The expression pattern of Notch2 and Dab1 in the postnatal kidney of Dab1-/- mice

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THE EXPRESSION PATTERN OF NOTCH2 AND DAB1 IN THE POSTNATAL KIDNEY OF DAB1 -/- MICE

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LIST OF ABBREVIATIONS

Notch ICD- Notch intracellular domain

Rbpj- recombination signal binding protein for immunoglobulin kappa J region

CBF-1- human C promoter Binding Factor

Dab1- Disabled-1 adaptor protein

Apoer2- Apolipoprotein E receptor 2

Vldlr- Very low-density lipoprotein receptor

SH2- Src Homology 2

DCT- distal convoluted tubule

PCT- proximal convoluted tubule

PGK-neocassette- Phosphoglycerate kinase neocassette

PI-PTB- Phosphotyrosine binding domain

PBS- Phosphate buffer saline

PFA- Paraformaldehyde

DAPI- 4′,6-diamidino-2-phenylindole

MAP- Mitogen-activated protein kinase

ERK- Extracellular signal-regulated kinases

Delta1- Delta-like 1

Hes1- Hairy and enhancer of split-1

LC3B- Microtubule-associated protein 1 light chain 3, isoform B

1. INTRODUCTION

1.1 The NOTCH signaling pathway

The NOTCH signaling pathway is a cell-cell communication mechanism present in all metazoans with a major role in cell identity signaling during development (1–3). All mammals have 4 *Notch* receptors labelled as *Notch 1-4* (4). *Notch*'s extracellular domain is involved in ligand binding and its intracellular domain is involved in signal transduction (2). In mammals, the ligand *jagged* binding to *Notch* receptors results in proteolytic release of the active form of *Notch- Notch ICD* (5). *Notch ICD* then translocates to the nucleus and prompts transcription of many target genes by establishing a transcriptional complex with a transcriptional factor *Rbpj* (or *CBF-1* as it is also known), that mediates canonical *Notch* signaling (5). This pathway has well understood defined roles in neurogenesis, for example cell elimination, by controlling apoptosis and dendrite morphogenesis (6,7).

1.2. NOTCH signaling and *Notch* receptors in the kidney

When referring to the kidney and its development however, previous studies have shown the importance of NOTCH signaling in nephron segmentation also (8,9). In the developing kidney, *Notch 1* and *Notch 2* are expressed, but *Notch 2* is the vital receptor for nephrogenic development without abnormalities (10). More specifically, the main role of *Notch2* receptors in nephron development is determining proximal epithelial fate. For example, overexpression of *Notch2* leads to direction of cells to fates in the proximal tubule and glomerular epithelium (11). Furthermore, in renal epithelial precursors, ablation of the *Notch2* receptor has a severe effect on renal development with loss of proximal epithelium including podocytes and proximal tubules (10,11). This is translated to humans as *Notch2* mutations specifically have been associated with an Alagille-like phenotype in patients who present with renal abnormalities, thus highlighting the importance of NOTCH signaling in specification of proximal epithelium in the kidney (11,12). With regard to kidney pathogenesis, through in vivo studies, it has been shown that *Notch* receptor activation may be a contributing factor in regeneration after acute kidney injury; however, *Notch* receptor overexpression is also causally connected with interstitial fibrosis, glomerulosclerosis, and clear renal cell carcinoma (11,13). All of the above emphasizes the role of NOTCH signaling in not only the development of the mammalian kidney, or its protective role when acute injury in fully mature nephrons occurs, but also its potential harmful effect when overexpressed.

1.3. *Disabled-1* protein, *reeler* and *yotari* mice

Disabled-1 (Dab1), a homolog of the Drosophila disabled protein, is an intracellular adaptor protein that has three main domains: C-terminal serine/threonine-rich region, N-terminal protein interaction/phosphotyrosine binding domain, and a tyrosine rich region (14). *Dab1* is located in cytoplasm and has a vital role as an adaptor protein especially associated with neuronal migration and polarization (14–16). This finding has been further supported by the *yotari* or *Dab1 -/-* mouse model. These mice are mutant neurological mice which have a phenotype that is very similar to that of *reeler* mice (17). The phenotype consists of ataxia and tremor, with *yotari* mice characteristically dying around the time of weaning (17,18). Whereas *reeler* mice are the result of mutations in the *reelin* gene, a gene important for brain maturation and development, *yotari* mice are the result of a mutation in the mouse equivalent *drosophila disabled* gene (19–21). Initially it was thought that *yotari* mice express mutated forms of *Dab1* messenger RNA but little or no *Dab1* protein in neuronal tissue (17), however, more recent literature has shown that the *yotari* mouse model does in fact still produce *Dab1* protein, although an aberrant form because it cannot be phosphorylated (22) .

1.4. The REELIN-DAB1 canonical pathway in neural tissue

The findings that *Dab1* is the product of *Reelin,* a large secreted glycoprotein, in a molecular signaling cascade in the brain, explains why *reeler* and *yotari* mice share almost identical neurological phenotypes (20). The REELIN-DAB1 canonical pathway begins with *Reelin* binding to its receptors, *Apoer2* and *Vldlr*, leading to induction of *Dab1* tyrosine phosphorylation, and subsequent tyrosine-phosphorylated *Dab1* recruitment of a variety of SH2 domain-containing proteins (19). This leads to initiation of several signaling cascades, which result in remodeling of cytoskeleton and exact positioning of neurons (14,19,23,24).

1.5. *Dab1* in the kidney

Outside of neural tissue however, *Dab1* has been confirmed to occur in rodent small intestine, mouse retina, human breast cancer, and in mouse kidneys with particular expression in podocytes and the distal convoluted tubule (DCT) (15,25–28). With regard to the kidney, expression of *Dab1* appears to be more important in the fetal period, and therefore kidney development, than in the postnatal period (28). This highlights that *Notch2* may not be the only

important signaling determinant in kidney development, but that *Dab1* may also have a less discovered, but still important role.

1.6. *Notch* receptor and *Dab1* adaptor protein interaction

More interestingly, *Notch* receptors and *Dab1* adaptor proteins have been shown to interact in other tissues to determine cell fate and specification via crosstalk between the REELIN pathway and the NOTCH pathway (20). As previously mentioned, the NOTCH pathway is known to have an important role in nephron cell determination, but also is important in several phases of neural development, cell fate specification, survival of neurons and synaptic plasticity (29,30). In Drosophila, a connection between *Dab1* adaptor proteins and *Notch* receptors was first revealed (6). When *Notch1* receptors are activated in mice it leads to the release and nuclear localization of their intracellular domains (*NICD*), facilitated by *gamma-secretase*, which ultimately regulates the transcription of target genes (20). Through biochemical studies of *reeler* mice it was shown that *Dab1* interacts downstream with *NICD* to correctly direct neuronal migration (5,7). The interaction of the REELIN and NOTCH pathways to effect neuronal migration via *Dab1* and *Notch* communication is not the only important finding, but also that their interaction plays a role in determining the radial glial characteristics of progenitor cells (7,31–34).

As *Dab1* and its downstream signaling interaction with the active form of *Notch (NICD)* are significant in both mice and humans in order to determine tissue cell fate in the brain, this interaction may potentially be apparent between *Notch2* receptors and *Dab1* adaptor proteins in mouse kidneys and deserves further investigation (5,7). As previously mentioned *Notch2* is known to be important for determination of kidney epithelial cell development, and *Dab1* has also been shown to be expressed in the DCT of the kidney, with a further emphasis on their actual signaling interplay found in podocytes (10,11,15,28). These findings suggest they may have a more central role in the kidney than previously anticipated. As the *yotari* mouse model has helped to accentuate the importance of DAB1 signaling cascades in neurons, and its possible downstream interaction with *Notch* receptors, the use of *yotari* postnatal kidney samples, along with samples from *Dab1* +/- *heterozygote* and *wildtype* +/+ mice for comparison, were chosen for this investigation.

2. OBJECTIVES

The aim of this study was to analyze the expression and localization of *Dab1* adaptor proteins and *Notch2* receptors in the nephrons of *yotari (Dab1* -/-), *heterozygote (Dab1 +/-),* and *Wildtype (Dab1 +/+)* mice in order to further develop their suggested importance not only in mammal kidneys overall, but also the significance they may have particularly in the *yotari* mouse nephron.

Hypotheses

Dab1 will be significantly positively expressed in the DCT across all genotypes, as shown by previous literature. *Dab1* and *Notch2* will be significantly positively expressed in the glomeruli of *heterozygote* and *yotari* genotypes. *Notch2* will not be significantly expressed in the PCT and glomeruli of *wildtype* samples as it is more important during development of the proximal epithelium.

3. MATERIALS AND METHODS

3.1. Ethics

The experimental protocol was approved by the Ethics Committee of the University of Split School of Medicine and conducted according to the Croatian Animal Welfare Act.

3.2. Experimental animals

Three groups of pups were observed according to their *Dab1* gene status: *yotari (Dab1* -/-), *heterozygotes (Dab1 +/-)* and *wildtype* (*Dab1 +/+)* controls. The *yotari* mice were produced by PGK-neo cassette which resulted in target disruption of the first 47 codons of the gene coding for the protein-interlacing domain (PI-PTB). *Heterozygotes* were produced by standard manipulation of blastocysts and mouse breeding. In standard polycarbonate cages at least one of each genotype of mouse were group-housed and raised. Their access to water and food was *ad libitum*, and their environment was a temperature-controlled (23±2°C) room with a 12-h light/dark cycle.

3.3. Tissue collection and immunohistochemistry

On the 4th postnatal day mice were anesthetized deeply with pentobarbital and were transcardially perfused with phosphate buffer saline (PBS, pH 7.2) and 4% paraformaldehyde (PFA) in 0.1 M PBS. The kidney samples were removed and then embedded in paraffin and cut transversely into 7 micrometer thick sections. Within each *yotari*, *heterozygote* and *wildtype* group, 4 slides consisting of 2 kidney sections were allocated per group to be further processed and analyzed. After the samples went through deparaffinization, the sections were rehydrated using ethanol and water, and then shortly rinsed with distilled water. Next, samples were heated in sodium citrate buffer (pH 6.0) for 20 min in the Epitope Retrieval Steamer, allowed to cool down at room temperature, washed in PBS, and separated with a PAP pen hydrophobic pencil. The sections were then incubated with primary antibodies after cooling to room temperature.

Rabbit polyclonal *Anti-Dab1* (ab 78200, Abcam, Cambridge, UK) was diluted 1:400 and Rabbit polyclonal *Anti-Notch2* (ab 8926, Abcam, Cambridge, UK) was diluted 1:200, both in Dako REAL antibody dilutent (Dako Denmark A/S, Glostrup, Denmark), and then applied to the sections. The primary antibodies were left overnight in a humidified chamber at room temperature, followed by rinsing the sections with PBS, where they were then incubated for 1h in a humidified chamber with secondary antibodies: Alexa Fluor 488 AffiniPure Donkey

Polyclonal Anti-Rabbit IgG (Jackson IR, 711-545-152) for *Dab1* primary antibody stained samples, and Alexa Fluor 488 AffiniPure Donkey Polyclonal Anti-Rabbit IgG (Jackson IR, 711-545-152) for *Notch2* primary antibody stained samples.

Sections were then rinsed a final time with PBS, and subsequently stained with 40,6-Diamidine-20-phenylindole dihydrochloride (DAPI), a stain specific for nuclei when using multicolour fluorescent techniques. Next, using a BX51 microscope (Olympus, Tokyo, Japan) equipped with a DP71 digital camera (Olympus), stained kidney sections were viewed and photographed. The images were then processed with CellA Imaging Software for Life Sciences Microscopy (Olympus). Three structures of the kidney sections were analyzed: glomeruli, proximal (PCT) and distal convoluted tubules (DCT) (*Figure 1*), within 15 non-overlapping fields taken at x40 objective magnification for each of the 4 slides per mouse group. Each field constituted one image. Within these microphotographs, 20 glomeruli, DCTs and PCTs were analyzed, using ImageJ software (National Institutes of Health, Bethesda, MD, USA), by counting the number of positive or immunoreactive cells and negative cells. Immunoreactive cells were determined by the colour staining intensity (green for both *Notch2* and *Dab1*) in the kidney tissue (*Figure 1*). Any form of nuclear, cytoplasmic, or membrane staining was noted as positive. The percentage of positive cells in the three kidney structures (glomeruli, PCT and DCT) was compared between the *yotari*, *heterozygote* and *wildtype* groups. No colocalization studies were carried out because rabbit antibodies (both green) were used for *Dab1* and *Notch2* staining respectively.

3.4. Statistics

For statistical analysis, a Kruskal-Wallis test was used in GraphPad (GraphPad Software, La Jolla, CA, USA) to examine differences in the 3 structures (glomeruli, PCT and DCT) between groups after testing the data distribution via a Kolmogorov-Smirnov test. The percentage of positive cells was expressed as the mean ± standard deviation (SD). Statistical significance was set at $P<0.05$.

4. RESULTS

The localization of positive expression and percentage of positive cells of *Dab1* and *Notch2* were analyzed in the PCT, glomeruli and DCT of 4 day old postnatal nephrons of *yotari, heterozygote, wildtype* samples. The percentage of positive cells between each group were then compared. *Figure 1*, shows examples of the localization and intensity of expression of *Dab1* and *Notch2* in the samples.

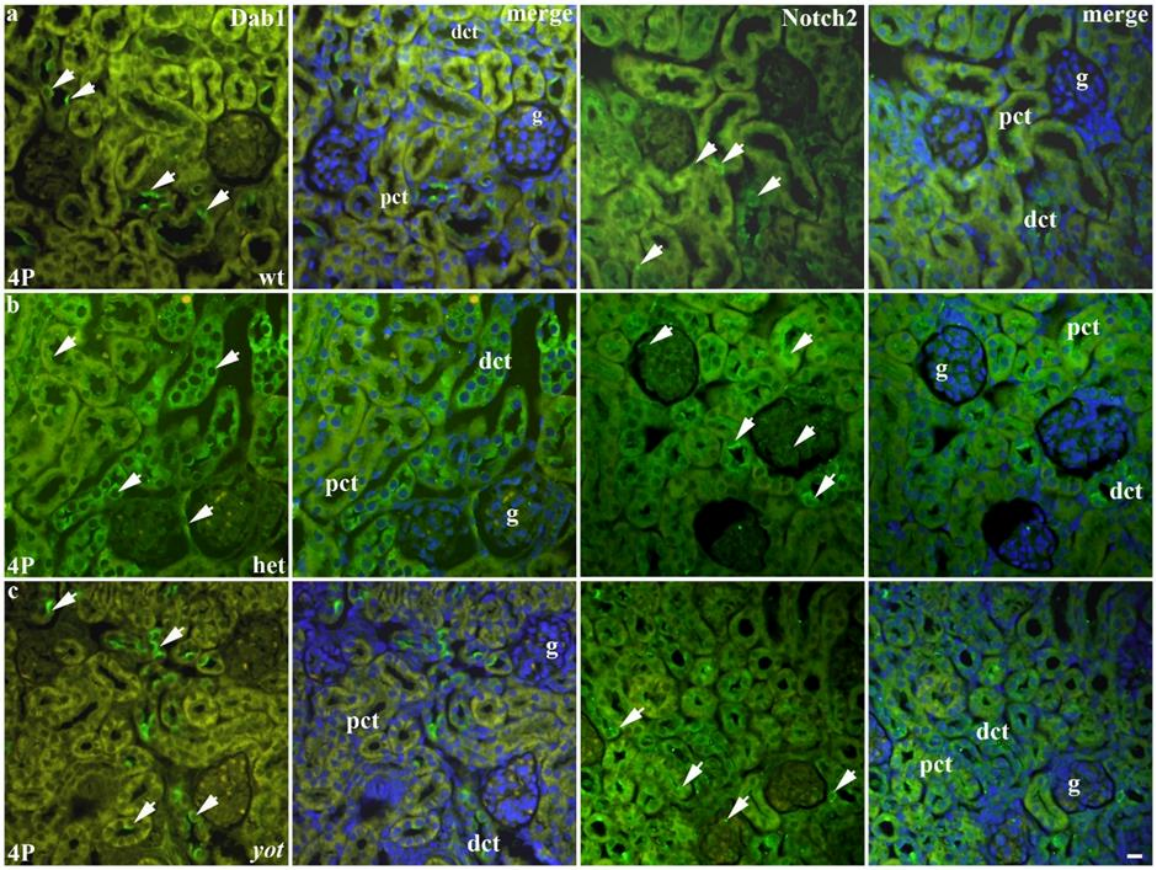

Figure 1: Double immunofluorescence of 4th postnatal day (4P) *wildtype* (wt), *heterozygote* (het) and *yotari* (yot) mouse kidney samples with *Dab1* and *Notch2* antibodies. Nuclear DNA DAPI staining merged with *Dab1* immunofluorescence in the second column and *Notch2* in the fourth column is shown in parallel to emphasize each cell type (merge). (a) In the 4P *wildtype* kidney samples, stained with *Dab1* antibodies, there was mostly strong expression on the membranes of the DCTs (arrowheads). In the 4P *wildtype* kidney samples stained with *Notch2* antibody, there was strong to intermediate expression in the cytoplasm and membrane of the DCT cells (arrowheads), intermediate staining in the cytoplasm of inconsistent PCT cells, and faint staining of the bowman's capsule of the odd glomeruli (arrowhead). (b) In the *heterozygote* kidney samples stained with *Dab1* there was strong expression of most DCT cell membranes and some of the cell cytoplasm (arrowhead), the occasional positive cell on the membrane of the PCT cells (arrowhead), and lastly, the occasional membrane of the parietal layer of the

Bowman's capsule. In the 4P *Notch2* antibody stained *heterozygote* kidney sample, there was strong expression in the cytoplasm of the DCT cells, PCT cells and in the glomeruli (arrowheads). (c) In the 4P *yotari* sample stained with *Dab1* antibodies, there was strong expression on the membranes and cytoplasm of the cells in the DCT (arrowheads), with very little positive expression in a few cells of the PCT, followed by barely any positive expression in glomeruli. In the 4P *yotari* samples stained with *Notch2* antibodies, there was intermediate expression on the membranes and in the cytoplasm of the DCT cells, some intermediate expression of the membranes of several cells of the PCT and the occasional strong signal in the glomeruli (arrowheads). The scale bar is 20 μm and refers to all images.

In the *yotari* group stained with *Dab1*, the DCT showed the highest number of positive cells at 60%, followed by the PCT with 5% and lastly by the glomeruli with 3% (*Figure 2)*. In the *heterozygote* group stained with *Dab1*, the DCT was again the structure with the highest number of positive cells at 95%, followed by the PCT with around 15%, and finally by the glomeruli with roughly 10% immunoreactive cells (*Figure 2)*. Finally, in the *Dab1* stained *wildtype* group the structure with the most immunoreactive cells was again the DCT at 30%, followed by the PCT at about 19% positive cells, and lastly the glomeruli with 2% positive cells (*Figure 2*).

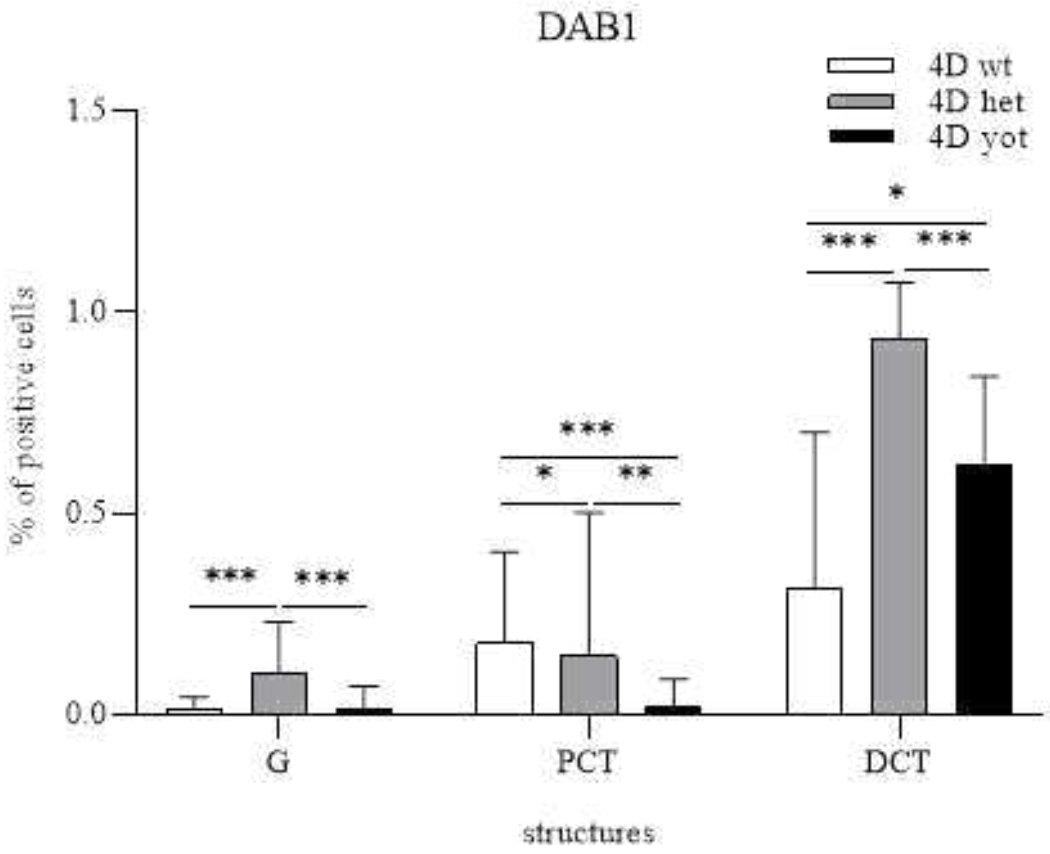

Figure 2: The distribution of percentages of DAB1 positive cells in the proximal convoluted tubules (PCT), distal convoluted tubules (DCT), and glomeruli (G) in 4th day postnatal kidneys of *wildtype* (wt), *heterozygote* (ht) and *yotari* (yot) genotypes. Data is presented as the mean ± standard deviation (SD) (vertical line). Significant differences between the PCT, DCT, and G in different genotypes are indicated by *P < 0.01, **P < 0.001, ***P < 0.0001 (Kolmogorov-Smirnov test for data distribution followed by Kruskal-Wallis test). In each genotype 20 PCT, DCT and glomeruli were assessed.

In the samples stained with *Notch2,* the *yotari* group's percentage of immunoreactive cells in the DCT was the highest at roughly 62%, followed by the PCT at about 20%, and lastly the least number of positive cells in the glomeruli at close to 18% *(Figure 3)*. In the *heterozygote* group the structure with the highest percentage of positive cells was the DCT at 98%, followed by both the PCT and glomeruli with around 95% positive cells *(Figure 3)*. Finally, in the *wildtype* samples the structure with the highest number of positive cells was the DCT with 22%, followed by the PCT with 10%, and ultimately the glomeruli with less than 1% positive cells *(Figure 3)*.

As can be seen in *Figures 2* and *3*, in all sample groups the DCT was the structure with the highest percentage of immunoreactive cells for both *Notch2* and *Dab1* (P< 0.05).

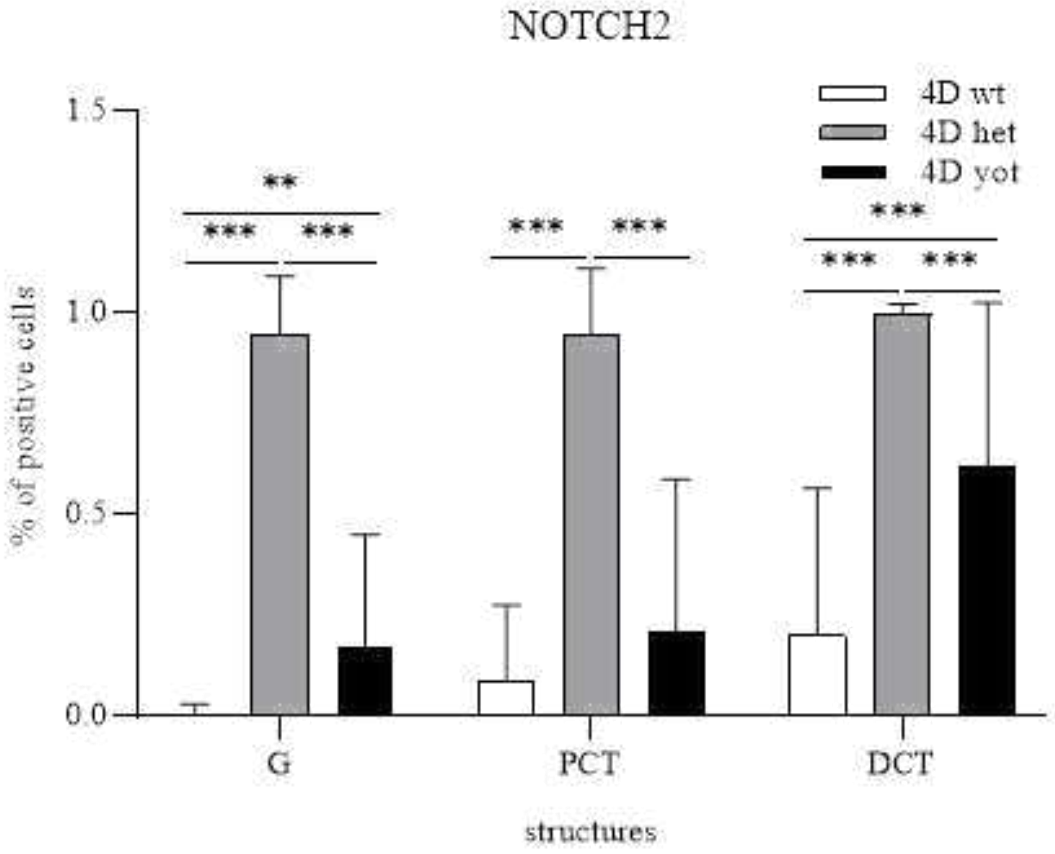

Figure 3: The distribution of percentages of *Notch2* positive cells in the proximal convoluted tubules (PCT), distal convoluted tubules (DCT), and glomeruli (G) in 4th day postnatal kidneys of *wildtype* (wt), *heterozygote* (ht) and *yotari* (yot) genotypes. Data is presented as the mean ± standard deviation (SD) (vertical line). Significant differences between the PCT, DCT, and G in different genotypes are indicated by *P < 0.01, **P < 0.001, ***P < 0.0001 (Kolmogorov-Smirnov test for data distribution followed by a Kruskal-Wallis test). In each genotype 20 PCT, DCT and glomeruli were assessed.

When comparing the percentages of positively expressed cells between groups of the *Dab1* immunofluorescence stained samples in the DCT, the *heterozygote* group had a statistically significant higher percentage of positive cells when compared against the *yotari* and *wildtype* group (P<0.001). Moreover, there was a statistically significant higher percentage of immunoreactive cells in the DCT of *yotari* mice in comparison to *wildtype* mice (P<0.05). In the PCT, there were the most statistically significant positive cells in the *wildtype* samples

than in the *yotari* groups (P< 0.001) (*Figure 2*). Next, there were more immunoreactive cells, of statistical significance, in the *heterozygote* PCT cells when compared to the *yotari* samples (P<0.01) (*Figure 2*). Lastly, as seen in *Figure 2*, there was a statistically significant higher percentage of positive cells in the PCT cells of the *wildtype* group than the *heterozygote* group (P<0.05). With regard to glomeruli, there was equally the highest percentage of positive cells with statistical significance in the *heterozygote* group compared to both *yotari* and *wildtype* groups (P<0.001) (*Figure 2*). There was no statistically significant difference between the percentage of positively expressed cells in the glomeruli of *yotari* and *wildtype* samples.

In the *Notch2* stained samples, when comparing between groups for the DCT, there was a statistically significant higher percentage of positive cells in the *heterozygote* group than in both the *wildtype* and *yotari* groups (P<0.001) (*Figure 3*). Also, as seen in *Figure 3*, there was a statistically significant greater percentage of immunoreactive cells in the *yotari* group when compared against the *wildtype* group (P<0.001). In the PCT, there was a statistically significant higher percentage of positive cells in the *heterozygote* group when compared to both the *wildtype* and *yotari* samples (P<0.001) (*Figure 3*). There was no statistically significant difference found between the *yotari* and *wildtype* group in the PCT. In the glomeruli, as seen in *Figure 3*, there was a greater percentage of immunoreactive cells with statistical significance in the *heterozygote* group than in both the *wildtype* and *yotari* samples (P<0.001). Lastly, there was also an increased percentage of positive cells with statistical significance in the *yotari* group when compared to the *wildtype* group in the glomeruli (P<0.01) (*Figure 3*).

5. DISCUSSION

The aim of this study was to determine the expression pattern of *Dab1* adaptor proteins and *Notch2* receptors in 4 day old postnatal nephrons of specifically *yotari* mice, in comparison to *heterozygote* and *wildtype* genotypes in order to further distinguish their role in kidney structure and function. After researching current literature, this study is one of the few which has looked further into the expression of *Dab1* in the kidney overall, not to mention in *yotari* mice at all. Moreover, this appears to be the only investigation into the expression of *Notch2* in the nephrons of *yotari* mice, especially when referring to the possible interaction of *Notch2* and *Dab1* in the mouse kidney.

In all mouse groups, the DCT showed the highest percentage of positive expression of *Dab1* out of all of the structures in the nephron, which supports the findings in other literature where *Dab1* was also found to be mostly expressed in the membranes of the cells of the DCT in human kidneys- particularly at different stages of embryonal development (28). As the cell membranes of the DCT have a large number of ion exchange protein channels required for fluid and ion homeostasis, it is possible that *Dab1* may activate pathways downstream, and therefore determine the expression and function of those transmembrane ion exchange proteins. In normal human kidneys it has been found that expression of *Dab1* in the DCT although still present, does decrease in expression level postpartum (28). This postnatal expression, even if decreased, of *Dab1* in the DCT of humans appears to also occur in the normal DCT of postnatal mice because *Dab1* also still showed positive expression in the normal *wildtype* genotype. Taking both of these together, the possible important signaling role of *Dab1* adaptor proteins with regard to transmembrane ion exchange proteins in the DCT, may be prominent in mature kidneys in humans and mice, and thus warrants further investigation as to which signaling cascades may be responsible for this that include *Dab1*.

At closer inspection, the *heterozygote* genotype, followed by that of *yotari* had significantly more immunoreactive cells in the DCT when compared to the *wildtype* samples with normal DCTs. Although this may seem counterintuitive, it is worth mentioning that the *heterozygote* mice have one normal copy of the DAB1 gene and one mutated copy, and although all *Dab1* proteins produced in *yotari* mice are not functional because of its inability to be phosphorylated due to genetic editing, abnormal proteins can still be expressed and therefore show immunoreaction. This correlates with continued expression of aberrant *Dab1* in the brain of *yotari* models, and suggests the use of *yotari* mouse models for determining the *Dab1* adaptor protein function in different tissues should in fact be used with caution (22). As to why there is positive expression of *Dab1* in nearly all the DCT cells in the *heterozygote* group of this study,

cannot be fully understood from this study alone, but an incidence of this has been found when looking at *Dab1* hemizygous models in the mouse brain, whereby there was a twofold increase in *Dab1* protein production in the cortical plate of the heterozygote group when compared to the *Dab1* homozygote and *wildtype* models (35). Both this study's result and that of the previous mentioned study, could be explained by a reduced rate of protein turnover because of a small decrease in the efficiency of signaling due to the presence of some aberrant *Dab1* intracellular adaptor proteins (35), but further investigation using *heterozygote* mouse models of *Dab1* are required to fully establish this hypothesis.

Different levels of expression of *Dab1* were also found in the PCT in all groups, with the highest percentage in that of the *wildtype* kidney samples. This finding highlights the role *Dab1* may have in signaling cascades of PCT cells in mice, which has also been shown to be expressed at different stages of human embryonic PCT development (28). Moreover, although this has not been anticipated yet in other literature using mouse models, *Reelin* has been found to be expressed in the PCT of the human embryonic nephron (28). As the REELIN-DAB1 signaling pathway is well established in neural tissue (19,20), the colocalization of *Reelin* and *Dab1* expression in maturing human PCTs at certain developmental weeks (28), plus the finding of *Dab1* protein expression in the current study, could implicate a possible role of a REELIN-DAB1 signaling cascade in the mouse PCT. However, *Dab1* could also be triggered due to factors other than REELIN signaling, as suggested by the fact that colocalization of *Dab1* and *Reelin* has not been confirmed during all developmental weeks in the human kidney (28). This stresses that *Dab1* can be triggered by some other factors rather than *Reelin*. Or it could be that *Dab1* itself signals other pathways altogether like the MAP kinase pathways (MAPK), such as p38MAPK and ERK (36). The expression of this pathway as a result of *Dab1* was confirmed during rat kidney growth and development, which also seems to partake in inflammation processes (36). Also, ERK seems to advocate nephrogenesis, with *p38* involved in kidney growth and nephrogenesis (36). Whether *Dab1* activation is the result of being triggered by a signaling cascade out of REELIN in the mouse PCT or *Dab1* itself initiates other known signaling pathways, this study highlighted the presence of *Dab1* intracellular adaptor proteins in mostly PCTs of normal postnatal *wildtype* and *heterozygote* mice, and very little expression in the *yotari* genotype samples which suggests the requirement of at least one functional copy of the DAB1 gene for significant expression of any kind in this structure. In a different stance, *Dab2* has been found to show expression in the mouse PCT, both adult and embryonic, where it is required for normal endocytosis (37). As *Dab1* and *Dab2* are related proteins, with similar

features of cytoplasmic adaptor proteins, such as protein binding domains, phosphorylation sites and the absence of catalytic domains, the finding of *Dab1* expression in the PCT of mouse kidneys is plausible in this sense (21,37,38). Overall, as *Dab1* is known to have an important role in signal transduction pathways of other tissues like developing neurons, it is possible that *Dab1* has a more distinguished role like *Dab2* in the kidney than previously anticipated (14,16). Thus, this discovery of *Dab1* expression in the mouse PCT is a starting point for further studies to develop.

Finally, the structure with the least percentage of immunoreactive cells across all *Dab1* stained mouse groups was the glomerulus, and almost non-existent in the *wildtype* and *yotari* genotypes. This was surprising especially considering the *wildtype* samples as previous studies have denoted the expression of *Dab1* in this structure at different stages of embryonic and postnatal development in the human kidney, along with its expression in cultured mouse podocytes (15,28). As this study only stained 4 day old mouse postnatal kidney samples, it is plausible that the importance of *Dab1* signaling in mouse nephrons is more established in glomerular development at embryonic stages and then re-expressed at more mature postnatal stages of glomerular function like shown in human kidneys, or its importance in mouse glomerular signaling for differentiation and function may be completely different entirely and therefore further study is required. Continuing on, this study looked at the expression of *Dab1* in postnatal glomeruli *in vivo,* instead of *in vitro* cultured mouse podocytes stimulated by angiotensin II in order to provoke apoptosis (15). Therefore, *Dab1* expression *in vivo* has to be taken into the context that there are a lot of different elements, both intracellular and extracellular, that could affect *Dab1* expression which are eliminated when *in vitro* studies are used, so the result of this study, where *Dab1* is not so prominently expressed could be seen as more representative.

Additionally, induction of glomerular injury in cultured podocytes (15) suggests expression of *Dab1* in the glomeruli as more of an important mediator in glomerular cell damage, like podocyte apoptosis in chronic kidney disease, rather than in normal postnatal glomerular cell function in mice. With this in mind, *Dab1* adaptor protein had very low expression in *yotari* glomeruli samples and low expression in *heterozygote* glomeruli samples, so if *Dab1,* even if in aberrant form, is looked at in the context of being a marker for glomerular damage, then the glomeruli may not be damaged in the *yotari* and *heterozygote* genotypes, and the idea that maybe *yotari* mice die as a result of kidney damage so soon after weaning is not accurate. However, further studies using electron microscopy for example are needed to look at glomerular cells and potential signs of injury, like podocyte effacement, in more detail in *yotari* and *heterozygote* genotypes in order to completely understand the integrity of their glomeruli. Moreover, other more distinguished markers of glomerular injury may be highly expressed in the glomeruli of *yotari* mice which were just not stained for in this study.

On another note, immunofluorescence staining revealed the highest percentage of positively expressed cells with *Notch2* receptors in the DCT across all genotypes. This corresponds with the highest percentage of *Dab1* immunoreactive cells being in the DCT across all samples as well, with almost matching percentages of *Notch2* and *Dab1* expression in each genotype. This high and corresponding percentage of co-occurrence of both *Dab1* adaptor protein and *Notch2* receptor positive expression in the DCT can be interpreted as possible evidence that they in fact do interact in this structure through some kind of signaling cascade, but further immunofluorescence colocalization investigation is needed in order to fully elucidate this interactive signaling relationship in the tubule structures. As mentioned previously in the introduction, downstream signaling from *Dab1*, via the REELIN pathway, to *Notch* receptors is prominent in other tissues, particularly in neural tissue during its embryonal development (5,7,31–33). Moreover, an interaction between the *Notch* receptor and its downstream signaling to *Dab1* adaptor proteins has been found to occur in the colon (39). It has been shown that coordinated activation of NOTCH signaling in colorectal cancer via RBPJ-dependent transcription of *Dab1* can promote the development of colorectal cancer (39). As can be interpreted from previous studies mentioned, whether there is downstream signaling from *Notch* to *Dab1* or vice versa, their important interaction in other tissues outside of the kidney is accepted, and therefore their similar percentages of co-expression in the mouse DCT of each genotype highlighted by this study opens to further investigation of their coordination in the DCT.

Notch2 receptor expression was also apparent in the PCT in all genotypes, but with a significantly higher percentage of expression in the *Dab1 heterozygote* group. It is known that *Notch2* receptors are required for the formation of the PCT in mice under normal circumstances (10), and then dissipates once maturation is achieved up to 7 days postpartum (11). This notion is supported by a small percentage of immunoreactive *Notch2* receptors in the PCTs of the *wildtype* genotype, as the samples are 4 days postpartum in age, and therefore nephrogenesis is near complete. However, as to why there is increased expression of *Notch2* receptors in *yotari* mice, and even more expression in the *Dab1 heterozygote* group is not as clear. If it is assumed that the *yotari* genotype and *heterozygote* do in fact have some form of PCT acute injury, the high expression of *Notch2* in the proximal tubules may be a reaction to this. This is supported by literature where *Notch2* signaling has been implicated in acute kidney injury using a rat ischemia-reperfusion injury model and cultured NRK-52E cells (40). When analysis of the whole kidney was done after injury, higher expression of *Delta1* and *Hes1* mRNA and protein, plus processed *Notch2* was seen (40). Further analysis of injured proximal tubule segments using confocal microscopy with specific antibodies showed that *Delta1*, cleaved *Notch2* and *Hes1* colocalized in the same segments (40). This mentioned study suggests that the DELTA1/NOTCH2/HES1 signaling pathway could regulate the regeneration and proliferation of renal tubules during acute kidney injury, which could be further supported by this study if the *heterozygote* and *yotari* genotype do in fact have some form of acute kidney injury affecting the proximal tubules as *Notch2* is significantly expressed, but further investigation is required to confirm this.

Although the DCT and PCT of the mouse nephron showed the presence of *Notch2* receptor expression across all genotypes, this finding was not consistent in the glomeruli. The expression of *Notch2* receptors was abundant in the glomeruli of the *heterozygote* genotype, followed by the *yotari* genotype and lastly almost nonexistent in the *wildtype* genotype. It is well known that *Notch2* expression is down-regulated when nephron maturation is achieved, except in conditions of renal injury, such as diabetic nephropathy and focal segmental glomerulosclerosis (11,41). This understood knowledge of the decreased role of *Notch2* receptors once maturation has been achieved, in an otherwise uninjured glomerulus, is further supported by this study's results where the normal postnatal *wildtype* genotype has almost no *Notch2* receptor expression in the glomerulus.

Exploring further the role of *Notch2* expression and activation in glomerular injury however, it has been elucidated that in the glomeruli of mice with adriamycin-induced nephrotic syndrome, *Notch2* receptors demonstrate a function which prevents nephrosis and loss of podocytes (42). Moreover, there are already a few findings that temporary increased *Notch2* activation is associated with a strong survival advantage for injured podocytes, but that this capacity is diminished in lasting disease models such as diabetic nephropathy (43). Therefore, the consensus that *Notch2* receptors have a signaling role in the early phase of damaged glomeruli, can also be further supported by the results of this study if the *heterozygote* and *yotari* genotypes are considered to have damaged glomeruli. This would mean excluding the lack of *Dab1* expression in the glomeruli, as previously mentioned in the above discussion, as a reflection of uninjured glomeruli and viewing *Notch2* expression as a more reliable one because of its more developed standing in literature in this field. This would also imply, that NOTCH2-DAB1 signaling interaction in the glomeruli is not of as great of importance as suggested by the positive expression showed in the DCT by this study, especially in the context of acute injury, and that other signaling cascades including *Notch2* are more substantial. As a side note, it is still unclear as to why *yotari* mice die so soon after weaning, but it could be severe glomerular injury, such as overwhelming nephrotic syndrome, with *Notch2* re-expression as a marker of this. However, further studies would need to be completed with other markers of disease process in the glomeruli, such as *LC3B* for autophagy (44), in *yotari* and *heterozygote* genotypes in order to give this hypothesis further weight.

Although this study is a sufficient starting point for *Dab1* and *Notch2* expression in the mouse kidney overall, not to mention in the unexplored *yotari* kidney, limitations should be highlighted. Firstly, co-localization of *Dab1* and *Notch2* expression within nephron structures could not be interpreted as both antibodies used were rabbit polyclonal antibodies, and therefore both green when expressed. Future immunofluorescence studies, should use different animal derived *Dab1* and *Notch2* antibodies to allow for co-localization in structures to be determined, which would mean results could more strongly support a possible *Dab1* and *Notch2* interaction. Secondly, only four prepared slides, each from a separate sacrificed mouse, with two kidney samples on each slide was used, which possibly limits the power of the results. Future investigations should include samples taken from more animals in order to avoid possible type II error. Lastly, only one investigator analyzed the images when counting positive cells, increasing the likelihood of human error and decreasing reproducibility. Future studies could negate this limitation by using at least three independent investigators to analyze the images.

To conclude, a double immunohistochemistry study of *Notch2* receptor and *Dab1* adaptor protein expression in 4 day old postnatal nephrons of *yotari, heterozygote* and *wildtype* mouse genotypes was carried out. The results showed that the DCT had prominent expression of *Dab1* across all genotypes, suggesting a possible important role of *Dab1* in DCT transmembrane ion exchange proteins important for fluid and ion homeostasis. Also, similar corresponding percentages of *Notch2* receptor expression in the DCT, implicates a possible interaction between the two in DCT cellular signaling, but co-localization studies are needed to further elucidate this. Interestingly, with regard to the PCT, *Dab1* was found to be expressed within *wildtype* and *heterozygote* samples and not so prominently in the *yotari* group. This implicates *Dab1* in proximal cell fate and determination in the mouse nephron under normal circumstances, but implies *yotari* mice cannot express *Dab1,* be it aberrant or not, in this structure for unknown reasons. Continuing on, decreased *Dab1* expression in the *wildtype* glomeruli further supported the role of *Dab1* adaptor proteins mainly in embryonic development of the glomeruli. Decreased *Dab1* glomerular expression in *yotari* samples was also seen, perhaps as an indication of normal glomeruli in *yotari* mice, but it is more likely they are injured because *Notch2* was significantly expressed in *yotari* glomeruli and is considered a more reliable marker of acute injury in this structure. This could be an indication that *yotari* animals may develop some type of glomerular injury, like nephrotic syndrome, as a potential cause of death during the weaning period. But to confirm these suggestions, it is necessary to perform further investigation of *yotari* mice since actual mechanisms leading to their death are still unknown. Furthermore, low to nonexistent expression of *Notch2* receptors in the PCT and glomeruli of the *wildtype* genotype reaffirmed that *Notch2* can be considered more important in embryological development of these structures, but also high *Notch2* expression in the *heterozygote* and substantial expression seen in *yotari* samples proposes that there may be PCT injury in these genotypes. Overall, *yotari* mice show significant *Dab1* expression in the DCT and *Notch2* receptor expression in the PCT, DCT and glomerulus.

6. CONCLUSIONS

1. The DCT had prominent cell membrane expression of *Dab1* across all genotypes.

2. Similar consistent percentages of *Dab1* adaptor protein and *Notch2* receptor expression in the DCT, implicates a possible signaling interaction between them.

3. In the PCT, *Dab1* was expressed within *wildtype* and *heterozygote* samples and not so prominently expressed in the *yotari* group.

4. Decreased *Dab1* expression in *wildtype* glomeruli further supported the role of *Dab1* adaptor proteins in embryonic development of the glomeruli.

5. Decreased *Dab1* glomerular expression in *yotari* samples implies normal glomeruli in *yotari* mice, but it is more likely they are injured because *Notch2* was significantly expressed in *yotari* glomeruli and is considered a more reliable marker of acute injury in this structure.

6. Low expression of *Notch2* receptors in the PCT and glomeruli of the *wildtype* genotype reaffirmed that *Notch2* can be considered more important in mouse embryological development.

7. Substantial *Notch2* expression in the *heterozygote* and *yotari* samples proposes PCT injury.

8. Overall, *yotari* mice showed significant *Dab1* adaptor protein expression in the DCT and variable *Notch2* receptor expression in the PCT, DCT and glomerulus.

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8. SUMMARY

Objectives: The expression and localization of *Dab1* adaptor proteins and *Notch2* receptors in the nephrons of *yotari* (*Dab1* -/-), *heterozygote* (*Dab1* +/-), and *wildtype* (*Dab1* +/+) mice was analyzed to further develop their suggested importance in mammal kidneys overall, but also their significance particularly in *yotari* mice nephrons.

Materials and methods: *yotari, heterozygote* and *wildtype* mice were sacrificed on the 4th postnatal day. Paraffin embedded kidney tissue sections were analyzed by immunofluorescence using the antibodies, *Notch2 and Dab1*. Kidney structures were examined by fluorescence microscope. The percentage of positive cells between each group were compared and analyzed by a Kruskal-Wallis test.

Results: In the DCT of all genotypes, a strong *Dab1* expression signal was seen mostly to be localized in the cell membranes and was the structure with the highest percentage of immunoreactive cells for both *Notch2* and *Dab1* ($P<0.05$). The highest percentage of positive cell expression for *Notch2* was in the *heterozygote* genotype for all structures ($P<0.05$). In *Dab1* immunofluorescence samples, the highest percentage of immunoreactive cells was observed in the DCT and glomeruli of *heterozygotes* and in the PCT of the *wildtype* genotype ($P<0.05$). Minimal positive cell expression in the *yotari* PCTs, and glomeruli of *yotari* and *wildtype* samples stained with *Dab1* antibodies was found. There was minimal positive expression in the *Notch2* stained *wildtype* glomeruli and PCT.

Conclusions: The expression patterns of *Dab1* and *Notch2* in the nephron structures of the three genotypes of mice implicate not only the potential importance of *Dab1* in DCT cellular signaling cascades for fluid and electrolyte homeostasis, but also suggests the possible signaling interaction of *Dab1* and *Notch2* in this structure. Overall, the *yotari* nephron showed significant DCT *Dab1* expression, and variable *Notch2* expression in all structures, with *Notch2* expression in the glomeruli implicating glomerular injury as a possible cause of death in these knockout mice.

9. CROATIAN SUMMARY

Naslov: PRIKAZ IZRAŽAJA NOTCH2 I DAB1 U POSTNATALNOM BUBREGU DAB1 -/- MIŠEVA

Ciljevi: Analizirali smo izražaj i lokalizaciju Dab1 adapterske bjelaničevine i Notch2 receptora u nefronima miševa yotari (Dab1 - / -), heterozigota (Dab1 +/-) i divljeg tipa (Dab1 +/+) kako bi se dalje utvrdio njihov predloženi značaj u bubrezima sisavaca općenito, ali i njihov značaj posebno u nefrona yotari miševa.

Materijali i metode: yotari, heterozigoti i divlji miševi žrtvovani su četvrtog postnatalnog dana. Parafinski rezovi bubrežnog tkiva analizirani su imunofluorescencijom pomoću protutijela, Notch2 i Dab1. Bubrežne strukture ispitivane su fluorescencijskim mikroskopom. Postotak pozitivnih stanica između svake skupine uspoređen je i analiziran Kruskal-Wallisovim testom.

Rezultati: U DCT svih genotipova imali smo snažni izražaj *Dab1* signala koji je uglavnom lokaliziran u staničnim membranama i bio je struktura s najvećim postotkom imunoreaktivnih stanica i za *Notch2* i *Dab1*. Izražaj *Notch2* imao je najveći postotak pozitivnih stanica bubrega u heterozogotnom genotipu za sve strukture. Imunofluorescencijski zražaj *Dab1* imao je najveći postotak imunoreaktivnih stanica zabilježen u DCT i glomerulama heterozigota i u PCT miševa divljeg tipa. Minimalni pozitivn izražaj *Dab1* nađen je u yotari miševa u PCT i glomerulima uzoracima divljeg tipa. Minimalan pozitivan izražaj *Notch2* nađen je u glomerulima i u PCT divljeg tipa.

Zaključci: Izražaj *Dab1* i *Notch2* u nefronskim strukturama tri genotipa miševa implicira ne samo potencijalnu važnost *Dab1* u DCT staničnim signalnim kaskadama za homeostazu tekućina i elektrolita, već također sugeriraju moguću signalnu interakciju *Dab1* i *Notch2* u ovim strukturama. Sveukupno, yotari nefron pokazao je značajan izražaj *Dab1* u DCT i promjenjiv *Notch2* izražaj u svim strukturama, gdje *Notch2* izražaj u glomerulima implicira glomerularnu ozljedu kao mogući uzrok smrti kod ovih knockout miševa.

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