: Institute of Biology and Medical Genetics,

Department of Experimental Genetics, Prague

Supervisor: doc MUDr.František Liška, Ph.D.

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Table of contents

Abstract:

The metabolic syndrome (MetS) is a worldwide highly prevalent disease defined as a clustering of at least of three of the following conditions: central obesity, hypertension, diabetes, high level of low-density lipoproteins, low level of high-density lipoprotein or high level cholesterol. In order to effectively fight the MetS pandemic, it is vital to dissect the genetic background and mechanisms that underlie MetS and its individual components, a goal that is profoundly benefitted by physiological and genetic studies in animal models of MetS. The aim of this thesis was to dissect the genetic background of metabolic syndrome by using genetically defined rodent models with distinct features of MetS. We present three separate transcriptomic experiments in order to unravel the genetic background of MetS. First, we performed comparative transcriptomic analysis of left ventricular tissue from SHR and SHR-derived minimal congenic strain PD5 with attenuated cardiac fibrosis. An overexpression of nuclear orphan receptor *Nur77/Nr4a1* in PD5 and dysregulation of *Nr4a3, Per1* and *Kcna5* were revealed. In the second experiment, we observed phenotypic changes in PD, SHR and BN rat strains, respectively, after high fat diet administration, with subsequent transcriptomic analysis of the liver to dissect the background of higher susceptibility of PD strain to MetS. We found a promising candidate gene *Acsm3* (coding for acyl-CoA-synthetase for medium-chain member 3), which belongs to a family of enzymes activating medium chain fatty acids (C4-C14) to beta-oxidation, and which was absent in liver of PD on both mRNA and protein levels. In the third experiment we tried to unravel mechanisms underlying differential liability of PD5 and SHR to glucocorticoid induced metabolic syndrome. We performed comparative transcriptomic analysis of PD5 and SHR liver tissue after dexamethasone treatment unraveling potential genetic determinants; furthermore, we performed a proteomic analysis unraveling potential targets of Plzf as a possible candidate gene responsible for this aspect of metabolic syndrome. Using defined rodent models and transcriptomic approach we mapped several key pathophysiological pathways accountable for development of MetS features and unraveled several candidate genes in the context of these pathways.

Key words: metabolic syndrome, hypertension, congenic strain, SHR, PD, PD5, candidate genes, *Nur77, Acsm3, Plzf*

Abstrakt:

Metabolický syndrom je celosvětově vysoce prevalentní komplexní onemocnění charakterizované přítomností alespoň tří z následujících znaků: abdominální obezity, hypertenze, diabetu mellitu, vyšší hladiny LDL cholesterolu či snížené hladiny HDL cholesterolu. Pochopení genetického pozadí metabolického za pomocí geneticky definovaných savčích modelů představuje jednu ze strategií ke zlepšení možností boje s MetS a jeho zdravotními následky. Cíl této disertační práce je poodkrýt genetické pozadí metabolického syndromu a to za pomoci geneticky definovaných potkaních modelů. K popisu genetické architektury metabolického syndromu jsme provedli tři nezávislé experimenty. Nejprve jsme pomocí komparativní transkriptomické analýzy mezi levými srdečními komorami kmene SHR a jeho minimálního kongenního kmene PD5, charakterizovaným mj. menší mírou fibrózy myokardu, identifikovali zvýšenou expresi genu *Nr4a1/Nur77* u kmene PD5 a dále dysregulaci genů *Nr4a3, Per1* a *KCNA5*. V druhém experimentu jsme pozorovali rozdíly fenotypových profilů potkaních kmenů PD, SHR a BN po podání vysokotukové diety za účelem odhalení genetické podstaty vyšší náchylnosti kmene PD k rozvoji metabolického syndromu. Identifikovali jsme slibný kandidátní gen *Acsm3* (acyl-CoA-synthetase for medium-chain member 3), který kóduje enzym patřící do rodiny syntetáz aktivujících středně dlouhé mastné kyseliny (C4-C14) pro vstup do beta-oxidace mastných kyselin. V jaterní tkáni kmene PD není tento gen prakticky exprimován, což jsme potvrdili na mRNA jakož proteinové úrovni. Cílem třetího experimentu byla identifikace patofyziologických mechanismů vedoucích k diferenciální citlivosti kmenů PD5 a SHR ke glukokortikoidy indukovanému metabolickému syndromu. I zde jsme provedli komparativní transkriptomickou analýzu jaterní tkáně potkanů PD5 a SHR před a po podání dexamethasonu odkrývající možné genetické determinanty, proteomická analýza nám poté pomohla určit potenciální cíle *Plzf* jakožto genu důležitého pro tento aspekt metabolického syndromu. Pomocí kombinovaného přístupu užití geneticky definovaných modelů a transkriptomických esejí jsme identifikovali několik metabolických drah s potenciální rolí v patogenezi metabolického syndromu a zároveň jsme odhalili několik kandidátních genů.

Klíčová slova: hypertenze, metabolický syndrom, kongenní kmen, SHR, PD, PD5, kandidátní geny, *Nur77, Acsm3, Plzf*.

Introduction:

Metabolic syndrome

Metabolic syndrome is defined as a clustering of visceral obesity, dyslipidemia, arterial hypertension, insulin resistance and related maladies such as prothrombotic state of sleep apnea syndrome. Metabolic syndrome is probably the most prevalent multifactorial non-communicable disease worldwide according to the WHO (Riley L. et al. 2016). The prevalence of many of the components of the "metabolic syndrome", particularly obesity and diabetes, has grown considerably throughout the Western World since this term was initially suggested by Haller in 1977 (Haller H. et al., 1977) which is an alarming condition given that the metabolic syndrome is an important precursor to cardiovascular disease and other chronic maladies (Grundi SM. et al., 2004; Pucci G. et al., 2017; Deboer MD. et al., 2018).

Genetic background of the Metabolic syndrome

MetS is a multifactorial disease with a polygenic heritability pattern. The evidence of the genetic component of metabolic syndrome was provided with family and twin studies (Lin HF. et al., 2005; Carmelli D. et al., 1994), but the challenge to uncover the genetic determinants of MetS has been largely faced with candidate gene association studies, linkage analysis and genome wide association studies GWAS. The candidate gene studies and GWAS in human and murine GWAS have revealed that each of the main components (insulin resistance, obesity, hypertension and dyslipidemia) has a complex genetic background affected by numerous genetic variants, with most individual loci affecting only one or some of the comorbidities that comprise MetS (Abou Ziki MD. et al., 2016). Given the complexity of the disease and exposure to environmental factors, studies in humans are still challenging (Brown AE. et al., 2016). For this very reason studies performed on experimental rodent models are still a mainstay in the research of polygenic diseases (Cox RD. et al., 2003; Rees DA. et al., 2005). We previously described an SHR derived minimal congenic counterpart SHR.PD-(D8Rat42-D8Arb23)/Cub (Rat Genome Database ID: 1641851; PD5 hereafter), (Šeda O. et al., 2005). PD5 strain has been established by introgression of a small segment of chromosome 8 from the PD/Cub strain (an inbred model of metabolic syndrome without hypertension, Šedová L. et al., 2000), on the genetic background of SHR by repeated backcrossing. Using the high definition marker-assisted approach and the congenic segment was identified as containing 788 kbp (chr8:51,897,776-52,685,422 according to the rat reference genome version 3.4), encompassing 7 genes: Plzf, Htr3a, Htr3b, Usp28, Zw10, Tmprss5, and Drd2. Sequencing the congenic segment a deletion in the noncoding sequence (with possible enhancer function) of Plzf was

revealed (Liška F. et al., 2014). From the phenotypic point of view PD5 displays significantly lower total body weight, lower blood pressure, heart weight (Křen V. et al., 1997), lesser tendency to myocardial fibrotization (Liška et al. 2014), lower triacylglycerol and cholesterol levels (Krupková M. et al., 2014) and tendency towards and enhanced glucose tolerance (Šeda O. et al., 2005) when compared to SHR, but dexamethasone administration led to significant elevation of TAG in SHR-Lx PD5 (Šeda O. et al., 2005). Plzf appears to function primarily as a transcriptional repressor by recruiting nuclear receptor co-repressors (N-CoRs) (Melnick et al., 2002) and was linked to several facets of metabolic syndrome. Plzf promotes gluconeogenic gene expression and hepatic glucose output leading to hyperglycemia and enhancing insulin resistance and stimulates hepatic glucose synthesis as Peroxisome proliferator-activated receptor-gamma coactivator (Chen S. et al., 2014). Data shows that Plzf has vital function for the correct development of natural killer T cells (Kovalovsky D. et al., 2008) and may thus be a reason for NAFLD development (Crosby CM. et al., 2018). Plzf also inhibits Na+ reuptake in renal epithelial cells upon aldosterone stimulation and hence makes up a negative feedback loop (Naray-Fejes-Toth A. et al., 2008). It was shown that SHR with TALEN targeted Plzf exhibited an amelioration of cardiac hypertrophy and increased sensitivity of adipose and muscle tissue to insulin action when compared with wild-type controls (Liška F. et al., 2017). This evidence marks Plzf as a candidate gene in the development of metabolic syndrome.

The aims of the thesis

The chief goal of this work was to identify genetic determinants underlying metabolic syndrome and to dissect the role of Plzf within the complex network of these determinants.

1. Elucidate the genetic background leading to amelioration of blood pressure and cardiac fibrosis in SHR minimal congenic strain PD5

2. Compare transcriptional and phenotypic changes in PD and SHR rat strains after high fat diet and unravel pathophysiological mechanisms underlying PD susceptibility to metabolic syndrome.

3. Compare transcriptional and phenotypic changes in PD5 and SHR rat strains before and after dexamethasone administration in order to fully uncover the role of Plzf in development of metabolic syndrome.

Methods:

Animal models:

In our experiments we used three established rodent models of metabolic syndrome. Animal experiments were conducted according to the guidelines of the Declaration of Helsinki, in compliance with the Animal Protection Law of the Czech Republic, and were approved by the Ethics Committee of the First faculty of Medicine, Charles University, Prague

Spontaneously hypertensive rat SHR

Spontaneously hypertensive rat (SHR/OlaIpcv, RGDID 631848) is a polygenic, highly inbred model of metabolic syndrome with marked hypertension, dyslipidemia, insulin resistance and is predisposed to left ventricular hypertrophy and myocardial fibrosis, in general however, is not considered obese. It was created in Japan in the sixties by crossing outbred Wistar Kyoto hypertensive rats (Okamoto K. et al., 1963) and has ever since been preserved by brother sister mating.

Polydactylous rat, PD strain

Polydactylous rat (PD/Cub, RGDID 728161) is a well defined luxate syndrome model with markedly high triglyceridemia and insulin resistance even in comparison with SHR, but lacks hypertension (Šedová L. et al., 2000). Polydactylous rat is particularly sensitive to aggravation of insulin resistance and serum TAG levels after a diet rich in carbohydrates, as opposed to Brown Norway. (Šeda O. et al., 2005).

PD5 rat strain

PD5 formally described as SHR.PD (D8Rat42-D8Arb23)/Cub (RGD ID: 1641851) is a minimal congenic strain derived from SHR rat strain. PD5 strain has been established by introgression of a small segment of chromosome 8 from the PD/Cub strain, on the genetic background of SHR by repeated backcrossing. By sequencing the congenic segment a deletion in the noncoding sequence (with possible enhancer function) of Plzf was revealed (Liška F. et al., 2014). From the phenotypic point of view PD5 displays significantly lower blood pressure, heart weight (Křen V. et al., 1997), lesser tendency to myocardial fibrotization (Liška F. et al., 2014) and lower triacylglycerol and cholesterol levels (Krupková M. et al., 2014), when compared to SHR.

Morphometric and metabolic assessments

Blood samples for metabolic and glycemic assessments were drawn after overnight fasting from the tail vein. The OGTT of males was performed at months of age. The blood samples were

obtained at intervals of 0,30, 60, 120, and 180 min after intragastric glucose gavage to conscious rats (3 g/kg body weight,30% aqueous solution; Ascensia Elite Blood Glucose Meter; Bayer HealthCare, Mishawaka, IN. The serum lipoprotein distribution was evaluated using gel-permeation high-performance liquid chromatography (GP-HPLC) and LipoSEARCH® algorithm (Toyobo Inc., Japan), serum levels of Free fatty acid before and after feeding HFD were analyzed using a commercially available acyl-CoA oxidase-based colorimetric kits (Roche Diagnostics, Basel, Germany), insulin and adiponectin levels before and after HFD feeding were determined using the Rat Insulin ELISA kit (Mercodia AB, Uppsala, Sweden) and Adiponectin ELISA kit (MyBioSource, San Diego, CA, USA), Levels of C-Peptide 2, GIP, GLP-1, glucagon, insulin, leptin, PP, and PYY before and after HFD were assessed via Milliplex Metabolic Hormone MAGNETIC kit using the BioPlex 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA, Merck Millipore Corp., Billerica, MA, USA), cytokine profiles of male rats before and after HFD were assessed via Bio-Plex Pro^{TM} Rat Cytokine 23-Plex Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA, Luminex Corporation, Austin, TX, USA) for levels of G-CSF, GM-CSF, GRO/KC, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17, IL-18, M-CSF, MCP-1, MIP-1α, MIP-3α, RANTES, TNF-α, and VEGF using the BioPlex system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). TAG content in liver tissue was determined by enzymatic assay (Erba-Lachema, Brno, Czech Republic). Hepatic diacylglycerols were separated using a modified Folche method followed by thin-layer chromatography, as previously described Hüttl M. et al., 2020), and its quantitative content was determined by enzymatic assay (Erba-Lachema, Brno, Czech Republic). In addition to metabolic assessments, the adult male rats were sacrificed to determine the weights of liver, heart, kidneys, adrenal glands, brown fat tissue, epididymal fat tissue, retroperitoneal fat tissue, soleus muscle, and diaphragm. Liver and left myocardial tissue were snap-frozen in liquid nitrogen in preparation for the transcriptome assessment.

Transcriptome assessment

For RNA isolation and purification we used RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Total RNA from relevant organs (liver, heart, kidneys, adrenal glands, brown fat tissue, epididymal fat tissue, retroperitoneal fat tissue, soleus muscle, and diaphragm) was used. The quality and integrity of the total RNA was evaluated on Agilent 2100 Bioanalyzer system (Agilent, Palo Alto, CA). The transcriptome was assessed using Affymetrix GeneChip® Rat Gene 2.1 arrays (Affymetrix, Santa Clara, CA, USA). O. For the first experiment 3 SHR and 3 PD5 male rats were used, in the second experiment we chose 4 male rats from each strain (SHR,

PD/Cub and BN), for the third experiment we used 3 male rats from each strain and diet (SHR, SHR + dexamethasone, PD5, PD5 + dexamethasone). The quality control of the chips was performed using Affymetrix Expression Console. Partek Genomics Suite (Partek, St. Louis, Missouri) was used for subsequent data analysis. After applying quality filters and data normalization by Robust Multichip Average (RMA) algorithm implemented in Affymetrix Expression Console, the set of obtained differentially expressed probesets was filtered by false discovery rate (FDR) method implemented in PARTEK Genomic Suite 6.6 (Partek, St. Louis, Missouri).

qPCR verification

The different expression of candidate genes was verified by quantitative real-time PCR (qPCR). Total RNA (1 μg) was reverse-transcribed with oligo-dT primers using the SuperScript III (Invitrogen). We used total RNA (c = 250 ng/μl) isolated from respective organs (heart, liver, liver, white and brown fat to prepare cDNA for the quantitative PCR validation of the microarray data. We used cDNA with c= 5 ng/μlPrimers for qPCR reactions were designed using PrimerBLAST (Ye J, et al. 2012) to span at least one exon–exon junction, with every amplicon within 70 to 200 bp range. All samples were used in triplicates. *Per1* and *Gadph* was amplified using the TaqMan® Gene Expression Master Mix (Applied Biosystems), *Acsl5, Acsm2a, Acsm3, Aox1, Apol, Casp12, Cd36, Ces2e, Crh, Cyp2b1, Cyp7b1,Doc2a, GCA, Insig1, KCNA5, Lepr, Lmod, Nox4, Npy, NR4a1, NR4a3,Oxt, Ppia, Pomc, Scd1, Rgs16, Sirt3, Slc17a2, Ugt2a3, Vas,* were amplified using the Power-up SYBRGreen master mix (Thermo Fisher Scientific, Waltham, MA, USA). Amplification was performed according to the respective manufacturer's instructions. Amplification was done in Applied Biosystems® 7900HT Real-Time PCR cycler system. Results were analyzed using the Livak (Livak K. et al., 2001) analysis method with every expression compared to glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) or cyclophilin (*Ppia*) as a reference gene expression.

DNA Sequencing

Sequencing of *Acsm3* in PD strain: Because of the impossibility of amplifying cDNA (absence of cDNA of *Acsm3* in liver tissue from PD), genomic DNA was used for sequencing of *Acsm3*. Long-range PCR products were sequenced on an Illumina MiSeq using the Nextera XT DNA Library Preparation Kit (Illumina). Bioinformatic analysis was done with the help of Galaxy (https://usegalaxy.org). Mapping was done by BWA-MEM (Burrows-Wheeler Aligner). Duplicated reads were removed by Picard (http://broadinstitute.github.io/picard/). FreeBayes

was used to identify sequence variants (Garrison E. et al., 2012). IGV (Integrated Genome Viewer) was used for data visualization.

Western Blotting

N-terminal rabbit monoclonal anti-Nra4a1 antibody (anti-Nur77; ab109180) was purchased from Abcam (Cambridge, UK), mouse monoclonal Acsm3 antibody (G-8; sc-377173) was purchased from Santa Cruz Biotechnologies, Inc., Dallas, TX, USA, N-terminal rabbit monoclonal anti-Plzf antibody (ab189849) was purchased from Abcam (Cambridge, UK). As controls mouse anti-αtubulin (B-5-2-1) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and mouse monoclonal Vinculin antibody (VLN01) was purchased from Thermo Fisher Scientific, Waltham, MA, USA. Tissue lysates (for every experiment 3 samples from each strain) were run on SDS-PAGE (12% separating gel), and proteins were blotted onto PVDF membranes Immobilon P (EMD Millipore Biosciences, Billerica, MA, USA). Membranes were incubated overnight at 4 °C with primary antibodies at a final dilution of 1:3000 NR4A1, 1:3000 PLZF, 1:10000 α-tubulin, 1:200 (Acsm3) and 1:10,000 (Vinculin). Secondary anti-mouse antibody and signal was detected using an ECL Prime chemiluminescent detection kit and Hyperfilm ECL (all from GE Healthcare Bio-Sciences, Chicago, IL, USA). Developed hyperfilms were scanned and densitometry was performed in ImageJ (Schneider CA. et al., 2012)

Luciferase assay

Five Plzf (RGD ID: 727921) regions denoted as L („left“), R („right“), A („all“), “out” and “full” were cloned from SHR genomic DNA. Constructs were cloned to pGL4.10 and pGL4.23 plasmids (Promega, Madison, WI, USA) respectively and electroporated into electrocompetent TOP10 E. coli cells (Thermofisher). Plasmids were sequenced on an Illumina MiSeq using the Nextera XT DNA Library Preparation Kit (Illumina). Bioinformatic analysis was done with the help of Galaxy (https://usegalaxy.org). Mapping was done by BWA-MEM (Burrows-Wheeler Aligner). Duplicated reads were removed by Picard (http://broadinstitute.github.io/picard/). FreeBayes was used to identify sequence variants (Garrison E. et al., 2012). IGV (Integrated Genome Viewer) was used for data visualization.

The firefly luciferase reporter pGL4.10 and pGL4.23 constructs were co-transfected with Renilla luciferase reporter vector pGL4.74 into HepG2 and HEK293 cells by ViaFect (E4981, Promega Madison, WI, USA), according to the manufacturer's protocol. Human cervical hepatocellular carcinoma (HepG2) cells purchased from Sigma-Aldrich (St. Louis,

MO, USA) were grown in D-MEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 mg/mL) at 37 °C in 5% CO_2 humidified atmosphere. Human embryonic kidney HEK 293 cells (Thermo Fisher Scientific, Waltham, MA) were grown in BalanCD HEK293 medium (Cat. 91165-1L, FUJIFILM Irvine Scientific, Wicklow, Ireland) n 4.5 mL medium at 1×10^6 cells/mL in 50 mL bioreactor tubes, incubated overnight at 37 °C, 8% CO_2, and agitated at 170 rpm in a humidified atmosphere. After 24 h cells were lysed and luciferase activity was measured according to Dual-Glo®Luciferase Assay System protocol (E2920, Promega, Madison, WI, USA), using the Infinite 200 luminometer (Tecan). Luminescence signal from empty vectors was set as threshold for background signal. Data were normalized to renilla luciferase activity and assay was performed in triplicate and repeated at least three times.

GST pull down assay

Three Plzf constructs encompassing whole gene Plzf, BTB and Zinc finger domains were prepared by standard PCR using cDNA obtained from BN heart tissues. The amplified constructs were ligated to pET42b plasmids and were in vivo amplified using E. coli cells. Grown cells were harvested by centrifugation, lysed and plasmids were isolated by standard alkaline method with QIAGEN® Plasmid Mini kit (Qiagen, Hilden, Germany). Plasmids were sequenced on an Illumina MiSeq using the Nextera XT DNA Library Preparation Kit (Illumina). The constructs were ligated in the pET42b plasmids which were transformed in a non-expression cells, isolated and re-transformed in the expression vector Rosetta(DE3)pLysS The expression of the recombinant constructs was induced by addition of 1mM IPTG. The fusion proteins were purified by Affinity chromatography using Glutathione HiCap Matrix (Quiagen, Hilden, Germany) and the concentration and purity of the protein samples was determined using spectrophotometry. The protein digestion was performed according to Rappsilber (Rappsilber J. et al., 2007) with subsequent nLC-MS 2 Analysis accoring to (Hebert AS. et al., 2014). All data were analysed and quantified with the MaxQuant software (version 2.0.2.0) (Cox J. et al., 2014). The false discovery rate (FDR) was set to 1% for both proteins and peptides and we specified a minimum peptide length of seven amino acids. The Andromeda search engine (Cox J. et al., 2011) was used for the MS/MS spectra search against the Rattus norvegicus database (downloaded from uniport.org in September 2022, containing 22 909) and database containing individual sequences of used protein constructs.

Results:

First experiment - Comparative transcriptomic analysis of left ventricular tissue in SHR and its minimal congenic sublime PD5 with ameliorated hypertension and cardiac fibrosis

Previously we revealed a decrease of blood pressure and an amelioration of cardiac fibrosis in a congenic line of spontaneously hypertensive rats (SHR), with a short segment of chromosome 8 of PD origin with a 2964bp intronic deletion within *Plzf* gene. To unravel the genetic background of this phenotype we compared heart transcriptomes between SHR rat males and this chromosome 8 minimal congenic line (PD5). We found 18 differentially expressed genes, which were further analyzed using annotations from Database for Annotation, Visualization and Integrated Discovery (DAVID). Four of the differentially expressed genes (Per1, Nr4a1, Nr4a3, Kcna5) belong to circadian rhythm pathways, aldosterone synthesis and secretion, PI3K-Akt signaling pathway and potassium homeostasis (Fig.1). We were also able to confirm Nr4a1 2.8x-fold upregulation in PD5 on protein level using Western blotting (Fig.2), thus suggesting a possible role of Nr4a1 in pathogenesis of the metabolic syndrome.

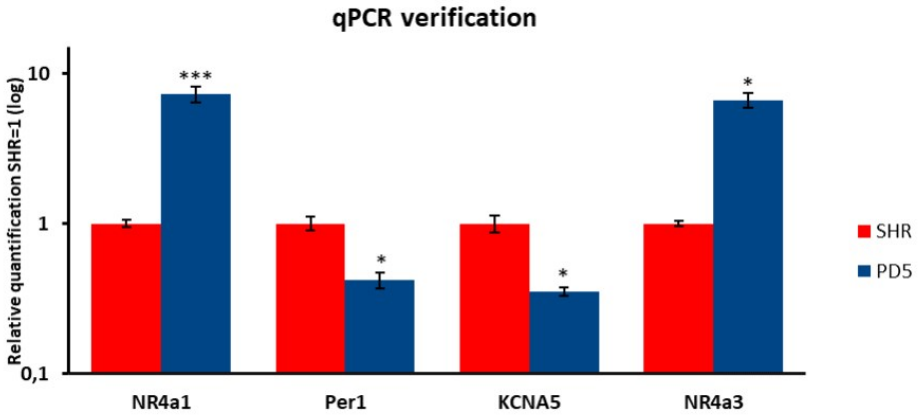

Fig. 1) qPCR validation of microarray results of heart tissue of SHR (n=6) and PD5 (n=6) rat strains: Nr4a1 – nuclear receptor subfamily 4 group A member 1, Nr4a3 – nuclear receptor subfamily 4 group A member 3, Kcna5 – potassium VoltageGated Channel Subfamily A Member 5, Per1 – period circadian regulator 1, Data are presented as arithmetic means ± SEM. Statistical significance levels for the factor strain of one-way ANOVA are indicated for pair-wise post hoc Tukey's test as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

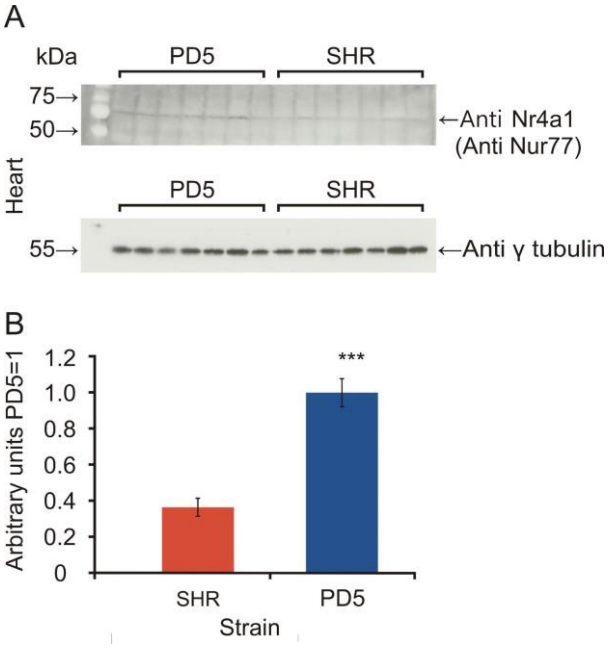

Fig.2) Western blot of Nr4a1 protein (nuclear receptor 4 alpha 1, also known as Nur77) shows upregulation in the minimal congenic strain PD5 compared to SHR. Western blotting using N-terminal antiNur77 antibody in the heart (A), Densitometry data (representing means ± S.E.M, ***=p<0.001; n=7 for both SHR and PD5 rat strains (B). Theoretical molecular weight of unmodified Nr4a1 is 64 kDa. We observed the signal consistently at slightly >60 kDa.

Second experiment - Comparative transcriptomic analysis of liver tissue after high fat diet admission in two established rodent models of metabolic syndrome PD and SHR strain

PD (Polydactylous rat) is an animal model of hypertriglyceridemia, insulin resistance, and obesity. To unravel the genetic and pathophysiologic background of this phenotype, we compared morphometric and metabolic parameters as well as liver transcriptomes among PD, spontaneously hypertensive rat, and Brown Norway (BN) strains fed a high-fat diet (HFD). After 4 weeks of HFD, PD rats displayed marked hypertriglyceridemia but without the expected hepatic steatosis (Fig. 3). Moreover, the PD strain showed significant weight gain, including increased weight of retroperitoneal and epididymal fat pads, and impaired glucose tolerance. In the liver transcriptome, we found 5480 differentially expressed genes, which were enriched for pathways involved in fatty acid beta and omega oxidation, glucocorticoid metabolism, oxidative stress, complement activation, triacylglycerol and lipid droplets synthesis, focal adhesion, prostaglandin synthesis, interferon signaling, and tricarboxylic acid cycle pathways. Interestingly, the PD strain, contrary to SHR and BN rats, did not express the Acsm3 (acyl-CoA synthetase medium-chain family member 3) gene in the liver (Fig. 4 and Fig. 5). Together, these results suggest disturbances in fatty acid utilization as a molecular mechanism predisposing PD rats to hypertriglyceridemia and fat accumulation.

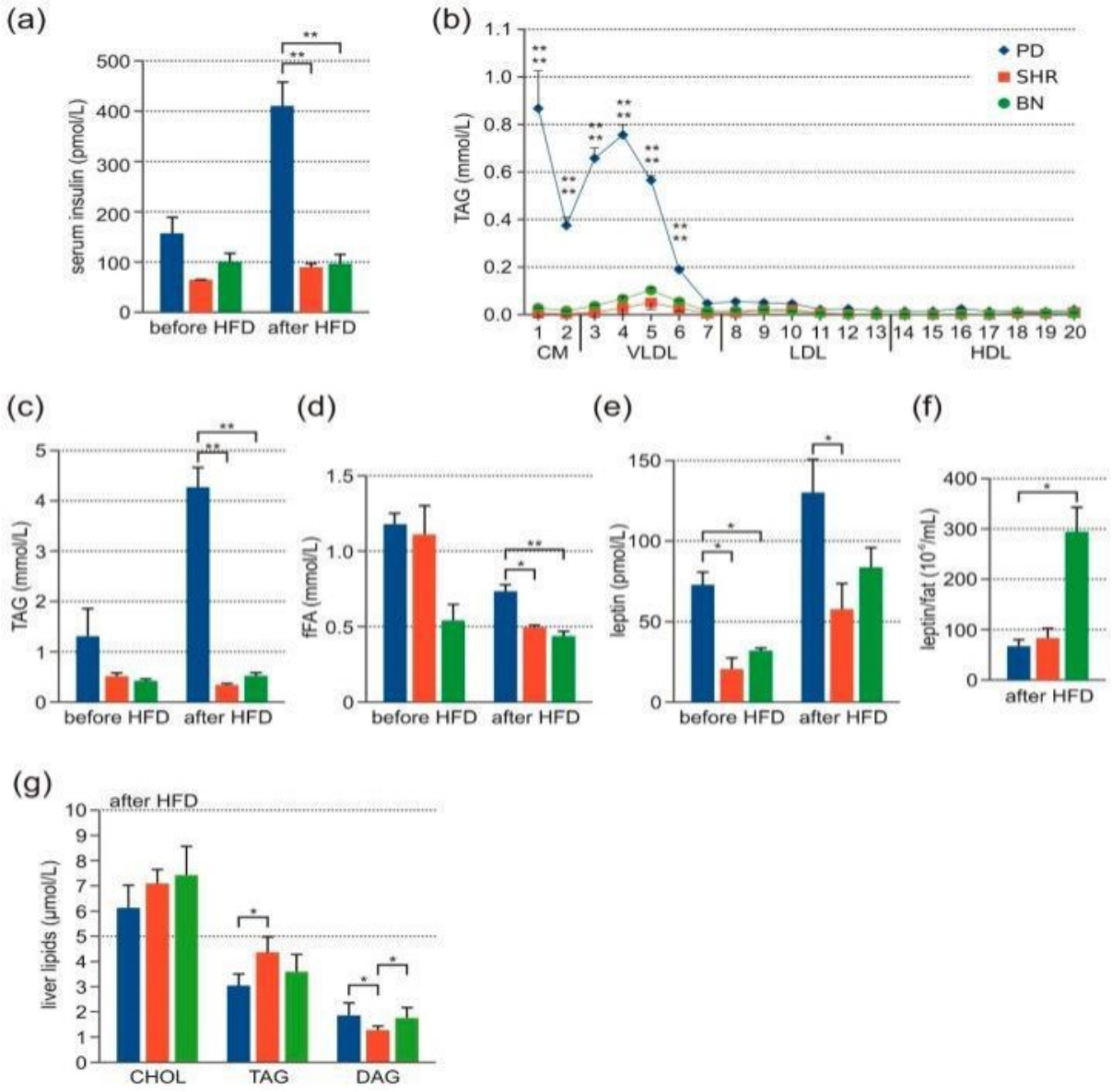

Fig.3) Laboratory findings in adult male rats-PD (blue, n = 3–7), SHR (red, n = 3–6), and BN (green, n = 3–8). (**a**) Insulin levels before and after HFD. (**b**) TAG levels after HFD in 20 lipoprotein fractions. (**c**) Total TAG levels before and after HFD. (**d**) Free FAs before and after HFD. (**e**) Absolute leptin blood level before and after HFD. (**f**) Relative leptin blood level (after correction to fat tissue amount) after HFD. (**g**) Cholesterol, TAG, and DAG levels in liver tissue. Data are presented as arithmetic means ± SEM. Statistical significance levels for the factor strain of repeated measurements ANOVA (cholesterol and TAG in 20 lipoprotein fractions) or one-way ANOVA (insulin levels, total TAG, fFAs, leptin, cholesterol, TAG, and DAG in liver tissue) are indicated for pair-wise post hoc Tukey's test as follows: * $p < 0.05$, ** $p < 0.01$. For post-hoc TAG levels, the upper symbol corresponds to PD vs. SHR comparison, the bottom symbol represents PD vs. BN comparison.

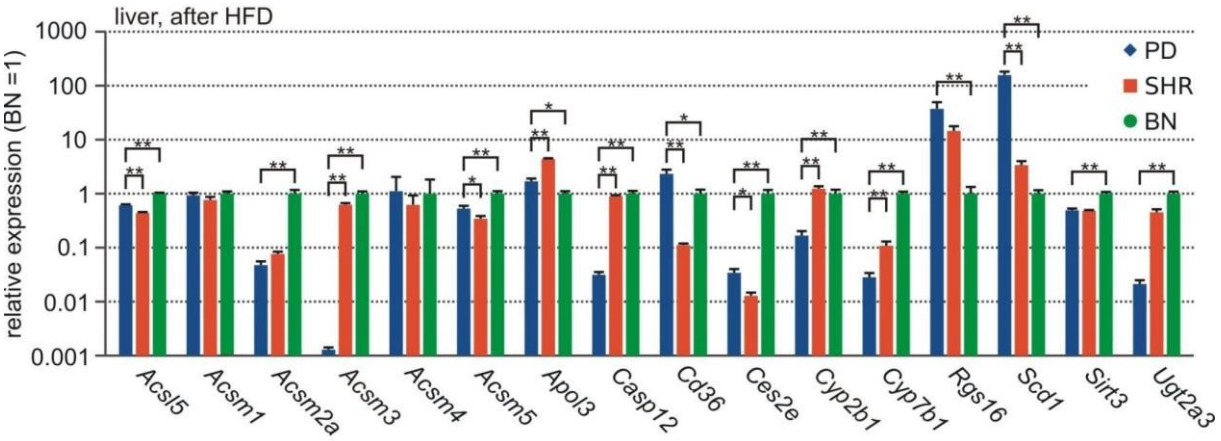

Fig. 4) Gene expression (qPCR) after HFD in adult male rats liver tissue. PD (blue, n = 7), SHR (red, n = 6), and BN (green, n = 8). Data are presented as arithmetic means ± SEM. Statistical significance levels for the factor strain of one-way ANOVA are indicated for pair-wise post hoc Tukey's test as follows: * $p < 0.05$, ** $p < 0.01$.

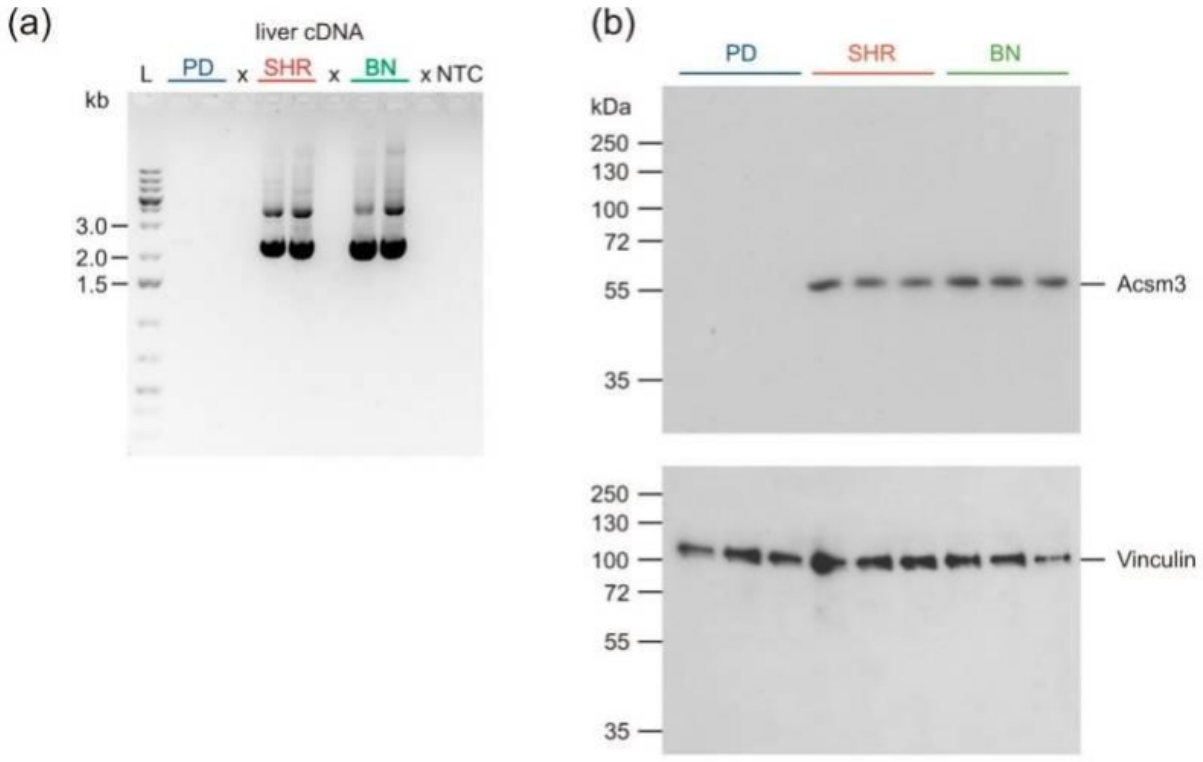

Fig. 5) Expression of *Acsm3* in the liver of adult male rats-PD (blue), SHR (red), and BN (green); after HFD. (**a**) RT-PCR of *Acsm3*, visualized on gel electrophoresis (two representative samples for each strain). Primers flank the complete coding sequence; the expected size of the PCR product is 2296 bp. (**b**) Western blotting of ACSM3 (expected molecular weight of the mature polypeptide 63.2 kDa). Vinculin (expected molecular weight 116.6 kDa) was used as the loading control (three representative samples for each strain).

Third experiment - Comparative transcriptomic analysis of liver tissue in SHR and its minimal congenic sublime PD5

We previously described attenuated features of metabolic syndrome, but greater sensitivity to steroid induced metabolic syndrome in minimal congenic strain SHR.lx PD5 (PD5) derived from spontaneously hypertensive rat (SHR). To unravel the genetic background of this phenotype we compared liver transcriptomes and physiologic parameters between SHR and PD5 strains without intervention and after dexamethasone administration. As opposed to its progenitor, PD5 rats displayed lower total body weight after dexamethasone administration, an observation contradictory to SHR where dexamethasone led to total body weight increase. On top of that, dexamethasone deteriorated OGTT in the same pattern in both strains, however this effect was pronounced more than two times more in SHR (AUC (mmol*min/L): SHR DEX / PD5 DEX 2.15-fold). Analyzing the transcriptome, we found 1739 differentially expressed genes, which were integrated into the global transcriptome context using Database for Annotation, Visualization and Integrated Discovery (DAVID), revealing involvement of these genes in glutathione metabolism, peroxisome pathway, Staphylococcus aureus infection, Protein processing in endoplasmic reticulum, Complement and coagulation cascades KEGG pathways. The transcriptomic data showed overexpression of *Plzf* in SHR after dexamethasone admission, in PD5 the expression increase was less pronounced; qPCR verification and Western-blot showed good consistency with microarray data (SHR transcript 4.06-fold, protein 4.08-fold; PD5 transcript 1.85-fold, protein 3.00-fold). We observed a shift in a phenotype after dexamethasone administration in PD5 rat strains, which was mirrored in the change of expression of *Plzf* gene as well as on proteomic level. However, the luciferase assay did not show a convincing direct effect of dexamethasone on the deleted intronic *Plzf* fragment, on the other hand it may appear, that according to luciferase data the *Plzf* intronic deletion may have enhancer activity.

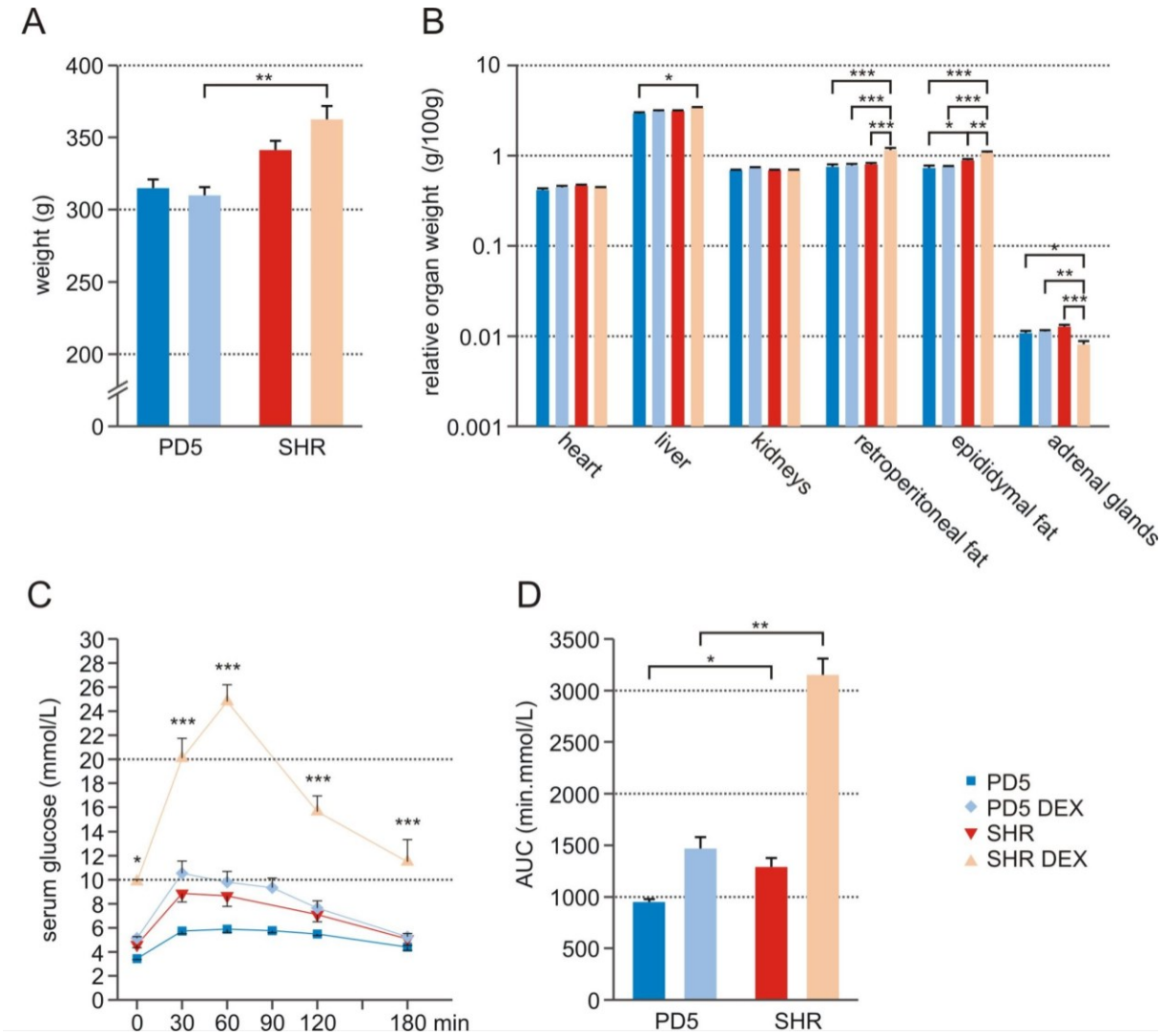

Fig.6) Morphometric profile and glucose tolerance of adult PD5 and SHR male rats fed with standard diet without dexamethasone (PD5, dark blue, n=10; SHR, red, n=7) and standard diet with dexamethasone treatment (DEX; PD5 DEX, light blue, n=9; SHR DEX, orange, n=7). **A**) Absolute weight gain. **B**) Relative tissue weights per 100 g body weight. **C**) Oral glucose tolerance test (OGTT) - time course of glucose level. **D**) The area under the curve (AUC) of OGTT.

Data are presented as arithmetic means ± SEM. Statistical significance levels for the factor strain and dexamethasone treatment of two-factor ANOVA (weight, relative organ weight, AUC) or repeated measurements ANOVA (OGTT) are indicated for pair-wise post hoc unequal N HSD test (honestly significant difference) as follows: * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$. The symbols in 1C (OGTT) correspond to PD5 DEX vs. SHR DEX comparison.

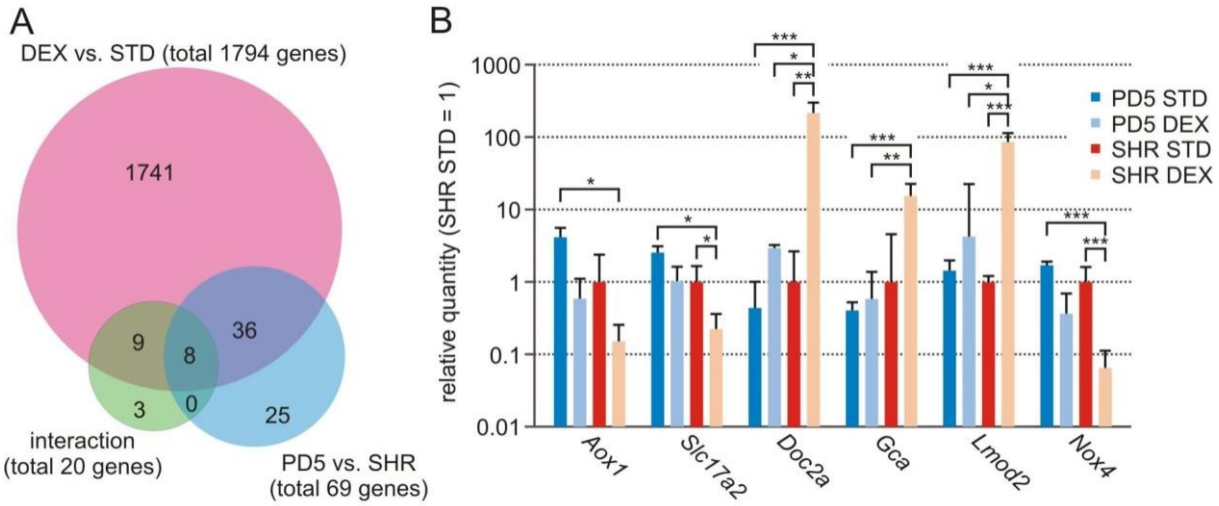

Fig.7) Hepatic transcriptome analysis. **A)** Venn diagram representing the overlapping numbers of significantly differentially expressed genes in individual comparisons – SHR vs. PD5 (blue), dexamethasone vs. standard diet (pink) and interaction between strain and dexamethasone treatment (green). Circle diameters correspond approximately to gene number on a logarithmic scale, overlapping regions are not to scale. **B)** Gene expression (qPCR) in PD5 (standard diet, dark blue; dexamethasone, light blue) and SHR (standard diet, red; dexamethasone, orange) liver tissue without intervention and after dexamethasone administration. Data are presented as arithmetic means ± SEM. Statistical significance levels for the factor strain and dexamethasone treatment of two-factor ANOVA are indicated for pair-wise post hoc unequal N HSD test (honestly significant difference) as follows: * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$.

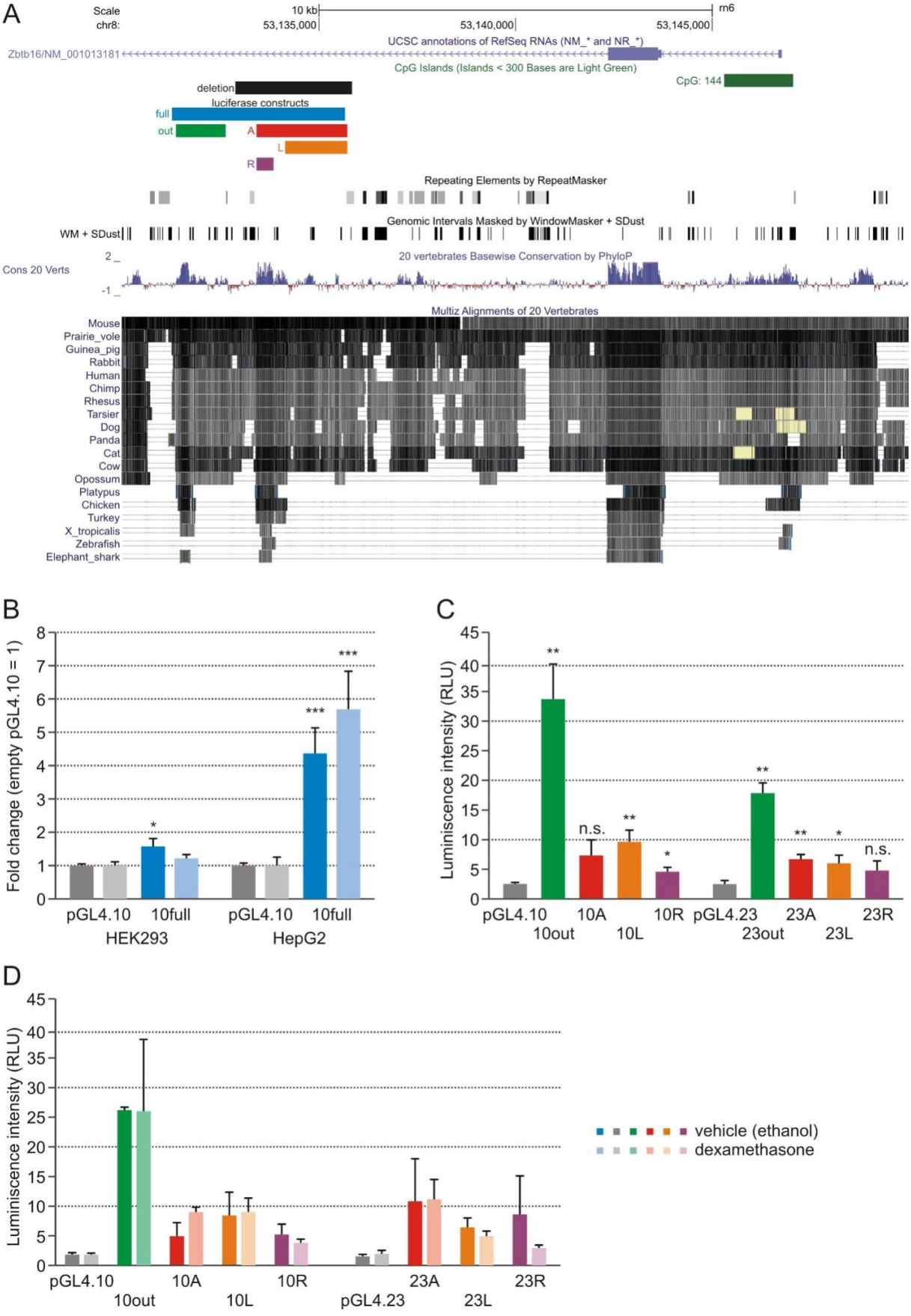

Fig.8) Luciferase reporter assay. **A)** Scheme of Plzf constructs used in luciferase reporter assay – A (red), L (orange), R (purple), out (green) and full (blue), and their respective relation to the *Plzf* sequence. Recombinant

constructs “A”, “L” and “R” span the intronic deletion of the *Plzf* gene. Construct R corresponds to the most conserved region of the intronic deletion, while construct L is congruent with the less conserved part of the *Plzf* deletion. Construct A correlates with the complete deleted sequence. Segment “full” spans the whole intronic deletion as well as the most conserved sequence 5´upstream of the intronic deletion; this part is also represented by the construct out. **B)** Intensities of firefly luminescence signal normalized to renilla luminescence signal. Empty vector (pGl4.10) resp. full vector ((pGl4.10 resp. pGl4.23 with constructs out, A, L, R) were transfected into HepG2 or HEK293 cells with / without dexamethasone. **C)** Intensities of firefly luminescence signal normalized to renilla luminescence signal. Empty vector (pGl4.10; pGl4.23) resp. full vector ((pGl4.10 resp. pGl4.23 with constructs out, A, L, R) were transfected into HepG2 cells. **D)** Intensities of firefly luminescence signal normalized to renilla luminescence signal. Empty vector (pGl4.10 resp. pGl4.23) resp. full vector ((pGl4.10 resp. pGl4.23 with constructs out, A, L, R) were transfected into HepG2 cells with / without dexamethasone.

Data are presented as arithmetic means ± SEM. Statistical significance levels of vector with construct (out, A, L, R) compared to empty vector (pGl4.10; pGl4.23) are indicated for post hoc Student's T test as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. – non significant. The luminescence signal for each vector was measured 3-times (Fig. B+D) and 4-times (figure C). All comparisons in Fig. 8D are non significant.

Discussion

Mounting evidence indicates an indisputable role of *Plzf* in the development of metabolic syndrome as well as highlighting its importance in the pathophysiology of every metabolic syndrome feature. The experiments that we conducted in this thesis are in line with this observation, underlining the pleiotropic character of *Plzf* across two tissue types - liver and heart tissue. Previous studies from our study group identified an intronic deletion of Plzf gene with pleiotropic metabolic and morphologic consequences such as alleviation of systolic hypertension, together with improvement of insulin resistance and lipidogram profile.

In our first experiment we performed comparative transcriptomic profiling of myocardial tissue between SHR and PD5 strain in order to dissect the genetic background responsible for attenuated myocardial fibrosis and metabolic syndrome features in PD5 strain which harbors the above mentioned intronic Plzf deletion and to dissect the transcriptomic pattern in relation to the Plzf gene. Promisingly, we identified overexpression of nuclear receptors NR4a1 in PD5 strain on both transcriptomic and proteomic level. There is a plethora of evidence showing a positive effect of NR4a1 on myocardial fibrosis involving a wide range of molecular and physiological mechanisms. Although the mitigated myocardial fibrosis in PD5 may be secondary to lower systolic blood pressure, we speculate that because of the pleiotropic functionality of NR4a1 the transcriptional changes in NR4a1 may at least to some extent be primary. Since the Plzf is transcriptional repressor our findings may indicate that the NR4a1 may well be under either direct or indirect repressor effect of Plzf.

In the next project we focused on nutrigenomic analysis of PD strain. PD is an established model of insulin resistance and metabolic syndrome. We compared the morphometric and metabolic parameters among PD, SHR and BN strains after a high fat diet. The most pronounced biochemical difference was markedly elevated TAG levels in PD rats compared to both SHR and BN. Interestingly, this hypertriglyceridemia was not accompanied by hepatic steatosis. PD is more prone to hypertriglyceridemia and insulin resistance than SHR, but unlike SHR is normotensive. The transcriptomic data revealed downregulation of *Acsm3* gene encoding a short to medium chain (isobutyrate and butyrate preferring) acyl-CoA synthetase in PD strain which was confirmed on proteomic level. We may suspect that lack of *Acsm3* expression may contribute to observed elevated TAG levels and insulin resistance deterioration after a high fat diet in PD strain.. PD harbors the same intronic *Plzf* deletion that is transferred to PD5 congenic strain; however, PD has a different genetic background than SHR or BN, with multiple functional genetic differences, so the resulting observed biochemical and transcriptomic differences among strains could not be put down to direct effect of *Plzf*. In our third experiment we focused on the functional characteristics of the *Plzf* intronic deletion. We observed only a mitigated effect of dexamethasone on aggravation of metabolic syndrome features in PD5 compared to its progenitor SHR. Since the PD5 harbors the "less-than-normal-functioning" *Plzf* with the intronic deletion we presumed that the deletion may be responsible for the mitigated effect of dexamethasone and it is still the most plausible hypothesis. As the luciferase assays indicated, the deleted Plzf segment displays intrinsic enhancer activity; however, there was no response to dexamethasone on the luciferase assays. Thus we deem that the effect of dexamethasone may be either indirect, or we were unable to uncover the effect due to the difference of the used cancer liver derived line HepG2 from the native hepatocytes. Glucocorticoid receptor is expressed in HepG2 cells (Lui WY. et al., 1993); however, the sensitivity can be lower, therefore higher dexamethasone concentration could be helpful in future. It needs to be stated here that the concentration used was already fairly high. In order to search for the possible missing interaction partners of Plzf that might be necessary for its interaction with dexamethasone, we established a GST pull down assay to capture the partners. The results of the pilot experiment demonstrated that we are able to express soluble Plzf-GST fusion proteins (both fragments and full-length), and the fusion proteins are captured by the glutathione resin; however, the data is insufficient to make any conclusion about Plzf interaction partners as yet.

Conclusion

The Plzf is a promising candidate gene in all facets of metabolic syndrome and in line with previous data we brought another evidence of its importance in development of metabolic syndrome.

The comparative transriptomic analysis in left ventricle myocardium in SHR and its minimal congenic strain PD5 harboring intronic *Plzf* deletion identified promising candidate gene *Nr4a1*, which we speculate has a protective role in the pathophysiology of cardiac hypertrophy and fibrosis and we managed to confirm the upregulation of this gene on proteomic level.

Next we performed two comparative transcriptomic analyses in two established rodent models of metabolic syndrome PD and PD5 after high fat diet admission and dexamethasone challenge respectively. We confirmed a higher sensitivity to glucocorticoid induced metabolic syndrome in PD5 strain and aggravation of metabolic syndrome features in PD strain after high fat diet admission. The transcriptomic data verified the polygenic etiology of metabolic syndrome and highlighted several dysregulated pathways. In order to fully elucidate the functional role of the *Plzf* intronic deletion we conducted luciferase assay experiments which identified a region with strong enhancer/promoter activity within a region in a close 5´vicinity of the deletion. The results also indicate an indirect glucocorticoid effect on the intronic deletion. For further proteomic studies we established Plzf-GST protein for further proteomic studies.

References:

ABOU ZIKI, Maen D.; MANI, Arya. Metabolic syndrome: genetic insights into disease pathogenesis. *Current opinion in lipidology*, 2016, 27.2: 162-171.

BROWN, Audrey E.; WALKER, Mark. Genetics of insulin resistance and the metabolic syndrome. *Current cardiology reports*, 2016, 18: 1-8.

CARMELLI, Dorit; CARDON, Lon R.; FABSITZ, Richard. Clustering of hypertension, diabetes, and obesity in adult male COX, Roger D.; BROWN, Steve DM. Rodent models of genetic disease. *Current opinion in genetics & development*, 2003, 13.3: 278-283.

COX, Jürgen, et al. Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Molecular & cellular proteomics*, 2014, 13.9: 2513-2526.

COX, Jurgen, et al. Andromeda: a peptide search engine integrated into the MaxQuant environment. *Journal of proteome research*, 2011, 10.4: 1794-1805.

CROSBY, C. M.; KRONENBERG, M. Tissue specific functions of invariant NKT cells. *Physiol Behav*, 2018, 176.1: DAVID, Galaxy. [(accessed on 8 February 2021)]; Available online: https://usegalaxy.org

DEBOER, Mark D.; FILIPP, Stephanie L.; GURKA, Matthew J. Use of a metabolic syndrome severity Z score to track risk during treatment of prediabetes: an analysis of the diabetes prevention program. *Diabetes Care*, 2018, 41.11: 2421-2430.

GARRISON, Erik; MARTH, Gabor. Haplotype-based variant detection from short-read sequencing. *arXiv preprint arXiv:1207.3907*, 2012.

GRUNDY, Scott M., et al. Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation*, 2004, 109.3: 433-438.

HALLER, H. Epidermiology and associated risk factors of hyperlipoproteinemia. *Zeitschrift fur die gesamte innere Medizin und ihre Grenzgebiete*, 1977, 32.8: 124-128.

HEBERT, Alexander S., et al. The one hour yeast proteome. *Molecular & Cellular Proteomics*, 2014, 13.1: 339-347.

HÜTTL, Martina, et al. Adverse effects of methylglyoxal on transcriptome and metabolic changes in visceral adipose tissue in a prediabetic rat model. *Antioxidants*, 2020, 9.9: 803.

CHEN, Siyu, et al. Control of hepatic gluconeogenesis by the promyelocytic leukemia zinc finger protein. *Molecular endocrinology*, 2014, 28.12: 1987-1998.

KOVALOVSKY, Damian, et al. The BTB–zinc finger transcriptional regulator PLZF controls the development of invariant natural killer T cell effector functions. *Nature immunology*, 2008, 9.9: 1055-1064.

KREN, Vladimir, et al. Genetic isolation of a region of chromosome 8 that exerts major effects on blood pressure and cardiac mass in the spontaneously hypertensive rat. *The Journal of clinical investigation*, 1997, 99.4: 577-581.

KRUPKOVÁ, Michaela, et al. Pharmacogenomic analysis of retinoic-acid induced dyslipidemia in congenic rat model. *Lipids in Health and Disease*, 2014, 13.1: 1-9.

LIN, H.-F., et al. Heritabilities of the metabolic syndrome and its components in the Northern Manhattan Family Study. *Diabetologia*, 2005, 48: 2006-2012.

LING, Charlotte; RÖNN, Tina. Epigenetics in human obesity and type 2 diabetes. *Cell metabolism*, 2019, 29.5: 1028-1044.

LIŠKA, František, et al. Deletion of a conserved noncoding sequence in Plzf intron leads to Plzf down-regulation in limb bud and polydactyly in the rat. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, 2009, 238.3: 673-684.

LIŠKA, František, et al. Downregulation of Plzf gene ameliorates metabolic and cardiac traits in the spontaneously hypertensive rat. *Hypertension*, 2017, 69.6: 1084-1091.

LIŠKA, František, et al. Plzf as a candidate gene predisposing the spontaneously hypertensive rat to hypertension, left ventricular hypertrophy, and interstitial fibrosis. *American journal of hypertension*, 2014, 27.1: 99-106.

LUI, Wing-Yiu, et al. Analysis of glucocorticoid receptors in human hepatocellular carcinoma and HepG2 cells. *Hepatology*, 1993, 18.5: 1167-1174.

MELNICK, Ari, et al. Critical residues within the BTB domain of PLZF and Bcl-6 modulate interaction with corepressors. *Molecular and cellular biology*, 2002, 22.6: 1804-1818.

NÁRAY-FEJES-TÓTH, Anikó; BOYD, Cary; FEJES-TÓTH, Géza. Regulation of epithelial sodium transport by promyelocytic leukemia zinc finger protein. *American Journal of Physiology-Renal Physiology*, 2008, 295.1: F18-F26.

OKAMOTO, Kozo; AOKI, Kyuzo. Development of a strain of spontaneously hypertensive rats. *Japanese circulation journal*, 1963, 27.3: 282-293.

Picard. [(accessed on 8 February 2021)]; Available online: http://broadinstitute.github.io/picard

PUCCI, Giacomo, et al. Sex-and gender-related prevalence, cardiovascular risk and therapeutic approach in metabolic syndrome: A review of the literature. *Pharmacological research*, 2017, 120: 34-42.

RAPPSILBER, Juri; MANN, Matthias; ISHIHAMA, Yasushi. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nature protocols*, 2007, 2.8: 1896-1906.

RILEY, Leanne, et al. The World Health Organization STEPwise approach to noncommunicable disease risk-factor surveillance: methods, challenges, and opportunities. *American journal of public health*, 2016, 106.1: 74-78.

SCHNEIDER, Caroline A.; RASBAND, Wayne S.; ELICEIRI, Kevin W. NIH Image to ImageJ: 25 years of image analysis. *Nature methods*, 2012, 9.7: 671-675.

ŠEDA, O., et al. A 14-gene region of rat chromosome 8 in SHR-derived polydactylous congenic substrain affects muscle-specific insulin resistance, dyslipidaemia and visceral adiposity. FOLIA BIOLOGICA-PRAHA-, 2005, 51.3: 53.

ŠEDOVÁ, L., et al. Rat Inbred PD/Cub Strain as a Model of Dyslipidemia and. *Folia Biologica (Praha)*, 2000, 46: 99-106.

Publications:

Publications, which serve as grounds for the PhD thesis:

Manuscript 1:

Mirchi LF, Chylíková B, Janků M, Šeda O, Liška F. Transcriptomic analysis of left ventricle myocardium in an SHR congenic line with ameliorated cardiac fibrosis. Physiol Res. 2019 Oct 25;68(5):747-755. doi: 10.33549/physiolres.934127. Epub 2019 Aug 19. PMID: 31424260.

Impact factor (2019). 1.736

Manuscript 2:

Junková K, **Mirchi LF**, Chylíková B, Janků M, Šilhavý J, Hüttl M, Marková I, Miklánková D, Včelák J, Malínská H, Pravenec M, Šeda O, Liška F. Hepatic Transcriptome Profiling Reveals Lack of *Acsm3* Expression in Polydactylous Rats with High-Fat Diet-Induced Hypertriglyceridemia and Visceral Fat Accumulation. Nutrients. 2021 Apr 25;13(5):1462. doi: 10.3390/nu13051462. PMID: 33923085; PMCID: PMC8147112.
Format:

Impact factor (2021): 6,706

Manuscript ahead of publication:

Mirchi LF, Procyková K., Chylíková B, Janků M, Vacík T., Talacko P., Šeda O., Šedová L. Pravenec M., Liška F. Plzf expression and transcriptomic footprint of dexamethasone-induced metabolic syndrome in SHR minimal congenic subline with enhanced glucose tolerance, ameliorated dyslipidemia and visceral adiposity.