Src64B phosphorylates Dumbfounded and regulates slit diaphragm dynamics: *Drosophila* as a model to study nephropathies

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**ABSTRACT**

*Drosophila* nephrocytes are functionally homologous to vertebrate kidney podocytes. Both share the presence of slit diaphragms that function as molecular filters during the process of blood and haemolymph ultrafiltration. The protein components of the slit diaphragm are likewise conserved between flies and humans, but the mechanisms that regulate slit diaphragm dynamics in response to injury or nutritional changes are still poorly characterised. Here, we show that Dumbfounded/Neph1, a key diaphragm constituent, is a target of the Src kinase Src64B. Loss of Src64B activity leads to a reduction in the number of diaphragms, and this effect is in part mediated by loss of Dumbfounded/Neph1 tyrosine phosphorylation. The phosphorylation of Duf by Src64B, in turn, regulates Duf association with the actin regulator Dock. We also find that diaphragm damage induced by administration of the drug puromycin aminonucleoside (PAN model) directly associates with Src64B hyperactivation, suggesting that diaphragm stability is controlled by Src-dependent phosphorylation of diaphragm components. Our findings indicate that the balance between diaphragm damage and repair is controlled by Src-dependent phosphorylation of diaphragm components, and point to Src family kinases as novel targets for the development of pharmacological therapies for the treatment of kidney diseases that affect the function of the glomerular filtration barrier.

**KEY WORDS:** Dumbfounded, Src64B, Slit diaphragm

**INTRODUCTION**

*Drosophila* nephrocytes, the cells involved in removal of waste products from the haemolymph, have a filtration diaphragm that shares similarities at the ultrastructural, molecular and functional levels with the vertebrate slit diaphragm (SD), the molecular filter of the vertebrate kidney glomerular filtration barrier (Prieto-Sánchez, 2009; Weavers et al., 2009; Zhuang et al., 2009). Nephrocytes are highly specialised cells whose surface is covered by extensive membrane invaginations, the labyrinthine channels, where most of their endocytic activity takes place. The entrance to these channels is capped by a filtration diaphragm, 40 nm wide and ultrastructurally similar to the SD, that restricts the passage of macromolecules according to their size. The extracellular domains of Dumbfounded (Duf, also known as Kirre) and Sticks and stones (Sns) [the *Drosophila* orthologues of Neph1 (also known as Kirrel) and nephrin, respectively], which are the main components of the filtration diaphragm, contribute to the structure of the porous filter. Their intracellular domains interact with other proteins of the SD protein complex in a way similar to that found in the vertebrate SD (Benzing, 2004; Patrakka and Tryggvason, 2007). Thus, Duf interacts with Polychateoid (Pyd), the orthologue of ZO-1, and Sns interacts with Cindr, the orthologue of CD2AP (Weavers et al., 2009).

In addition to its well-established structural role, the vertebrate SD also functions as a signalling node that controls fundamental aspects of podocyte biology such as transcriptional regulation, cytoskeleton dynamics, cell survival and endocytosis (reviewed by Aaltonen and Holthöfer, 2007; Benzing, 2004; Garg et al., 2007a; Patrakka and Tryggvason, 2010). The importance of SD integrity for kidney ultrafiltration is highlighted by the fact that congenital and acquired glomerular diseases that affect the SD severely alter the morphology of the podocytes, inducing the regression of the foot processes, a phenomenon known as foot process effacement, that results in massive proteinuria (Patrakka and Tryggvason, 2009). However, the molecular mechanisms that lead to foot process effacement and proteinuria are far from being understood (Kriz et al., 2013). It is known that podocyte injury associates with changes in the levels of nephrin and Neph1 phosphorylation. However, published data are contradictory, as some authors claim that podocyte injury results in an increase in nephrin and Neph1 tyrosine phosphorylation, whereas others, using the same experimental model of induced nephrosis, found a correlation between podocyte damage and a decrease in phosphorylation (reviewed by Hattori et al., 2011). Therefore, it is not clear whether increase or decrease of nephrin and Neph1 phosphorylation is associated with injury and whether injury is the cause or the consequence of changes in nephrin and Neph1 phosphorylation. In *Drosophila*, mutations in *duf* and *sns* also result in loss of filtration diaphragms and in radical changes in nephrocyte architecture and function (Prieto-Sánchez, 2009; Weavers et al., 2009; Zhuang et al., 2009). Taking into account all the similarities previously described, we proposed that the nephrocyte filtration diaphragm could be a suitable model with which to study the formation, maintenance and repair of the vertebrate SD (Weavers et al., 2009) (a view supported by Dow and Romero, 2010; Simons and Huber, 2009; Zhang et al., 2013).

In this study, we investigate whether regulation by phosphorylation of one of the main constituents of the diaphragm occurs in *Drosophila* nephrocytes. We find that Src64B is responsible for Duf tyrosine phosphorylation in nephrocytes and that its activity regulates Duf association with the actin regulator Dock. Using loss- and gain-of-function experiments, we show that filtration diaphragm stability requires physiological levels of Src64B activation. Thus, *Src64B* loss-of-function mutant nephrocytes present less SD and altered morphology, including nephrocyte agglutination and partial regression of labyrinthine channels, whereas hyperactivation of Src64B leads to massive loss of SD. Interestingly, diaphragm injury caused by both attenuation or hyperactivation of Src64B can be repaired upon return to physiological levels of Src64B activity. We also show that the
puromycin aminonucleoside (PAN) model of induced nephrosis, which is extensively used in vertebrates, is applicable to Drosophila and that the resulting injury to nephrocytes correlates with increased activity of Src64B. Our results imply that hyperactivation of Src family kinases (SFK), and hence hyperphosphorylation of SD constituents, is a cause of diaphragm injury. In this manner, our findings suggest that Src family kinases (SFK) should be considered as novel targets for the development of new therapies in the treatment of congenital and acquired glomerular diseases associated to dysfunction of the SD.

RESULTS

Dumbfounded is tyrosine phosphorylated by Src64B

In vertebrates, the slit diaphragm components nephrin and Nep1 are transiently phosphorylated on tyrosine residues during development and after podocyte injury (Harita et al., 2008; Lahdenperä et al., 2003; Verma et al., 2006; Verma et al., 2003). We therefore anticipated that Duf, the Drosophila orthologue of Nep1, might also be a substrate for tyrosine phosphorylation. This was indeed the case, as Duf was detected with anti-phosphotyrosine antibodies in protein extracts of Drosophila S2 cells transiently transfected with Duf-GFP.

Src64B is responsible of Duf phosphorylation in nephrocytes

To identify the tyrosine kinases responsible for endogenous Duf phosphorylation, we analysed whether the two Drosophila Src orthologues Src42A and Src64B, as well as the cytoplasmic kinases Btk29A (Btk family kinase), Csk (C-terminal Src kinase)
and Shark (Src homology2, ankyrin repeat, tyrosine kinase) were expressed in third instar larval nephrocytes. We found that Src42A, Src64B and Btk29A were strongly expressed at this stage, whereas Csk and shark were expressed at low or undetectable levels (supplementary material Fig. S1). Next, we used UAS-RNAi lines for these kinases to attenuate their expression in larval nephrocytes (see supplementary material Fig. S1 for control of Src42A and Src64B attenuation). To identify the requirements for Src42A, Src64B and Btk29A, we analysed the consequences of loss-of-function conditions for these genes on the distribution of both Duf and Pyd in nephrocytes. We expected that interfering with Duf phosphorylation could have an effect on the regulation and/or stability of the Duf-Pyd complex (Prieto-Sánchez, 2009; Weavers et al., 2009). Expression of UAS-RNAi-Src42A or UAS-RNAi-Btk29A in nephrocytes (by using Pros-Gal4 that drives expression in garland nephrocytes from stage 16 onwards) (Weavers et al., 2009) did not significantly affect Duf or Pyd localisation (supplementary material Fig. S1 and not shown). However, attenuation of Src64B induced nephrocyte agglutination and redistribution of both Duf and Pyd proteins. Thus, in contrast to control nephrocytes (Fig. 1D), Src64B attenuated nephrocytes maintained many cell contacts between them and although Duf and Pyd were still present at the cell surface, both proteins accumulated strongly at cell contact membranes and could be also detected intracellularly (arrowheads and arrows in Fig. 1E). Moreover, Duf from Src64B attenuated nephrocytes showed a strong reduction in tyrosine phosphorylation relative to wild type (Fig. 1F). To verify that Src64B phosphorylates Duf, S2 cells were transiently co-transfected with tagged full-length Duf (Duf-GFP) and either wild-type Src64B, a kinase-constitutively active form (Y547F) or a kinase-dead variant (K312R) (Kussick and Cooper, 1992). We observed an increase in tyrosine phosphorylation of Duf in Src64BKO nephrocytes. The asterisks in A-B′ show the cell enlarged in the insets; the bars in C and D show the extension of the cortical region; the rectangles in C-E show the regions enlarged in C′,D′ (1), D′ (2) and E′. N, nucleus; e, cisterna of rER; v, vacuole. n refers to number of larvae examined, each containing ~20 nephrocytes.
Src64B is required to maintain the integrity of the nephrocyte filtration diaphragm

To further evaluate the requirement for Src64B-mediated phosphorylation in nephrocytes, we analysed amorph Src64BKO mutants (O’Reilly et al., 2006). The observed defects were more severe than those induced by RNAi-mediated attenuation (Fig. 2A,B). The close apposition of mutant nephrocytes (Fig. 2B,E) could be indicative of a fusion event. However, examination of these cells revealed that they were separated by cell membranes (arrowheads in Fig. 2B′,E′) that accumulated both Duf and Pyd (Fig. 2B′). In addition, the nephrocyte membranes exposed to haemolymph showed reduced amounts of Duf and Pyd complexes (arrowheads in insets, Fig. 2B,B′) which suggested a lower density of filtration diaphragms. Furthermore, Duf and Pyd were ectopically present inside the nephrocyte (arrows in Fig. 2B′) and in some instances both proteins could be detected in internal protrusions at the cortical region (arrowheads in Fig. 2B″).

These phenotypes were further analysed at the ultrastructural level. Transmission electron micrographs of Src64KO nephrocytes revealed changes in their overall morphology (Fig. 2C-E). The most significant were the shortening and reduction in numbers of labyrinthine channels (bars in Fig. 2C,D, details in 2C′,D′ and quantification in 2F) with the consequent displacement of vacuoles and organelles towards the periphery of the cell and nephrocyte agglutination (Fig. 2E). We also observed regions practically devoid of diaphragms (arrowheads in Fig. 2D′) and more than one diaphragm closing the entrance of the labyrinthine channels (arrowheads in Fig. 2D″). The formation of ectopic diaphragms, which is never observed in wild-type nephrocytes, correlates with the accumulation of Duf and Pyd at internal protrusions from the membrane (Fig. 2B″) and could reflect their internalisation. In addition, electron-dense material, reminiscent of adherens junctions, was observed at the membrane in regions of nephrocytes apposition (Fig. 2E′). Scanning electron microscopy of Src64KO nephrocytes showed a surface smoother than in wild-type cells (Fig. 2G,H), confirming a reduction of labyrinthine channels. Finally, tyrosine phosphorylation of Duf in Src64BKO nephrocytes was significantly reduced (Fig. 2I). Together, these results indicate that Src64B is the SFK that phosphorylates Duf in nephrocytes and that Src64B is required to maintain nephrocyte diaphragm stability, regulating the localisation of the Duf-Pyd complex at the membrane exposed to haemolymph.

Phosphorylation of Tyr810 is critical for Duf function in nephrocytes

Duf contains five tyrosine residues susceptible to being phosphorylated (see Materials and methods). When each of these...
tyrosines was mutated to alanine and transfected into S2 cells, only Duf-Y638A, Duf-Y724A and Duf-Y810A showed reduced tyrosine phosphorylation (40%, 20% and 30% relative to wildtype, respectively), suggesting that these residues are phosphorylated in S2 cells (Fig. 3A; data not shown). Significantly, Tyr810 (FSAIYGNPY) fulfils the consensus for a Src kinase substrate motif (pY[A/G/S/T/E/D]) and also for a -SH2 domain binding motif (pYXXN).

Overexpression (Pros-Gal4) of Duf-Y638A, Duf-Y705A, Duf-Y724A and Duf-Y814A caused no apparent effect, as is the case for the wild-type Duf protein (Fig. 3B and data not shown). However, overexpression of Duf-Y810A induced nephrocyte agglutination (Fig. 3C) similar to that generated by overexpression of a Duf protein lacking its intracellular region (Duf-ext; Fig. 3D). As these transgenic lines are inserted at the same chromosomal site, the differences in phenotype cannot be attributed to different levels of overexpression, and therefore the similarity between Duf-Y810A and Duf-ext overexpression suggest that impeding Duf phosphorylation at Tyr810 affects duf function in a similar manner to the deletion of its intracellular region. The contribution of Tyr810 phosphorylation to duf function was verified in rescue experiments in a duf loss-of-function alleles, we resorted to RNAi-mediated attenuation to analyse its role in nephrocytes. Dysregulation of Src64B induces occasional agglutination of nephrocytes (arrowhead) and the delocalisation of Duf and Pyd towards the interior of the cell (n=12).

Fig. 4. Dock associates with Duf phosphorylated in Tyr810. (A) dock expression in wild-type larval nephrocytes (n=5). (B) Immunoblot analysis of S2 cells co-transfected with either the wild-type or the Y810A Duf variant and Dock in the presence of wild-type Src64B or the kinase-dead variant Src64B K312R as indicated. Dock co-immunoprecipitates with wild-type Duf (lanes 1, 2) in a phosphorylation-dependent way: the amount of Dock immunoprecipitated in S2 cells increases in the presence of ectopic Src64B (lane 2) in comparison with endogenous S2 Src64B (lane 1), whereas there is no interaction in the presence of Duf Y810A (lane 3) or Src64B K312R (lane 4). (C) Attenuation of dock in nephrocytes induces occasional agglutination of nephrocytes (arrowhead) and the delocalisation of Duf and Pyd towards the interior of the cell (n=12). Duf and Pyd granules are segregated (arrows) in the medial sections. The right panels show higher magnifications of the nephrocyte depicted in the top-left panel by an asterisk. n refers to number of larvae examined, each containing ~20 nephrocytes.

Phosphorylation of Tyr810 is crucial for Duf interaction with Dock

Next, we searched for candidate proteins that bind to Duf upon tyrosine phosphorylation. As Tyr810 fulfils the consensus for an SH2 domain-binding motif, we investigated the SH2-containing adaptor proteins Drk (Downstream of receptor kinase) and Dock (Dreadlocks), the Drosophila orthologues of Grb2 and Nck, on the basis of their ability to associate with Neph1 and nephrin, respectively (Harita et al., 2008; Jones et al., 2006; Verma et al., 2006). As dock and drk were expressed in larval nephrocytes (Fig. 4A and not shown), we examined whether their products associated with Duf in a phosphorylation-dependent way. S2 cells were co-transfected with GFP-tagged Duf (wild-type or duf-Y810A) and either V5-tagged Drk or V5-Dock, together with or without Src64B. Dock (Fig. 4B), but not Drk (not shown), was co-immunoprecipitated with phosphorylated Duf. Strong interaction was found only in the presence of exogenous Src64B kinase (lane 2). In its absence (lane 1) or in the presence of the kinase-dead variant Src64B K312R (lane 4), weak or no interaction was detected, respectively. Furthermore, replacing Tyr810 by Ala completely abolished Duf-Dock interaction (lane 3). Hence, Tyr810 and its phosphorylation are crucial for Duf-Dock association.

Drosophila Dock acts as an adaptor of the actin cytoskeleton (Rao and Zipursky, 1998); thus, a role for Dock for nephrocyte cell architecture could be anticipated. Owing to the embryonic lethality of dock loss-of-function alleles, we resorted to RNAi-mediated attenuation to analyse its role in nephrocytes. dock-attenuated nephrocytes showed fewer Duf-Pyd complexes at the membrane. Most interestingly, Duf/Pyd complexes redistributed towards the interior of the cell (Fig. 4C), suggesting that Dock recruitment by Duf mediates cytoskeleton rearrangements that are important for the stability of the filtration diaphragm.

Hyperactivation of Src64B induces nephrocyte agglutination and loss of filtration diaphragms

After establishing the importance of Duf tyrosine phosphorylation for the stability of the diaphragm, we examined the effect of Src64B hyperactivity. Overexpression of the constitutively active form of Src64B (UAS-Src64BY547F) induced extreme nephrocyte agglutination (Fig. 5A,B). This was accompanied by severe loss of filtration diaphragms, visualised as a large decrease of Duf and Pyd at the nephrocyte surface and their accumulation in the interior of these cells (Fig. 5A). Moreover, Duf from these experimental nephrocytes displayed increased tyrosine phosphorylation (Fig. 5C; Fig. 7). The severity of the phenotype might indicate cell death.
However, when UAS-Src64BY547F was co-expressed with UAS-Apoliner, a vital reporter of apoptosis (Bardet et al., 2008), no signals of nephrocyte apoptosis were evident (supplementary material Fig. S3). We further studied the effect of Src64B hyperactivity on nephrocyte architecture by transmission electron microscopy (Fig. 5D,E). Sections at the nuclei level revealed dramatic changes in nephrocyte organisation: the cells lacked a cortical region (Fig. 5D), due to the absence of labyrinthine channels and filtration diaphragms (Fig. 5D′); the organelles appeared close to the membrane (Fig. 5D′′); the basement membrane was thicker with occasional inclusions (Fig. 5D′′′ bar and arrow, compare with 5F); and the cytoplasm contained intracellular structures composed of membrane fragments (arrow in Fig. 5D′′). Furthermore, there were membranes separating adjacent nephrocytes with regions rich in adherens junctions (arrow in Fig. 5D′), which accumulate Duf and Pyd (Fig. 5E). In summary, either reduced (as in Src64BKO mutants) or increased (overexpression of activated Src64B) Src64B activity in nephrocytes causes destabilisation of the filtration diaphragm complex and changes nephrocyte architecture, including loss of labyrinthine channels and agglutination of adjacent nephrocytes. These changes are concomitant with changes in the levels of Duf tyrosine phosphorylation and redistribution of Duf-Pyd complexes from the nephrocyte surface towards the interior of the cell and regions of cell-cell contact.

Nephrocyte injury correlates with Src64B hyperactivation and its recovery requires physiological activity of Src64B

Puromycin aminonucleoside-induced nephrosis (PAN) in rodents is a well-established model of renal injury that causes characteristic morphological changes, which are associated with rearrangement of the cytoskeleton, and proteinuria (Caulfield et al., 1976; Luimula et al., 2002). These changes have been correlated with alterations in the
levels of phosphorylation of nephrin and NepH1, although there is disagreement over whether injury is associated with an increase (Garg et al., 2007b; Harita et al., 2008) or decrease (Jones et al., 2006) of phosphorylation. To examine the relationship between nephrocyte injury and levels of Src64B activation, we reproduced the PAN model of nephrosis in flies. We found that nephrocytes dissected from third instar larvae cultured in food supplemented with puromycin aminonucleoside showed a mild degree of agglutination (Fig. 6A,C, compare with Fig. 2A) and Duf/Pyd complexes delocalised from the membrane towards more internal regions (arrowheads in Fig. 6A′), most likely owing to internalisation. Unequivocally, these structural changes were accompanied by Src64B hyperactivity, revealed by anti-pY434 Src64B staining, specific for activated Src64B (Fig. 6C, compare with 6B). Next, we used the TARGET system (McGuire et al., 2003), which permits spatiotemporal control of gene expression, to induce conditional damage to nephrocytes by Src64B hyperactivation. Tubulin-GAL80Δ; Pros-Gal4::UAS-Src64BY547F individuals were raised at permissive temperature until the onset of third instar larval stage (T0). Gal80 was inactivated by shifting to 29°C for 10 hours. Nephrocytes were dissected 1 hour after returning to 17°C (T1) and every 24 hours thereafter (T2-T4). (B) Immunostaining of nephrocytes with anti-Duf and anti-Pyd antibodies. T0 nephrocytes were undistinguishable from the wild type (n=6). T1 nephrocytes presented strong agglutination and loss of filtration diaphragms (n=9, see also supplementary material Fig. S2). T2 nephrocytes showed a clear recovery, manifested by mild agglutination and partial re-localisation of Duf/Pyd complexes at the outer membrane, n=14. Recovery was almost complete at T3 (n=10) and totally achieved at T4 (n=7). Left panels show z-stack projections of representative strings of nephrocytes; right panels show cortical and medial sections of the nephrocyte, indicated by an asterisk in the left panels. Arrowheads indicate the outer membrane. (C) Profile of Duf tyrosine phosphorylation and Src64B activation at different experimental times. Western blot and immunoblot analyses showed that overexpression of Src64BY547F resulted in a strong hyperactivation of Src64B and in Duf hyperphosphorylation (see graph), and in a dramatic reduction in the levels of Duf in nephrocytes. Return to physiological activity of Src64B resulted in recovery of Duf levels and in the state of phosphorylation. n refers to number of larvae examined, each containing ~20 nephrocytes.

Fig. 7. Hyperactivation of Src64B induces reversible nephrocyte damage that correlates with hyperphosphorylation of the filtration diaphragm component Duf. (A) Experimental procedure used to manipulate Src64BY547F expression temporally in Tubulin-GAL80Δ; Pros-Gal4::UAS-Src64BY547F individuals. Data presented refer to three independent experiments. At 17°C, Gal80 prevents GAL4-dependent activation of UAS-Src64BY547F. Individuals were raised 17°C until the onset of third instar larval stage (T0). Gal80 was inactivated by shifting to 29°C for 10 hours. Nephrocytes were dissected 1 hour after returning to 17°C (T1) and every 24 hours thereafter (T2-T4).
DISCUSSION

The dissection of the molecular mechanisms involved in the regulation of SD stability in mammals has been hindered by access to podocytes in vivo and by the requirement of podocyte functional integrity for their viability. Thus, it is still unknown how SD assemblies during development and after injury, and which are the major players that regulate the dynamic behaviour of SD. Both issues are fundamental to understanding the molecular basis for most diseases that lead to end-stage renal failure (Patrakka and Tryggvason, 2009). Given the high degree of similarity between the podocyte slit diaphragm and the nephrocyte filtration diaphragm at the molecular, ultrastructural and functional levels (Prieto-Sánchez, 2009; Weavers et al., 2009; Zhuang et al., 2009), we decided to use the fly nephrocyte to study in the whole organism which aspects of podocyte development and behaviour affect SD integrity under physiological and pathological conditions. In this work, we show that the stability of the filtration diaphragm is regulated by the activity of a tyrosine kinase of the Src family, Src64B, which at least phosphorylates the main SD constituent, Duf, and controls its association to the actin regulator Dock.

Src64B phosphorylates Duf and regulates the structural integrity of the nephrocyte filtration diaphragm

Many of the vertebrate slit diaphragm proteins are tyrosine phosphorylated in normal glomeruli (Zhang et al., 2010). Furthermore, tyrosine phosphorylation of nephrin and Neph1 by the SFK Fyn is crucial for the stability of this protein complex and therefore for glomerular filtration function (reviewed by Hattori et al., 2011). Thus, upon phosphorylation, the cytoplasmic regions of nephrin and Neph1 recruit the intracellular adaptors Nck and Grb2, among others, that in turn regulate actin cytoskeleton reorganisation (Harita et al., 2008; Jones et al., 2006; Tryggvason et al., 2006; Verma et al., 2003). We found that the post-translational regulation by phosphorylation of Duf, the orthologue of Neph1, is conserved and that Src64B, a member of the non-receptor Src family tyrosine kinases, is responsible for Duf tyrosine phosphorylation in nephrocytes. Furthermore, Src64B function is necessary for the structural integrity of the filtration diaphragm and for normal nephrocyte morphology. In Src64B loss-of-function or knockdown conditions, there is a reduction in the density of filtration diaphragms at the nephrocyte cell membrane. Presumably, this is due in part to their internalisation, as suggested by the accumulation of both Duf and Pyd at protrusions extending inwards from the membrane and the presence in these locations of structures dense to electrons reminiscent of filtration diaphragms. In addition, we observe delocalisation of Duf/Pyd complexes to cell contact membranes that, at the ultrastructural level, are rich in adherens junctions. The presence of adherens junctions in apposed membranes is never found in wild-type mature nephrocytes but is characteristic of embryonic nephrocytes prior to the formation of filtration diaphragms (Prieto-Sánchez, 2009). All these alterations are concomitant with changes in nephrocyte architecture, revealed by their smoother surface and their agglutination. As the loss of filtration diaphragms result in regression of labyrinthine channels, and this is always associated with nephrocyte agglutination, we interpret these morphological changes as being due to reallocation of Duf/Pyd complexes from SD to adherens junctions. We suggest that these morphological alterations are the nephrocyte equivalent of podocyte foot process effacement, a feature common to all proteinuric diseases and believed to be initiated by changes in the actin cytoskeleton.

Scr64B-mediated phosphorylation of Duf regulates its coupling to the cytoskeleton

Although Src64B might be involved in phosphorylation of other components of the filtration diaphragm, including the fly nephrin orthologue Sns, our data suggest that Duf is a main target of Src64B-mediated phosphorylation and participates in the reorganisation of the actin cytoskeleton. We find that, upon phosphorylation of residue Tyr810, Duf can associate with the adaptor protein Dock, the Drosophila orthologue of Nck, and that phosphorylation of this residue is important for Duf function, thus reproducing in nephrocytes the connection between SD and actin cytoskeleton existing in podocytes. Our data also show that attenuation of dock leads to dissociation of the Duf/Pyd complex, although further studies will be necessary to reveal the molecular basis of these observations. These results indicate that, through the regulation of Duf-Dock interactions, Src64B controls the coupling of SD protein complex to the actin cytoskeleton, and point to the modulation of SFKs activity as a promising therapeutic target for the treatment of proteinuric renal diseases.

Drosophila nephrocytes as a model of induced nephrosis

The advance in the knowledge of the molecular basis of human diseases and their treatment constantly demands the development of reliable and genetically amenable preclinical animal models, valid for genetic screenings and drug discovery. The success of such preclinical models relies on the rigorous assessment of the reproducibility of the human disease under study in the animal model. We show here that this is the case for Drosophila melanogaster and the study of some nephropathies. The PAN model, which induces foot process effacement and proteinuria, has become a mainstay in the study of podocyte dysfunction (Pippin et al., 2009). Thus, the early response of podocytes to PAN is to change the levels and distribution of SD proteins such as nephrin and podocin, followed by flattening of foot processes and proteinuria (Guan et al., 2004; Luimula et al., 2002). We reasoned that if the analogy between podocyte and nephrocyte filtration diaphragms was genuine and the earliest targets of PAN were SD components, then the nephrocyte diaphragm should react to PAN treatment in a similar way. Interestingly, this is what we observed. Drug treatment induced delocalisation of Duf and Pyd from the outer membrane and a mild degree of nephrocyte agglutination. Moreover, this treatment has allowed us to firmly establish a correlation between induced nephrocyte damage, Src64B hyperactivity and an increase in the phosphorylation of SD components, in agreement with some vertebrate data (Garg et al., 2007b; Harita et al., 2008). These results confirm the similarity between both cell types and validate the use of Drosophila to study SD behaviour during development and in pathological conditions.

Scr64B hyperactivity causes reversible nephrocyte damage

PAN treatment did not allow us to solve the controversial subject of the causality between diaphragm injury and changes in phosphorylation of SD components, as continuous exposure to the drug could mask possible attempts of diaphragm repair. Therefore, we used the advantages of Drosophila to manipulate gene function spatiotemporally to induce the expression of the constitutively active form of Src64B for a short temporal window and check whether it provoked nephrocyte damage. This allowed us to establish a causal relationship between Src64B hyperactivity and injury, and returning to permissive temperature permitted analysis of diaphragm recovery in the absence of further induced damage. In this way we could establish that Src64B hyperactivity causes an increase in Duf phosphorylation and a dramatic decrease in the total amount of this
redistribution to cell contact membranes, where they contribute to take place, there is an internalisation of the SD components and their association between SD and the cytoskeleton is weak or does not membrane, sealing the labyrinthine channels. We suggest that when which could be necessary to stabilise the complex at the outer link between the diaphragm protein complex and the cytoskeleton, the main diaphragm components (Sns, Duf, Pyd) but in regulating the physiological levels present in mature nephrocytes.

In summary, our results indicate that, in Drosophila, the stability of the filtration diaphragm depends on the activity of the SFK Src64B, which phosphorylates Duf. Phosphorylation of Duf creates a new docking site for its association with Dock, a regulator of actin cytoskeleton. This interaction stabilises the slit diaphragm complex at the outer membrane, capping the entrance to the labyrinthine channels. (B) In Src64B flies, failure to establish a stable link between the slit diaphragm complex and the cytoskeleton entails the mobilisation of their constituents towards sites of cell contact, inducing nephrocyte agglutination. (C) Src64B hyperactivation, induced by overexpression of activated Src64B or as result of external agents that result in nephrocyte injury, leads to an increase in the levels of Duf phosphorylation, its mobilisation towards cell contact membranes and its internalisation for degradation.

Materials and methods
Fly stocks
Flies were reared at 25°C, except when specifically indicated. The following Drosophila strains were used: Oregon R, Df(1)Dufapos (deficiency made using the P elements P[XP]B2829 and pBAC[RB]P0355 that eliminates 52 kb of DNA, including Duf coding region) (Prieto-Sánchez, 2009), Src64Bapo, UAS-Src64B Y547F (A. O’Reilly, Fox Chase Cancer Center, USA), UAS-apolliner (J. P. Vincent, MRC, UK), UAS-Src64B RNAi (DGRC, Japan), UAS-Src64B K312R (L. G. Fradkin, LUMC, The Netherlands), UAS-Src42A RNAi, UAS-DufK29 RNAi, UAS-DufK29 RNAi and UAS-Nck RNAi (VDRC, Austria), Apterix-GAL4, UAS-GFP, prospero-GAL4 (C. Doe, HHMI, USA), tubulin-GAL80: prospero-GAL4 and sns-GC-GAL4 (S. Abmayr, SIMR, USA).

Cell culture and transfections
Drosophila Schneider (S2) cells were grown at 25°C in Insect-X press (BioWhittaker, MD, USA) containing 7% fetal bovine serum. Cells were transfected with 2 μg of the DNAs required using the Nucleofector Amaxa procedure, as indicated in the manufacturer’s protocol (BioWhittaker). After transfection, cells were plated in six-well plates (2×10^6 cells per well) and incubated at 25°C for 48 hours. In specific cases, cells were treated with 10 mM orthovanadate (Sigma) or 5 mM PP2A inhibitor (Sigma) for 30 minutes before harvesting the cells.

Co-immunoprecipitations and western blots
Transfected S2 cells or larval nephrocytes were washed and lysed in modified ice-cold RIPA lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% DOC] supplemented with phosphatase inhibitor (1 mM NaVO_4 and 25 mM NaF) plus proteases inhibitors for 60-90 minutes at 4°C. After centrifugation (15,000 g, 15 minutes), lysate aliquots were taken to assess protein overexpression and the rest of cellular extracts were immunoprecipitated with the desired antibodies by overnight incubation at 4°C. After incubation with protein A or G agarose and extensive washing with lysis buffer, lysates and immunoprecipitates were resolved by SDS-PAGE, and the gel was transferred to nitrocellulose membranes to be probed with specific antibodies. Densitometry analysis was carried out using Bio-Rad Quantity One software. The following antibodies were used: anti-GFP (1:1000, Roche), anti-phosphotyrosine (1:1000, Millipore), anti-Duf (1:100), anti-Src64B (1:500, A. O’Reilly) or anti-V5 (1:5000, Invitrogen).

Plasmid constructs
Online prediction servers NetPhos 2.0 (Blom et al., 1999), DISPHOS 1.3 (Iakoucheva et al., 2004) and PhosphoMotif Finder (Amanchy et al., 2007) were used to identify Duf tyrosines residues susceptible to phosphorylation. Duf mutant variants Y638A, Y705A, Y724A, Y810A and Y814A were generated by PCR using as DNA template pBS-DufGFP-wt with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). PCR products were sequenced and subcloned into pAC5.1 for cell culture experiments and pUAST-attB vectors for generation of transgenic lines. EST clones LD42588 and LD12029 were used to clone V5-tagged versions of Dock and Drk, respectively, in pAC5.1 vector. Src64B wt, Src64B K312R and Src64B Y547F were gifts from J. A. Cooper (FHCRC, WA, USA).

Fig. 8. Effect of Src64B activity on the stability of the nephrocyte slit diaphragm. (A) In wild-type larval nephrocytes, Src64B phosphorylates Duf at Tyr 810, creating a novel docking site for its association with Dock, a regulator of actin cytoskeleton. This interaction stabilises the slit diaphragm complex at the outer membrane, capping the entrance to the labyrinthine channels. (B) In Src64B flies, failure to establish a stable link between the slit diaphragm complex and the cytoskeleton entails the mobilisation of their constituents towards sites of cell contact, inducing nephrocyte agglutination. (C) Src64B hyperactivation, induced by overexpression of activated Src64B or as result of external agents that result in nephrocyte injury, leads to an increase in the levels of Duf phosphorylation, its mobilisation towards cell contact membranes and its internalisation for degradation.
RNA in situ hybridisation and immunohistochemistry

Larval nephrocytes were processed for in situ hybridisation and immunohistochemistry as indicated by Weavers et al. (W. et al., 2009). The following primary antibodies were used: anti-Duf (1:100), anti-Pyd (1:100), anti-Fas3 (1:50, DSHB, IA, USA), anti-pSrc64B (1:500; A. O’Reilly) and anti-Src42A (1:1000; S. Hayashi, RIKEN Center, Japan). A 15-minute counterstain with TO-PRO-3 (1:1000, Invitrogen) was used to visualise nuclei. Confocal images were acquired using a Zeiss LSM 510 Meta or Zeiss LSM 710 microscope and processed using Adobe Photoshop CS and ImageJ software.

Electron microscopy

Samples for transmission and scanning electron microscopy were processed using standard techniques modified as described previously (Weavers et al., 2009). For immunoelectron microscopy, dissected third instar larval nephrocytes were fixed in 4% formaldehyde + 0.05% glutaraldehyde, embedded in gelatin, cryosectioned and incubated with anti-Duf (1:5) and anti-Pyd (1:5); 5 nm and 10 nm gold-conjugated secondary antibodies were used. Samples for transmission electron microscopy were observed in a Jem1010 (JEOL) instrument working at 80 kV.

Pyruvycin aminonucleoside treatment

Adult Oregon R flies were collected within the first 24 hours of their emergence and fed with standard fly food supplemented with 400 µg/ml pyruvycin aminonucleoside (Sigma). Wandering third instar larvae from their progeny were dissected for nephrocyte analysis.

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Competing interests

The authors declare no competing financial interests.

Author contributions

M.R.-G. and A.S.T. conceived and designed the experiments. A.S.T. and S.P.-S. performed the experiments. A.S.T., S.P.-S. and M.R.-G. analysed the data. M.R.-G. wrote the paper.

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Supplementary material

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