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Diploma Thesis

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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 \textit{Dab1} may also have a less discovered, but still important role.

1.6. \textit{Notch} receptor and \textit{Dab1} adaptor protein interaction

More interestingly, \textit{Notch} receptors and \textit{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 \textit{Dab1} adaptor proteins and \textit{Notch} receptors was first revealed (6). When \textit{Notch1} receptors are activated in mice it leads to the release and nuclear localization of their intracellular domains (\textit{NICD}), facilitated by \textit{gamma-secretase}, which ultimately regulates the transcription of target genes (20). Through biochemical studies of \textit{reeler} mice it was shown that \textit{Dab1} interacts downstream with \textit{NICD} to correctly direct neuronal migration (5,7). The interaction of the REELIN and NOTCH pathways to effect neuronal migration via \textit{Dab1} and \textit{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 \textit{Dab1} and its downstream signaling interaction with the active form of \textit{Notch} (\textit{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 \textit{Notch2} receptors and \textit{Dab1} adaptor proteins in mouse kidneys and deserves further investigation (5,7). As previously mentioned \textit{Notch2} is known to be important for determination of kidney epithelial cell development, and \textit{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 \textit{yotari} mouse model has helped to accentuate the importance of DAB1 signaling cascades in neurons, and its possible downstream interaction with \textit{Notch} receptors, the use of \textit{yotari} postnatal kidney samples, along with samples from \textit{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 \textit{Dab1} gene status: \emph{yotari} (\textit{Dab1} \textit{-/-}), \emph{heterozygotes} (\textit{Dab1} +/-) and \emph{wildtype} (\textit{Dab1} +/-) controls. The \emph{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). \emph{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 \emph{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 4\textsuperscript{th} 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 \emph{yotari}, \emph{heterozygote} and \emph{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 \textit{Anti-Dab1} (ab 78200, Abcam, Cambridge, UK) was diluted 1:400 and Rabbit polyclonal \textit{Anti-Notch2} (ab 8926, Abcam, Cambridge, UK) was diluted 1:200, both in Dako REAL antibody diluent (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 \textit{Dab1} and \textit{Notch2} were analyzed in the PCT, glomeruli and DCT of 4 day old postnatal nephrons of \textit{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 \textit{Dab1} and \textit{Notch2} in the samples.

\textbf{Figure 1:} Double immunofluorescence of 4th postnatal day (4P) \textit{wildtype} (wt), \textit{heterozygote} (het) and \textit{yotari} (yot) mouse kidney samples with \textit{Dab1} and \textit{Notch2} antibodies. Nuclear DNA DAPI staining merged with \textit{Dab1} immunofluorescence in the second column and \textit{Notch2} in the fourth column is shown in parallel to emphasize each cell type (merge). (a) In the 4P \textit{wildtype} kidney samples, stained with \textit{Dab1} antibodies, there was mostly strong expression on the membranes of the DCTs (arrowheads). In the 4P \textit{wildtype} kidney samples stained with \textit{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 \textit{heterozygote} kidney samples stained with \textit{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*).
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.
7. REFERENCES


35. Herrick TM, Cooper JA. High affinity binding of Dab1 to Reelin receptors promotes normal positioning of upper layer cortical plate neurons. Mol Brain Res. 2004;126(2):121–8.


**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.


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 bubregu 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 pozitivni 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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