Haemocyte-derived SPARC is required for collagen-IV-dependent stability of basal laminae in *Drosophila* embryos

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Further characterization of the *SPARC*-null fly line *Df(3R)nm136*, generated by *P*-element mutagenesis, has revealed that the line carries a secondary mutation in the neuralized locus, in addition to an absence of SPARC. We have confirmed in a wild-type neuralized background that the absence of SPARC leads to a reduction of collagen IV in basal lamina in stage 17 mutant embryos. The absence of SPARC does not affect neural development during embryogenesis.

Haemocyte-derived SPARC is required for collagen-IV-dependent stability of basal laminae in *Drosophila* embryos

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Summary

SPARC is an evolutionarily conserved collagen-binding extracellular matrix (ECM) glycoprotein whose morphogenetic contribution(s) to embryonic development remain elusive despite decades of research. We have therefore used *Drosophila* genetics to gain insight into the role of SPARC during embryogenesis. In *Drosophila* embryos, high levels of SPARC and other basal lamina components (such as network-forming collagen IV, laminin and perlecan) are synthesized and secreted by haemocytes, and assembled into basal laminae. A *SPARC* mutant was generated by *P*-element mutagenesis that is embryonic lethal because of multiple developmental defects. Whereas no differences in collagen IV immunostaining were observed in haemocytes between wild-type and *SPARC*-mutant embryos, collagen IV was not visible in basal laminae of *SPARC*-mutant embryos. In addition, the laminin network of *SPARC*-mutant embryos appeared fragmented and discontinuous by late embryogenesis. Transgenic expression of SPARC protein by haemocytes in *SPARC*-mutant embryos restored collagen IV and laminin continuity in basal laminae. However, transgenic expression of SPARC by neural cells failed to rescue collagen IV in basal laminae, indicating that the presence of collagen IV deposition requires SPARC expression by haemocytes. Our previous finding that haemocyte-derived SPARC protein levels are reduced in collagen-IV-mutant embryos and the observation that collagen-IV-mutant embryos showed a striking phenotypic similarity to *SPARC*-mutant embryos suggests a mutual dependence between these major basal laminae components during embryogenesis. Patterning defects and impaired condensation of the ventral nerve cord also resulted from the loss SPARC expression prior to haemocyte migration. Hence, SPARC is required for basal lamina maturation and condensation of the ventral nerve cord during *Drosophila* embryogenesis.

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Key words: SPARC, Collagen IV, Basal lamina, Ventral nerve cord, Haemocyte

Introduction

Metazoan radiation gave rise to a complex variety of organisms with distinctive body plans, adaptations and survival strategies. This necessitated the co-evolution of specialized extracellular matrix (ECM) macromolecules capable of forming elaborate matrices that provide tissues with their unique biomechanical, biochemical and functional properties (Patthy, 2003). Among the most ancient ECM molecules are those that comprise the basal lamina, a specialized, cell-surface-associated ECM sheet underlying epithelial and endothelial cells and surrounding muscle, neural and adipose tissues (Hutter et al., 2000; Inoue et al., 1989; Merker, 1994). In addition to serving as adhesive substrata for cell adhesion and migration, basal laminae regulate signal transduction pathways through interactions with cell-surface receptors, such as members of the integrin superfamily (Bokel and Brown, 2002). Whereas the molecular complexity of basal laminae varies among tissues, the most broadly distributed components include laminin, collagen IV, perlecan, nidogen and SPARC (Erickson and Couchman, 2000; Martinek et al., 2002; Quondamatteo, 2002). Mammalian genomes encode six genetically distinct collagen IV α chains (Boutaud et al., 2000). The major embryonic and most broadly distributed isoform of collagen IV is a heterotrimer composed of two α1(IV) and one α2(IV) chain, designated as $\alpha1(IV)_2\alpha2(IV)$ (Hudson et al., 1993; Timpl, 1989). The folding and maturation of collagen IV is dependent on molecular chaperones such as the endoplasmic reticulum (ER)-resident 47-kDa heat shock protein (HSP47) (Lamandé and Bateman, 1999; Nagata, 2003). Even though embryonic expression of collagen IV begins in mouse embryos at day 5 post-coitus (Adamson and Ayers, 1979), mutations in collagen IV do not lead to developmental arrest until embryonic day (E) 10.5-11.5 (Poschl et al., 2004). Since embryonic lethality is coincident with the onset of muscle contractions, it has been hypothesized that collagen IV is required at this stage of development to provide tensile strength to basal laminae, enabling them to withstand contractile forces associated with embryonic movements (Yurchenco et al., 2004). However, the underlying cause of lethality is likely to be more complex because dynamic interactions exist between collagen IV and other basal laminae components that affect multiple signaling pathways during embryogenesis.

SPARC is a 32-35 kD Ca^{2+}-binding matricellular glycoprotein whose modular organization is phylogenetically conserved (Martinek et al., 2002). Biochemical studies indicate that SPARC binds to several collagenous and non-collagenous ECM molecules, including a Ca^{2+}-dependent interaction with network-forming collagen IV (Maurer et al., 1997; Rosenblatt et al., 1997). The

binding of SPARC to collagen IV might serve to concentrate SPARC in a subset of embryonic basal laminae (Mayer et al., 1991; Wewer et al., 1988) and basal lamina EHS tumors (Dziadek et al., 1986). However, other studies indicate that SPARC is either associated with the plasma membrane or concentrated at the interface between epithelial and basal lamina (Hunzelmann et al., 1998; Kim et al., 1997; Sage et al., 1989). Whereas the precise role of SPARC in vertebrate basal lamina assembly and maturation is poorly understood, in vivo studies indicate that the stability of the lens capsule is compromised in *SPARC*-null mice (Yan et al., 2002). The lens capsule (hereafter referred to as a basement membrane) is a continuous thick avascular collagen-IV-rich specialized basal-lamina-like matrix that surrounds the lens. In *SPARC*-null mice, cataract formation is preceded by disruptions in the ultrastructural organization of capsular collagen IV and laminin networks (Yan et al., 2002; Yan et al., 2005). Coincident with the altered matrix organization is the presence of filopodia-like cellular extensions in the lens capsule derived from cells that form the lens capsule (Norose et al., 2000; Yan et al., 2003; Yan et al., 2002).

SPARC is an integral component of most embryonic laminae in invertebrates. In the nematode *Caenorhabditis elegans*, SPARC protein is distributed in basal laminae body wall and sex muscles and overlaps with the distribution of collagen IV (Fitzgerald and Schwarzbauer, 1998). The reduction of SPARC protein production by RNA interference results in embryonic and larval lethality. We have previously reported that SPARC is a component of embryonic basal laminae in *Drosophila melanogaster* (Martinek et al., 2002). In collagen-IV-α1-mutant embryos, the level of SPARC immunostaining within haemocytes was dramatically decreased and present at very low levels in the basal laminae. We now report that inhibition of *SPARC* expression in *Drosophila* leads to several developmental anomalies, impaired ventral nerve cord (VNC) condensation and the absence of collagen IV from haemocyte-derived embryonic basal laminae.

Results

Expression of *SPARC* during embryogenesis

SPARC transcripts were first detected in cellularizing embryos by reverse transcriptase (RT)-PCR (data not shown). In situ hybridization and immunostaining revealed that *SPARC* was expressed at the onset of gastrulation and became enriched within the invaginating mesoderm (Fig. 1A). During gastrulation, expression was restricted to the mesoderm and invaginating endoderm (Fig. 1B,C). By embryonic stage (ES) 11, *SPARC* transcript was observed in the developing anterior and posterior midgut rudiments (Fig. 1D,E). During germ-band retraction, *SPARC* was expressed by migrating midgut rudiments and haemocytes (Fig. 1F) (Tomancak et al., 2005). Haemocytes are easily distinguishable from other embryonic cells by their large size and vacuoles filled with dark inclusions as a result of ingesting apoptotic cells. Embryonic haemocytes are also identified by GFP expression using the haemocyte-specific *SrpHemo-GAL4* (Bruckner et al., 2004) and *collagen-GAL4* drivers (Asha et al., 2003) (Fig. 3D-G). By ES15, the basal laminae surrounding the brain (Fig. 1J), VNC (Fig. 1L), along the ventral epidermis and the ventral cord channels (Fig. 1M,N) were enriched with SPARC secreted by migrating haemocytes. Whereas haemocytes continued to express SPARC until the end of embryogenesis, SPARC expression by the fat body

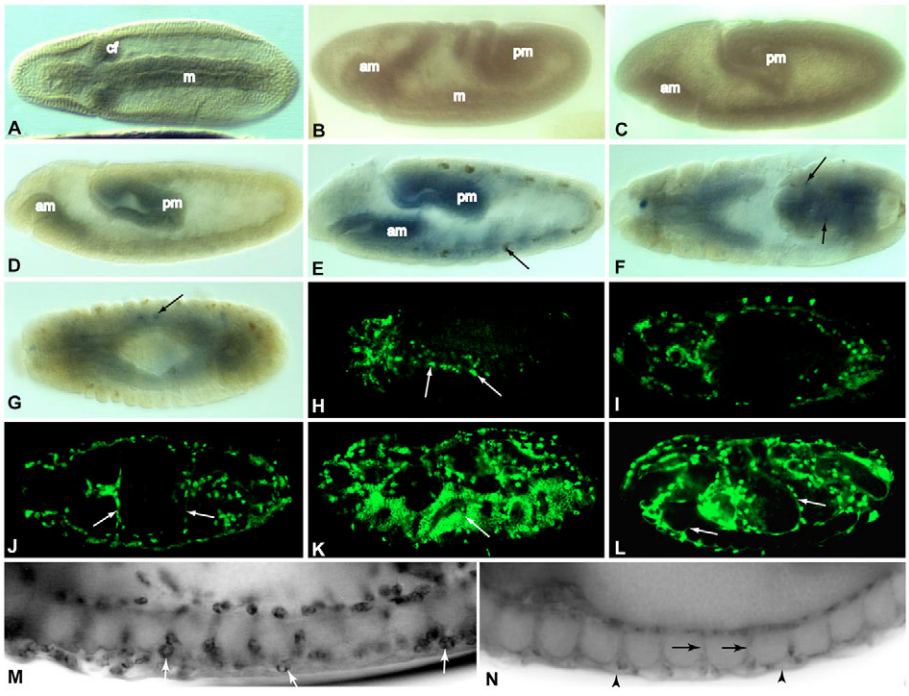

Fig. 1. Expression of *SPARC* transcript and SPARC protein during embryonic development. Transcript and protein expression overlap during gastrulation. (A-N) Wild-type embryos; A, D-G are in situ hybridizations (blue) and B, C, H-N are immunostains. (A) The ventral view of an ES7 embryo shows that *SPARC* mRNA is expressed by invaginating mesoderm (m) along the ventral midline and the cephalic furrow (cf). (B) Lateral view of an ES8 embryo shows SPARC protein expression (brown) in mesoderm (m), anterior midgut (am) and posterior midgut (pm) primordia. (C) Lateral view of an ES9 embryo shows SPARC protein expression (brown) throughout mesoderm and anterior (am) and posterior (pm) midgut primordia. (D) Lateral view of an ES11 embryo shows *SPARC* expression (blue) in midgut rudiments as they begin to migrate along the underlying mesoderm. (E) Lateral view of an early ES12 embryo shows *SPARC* expression at the onset of germ band retraction in growing midgut rudiments and mesoderm. Several neurons are also highlighted at this stage by 22C10 immunostaining (brown, arrow). (F) Dorsal view of an ES12 embryo during germ band retraction. *SPARC* is observed in migrating midgut rudiment epithelia (blue) and haemocytes (arrows). (G) Dorsal view of an ES13 embryo with fused midgut and haemocytes (arrow) expressing *SPARC*. (H) Ventral lateral view of haemocytes in an ES13 wild-type embryo. Haemocytes populate the head and begin posterior migration along the ventral nerve cord (VNC) (arrows). (I) The dorsal view of an ES14 embryo shows SPARC-positive haemocytes present throughout the embryo. (J) SPARC immunostains basal laminae around the developing brain (left arrow) and midgut (right arrow) at ES15. (K) Lateral view of an ES17 embryo expressing SPARC in the fat body (arrow) and haemocytes. (L) The same embryo as in K shows SPARC immunostaining in the basal lamina around the midgut (upper arrow), VNC channels and around the VNC (lower arrow). (M,N) Magnified views of an ES14 wild-type embryo immunostained with SPARC antibody (lateral view). (M) Haemocytes (arrows) migrating along the VNC express SPARC. Low to no SPARC immunostaining is observed in haemocyte nuclei. (N) A different focal plane reveals that SPARC colocalizes with VNC channels (arrows) and ventral epidermal (arrowheads) basal laminae. All confocal images are single sections.

was detected at ES16 (Fig. 1K), likely contributing to the accumulation of SPARC in basal laminae. SPARC was also detected in channel glia (Fig. 3A,F). The pattern of *SPARC* mRNA expression prior to ES11 has not been previously shown possibly due to the absorption of SPARC probe by the haemocytes and fat body, which express *SPARC* at high levels during late embryogenesis. The expression pattern of SPARC in haemocytes is consistent with other studies demonstrating that haemocytes secrete ECM components, such as collagen IV, laminin, tiggrin, glutactin, peroxidasin, and papilin (Fessler and Fessler, 1989; Fogerty et al., 1994; Mirre et al., 1988; Kramerova et al., 2003; Yasothornsrikul et al., 1997).

Loss of *SPARC* function results in embryonic lethality

A *P*-element mutagenesis screen was undertaken to generate a *SPARC* loss-of-function allele. The nearest *P*-element insertion to the *SPARC* locus was located within the 5′ untranslated region of the adjacent *His2A variant* gene ($H2Av^{L1602}$), ~5 kb downstream from the *SPARC* transcriptional start site (supplementary material Fig. S1A). The *H2Av* gene is maternally transcribed and zygotically expressed, and homozygotes for a deletion within the *H2Av* gene ($H2Av^{810}$) fail to pupate (Clarkson et al., 1999). We generated an excision mutation, *Df(3R)nm136*, that resulted in the loss of SPARC protein expression and embryonic lethality in 100% of homozygous embryos. Sequence analysis revealed that the *P*-element-excision event removed 1936 bp from the adjacent *H2Av* gene that is located downstream of the *SPARC* transcriptional unit (supplementary material Fig. S1A) but, surprisingly, did not extend into the *SPARC* transcriptional unit. Moreover, PCR analysis of the *Df(3R)nm136* putative promoter region with *P*-element-specific primers failed to detect *P* element signatures present after a transposition event (Castro and Carareto, 2004). Analysis of a mutant containing a shorter *P*-element-excision-induced deletion (named 72b) that did not silence the *SPARC* locus indicates that cis-regulatory element(s), located within the last exon of *H2Av* transcriptional unit are essential for *SPARC* expression (supplementary material Fig. S1A). To generate a *SPARC*-specific allele, *H2Av* expression was restored by two different transgenic lines, $H2AvD^{+t4.0}$ and *H2AvDGFP*, that have been shown previously to functionally rescue *H2Av* null mutations (Clarkson and Saint, 1999; Clarkson et al., 1999). Consistent with a phenotype due to the absence of SPARC expression, *H2Av*-rescued embryos still showed fully penetrant embryonic lethality and western blotting indicated the absence of SPARC protein in extracts from homozygous mutant embryos (supplementary material Fig. S1B). Holes in the ventral cuticle were observed in cuticle preparations of *SPARC*-mutant embryos (Fig. 2M) and were often accompanied by a loss of anterior cuticle and head-involution defects (Fig. 2L). Tracheal defects were also associated with *SPARC*-mutant embryos as they contained constricted or broken dorsal-longitudinal tracheal trunks, suggestive of a loss in tracheal integrity (supplementary material Fig. S2A-C). Additionally, ES17 *SPARC*-mutant embryos contained enlarged and distorted VNCs (Fig. 2E-H,J; Fig. 3C). Despite the hyperplastic appearance of the VNCs, immunostaining against phosphorylated histone did not differ between VNCs from wild-type or *SPARC*-

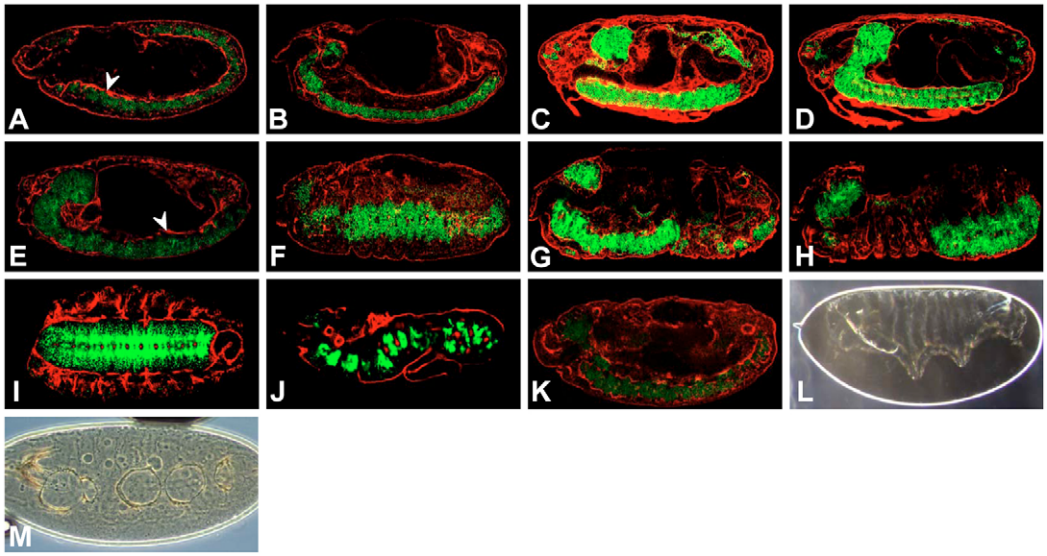

Fig. 2. Loss of *SPARC* function results in embryonic lethality and compromised basal lamina stability. (A-H) A-D Wild-type embryos A-D and *SPARC*-mutant embryos (E-H) immunostained with anti-laminin (red) and anti-neurotactin (green) antibodies to highlight the developing central nervous system (CNS). (A) Lateral view of a late ES11 wild-type embryo shows that laminin accumulates along the mesoderm-neuroepithelium interface (arrowhead). (B) Laminin sheets form around the CNS and internal organs by ES13. (C) Thicker laminin sheets surround the CNS, body-wall muscles and the digestive tract by ES17. (D) A different confocal view of the embryo shown in C. Note that the entire VNC can be seen in a single confocal section. (E) Laminin is loosely associated with the VNC because spaces are observed between the deformed CNS and the discontinuous laminin sheet (arrow) at ES15. (F) The ventral view of an ES16 embryo shows fragmented laminin immunostaining around the VNC. (G) Laminin immunostaining around the CNS and other tissues is discontinuous at ES17 in the *SPARC*-mutant embryo. (H) A different confocal plane of the embryo in shown in G shows a distorted and uncondensed VNC. (I) Ventral view of an ES17 wild-type embryo immunostained with anti-perlecan antibodies (red) shows continuous perlecan distribution around the condensed VNC. Commissures of the VNC are highlighted by anti-CNS axons antibodies (green). Perlecan immunostaining around the ventral cord is continuous. (J) The ventral view of an ES17 *SPARC*-mutant embryo also shows continuous perlecan immunostaining around the VNC. (K) Lateral view of an ES16 deficiency embryo that lacks both collagen IV genes immunostained with laminin. Laminin distribution is also discontinuous around the VNC (green). (L) Lateral view of a *SPARC*-mutant embryo showing a complete absence of ventral and head cuticle. (M) Ventral view of *SPARC*-mutant embryonic cuticle highlighting cuticle holes along the ventral surface. All images of immunostains are single confocal sections.

mutant flies (data not shown). This observation suggests that the enlarged VNC resulted from impaired VNC condensation rather than from increased cell proliferation along the VNC. Defects in VNC condensation have been observed previously in embryos with haemocyte migration defects and result in the absence of collagen IV α2/Vkg-GFP deposition in the basal lamina that surrounds the VNC (Olofsson and Page, 2005).

The *SrpHemo-GAL4* and *collagen-GAL4* driver lines were used to knock down SPARC expression in haemocytes when crossed with UAS-SPARC RNA interference (RNAi) transgenic lines. All UAS-SPARC RNAi lines tested resulted in the disappearance of SPARC protein from haemocytes but not from fat body and channel glia (Fig. 3D,F-G). However, the fat body underwent progressive fragmentation from late ES16 onwards, indicative of a loss of tissue integrity (Fig. 3F,G). Moreover, collagen IV immunostaining was punctate and only observed in circulating haemocytes (Fig. 3E, inset). SPARC also immunostained the fat body of *SrpHemo-Gal4* embryos that lack haemocytes because of the ectopic expression of *UAS-ricin* (Fig. 3H). VNC condensation defects also occurred but the VNC did not become distorted by ES17 (data not shown) unlike the *SPARC* mutant VNC. Knockdown of SPARC using *da-GAL4* also resulted in embryonic lethality and similar neural scaffold defects were observed in *SPARC*-mutant embryos (supplementary material Fig. S2D). These particular neural defects have not been previously reported in embryos with impaired haemocyte migration or absent collagen IV deposition around the VNC, which indicates that SPARC has a role in neural and/or glia differentiation before SPARC is deposited by haemocytes.

Collagen IV fails to assemble in basal laminae of *SPARC*-mutant embryos

We have shown previously that SPARC protein expression in haemocytes is dramatically reduced in collagen IV hypomorphs and that the protein is absent from basal laminae (Martinek et al., 2002). To determine whether the assembly of basal lamina is affected by the absence of SPARC, the distribution of other basal lamina components was analyzed. Laminin is expressed by a broad range of tissues during embryogenesis and, similar to SPARC and collagen

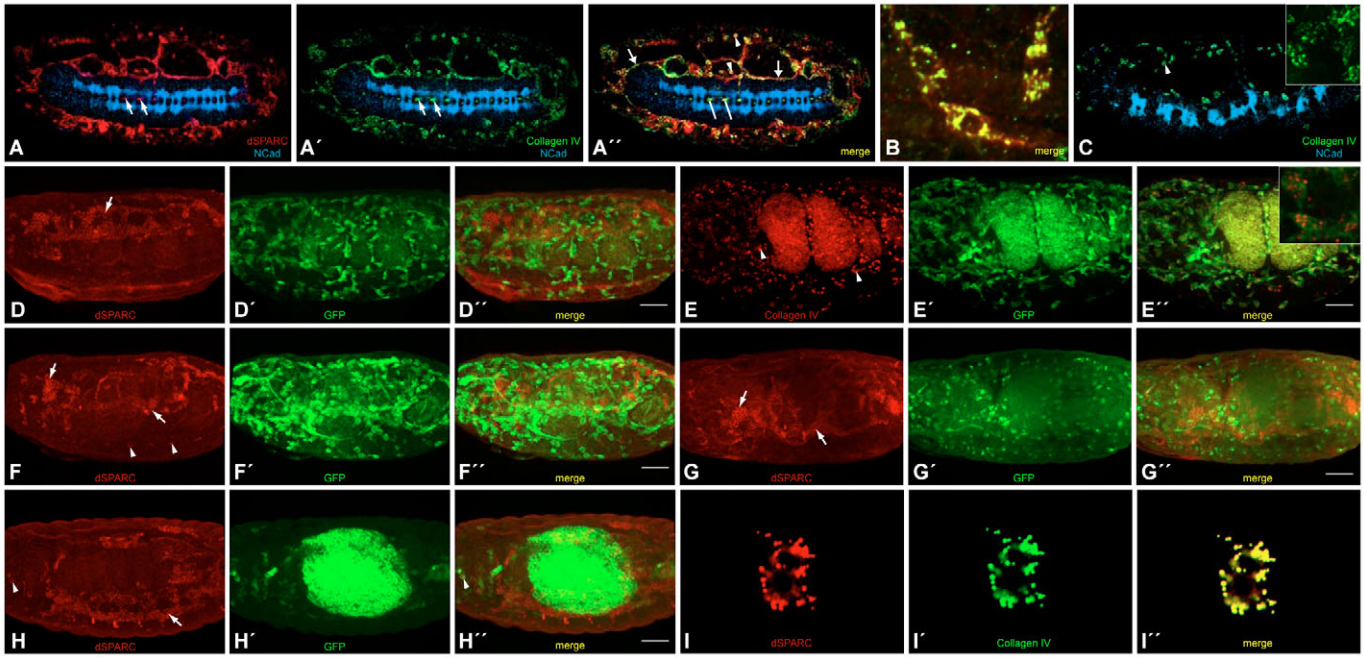

Fig. 3. Collagen IV is absent from basal laminae in SPARC-mutant embryos. (A) ES15 wild-type embryo immunostained with DN-cadherin (blue), SPARC (red) and collagen IV (A´, green). Channel glia are highlighted by arrows. A´´. The merged image shows that SPARC and collagen IV colocalize in the basal lamina surrounding the VNC (arrows), haemocytes (arrowheads) and channel glia (lines). Images in A and C are single confocal sections. (B) Projection of a magnified view of haemocytes from wild-type embryos coimmunostained with SPARC and collagen IV. (C) In an ES17 *SPARC*-mutant embryo, collagen IV is only observed in haemocytes (arrowhead). DN-cadherin highlights the deformed commissures. The intensity of collagen IV immunostaining in haemocytes (arrowheads) is similar to that in wild-type embryonic haemocytes. The inset shows a higher magnification of collagen IV-positive haemocytes. Collagen IV appears as puncta within haemocytes. (D) SPARC immunostains the fat body (arrow) *in SrpHemo-GAL4* embryos expressing the *UAS-SPARC RNAi* transgene. (D´) GFP is expressed under the control of the *SrpHemo-GAL4* driver only in haemocytes. (D´´) The merged image shows the absence of SPARC protein from haemocytes. (E) Collagen IV immunostaining (red) is only detected in haemocytes (arrowheads) in embryos of the same genotype as D. (E´) The same embryo as D shows GFP-positive haemocytes (green). (E´´) Collagen IV immunostaining colocalizes with haemocytes and basal lamina immunostaining is not observed. The inset shows a higher magnification of collagen IV puncta (red) in haemocytes (green). (F) The SPARC-positive fat body (arrows) is disorganized and fragmented in an ES17 embryo of the same genotype as D. Expression of SPARC is also observed in the channel glia (arrowheads). (F´) Haemocytes are GFP positive (green). (F´´) The merged image shows that SPARC is not expressed by haemocytes. (G) The SPARC-positive fat body (arrows) is disorganized and fragmented in the ES16 SPARC RNAi embryo. (G´) GFP is expressed exclusively in the haemocytes under the control of a *collagen-GAL4* driver. (G´´) The merged image shows that SPARC is not expressed by haemocytes. (H) A ES16 *SrpHemo-GAL4* embryo expressing *UAS-ricin*. SPARC (red) immunostaining in fat body (arrow) and the remaining haemocytes (arrowhead). (H´) Faint GFP expression is observed in a decreased population of haemocytes (green). (H´´) The merged image shows SPARC immunostaining in a few haemocytes (arrowhead). Images D-H are confocal projections. Scale bar, 10 μm. (I) S2R+ cells immunostained with SPARC (red, I) and collagen IV (green, I´) show colocalization of SPARC and collagen IV in intracellular vesicles (yellow, I´´). All images in I are single confocal sections. The following genotypes were used: C: *w;; H2AvD-GFP, Df (3R)nm136/ H2AvD-GFP, Df (3R)nm136* D-F: *w; SrpHemo-GAL4, UAS-eGFP/+; UAS-SPARC RNAi/+* G: *w; Collagen-GAL4, UAS-GFP/+; UAS-SPARC RNAi/+*. H: *w; SrpHemo-GAL4, UAS-eGFP/UAS-ricin.*

IV, is synthesized by haemocytes (Kusche-Gullberg et al., 1992). The laminin antibodies used in this study only recognized fully assembled, extracellular laminin (Fessler et al., 1987) and, thus, haemocyte immunostaining of laminin was not observed. Immunostaining of ES12 wild-type embryos revealed a laminin network that overlies the neuroepithelium (Fig. 2A). By ES14, laminin immunostaining was observed around the brain and VNC (Fig. 2B), and continued to increase in intensity such that by ES17, a uniform, continuous sheet had formed around the CNS and internal organs (Fig. 2C,D). In ES12 *SPARC*-mutant embryos, the laminin network overlying the developing VNC was indistinguishable from wild type. By ES15, however, the laminin network appeared loosely associated with the underlying VNC because gaps were present at the laminin and VNC boundary (Fig. 2E). Additionally, by ES17, the laminin network surrounding the deformed brain and VNC appeared less uniform, characterized by discontinuous laminin immunostaining along the VNC in 100% of the homozygotes (Fig. 2F-H). The distribution of perlecan, another major component of basal laminae, was also analyzed in *SPARC*-mutant embryos. Perlecan is widely distributed throughout late embryogenesis and is also observed along motorneurons and sensory organs (data not shown). Perlecan immunostaining around the VNC of both wild-type and *SPARC*-mutant embryos was unaltered by the end of embryogenesis (Fig. 2I,J). The absence of SPARC also did not affect the distribution of nidogen around the VNC (data not shown). By contrast, tissue abnormalities were not observed along the dorsal region of late-stage *SPARC*-mutant embryos.

The discontinuous distribution of laminin in *SPARC*-mutant embryos suggested that the stability of basal laminae was compromised. Since one of the principal functions of collagen IV is to enhance the tensile strength of basal laminae, we analyzed the expression and distribution of collagen IV in *SPARC*-mutant embryos. In wild-type embryos, SPARC and collagen IV expressed by haemocytes colocalized to the basal lamina surrounding the VNC at ES15 (Fig. 3A,B and Fig. 4A). Moreover, channel glia, which lie between each commissure along the ventral midline, also coexpressed SPARC and collagen IV (Fig. 3A). Although collagen IV was observed in haemocytes of *SPARC*-mutant embryos, immunostaining was not detected in basal laminae, indicating that SPARC does not affect the expression and synthesis of collagen IV by haemocytes (Fig. 3C). Furthermore, knockdown of SPARC by the *SrpHemo-GAL4* driver also resulted in the accumulation of collagen IV in hemocytes and the absence of collagen IV from basal laminae (Fig. 3E). In haemocyte-derived S2R+ cells, SPARC and collagen IV colocalized to intracellular vesicles (Fig. 3I), consistent with a functional relationship between SPARC and collagen IV.

To further distinguish the phenotypes associated with the absence of *SPARC* from those caused by the absence of collagen IV from the basal lamina, we examined $DCg1^{412}$, $DCg1^{234}$ and *Df(2L) sc19-8* embryos. $DCg1^{412}$ and $DCg1^{234}$ are collagen IV α1 hypomorphic alleles that are embryonic lethal (Gellon et al., 1997; Martinek et al., 2002) and *Df(2L)sc19-8* is a deficiency lacking both collagen IV genes (*DCg1* and *viking*). Cuticle preparations of $DCg1^{412}$ (Gellon et al., 1997) homozygous embryos revealed lesions along the ventral and lateral regions, deformed mouth hooks and collapsed tracheae (supplementary material Fig. S2E). Discontinuous laminin deposits were observed around the VNC of *Df(2L)sc19-8* homozygous embryos (Fig. 2K). The VNC failed to condense and appeared kinked in $DCg1^{234}$ mutant embryos by late embryogenesis (supplementary material Fig. S2F). As in *SPARC*-mutant embryos, collagen IV was absent from basal laminae and laminin distribution around the VNC was discontinuous in all collagen-IV-homozygous embryos analyzed (Fig. 2K, supplementary material Fig. S2F).

Collagen IV deposition in basal laminae is restored by transgenic SPARC expression in *SPARC*-mutant embryos

To determine whether the absence of collagen IV deposition and discontinuous laminin distribution in basal laminae can be attributed to the absence of SPARC expression by haemocytes, SPARC was transgenically expressed by two haemocyte-specific driver lines in the *SPARC*- mutant background. Haemocytes, which originate from the haemocyte anlagen in the cellular blastoderm, are first distinguished at ES8 in the head mesoderm (Holz et al., 2003; Paladi and Tepass, 2004; Tepass et al., 1994). Of all embryonic haemocytes, ~95% are plasmatocytes that migrate and differentiate into macrophages (Lebestky et al., 2000; Milchanowski et al., 2004; Tepass et al., 1994). Differentiation of haemocyte precursors into plasmatocytes is dependent upon the expression of the *Glial Cell Missing* (*gcm*) transcription factor (Bernardoni et al., 1997; Hosoya et al., 1995). The *gcm-GAL4* driver is activated just prior to ES8 in haemocyte progenitors (Paladi and Tepass, 2004) and, thereafter, in macrophages and glial cells . Similarly, *SrpHemo-GAL4* is active in haemocyte progenitors at ES9 (Bruckner et al., 2004) and, thereafter, in macrophages. Use of both these drivers to overexpress SPARC in haemocytes in *SPARC*-mutant embryos restored SPARC immunostaining in basal laminae of the gut and CNS (Fig. 4B-F). Although transgenic expression of SPARC by haemocytes in *SPARC*-mutant embryos failed to rescue embryonic lethality, ventral cuticle morphology was restored (supplementary material Fig. S2G). Moreover, tracheal defects were suppressed as rescued embryos contained intact tracheal tubes. Transgenic expression of *SPARC* in haemocytes also restored the association of collagen IV with basal laminae (Fig. 4C-F). Collagen IV colocalized with SPARC in basal laminae of the brain and ventral cord, CNS channels, midgut and hindgut (Fig. 4D-F). Moreover, the laminin network remained continuous throughout embryogenesis (Fig. 4G). These combined data demonstrate that SPARC is required for secretion and integration of collagen IV into basal laminae, which in turn, enhances the stability of the laminin network. The failure to completely rescue embryonic lethality is probably owing to non-basal lamina activities of SPARC.

To confirm that the deposition of collagen IV in embryonic basal laminae is dependent on SPARC expression by haemocytes, SPARC was ectopically expressed in neural tissues of *SPARC*-mutant embryos. Expression of SPARC by the *sca-GAL4* driver line was first observed in the proneural clusters of ES11 embryos (data not shown). Ectopic *SPARC* expression in *SPARC*-mutant embryos by using this driver did not promote expression of collagen IV in neural tissues, its deposition by haemocytes into basal laminae, or its rescue of the *SPARC* mutant phenotype (Fig. 4H). Therefore, the data indicate that the deposition of collagen IV in embryonic basal laminae is dependent upon the coexpression of collagen IV and SPARC in haemocytes, suggesting an intracellular role for SPARC in promoting collagen IV secretion. However, our data do not eliminate the possibility that collagen IV is secreted but rapidly degraded by matrix-remodeling proteinases in the absence of SPARC.

Discussion

Our studies indicate that SPARC is required for normal embryonic development in *Drosophila*. In the absence of SPARC, haemocyte-

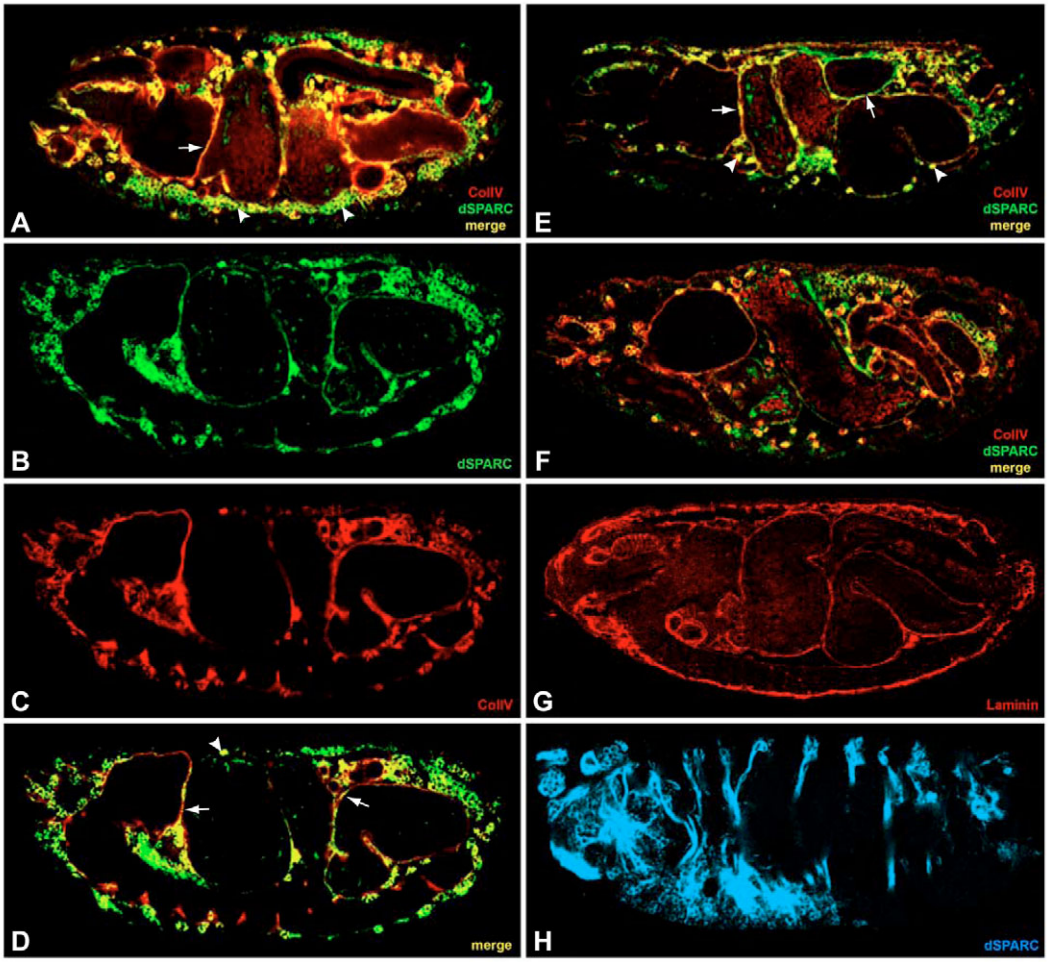

Fig. 4. Collagen IV associates with basal laminae in *SPARC* mutants that express transgenic SPARC. Embryos transgenically expressing SPARC were immunostained with anti-collagen IV (ColIV, red) and anti-SPARC (dSPARC, green) antibodies. (A) ES16 embryo expressing the *SPARC* transgene in a *SPARC* mutant background in haemocytes (*UAS-SPARC; gcm-GAL4*) shows SPARC colocalization with collagen IV in basal laminae (arrow, yellow). No morphological defects are observed. Endogenous SPARC expression and colocalization with collagen IV is observed in the fat body (arrowhead). (B) ES16 rescued embryo (*UAS-SPARC/+ or y; gcm-GAL4/+; H2Av-GFP, Df(3R)nm136/H2Av-GFP, Df(3R)nm136*) immunostained for SPARC (green). (C) The same embryo as in B immunostained for collagen IV (red). GFP expression is not shown. (D) The merged image (yellow) shows their colocalization in haemocytes (arrowhead) and basal laminae (arrows) around the brain, VNC, midgut chambers and along the VNC channels. (E) Lateral view of an ES16 *SPARC*-mutant embryo that expresses transgenic SPARC in haemocytes (*UAS-SPARC/+ or y; SrpHemo-GAL4 UAS-GFP/+; H2Av-GFP, Df(3R)nm136/ H2Av-GFP, Df(3R)nm136*) immunostained for collagen IV (red) and SPARC (green). Intense coimmunostaining is observed in haemocytes (arrowheads) and basal laminae (arrows). (F) ES17 embryo with the same genotype as in E. Collagen IV and SPARC colocalize in basal laminae. (G) *UAS-SPARC/+ or y; gcm-GAL4/+; H2Av-GFP, Df(3R)nm136/H2Av-GFP, Df(3R)nm136* embryos immunostained for laminin (red). Laminin distribution is continuous around the VNC. (H) Lateral view of an ES17 *SPARC*-mutant embryo expressing SPARC (blue) in all neuroblast- and glia-derived cells and sensory organ precursor cells (*UAS-SPARC/+ or y; sca-GAL4/+; H2Av-GFP, Df(3R)nm136/H2Av-GFP, Df(3R)nm136)*. Motorneurons are disorganized and the VNC twists out of focus. All images are single confocal sections.

derived collagen IV is not observed in basal laminae during mid- to late embryonic development. The absence of collagen IV leads to discontinuous laminin distribution during late embryonic development, indicative of decreased basal lamina stability. That SPARC selectively affects the presence of collagen IV in basal laminae is further supported by data demonstrating that collagen-IV-mutants have phenotypic similarities to *SPARC*-mutant embryos (Fig. 2K, supplementary material Fig. S2E).

Studies using vertebrates and invertebrates have shown that laminin is the first basal lamina component to be expressed and secreted during embryonic development (Huang et al., 2003; Smyth et al., 1999; Yurchenco et al., 2004). The expression and deposition of laminin along cell surfaces are promoted by its binding to cell-surface receptors such as α1-integrin and β-dystroglycan. In *SPARC*-mutant embryos, the association of laminin with cell surfaces is unaffected until late embryogenesis, a stage in development when collagen IV and SPARC have been integrated into basal laminae of wild-type embryos. In support of the proposal that the discontinuous laminin network observed in *SPARC* mutants is because collagen IV is absent from the basal lamina, discontinuous laminin networks are also observed in late-stage collagen-IV-mutant embryos (Fig. 2K). Laminin networks are likewise disrupted in mouse and *C. elegans* mutants that lack the expression of collagen IV (Guo et al., 1991; Guo and Kramer, 1989; Poschl et al., 2004). The data indicate that the compromised structural integrity of the laminin network is probably owing to the absence of collagen IV in basal lamina rather than a molecular interaction between SPARC and laminin. However, the presence of a thicker laminin network

in lens capsules of SPARC-null mice might reflect a more complex relationship between laminin and SPARC (Yan et al., 2005).

Molecular interactions have not been demonstrated between SPARC and perlecan or nidogen, two other universal components of basal laminae. Our data indicate that absence of SPARC does not affect the distribution of perlecan and nidogen in basal laminae during embryogenesis. A potential explanation is that nidogen and perlecan do not form extended crosslinked polymers such as laminin and collagen IV (Yurchenco and O'Rear, 1994; Yurchenco et al., 2002). Hence, they are expected to be less susceptible to distortion by mechanical forces associated with late embryonic development. Another possibility is that, whereas perlecan and nidogen bind to, and bridge with, laminin and collagen IV, their interactions with transmembrane receptors (Dedhar et al., 1992; Dong et al., 1995; Henry et al., 2001) promotes pericellular associations that are independent of laminin and collagen IV networks.

Whereas our data indicate that SPARC and collagen IV are integral components of the majority of embryonic basal laminae in *Drosophila*, we did not detect SPARC in basal laminae overlying the dorsal vessel and somatic muscles of wild-type embryos, which suggests that molecules other than SPARC promote the deposition of collagen IV molecules in these basal laminae. Interestingly, pericardial cells only express the α2 chain of collagen IV (N.N.M., unpublished), raising the possibility that the basal lamina overlying the dorsal vessel is composed of collagen IV α2 homotrimers (Tomancak et al., 2002). Adding to the complexity of this basal lamina, Pericardin, a collagen-IV-like ECM molecule is also required for proper dorsal vessel formation (Chartier et al., 2002). Hence, diverse regulatory factors and mechanisms are likely to control collagen IV deposition and/or stability during development, consistent with cumulative data indicating that the precise molecular composition and function of basal laminae varies between tissues and at different stages of development.

A direct Ca^{2+}-dependent interaction has been demonstrated between collagen IV and the EC domain of SPARC. Phylogenetic analysis reveals a striking evolutionary conservation of amino acids in the EC domain essential for collagen binding in organisms ranging from nematodes to mammals. Site-directed mutagenesis of these conserved amino acids results in a loss of binding between SPARC and collagen triple helices (Maurer et al., 1995; Mayer et al., 1991; Martinek et al., 2002; Martinek et al., 2007; Pottgiesser et al., 1994). Since we have demonstrated that the presence of collagen IV in basal laminae requires SPARC, we examined whether mutations in collagen IV generate a similar phenotype as *SPARC* mutants to further substantiate their proposed interrelationship.

We partially characterized alleles of the gene encoding the α1 subunit of collagen IV (*DCg1*^{412} and *DCgl*^{234}) and a deficiency line that lacks both collagen IV genes (*Df(2L)sc19-8*). Mutant embryos homozygous for collagen IV show reduced protein expression of collagen IV and, similar to *SPARC*-mutant embryos, are embryonic lethal. As in *SPARC*-mutant embryos, ventral cuticle holes are observed in these collagen-IV-mutant embryos; however, the holes are smaller in the latter (Fig. 2L,M and supplementary material Fig. S2E). In both *SPARC*- and collagen-IV-mutants, tracheal integrity is also compromised (supplementary material Fig. S2A-C,E). A major function of collagen IV is to provide tensile strength to basal laminae, a biomechanical contribution that increases in importance during late embryogenesis due to an increase in the frequency and strength of muscle contractions. The discontinuous laminin network surrounding the ventral nerve cord and other organs by late embryogenesis in *collagen IV* and *SPARC* mutants, as previously stated, is probably owing to the absence of collagen IV from basal laminae.

A similarity between *SPARC*-mutant and collagen-IV-mutant embryos during late embryogenesis is the absence of VNC condensation. VNC condensation has been shown by a variety of genetic approaches to be dependent on the deposition of collagen IV in basal laminae and on electrical conductivity (Olofsson and Page, 2005). Hence, failure to undergo VNC condensation in *SPARC*-mutant embryos is probably because of the absence of collagen IV from basal lamina surrounding the VNC. Whereas the molecular and cellular events regulating VNC condensation are poorly understood, intracellular signaling events are affected by integrins binding to collagen IV during late embryogenesis (Fessler and Fessler, 1989). These data suggest both a biomechanical and regulatory role for collagen IV that is crucial in VNC condensation. Transgenic expression of SPARC in haemocytes and glia (under the control of *gcm-GAL4*) as well transgenic expression only in haemocytes (under the control of *SrpHemo-GAL4*) in a *SPARC* mutant background, restored the presence of collagen IV in the basal lamina surrounding the VNC, but did not promote its condensation (Fig. 4D-G). The combined data indicate that SPARC plays a role in neural patterning that is independent of its contribution to the deposition of collagen IV in basal laminae.

The coexpression of SPARC and collagen IV in haemocytes, combined with the direct demonstrated biochemical interactions (Maurer et al., 1995; Mayer et al., 1991; Pottgiesser et al., 1994), raises the possibility that SPARC and collagen IV form a complex in the ER that promotes the proper folding and secretion of collagen IV. In support of this hypothesis, the presence of collagen IV in basal laminae is restored when haemocyte expression of SPARC is rescued transgenically (Fig. 4C). Ectopic expression of SPARC by neuroblasts or glia in *SPARC*-mutant embryos does not induce collagen IV expression by neural and glial cells, nor does it induce the presence of haemocyte-derived collagen IV in basal laminae (Fig. 4H). Whereas collagen IV and SPARC colocalize in basal laminae of tissues that do not express either protein, their coexpression by haemocytes appears to be required for their proper integration into basal laminae.

Our data indicate that inhibition of SPARC expression leads to the absence of collagen IV in the basal laminae during *Drosophila* embryogenesis, without affecting the secretion and deposition of the other major basal lamina components. The combined data raise the possibility that SPARC functions intracellularly to promote correct folding and secretion of collagen IV and/or its stability in basal laminae during *Drosophila* embryogenesis. Consistent with a collagen-chaperone-like activity is the recent report that SPARC affects the processing of fibrillar collagen I at the plasma membrane, which could in part account for the distinct collagen phenotype between wild-type and *SPARC*-null mice (Rentz et al., 2007). Moreover, it is also possible that collagen IV is not properly assembled extracellularly into a stable network and is therefore rapidly degraded by matrix remodeling proteases. Whereas this possibility cannot be discounted on the basis of our data, proteases capable of selectively degrading collagen IV during *Drosophila* embryogenesis have yet to be identified. Moreover, as stated above, the secretion of SPARC by non-haemocyte cells does not rescue the association of collagen IV with basal laminae, which indicates that the formation of a stable collagen IV network is not generated by an extracellular interaction with SPARC. Whereas we cannot eliminate a potential role for SPARC in regulating the maturation

of collagen IV in extracellular membrane compartments, the vesicular colocalization of SPARC and collagen IV in haemocytes is indicative of an intracellular functional relationship.

The folding, assembly and processing of collagens from cells via the secretory pathway is dependent on molecular chaperones. Misfolded or incompletely assembled proteins are retained in the ER and are eventually targeted for degradation. In vertebrates, heat shock protein 47 (Hsp47) is a 47 kD collagen-specific protein that binds to and promotes the maturation of collagen molecules (Ishida et al., 2006; Marutani et al., 2004; Nagata, 2003). In the absence of Hsp47, both fibril-forming collagen I, and network-forming collagen IV secretion and assembly into matrices are severely compromised, leading to embryonic lethality at ES10.5-ES11.5 in mice (Marutani et al., 2004). Immunoelectron microscopy shows that collagen IV accumulates within the dilated ER of mutant cells. The accumulation of misfolded or unfolded protein within the ER activates an ER-stress response, in which the expression of molecular chaperones is induced. In Hsp47-null mouse embryos, massive apoptotic cell death occurs just before the death of the embryo at ES10.5. Collagen molecules that bypass the ER-quality control in mouse Hsp47-null fibroblasts and embryonic stem (ES) cells show increased sensitivity to protease degradation, indicative of incorrectly folded procollagen molecules (Marutani et al., 2004; Matsuoka et al., 2004). Since an Hsp47 ortholog is not encoded by invertebrate genomes, it is possible that one or more alternative chaperones ensure correct collagen assembly, maturation and secretion.

Studies have indicated that the basal lamina components are highly conserved in metazoans. Our data and findings from other laboratories indicate that a functional relationship between SPARC and collagens is also evolutionarily conserved. Analyses of *SPARC*-null mice demonstrate that SPARC affects the supramolecular assembly of both network and fibrillar collagens (Bradshaw et al., 2003; Norose et al., 2000; Sangaletti et al., 2003). Two months after birth, *SPARC*-null mice develop early onset cataracts, which suggest of a role for SPARC in lens transparency (Gilmour et al., 1998). Ultrastructural analysis of the lens capsule revealed that cellular extensions from the lens epithelium penetrate and invade the overlying basal lamina, and that the lens capsule contains an altered distribution of collagen IV and laminin (Yan et al., 2002). Therefore, the early onset cataracts observed in *SPARC*-null mice probably result from compromised assembly and stability of the lens basal lamina. Our data indicate that, in *Xenopus*, decreased SPARC expression during embryogenesis also leads to the formation of cataracts (M.J.R., unpublished).

In this study we observed that early loss of SPARC expression in *SPARC*-mutant embryos and SPARC knockdown using *da-GAL4* prior to haemocyte migration produces a variety of patterning defects within the developing nervous system that cannot be rescued by SPARC expression in haemocytes. Moreover, loss of tracheal, fat-body and ventral-epidermal integrity were observed by the end of embryogenesis together with disorganized neurons and glia (our unpublished observations). These observations suggest that SPARC has a non-cell-autonomous role in the development of the CNS that impacts on guidance of muscles, neurons, glia and the tracheal system.

The novel neural phenotype observed in *SPARC*-mutant embryos points to a role for *SPARC* in CNS patterning that is independent of collagen IV. This is not surprising in light of vertebrate studies that lend strength to the idea that SPARC is a multifunctional glycoprotein with both extracellular and intracellular functions (Barker et al., 2005; Huynh et al., 2004; Sodek et al., 2002).

Materials and Methods

Drosophila strains

Flies were raised on standard *Drosophila* media at 25°C and Oregon R was used as wild type. *w; P(lacW)His2AvL1602/TM3, Ser* flies (A. Spradling), which contain a *P* element insertion within the *His2AvD* regulatory region were crossed *to w1118; e Sb P(lacW)Δ2-3/TM6,Ubx* (Bloomington Stock Center) to generate a deletion of *SPARC* by imprecise excision alleles. Lethal excision mutations were balanced and tested in trans to the deficiency line *Df(3R) Tl-P*, which covers *SPARC* (Anderson et al., 1985), for embryonic lethality. Deletion mutations were identified using PCR on pooled excision lines and immunostained with the anti-SPARC antibody (Martinek et al., 2002) to identify *SPARC* mutants. The isolated *SPARC* deletion (*Df(3R)nm136*) was maintained as *Df(3R)nm136*/TM3, Ser and was outcrossed for several generations to remove potential second-site mutations. *Df(3R)nm136* will be referred to as the *SPARC* mutation. *P(His2AvD+t4.0)*and *P(His2AvT:Avic\GFP-S65T)62A* were used to compensate for the deletion of *His2Av* in *Df(3R)nm136* flies and for the null allele $His2Av^{810}$ for phenotypic analysis (Clarkson and Saint, 1999; Clarkson et al., 1999). A recombinant chromosome *P(His2AvT:Avic\GFP-S65T)62A, Df(3R)nm136* was generated by classic genetic techniques. The $DCg1^{412}$ and $DCg1^{234}$ allele of the *DCg1* gene (Gellon et al., 1997) were obtained from Nadine McGinnis (University of California, San Diego, CA) and *Df(2L)sc19-8* flies were obtained from the Bloomington Stock Center. Transgenic lines of *UAS-SPARC* were generated by subcloning the full length *dsparc* cDNA (915 bp) into the pUAST vector (Brand and Perrimon, 1993). Full-length *dsparc* cDNA was obtained by performing reverse transcription and the polymerase chain reaction (RT-PCR) on total RNA extracted from wild-type embryos. The full-length *SPARC* cDNA sequence was confirmed by sequencing. Fly transformation was performed according to standard procedures to generate *UAS-SPARC* transgenic lines. UAS-*SPARC RNAi* lines were obtained from Barry Dickson (Vienna Drosophila RNAi Centre). The following driver lines were used in this study: *gcm-GAL4* (Paladi and Tepass, 2004), *SrpHemo-GAL4* (Bruckner et al., 2004), *sca-GAL4* (Guo et al., 1996), *da-GAL4* (Giebel et al., 1997), *collagen-GAL4* (Asha et al., 2003). The following genotypes were examined in *SPARC* mutant rescue experiments: (1) *UAS-SPARC/+ or y; gcm-GAL4/+; H2Av-GFP Df(3R)nm136/H2Av-GFP Df(3R)nm136,* (2) *UAS-SPARC/+ or y; SrpHemo-Gal4 UAS-srcEGFP/+; H2Av-GFP Df(3R)nm136/H2Av-GFP Df(3R)nm136* and (3) *UAS-SPARC/+ or y; sca-GAL4/+; H2Av-GFP Df(3R)nm136/H2Av-GFP Df(3R)nm136*.

Cuticle preparation, in situ hybridization and antibody staining

For each batch of embryos analyzed (ranging on average in number from 80-150), ~25% of the embryos were embryonic lethal, indicating 100% penetrance of the phenotype. All genetic crosses and experiments were carried out at least four times. For cuticle preparation, embryos were collected and allowed to develop for 24 hours. Dechorionated embryos were transferred to slides containing a 1:1 mixture of Hoyer's medium and 85% lactic acid and then incubated at 65°C between 3 hours and overnight. In situ hybridization was performed as described in Martinek et al. using a digoxigenin-labeled *SPARC* cDNA as a probe on fixed embryos (Martinek et al., 2002). Whole-mount immunostaining was carried out using standard procedures with antibodies obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242 against the following targets: DN-cadherin (DN-Ex number 8), Neurotactin (BP106), Futsch (22C10), CNS axons (BP102), Crumbs (Cq4) and Repo (8D12). The following antibodies were also used: monoclonal antibody against *Drosophila* collagen IV (Fessler et al., 1994) and polyclonal antibodies against *Drosophila* SPARC (Martinek et al., 2002), *Drosophila* laminin (α3,5/β1/γ1) (Fessler et al., 1987), *Drosophila* perlecan (Friedrich et al., 1999) and hunchback (Kosman et al., 1998). Secondary antibodies conjugated to Alexa Fluor 488, Cy3, Cy5 (Molecular Probes) or HRP (Jackson Laboratories) were used. Embryos were mounted in Antifade (Sigma) or 2:1 Permount:methyl-salicylate. Images using Nomarski optics and phase contrast were recorded with a Zeiss light microscope using the Zeiss Axiophot2 software. Confocal images were obtained with a Zeiss LSM510 confocal microscope using a Plan-Neofluar 40×/1.30 oil lens and Olympus FV1000 using FV1000 Version 1.6a software. Images were processed with NIH ImageJ software and Adobe® Photoshop® version 7. Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997).

S2R+ cell culture

Drosophila S2R+ cells were grown in Shields and Sang M3 medium containing 10% fetal bovine serum at 25°C. *Drosophila* Schneider-2R+ cells were seeded in an eight-well chamber slides coated with poly-L-lysine and grown until confluent. Cells were fixed for 15 minutes with 3.7% formaldehyde in PBS/0.1% Triton X-100 and immunostained with anti-SPARC and anti-*Drosophila* collagen IV antibodies as above.

Western blotting

One-hundred dechorionated and handpicked embryos of each genotype were placed in microcentrifuge tubes, snap-frozen in liquid nitrogen and ground to powder with a fitted pestle. Protein sample buffer (100 mM Tris pH 6.8, 100 mM DTT, 10% glycerol, 0.01% Bromophenol Blue and 2% SDS) was added to each sample which was boiled for three minutes. Lysates were electrophoresed on a 12% polyacrylamide gel and transferred to PVDF membranes (Amersham). Membranes were blocked in

5% nonfat milk in PBT (phosphate-buffered saline plus 0.05% Tween-20) and probed with anti-SPARC antisera diluted 1:1000, anti-β-tubulin (E7) (1:2000) then with HRP-conjugated goat anti-rabbit secondary antibodies (1:3000). Proteins on membranes were visualized using the ECL system (Amersham).

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