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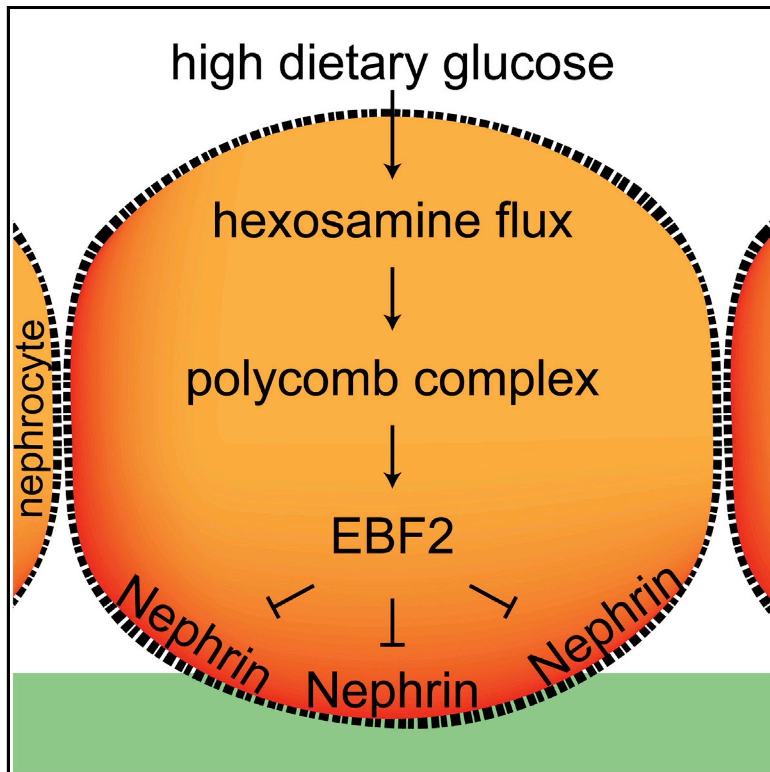
Diet-Induced Podocyte Dysfunction in *Drosophila* and Mammals

Graphical Abstract



Highlights

- Diabetic nephropathy is a growing health problem worldwide
- High dietary sugar led to loss of proper renal (nephrocyte) function in *Drosophila*
- An OGT-Polycomb-Knot-Nephrin pathway mediates nephrocyte dysfunction
- The Knot ortholog EBF2 mediates aspects of podocyte dysfunction in mice and humans

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In Brief

Diabetes-related kidney failure is a major source of morbidity and mortality worldwide. Na et al. provide evidence that glucose, OGT, Polycomb, EBF2, and Nephrin connect chronic high dietary sugar to loss of proper filtration in the *Drosophila* nephrocyte and mammalian podocyte.

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Diet-Induced Podocyte Dysfunction in *Drosophila* and Mammals

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SUMMARY

Diabetic nephropathy is a major cause of end-stage kidney disease. Characterized by progressive microvascular disease, most efforts have focused on injury to the glomerular endothelium. Recent work has suggested a role for the podocyte, a highly specialized component of the glomerular filtration barrier. Here, we demonstrate that the *Drosophila* nephrocyte, a cell analogous to the mammalian podocyte, displays defects that phenocopy aspects of diabetic nephropathy in animals fed chronic high dietary sucrose. Through functional studies, we identify an OGT-Polycomb-Knot-Sns pathway that links dietary sucrose to loss of the Nephrin ortholog Sns. Reducing OGT through genetic or drug means is sufficient to rescue loss of Sns, leading to overall extension of lifespan. We demonstrate upregulation of the Knot ortholog EBF2 in glomeruli of human diabetic nephropathy patients and a mouse *ob/ob* diabetes model. Furthermore, we demonstrate rescue of Nephrin expression and cell viability in *ebf2*$^{-/-}$ primary podocytes cultured in high glucose.

INTRODUCTION

Diabetic nephropathy is a growing global health challenge, representing the most common cause of end-stage renal disease in the developed world (Zimmet et al., 2001). Diabetic nephropathy including progressive proteinuric kidney disorder primarily affects the glomerulus and involves all cellular compartments. It is characterized histologically by arterial hyalinosis, collagen deposition, thickening of the glomerular basement membrane, mesangial expansion, glomerular endothelial cell injury and podocyte loss and hypertrophy, tubular epithelial atrophy, accumulation of activated myofibroblasts and matrix, influx of inflammatory cells, and capillary rarefaction (Jefferson et al., 2008; Reidy et al., 2014).

Recent studies have emphasized the pathogenic targeting of podocytes, a layer of cells that encircle the glomerular capillaries (Doublier et al., 2003; Reidy et al., 2014). Neighboring podocyte foot processes interdigitate to form the size and charge selective slit diaphragm. This modified adherens junction, composed mostly of the transmembrane proteins Nephrin and Neph1, serves as the final barrier to urinary protein loss. Diabetes-related injury to podocytes—occurring by poorly understood mechanisms—results in reorganization of the podocyte actin cytoskeleton characterized by effacement of the foot processes on electron microscopy and the clinical appearance of albuminuria. Currently, no cell-specific therapy is available for proteinuric kidney disorders in general or diabetic nephropathy in particular, though strategies such as angiotensin II blockade may slow disease progression. This emphasizes the need for a better understanding of the relationship between diet-induced metabolic defects and progressive podocyte dysfunction.

Drosophila has a set of cells analogous to the podocyte, the pericardial nephrocytes. They play a similar function, filtering the *Drosophila* hemolymph. In this article, we explore the effects of feeding flies chronic high dietary sucrose. We found evidence for nephrocyte abnormalities that reflect aspects of podocyte dysfunction in diabetic nephropathy patients. These include a severely compromised "nephrocyte diaphragm" due to loss of the Nephrin-like protein Sns. We provide evidence for a pathway that includes flux through the hexosamine biosynthetic pathway and the Polycomb gene complex, which in turn regulates the transcription factor Knot to regulate Sns expression. In cultured mouse primary podocytes, the Knot ortholog EBF2 similarly mediated response by Nephrin to high dietary sucrose. Finally, we demonstrate how a chemical inhibitor of hexosamine flux can improve the whole-animal response to high dietary sucrose, providing a guideline for candidate therapeutics.

RESULTS

Drosophila Adult Pericardial Nephrocytes Are Podocyte-like Cells

The *Drosophila* pericardial nephrocyte (Figures 1A and 1B) is a podocyte-like cell with additional properties shared with the mammalian proximal tubules (Weavers et al., 2009; Zhang et al., 2013a, 2013b; Zhuang et al., 2009). Associated with the heart, its primary function is to filter *Drosophila* hemolymph, a fluid analogous to mammalian blood. The first steps in filtration occur at the surface of the nephrocyte as hemolymph passes through the basement membrane and nephrocyte diaphragm,

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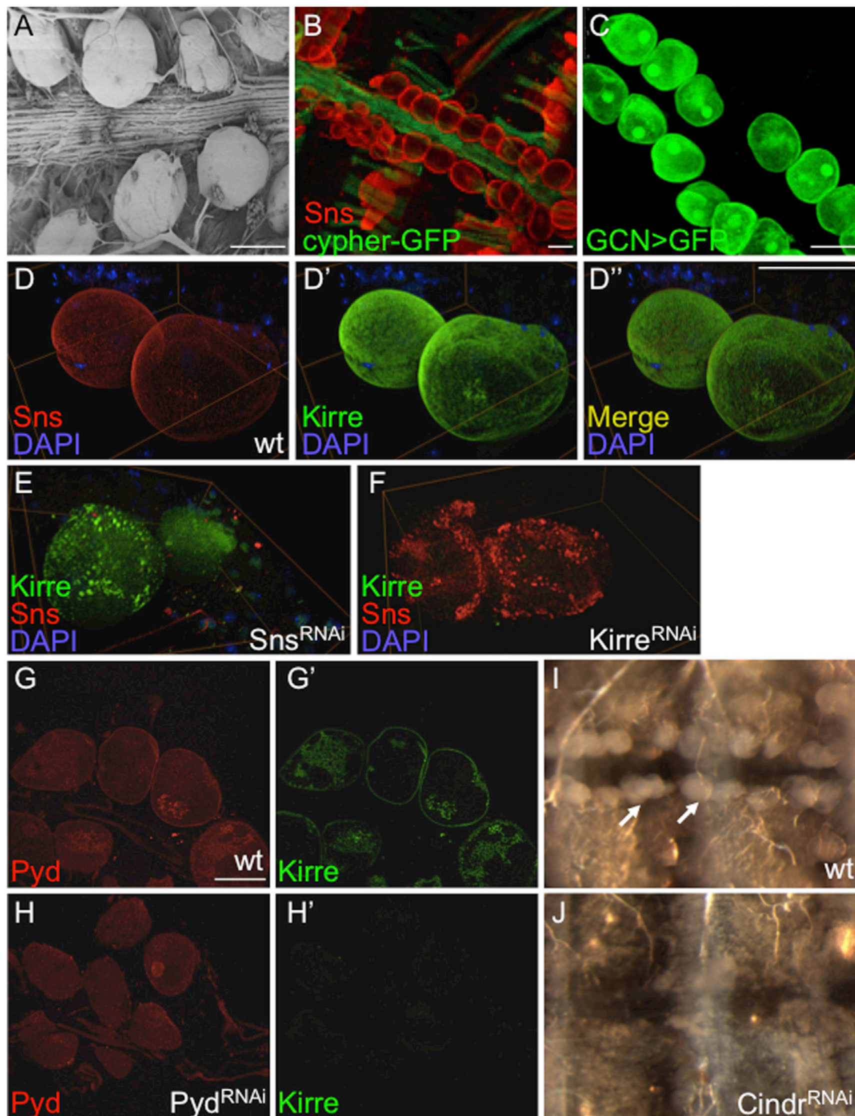

Figure 1. *Drosophila* Adult Pericardial Nephrocytes Are Podocyte-like Cells

(A) SEM of *Drosophila* adult pericardial nephrocytes.

(B) Pericardial nephrocytes (Sns antibody, red) are localized along the heart tube (Cypher-GFP, green). Also visible is Cypher-GFP in the body wall muscle.

(C) The Sns reporter *GCN>GFP* is expressed specifically in pericardial nephrocytes.

(D–D″) Confocal-based reconstruction. Sns (red) and Kirre (green) are co-localized to the pericardial nephrocytes. The paralogs Roughest and Hibris are not expressed at detectable levels in nephrocytes, as shown in Figure S1.

(E) Kirre (green) was mislocalized in nephrocytes with reduced Sns.

(F) Sns (red) was mislocalized in nephrocytes with reduced Kirre.

(G and G′) Pyd (red) co-localized with Kirre (green).

(H and H′) Kirre (green) was mislocalized in nephrocytes with reduced Pyd.

(I) Nephrocytes (e.g., arrows) are visible in wild-type flies visualized with light microscopy.

(J) Reducing Cindr expression led to absence of nephrocytes.

Scale bars represent 50 μm (A–C and G).

a slit-diaphragm-like structure composed primarily of the Nephrin ortholog Sticks and Stones (Sns) and the Neph1 ortholog Kin of Irre (Kirre) (Weavers et al., 2009; Zhang et al., 2013a, 2013b; Zhuang et al., 2009).

Similar to the larval nephrocyte, Sns is expressed in the adult pericardial nephrocyte and is localized to its surface within the nephrocyte diaphragm, as assessed by antibody localization and by the transcriptional reporter *sns-GCN-gal4;uas-GFP* (abbreviated *GCN>GFP*; Figures 1B and 1C; Zhuang et al., 2009). Kirre protein co-localized with Sns (Figures 1D–1D″). RNAi-mediated knockdown (Dietzl et al., 2007) of Sns (*GCN>sns(RNAi)*) led to mislocalization of Kirre; we also observed the converse (Figures 1E and 1F). We also examined the orthologs of two proteins associated with Nephrin and Neph1. The structural protein Polychaetoid (Pyd), an ortholog of mammalian ZO-1, was confirmed as co-localized with Kirre in nephrocytes (Figures 1G and 1G′; Seppa et al., 2008). Reducing Pyd levels (*GCN>pyd(RNAi)*) depleted Kirre from the nephrocyte plasma membrane (Figures 1H and 1H′). Reducing the CD2AP ortholog Cindr (*GCN>cindr(RNAi)*; Johnson et al., 2008) led to complete loss of nephrocytes from an early developmental stage (Figures 1I and 1J). This reflects a stronger phenotype than loss of CD2AP in murine podocytes, which led to foot process defects in podocytes (Shih et al., 1999), perhaps reflecting the single CD2AP ortholog in *Drosophila* (Johnson et al., 2008). *Drosophila* contains two additional orthologs of Nephrin and Neph1, Hibris and Roughest, respectively; neither is expressed in nephrocytes (Figure S1).

Sns Was Downregulated upon High-Sucrose and High-Glucosamine Feeding

The striking similarities between the *Drosophila* nephrocyte and the human podocyte indicated it could be useful as an in vivo tool for studying diabetic nephropathy. While important changes have been identified in the kidney endothelium, previous expression work also noted downregulation of Nephrin in diabetic nephropathy patients (Doublier et al., 2003; Langham et al., 2002). In mice, long-term, sustained reduction of Nephrin led to mild defects in adult glomerular structure and function (Li et al., 2015). We therefore examined nephrocytes in animals fed high dietary sucrose (HDS).

Our previous work demonstrated that adult flies fed chronic HDS develop a series of diabetes-like phenotypes including hyperglycemia, hyperlipidemia, insulin resistance, and aspects of

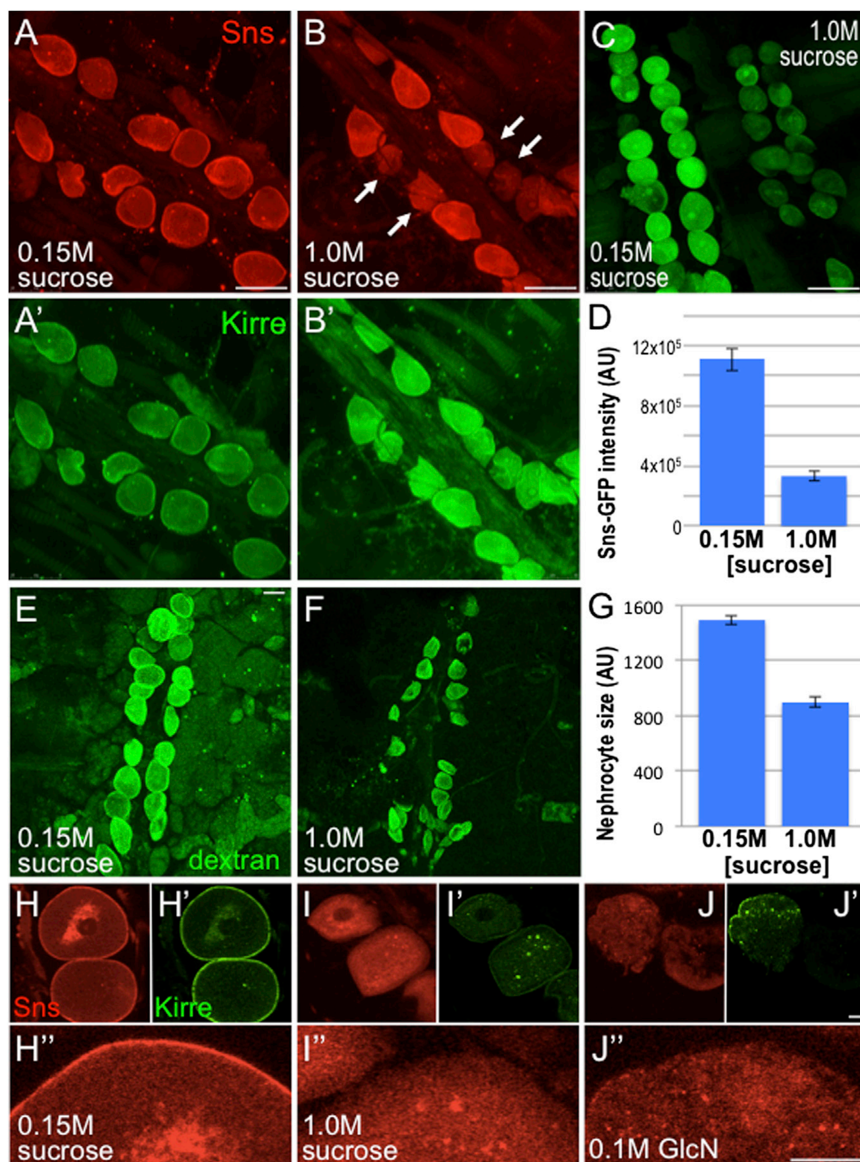

Figure 2. High-Sugar and High-Glucosamine Diets Downregulate Nephrocyte Sns Levels

(A and A′) Sns (red) and Kirre (green) were expressed in the nephrocytes of flies fed a control diet for 3 weeks.

(B and B′) Sns (red) expression was lost in some nephrocytes (e.g., arrows) of flies fed HDS for 3 weeks; Kirre (green) was unchanged. Loss of Sns was not observed in the developing pupal eye (Figure S2).

(C) The Sns reporter *GCN>GFP* in animals fed control diet and HDS for 3 weeks.

(D) Quantification of *GCN>GFP* in situ fluorescence levels in animals fed a control diet and HDS. Each measurement represents the average intensity of 20–30 cells; error bars represent the SE within each group.

(E and F) 10 kDa Dextran was used to label nephrocytes, emphasizing the decreased size of nephrocytes exposed to HDS. Nephrocyte size was measured with Adobe Photoshop software for (G).

(G) Quantification of nephrocyte size difference between flies fed control diet versus HDS. Each measurement represents the average size of 20–30 cells; error bars represent the SE within each group.

(H–H″) Sns (red) and Kirre (green) are localized primarily at the plasma membrane in flies fed with a control diet.

(I–I″) Sns (red) is depleted from the plasma membrane, but Kirre is still localized at the plasma membrane in flies fed with HDS.

(J–J″) Sns (red) is depleted from the plasma membrane, but Kirre is partially localized at the plasma membrane in flies fed with HDS.

Scale bars represent 50 μm (A, B, and D) and 10 μm (J′ and J″).

diabetic cardiomyopathy (Na et al., 2013). In animals fed a diet supplemented with 1 M sucrose, Sns protein was strongly downregulated; Kirre protein was unaffected (Figures 2A–2B′). Loss of Sns was somewhat variable between nephrocytes, ranging from partial loss to complete absence of detectable protein (Figure 2B and data not shown). Loss of Sns expression was confirmed by confocal microscopy (Figures 2H and 2I). Loss was also confirmed by western analysis (Figure S1D); HDS led to loss of nephrocytes from the heart structures, making accurate western-based quantification difficult. Strong downregulation of the *GCN>GFP* reporter indicated that decreased Sns levels upon high-sucrose feeding is regulated at least in part at the transcriptional level (Figures 2C and 2D).

Interestingly, we observed a consistent and significant decrease in overall nephrocyte volume that directly reflected loss of Sns, often to two-thirds normal volume (Figures 2E–2G). This decrease maintained close to normal concentrations of Sns protein on the surface and may reflect a mechanism to account for fluctuating Sns levels. However, extensive loss of Sns led to complete loss at the surface, perhaps reflecting a limit to the extent by which nephrocyte volume can be reduced.

Several metabolic pathways that process glucose have been shown to be involved in the pathogenesis of diabetes, including the polyol, advanced glycation end products (AGE), PKC, and hexosamine biosynthetic pathways (Brownlee, 2005). Our and others' previous work defined a role for flux through the hexosamine biosynthetic pathway in diet-induced metabolic dysfunction in fly and rat models (Erickson et al., 2013; Na et al., 2013). Hexosamine flux is regulated in part by the rate-limiting enzymes GFAT and O-GlcNAc transferase (OGT); the latter enzyme catalyzes the final step of transferring an O-GlcNAc moiety onto downstream targets (Kreppel et al., 1997). O-glycosylation is stimulated by high glucose levels in glomeruli and mesangial cells (Degrell et al., 2009; Goldberg et al., 2006), and genomic studies have linked the GFAT2 gene region with diabetic

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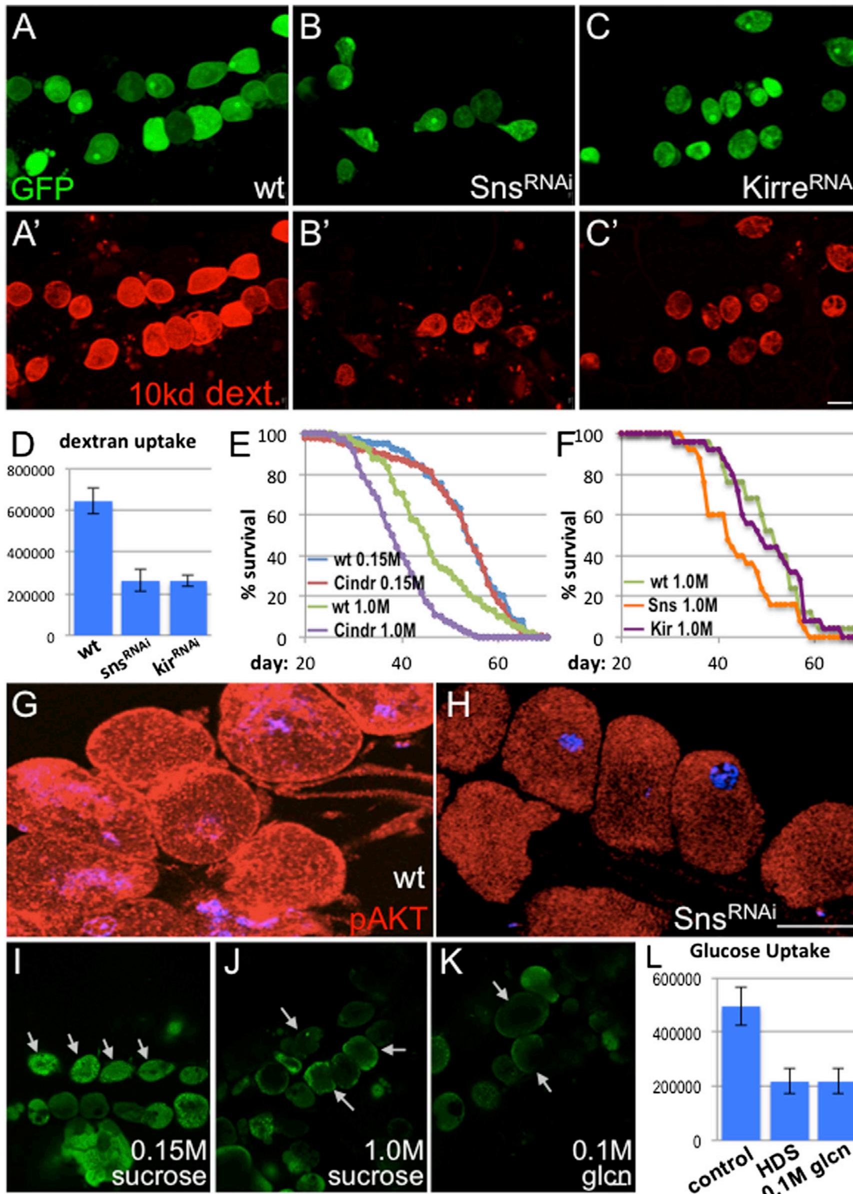

Figure 3. HDS Disrupts Nephrocyte Function

(A–C′) 10 KD Dextran uptake in nephrocyte from control flies (A and A′), Sns-knockdown flies (B and B′), and Kirre-knockdown flies (C and C′).

(D) Quantitation of dextran uptake. Each measurement represents the average intensity of 20–30 cells; error bars represent the SE within each group.

(E) Lifespan of control or Cindr knockdown flies in control diet or HDS.

(F) Lifespan of control, Sns knockdown or Kirre knockdown flies fed HDS.

(G) Phospho-AKT staining of nephrocytes from wild-type flies challenged with 5 μM human insulin.

(H) Phospho-AKT staining of nephrocytes from Sns knockdown flies challenged with 5 μM human insulin.

(I–K) 2-NBDG uptake by nephrocytes from flies fed with control food (I), HDS (J), or 0.1 M glucosamine diet (K).

(L) Quantitation of glucose uptake as assessed by 2-NBDG uptake. Each measurement represents the average intensity of 20–30 cells; error bars represent the SE within each group.

Scale bars represent 50 μm (C′, H, and K).

nephropathy including an increase in mRNA levels (Zhang et al., 2004). We directly stimulated flux through the hexosamine pathway by supplementing the fly's diet with 0.1 M glucosamine. Dietary glucosamine led to strong loss of Sns from the nephrocyte plasma membrane, similar to the effects of HDS (Figure 2J). Concomitant with Sns protein loss, nephrocyte morphology was strongly disrupted. Interestingly and similar to high sugar feeding, Sns levels in other tissues such as the eye were unaffected (Figure S2).

High Dietary Sugar Disrupted Nephrocyte Function

Loss of Sns had consequences for nephrocyte function. Dextran uptake is a direct measure of functional uptake by nephrocytes (Weavers et al., 2009). Knockdown of Sns or Kirre strongly affected the ability of nephrocytes to internalize fluorescent dextran (Figures 3A–3D). To determine the consequence of reduced nephrocyte function on flies, we ablated the nephrocytes at an early stage by targeted knockdown of Cindr (Figures 1I and 1J). Surprisingly, *GCN>cindr(RNAi)* flies exhibited no difference in lifespan compared to *GCN-gal4* controls when both were fed a control diet (Figure 3E). However, when challenged with HDS, the lifespan of *GCN>cindr(RNAi)* adults was significantly reduced (Figure 3E). The lifespan of *GCN>sns(RNAi)* flies was also shortened in HDS, while the *GCN>kirre(RNAi)* lifespan was unaffected when fed HDS despite the compromise in nephrocyte diaphragm integrity (Figure 3F). These data suggest that reduced nephrocyte function affects overall fly homeostasis in a manner at least partially independent of hemolymph filtration. It further indicates that Sns has a primary role in nephrocyte response to HDS, including activities outside of slit-diaphragm integrity.

In addition to a role in slit-diaphragm stability, Nephrin is also likely required for normal Insulin activity in human glomerular podocytes (Coward et al., 2007). Consistent with this view, we observed compromised activity of the downstream Insulin pathway effector AKT at the membrane of animals fed HDS or glucosamine (Figures 3G and 3H). We previously demonstrated a role for Insulin pathway activity in regulating cellular glucose uptake in flies (Hirabayashi et al., 2013). As anticipated, uptake of the fluorescent glucose analog 2-NBDG by nephrocytes was compromised in flies fed HDS or glucosamine (Figures 3I–3L). We conclude that reduced

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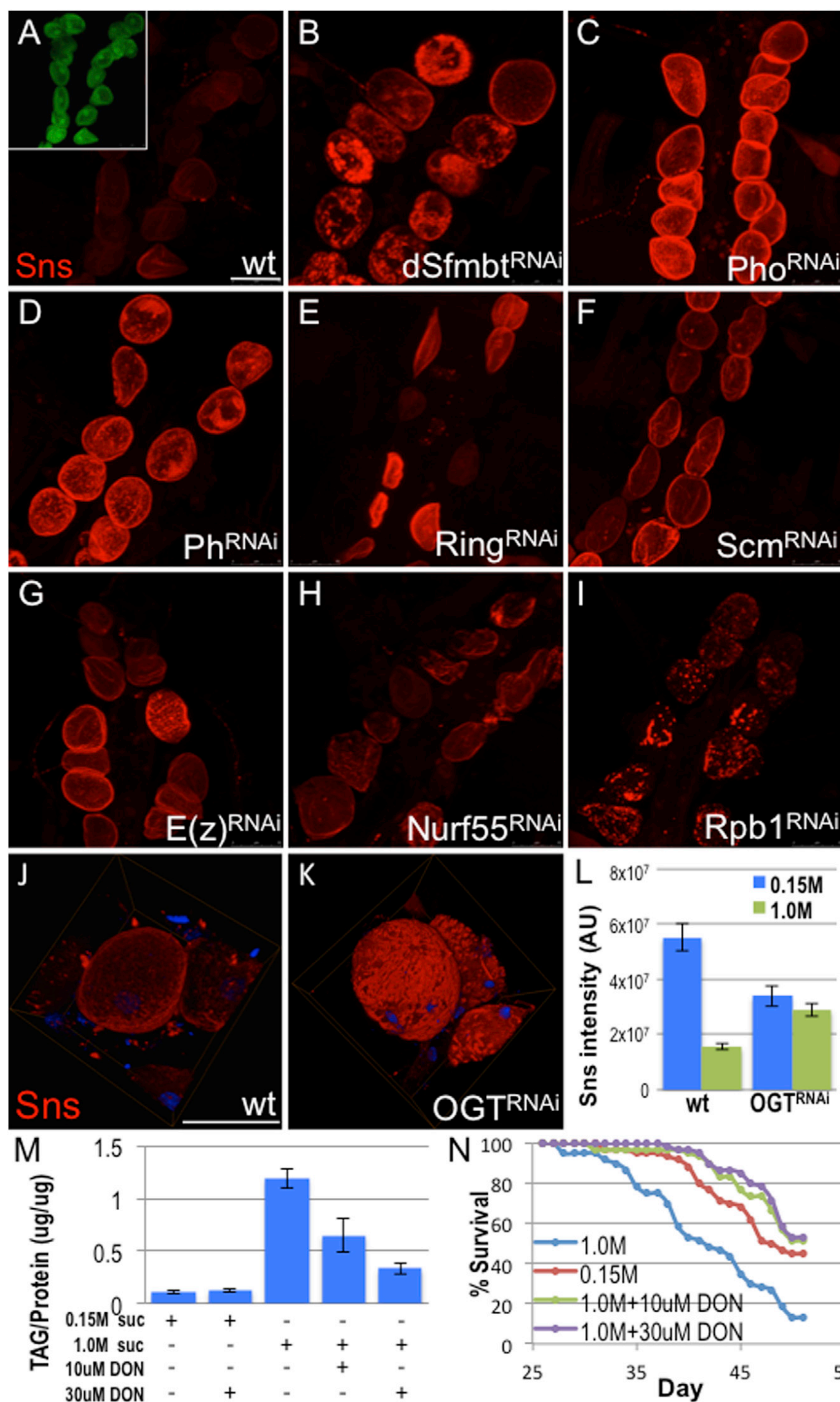

Figure 4. OGT-Polycomb-Knot Regulates Sns Level

(A–I) Sns (red) in nephrocytes of wild-type or *GCN*-targeted knockdown of the indicated Pc-G complex components. Inset in (A) shows the Sns reporter *GCN>GFP* to visualize cells. Data are quantified in Figure S2.

(J) Sns (red) staining in nephrocytes of wild-type flies fed HDS.

(K) Sns (red) expression is rescued in nephrocytes of *GCN>OGT(RNAi)* flies.

(L) Quantification of Sns level in nephrocytes of wild-type or *GCN>OGT(RNAi)* flies raised on control diet or HDS. Evidence for O-GlycNAcylation is shown in Figure S5. Each measurement represents the average fluorescence intensity of 20–30 cells; error bars represent the SE within each group.

(M) DON rescued TAG levels in flies fed with HDS. TAG quantitation was normalized to protein level; each measurement is the average of three experiments. The error bar represents the SD within each group.

(N) DON rescued lifespan of flies fed with HDS. Control food, 0.15 M sucrose.

Scale bars represent 50 μm (A and J).

Sns affects nephrocyte function, adult homeostasis, and longevity.

Evidence that OGT Acts through the Polycomb Gene Complex

The *Drosophila* OGT ortholog, encoded by *super sex combs* (*sxc*), was originally identified as a gene required in a broad cross section of developmental processes that acts through the Polycomb group (Pc-G) complex of epigenetic factors (Sinclair et al., 2009). The Pc-G consists of three protein complexes—PRC1, PRC2, and PhoRC—that act together to regulate transcription (Ringrose and Paro, 2004; Steffen and Ringrose, 2014). Sxc directly O-GlycNAcylates the PRC1 subunit Polyhomeotic (Ph), which is essential for Polycomb repression (Gambetta et al., 2009). We therefore used targeted RNAi-mediated knockdown to examine several Pc-G components including the PRC1 subunits Ph, Ring, and Scm; the PRC2 subunits E(Z) and Nurf55; and the PhoRC subunits dSfmbt and Pho.

In animals fed HDS, targeted reduction of Pho or Ph strongly rescued loss of Sns; reducing Ring, Scm, or E(Z) led to partial rescue (Figures 4A–4G). Nurf55 and Rpb1—also demonstrated Ogt targets (Kelly et al., 1993)—are involved in general transcription regulation; reducing activity of either led to aberrant nephrocyte morphology (Figures 4H and 4I), suggesting they have pleiotropic roles. Reducing dSmfbt activity also led to increased Sns protein, but the protein was mislocalized away from the surface; this may reflect subtle nephrocyte structural defects (Figure 4B). Sns expression levels are quantified in Figure S2. We conclude that Pc-G complexes mediate sugar-dependent regulation of Sns.

Knot Is a Transcription Factor that Regulates Sns Levels

Our results indicate that a primary effect of chronic HDS is compromise of nephrocyte function. This would support the view that effects on podocytes contribute to diabetic nephropathy (Lemley et al., 2000; Meyer et al., 1999; Pagtalunan et al., 1997; Steffes et al., 2001). Our experiments point to control of Sns expression as a key step in progressive nephrocyte dysfunction. To better understand the mechanism behind sugar-dependent nephrocyte dysfunction, we examined the Sns promoter region. Aligning the *Drosophila melanogaster* sequence to seven related *Drosophila* species, we identified a strong enrichment in consensus ''E-box'' sequences as assessed with TRANSFAC (Wingender, 2008), which are the sites of binding by basic-helix-loop-helix (bHLH) transcription factors (Figure S3A). To identify candidate bHLH factors that regulate sugar response through hexosamine flux, we performed an expression microarray analysis on animals fed control versus 0.1 M glucosamine: we identified seven bHLH factors as significantly altered in the presence of glucosamine (Figure S3B). Knot was among the highest differentially regulated genes. Furthermore, data from the *Drosophila* modENCODE project (Nègre et al., 2011) identified direct Knot binding to sequences upstream of the *sns* transcriptional start site.

Knot is a member of the early B cell factor (EBF) superfamily of transcription factors, which are evolutionarily conserved between species (Liberg et al., 2002). The mouse has four members: EBF1–EBF4. They are involved in the differentiation of cells originating from all three embryonic germ layers including nervous system, B lymphocyte, and adipocyte development (Liberg et al., 2002). Recently, EBF1 has been linked to late glomerular maturation and subsequent proper induction of the endothelia (Fretz et al., 2014); a role for the remaining family members has not been reported. The *Drosophila* EBF family member Knot is also required for multiple aspects of development including head, embryonic muscle, and wing patterning (Crozatier et al., 1996, 1999; Crozatier and Vincent, 1999; Vervoort et al., 1999).

Despite reduced overall *knot* expression (Figure S3B), histological analysis indicated a significant increase in detectable nuclear Knot (Figures 6A and 6B). Reducing Knot activity protected flies from several aspects of chronic HDS: genotypically *knot*^{EY09641} mutant flies failed to accumulate fat or develop hyperglycemia when fed HDS (Figures S4A and S4B). Further, the size of *knot*^{EY09641} nephrocytes was protected from reduced volume in response to HDS (Figures S4C–S4E). To determine if this rescue of the nephrocyte phenotype was due to a requirement for Knot activity within the nephrocyte itself, we used a targeted knockdown approach. *GCN>knot(RNAi)* adult nephrocytes exhibited a strongly diminished response to HDS; Sns expression levels were virtually unaffected by HDS (Figures 5A–5C). In contrast, targeting overexpression of Knot protein to nephrocytes led to strongly aberrant morphology (Figure 5E).

To further explore its regulation of Sns expression, we overexpressed Knot in the developing *Drosophila* eye; Sns is expressed dynamically during pupal eye development (Bao et al., 2010; Fischbach et al., 2009). Broad overexpression of Knot led not only to reduced Sns expression throughout the eye but also to severe ommatidial mispatterning (Figures 5F and 5G). Expressing Knot in discrete clones of cells (Ito et al., 1997), we observed autonomous loss of Sns expression (Figure 5H). Based on these results we conclude that Knot is a suppressor of Sns and that, in the nephrocyte, Knot controls Sns in response to HDS. Considering Knot protein has been demonstrated to bind a region just upstream of *sns*, this regulation may be direct.

Reducing OGT Activity Rescued Sns Levels and Improved Lifespan

Our data are consistent with a model in which high dietary sugar is processed by elevated flux through the hexosamine biosynthetic pathway, leading to an OGT-Polycomb-Knot-Sns pathway that controls nephrocyte response to HDS. This model suggests that reducing a key rate-limiting step in this process, the activity of Ogt/Sxc, should improve the fly's response to HDS. We targeted knockdown of the rate-limiting enzyme OGT specifically to the nephrocyte and challenged the flies with HDS. *GCN>ogt(RNAi)* adult nephrocytes were protected from HDS, and Sns levels remained near normal (Figures 4J–4L).

We previously demonstrated a role for hexosamine flux in a fly model of diabetic cardiomyopathy, highlighting the importance of this biosynthetic pathway on the fly's response to HDS. We assessed whether broad but moderate reduction of OGT activity improved overall survival by orally administering the GFAT inhibitor 6-diazo-5-oxo-norleucine (Don). Added to HDS food at 10 μM or 30 μM, Don significantly lowered hemolymph triacylglyceride (TAG) levels (Figure 4M). Importantly, at these concentrations Don significantly improved lifespan in the presence of HDS (Figure 4N).

Knot/EBF2 Accumulates in the Nuclei of Diabetic Glomeruli

Next, we examined mammalian models of diabetic nephropathy. During the course of our *Drosophila* studies, we noted that Knot protein displayed increased nuclear localization in response to HDS (Figures 6A and 6B), providing a visual assay for Knot activity. We performed microarray-based gene expression analysis of human kidney tissue samples (n = 22). These samples were microdissected to isolate glomeruli from tubular tissue and have been previously described (Woroniecka et al., 2011). Expression of the Knot ortholog EBF2 was significantly elevated in glomerular samples from patients with documented diabetic kidney disease (DKD) as defined by histological lesions and glomerular filtration rate less than 60 cc/min, when compared to control samples (Figure 6C; Woroniecka et al., 2011). Expression of the Nephrin transcript NPHS1 in these samples displayed a negative correlation with EBF2 expression ($r = -0.359$, $p = 0.0492$; Figure 6D). Immunohistochemical analysis confirmed the increase in EBF2 protein expression. Human glomerular EBF2 protein was primarily nuclear; in diabetic patients, nuclear EBF2 was significantly increased both within podocytes and elsewhere in the glomerulus (Figures 6E and 6F).

We also examined the expression and regulation of EBF2 in mouse models of DKD, comparing genotypically *ob/ob* mice and wild-type animals on a BTBR genetic background. The *ob/ob* mice lack functional Leptin activity; the result is poor appetite control, obesity, insulin resistance, and progressive aspects of diabetic nephropathy (Hudkins et al., 2010; Ingalls et al., 1950; Zhang et al., 1994). Nuclear EBF2 expression was

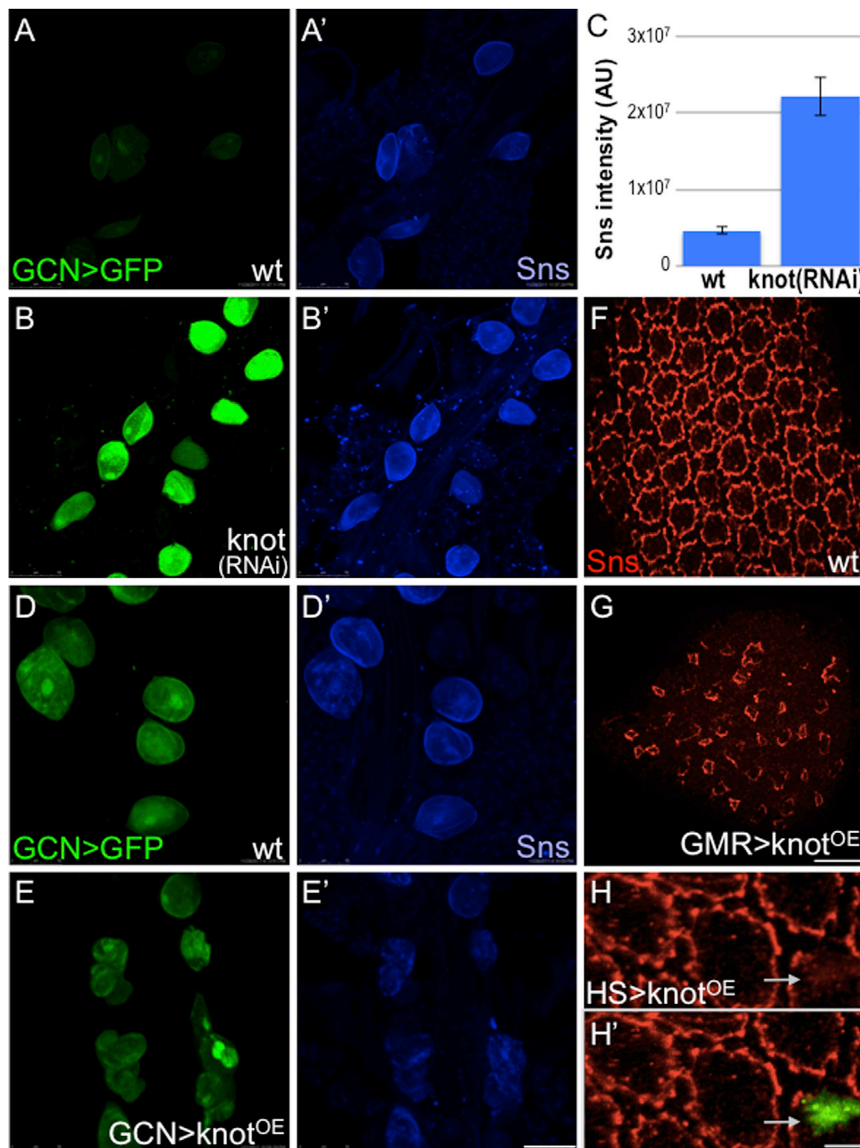

Figure 5. Knot Is a Transcriptional Factor that Regulates Sns Levels

(A and A′) *GCN>GFP* (green) and Sns staining (blue) in nephrocytes of wild-type flies raised in HDS for 3 weeks.

(B and B′) *GCN>GFP* (green) and Sns staining (blue) in nephrocytes of *knot(RNAi)* knockdown flies raised in HDS for 3 weeks.

(C) Quantification of Sns level in wild-type and *knot(RNAi)*-knockdown nephrocytes. Each measurement represents the average intensity of 20–30 cells; error bars represent the SE within each group.

(D and D′) *GCN>GFP* (green) and Sns staining (blue) in nephrocytes of wild-type flies raised in control food for 3 weeks.

(E and E′) *GCN>GFP* (green) and Sns staining (blue) in nephrocytes of *GCN>knot* flies raised on HDS for 3 weeks. Overexpression of Knot led to aberrant nephrocyte structure.

(F and G) Sns (red) staining as well as ommatidial patterning was strongly disrupted in *GMR>knot^{OE}* pupal eyes.

(H and H′) Sns (red) staining in small clones of cells in the eye expressing exogenous Knot^{OE} (genotype: *actin5C>y+>gal4, UAS-GFP;hs-flp*; *UAS-knot^{OE}*). GFP (green) marks the overexpression clone; note autonomous reduction of Sns expression (arrow).

Scale bar represents 50 μm (E′), 20 μm (G), and 5 μm (H′). See Figure S4 for additional *knot* phenotypes.

significantly increased in glomeruli of *ob/ob* mice compared to wild-type (Figures 6G and 6H). In summary, EBF2 expression was increased in patient samples and mouse models with DKD, suggesting the testable hypothesis that EBF2 was mediating aspects of glomerular damage in animals induced by hyperglycemia.

EBF2-Null Mutant Mouse Podocytes Are Resistant to High-Glucose Treatment

Immortalized mouse podocytes do not express detectable levels of Nephrin and, as expected, small hairpin RNA (shRNA)-mediated knockdown of EBF2 had no effect on Nephrin expression (data not shown). We therefore established short-term primary cultures from podocytes isolated directly from the glomeruli of *ebf2$^{-/-}$*-null mice (Wang et al., 2014) and control mice. Assessment of the adult kidneys from unchallenged control and *ebf2$^{-/-}$* mice did not show any appreciable differences between gross glomerular morphology or in the relative amount and localization of Nephrin protein as measured by immunofluorescence, though Nephrin mRNA levels were reduced (Figures S6A and S6B). Isolated podocytes were permitted to settle and attach to the culture dish (~3 days) and then challenged with media containing high glucose.

After 3 days of culturing in the presence of 25 mM glucose, control podocytes exhibited Nephrin localized almost exclusively to the cytoplasm, primarily in cytoplasmic particles (Figure 7A). Six days after culturing in high glucose, Nephrin localization became more diffuse but remained cytoplasmic (Figure 7C). Nephrin has been previously linked to stabilization of podocyte actin filaments (Zhu et al., 2008) and, indeed, we observed progressive loss of phalloidin staining (Figures 7A′ and 7C′). In contrast, a significant proportion of Nephrin in *ebf2$^{-/-}$* podocytes was observed at the plasma membrane at both three and six days after high glucose treatment (Figures 7B and 7D). Loss of EBF2 activity also prevented significant loss of actin filaments (Figures 7B′ and 7D′). Our observations phenocopied those reported for loss of Nephrin in related systems (e.g., Babayeva et al., 2011; Saleem et al., 2002; Schiwek et al., 2004).

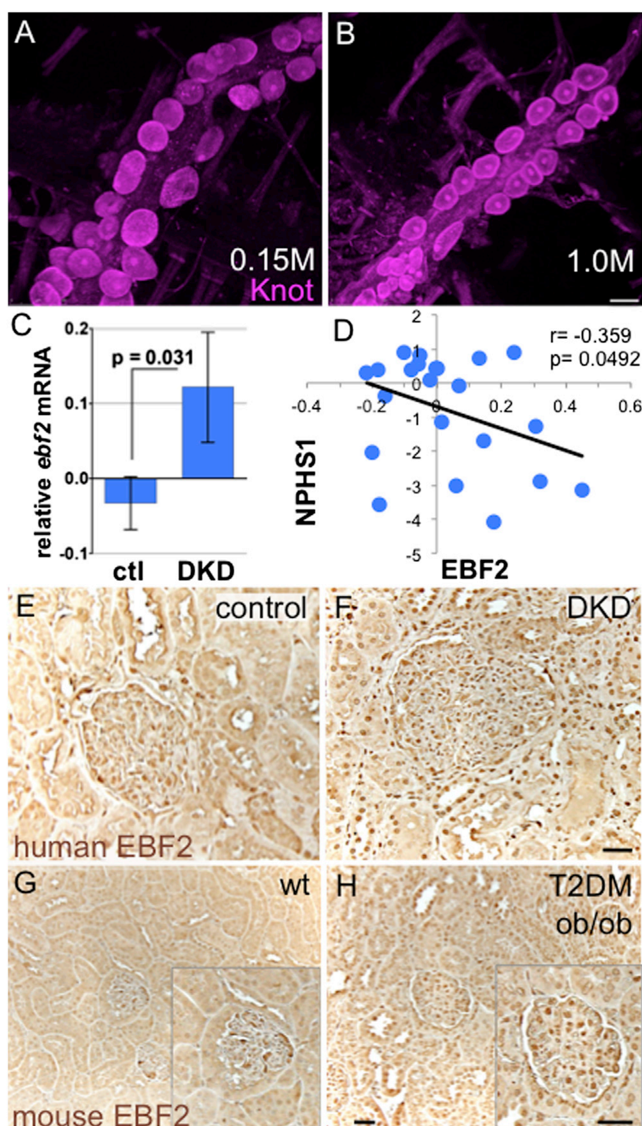

Figure 6. Knot/EBF2 Accumulated in the Nuclei of Nephrocytes or Podocytes in Diabetic Models and Patient

(A and B) Despite reduction of *knot* transcript in the presence of glucosamine (Figure S3B), nuclear Knot staining was elevated in wild-type flies fed with HDS.

(C) *ebf2* transcript levels were elevated in the glomeruli of diabetic kidney disease (DKD) patients. Scale bars represent SE.

(D) Negative correlation between Nephrin and EBF2 transcript levels in human control and diabetic glomeruli.

(E and F) Nuclear EBF2 was elevated in glomeruli of DKD patients compared to tissue from control patients as assessed by EBF2 antibody.

(G and H) EBF2 staining was strongly elevated within the glomeruli of *ob/ob* mice compared to controls. Insets show enlarged views.

Scale bar represents 50 μm (B) and 20 μM (F and H).

RT-PCR studies confirmed that, similar to *Drosophila* nephrocytes, loss of Nephrin was at the level of transcription (Figure 7E). Within 1 day of culturing in 25 mM glucose, we observed a strong downregulation of Nephrin that was rescued in genotypically *ebf2*$^{-/-}$ podocytes. This did not reflect a generalized reduction in expression of podocyte or glomerular proteins, as transcript levels of *podocin*, *p-cadherin*, and *vegf*α were unaffected (Figure 7F; Figure S6C). Further, loss of Nephrin was not due to osmotic shock, as supplementing control media with 19.5 mM mannitol did not downregulate Nephrin (Figure 7E). We conclude that, analogous to *Drosophila*, EBF2 mediates the loss of Nephrin as podocyte respond to high sugar levels.

Control cultured mouse podocytes were highly sensitive to increasing doses of glucose treatment, leading to a progressive reduction in the number of viable cells over 3 days (Figures 7G–7I). In contrast, *ebf2*$^{-/-}$ podocytes were less sensitive to high glucose; many podocytes survived 50 mM glucose, a concentration well above physiological levels (Figures 7J–7L). We have observed similar loss of nephrocytes in our whole-*Drosophila* HDS models, which was rescued by reducing Knot function (not shown).

DISCUSSION

In this article, we use a *Drosophila* model of diet-induced metabolic dysfunction to explore the link between HDS and renal dysfunction. Together with previous studies, our work suggests a model (Figure 7M) in which chronically high dietary sugar promotes OGT-mediated O-GlycNAcylation of Polyhomeotic and subsequent Pc-G activation of the transcription factor Knot/EBF2—potentially by regulating its nuclear localization—reduces Sns/Nephrin expression to compromise slit-diaphragm integrity. We propose that this pathway acts with previously identified mechanisms that compromise endothelial function (Stehouwer et al., 1991, 1992) to promote progressive podocyte and kidney dysfunction.

Previous work has led to the suggestion that podocytes are the "weakest link" in the development of DKD (Reidy et al., 2014). Indeed, podocyte depletion is strongly correlated with proteinuria and progression of renal disease in type I and type II diabetics (Meyer et al., 1999; Pagtalunan et al., 1997; Steffes et al., 2001; White et al., 2002). Our work demonstrates that lost or abnormal nephrocytes—associated with compromised filtration—is sufficient to reduce the lifespan of adult flies subsisting on HDS (Figures 3E and 3F), further highlighting the links among dietary sugar, podocyte function, and longevity.

Functional Consequences of Nephrin Loss

In addition to compromised filtration, loss of Nephrin affects insulin signaling. Nephrin has been previously shown to be required for the action of insulin on human podocytes (Coward et al., 2007), and mice with targeted deletion of the insulin receptor in podocytes developed significant albuminuria together with histological features of DKD (Welsh et al., 2010). Our data further support the view that reduced Nephrin, a result of HDS, is sufficient to impair insulin pathway activity (Figures 3G–3L). Whether chronically high dietary sugar also contributes to insulin resistance in podocytes by mechanisms active in other cell types remains to be determined.

One controversial idea regarding DKD progression and podocyte loss is the concept that the remaining podocytes use a variety of mechanisms to retain protection of the basement membrane. This can include activation of small GTP binding

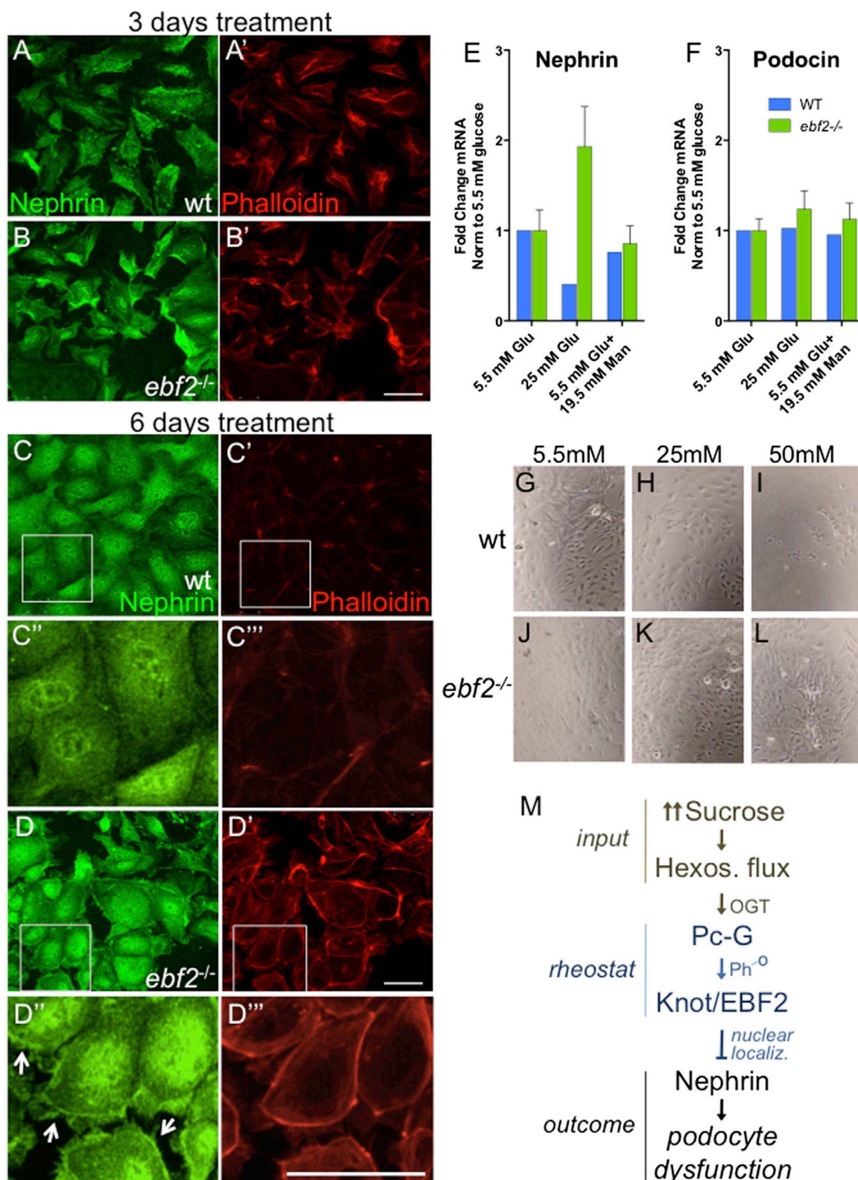

Figure 7. EBF2-Null Mutant Mouse Podocytes Were Resistant to High-Glucose Treatment

(A–B′) Nephrin (green) and phalloidin (red) staining in wild-type versus genotypically *ebf2*-null mouse podocytes cultured for 3 days. Scale bar in (B) represents 50 μm.

(C–D′′′) Nephrin (green) and phalloidin (red) staining in wild-type versus ebf2-null mouse podocytes cultured for 6 days. Quantification of NPHS1 transcript expression is shown in Figure S6A.

(E and F) RT-PCR quantification of Nephrin and Podocin RNA in wild-type and *ebf2*-null mutant mouse podocytes. Control, high-glucose, and control plus high-mannose media conditions are shown. Similar data for P-cadherin and VEGFα are shown in Figure S6C. Scale bars represent SE.

(G–I) Light micrograph image of wild-type podocytes growing in media containing 5.5 mM (G), 25 mM (H), and 50 mM (I) glucose.

(J–L) Light micrograph image of *ebf2*-null podocytes growing in media containing 5.5 mM (J), 25 mM (K), and 50 mM (L) glucose.

(M) Model of podocyte response to chronic high sugar. An *input* (sugar) is interpreted by a *rheostat* (Pc-G, EBF2) to direct downregulation of Nephrin and podocyte dysfunction.

proteins, loss of Nephrin expression, and reorganization of the actin cytoskeleton (Doublier et al., 2003; Garg and Holzman, 2012; Woroniecka et al., 2011). In *Drosophila* we observed clear evidence for compensation: nephrocytes displayed consistent compensatory shrinkage in response to diet or genetic reduction of Sns. This resulted in retention of near normal surface Sns and associated nephrocyte diaphragms (Figure 2B). Presumably, this mechanism ensures consistent filtration in the face of constantly varying blood sugar. Reducing Sns had other consequences as well, including loss of Pyd and mislocalization of Kirre (Figures 1E and 1G); these results roughly mirror data in mammals (Doné et al., 2008). These factors are linked to components of actin remodeling complexes (Jones et al., 2006; Uchida et al., 2008; Verma et al., 2006), which may contribute to podocyte remodeling and potentially to Insulin pathway activity.

Multiple Points of Nephrin Regulation by the Hexosamine Biosynthetic Pathway

We have identified a key role for the hexosamine biosynthetic pathway in the fly's response to HDS, an observation consistent with a role in mammalian diabetic nephropathy (Degrell et al., 2009; Goldberg et al., 2006; Zhang et al., 2004). This includes overall insulin resistance, cardiac dysfunction, and nephrocyte dysfunction. Here, we demonstrate a key role for hexosamine flux in regulating the Pc-G complex. Previous work demonstrated direct O-GlycNAcylation of Polyhomeotic; our data demonstrate the consequences of this step in Sns expression. In the course of our studies with HDS we also observed strongly increased O-GlycNAcylation of Sns itself that is dependent on Sxc (Figures S5A–S5C); our data further identified an OGT/Sns complex (Figure S5D). This observation may explain why, in animals fed HDS, overexpression of exogenous Sns protein failed to stably localize to the nephrocyte surface (data not shown). Detailed structure/function studies will be required to determine whether hexosamine flux regulates Sns at the level of post-translation modification as well as transcriptionally.

One puzzling difference between mammals and flies is the expression of EBF2 and Knot. In mice and humans, EBF2 protein is upregulated and, at least in mice, this reflects upregulation of the *ebf2* transcript; both accumulate EBF2 protein in the nucleus (Figure 6). *Drosophila* fed HDS also exhibited upregulation of

Knot protein in the nucleus in HDS (Figure 6B) but, surprisingly, downregulation of *knot* transcript (Figure S3). The latter is especially puzzling, as *knot* knockdown reversed the effects of HDS, indicating that Knot is functionally upregulated in response to HDS. Perhaps this reflects an alternative splice form of *knot* that mediates sugar response or feedback downregulation of the transcript. We also note that genotypically *ebf2*$^{-/-}$ mice did not display gross morphological or functional defects in contrast to loss of *ebf1* (Fretz et al., 2014). This suggests that either the two paralogs have different roles in glomerular development and function or redundancy masks some of Ebf2's activities.

Current treatments for DKD focus on control of blood glucose and blood pressure to reduce microvascular injury, hypertension, and proteinuria. Therapies include angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs). These drugs slow, but do not halt, progression of the disease. Emerging evidence suggests that "metabolic memory" involving prior exposure of target cells to high blood glucose may play a major role in diabetes and its complications (Ling et al., 2008; Villeneuve and Natarajan, 2010; Villeneuve et al., 2008). Metabolic memory presumably reflects epigenetic chromatin regulation; Pc-G may serve as a link between diet (O-glycosylation) and epigenetic regulation (Gambetta et al., 2009). Administering factors such as the Don compound that reduce hexosamine flux could mitigate cardiovascular and renal damage by opposing the O-GlycNAcylation effects of hyperglycemia.

EXPERIMENTAL PROCEDURES

Additional experimental procedures are provided in the Supplemental Experimental Procedures.

Human Diabetic Specimens

Samples were collected without patient identifiers from archived kidney biopsy specimens and from nephrectomies at the Perelman School of Medicine at the University of Pennsylvania. We selected cases with biopsy-proven diabetic nephropathy and focal segmental glomerulosclerosis with significant proteinuria. The study was approved by the institutional review board.

Primary Podocyte Isolation and Cell Culture

Primary glomeruli from wild-type and Ebf2-deficient mice were isolated using Dynabeads as described previously (Krtil et al., 2007; Takemoto et al., 2002). Podocytes were cultured for 3 days, then cultured with glucose for 1 day (RT-PCR) or 3 or 6 days (imaging). See the Supplemental Experimental Procedures for details. The study was approved by the institutional animal care and use committee.

Dextran Uptake Assay

Pericardial nephrocytes were dissected from adult *Drosophila*, incubated with Alexa-488- or Alexa-Fluor-568-Dextran beads for 15 min, rinsed, and imaged. See the Supplemental Experimental Procedures for details.

Microarray Data

See Woroniecka et al. (2011) for human microarray data (accession GEO: GSE-30122). *Drosophila* glucosamine microarray analysis is described in the Supplemental Experimental Procedures

ACCESSION NUMBERS

Microarray data have been deposited to the NCBI GEO and are available under accession number GEO: GSE-69815.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.06.056.

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