## Inventory of Supplemental Information

1. Figure S1, supplements Figure 1 and demonstrates impaired junctional maturation and function in immortalized Nf2-deficient keratinocytes. Additionally, this figure provides evidence that the levels of actin associated with the E-cadherin complex are decreased in Nf2-shRNA expressing keratinoctyes while the levels and composition of the core AJ complex are unaffected.
2. Figure S2, supplements Figure 1 and demonstrates that Merlin binds to the VH1 region of α-catenin *in vitro*. Additionally, this figure shows that Ezrin does not bind α-catenin *in vitro*.
3. Figure S3, supplements Figure 3 and demonstrates an impaired inside-out barrier function in the *K14-Cre*;$Nf2^{lox/lox}$ epidermis. This figure also shows that the formation of desmosomes, hemidesmosomes and the localization of α-catenin is indistinguishable between wild-type and *K14-Cre*;$Nf2^{lox/lox}$ skin.
4. Figure S4, supplements Figure 6 and provides evidence that Par3 can associate with both the N-terminal and C-terminal region of Merlin in cells. Additionally, this figure demonstrates that Merlin binds the region of Par3 that contains PDZ3. This figure also shows that Par3 does not directly bind AJ proteins *in vitro*, and that endogenous Merlin can associate with α-catenin, Par3 and actin in keratinoctyes.
5. Figure S5, supplements Figure 7 and demonstrates that Merlin is necessary to retain Par3 in an insoluble compartment. *In situ* extraction of Nf2-shRNA expressing keratinocytes removes membrane co-localization of Par3 and α-

catenin while control keratinocytes maintain Par3 and α-catenin co-localization following *in situ* extraction.

6. Supplemental materials and methods describe additional materials used and methods performed in this study.
7. Supplemental references lists articles referenced in the supplemental figure legends or materials and methods.

# Figure S1, related to Figure 1, Gladden *et al.*

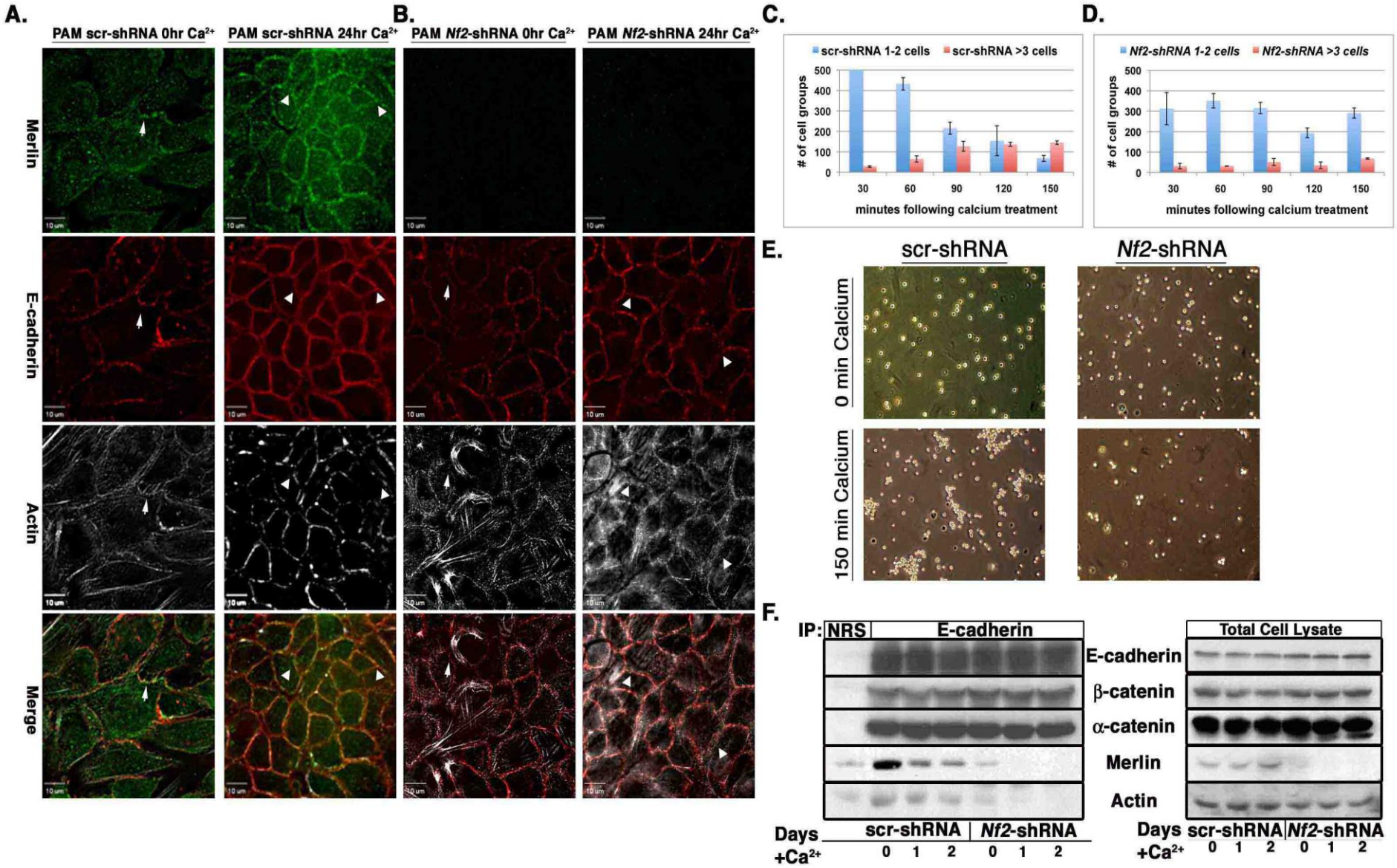


**Figure S2, related to Figure 1, Gladden *et al.***

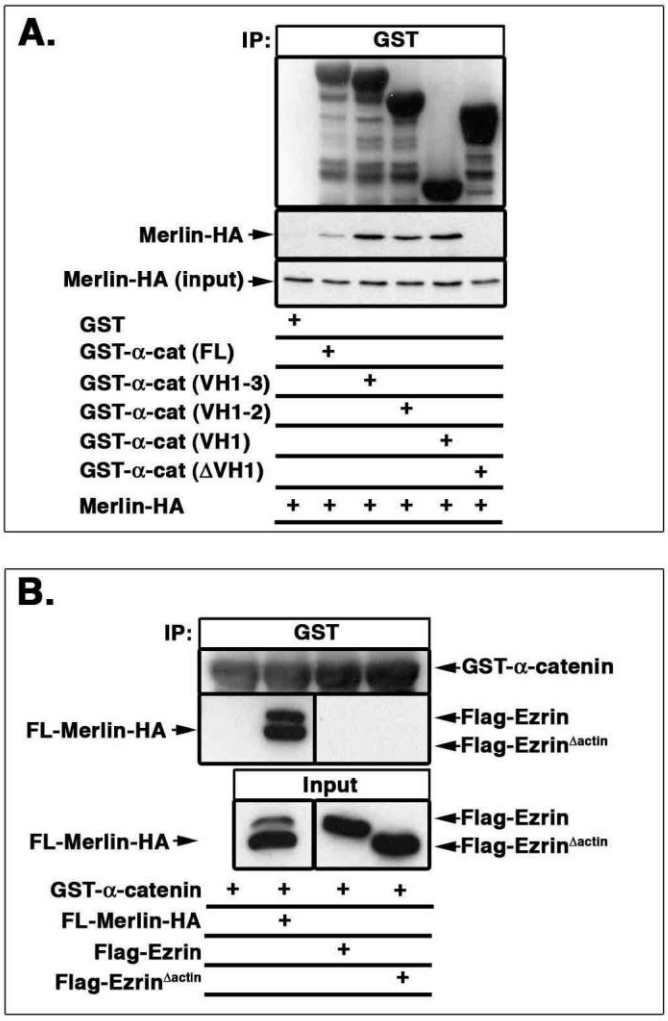


**Figure S3, related to Figure 3, Gladden *et al.***

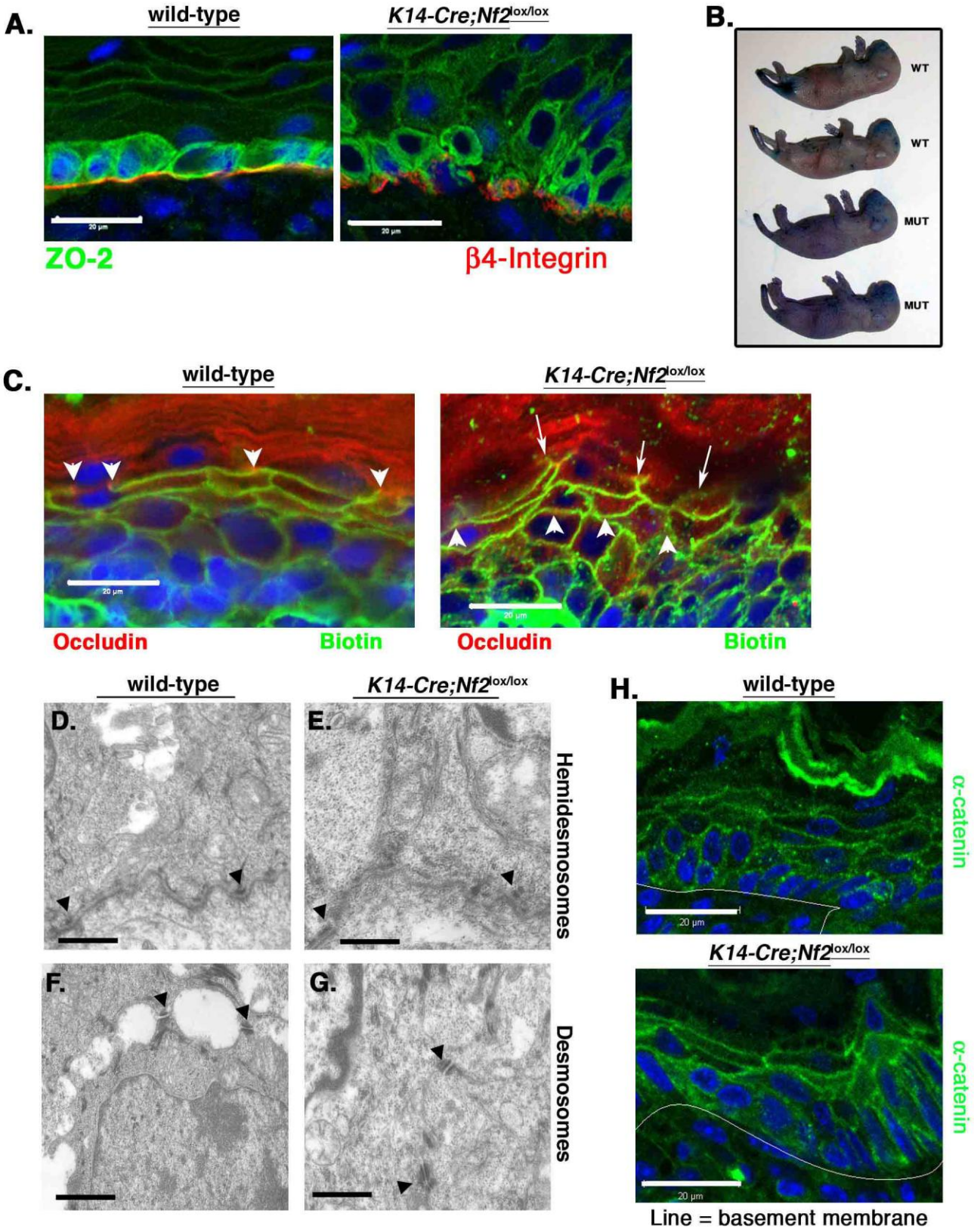


**Figure S4, related to Figure 6, Gladden *et al.***

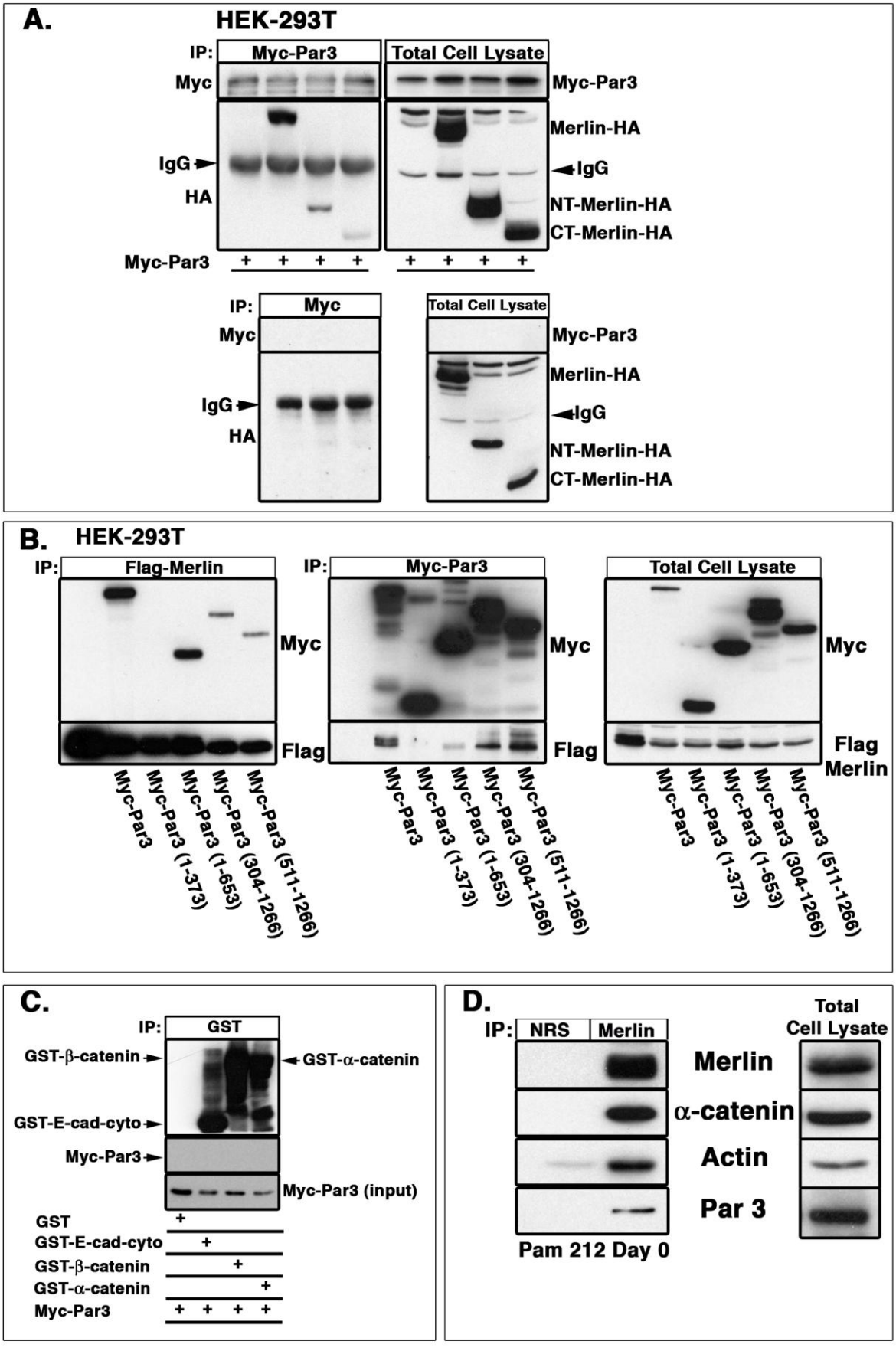
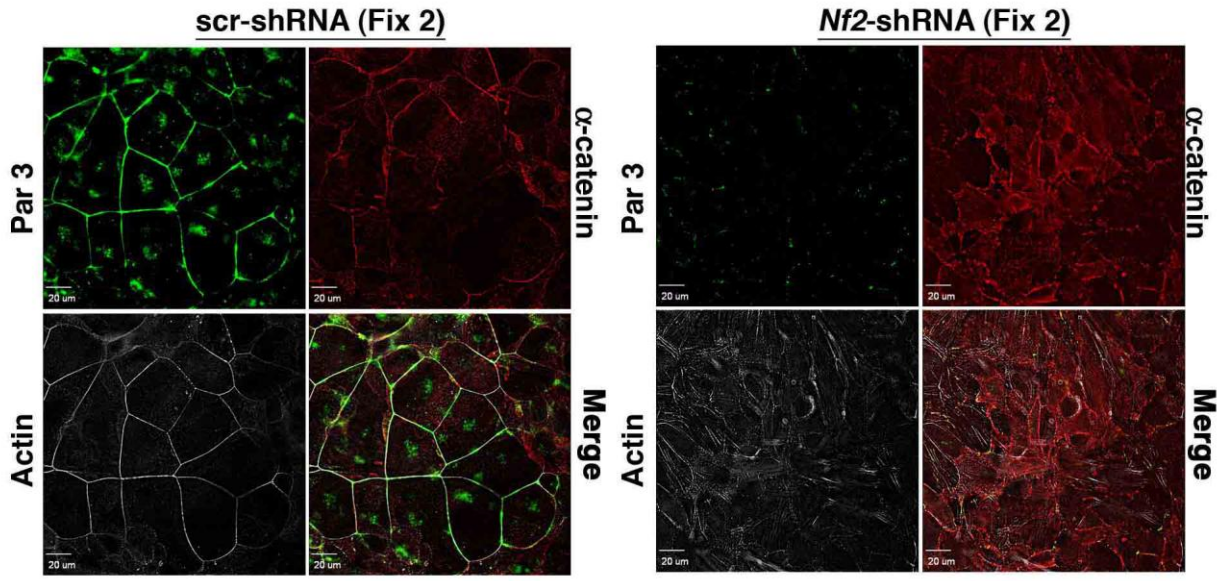


**Figure S5, related to Figure 7, Gladden *et al.***



**Supplemental Figure S1. Impaired junctional maturation and function in immortalized Nf2-deficient keratinocytes .**

Immortalized keratinocytes (PAM212) were infected with (A) control (scr-shRNA-expressing) or (B) *Nf2*-shRNA-expressing lentiviruses and cultured in calcium-depleted or calcium-containing media (1.5mM) for 24 hours. Staining of control cells with antibodies specific for Merlin (green), E-cadherin (red), or actin (white) revealed colocalization of Merlin, E-cadherin and actin at PAs (arrows) and AJ belts (arrowheads) as observed in primary keratinocytes (Figure 1A). Likewise, staining of *Nf2*-shRNA expressing PAM212 cells revealed similarly defective junctional maturation as observed in *K14-Cre;Nf2*$^{lox/lox}$ keratinocytes (Figure 1B). (C) Hanging drop assays were performed on (C) scr-shRNA- or (D) *Nf2*-shRNA-expressing PAM212 keratinocytes. Single-cell suspensions were placed in calcium-containing media (1.5mM) for the indicated times. Clusters containing increasing numbers of cells were observed over time in scr-shRNA- but not in *Nf2*-shRNA-expressing cells. Instead, *Nf2*-shRNA-expressing cells formed only 1-2 cell clusters at all times after calcium-stimulation indicating impaired cell adhesion in the absence of Merlin. Values = mean +/- SD. (E) Representative pictures of cells in C and D at the start (0 min) and end (150 min) of the assay. (F) scr-shRNA-expressing or *Nf2*-shRNA-expressing keratinocytes were cultured in calcium-depleted (day 0) or calcium-containing media for the indicated times followed by immunoprecipitation of E-cadherin and immunoblotting using anti-E-cadherin, -β-catenin, -α-catenin, -actin, or -Nf2 antibodies. As a negative control, immunoprecipitations were performed using normal rabbit serum (NRS). The levels of β-catenin and α-catenin that associate with E-cadherin are not affected by the absence of

Merlin whereas the amount of actin associated with E-cadherin was decreased in the absence of Merlin.

**Supplemental Figure S2. Merlin, but not Ezrin, binds the VH1 region of α-catenin.** (A) Full length Merlin-HA produced by IVTT was mixed with bacterially produced GST-α-catenin (FL), (VH1-3), (VH1-2), (VH1), or (ΔVH1). Merlin-HA associates with GST-α-catenin products that contain the VH1 domain but not with GST-α-catenin (ΔVH1) that lacks the VH1 domain. Merlin-HA input was monitored by western blot of 10% of the Merlin-HA used in the binding assay. (B) Neither full length Flag-Ezrin nor Flag-Ezrin$^{\Delta actin}$, which adopts a constitutively open conformation with an exposed FERM domain, binds full length GST-α-catenin *in vitro*.

**Supplemental Figure S3. The *K14-Cre;Nf2$^{lox/lox}$* epidermis exhibits an impaired inside-out epidermal barrier function but displays normal hemidesmosome/desmosome formation and α-catenin localization.**

(A) Sections of wild-type (left panel) or *K14-Cre;Nf2$^{lox/lox}$* (right panel) neonatal skin were stained with ZO-2 (green), β4-integrin (red), and DAPI. ZO-2 localizes to cell-cell boundaries in suprabasal cells of the wild-type epidermis but displays a ragged diffuse staining pattern in *K14-Cre;Nf2$^{lox/lox}$* epidermis. Bars, 20 μm. (B) Blue dye penetration ('outside-in') assay performed on E19.5 wild-type (top) or *K14-Cre;Nf2$^{lox/lox}$* (bottom) embryos shows little change in penetration of toluidine blue dye indicating an intact stratum corneum. (C) Biotin permeability assay performed on newborn wild-type (left) or

*K14-Cre;Nf2*$^{lox/lox}$ (right) epidermis reveals the abnormal penetration of biotin (arrows) beyond the onset of strong occludin membrane staining (arrowheads) in the absence of Merlin; in contrast, biotin penetration abruptly stops at this point in the wild-type epidermis. This defect is typical of mice with an impaired inside-out epidermal barrier function. Bars 20 μm. (D, E) Transmission electron microscopy reveals normal hemidesmosome formation (arrowheads) at the dermal:epidermal junction within the epidermis of both wild-type (D) and *K14-Cre;Nf2*$^{lox/lox}$ (E) newborn (P0) littermates. Bars, 500 nm. (F, G) Similarly, a normal appearance and distribution of desmosomes (arrowheads) are observed between suprabasal cells of the wild-type (F) and *K14-Cre;Nf2*$^{lox/lox}$ (G) newborn (P0) littermates. Bars, 500 nm. (H) The localization of α-catenin to cell:cell boundaries is similar in the newborn (P0) wild-type and *K14-Cre;Nf2*$^{lox/lox}$ epidermis. The white line in each panel denotes the location of the basement membrane. Bars, 20 μm.

**Supplemental Figure S4. Merlin links Par3 to α-catenin via a region of Par3 necessary for TJ formation.**

(A) HEK293T cells were transfected with a Myc-Par3-expressing plasmid alone or in combination with plasmids expressing full-length Merlin-HA, N-terminal (NT) Merlin-HA, or C-terminal (CT) Merlin-HA. Immunoprecipitation using an anti-Myc antibody followed by immunoblotting with an anti-HA antibody revealed that, as *in vitro*, full-length Merlin as well as the N- and C-terminal portions of the protein, can associate with Par3 (top panel). In contrast, transfection of full-length, N-terminal or C-terminal Merlin-HA alone followed by immunoprecipitation using an anti-Myc antibody and

immunoblotting with an anti-HA antibody revealed only the IgG-heavy chain confirming the specificity of the IPs (bottom panel). (B) HEK293T cells were transfected with a Flag-Merlin-expressing plasmid alone or in combination with plasmids expressing the indicated Myc-Par3 deletion constructs. Immunoprecipitation using anti-Flag (left panel) or anti-Myc (middle panel) antibodies followed by immunoblotting suggest that the central region of Par3 containing the third PDZ domain (residues 511-653) and previously shown to be important in TJ formation is necessary for Merlin-association. Note that these Par3 expression constructs lack the aPKC binding site and have been described in (Chen and Macara, 2005). Expression of each protein was detected by immunoblotting of the total cell lysate as shown in the right panel. (C) Myc-Par3 produced in Sf9 cells was mixed with either GST-E-cadherin cytoplasmic domain (E-cad-cyto), GST-β-catenin, or GST-α-catenin; isolation, separation and immunoblotting of GST-containing complexes revealed that Par3 cannot directly bind to AJ proteins. (D) Immunoprecipitation of endogenous Merlin from PAM212 keratinocytes cultured in calcium-depleted media (day 0) followed by immunoblotting revealed that endogenous Merlin associates with endogenous α-catenin, actin and Par3 (180kd isoform).

**Supplemental Figure S5. Merlin is necessary for the retention of Par3 in an insoluble α-catenin- and actin-containing membrane compartment.**

scr-shRNA- (left panel) or *Nf2*-shRNA-expressing PAM212 keratinocytes (right panel) were cultured in calcium-containing media for 8 hours followed by *in situ* extraction (Fix 2) to enrich for Triton insoluble proteins. Indirect immunofluorescence using anti-Par3, -

α-catenin and -actin specific antibodies revealed that the detergent-resistant localization of Par3 to an α-catenin- and actin-containing membrane compartment requires Merlin.

**Supplemental Materials and Methods**

**Hanging Drop Assay**

The assay was performed as described previously (Kim et al., 2000). In brief, immortalized PAM212 keratinocytes infected with scr-shRNA or *Nf2*-shRNA were grown in calcium depleted media and then trypsinized and resuspended in high calcium media (1.5mM calcium, 5% serum) as single-cell suspensions at 1 x $10^6$ cells/ml. Single-cell suspensions were pipetted in 50μl drops onto the inside surface of a 60-mm culture dish lid, and media was added to the dishes to prevent evaporation. At each time point the lids were inverted and the drop was titurated 30 times through a 40μl pipet. Following tituration cell clusters were counted and photographed.

**Production of GST-α-catenin deletion mutants**

GST-α-catenin deletion mutants were created by PCR cloning using full-length (amino acids 1-906) α-catenin (a kind gift from William I. Weis) as a template. The following α-catenin mutants were cloned in-frame into the EcoRI site of pGex4T-1 for expression in bacteria and GST purification: full-length (amino acids 1-906); VH1-3 (amino acids 1-849); VH1-2 (amino acids 1-633); VH1 (amino acids 1-261); and ΔVH1 (amino acids 262-906).

**Barrier function assays**

Blue dye exclusion assay was performed as previously described (Koch et al., 2000). Outside-in barrier assay using toluidine blue dye was performed on E19.5 embryos by immersing embryos in 0.0125% toluidine blue in PBS followed by washing with PBS and photographing specimen. Inside-out barrier assay was performed as previously described (Furuse et al., 2002). Briefly, 50μl of 10 mg/ml freshly made biotin in PBS containing 1 mM $CaCl_2$ was injected intradermally into newborn mice and incubated for 30 minutes at room temperature. Back skin was collected, embedded in OCT and cryosectioned. Sections were stained with streptavidin-FITC and occludin followed by a Cy3-labelled secondary antibody (Jackson ImmunoResearch).

**Ultrastructural analysis**

Newborn back skin was collected and immersed in 2.5% paraformaldehyde/2.5% glutaraldehyde/0.1M sodium cacodylate/2mM $CaCl_2$ for 3 hours. Samples were then transferred to 0.1M sodium cacodylate/2mM $CaCl_2$. Samples were dehydrated and embedded in Epon. Thin sections were stained with lead citrate and uranyl acetate and examined in a Philips 300 electron microscope.

**Supplemental References**

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Koch, P.J., de Viragh, P.A., Scharer, E., Bundman, D., Longley, M.A., Bickenbach, J., Kawachi, Y., Suga, Y., Zhou, Z., Huber, M., *et al.* (2000). Lessons from loricrin-deficient mice: compensatory mechanisms maintaining skin barrier function in the absence of a major cornified envelope protein. J Cell Biol *151*, 389-400.