Pathways to proteinuria

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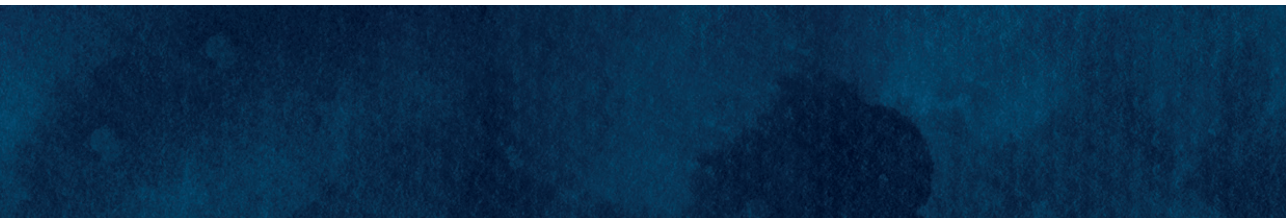

CHAPTER 3

Glomerular permeability is not affected by heparan sulfate glycosaminoglycan deficiency in zebrafish embryos

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Abstract

Proteinuria develops when specific components in the glomerular filtration barrier have impaired function. Although the precise components involved in maintaining this barrier have not been fully identified, heparan sulfate proteoglycans are believed to play an essential role in maintaining glomerular filtration. Although *in situ* studies showed that a loss of heparan sulfate glycosaminoglycans increases the permeability of the glomerular filtration barrier, recent studies using experimental models showed that podocyte-specific deletion of heparan sulfate glycosaminoglycan assembly does not lead to proteinuria. However, tubular reabsorption of leaked proteins might have masked an increase in glomerular permeability in these models. Furthermore, not only podocytes, but also glomerular endothelial cells are involved in heparan sulfate synthesis in the glomerular filtration barrier. Therefore, we investigated the effect of a global heparan sulfate glycosaminoglycan deficiency on glomerular permeability.

We used a zebrafish embryo model carrying a homozygous germline mutation in the *ext2* gene. Glomerular permeability was assessed with a quantitative dextran tracer injection method. In this model, we accounted for tubular reabsorption. Loss of anionic sites in the glomerular basement membrane was measured using polyethyleneimine (PEI) staining.

Although mutant animals had significantly fewer negatively charged areas in the glomerular basement membrane, glomerular permeability was unaffected. Moreover, heparan sulfate glycosaminoglycan–deficient embryos had morphologically intact podocyte foot processes.

Glomerular filtration remains fully functional despite a global reduction of heparan sulfate.

Keywords: glomerular basement membrane, glomerular filtration barrier, heparan sulfate, zebrafish, proteinuria

Introduction

Proteinuria is associated with a wide variety of renal diseases. Proteinuria is an independent risk factor for loss of renal function, renal failure, and cardiovascular mortality.(1, 18) Proteinuria occurs when proteins pass the glomerular filtration barrier (GFB) and are not fully reabsorbed by tubular epithelial cells; proteinuria can also develop when the reabsorption system is saturated. The GFB is comprised of fenestrated endothelial cells covered by a glycocalyx, the glomerular basement membrane (GBM), and podocyte foot processes.(19) The GFB filters molecules based on size and charge.(20)

The GFB's charge selectivity has long been attributed primarily to heparan sulfate proteoglycans (HSPGs) in the GBM.(8) HSPGs consist of a core protein to which heparan sulfate glycosaminoglycan (HS-GAG) chains are covalently attached. The HS-GAG chain is polymerized by enzymes encoded by the *EXT1* and *EXT2* genes. A defect in either is considered to be sufficient severely disrupt HS-GAG synthesis. Both during and after polymerization, the chain can be modified by several sulfotransferases and an epimerase, resulting in the addition of sulfate groups, which give the HS-GAGs and thus the whole HSPG a negative net charge.(9, 21)

HS-GAGs have been considered to be essential for GFB permeability as far back as 1980, when Kanwar *et al.* showed that enzymatic removal of HS-GAGs resulted in increased GBM permeability.(7, 8) These early results were supported by the finding that HSPG expression is reduced in several proteinuric renal diseases.(22) However, recent findings in various mouse models have challenged the notion that HS-GAGs are essential for maintaining GFB permeability; these models include podocyte-specific knockouts of perlecan and agrin (two specific types of HSPGs),(23, 24) *Ext1*,(25) *Extl3*,(26) and *Ndst1* (an enzyme involved in GAG sulfation).(27) . Although most of these models have a loss of anionic charge in the GBM, they do not develop significant proteinuria. However, HSPGs are also synthesized by mesangial cells and by glomerular endothelial cells.(28, 29) HSPGs produced by glomerular endothelial cells have been shown to be important for GFB function, as removal of HS-GAG from glomerular endothelial cells results in increased permeability to albumin in *in vitro* experiments.(29) The effects of an *in vivo* global HS-GAG deficiency on glomerular permeability remains unknown. Thus, the primary goal of this study was to investigate the effect of a global (in contrast to a podocyte-specific) HS-GAG deficiency on glomerular permeability. In addition, although various experimental models using podocyte-specific HS-GAG deletion do not develop proteinuria, they may have increased glomerular permeability. Indeed, Chen *et al.* hypothesized that a relatively moderate increase in tubular reabsorption may

prevent proteinuria despite reduced glomerular permeability.(25) To test this hypothesis, we performed quantitative analyses using a zebrafish embryo model in which we could account for tubular reabsorption capacity.

Materials and methods

Animals

Zebrafish (*Danio rerio H)* were maintained as described by Westfield (1995). Embryos were collected from natural crosses and kept at 28.5°C in E3 medium. Wild-type (WT) AB/TL strain zebrafish embryos and *dackel* mutant (*dak/ext2to273b*, referred to as "*dak/ext2*") zebrafish embryos were used for this study. Homozygous *dak/ext2* mutants contain a biallelic premature stop codon in the *ext2* gene. This mutation globally reduces zygotic *ext2* expression in homozygous *dak/ext2* mutants, resulting in impaired HS-GAG chain polymerization. Thus, HSPGs in homozygous mutants have truncated, functionally impaired HS-GAG side chains that are neither sulfated nor negatively charged.(9) This model has been characterized previously with respect to HSPG distribution.(21, 30-32) The total decrease of HS-GAGs in *dak/ext2* zebrafish embryos has previously been reported to be over 80%.(21, 30)

Homozygous *dak/ext2* mutants were sorted at 3 days post-fertilization (dpf) based on cranofacial, ear, and fin phenotypes as described previously.(33, 34) Embryos from separate crosses were used for each experiment.

All experiments were performed on embryos prior to the free-feeding stage and therefore did not fall under the animal experimentation law in accordance with to EU Animal Protection Directive 2010/63/EU.

Electron microscopy

WT and homozygous *dak/ext2* embryos at 3 and 5 dpf were anesthetized with 4% tricaine methanesulfonate (4 mg/ml), chemically fixed for one hour in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), rinsed twice with 0.1 M sodium cacodylate, incubated for 1 hour in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated sequentially in 70%, 80%, 90%, and 100% ethanol, and then immersed in 1:1 propylene oxide:epon LX-112 solution for 1 hour. The fish were then washed, infiltrated with pure epon for 2 hours, embedded in epon LX-112, and polymerized at 60°C for 2 days.

Ultrathin sections (100 nm) were mounted on copper slot grids (Storck Veco B.V., Eerbeek, The Netherlands), covered with formvar film and carbon layer, and then stained

with an aqueous solution of 7% uranyl acetate for 20 minutes, followed by Reynold's lead citrate for 10 minutes. Specimens were imaged at an acceleration voltage of 120 kV using a Tecnai 12 BioTWIN transmission electron microscope (FEI, Eindhoven, The Netherlands), equipped with a FEI 4k Eagle CCD camera. Virtual slides of zebrafish glomeruli were recorded at 18500x magnification, corresponding to a 1.2 nm pixel size at the specimen level, using automated data acquisition and stitching software.(35)

Loss of anionic sites in the GBM

The number of anionic sites in the GBM was measured using polyethylenimine (PEI) staining as described previously.(36, 37) Specifically, we measured the number of PEI particles per µm of GBM. PEI is a cationic polymer that binds to anionic sites. It was used a surrogate marker for HS-GAG, as HS-GAG are highly sulfated and therefore negatively charged. PEI is not specific to HS-GAG, as it binds to all anionic sites. Two samples each of homozygous *dak/ext2* and WT embryos were incubated in 1% PEI (PEI-600, Polysciences, Warrington, PA) in 0.1 M sodium cacodylate buffer (pH 7.4) for 6 hours with constant agitation. The samples were washed after incubation and subsequently fixed with 2% phosphotungstic acid and 1% glutaraldehyde. The samples were then stained with 1% osmium tetroxide and embedded in epon LX-112. A selection of representative images containing at least 13 µm of GBM was analyzed using transmission electron microscopy.

Foot process width

Foot process width was analyzed using a selection of representative EM images obtained from three different homozygous *dak/ext2* mutants and three different WT embryos. All images contained at least 9 µm of contiguous GBM. The formula $\frac{\pi}{4} * \frac{\Sigma \text{GBMlength}}{\Sigma \text{foot processes}}$ was used to calculate foot process width.(38)

Assessing glomerular permeability

The quantitative dextran tracer injection method (adapted from the qualitative method described by Ebarasi *et al.*(39)) was used to measure glomerular permeability. This method is not used for an assessment of charge- or size-selectivity, but for analyzing functionally significant alterations to the global permeability of the GFB. A total of 32 homozygous *dak/ext2* and 35 WT embryos were used for four experiments as follows: at 5 dpf, the zebrafish embryos were anesthetized with 4% tricaine methanesulfonate (4 mg/ml), injected intravenously with 1 nl of a mixture containing FITC-labeled lysine-fixable 70-kDa dextran (25 mg/ml; Invitrogen, Waltham, MA) and TRITC-labeled 3-kDa dextran (100 mg/ml; Invitrogen). As a positive control, WT zebrafish embryos

were also injected with puromycin aminonucleoside (PAN, Sigma-Aldrich, St. Louis, MO) at 4 dpf.(40) One hour after the dextrans were injected, the samples were fixed in 10% formalin for 24 hours and stored in 70% ethanol until further processing. The samples were then embedded in paraffin, sectioned at 4-μm thickness, and examined using immunofluorescence microscopy. The samples were analyzed in a blinded manner.

Under physiological conditions, 3-kDa dextran can readily pass the GFB, whereas 70-kDa dextran does not. If the integrity of the GFB is sufficiently compromised, the 70-kDa dextran can pass the GFB, after which it is reabsorbed by proximal tubule epithelial cells in endosomes. Therefore, we measured the number of 70-kDa dextran particles in the proximal tubule cells as a measure of glomerular permeability. To confirm functional tubular reabsorption, we also measured the number of 3-kDa dextran particles in the proximal tubule cells.

Statistical analyses

Statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY). The proportion of zebrafish embryos with pericardial edema was analyzed using Fisher's exact test. Dextran tracer measurements of glomerular permeability, foot process width, and the presence of anionic sites were analyzed using the Student's unpaired *t*-test. Differences with a p-value <0.05 were considered significant.

Results

The dackel mutant phenotype

Homozygous *dak/ext2to273b* (referred to hereafter as simply "*dak/ext2")* mutants develop a clear phenotype, as described before.[15] Consistent with previous reports, compared to wild-type (WT) embryos, mutant embryos lacked pectoral fins and had a protruding jaw, a non-inflated swim bladder, and a more concave body shape.(34, 36) In addition, homozygous *dak/ext2* mutants had a significantly higher prevalence of pericardial edema compared to WT embryos (31/34 versus 1/140, respectively; $p<0.0001$). An example of morphological changes and pericardial edema in a mutant embryo is shown in Figure 1.

Loss of anionic sites in the GBM

Because HS-GAGs are believed to be the primary contributor to the negative charge in the GBM(7), we expected that the loss of HS-GAGs in the homozygous *dak/ext2* mutants would result in reduced numbers of negatively charged sites in the GBM. We therefore tested this hypothesis using polyethylenimine (PEI) staining;(41) PEI molecules bind to

negatively charged sites and form electron-dense deposits. Consistent with our hypothesis, the mean number of electron-dense deposits per μm of GBM was significantly lower in homozygous *dak/ext2* mutants compared to WT embryos (0.80, SD 0.39, $n = 2$ versus 2.2, SD 0.72, $n = 2$, respectively; $p<0.05$) (Figure 2).

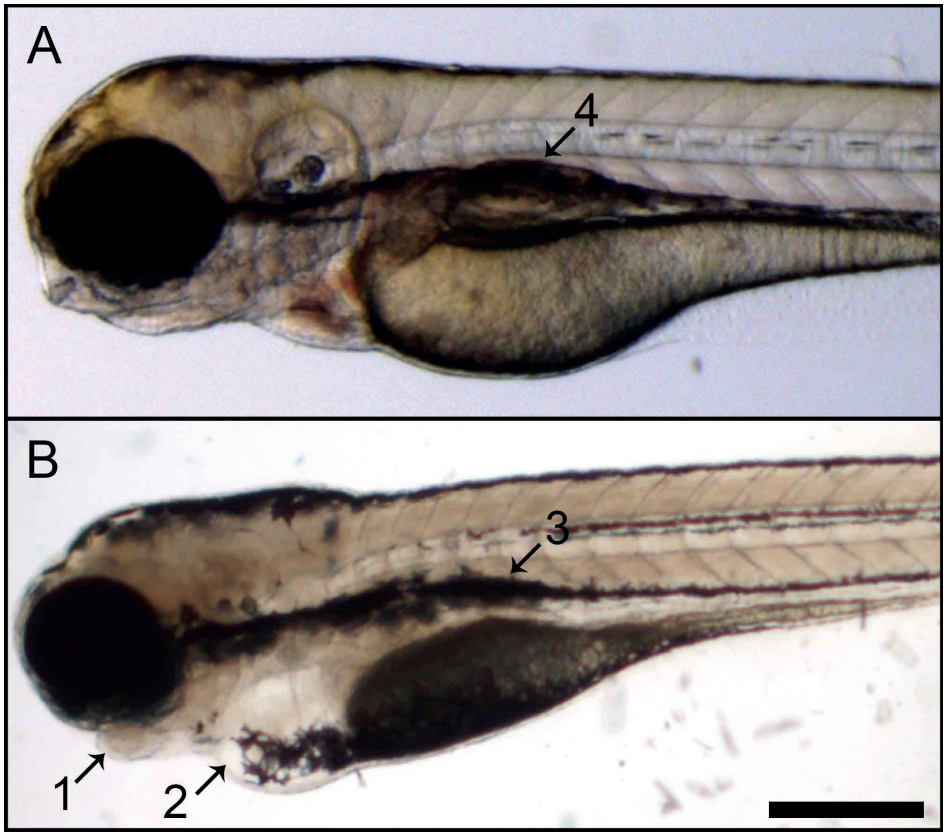

Figure 1. Homozygous dak/ext2 mutant zebrafish develop morphological changes
Shown are examples of a wild-type (A) and homozygous *dak/ext2* (B) embryo at 5 dpf, with several morphological changes in the mutant embryos. Arrows 1, 2, and 3 indicate a protruding jaw, pericardial edema, and a non-inflated swim bladder, respectively; arrow 4 shows a normally developed swim bladder in the WT embryo. Scale bar = 250 μm.

Foot process width

Ultrastructural analysis of the GFB using transmission electron microscopy revealed normal foot processes (Figure 2) in homozygous *dak/ext2* mutants. The mean width of foot process did not differ significantly between homozygous *dak/ext2* mutants and WT embryos (0.181. SD 0.025, $n = 3$ versus 0.176, SD 0.012, $n = 3$; $p=0.76$).

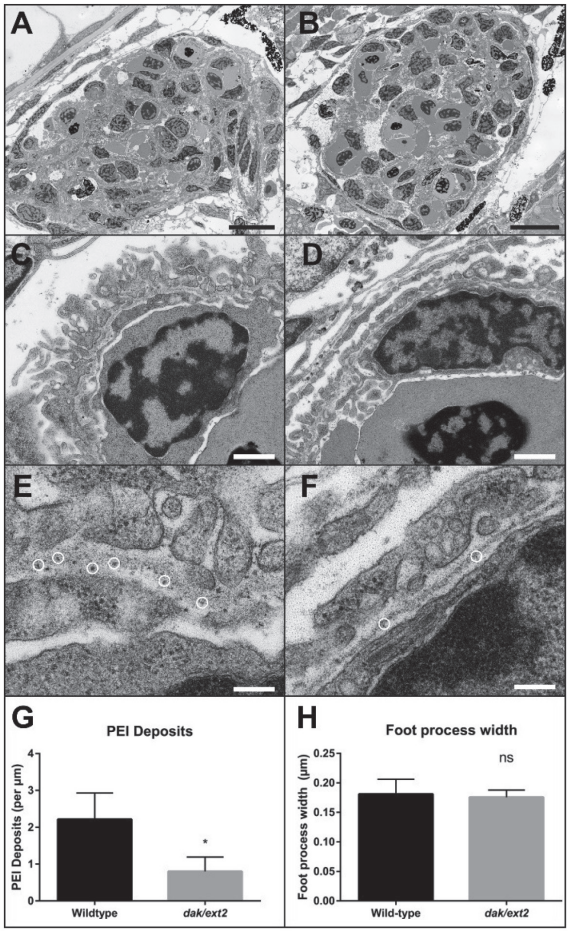

Figure 2. Homozygous dak/ext2 mutant zebrafish have significantly fewer electron dense-deposits in the GBM, but normal podocyte foot process width

Transmission electron micrographs of the glomerulus (A and B), a capillary loop (C and D) and representative sections of GBM (E and F) in a wild-type (A, C, and E) and homozygous *dak/ext2* mutant (B, D, and F) embryo. Sections were stained with PEI particles, which bind to negatively charged molecules in the GBM, forming electron-dense deposits (examples indicated by white circles in E and F). (G) Summary of the number of electron-dense PEI-containing deposits in the GBM; **p*<0.05. (H) Summary of the foot process width; ns, *p*>0.05. Scale bars = 10 µm (A and B), 1 µm (C and D) and 200 nm (E and F).

Glomerular permeability

Next, we analyzed the reabsorption of injected dextran particles (Figure 3). We found no significant difference between homozygous *dak/ext2* mutants and WT embryos with respect to the mean number of reabsorbed 3-kDa dextran droplets (22.69, SD 10.22, *n* = 32 versus 24.33 SD 12.51, *n* = 21; *p*=0.60). PAN-injected WT embryos, which were used as a positive control, had a similar number of 3-kDa dextran droplets (25.79, SD 13.33,

n = 14; *p*=0.75 versus WT and *p*=0.39 versus homozygous *dak/ext2*). As small particles can pass readily through the GFB, the similar number of 3-kDa dextran droplets indicates fully functional tubular reabsorption in all groups.

We also found no significant difference between homozygous *dak/ext2* mutants and WT embryos with respect to 70-kDa dextran particles (the mean number of droplets was 8.56, SD 5.33, *n* = 32 and 9.27, SD 3.72, *n* = 21 respectively; *p*=0.59) However, WT PAN-injected embryos had significantly more 70-kDa droplets (16.93, SD 5.41, *n* = 14) compared to both WT embryos (*p*<0.0001) and *dak/ext2* mutants (*p*<0.0001). Taken together, these data indicate that 70-kDa particles pass the GFB in similar quantities under physiological conditions and in an HS-GAG-free environment.

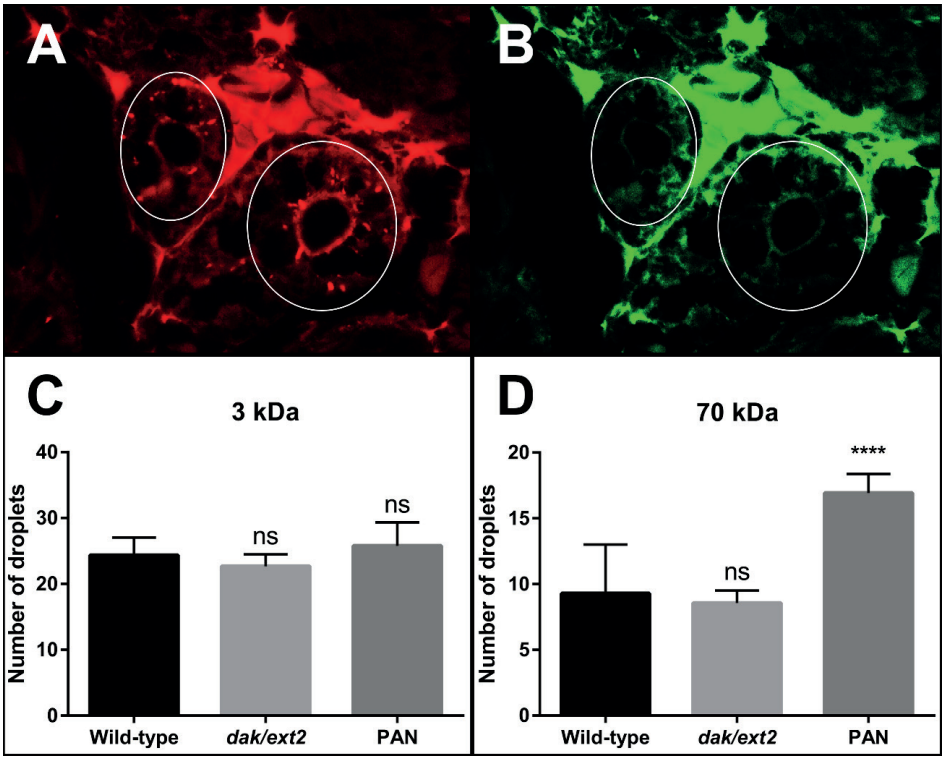

Figure 3. Homozygous dak/ext2 mutant zebrafish have normal glomerular permeability and tubular reabsorption capacity

Panels A and B show transverse sections through the proximal tubular epithelium of homozygous *dak/ext2* mutant embryos injected with red fluorescent 3-kDa dextran (A) and green fluorescent 70-kDa dextran (B). The white ellipse highlights the proximal tubule epithelial cells. The bright area between the circle reflects background fluorescence, partly due to auto-fluorescence. (C and D) Summary of the number of 3-kDa (C) and 70-kDa (D) dextran droplets in wild-type, homozygous *dak/ext2* mutant, and PAN-injected wild-type embryos. ns, *p*>0.05 versus wild-type; ****$p<0.0001$ versus wild-type. A digital high-pass filter has been placed over panels A and B to enhance the contrast between reabsorption droplets and the surrounding tissue. Scale bar = 5 μm.

Discussion

Here, we investigated whether HS-GAGs play an essential role in glomerular permeability using homozygous *dak/ext2* mutant zebrafish in which HS-GAGs are lacking in the entire embryo. We found that homozygous *dak/ext2* mutants develop an overall phenotype similar to previous reports.(34, 36) PEI staining of the GBM revealed significantly fewer anionic sites in homozygous mutants compared to WT embryos; however, despite having fewer negative sites in the GBM, homozygous *dak/ext2* mutants had normal glomerular permeability. Furthermore, our ultrastructural analysis revealed no difference between homozygous *dak/ext2* mutants and WT embryos with respect to podocyte foot width.

The classic paradigm based on the results of Kanwar *et al.*(8, 42) suggests that HS-GAGs are essential for normal glomerular filtration. In their study, the authors found that glomerular permeability increased after perfusing the kidney with heparinase III, an enzyme that cleaves HS-GAG chains. However, several other studies showed that podocyte-specific knockout of HS-GAGs did not result in proteinuria, suggesting that HS-GAGs may not necessarily be required for normal glomerular filtration.(23-27) Consistent with this notion, our results show that even when HS-GAGs are deleted in the entire embryo (and not specifically in podocytes), glomerular filtration is essentially intact. It remains possible that the size- and charge-selective properties of the GFB are altered in HS-GAG deficiency. However, overall glomerular permeability remains intact despite a severe and global loss of HS-GAG, as was hypothesized.

Previous *in vivo* models of HS-GAG deficiency used proteinuria as a readout of glomerular permeability; thus, albumin retrieval by proximal tubule epithelial cells may have masked changes in glomerular permeability, as suggested by Chen *et al.*(25) Therefore, we used tubular reabsorption of 70-kDa dextran particles as our readout of glomerular permeability; this method has previously been reported as providing a reliable measure of overall glomerular permeability.(40, 43) Although dextrans are known to move through a network as chains rather than as hydrodynamic spheres, dextrans have been validated as a sensitive marker for loss of glomerular permeability in various zebrafish models.(40, 43-46) Tubular reabsorption mechanisms were found to be fully functional in homozygous *dak/ext2* mutants, as a similar number of 3-kDa dextran droplets, which readily pass the GFB, was found between mutant and wild-type embryos.

Pericardial edema has previously been used as an indicator of renal damage in zebrafish.(43) Interestingly, although homozygous *dak/ext2* mutants lack HS-GAGs throughout the entire body and develop pericardial edema, they have essentially normal glomerular permeability. We therefore speculate that the pericardial edema observed in homozygous

dak/ext2 mutants is not caused by renal damage. For example, HS-GAG deficiency may have had an effect on the permeability of systemic capillaries because of an altered glycocalyx composition. Such a correlation between the endothelial glycocalyx of systemic capillaries and vascular permeability has been reported previously.(47)

An important difference between Kanwar *et al.*'s study and our approach using a germline mutation is that Kanwar *et al.* investigated the acute loss of HS-GAG by enzymatic activity, whereas our approach deleted HS-GAGs for an extended period. This difference may account for the difference in results between the two studies. For example, with a germline mutation, the effects will be present throughout the animal's life; therefore, other GAGs may have functionally replaced the deleted HS-GAGs. In the *in situ* set-up used by Kanwar *et al.,* loss of HS-GAG is acute and there is not enough time for such a compensatory mechanism to take place. Another possible explanation for the different results is that the enzymes used by Kanwar *et al.* may have caused collateral damage to other components of the GFB, which could have led to their observed increase in glomerular permeability.

The charge-selective nature of the GBM has long been attributed to the presence of HS-GAG.(7) In contrast, Miner proposed that charge selectivity is not an essential factor in glomerular permeability, as several studies found that a decreased number of anionic sites does not necessarily coincide with proteinuria.(48) Consistent with this notion, we found that glomerular permeability was not affected in homozygous *dak/ext2* mutants, despite the fact that these animals had significantly fewer PEI particles in the GBM. It is important to note that although negatively charged sites were significantly reduced in the GBM of homozygous *dak/ext2* mutants, these sites were not completely absent. Thus, these residual anionic sites could reflect negatively charged particles other than HS-GAGs. For example, type IV collagen, which is one of the primary constituents of the GBM, contains negatively charged sialic acid residues.(49) Therefore, we cannot exclude the possibility that the residual negatively charged sites in the GBM are sufficient for conferring charge selectivity.

Our ultrastructural analysis of the glomeruli in zebrafish embryos revealed that homozygous *dak/ext2* mutants have normal podocyte foot processes. Although similar results were reported in podocyte-specific agrin-deficient and perlecan-deficient mice, podocyte-specific *Ext1* knockout mice have foot process effacement.(23-25) The difference between our global *ext2* deficient zebrafish model and the podocyte-specific *Ext1* knockout mice may be due to differential effects of Ext1 and Ext2 on podocyte cytoskeletal organization. Both EXT1 and EXT2 have been reported to influence cytoskeletal organization in

chondrocytes obtained from patients with EXT1 or EXT2 mutations,(50) in which the specific mutation seemed to determine the effect on cytoskeletal organization. Whether EXT1 and/or EXT2 directly affect cytoskeletal organization in podocytes, independently of HS-GAG side chains, remains an open question.

This study has several limitations. First, a direct local decrease in HS-GAGs could not be shown by immunohistochemistry. However, *dak/ext2* mutants had significantly less anionic sites in the GBM than WT animals in our PEI labelling experiment. This is indirect proof of a decrease in HS-GAGs. Thus, the degree of HS-GAG deficiency in the glomerulus is at least 63%, as this is the relative difference in PEI particles. These particles could reflect HS-GAGs, other sulfated GAGs or other negatively charged areas. Also, the total decrease of HS-GAGs in *dak/ext2* zebrafish embryos has previously been reported to be over 80%.(21, 30) Although a relative and significant decrease in PEI-particles was observed, the absolute extent of PEI-labeling is less than in mammalian systems. A possible explanation is that this is due to a methodological difference, as we did not perform an *in vivo* labelling.(25) Also, it could be a reflection of interspecies variation in anionic site distribution in the GBM.

Furthermore, the *dak/ext2* model does not have a complete loss of HS-GAG. Theoretically, a double knock out of ext1 and ext2 would result in even more pronounced loss of HS-GAG. As zebrafish have three ext1 genes (ext1a, ext1b and ext1c) and up to date, characterized mutants lines are not available for any of these semi-orthologues, this could not be confirmed experimentally. To the best of our knowledge, this is the first study to examine the effect of a global HS-GAG deficiency on glomerular permeability. Globally deleting HS-GAGs in most vertebrates causes extremely early lethality;(51) Thus, the homozygous *dak/ext2* mutant zebrafish is currently the only viable model for global loss of HS-GAGs that survives gastrulation when both copies of the ext2 gene are impaired and that gives unique opportunity to study glomerular filtration that cannot be rescued by HS-GAGs produced by other type of cells. Although homozygous *dak/ext2* mutants do not survive to adulthood, Lee *et al.* hypothesized that these mutants survive the first part of the embryonic phase due to the maternal contribution of Ext2;(30) by 5 dpf, maternal contribution of Ext2 decreases to non-detectable levels.(30)

In conclusion, we provide the first report that glomerular permeability is not affected by a global HS-GAG deficiency. These results support the growing body of evidence that HS-GAGs do not play an essential role in mediating glomerular permeability.

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Disclosures

The authors declare no competing interests.

Author's contributions

All authors contributed to conception or design, or analysis and interpretation of data, provided intellectual content of article drafting or revision and have provided final approval of the version to be published.

RK designed and performed experiments, analyzed data, and wrote the paper. RAL performed experiments and analyzed data, MIW and CMA designed and performed experiments, AJK, HPS, JAB, and PCW designed experiments and provided conceptual advice. HJB designed experiments, provided technical support and conceptual advice.

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