

## RESEARCH ARTICLE

# 14-3-3 $\gamma$ -mediated transport of plakoglobin to the cell border is required for the initiation of desmosome assembly *in vitro* and *in vivo*

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**ABSTRACT**

The regulation of cell–cell adhesion is important for the processes of tissue formation and morphogenesis. Here, we report that loss of 14-3-3 $\gamma$  leads to a decrease in cell–cell adhesion and a defect in the transport of plakoglobin and other desmosomal proteins to the cell border in HCT116 cells and cells of the mouse testis. 14-3-3 $\gamma$  binds to plakoglobin in a PKC $\mu$ -dependent fashion, resulting in microtubule-dependent transport of plakoglobin to cell borders. Transport of plakoglobin to the border is dependent on the KIF5B–KLC1 complex. Knockdown of KIF5B in HCT116 cells, or in the mouse testis, results in a phenotype similar to that observed upon 14-3-3 $\gamma$  knockdown. Our results suggest that loss of 14-3-3 $\gamma$  leads to decreased desmosome formation and a decrease in cell–cell adhesion *in vitro*, and in the mouse testis *in vivo*, leading to defects in testis organization and spermatogenesis.

**KEY WORDS:** 14-3-3 $\gamma$ , Desmosome, Plakoglobin, KIF5B, Spermatogenesis

**INTRODUCTION**

Desmosomes are adherens-like junctions that anchor intermediate filaments, leading to the generation of a tissue-wide intermediate filament network. Three different protein families contribute to desmosome structure and function – the desmosomal cadherins desmocollins (DSCs) and desmogleins (DSGs), the armadillo (ARM) proteins and the plakins (Green and Gaudry, 2000). Desmosome composition varies with respect to tissue type and differentiation status, as the cadherins and the associated ARM family members show tissue- and cell-type-specific expression (Bass-Zubek et al., 2009; Dusek et al., 2007), leading to changes in the organization and function of desmosomes in different tissues.

The ARM proteins participate in the regulation of desmosome assembly and cell–cell adhesion (Marcozzi et al., 1998; Palka and Green, 1997). Plakoglobin (encoded by *JUP*) localizes to both

desmosomes and adherens junctions and is required for the initiation of desmosome formation by adherens junctions (Acehan et al., 2008; Knudsen and Wheelock, 1992; Lewis et al., 1997). Decreases in DSG3, the density of the plaque and the levels of plakophilin 1 (PKP1) at the cell border have been observed in plakoglobin-null keratinocytes (Acehan et al., 2008; Caldelari et al., 2001), suggesting that plakoglobin is required for desmosome formation and function in cultured keratinocytes. Plakoglobin-knockout mice die during embryogenesis owing to defects in desmosome formation in cardiac tissue (Ruiz et al., 1996). Some discrepancies exist in the literature regarding the effects of plakoglobin loss on desmosome formation in the epidermis. Ruiz and colleagues have reported that the epidermis of embryos at 11.5 days post coitum are normal upon loss of plakoglobin (Ruiz et al., 1996), whereas others have reported that defects in epidermal organization and desmosome function are observed in mice lacking plakoglobin at 17.5 days post coitum (Bierkamp et al., 1996). Plakoglobin has been reported to form a complex with both P-cadherin and E-cadherin, and the total levels of the classical cadherins dictate desmosome formation and organization (Lewis et al., 1997; Michels et al., 2009; Tinkle et al., 2008). These results suggest that plakoglobin and other ARM proteins might serve as a link between adherens junction formation and desmosome formation. Consistent with this hypothesis, plakoglobin and E-cadherin are independently required for the recruitment of plakophilin 3 (PKP3) to the cell border in order to initiate desmosome formation in HCT116 cells (Gosavi et al., 2011), whereas plakoglobin and the plakophilin family members collaborate with the desmoplakin N-terminus to regulate the clustering of the desmosomal cadherins at the cell surface (Chen et al., 2002). These data are indicative of plakoglobin being required for the initiation of desmosome formation and maintenance. However, the mechanisms by which the ARM proteins are transported to the cell border to initiate the process of desmosome formation remain unclear.

Spermatogenesis occurs in seminiferous tubules in the testis and is intrinsically dependent upon cell–cell adhesion between spermatocytes and Sertoli cells, and the formation of the blood–testis barrier between two Sertoli cells (Russell et al., 1990). These adhesive interactions are crucial for the progression of spermatogenesis. The blood–testis barrier comprises tight junctions, basal ectoplasmic specializations, gap junctions and desmosome-like junctions (Cheng and Mruk, 2002; Lie et al., 2011). Disruption of cell–cell adhesion perturbs the normal progression of spermatogenesis (Wong et al., 2004). Importantly,

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decreasing the expression of PKP2, DSG2 and DSC2 affects cell–cell adhesion, indicating that the development of spermatozoa is regulated by the formation of desmosome-like junctions in the testis (Li et al., 2009; Lie et al., 2010).

The 14-3-3 protein family is a family of small acidic proteins (Yaffe, 2002) that bind to proteins that contain a phosphorylated serine residue in a consensus motif (Muslin et al., 1996; Yaffe et al., 1997). Loss of 14-3-3 $\epsilon$  and 14-3-3 $\gamma$  leads to the overriding of checkpoint function and premature entry into mitosis (Hosing et al., 2008; Telles et al., 2009). Therefore, we wanted to determine whether loss of 14-3-3 $\gamma$  in the mouse led to defects in checkpoint function. When we attempted to generate 14-3-3 $\gamma$ -knockdown mice by using a novel transgenic protocol that had been developed in our laboratory (Sehgal et al., 2011), we observed that loss of 14-3-3 $\gamma$  led to sterility in male mice due to a decrease in cell–cell adhesion and a defect in the transport of plakoglobin and other desmosomal proteins to the cell border in the seminiferous tubules of mice. Similar results were obtained in the human HCT116 colorectal cancer cell line. Furthermore, our results demonstrate that 14-3-3 $\gamma$  might load plakoglobin onto the KIF5B–KLC1 complex in order to transport plakoglobin to the cell border to initiate desmosome formation, both in HCT116 cells in culture and in the mouse testis, thus demonstrating that 14-3-3 $\gamma$  is required for desmosome formation.

## RESULTS

### Loss of 14-3-3 $\gamma$ leads to sterility in male mice

To determine whether loss of 14-3-3 $\gamma$  leads to a loss of checkpoint regulation *in vivo*, we attempted to generate knockdown mice for 14-3-3 $\gamma$  by using a sperm-mediated gene transfer protocol that was developed in our laboratory (Sehgal et al., 2011). However, when mice that had been injected with viruses expressing the shRNA construct against 14-3-3 $\gamma$  were mated with female mice, no pups were obtained. The levels of 14-3-3 $\gamma$  were substantially decreased in the testis of mice that had been injected with viruses expressing the shRNA construct against 14-3-3 $\gamma$  (sh14-3-3 $\gamma$ ) in comparison with the mice that had been injected with the vector control (Vec, Fig. 1A). Loss of 14-3-3 $\gamma$  led to an almost complete absence of mature spermatozoa in the epididymis in comparison with the control mice (Fig. 1B,C). In addition, the organization of the seminiferous tubule was severely disrupted upon 14-3-3 $\gamma$  knockdown, as evidenced by individual sections of the seminiferous tubule being dissociated from one another in comparison with those of control mice (Fig. 1B). Furthermore, primary germ cells and Sertoli cells were detached from the basal lamina. This did not lead to a large increase in transferase dUTP nick end labeling (TUNEL)-positive cells (supplementary material Fig. S1A). Finally, histological examination revealed an abrogation of cell–cell adhesion between Sertoli cells, and between Sertoli cells and germ cells, in the testis, upon knockdown of 14-3-3 $\gamma$  (Fig. 1D), which was confirmed by electron microscopy (Fig. 1E). Thus, these results suggest that loss of 14-3-3 $\gamma$  leads to a decrease in cell–cell adhesion *in vivo*.

### 14-3-3 $\gamma$ loss leads to defects in cell adhesion and desmosome assembly

To identify the mechanisms that lead to a decrease in cell–cell adhesion, we used a HCT116 cell line model in which 14-3-3 $\gamma$  had been knocked down (sh14-3-3 $\gamma$ ) (described previously by Hosing et al., 2008). 14-3-3 $\gamma$  mRNA and protein levels were lower in the sh14-3-3 $\gamma$  cells in comparison with the control cells

(Fig. 2A,B). The protein levels of 14-3-3 $\epsilon$  and 14-3-3 $\sigma$ , or the mRNA levels of 14-3-3 $\epsilon$ , 14-3-3 $\beta$ , 14-3-3 $\tau$  and 14-3-3 $\zeta$  (Fig. 2A,B) were not altered in the sh14-3-3 $\gamma$  cells in comparison with vector control cells (Fig. 2A). In comparison with the control cells, the sh14-3-3 $\gamma$  cells showed a decrease in cell–cell adhesion in hanging-drop assays (Fig. 2C,D), and cell adhesion to fibronectin and collagen IV was also diminished in these cells (supplementary material Fig. S1B), which is consistent with the detachment of cells in the testis from the basal lamina.

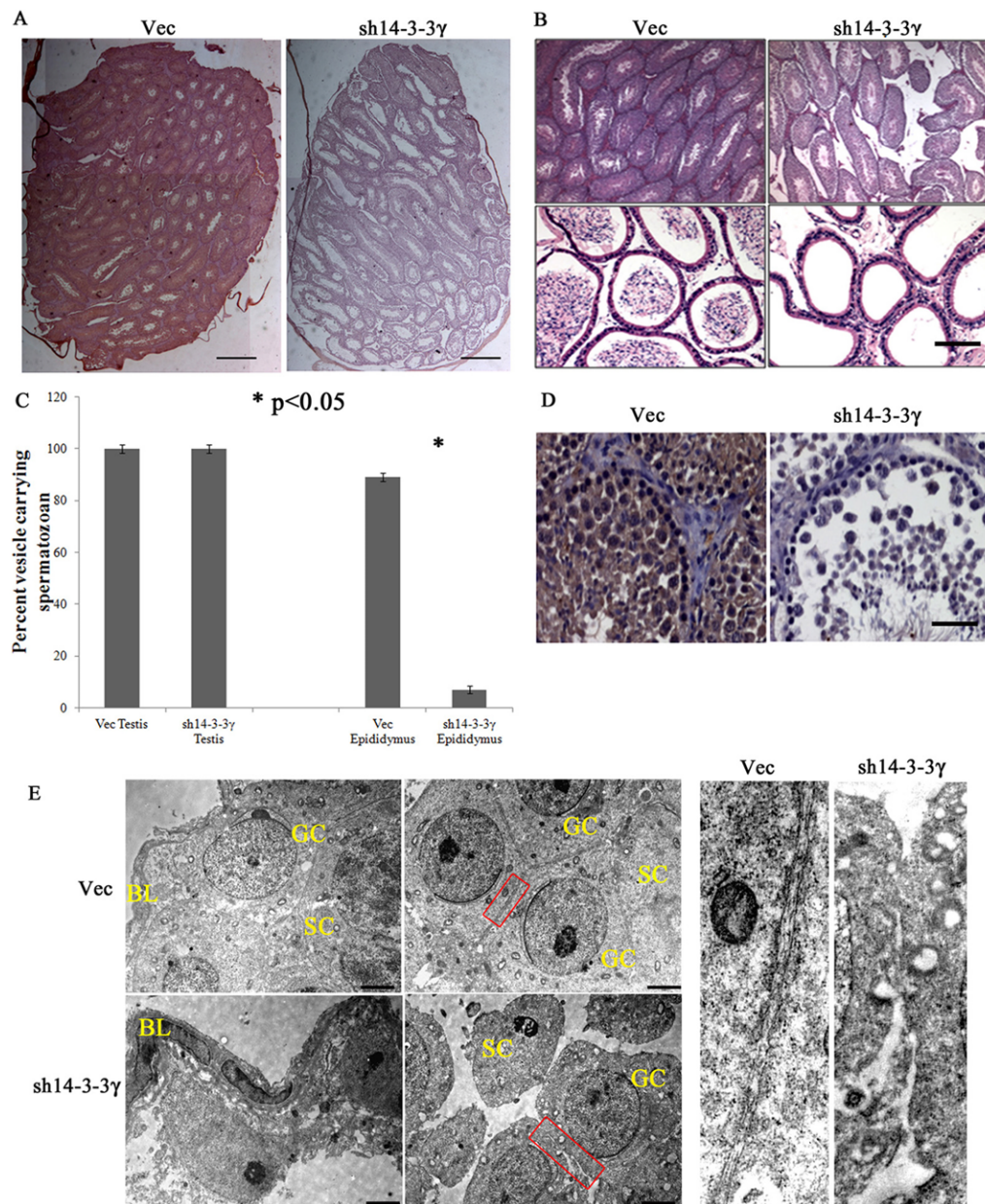
To determine whether the defect in cell–cell adhesion was induced specifically by the loss of 14-3-3 $\gamma$ , cells that had been subjected to 14-3-3 $\epsilon$  knockdown were generated (sh14-3-3 $\epsilon$ ). Western blot analysis demonstrated that, although 14-3-3 $\epsilon$  protein levels were decreased in sh14-3-3 $\epsilon$  cells, the levels of 14-3-3 $\gamma$  were unaltered. No substantial difference was observed in cell–cell adhesion in the sh14-3-3 $\epsilon$ -knockdown cells in comparison with the control cells (Fig. 2F,G). These results suggest that the differences in cell–cell adhesion that are observed upon knockdown of 14-3-3 $\gamma$  are specific to the 14-3-3 $\gamma$  isoform.

To determine the causes of the decrease in cell–cell adhesion, the levels of adhesion proteins in the control and sh14-3-3 $\gamma$  cells were determined by western blot analysis or reverse transcription PCR (RT-PCR) (Fig. 3A–C). The levels of these proteins or mRNAs were not decreased upon 14-3-3 $\gamma$  knockdown. However, in the case of DSG2, and DSC2 and DSC3, increased protein levels were observed in the 14-3-3 $\gamma$  knockdown cells (Fig. 3B), although no substantial increase was observed in mRNA levels (Fig. 3C). PKP2 mRNA levels were not altered substantially in the sh14-3-3 $\gamma$  cells in comparison with the control cells (Fig. 3C), and our previous reports have suggested that HCT116 cells do not express PKP1 (Kundu et al., 2008). Notably, the levels of the desmosomal proteins plakoglobin, PKP3, desmoplakin, DSC2 and DSC3, DSG2 and PKP2 were significantly lower at the cell borders in sh14-3-3 $\gamma$  cells than in control cells (Fig. 3E,F), even though the total levels of these proteins were unaltered in the sh14-3-3 $\gamma$  cells. Intensity profiles for the staining are shown in supplementary material Fig. S2. Importantly, no change in the detergent solubility of the desmosomal proteins was observed in the 14-3-3 $\gamma$ -knockdown cells when compared with the control cells (supplementary material Fig. S4D).

By contrast, the levels of adherens junction components [E-cadherin (also known as CDH1), P-cadherin (also known as CDH3),  $\beta$ -catenin, p120-catenin (also known as CTNND1) and  $\alpha$ -E-catenin], tight junction components (ZO-1) and polarity proteins (Par-3, also known as PARD3) were not reduced at the cell border in sh14-3-3 $\gamma$  cells (supplementary material Fig. S1D,E). Loss of 14-3-3 $\epsilon$  did not result in a decrease in the levels of plakoglobin at the cell border (Fig. 3D). HCT116 cells that lacked both copies of 14-3-3 $\sigma$  (Chan et al., 1999) showed a decrease in plakoglobin levels (supplementary material Fig. S4A) and a decrease in cell–cell adhesion (supplementary material Fig. S4B). However, there was no defect in localization of plakoglobin to the border in these cells (supplementary material Fig. S4C), suggesting that 14-3-3 $\sigma$  is required to maintain plakoglobin protein levels, but not plakoglobin localization to the border.

Expression of a green fluorescent protein (GFP)-tagged shRNA-resistant 14-3-3 $\gamma$  cDNA (GFP-14-3-3 $\gamma$ R) resulted in the recruitment of plakoglobin to the cell border in sh14-3-3 $\gamma$  cells in contrast with cells transfected with GFP alone (Fig. 4A). To determine whether the desmosomal proteins formed a complex with 14-3-3 $\gamma$ , protein extracts from HCT116 cells



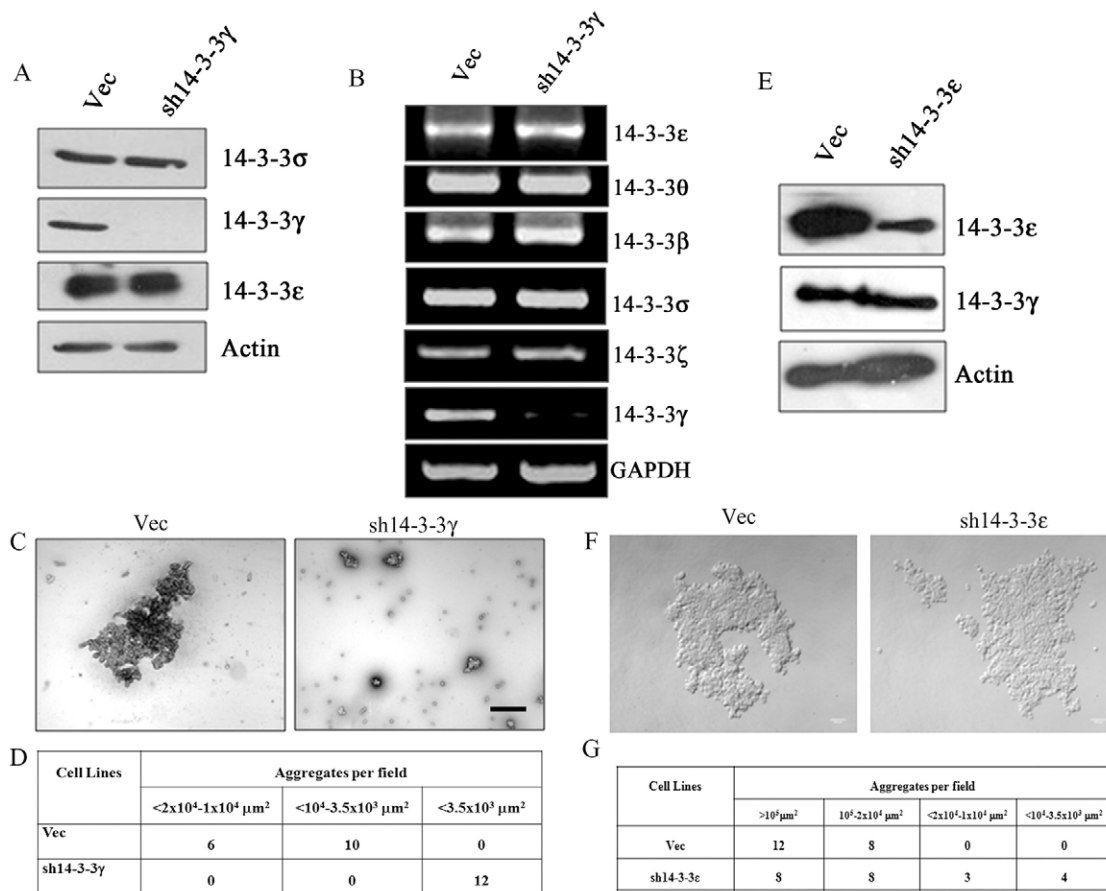


**Fig. 1. Loss of 14-3-3 $\gamma$  leads to disruption of cell-cell adhesion.** (A–D) Tissue sections from mouse testis that had been injected with either the 14-3-3 $\gamma$ -knockdown construct (sh14-3-3 $\gamma$ ) or the vector control (Vec) were stained with antibodies against 14-3-3 $\gamma$  and visualized by using light microscopy (A shows the entire testis, D shows a single seminiferous tubule), or were stained with hematoxylin and eosin (B) to visualize either the seminiferous tubules (top panels) or the epididymis (bottom panels). The percentage of vesicles that contained spermatozoa in the epididymis is shown and the error bars represent the mean  $\pm$  s.d. for three different animals (C). (E) Electron micrographs of testis that had been injected with either the 14-3-3 $\gamma$  knockdown virus or the vector control. Sertoli cells (SC), and germ cells (GC) are indicated. The panels on the far right are higher magnification images of the boxed areas indicated. Scale bars: 5  $\mu$ m (A,B,D); 2  $\mu$ m (E).

were incubated with either glutathione S-transferase (GST) alone or GST–14-3-3 $\gamma$ . 14-3-3 $\gamma$  formed a complex with plakoglobin, PKP3 and desmoplakin but not with DSG2 or adherens junction proteins, such as E-cadherin (Fig. 4B). Our previous results have suggested that plakoglobin is present at the cell border in low-calcium medium, and that plakoglobin is required for the recruitment of other desmosomal proteins to the cell border in HCT116 cells and for the initiation of desmosome formation upon the addition of calcium (Gosavi et al., 2011). Therefore, to determine whether 14-3-3 $\gamma$  is required for the initiation of desmosome formation, calcium-switch assays were performed. Plakoglobin was present at the cell border in low-calcium medium and the levels at the border increased upon the addition of calcium in the vector control cells. DSC2 and DSC3 were not detectable at the border in the control cells in low-calcium medium. However, DSC2 and DSC3 localized to the border 60 minutes after the addition of calcium in the control cells. By contrast, in the sh14-3-3 $\gamma$  cells, plakoglobin, DSC2 and DSC3

were not present at the border in low-calcium medium and accumulated at significantly lower levels at the border upon the addition of calcium in comparison with the control cells (Fig. 4C). E-cadherin levels at the border were unaffected in the sh14-3-3 $\gamma$  cells in comparison with the control cells (Fig. 4C). These results suggest that 14-3-3 $\gamma$  specifically is required for the localization of plakoglobin to cell borders and is required for the initiation of desmosome formation.

We observed that, although the levels of DSC2 and DSC3 at the cell borders were lower in 14-3-3 $\gamma$ -knockdown cells than in the control cells, a substantial fraction of DSC2 and DSC3, nevertheless still localized to the borders in the knockdown cells. These results suggested that, to some extent, localization of DSC2 and DSC3 to the border might be independent of the presence of 14-3-3 $\gamma$  and plakoglobin at the cell border. We have previously shown that, in this cell system, plakoglobin is required to recruit PKP3 and desmoplakin to the cell border (Gosavi et al., 2011). To determine whether plakoglobin is required for the recruitment of



**Fig. 2. Loss of 14-3-3 $\gamma$  leads to a decrease in cell–cell adhesion.** (A) Protein extracts from the vector control (Vec) and 14-3-3 $\gamma$ -knockdown (sh14-3-3 $\gamma$ ) cells were resolved on SDS-PAGE gels followed by western blotting with the indicated antibodies. Actin served as a loading control. (B) mRNA was prepared from the control and sh14-3-3 $\gamma$  cells and RT-PCR reactions were performed with oligonucleotide pairs specific for the indicated genes. GAPDH served as a reaction control. (C,D) Hanging-drop assays were performed on the control and sh14-3-3 $\gamma$  cells. The images (C) of the clumps and the quantification (D) of cluster number and size are shown. (E) Protein extracts from the control and sh14-3-3 $\epsilon$  cells were resolved on SDS-PAGE gels followed by western blotting with the indicated antibodies. Actin served as a loading control. (F,G) Hanging-drop assays were performed on the control and sh14-3-3 $\epsilon$  cells. The images (F) of the clumps and the quantification (G) of cluster number and size are shown. Scale bar: 200  $\mu\text{m}$ .

desmosomal cadherins to the border, the localization of these cadherins was studied in HCT116-derived plakoglobin-knockdown cells, which have been described previously (Gosavi et al., 2011). The levels of DSC2 and DSC3, were not reduced at the border in the plakoglobin-knockdown cells (supplementary material Fig. S1C), suggesting that 14-3-3 $\gamma$ , in addition to being required for plakoglobin localization to the border, might have other functions in desmosome formation. These data are consistent with the observation that 14-3-3 $\gamma$  forms a complex with PKP3 and desmoplakin (Fig. 4B).

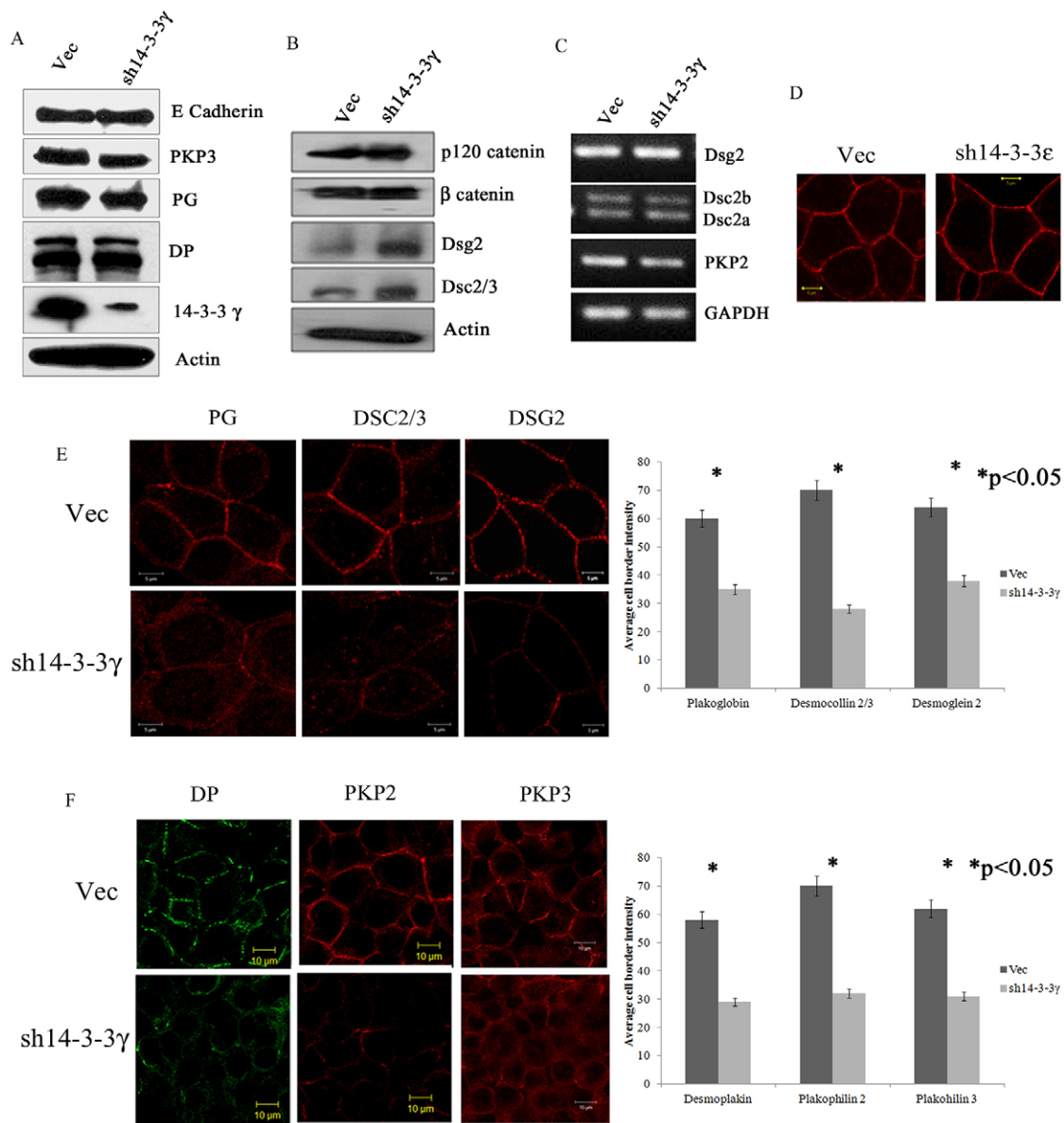
#### Complex formation between plakoglobin and 14-3-3 $\gamma$ requires PKC $\mu$

Analysis of the plakoglobin amino acid sequence led to the identification of a potential 14-3-3 binding site at serine residue 236 (S236) (supplementary material Fig. S3A) (Obenauer et al., 2003). To determine whether S236 is required for an interaction between plakoglobin and 14-3-3 $\gamma$  and to mediate the targeting of plakoglobin to the surface, residue S236 was replaced with alanine (S236A), and the ability of this mutant to bind to 14-3-3 $\gamma$  and to localize to the border was investigated. GST–14-3-3 $\gamma$  formed a complex with wild-type plakoglobin but not with that of the S236A mutant (Fig. 5A). In contrast with wild-type plakoglobin, which

localized to the cell border, we observed reduced levels of the S236A protein at the border and an increased pan-cellular localization (Fig. 5B). Although some of the S236A mutant protein still localized to the border, the localization to the border was attenuated in comparison with that of the wild-type protein, suggesting that binding of plakoglobin to 14-3-3 $\gamma$  is required for the efficient localization of plakoglobin to the border.

The S236 residue is a potential site for phosphorylation by PKC $\mu$  (supplementary material Fig. S3A), suggesting that phosphorylation of plakoglobin by PKC $\mu$  is required for plakoglobin localization to the border. HCT116 cells were treated with the vehicle control dimethyl sulfoxide (DMSO), a pan PKC inhibitor that doesn't inhibit PKC $\mu$  (bisindolylmaleimide I, BisI) or an inhibitor that is specific for PKC $\mu$  and PKC $\alpha$  (Go6976). A GST pull-down assay was then performed using 14-3-3 $\gamma$ . Importantly, GST–14-3-3 $\gamma$  was unable to pull down plakoglobin from protein extracts that had been prepared from cells treated with Go6976, in contrast to cells that had been treated with DMSO (Fig. 5C), suggesting that the activity of either PKC $\alpha$  or PKC $\mu$  is required for complex formation with 14-3-3 $\gamma$  and localization of plakoglobin to the cell border. However, 14-3-3 $\gamma$  formed a complex with desmoplakin and PKP3 in extracts that had been derived from cells treated with the inhibitor (Fig. 5C),



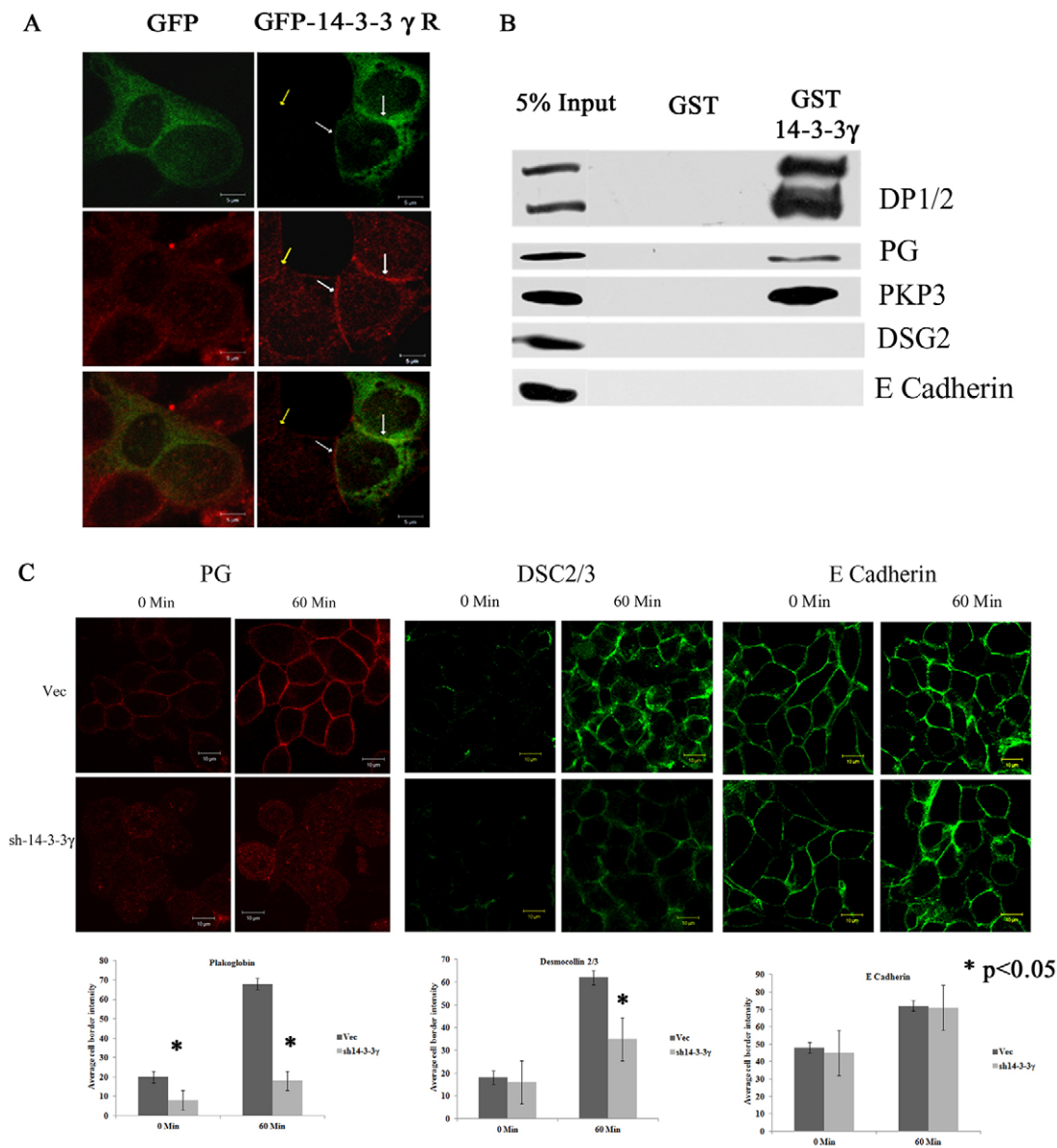


**Fig. 3. Localization of plakoglobin is altered in sh14-3-3 $\gamma$  cells.** (A,B) Protein extracts from the vector control (Vec) and 14-3-3 $\gamma$ -knockdown (sh14-3-3 $\gamma$ ) cells were resolved on SDS-PAGE gels followed by western blotting with the indicated antibodies. Western blotting for actin served as a loading control. (C) mRNA was prepared from the control and sh14-3-3 $\gamma$  cells, and RT-PCR reactions were performed with oligonucleotide pairs specific for the indicated genes. The oligonucleotides used for DSC2 amplified both splice isoforms, DSC2a and DSC2b, as indicated. GAPDH served as a reaction control. (D) Plakoglobin (PG) levels at the cell borders were determined in the vector control and 14-3-3 $\gamma$  knockdown cells. Note that plakoglobin levels did not change at the cell border upon knockdown of 14-3-3 $\epsilon$  (sh14-3-3 $\epsilon$ ). (E,F) Control and sh14-3-3 $\gamma$  cells were stained with the indicated antibodies, and the staining was observed by using confocal microscopy. Representative images are shown. The border intensity was measured for at least 20 cells in three different experiments. The mean  $\pm$  s.d. is shown for three independent experiments. Scale bars: 5  $\mu$ m (D,E); 10  $\mu$ m (F). DP, desmoplakin.

suggesting that the activity of PKC $\mu$  or PKC $\alpha$  is not required for the association of desmoplakin or PKP3 with 14-3-3 $\gamma$ . In agreement with these results, immunofluorescence analysis using antibodies against plakoglobin demonstrated that the treatment of cells with Go6976 decreased the localization of plakoglobin at the cell border in comparison with that of cells that had been treated with either DMSO or BisI (Fig. 5D).

Because the motif scan software identified S236 as a potential site for phosphorylation by PKC $\mu$ , we inhibited the expression of PKC $\mu$  using vector-driven RNA interference (RNAi) in HCT116 cells using a previously described sequence that inhibits PKC $\mu$  expression but not PKC $\alpha$  expression (Park et al.,

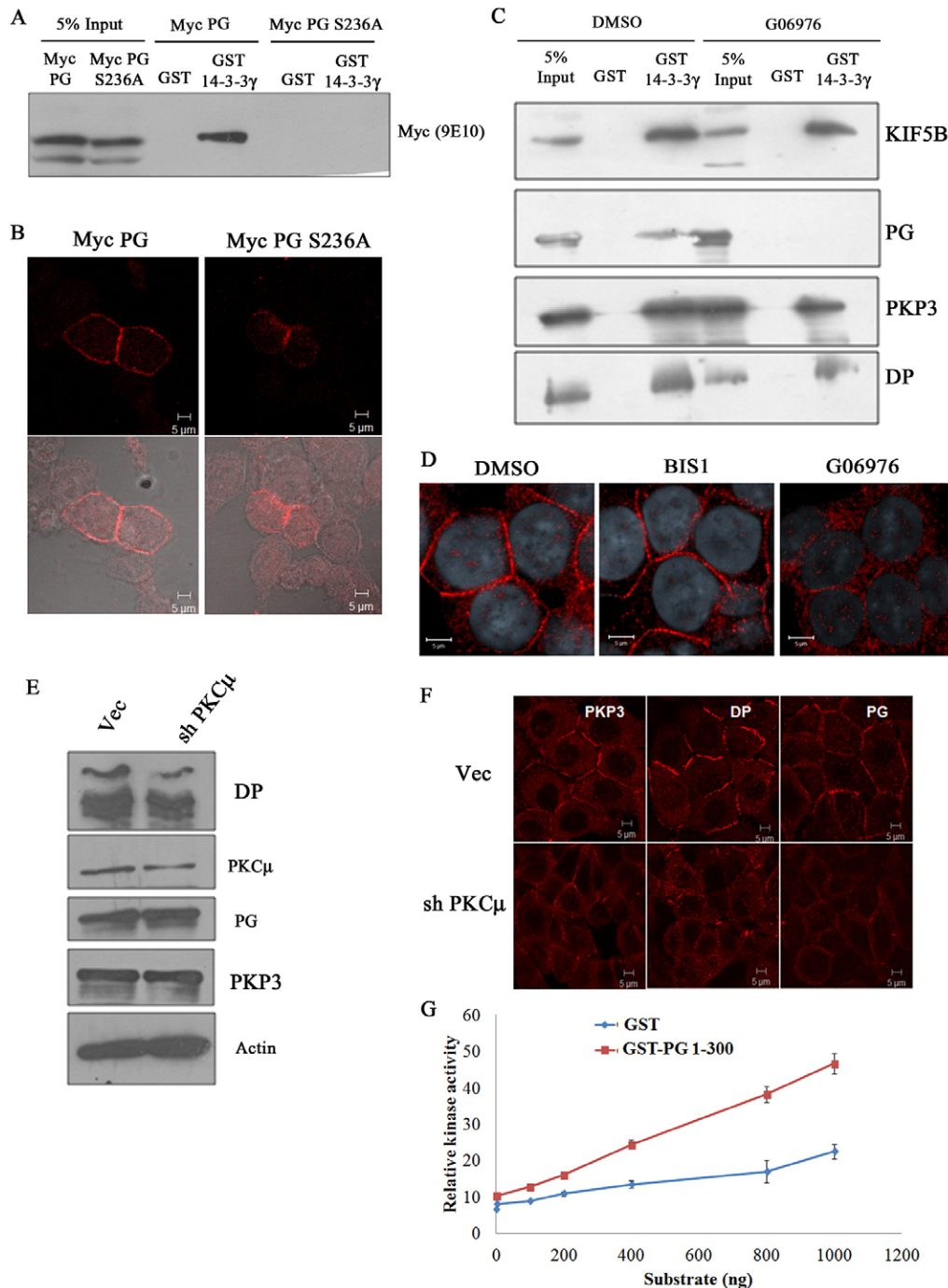
2009). HCT116 cells were transfected with either the vector plasmid or a plasmid that expressed shRNA sequences that target PKC $\mu$  (shPKC $\mu$ ). Forty-eight hours post transfection, the cells were transferred to medium containing puromycin in order to enrich for transfected cells. Western blot analysis demonstrated that PKC $\mu$  levels were reduced, as expected; however, plakoglobin and desmoplakin protein levels were reduced in cells that had been transfected with the shRNA against PKC $\mu$ , suggesting that PKC $\mu$  might also regulate the stability of these proteins (Fig. 5E). No change in the levels of PKP3 was observed, and western blots for actin were performed as loading controls. The levels of plakoglobin, desmoplakin and



**Fig. 4. 14-3-3 $\gamma$  is required to initiate desmosome formation.** (A) 14-3-3 $\gamma$ -knockdown (sh14-3-3 $\gamma$ ) cells were transfected with either GFP alone or a GFP-14-3-3 $\gamma$  construct that was resistant to RNAi (GFP-14-3-3 $\gamma$ R). The cells were stained with antibodies to plakoglobin (PG, red) and analyzed by using confocal microscopy. (B) Protein extracts of HCT116 cells were incubated with either GST or GST-14-3-3 $\gamma$ . The reactions were resolved on SDS-PAGE gels, and followed by western blotting with the indicated antibodies. (C) Vector control (Vec) and sh14-3-3 $\gamma$  cells were incubated in low-calcium medium for 24 hours (0 min). After calcium addition for 60 minutes, the cells were fixed and then stained with the indicated antibodies and analyzed by using confocal microscopy. Note that the levels of the desmosomal proteins do not increase at the border in sh14-3-3 $\gamma$  cells in comparison with control cells. No change in E-cadherin staining was observed. The border intensity was measured for at least 20 cells in three different experiments. The mean and standard deviation for three independent experiments is shown. Scale bars: 5  $\mu$ m (A); 10  $\mu$ m (C).

PKP3 at the cell borders were decreased in cells that had been transfected with the PKC $\mu$  shRNA in comparison with cells that had been transfected with the vector control, and the remaining protein in these cells was not localized to the border (Fig. 5F). These results suggest that, in addition to the decreased protein levels, there is a decrease in the localization of the desmosomal components to the cell borders in the absence of PKC $\mu$ . This is in contrast with the results obtained for cells that lacked 14-3-3 $\sigma$ , in which a decrease in the levels of plakoglobin was observed but there was no defect in plakoglobin localization to the border (supplementary material Fig. S4C). To determine whether PKC $\mu$  phosphorylated plakoglobin directly, the first 300 amino

acids of plakoglobin, which comprise the putative 14-3-3-binding site, were purified from bacteria as a GST fusion protein and used as a substrate in an *in vitro* kinase assay using purified PKC $\mu$ . A peptide derived from CREB (catalog number C50-58, Signal Chem) was used as a positive control in these assays. As shown in Fig. 5G, the GST-PG1-300 fusion protein was phosphorylated *in vitro* by PKC $\mu$ , and the level of phosphorylation increased markedly with an increase in substrate concentration, in contrast with the results observed with GST alone. These results suggest that PKC $\mu$  regulates localization of plakoglobin to the cell border, and plakoglobin expression or stability.



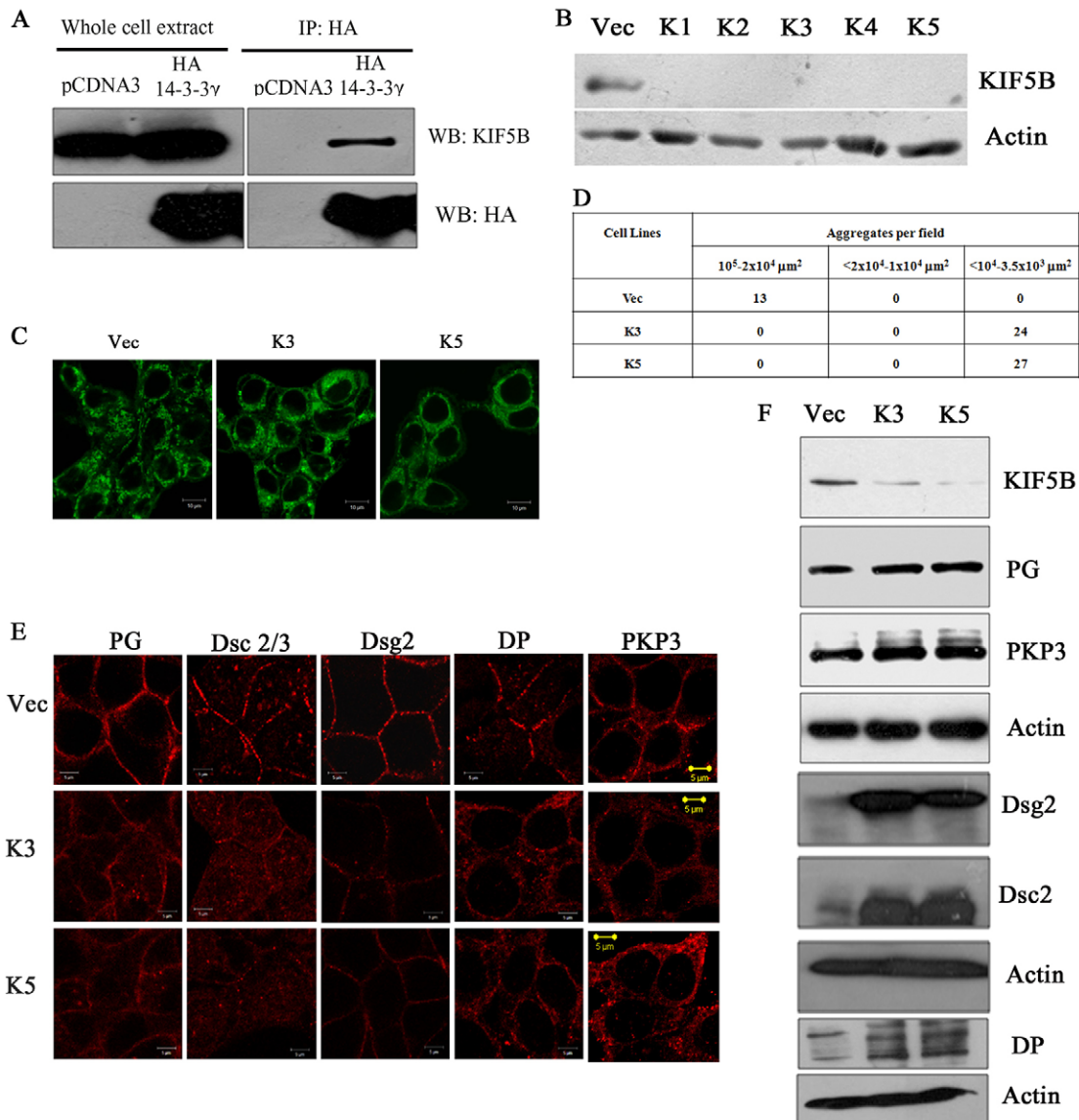
**Fig. 5. The association of 14-3-3 $\gamma$  with plakoglobin requires PKC $\mu$  activity.** (A,B) HCT116 cells were transfected with either MYC-epitope-tagged wild-type plakoglobin (PG) or the S236A mutant of plakoglobin. 48 hours post transfection, protein extracts were incubated with GST or GST–14-3-3 $\gamma$  followed by western blotting with antibodies against MYC (A), or the cells were stained with antibodies against the MYC-epitope (B). Differential interference contrast images for the fields are shown in the lower panels. (C,D) HCT116 cells were treated with either the vehicle control (DMSO), the pan-PKC inhibitor (Bis1), or the PKC $\alpha$ - and PKC $\mu$ -specific inhibitor (Go6976). Protein extracts from these cells were incubated with either GST or GST–14-3-3 $\gamma$ , the reactions were resolved on SDS-PAGE gels, and western blots were performed with the indicated antibodies (C). The cells were also stained with antibodies against plakoglobin (D). (E,F) Protein extracts from vector control (Vec) or PKC $\mu$ -knockdown cells (shPKC $\mu$ ) were resolved on SDS-PAGE gels, and western blots were performed with the indicated antibodies. Note that there is a decrease in plakoglobin and desmoplakin (DP) levels upon PKC $\mu$  knockdown, but no difference in PKP3 levels is observed. (F) Control cells and shPKC $\mu$  cells were fixed and then stained with the indicated antibodies. (G) The PG1-300 construct, comprising the first 300 amino acids of plakoglobin, was produced in bacteria as a GST fusion protein, and kinase assays were performed using recombinant PKC $\mu$ . GST alone was used as a negative control in this assay. Different concentrations of the substrate are shown on the x-axis and enzyme activity is recorded on the y-axis. The mean  $\pm$  s.d. is shown. Note that an increase in kinase activity is observed upon an increase in the concentration of PG1-300 but not with an increase in the concentration of GST alone. Scale bars: 10  $\mu$ m (B); 5  $\mu$ m (D,F).



### KIF5B binds to 14-3-3 $\gamma$ and is required for desmosome assembly

A proteomic screen conducted in our laboratory identified the kinesin 1 family member KIF5B (Cross and Carter, 2000) as a potential ligand for 14-3-3 $\gamma$  (data not shown). As kinesin motor proteins are required for the transport of proteins to the cell border (Cross and Carter, 2000) and have been shown to be required for the transport of the desmosomal cadherins to the cell border (Nekrasova et al., 2011), we hypothesized that plakoglobin was transported to the border by KIF5B. To determine whether

14-3-3 $\gamma$  forms a complex with KIF5B, HCT116 cells were transfected with either the vector control (pcDNA3) or hemagglutinin (HA)-tagged 14-3-3 $\gamma$ , and immunoprecipitation reactions were performed using antibodies to the HA epitope. 14-3-3 $\gamma$  formed a complex with KIF5B, suggesting that 14-3-3 $\gamma$  might load plakoglobin onto KIF5B and lead to the transport of plakoglobin to the cell border (Fig. 6A). To test this hypothesis, KIF5B expression was stably downregulated using vector-driven RNAi. Five clones (K1–K5) were isolated that showed a decrease in KIF5B protein expression in comparison with the vector



**Fig. 6. KIF5B is required for the transport of plakoglobin to the cell border.** (A) HCT116 cells were transfected with the vector control (pcDNA3) or HA-14-3-3 $\gamma$  and immunoprecipitations (IP) were performed with antibodies against the HA epitope. The reactions were resolved on SDS-PAGE gels, and then western blots (WB) were performed with the indicated antibodies. (B) HCT116 cells were transfected with constructs expressing an shRNA targeting KIF5B. Individual cell clones were expanded, and protein extracts from these clones were resolved on SDS-PAGE gels followed by western blotting with antibodies to KIF5B. A western blot for actin was performed as a loading control. Note that the knockdown clones (K1–K5) have a lower level of KIF5B than the vector control (Vec). A western blot for actin served as a loading control. (C) Vector control or the KIF5B-knockdown clones K3 and K5 were incubated with Mitotracker Green FM. (D) Hanging-drop assays were performed to determine cell–cell adhesion. K3 and K5 formed fewer and smaller clumps in comparison with the control cells. (E) Control, K3 and K5 cells were stained with antibodies against plakoglobin (PG), DSC2 and DSC3, DSG2, desmoplakin (DP) and PKP3 and the intensity of the surface staining was quantified by using confocal microscopy. The total magnification was  $\times 630$  with  $\times 2$  optical zoom. Bars correspond to 5  $\mu$ m. (F) Protein extracts prepared from the control, K3 and K5 cells were resolved on SDS-PAGE gels and then analyzed by western blotting with the indicated antibodies. Scale bars: 10  $\mu$ m (C); 5  $\mu$ m (E).

control cells (Fig. 6B). Loss of KIF5B led to a perinuclear accumulation of mitochondria, as reported previously (Tanaka et al., 1998) (Fig. 6C). Furthermore, hanging-drop assays demonstrated that loss of KIF5B led to a decrease in cell–cell adhesion in comparison with the control cells (Fig. 6D; supplementary material Fig. S3B).

To determine whether the decrease in cell–cell adhesion that was observed upon KIF5B knockdown was accompanied by a decrease in the localization of the desmosomal proteins to the cell borders, the control cells and the KIF5B knockdown clones K3 and K5 were stained with antibodies against components of desmosomes or adherens junctions. Similar to that observed in the sh14-3-3 $\gamma$  cells, the levels of plakoglobin, PKP3, desmoplakin, DSC2 and DSC3, and DSG2 substantially decreased at the border in K3 and K5 KIF5B-knockdown cells – a phenotype similar to that of the control cells (Fig. 6E,F). Intensity profiles for the staining are shown in supplementary material Fig. S2. By contrast, knockdown of KIF5B did not lead to a decrease in the levels of E-cadherin, p120 catenin,  $\alpha$ -E-catenin and  $\beta$ -catenin at the cell border (supplementary material Fig. S3C,F,G). The decrease in the levels of desmosomal proteins at the border was not due to a decrease in protein expression levels, as indicated by western blot analysis. In contrast with the results that were observed for plakoglobin, PKP3 and desmoplakin, a large increase in the levels of DSC2 and DSC3, and DSG2 were observed in the kinesin-knockdown cells (Fig. 6F), although this was not observed at the mRNA level (supplementary material Fig. S3E). In cells that had been fixed with methanol, we observed low levels of DSC2 and DSC3, and DSG2, in the cytoplasm (Fig. 6E); however, fixation with paraformaldehyde revealed high levels of DSC2 and DSC3, and DSG2, in the cytoplasm (supplementary material Fig. S4E), suggesting that these proteins accumulate in the cytoplasm upon loss of KIF5B. PKP2 mRNA levels were not altered upon knockdown of kinesin (supplementary material Fig. S3E).

Because kinesin motor proteins transport their cargo on microtubules, we investigated whether disruption of the microtubule network would lead to a decrease in the concentration of plakoglobin at the cell border. Treatment with nocodazole, but not the vehicle control, did indeed lead to a decrease in the levels of plakoglobin at the border (supplementary material Fig. S3D). To then determine whether complex formation between KIF5B and plakoglobin is dependent upon PKC $\mu$ , protein extracts from Go6976- or DMSO-treated HCT116 cells were incubated with GST–14-3-3 $\gamma$ . GST–14-3-3 $\gamma$  formed a complex with KIF5B under both conditions but did not interact with plakoglobin in protein extracts from cells that had been treated with Go6976 (Fig. 5C), indicating a requirement for active PKC $\mu$  for the association between plakoglobin and 14-3-3 $\gamma$  but not for the association between KIF5B and 14-3-3 $\gamma$ .

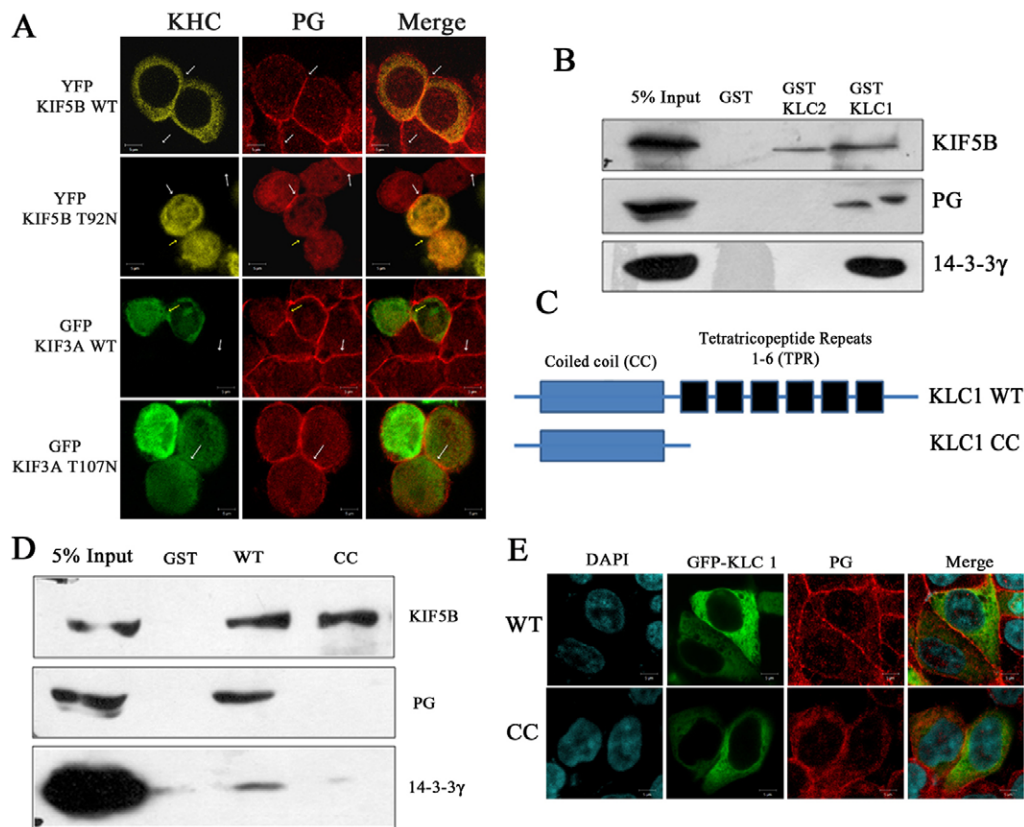
To further confirm the role of KIF5B in transporting plakoglobin to the border, we determined whether a dominant-negative KIF5B construct could inhibit transport of plakoglobin to the cell border. The dominant-negative kinesin heavy chain constructs used here have a point mutation in the ATPase domain (see Materials and Methods), which results in an inability of the proteins to move along the microtubules but preserves the ability of kinesins to bind to cargo (Cross and Carter, 2000). Yellow fluorescent protein (YFP)-tagged versions of wild-type and dominant-negative (mutation T92N) KIF5B, or the GFP-tagged wild-type and dominant-negative (mutation T107N) kinesin 2 family member KIF3A (Cross and Carter, 2000) constructs were

transfected into HCT116 cells. After transfection, the cells were stained with antibodies against plakoglobin and visualized by using confocal microscopy. Cells that expressed wild-type YFP–KIF5B showed border staining for plakoglobin. By contrast, plakoglobin was not localized at the cell border in cells that expressed dominant-negative YFP–KIF5B (Fig. 7A). Cells that had been transfected with either of the KIF3A constructs showed border staining for plakoglobin. Cells that had been transfected with either of the dominant-negative constructs had round edges and showed a morphology that was different from that of cells that had been transfected with either of the wild-type constructs, presumably because overexpression of the dominant-negative constructs affects other cellular processes, such as microtubule organization (Silver and Harrison, 2011). However, despite the change in morphology, only the cells that had been transfected with the dominant-negative KIF5B construct, and not those transfected with the dominant-negative KIF3A construct, showed a disruption of plakoglobin localization (Fig. 7A). These results suggest that KIF5B specifically is required for transport of plakoglobin to the border.

Kinesin motor proteins are heterodimers that consist of two heavy chains and two light chains (Cross and Carter, 2000). KIF5B associates with two different kinesin light chains (KLCs) – KLC1 and KLC2 (Verhey and Hammond, 2009). To determine which of these is required for plakoglobin transport to the cell border, bacterially produced GST-tagged KLC1 and KLC2 were incubated with protein extracts that had been prepared from HCT116 cells, and the reactions were resolved on SDS-PAGE gels followed by western blotting with antibodies against plakoglobin, KIF5B and 14-3-3 $\gamma$ . Both KLC1 and KLC2 formed a complex with KIF5B; however, only KLC1 could form a complex with both plakoglobin and 14-3-3 $\gamma$  (Fig. 7B). KLCs contain a coiled-coiled domain, which is required for complex formation with the kinesin heavy chain, and a cargo-binding domain [which comprises tetratricopeptide repeats (TPRs)] (Verhey and Hammond, 2009) (Fig. 7C). To determine whether the coiled-coiled domain of KLC1 could form a complex with plakoglobin, protein extracts from HCT116 cells were incubated with either GST–KLC1 or the GST-tagged coiled-coiled domain of KLC1. GST–KLC1 could form a complex with KIF5B, plakoglobin and 14-3-3 $\gamma$ , whereas the coiled-coiled domain bound to KIF5B but not to plakoglobin and 14-3-3 $\gamma$  (Fig. 7D). Therefore, the coiled-coiled domain of KLC1 might be acting as a dominant-negative mutant because it should bind to the heavy chain but fail to form a complex with plakoglobin and 14-3-3 $\gamma$ . To test this hypothesis, HCT116 cells were transfected with either GFP–KLC1 or the GFP-tagged KLC1 coiled-coiled domain only and stained with antibodies against plakoglobin. Overexpression of the KLC1 coiled-coiled domain disrupted the transport of plakoglobin to the cell border, whereas cells that expressed the wild-type protein did not show any alteration in plakoglobin localization (Fig. 7E). These results suggest that the KIF5B–KLC1 complex is required for the transport of plakoglobin to the cell border.

#### Loss of KIF5B leads to sterility in male mice

The results described above suggest that 14-3-3 $\gamma$  and KIF5B are required for desmosome formation in epithelial cells. To determine whether loss of KIF5B leads to a decrease in cell–cell adhesion in the seminiferous epithelium, KIF5B expression was inhibited in the testis, as described previously (Sehgal et al., 2011). Loss of KIF5B in the testis led to a phenotype similar to that observed for



**Fig. 7. Dominant-negative mutants of KIF5B and KLC1 inhibit plakoglobin transport to the border.** (A) HCT116 cells were transfected with kinesin heavy chain constructs (KHC) – YFP-tagged wild-type (WT) or dominant-negative (T92N) KIF5B, or GFP-tagged wild-type or dominant-negative (T107N) KIF3A. Post transfection, the cells were fixed and stained with antibodies against plakoglobin (PG). (B) Protein extracts from HCT116 cells were incubated with GST alone or GST–KLC1 or GST–KLC2. The reactions were resolved on SDS-PAGE gels followed by western blotting with the indicated antibodies. (C) The KLC1 mutant KLC1-CC comprises only the coiled-coiled domain and not the tetratricopeptide repeat domain (TPR). (D) Protein extracts from HCT116 cells were incubated with GST alone, wild-type GST–KLC1 and GST–KLC1-CC. The reactions were resolved on SDS-PAGE gels followed by western blotting with the indicated antibodies. (E) GFP–KLC1 or GFP–KLC1-CC were transfected into HCT116 cells. Forty-eight hours post transfection, the cells were fixed and then stained with antibodies against plakoglobin. The nuclei were stained with DAPI. Total magnification is  $\times 630$  with  $\times 2$  optical zoom. Scale bars: 5  $\mu$ m.

the loss of 14-3-3 $\gamma$  (Fig. 8A–C), suggesting that both KIF5B and 14-3-3 $\gamma$  are independently required to regulate cell–cell adhesion in the testis. Importantly, an immunohistochemical analysis demonstrated that loss of either KIF5B or 14-3-3 $\gamma$  did not lead to a decrease in the levels of the other protein (Fig. 8B). Loss of 14-3-3 $\epsilon$  did not lead to a decrease in cell–cell adhesion and spermatogenesis, suggesting that the effects observed upon knockdown of 14-3-3 $\gamma$  are specific to 14-3-3 $\gamma$  (Fig. 8A,B), a result that is consistent with those observed in HCT116 cells (Fig. 2). In addition, loss of either 14-3-3 $\gamma$  or KIF5B led to a detachment of the cells from the basal lamina. To determine whether the detachment of the primary germ cells and the Sertoli cells from the basal lamina led to an increase in cell death, the testis sections were stained using a TUNEL staining kit. As shown in supplementary material Fig. S1A, treatment of the positive-control sections with DNase resulted in a strong positive signal in the TUNEL assay. Testis sections from mice that had been injected with the 14-3-3 $\gamma$ -knockdown construct showed low levels of TUNEL positivity in comparison with testis sections from the control or KIF5B-knockdown mice. Given that the testis morphology of the KIF5B-knockdown mice and the 14-3-3 $\gamma$ -knockdown mice was very similar, it is likely that the loss of cell–matrix adhesion does not lead to cell death, which is consistent

with our results in the cell line model. These results suggest that cell–cell adhesion in the testis requires both 14-3-3 $\gamma$  and KIF5B and that loss of either protein leads to defects in cell–cell adhesion.

To determine whether desmosome organization was altered in the 14-3-3 $\gamma$ - and KIF5B-knockdown testis, testis sections were stained with antibodies against plakoglobin, PKP3, DSC2 and DSC3, N-cadherin and E-cadherin. Plakoglobin, PKP3 and DSC2 and DSC3 localized to the border in testis that had been injected with the control virus; however, the levels of these proteins at the cell border were greatly diminished in the 14-3-3 $\gamma$ - and KIF5B-knockdown testis (Fig. 8D). By contrast, there was no change in E-cadherin localization in the 14-3-3 $\gamma$ - and KIF5B-knockdown testis in comparison with testis sections that had been injected with the vector control, a phenotype similar to that observed in HCT116 cells in culture. In contrast with the results obtained for E-cadherin, it was observed that N-cadherin localized to the border in the vector control and 14-3-3 $\gamma$ -knockdown testis but not in the KIF5B-knockdown testis (Fig. 8D). These results are consistent with our observations that the KIF5B-knockdown testis showed a more severe adhesion phenotype than the 14-3-3 $\gamma$ -knockdown testis and that KIF5B might be required for the transport of other cell–cell adhesion molecules to the border, in addition to desmosomal proteins. Overall, our results suggest that



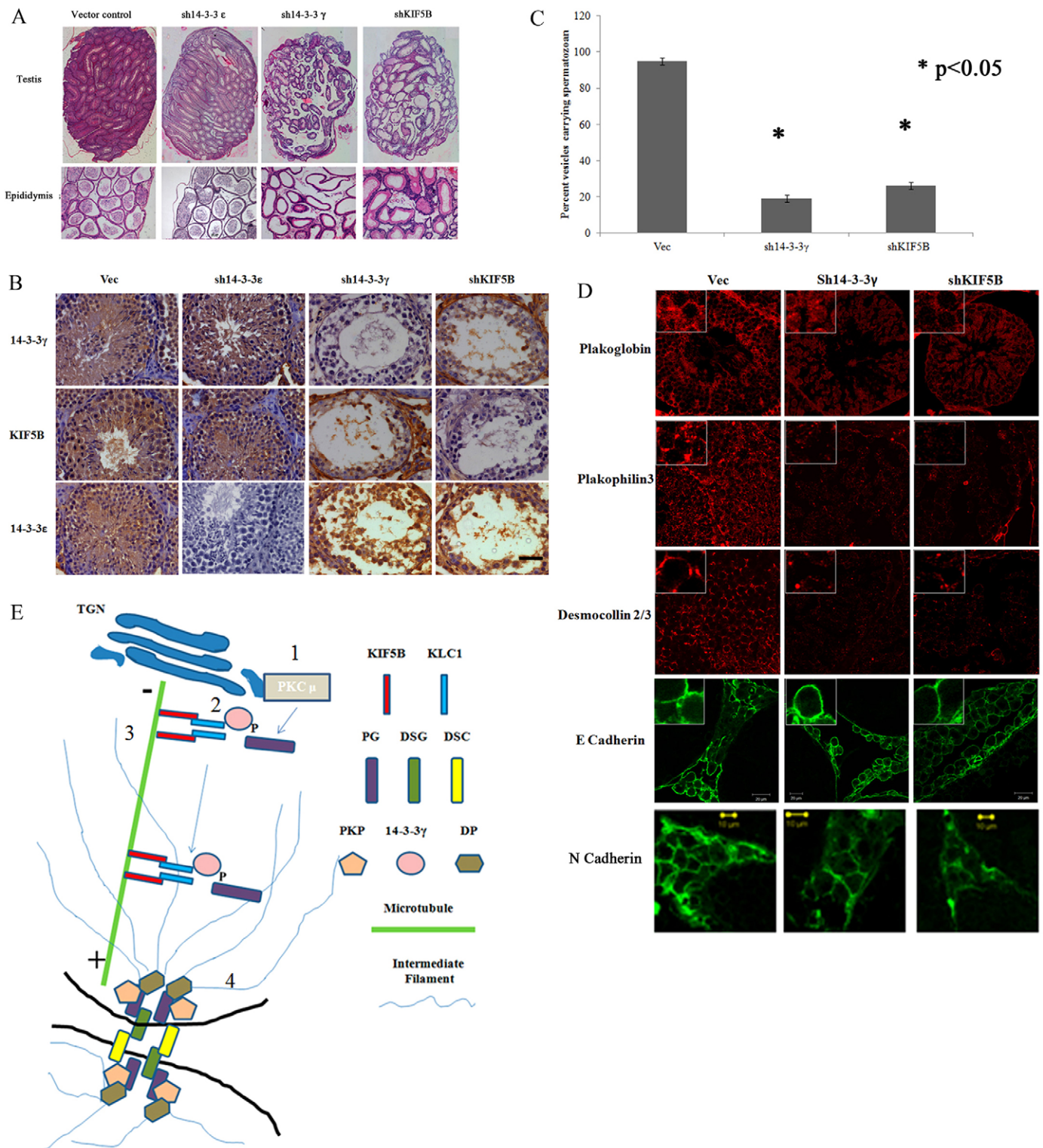


Fig. 8. See next page for legend.

14-3-3γ and KIF5B are required for the formation of desmosomes *in vivo*.

**DISCUSSION**

Our results suggest that 14-3-3γ and the KIF5B–KLC1 complex are required for the transport of plakoglobin to cell borders in

human cell lines and in the mouse testis, and disruption of this interaction with 14-3-3γ leads to a defect in the localization of plakoglobin. In addition, loss of 14-3-3γ leads to a disruption of cell–matrix adhesion, which might affect cell–cell adhesion *in vitro* and *in vivo*. The association of plakoglobin with 14-3-3γ, and the transport of plakoglobin to the border is dependent on

**Fig. 8. Loss of KIF5B and 14-3-3 $\gamma$  in the testis leads to a disruption of desmosome formation and sterility.** (A–D) Lentiviruses encoding the vector control or shRNAs targeting 14-3-3 $\epsilon$ , 14-3-3 $\gamma$  or KIF5B were injected into the testes of Swiss mice (sh14-3-3 $\epsilon$ , 14-3-3 $\gamma$  and shKIF5B, respectively). 35 days post-injection the mice were killed, sections of the epididymis and testes were stained with hematoxylin and eosin and examined by using microscopy (A). Immunohistochemical analysis using antibodies against the different proteins that were knocked down demonstrated that the expression of 14-3-3 $\epsilon$ , 14-3-3 $\gamma$  and KIF5B is inhibited in the testis that had been injected with the appropriate lentivirus (B). The percentage of epididymal vesicles that showed the presence of mature spermatozoa in three different animals was determined and the means  $\pm$  s.d. are shown. (C) Testis sections were stained with antibodies against plakoglobin (PG), plakophilin 3 (PKP3), desmocollins (DSC)2 and DSC3, N-cadherin and E-cadherin. (D) The total magnification was  $\times$ 630 with  $\times$ 2 optical zoom. The inset images show a higher magnification of the cells to demonstrate border localization. (E) Desmosome assembly based on the experimental findings presented here. (1) PKC $\mu$  phosphorylates plakoglobin, thereby, allowing association with 14-3-3 $\gamma$  and resulting in the loading of plakoglobin onto the KIF5B–KLC1 complex. (2) The motor protein complex containing plakoglobin moves along microtubules (3) resulting in desmosome assembly at the border (4). TGN, trans-Golgi network. Scale bars: 20  $\mu$ m (B); 10  $\mu$ m (C).

PKC $\mu$  activity; thus, loss of either 14-3-3 $\gamma$  or KIF5B inhibits plakoglobin transport, resulting in a decrease in desmosome formation and cell–cell adhesion in HCT116 cells and the testis, leading to male sterility. The decrease in the levels of N-cadherin at cell borders in KIF5B-knockdown testis resulted in a more drastic phenotype in these animals than in those that had been subjected to knockdown of 14-3-3 $\gamma$ , suggesting that N-cadherin is required for cell–cell adhesion in the testis as previously reported (Andersson et al., 1994; Lee et al., 2003).

Previous results have shown that loss of plakoglobin leads to the depletion of desmosomal proteins from the cell border and to defects in desmosome formation (Acehan et al., 2008; Gosavi et al., 2011; Knudsen and Wheelock, 1992; Lewis et al., 1997). Although the presence of a classical cadherin seems to be required for plakoglobin recruitment to the cell border (Michels et al., 2009; Tinkle et al., 2008), the mechanisms by which plakoglobin is transported have not been identified. The results presented here suggest that phosphorylation of plakoglobin by PKC $\mu$  at residue S236 leads to the generation of a binding site for 14-3-3 $\gamma$ , and that 14-3-3 $\gamma$  is required for the transport of plakoglobin to the cell border in order to initiate desmosome formation, presumably in a complex with a classical cadherin (Michels et al., 2009; Tinkle et al., 2008). 14-3-3 $\gamma$  might be required to load plakoglobin onto the KIF5B–KLC1 complex, which precedes the transport of plakoglobin on microtubules (Fig. 8E). This is consistent with previous observations that showed that PKC $\mu$  localizes to the Golgi complex (Hausser et al., 2002; Prestle et al., 1996) and is required for the fission of vesicles carrying cargo to the cell border (Liljedahl et al., 2001). A 14-3-3 $\gamma$  dimer is required for carrier formation at the Golgi complex along with PKC $\mu$  (Valente et al., 2012), suggesting that the loss of 14-3-3 $\gamma$  could disrupt desmosome formation owing to defects in plakoglobin transport.

The treatment of cells with an inhibitor that inactivates both PKC $\alpha$  and PKC $\mu$  led to a decrease in the localization of plakoglobin at cell borders and abolished complex formation between plakoglobin and 14-3-3 $\gamma$ . However, treatment with the inhibitor does not inhibit the interaction between 14-3-3 $\gamma$  and other desmosomal proteins or KIF5B. These results suggest that phosphorylation of plakoglobin by PKC $\mu$  is required for complex formation between plakoglobin and 14-3-3 $\gamma$  and the loading of

plakoglobin onto KIF5B, which is essential for the transport of plakoglobin. Alternatively, it is possible that plakoglobin, desmoplakin and PKP3 are transported independently to the border and that the absence of plakoglobin at the border prevents the formation of a functional desmosome. Furthermore, it was observed that loss of plakoglobin did not affect the localization of DSC2 and DSC3, and DSG2 to cell borders, unlike the loss of 14-3-3 $\gamma$  or KIF5B. This might be because 14-3-3 $\gamma$  is required for the localization of PKP3, desmoplakin and plakoglobin; therefore, the defects in the localization of the desmosomal cadherins that is observed upon 14-3-3 $\gamma$  loss are not observed upon plakoglobin loss. Plakoglobin is probably required to stabilize and maintain the organization of the desmosome, which is why loss of plakoglobin leads to a decrease in cell–cell adhesion in these cells as previously reported (Gosavi et al., 2011). PKC $\mu$  was also shown to directly phosphorylate a peptide comprising the first 300 amino acids in plakoglobin, which contains the S236 residue, consistent with the notion that S236 serves as site for phosphorylation by PKC $\mu$ . This does not exclude the possibility that PKC $\mu$  phosphorylates other residues in plakoglobin and might provide an explanation for the observation that plakoglobin protein levels decreased when PKC $\mu$  expression was inhibited. It is possible that PKC $\mu$  regulates other aspects of plakoglobin function that are not limited to the transport of plakoglobin to the cell border, such as the retention of plakoglobin at the border or the stability of the plakoglobin protein.

In the absence of KIF5B, or upon expression of a dominant-negative KIF5B construct, plakoglobin does not localize to the cell border, leading to a decrease in the recruitment of other components of the desmosome, as previously reported (Gosavi et al., 2011; Lewis et al., 1997). Previous experiments have suggested that inhibiting KIF5B expression does not affect the localization of plakoglobin to the border in SCC9 cells (Nekrasova et al., 2011). Our results, however, suggest that loss of KIF5B in HCT116 cells and the testis results in an inhibition of plakoglobin transport to the border. Consistent with the results reported by Nekrasova and colleagues (Nekrasova et al., 2011), we did not observe any defects in plakoglobin localization upon expression of a dominant-negative KIF3A mutant. The discrepancy in these two reports might be due to the use of different cell types; other kinesin-family members could regulate plakoglobin transport in SCC9 cells in the absence of KIF5B. Similarly, Pasdar and colleagues have reported that a microtubule network is not essential for desmosome formation (Pasdar et al., 1991); however, the results from this report and others suggest that the transport of desmosomal components to the border is dependent on an intact microtubule network (Gloushankova et al., 2003; Nekrasova et al., 2011). This is consistent with reports that suggest that desmosome organization, function and composition vary in different cell types (reviewed in Cross and Carter, 2000; Garrod and Chidgey, 2007; Getsios et al., 2004; Green and Gaudry, 2000; Hatzfeld, 2007) and with the observations that loss of the different desmosomal components in the mouse leads to a vast variety of phenotypes (Chidgey et al., 2001; Gallicano et al., 1998; Grossmann et al., 2004; Koch et al., 1997; Lechler and Fuchs, 2007; Ruiz et al., 1996; Sklyarova et al., 2008; Vasioukhin et al., 2001).

Although 14-3-3 $\gamma$  seems to be required for the transport of plakoglobin, it might also be required for the transport of PKP3 and desmoplakin to the cell border in a manner that is independent of plakoglobin transport. This is consistent with our observation that inhibition of both PKC $\alpha$  and PKC $\mu$  did not



lead to the disruption of the interaction between 14-3-3 $\gamma$  and desmoplakin or PKP3. The transport of DSG2 to the border is dependent on KIF5B, whereas the transport of DSC2 requires KIF3A and PKP2 (Nekrasova et al., 2011). Our results suggest that plakoglobin transport to the border is dependent on KIF5B, but not KIF3A, suggesting that the transport of desmosomal cadherins and plaque proteins to the border occurs through independent microtubule-dependent pathways. Alternatively, because 14-3-3 proteins bind to their ligands as dimers, with each member of the dimer forming a complex with a phosphopeptide (Brunet et al., 2002; Yaffe et al., 1997), it is possible that 14-3-3 $\gamma$  bridges interactions between the different desmosomal plaque proteins, thereby allowing the formation of an intact desmosome, as previously postulated by Bonn e and colleagues (Bonn e et al., 2003). The only argument against this hypothesis is that, according to our study, 14-3-3 $\gamma$  does not localize to the desmosome (Fig. 4A). In fact, the lack of any colocalization between 14-3-3 $\gamma$  and plakoglobin suggests that any function that 14-3-3 $\gamma$  performs, with respect to plakoglobin localization, is transient in nature; therefore, we favor the hypothesis that 14-3-3 $\gamma$  is required for loading plakoglobin onto the KIF5B–KLC1 complex.

In contrast with the results reported here, previous work has suggested that 14-3-3 $\gamma$ -knockout mice are viable, and no adhesion or sterility defects have been reported in these mice (Steinacker et al., 2005). Plakoglobin-knockout mice die during embryogenesis owing to cardiac defects arising as a result of decreased desmosome formation, and, as these mice die before the testis is formed, no information is available on the effects of loss of plakoglobin in the testis (Ruiz et al., 1996). Taken together, these previously published reports suggest that the functions of plakoglobin that are required for desmosome formation are not altered in the 14-3-3 $\gamma$ <sup>-/-</sup> mice. Our results indicate that 14-3-3 $\gamma$  regulates desmosome formation in multiple cell types, as loss of 14-3-3 $\gamma$  in HCT116 cells, which are derived from the colon, and in the seminiferous epithelium leads to a decrease in cell–cell adhesion and in desmosome formation. Our results are also consistent with the previously reported role of 14-3-3 $\gamma$  in the transport of proteins from the Golgi complex to the cell border (Valente et al., 2012). One reason for the differences in these results and those reported by Steinacker and colleagues (Steinacker et al., 2005) could be that another 14-3-3 family member binds to plakoglobin and stimulates desmosome formation in the 14-3-3 $\gamma$ <sup>-/-</sup> mice, and that this compensation is not observed upon shRNA-mediated knockdown in the testis. Another possibility is that these are strain-specific variations that are due to differences in the genetic background of the mice used in the two studies. The generation of an inducible knockdown of 14-3-3 $\gamma$ , in either the Swiss mice used in this study or in other mouse strains, could help determine whether loss of 14-3-3 $\gamma$  leads to defects in desmosome formation and cell–cell adhesion in other tissues and not just in the testis.

The results described above point to the following model. 14-3-3 $\gamma$  binds to plakoglobin that has been phosphorylated at residue S236 by PKC $\mu$  and loads plakoglobin onto the KIF5B–KLC1 complex for transport to the cell border (Fig. 8E). Loss of either 14-3-3 $\gamma$  or KIF5B, or the dominant-negative versions of KIF5B and KLC1, inhibit the transport of plakoglobin to the border. Because 14-3-3 $\gamma$  forms a complex with PKP3 and desmoplakin, loss of 14-3-3 $\gamma$  might also lead to defects in transport of these proteins to the border. As loss of plakoglobin does not substantially affect the localization of the cadherins to the

border, it is possible that loss of plakoglobin leads to a defect in cadherin retention or the formation of an intact desmosome at the border in the absence of 14-3-3 $\gamma$ . Furthermore, loss of KIF5B in the testis led to sterility and a decrease in desmosome formation. This is consistent with previously reported results that showed that disruption of cell–cell adhesion (Cheng and Mruk, 2002) and desmosome-like junction formation in the testis leads to an increase in sterility (Li et al., 2009; Lie et al., 2010).

To conclude, 14-3-3 $\gamma$  and the KIF5B–KLC1 complex are required for regulating the transport of plakoglobin to the cell border. A decrease in 14-3-3 $\gamma$  levels leads to a decrease in desmosome formation and the recruitment of other desmosomal proteins to the border. 14-3-3 $\gamma$  might be required to maintain cell–cell adhesion in multiple tissues; however, this can only be confirmed by additional experiments *in vivo*.

## MATERIALS AND METHODS

### Animals

Swiss mice Crl:CFW(SW) were bred and maintained in the laboratory animal facility of the Advanced Centre for Treatment Research and Education in Cancer (ACTREC). Protocols for the experiments were approved by the Institutional Animal Ethics Committee of ACTREC. The animal study proposal number is 11/2008 dated August 19, 2008. The testis injections were performed as previously described (Sehgal et al., 2011).

### Plasmids

Details of the oligonucleotides used in this study are available in supplementary material Table S1. The GST–14-3-3 $\gamma$ , HA-epitope-tagged 14-3-3 $\gamma$  and shRNA-resistant GFP–14-3-3 $\gamma$  constructs have been previously described (Hosing et al., 2008). Site-directed mutagenesis (Stratagene) was used to generate the MYC–PG-S236A construct. The full-length KLC1 and KLC2 cDNA (Rahman et al., 1998), KLC-1 deletion (GST–KLC1 WT, GST–KLC1-CC and GST–KLC1-TPR) (Aoyama et al., 2009), GFP–KIF3A (Haraguchi et al., 2006), wild-type YFP–KIF5B (Gu et al., 2006), dominant-negative kinesin (GFP–KIF3A-T107N and YFP–KIF5B-T92N) (Wiesner et al., 2010), wild-type GFP–KLC1 and the GFP-tagged KLC1 coiled-coiled domain (Araki et al., 2007) constructs have been described previously. To generate the shRNA constructs against KIF5B and PKC $\mu$ , oligonucleotide pairs (supplementary material Table S1) were ligated into the pLKO.1 Puro or pLKO.1 EGFP-f Puro vectors as described previously (Sehgal et al., 2011). GST–PG1-300 was generated by amplifying the first 300 amino acids of plakoglobin (supplementary material Table S1) and cloning the fragment into the pGEX4T1 vector (Amersham).

### Cell lines and transfections

The HCT116 (American Type Culture Collection) and the HCT116-derived stable cell lines were cultured as described previously (Hosing et al., 2008). To generate the KIF5B-knockdown clones in the HCT116 cell line, the cells were transfected with 1  $\mu$ g of the shRNA constructs that targeted human KIF5B. Sixty hours post transfection, the cells were transferred to medium that contained 0.5  $\mu$ g/ml of puromycin (Sigma) to generate single-cell clones. The clones K3 and K5 were used for the indicated experiments. The HCT116-derived plakoglobin-knockdown clones have been described previously (Gosavi et al., 2011).

### Antibodies and western blotting

The antibodies against PKP3, both DSC2 and DSC3, DSG2, plakoglobin, desmoplakin, K8 (keratin 8), actin, E-cadherin,  $\beta$ -catenin and  $\alpha$ -E-catenin were used in western blots as previously described (Gosavi et al., 2011; Khapare et al., 2012; Kundu et al., 2008). Tissue culture supernatants of the antibodies against HA (12CA5), 14-3-3 $\gamma$  (CG31) and 14-3-3 $\sigma$  (CS112) were used at a dilution of 1:50. The antibodies against 14-3-3 $\epsilon$  (T-16, Santa Cruz, dilution of 1:2000), p120 catenin (mouse monoclonal from BD Transductions, catalog number 610134, dilution of 1:1000) and



PKC $\mu$  (rabbit monoclonal obtained from Abcam, catalog number 3146-1, dilution of 1:500) were used for western blot analysis. The secondary antibodies against mouse and rabbit IgGs were conjugated to horseradish peroxidase and used at a dilution of 1:1000 (Invitrogen) and 1:5000 (Pierce), respectively.

### Immunofluorescence and calcium-switch assays

The cells were cultured on chromic-acid-treated, poly-L-lysine-coated glass coverslips at a confluence of 70–80%. Before fixation, the cells were carefully washed twice with  $1\times$  PBS. HCT116-derived clones were fixed in absolute methanol for 10 minutes at  $-20^{\circ}\text{C}$  to detect  $\alpha$ -tubulin, KIF5B, PKP2, Par3, ZO1, P-cadherin, desmoplakin, plakoglobin, DSC2 and DSC3, DSG2, E-cadherin and PKP3. In some experiments, cells were fixed in 4% paraformaldehyde and permeabilized with Triton X-100 as described previously (Gosavi et al., 2011). The antibodies against PKP3, both DSC2 and DSC3, DSG2, plakoglobin, desmoplakin, K8, actin, E-cadherin,  $\beta$ -catenin, ZO-1 and  $\alpha$ -E-catenin were used in immunofluorescence analysis as described previously (Gosavi et al., 2011; Khapare et al., 2012; Kundu et al., 2008). Antibodies against PKP2 (BD Clontech, dilution 1:25), KIF5B (Abcam, dilution 1:100),  $\alpha$ -tubulin (Abcam, dilution 1:150), Par3 (Millipore, dilution 1:50), ZO-1 (Abcam, dilution 1:100), P-cadherin (BD Transduction Laboratories, dilution 1:100), HA (12CA5, supernatant), p120 catenin (BD Transductions, dilution 1:100), N-cadherin (Life Technologies, catalog number 33-3900, dilution 1:50),  $\alpha$ -E-catenin (Santa Cruz Biotechnology, dilution 1:25) and E-cadherin (clone 36/E-cadherin, mouse monoclonal, BD Transduction Laboratories, dilution 1:100) were incubated with the cells for 1 hour at room temperature at the indicated dilutions as described previously (Gosavi et al., 2011). To stain mitochondria, Mitotracker Green FM (Invitrogen) was used at a concentration of 100 nM to stain live cells. Confocal images were obtained by using a LSM 510 Meta Carl Zeiss confocal system with argon 488-nm and HeNe 543-nm lasers. All images were obtained by using an Axio Observer Z.1 microscope (numerical aperture 1.4) at a magnification of  $\times 630$  ( $\times 63$  objective and  $\times 10$  eyepiece) with a  $\times 2$  or  $\times 4$  optical zoom. The surface intensity of staining was measured for the different proteins in a minimum of 24 cells using the Axiovision software, and the mean and standard deviation were plotted.

### Hanging-drop assays

Hanging-drop assays were used to measure cell adhesion as described previously (Kundu et al., 2008).

### GST-pulldown and immunoprecipitation assays

These assays were performed as described previously (Dalal et al., 1999).

### GST-plakoglobin production and kinase assays

GST alone or GST-PG1-300 was purified from bacteria as described previously (Dalal et al., 2004). The purified proteins were used in kinase assays with recombinant PKC $\mu$  and a peptide that had been derived from CREB as a positive control (Signal Chem). Kinase activity was determined by using the ADP glow assay kit (Promega) according to the manufacturer's instructions.

### Histology and immunohistochemistry

Mouse testes were fixed in 10% formaldehyde overnight and processed for histology as described previously (Kundu et al., 2008). TUNEL assays were performed as per the manufacturer's instructions (Promega).

### Electron microscopy

Wild-type and 14-3-3 $\gamma$ -knockdown testes were fixed with 3% glutaraldehyde and post-fixed with 1% osmium tetroxide (Tedpella). Grids were contrasted with alcoholic uranyl acetate for 1 minute and lead citrate for 30 seconds. The grids were observed under a Carl Zeiss LIBRA120 EFTEM transmission electron microscope at an accelerating voltage of 120 kV and at  $\times 325,000$  magnification. Images were captured using a Slow Scan CCD camera (TRS, Germany).

### Statistical Analysis

All *P*-values were generated using a Student's *t*-test.

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### Competing interests

The authors declare no competing interests.

### Author contributions

L.S., M.M.V., F.S., U.K. and S.N.D. designed experiments and wrote the manuscript. L.S. performed the majority of the experiments with contributions from A.R., N.K., M.S., K.B., S.A. and N.A. A.M. determined that 14-3-3 $\gamma$  forms a complex with KIF5B, S.S.V. performed the PKC $\mu$ -knockdown experiments and solubility assays. L.B. performed electron microscopy, R.M. performed the PKC $\mu$  kinase assays, M.G. and S.B. performed the RT-PCR assays and helped with confocal microscopy, H.A. performed the cell–matrix adhesion assays, R.T. performed the testis injections and A.S.H. generated the 14-3-3 $\epsilon$ -knockdown clones.

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### Supplementary material

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