F–actin-bundling sites are conserved in proteins with villin-type headpiece domains

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ABSTRACT **Villin is a major actin-bundling protein that assembles the brush border of intestinal and renal epithelial cells. The villin "headpiece" domain and the actin-binding residues within it regulate its actin-bundling function. Substantial experimental and theoretical information about the three-dimensional structure of the isolated villin headpiece, including a description of the actin-binding residues within the headpiece, is available. Despite that, the actin-bundling site in the full-length (FL) villin protein remains unidentified. We used this existing villin headpiece nuclear magnetic resonance data and performed mutational analysis and functional assays to identify the actin-bundling site in FL human villin protein. By careful evaluation of these conserved actin-binding residues in human advillin protein, we demonstrate their functional significance in the over 30 proteins that contain a villin-type headpiece domain. Our study is the first that combines the available structural data on villin headpiece with functional assays to identify the actin-binding residues in FL villin that regulate its filament-bundling activity. Our findings could have wider implications for other actin-bundling proteins that contain a villin-type headpiece domain.**

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INTRODUCTION

Villin-1 (henceforth referred to as villin) is an actin-regulatory protein with a gelsolinlike "core" that regulates the actin-severing, -nucleating, and -capping functions of the protein (Khurana, 2006). In addition, villin contains a distinct carboxyl-terminal "headpiece" domain that is required for its actin-bundling function (Khurana, 2006). The headpiece of villin is an actin-binding module that binds F-actin at a molar ratio of 1 headpiece:1 actin and retains high-affinity F–actin-binding activity, even when cleaved from the core (Glenney *et al.*, 1981; Vardar *et al.*, 2002; George *et al.*, 2007). The headpiece was first identified in villin, but since then multiple headpiece domain-containing proteins have been identified in a variety of tissue and with varying cellular locations. This indicates a wide range of functions for proteins containing this functional domain. Even though most of these headpiece domains share ~40% sequence identity, they are parts of otherwise completely unrelated proteins. For example, dematin, which contains a headpiece domain, plays a major role in regulating erythrocyte membrane integrity. Accordingly, loss of dematin and its actin-bundling function are associated with severe hemolytic anemia, autosomal-dominant Marie Unna hereditary hypotrichosis disease, while heterozygosity of this gene is thought to play a role in prostate cancer progression (Khanna *et al.*, 2002; Mohseni and Chishti, 2008a,b; Lu *et al.*, 2016). Similarly, talin, a ubiquitous cytosolic protein found in high concentrations in focal adhesions, also contains a villin-type headpiece domain (Zhang *et al.*, 1996; Tsujioka *et al.*, 1999). Other actin-bundling proteins that contain a headpiece domain include advillin, villinlike protein, flightless 1, ABLIM1/2/3 (also called limatin), and supervillin. In all these proteins, the headpiece is found at the extreme carboxyl-terminus

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Author contributions: all in vitro studies to identify F-actin bundling residues in villin and advillin were performed by A.E. and S.P.G.; all mutations were made by A.E., S.P.G., and S.R.; the actin assay was performed by A.E. and S.P.G.; all cell work was performed by A.E. and S.P.G.; all experiments were conceived and designed by S.K.; data were analyzed by A.E., S.P.G., S.R., and S.K.; all authors reviewed the manuscript.

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Abbreviations used: DFDNB, 1,5-Difluoro-2,4-dinitrobenzene; DSS, disuccinimidyl suberate; EGFP, enhanced green fluorescent protein; EGS, ethylene glycol bis(succinimidyl syccinate; FBS, fetal bovine serum; FL, full length; GST, glutathione S-transferase; NMR, nuclear magnetic resonance; villin, Villin-1.

and all headpieces show high-sequence similarity in the putative F–actin-binding subdomain identified in the villin headpiece (Vardar *et al.*, 1999; Frank *et al.*, 2004; Vermeulen *et al.*, 2004).

The headpiece of villin and dematin have been studied most extensively and their findings inform what we know about this structural and functional domain. Vermeulen and colleagues used nuclear magnetic resonance (NMR) to show that the isolated human villin and human advillin headpiece proteins have very similar three-dimensional structures that were also comparable to the structure of the isolated chicken villin headpiece protein (McKnight *et al.*, 1997; Vermeulen *et al.*, 2004). Such studies validated the high-sequence and structural homology within this region among different headpiece-containing proteins. A summary of all the structural analysis on the isolated headpiece proteins studied thus far specifies that the headpiece structure can be further divided into two subdomains, the amino-terminal and carboxyl-terminal subdomains of the headpiece (McKnight *et al.*, 1997). Additionally, the structure of the carboxyl-terminal subdomain has been determined to be the same whether the amino-terminal subdomain of the headpiece is present or not (Vardar *et al.*, 1999). As a result, much like the isolated headpiece protein itself, the isolated carboxyl-terminal subdomain of the headpiece also maintains its structure independent of the rest of the villin protein. All the residues that directly interact with F-actin have been localized to this carboxyl-terminal subdomain of the villin headpiece. Based on these and similar studies, a three-dimensional structure of the villin headpiece has been put forth represented as a compact structure with a "hydrophobic patch" that docks the villin headpiece to F-actin and a "crown" with charged residues that form electrostatic interactions necessary for actin binding to the hydrophobic patch (McKnight *et al.*, 1996, 1997; Vardar *et al.*, 1999, 2002). Many of the residues identified in the hydrophobic patch are also conserved among the various headpiece sequences that are available from different proteins.

A cysteine-scanning mutagenesis approach was used to identify the F–actin-binding residues within the isolated human villin headpiece protein and included the following residues: K789, K790, K816, K822, L826, and F827 (Doering and Matsudaira, 1996). Vardar and colleagues performed the NMR analysis of the 67 amino acid construct of the chicken villin headpiece and identified three structural features required for actin binding, namely, a "hydrophobic cap" (W815), a "charged crown" (R788, K816, K821, E823, and F827) and a patch below the crown (K789, K824) (Vardar *et al.*, 1999). This model was further improved by Rossenu *et al.* (2003), who demonstrated that at least two charged residues within the crown were dispensable for actin binding and, more importantly, that most residues, with the exception of K821, in the putative actin-binding motif ^{820}LKKEKG^{825}, identified by Louvard's group including E823 and K824, were dispensable for F-actin binding (Friederich *et al.*, 1992; Rossenu *et al.*, 2003). Finally, Vermeulen and colleagues using proton NMR spectroscopy (H-NMR) analysis of the headpiece from human villin and human advillin proteins validated the significance of the single hydrophobic residue, W815, which was identified previously by Vardar and colleagues (Vardar *et al.*, 1999; Vermeulen *et al.*, 2004). Furthermore, their study made the distinction between residues that directly bind F-actin (i.e., K816, K821, and F827) versus those that are required to maintain the conformation of the villin headpiece (i.e., K789) (Vermeulen *et al.*, 2004). By analyzing the advillin headpiece, they also confirmed the prevailing notion that the actin-binding residues are conserved in other proteins with a villin-type headpiece. We note that, despite these well-resolved NMR structures of the villin headpiece, knowledge of the headpiece residues that bind F-actin and are required for actin bundling by villin has remained elusive. This is, in large part, because none of the structural data have been verified by functional assays to demonstrate actin binding. Even more remarkably, none of the structural data have been verified in the context of the full-length (FL) villin or advillin proteins. Louvard's group used mutants of FL villin protein to identify the headpiece motif ^{821}KKEK^{824}, as the actin-bundling site in villin (Friederich *et al.*, 1992; Friederich *et al.*, 1999). However, their studies were performed before the structural analysis of Rossenu *et al.* (2003) demonstrated that with the exception of K821 the rest of the KKEK motif does not contain an F–actin-binding residue (Rossenu *et al.*, 2003). Ectopic, transient, and overexpression of FL villin in cells that endogenously lack villin results in the assembly of microvillilike or filopodialike cell surface protrusions (Friederich *et al.*, 1989, 1992; Franck *et al.*, 1990). The assembly of these filopodialike cell surface protrusions has been attributed to the F-actin-bundling function of villin (Friederich *et al.*, 1989; Franck *et al.*, 1990). Friederich *et al.* (1992) used this property to demonstrate that a double amino acid substitution in the motif KKEK (i.e., KEEE) impairs the ability of villin to promote microvilli/filopodia growth in CV-1 cells (Friederich *et al.*, 1992). Using synthetic peptides, they likewise demonstrated that substitution of any one residue within the KKEK motif resulted in a decrease in F-actin binding by the peptide while substitution of two residues within this motif abolished F-actin binding by the peptide. Based on that, the authors concluded that all four residues within this headpiece motif ^{821}KKEK^{824} of villin are required for F-actin binding and for filopodia growth in CV-1 cells. Additionally, they identified a small but significant role for the last three amino acids of the human villin headpiece (^{825}GLF^{827}). Although not supported by statistical analysis, in a subsequent study using recombinant FL and mutant villin proteins, Friederich *et al.* (1999) reported an approximately 30% decrease in the F–actin-binding affinity of the double mutant (KEEE) compared with FL villin protein (Friederich *et al.*, 1999). Furthermore, they attributed the impaired ability of the KEEE mutant to assemble cell surface protrusions in CV-1 to this loss in F–actin-binding affinity of the recombinant mutant protein (Friederich *et al.*, 1992, 1999). However, prior to this study by Friederich and colleagues, Doering and Matsudaira reported that replacement of at least one of these residue (K824) with cysteine had no effect on the in vitro F–actin-binding function of villin headpiece protein (Doering and Matsudaira, 1996). Subsequently, structural data on the isolated villin headpiece protein provided by Rossenu *et al.* (2003) not only validated the findings of Doering and Matsudaira but also made the determination that only K821 within this ^{821}KKEK^{824} motif is an F–actin-binding residue (Rossenu *et al.*, 2003). NMR studies also identified F827 as an F–actin-binding residue within the charged crown domain of the villin headpiece protein (Vardar *et al.*, 1999). Based on that, one could conclude that with the exception of K821 and F827, most residues within the two sites (^{821}KKEK^{824} and ^{825}GLF^{827}) identified by Friederich and colleagues should be dispensable for F-actin binding and F-actin bundling by villin. Justified by these previously published studies, we recognized significant gaps in our knowledge of the headpiece F–actin-binding residues that regulate the actin-binding and actin-bundling activity of villin and potentially other headpiece-containing proteins.

Advillin is the closest homolog of villin and shares over 75% homology with villin including within the headpiece domain (Ravenall *et al.*, 2002). Advillin is expressed in sensory neurons, in chemosensory cells such as taste bud receptor cells, and in mechanosensory cells such as Merkel cells (Ravenall *et al.*, 2002; Rao *et al.*, 2017; Chuang *et al.*, 2018; Hunter *et al.*, 2018). More recently, we have identified advillin as a major protein expressed in the chemosensory

tuft/brush cells of the intestinal and respiratory epithelium that play a major role in the regulation of the body's response to pathogens and allergens, respectively (Esmaeilniakooshkghazi *et al.*, 2020). The actin-bundling function of advillin is required for neurite outgrowth in vitro and for axon regeneration in vivo (Chuang *et al.*, 2018; Ravenall *et al.*, 2002). We have shown that mutations in advillin that disrupt its actin-bundling activity are linked to steroid-resistant nephrotic syndrome (Rao *et al.*, 2017). The NMR structure of the human advillin headpiece is also available and reveals significant structural homology with the human villin headpiece including conserved actin-binding residues within its headpiece (Vermeulen *et al.*, 2004). However, like the villin headpiece, the actin-binding residues in the advillin headpiece have not been validated in functional assays or in the context of the FL protein.

With that in mind, we elected to identify the F–actin-binding residues in the villin and advillin proteins that are required for their actin-binding and actin-bundling activity. Almost four decades ago, villin was identified as a major actin-bundling protein that is required to assemble the brush border of intestinal epithelial cells (Bretscher and Weber, 1979). To the best of our knowledge, our study is the first that combines the structural data obtained from existing NMR studies of the isolated headpiece with the functional analysis of the FL protein to identify the actin-bundling site in the villin protein. Furthermore, our detailed comparison of the villin and advillin proteins defines the function of these conserved F–actin-binding residues in other headpiece-containing, actin-bundling proteins.

RESULTS AND DISCUSSION

Since villin and advillin can bundle actin in vitro, we elected to assess the function of the putative F–actin-binding residues using this functional assay (Rao and Craig, 2000; George *et al.*, 2007; Rao *et al.*, 2017; Esmaeilniakooshkghazi *et al.*, 2020). Based on the last revised model of the F–actin-binding residues in the villin and advillin headpiece structure, we elected to individually substitute the following residues in human villin: W815, K816, K821, and F827 and the following conserved residues in human advillin protein: W807, K808, K813, and F819 with alanine (Figure 1A, shown in red). All four-point mutants and the FL villin and advillin proteins were expressed as glutathione S-transferase (GST)-tagged recombinant proteins (Figure 1B). FL and each of these point mutants were tested for their ability to bundle F-actin in vitro using a sedimentation assay described previously (George *et al.*, 2007). As shown in Figure 1C, compared with FL villin protein, actin bundling by each of the four mutant proteins was ineffectual. Similarly, compared with FL advillin protein, actin bundling by each of the four advillin point mutants was also disrupted by the loss of the actin-binding residues (Figure 1D). Control refers to actin bundling in the absence of any protein. As reported previously and as shown here, the GST tag does not affect the actin-bundling activity of villin or advillin proteins (Figure 1C) (George *et al.*, 2007; Rao *et al.*, 2017). Using synthetic peptides, recombinant proteins, and transfection of CV-1 cells to express wild-type and mutant villin proteins, Friederich *et al.* (1992, 1999) demonstrated that a conserved COOH terminal cluster of amino acids (^{821}KKEK^{824}) are crucial for villin's F–actin-binding function and for its function in promoting growth of microvilli/filopodia when overexpressed in CV-1 cells (Friederich *et al.*, 1992, 1999). In these studies, they demonstrated that individually as well as a double amino acid substitution in the motif KKEK (i.e., KEEE) impaired the ability of villin to bind F-actin and to promote cell surface protrusions in CV-1 cells. To integrate the functional data from Friederich and colleagues with the structural data obtained from the isolated villin headpiece protein (which suggests only the first lysine [K821] in this motif is an actin-binding residue), we generated a villin mutant with three of the four residues (Figure 1A, shown in green) mutated to alanine (namely, the KAAA mutant). For our studies, FL villin and the KAAA mutant of villin were expressed as GST-tagged recombinant proteins (Figure 1E). Using the in vitro sedimentation assay, we now demonstrate that the KAAA mutant bundles F-actin like the FL villin protein (Figure 1E). Control refers to the actin bundling assay in the absence of any protein, and GST protein alone had no effect on actin bundling as previously reported and as shown here (George *et al.*, 2007). Our data concur with the most recent structural data on the isolated villin headpiece provided by Rossenu *et al.* (2003) and that previously reported by Doering and Matsudaira (1996), namely, that only the first lysine K821 in the KKEK motif of villin headpiece is required for F-actin binding and F-actin bundling by villin (Doering and Matsudaira, 1996; Rossenu *et al.*, 2003). The only notable difference between our study and that previously reported by Friederich *et al.* (1992, 1999) is that we substituted all three amino acids within this motif with the hydrophobic amino acid alanine (KAAA), while Friederich *et al.* (1992, 1999) substituted only two lysines, K822 and K824, with the charged amino acid, glutamic acid (KEEE). The villin-like headpiece domain is found in over 30 plant and animal cell proteins. Sequence alignment of the carboxyl-terminal subdomain of the villin headpiece with the carboxyl-terminal headpiece subdomains of some of these proteins show that all four residues identified in this study are well conserved in other proteins that contain a villin-type headpiece domain (Figure 2, highlighted in yellow). As shown in Figure 2, with the exception of the first lysine, the remainder of the KKEK motif is not well conserved in villin proteins from other species, in advillin proteins from different species, or in unrelated actin-bundling proteins that contain a villin-type headpiece domain, for example, dematin. Based on that and our findings with the villin mutants, we suggest that other than K813, no residue within the ^{813}KKEK^{816} motif of advillin is likely to be required for F-actin bundling by advillin. This would coincide with the existing structural data on the isolated advillin headpiece obtained by Vermeulen *et al.* (2004).

We have previously reported that villin self-associates and that villin dimers bundle actin using the single F–actin-binding site in the headpiece domain (George *et al.*, 2007). Based on the structural homology of the headpiece domains of villin and advillin, we hypothesized a similar molecular mechanism for actin bundling by advillin. For these studies, the GST tag in FL villin and FL advillin proteins was cleaved by thrombin digestion, as described in *Materials and Methods.* As reported previously and as shown here, under nonreducing conditions, recombinant villin protein forms monomers and dimers with disuccinimidyl suberate (DSS), 1,5-Difluoro-2,4-dinitrobenzene (DFDNB), and to a lesser extent with ethylene glycol bis(succinimidyl syccinate) (EGS) (George *et al.*, 2007). Using chemical cross-linkers of varying spacer length, we have previously reported that villin self-associates with most cross-linkers including DTNB, which suggested to us that in villin dimers, two thiol groups must come as close as 2Å (the length of a disulfide bond) (George *et al.*, 2007). In data shown here, we demonstrate, as reported before, that under nonreducing conditions, villin self-associates with the cleavable (EGS, DFDNB) and the noncleavable (DSS) cross-linkers of varying lengths (EGS, 16.1 Å; DSS, 11.4 Å; and DFDNB, 3.0 Å), including DFDNB, which has short spacer arm length. Additionally, we have shown that under similar conditions, a villin mutant that lacks the dimerization site, as well as the villin homolog gelsolin, do not self-associate (George *et al.*, 2007). Furthermore, we reported that under similar conditions, the actin-binding protein ezrin, which is known to self-associate, forms dimers and

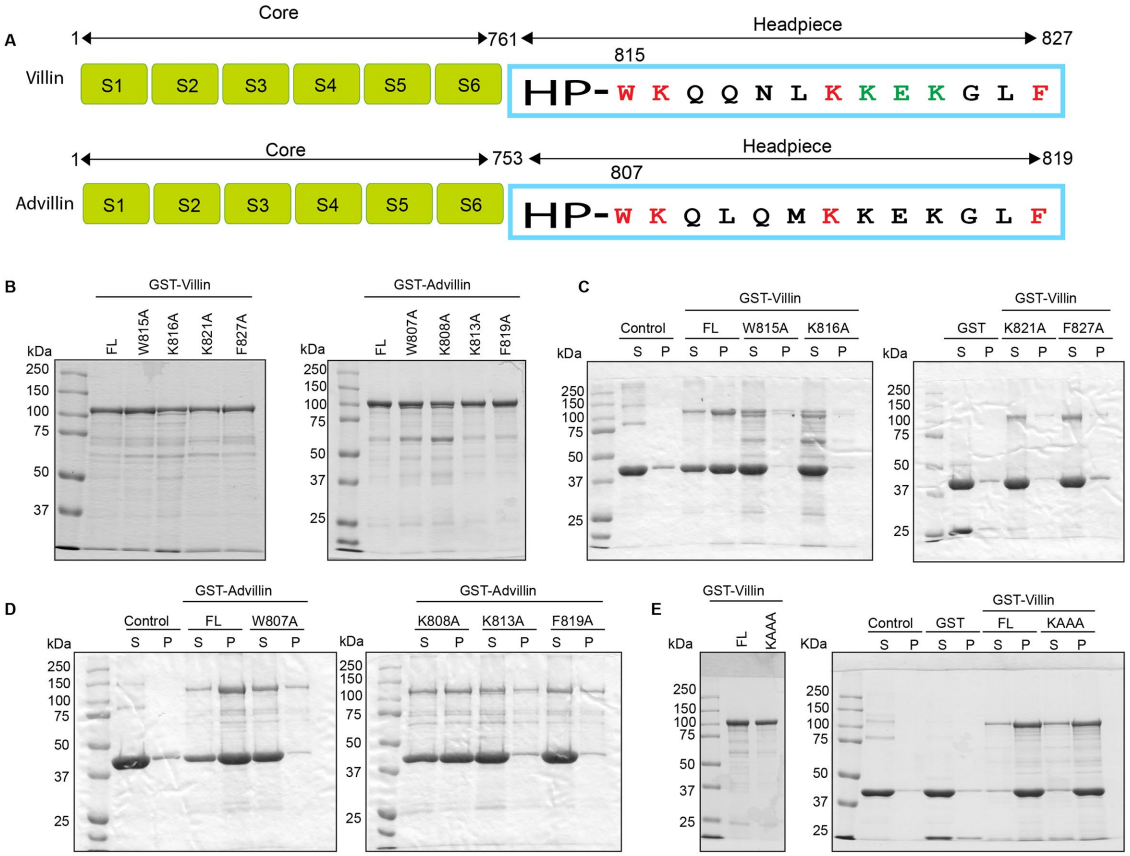

FIGURE 1: Identification of the F–actin-bundling site in recombinant human villin and human advillin proteins. (A) Schematic map comparing the amino-terminal core (S1–S6) and carboxyl-terminal headpiece (HP) of human villin and advillin proteins. S1–S6 represent six homologous domains conserved between villin and other proteins of its family including advillin and gelsolin. The actin-binding residues identified in the structure of isolated villin headpiece and isolated advillin headpiece domain are highlighted in red. The "KEK" residues in the actin-binding motif ^{821}KKEK^{824} identified in FL villin are shown in green. (B) Human recombinant FL villin, point mutants of villin (W815A, K816A, K821A, F827A), human recombinant FL advillin, and point mutant of advillin (W807A, K8808A, K813A, F819A) were expressed as GST-tagged proteins. Proteins were purified using glutathione-Sepharose 4B beads, separated by SDS–PAGE and visualized by GelCode Blue staining. (C) FL and mutant villin proteins (1.0 μM) were incubated with 0.76 μM F-actin and centrifuged at 10,000 × *g* for 15 min. Actin distribution in the supernatant (S) and pellet (P) fractions was analyzed by SDS–PAGE and GelCode Blue staining. Control refers to F-actin bundling assay performed in the absence of any protein. GST protein has no effect on F-actin bundling. (D) FL and mutant advillin proteins (1.0 μM) were incubated with 0.76 μM F-actin and centrifuged at 10,000 × *g* for 15 min. Actin distribution in the supernatant (S) and pellet (P) fractions was analyzed by SDS–PAGE and GelCode Blue staining. Control refers to F-actin bundling assay performed in the absence of any protein. (E) Mutant villin proteins with the ^{822}KEK^{824} residues mutated to ^{822}AAA^{824} (KAAA); 1.0 μM) were expressed as a GST-tagged protein. Proteins were purified using glutathione-Sepharose 4B beads, separated by SDS–PAGE, and visualized by GelCode Blue staining. The purified KAAA recombinant protein was incubated with 0.76 μM F-actin and centrifuged at 10,000 × *g* for 15 min. Actin distribution in the supernatant (S) and pellet (P) fractions was analyzed by SDS–PAGE and GelCode Blue staining. Control refers to F-actin bundling assay performed in the absence of any protein. GST protein had no effect on F-actin bundling. All data shown are representative of three independent experiments.

higher oligomers (George *et al.*, 2007). We now show that under similar experimental conditions, recombinant advillin self-associates with DSS and somewhat with DFDNB but does not self-associate in the presence of the EGS (Figure 3A). These data imply a defined molecular structure of the advillin dimer with a particular spacing of the reactive groups in the cross-linked protein (~11.4 Å but less than 16.1 Å). DMSO-treated control shows only monomers of villin and advillin protein. The DMSO controls also reveal that the degraded protein fractions seen in this Western blot below 100 kDa have no effect on protein self-association. To validate these findings, we expressed enhanced green fluorescent protein (EGFP)-tagged villin and EGFP-advillin in the epithelial cell line, MDCK (Figure 3B). Western analysis shows relative levels of protein expression in these cells. Tubulin was run as a loading control. Under reducing conditions in MDCK cells, we have previously shown that villin self-associates best with DFDNB, DSS, and EGS (George *et al.*, 2007). Similarly, we now show that under reducing conditions the cleavable cross-linker EGS leads to villin self-association (Figure 3C). Under similar conditions, we now show that advillin in MDCK cells self-associates in the presence of both the cleavable (DFDNB) and the noncleavable (DSS) cross-linkers (Figure 3C). Recombinant advillin did not cross-link in the presence of EGS; hence, we elected not to study advillin self-association in cells treated with EGS. DMSO-treated control shows only monomers of villin and advillin protein. These findings in MDCK

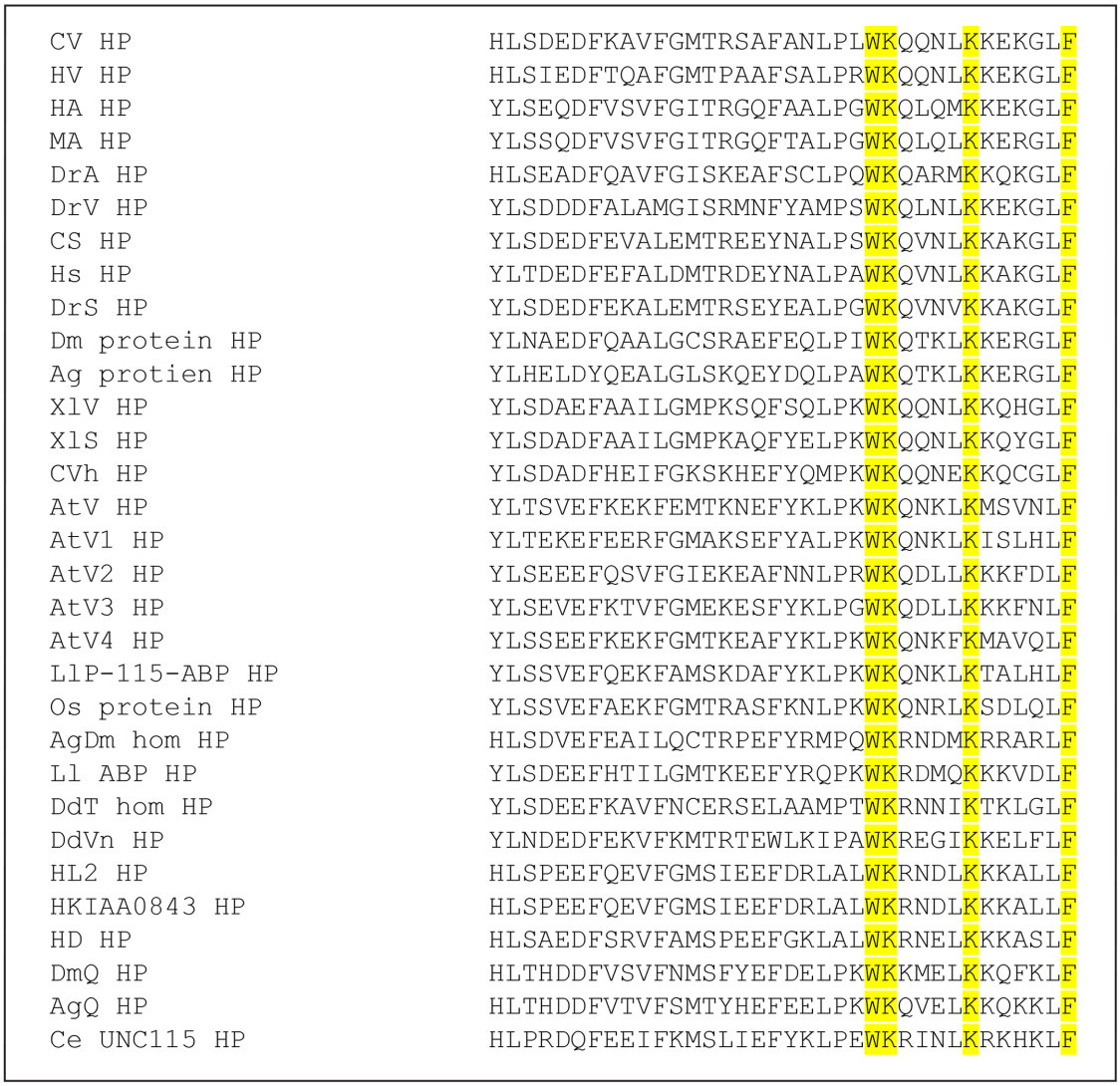

FIGURE 2: The actin bundling site in villin headpiece is well conserved in other proteins that contain a villin-type headpiece domain. Sequence alignment of the headpiece domain of villin proteins from different species and unrelated actin-bundling proteins that contain a villin-type headpiece domain. The four F–actin-binding residues required for actin bundling by villin (highlighted in yellow) are well conserved in these proteins. Sequences shown include: chicken villin headpiece (CV HP), human villin headpiece (HV HP), human advillin headpiece (HA HP), *Mus musculus* advillin headpiece (MA HP), *Danio rerio* advillin headpiece (DrA HP), *D. rerio* villin headpiece (DrV HP), chicken supervillin headpiece (CS HP), human supervillin headpiece (HS HP), *D. rerio* supervillin headpiece (DrS HP), *Drosophila melanogaster* headpiece-containing protein (Dm protein HP), *Anopheles gambiae* headpiece-containing protein (Ag protein HP), *Xenopus laevis* villin headpiece (XlV HP), *X. laevis* supervillin headpiece (XlS HP), chicken villin homolog (CVh HP), *Arabidopsis thaliana* villin headpiece (AtV HP), *A. thaliana* villin 1 headpiece (AtV1 HP), *A. thaliana* villin 2 headpiece (AtV2 HP), *A. thaliana* villin 3 headpiece (AtV3 HP), *A. thaliana* villin 4 headpiece (AtV4 HP), *Lilium longiflorum* actin-binding protein P-115-ABP headpiece (Ll P-115-ABP HP), *Oryza sativa* headpiece-containing protein (Os protein HP), *A. gambiae* homolog of *D. melanogaster* Unc-115l (AgDm hom HP), *L. longiflorum* actin-bundling protein ABP135 (Ll ABP HP), *Dictyostelium discoideum* talin homolog (DdT hom HP), *D. discoideum* villidin headpiece (DdVn HP), human actin-binding LIM2 protein headpiece (HL2 HP), human KIAA0843 headpiece (HKIAA0843 HP), human dematin headpiece (HD HP), *D. melanogaster* Quail headpiece (DmQ HP), *A. gambiae* Quail hom headpiece (AgQ HP), and *Caenorhabditis elegans* UNC115 protein headpiece (Ce UNC115 HP).

cells support our hypothesis that a defined molecular structure with a particular spacing of the reactive groups in cross-linked advillin protein is required (~11.4 Å but less than 16.1 Å). Nonetheless, we also note that while recombinant advillin does not self-associate well in the presence of DFDNB, in MDCK cells, DFDNB treatment cross-links advillin protein just as well as EGS. One conceivable explanation for this is that in cells, advillin can associate with F-actin and this may generate a different molecular structure in which the intermolecular spacing between reactive groups of ~3.0 Å can be achieved. We have previously shown that villin self-association is regulated by F-actin (George *et al.*, 2013). It is also possible that a similar molecular mechanism regulates advillin dimerization in cells, where DFDNB works well to promote advillin self-association because of the presence of high F-actin levels; these are conditions that cannot

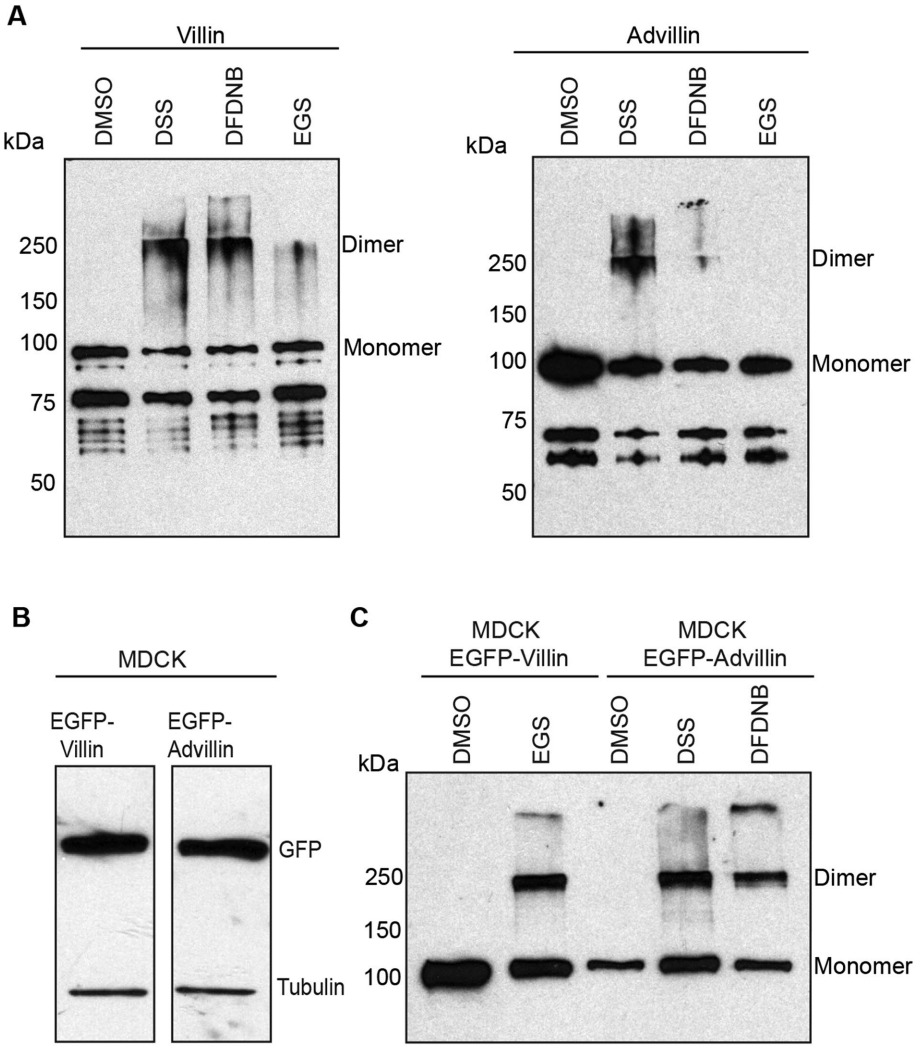

FIGURE 3: Villin and advillin self-associate. (A) Recombinant FL villin and advillin proteins (20 nM each) were incubated with different cross-linkers (50-fold M excess) for 90 min at room temperature. Western analysis shows chemically cross-linked proteins analyzed under nonreducing conditions. DMSO-treated proteins were used as negative controls. (B) The expression of EGFP-villin and EGFP-advillin proteins in MDCK cells. To compare protein expression, Western analysis was performed using tubulin as a loading control. (C) MDCK cells expressing EGFP-villin or EGFP-advillin were incubated with different cross-linkers (2.5 mM at room temperature for 30 min) and cell extracts were analyzed under reducing conditions. Self-association of proteins was analyzed by Western analysis using villin and advillin antibodies. DMSO-treated cells were used as a negative control. All data shown are representative of three independent experiments.

be mimicked when DFDNB is used to cross-link recombinant advillin protein. Our data also indicate other similarities between the advillin and the villin dimers namely, the distance between the conjugated molecules as small as 3.0 Å can be reached.

Overexpression of villin in fibroblasts and other cells that lack endogenous villin results in villin recruiting F-actin from microfilament-containing structures (e.g., stress fibers) to form cell surface protrusions that resemble microvilli or filopodia (Friederich *et al.*, 1989, 1992; Franck *et al.*, 1990; Tomar *et al.*, 2004). While the in vitro studies show that villin can only associate with F-actin filaments with a single polarity, when overexpressed in cells, villin has the intrinsic ability to reorganize the microfilament structure into ordered bundles of actin filaments with a single polarity within these cell surface protrusions (Pringault *et al.*, 1986; Shibayama *et al.*, 1987; Friederich *et al.*, 1989). Likewise, advillin expression in the developing nervous system has been associated with neuronal cell surface projections (Rao and Craig, 2000). Moreover, we have previously shown that overexpression of advillin assembles cell surface protrusions that require the actin-bundling activity of advillin (Rao *et al.*, 2017). Here, we use this property of villin and advillin proteins to validate the F–actin-bundling site in these proteins. For these studies, FL human villin and FL human advillin were transiently expressed as EGFP-tagged proteins in the epithelial cell line, HeLa. Alternatively, EGFP-tagged point mutants of villin and advillin proteins were expressed in HeLa cells. We elected to use HeLa cells because unlike MDCK cells, they form exceptionally long filopodia which permits easy detection of changes in the size and number of these cell surface structures as well as easy detection of changes in the localization of proteins to these cell surface structures. The expression of FL and mutant villin proteins in HeLa cells is shown in Figure 4A. Tubulin was used as a loading control. As expected, HeLa cells expressing FL villin protein form long and multiple filopodia like cell surface protrusions in which villin colocalizes with F-actin (Figure 4Ba; Supplemental Figure S1, A–C). In contrast, two mutants of villin, W815 and F827, form filopodia, the length of which is indistinguishable from those assembled by FL villin; however, they form fewer of these cell surface protrusions and the mutant villin proteins colocalize poorly with the F-actin in these cell surface protrusions (Figure 4B, b and e; Supplemental Figure S1, A–C). A similar behavior of villin headpiece mutant proteins has been described previously and suggests a cooperative role for the multiple F–actin-binding residues for effectual actin-bundling function (Friederich *et al.*, 1992). In contrast, villin mutants K816A and K821A did not form the typical long cell surface protrusions seen with FL villin protein (Figure 3B, c and d; Supplemental Figure S1A) and unlike the FL villin protein, the mutant proteins formed fewer of these filopodia and these mutant proteins were largely excluded from these stubby cell surface structures (Supplemental Figure S1, B–C). HeLa cells expressing the villin mutant KAAA behaved like FL villin protein, assembling filopodia in which the mutant protein and F-actin colocalized (Figure 4, A and Bf; Supplemental Figure S1, A–C). We used a similar approach to overexpress FL and mutant advillin proteins in HeLa cells. Figure 5A shows the relative expression of the proteins in HeLa cells. For these Western analyses, tubulin was used as a loading control. In keeping with our in vitro data and our previously published findings, we noted that HeLa cells expressing FL human advillin protein form long cell surface filopodia in which advillin colocalizes with the F-actin in these structures (Figure 5Ba; Supplemental Figure S2, A–C) (Rao *et al.*, 2017). In contrast, HeLa cells expressing the advillin point

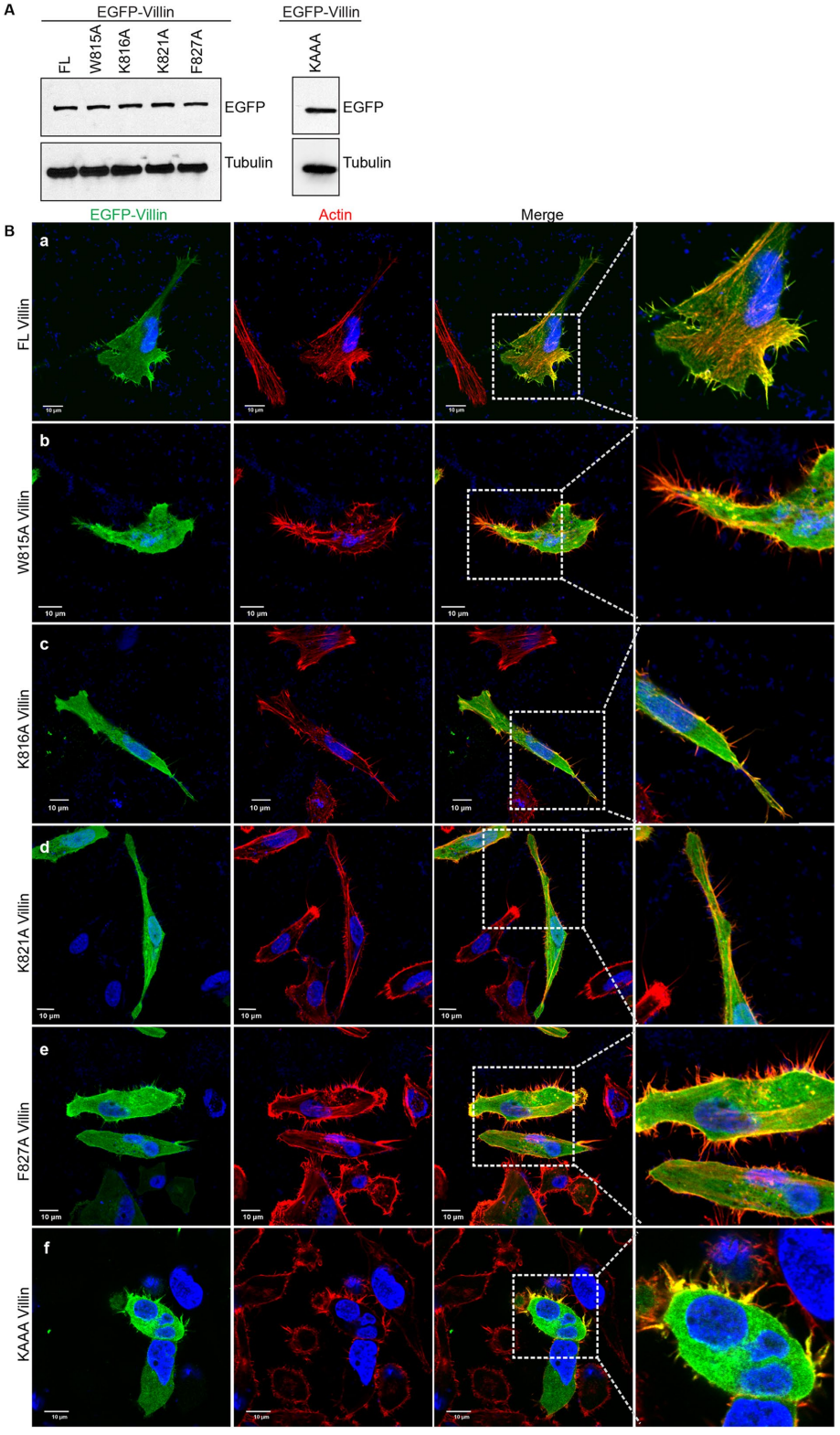

FIGURE 4: Validation of human villin F–actin-bundling site in HeLa cells. (A) Western analysis of HeLa cells transiently transfected with EGFP-tagged FL or point mutants (W815A, K816A, K821A, F827A) of villin. Also shown is Western analysis of HeLa cells transiently transfected with EGFP-tagged villin mutant KAAA. Tubulin was used as a loading control. (B) The cell morphology and the distribution of EGFP-tagged FL or mutant villin proteins was assessed by confocal microscopy. F-Actin staining was monitored using Alexa Fluor 568 Phalloidin. Merged images show colocalization of villin and F-actin. Nuclei were imaged using DAPI. Right panels are higher magnifications of the boxed areas. Scale bar represents 10 µm. All data shown are representative of three independent experiments.

mutants K807A, K808A, K813A, and F819A displayed very short and very few filopodia, and the mutant advillin protein distribution to these cell surface structures was significantly reduced (Figure 5 B, b–e; Supplemental Figure S2, A–C). Data shown in Figures 4A and 5A demonstrate comparable levels of protein expression for FL and mutant villin and advillin proteins. These data reveal that the differences in the cell surface protrusions seen with mutant villin or advillin proteins are unlikely to be due to differences in mutant villin or advillin protein to F-actin ratios when compared with FL villin or advillin to F-actin ratios. Together, these mutants identify actin-binding residues in villin and advillin headpiece that are required for F-actin bundling and for the assembly of long cell surface protrusions in cells.

F-actin filaments are bundled in the intestinal microvilli by actin-bundling proteins that include villin and fimbrin (Bretscher, 1983). Villin is the first actin cross-linking protein that is recruited to the apical surface of intestinal epithelial cells, although actin filaments bundled by villin alone are not as well organized as those bundled by both villin and fimbrin (Matsudaira *et al.*, 1983). Neither protein competes with the other for F-actin binding and both proteins appear to work together to assemble the intestinal and renal microvillar structure (Hampton *et al.*, 2008). While villin's actin-bundling function may be largely dispensable for intestinal microvillar assembly, villin's actin-bundling functions required for filopodia assembly and directional cell migration are not (Pinson *et al.*, 1998; George *et al.*, 2013; Ubelmann *et al.*, 2013). Villin has been shown to contain two F–actin-binding sites, one in the villin core in the S2–S3 region (Figure 1A) that only binds F-actin in the presence of high concentrations of calcium (>200 µM) and the second in the headpiece domain (Pope *et al.*, 1994). Previous biochemical studies have shown that the villin core (S1–S6; Figure 1A) or the amino-terminal proteolytic fragment 44T (S1–S3; Figure 1A) does not bind F-actin in the absence of Ca^{2+} (Glenney *et al.*, 1981; Matsudaira *et al.*, 1985). This has been attributed to the inaccessibility of the core F–actin-binding site either due to protein folding or due to low affinity for F-actin binding in the absence of high Ca^{2+}. We have also reported previously that deletion of the amino-terminal high Ca^{2+} F–actin-binding site has no effect on the actin-bundling function of villin protein (George *et al.*, 2007). Furthermore, we have shown that villin self-associates and villin dimers bundle actin using the single F–actin-binding site

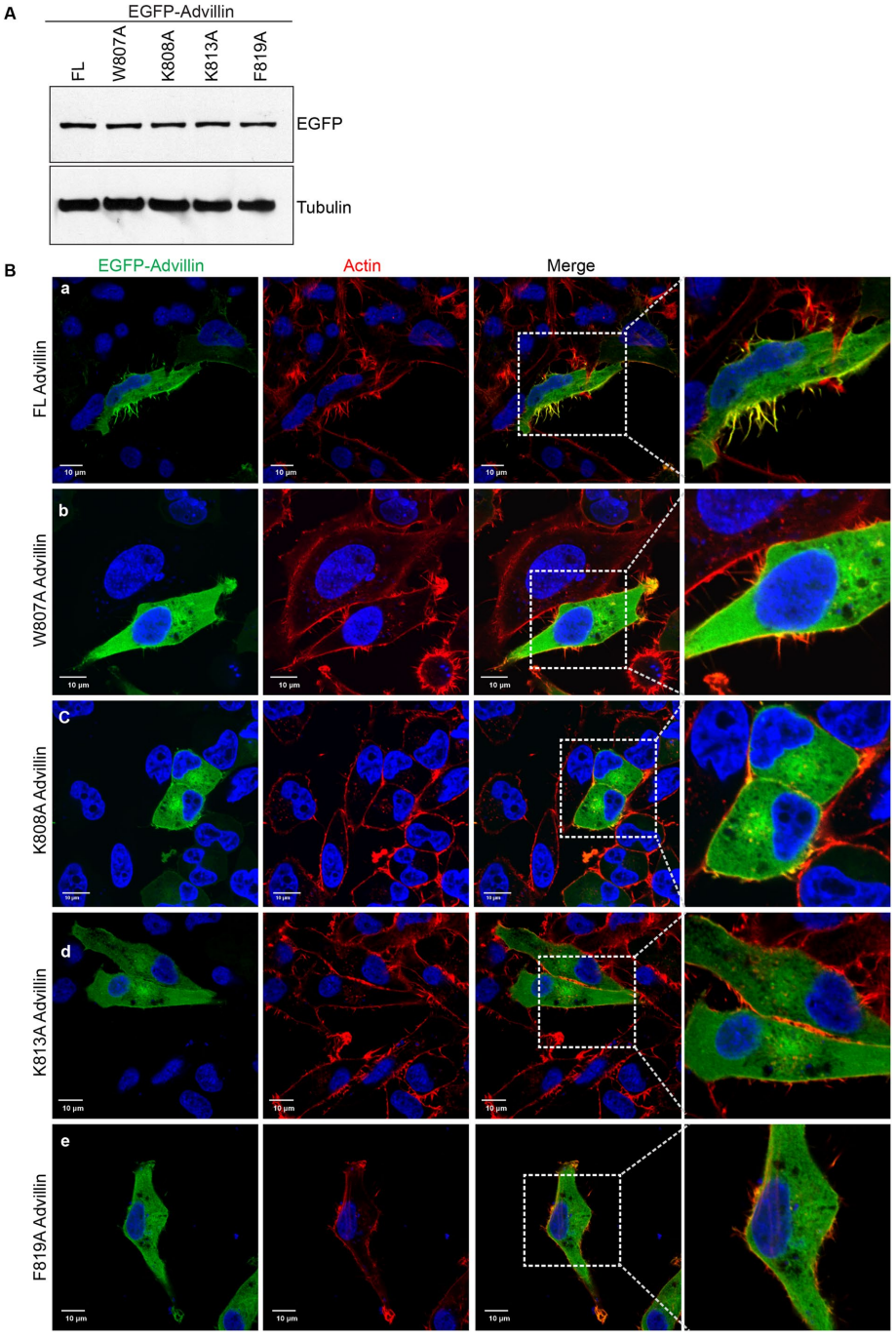

FIGURE 5: Validation of human advillin F–actin-bundling site in HeLa cells. (A) Western analysis of HeLa cells transiently transfected with EGFP-tagged FL or point mutants of advillin. Tubulin was used as a loading control. (B) HeLa cells expressing EGFP-tagged FL or EGFP-tagged mutant advillin proteins were examined by confocal microscopy to assess filopodial assembly and intracellular distribution of advillin proteins and F-actin. F-Actin staining was monitored using Alexa Fluor 568 Phalloidin. Merged images show colocalization of advillin with F-actin. Nuclei were visualized using DAPI staining. Right panels are magnifications of the boxed areas. Scale bar represents 10 µm. All data shown are representative of three independent experiments.

contained within the headpiece domain (George *et al.*, 2007). Our data shown here corroborate this model put forward by us previously in which only the headpiece F–actin-binding residues can bundle actin and villin dimers provide the two actin-binding sites required for this function (George *et al.*, 2007). It is noteworthy that none of the proteins of the villin family that share structural homology with the villin core and lack a headpiece self-associate, for example, gelsolin (George *et al.*, 2007). However, it appears that proteins that contain a villin-type headpiece and bundle F-actin self-associate, as shown here for advillin and as reported previously for dematin (Khanna *et al.*, 2002). Our findings validate a molecular mechanism in which dimerization rather than bivalent binding to F-actin is used by headpiece-containing proteins in order to bundle F-actin. Taken together, our study provides a new level of understanding of how cytoskeletal proteins with a villin like headpiece structure bind and bundle F-actin.

MATERIALS AND METHODS

Reagents

Quick-change II XL Site-Directed Mutagenesis Kit was purchased from Agilent Technologies. Actin Polymerization Biochem Kit was from Cytoskeleton. Alexa Fluor 568 Phalloidin and lipofectamine 2000 were purchased from Thermo Fisher Scientific. The antibodies used in this study were purchased from the following sources: advillin (NBP2-34118) from Novus Biologicals, tubulin (ab131205) and GFP (ab6556) from Abcam, and villin (610359) from BD Transduction. HeLa cells and MDCK cells were a kind gift from Keith Mostov (University of California, San Francisco, San Francisco, CA).

Expression and purification of FL and mutant recombinant human villin and human advillin proteins

Human villin and human advillin were cloned in the prokaryotic expression vector pGEX-4T3 as described previously (George *et al.*, 2007). Villin and advillin mutants lacking the actin-binding residues were constructed using the Quik-Change Site-Directed Mutagenesis Kit. GST-tagged villin and advillin proteins were purified as described before (Panebra *et al.*, 2001; Rao *et al.*, 2017). The GST tag was cleaved by thrombin digestion (1 U/100 mg of protein at room temperature, 16 h) and removed by incubation with glutathione-Sepharose 4B.

Cloning of FL and mutant villin and advillin proteins

FL human villin and advillin were cloned in pBudCE4.1 vector. EGFP-tagged villin and advillin were made by subcloning EGFP into FL villin and advillin cloned in pBudCE4.1 vector as described before (George *et al.*, 2007). The sequence and frame of the inserts were checked by sequencing. Quick-change II XL Site-Directed Mutagenesis Kit was used to generate each of the four-point mutants of human villin and advillin proteins.

Transfection of HeLa and MDCK cells

MDCK cells were stably transfected with EGFP-tagged villin or EGFP-tagged advillin proteins as described previously (Wang *et al.*, 2016). Transfected cells were cultured in DMEM containing Zeocin B (50 μg/ml), and 10% fetal bovine serum (FBS). HeLa cells were transiently transfected using lipofectamine 2000 and transfected cells expressing FL or mutant villin or advillin proteins were cultured in DMEM containing 10% FBS.

Measurement of actin bundling activities of villin and advillin proteins

A low-speed actin bundling assay was performed by incubating F-actin (0.76 μM) with recombinant FL or mutant villin and advillin proteins (1 μm), or GST protein alone (control; 1 μm) in bundling buffer (25 mM HEPES buffer, pH 7.4, 100 mM KCl, 2 mM EGTA for 60 min). The samples were centrifuged at low speed (10,000 × *g* for 15 min), the supernatant and pellet fractions were analyzed by SDS–PAGE, and distribution of F-actin in these two fractions was determined using GelCode Blue staining.

Chemical cross-linking of villin and advillin proteins

In vitro cross-linking of purified recombinant human villin and human advillin proteins (20 nM each) with chemical cross-linkers was performed with 50-fold M excess of the following cross-linkers, DSS, EGS, and DFDNB in phosphate-buffered saline at room temperature for 90 min. The chemically cross-linked proteins were analyzed by Western analysis under nonreducing conditions. Cross-linking reactions were stopped by the addition of 20 mM lysine, pH 7.4. In MDCK cells expressing FL villin or FL advillin, proteins were cross-linked by incubating the cells at room temperature with 2.5 mM of each of the following cross-linkers: EGS, DSS, and DFDNB for 30 min. Villin and advillin self-association was analyzed under reducing conditions by Western analysis with villin and advillin antibodies.

Confocal microscopy

Confocal imaging of HeLa and MDCK cells was performed using Fluor 568 Phalloidin to image F-actin, and nuclei were counterstained with DAPI. All images were acquired on an Olympus Fluoview FV1200 Laser Scanning confocal microscope with a 60× NA 1.35 objective.

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