# KazrinE is a desmosome-associated liprin that colocalises with acetylated microtubules

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## Summary

**Kazrin is a widely expressed, evolutionarily conserved cytoplasmic protein that binds the cytolinker protein periplakin. Multiple functions of kazrin have been reported, including regulation of desmosome assembly, embryonic tissue morphogenesis and epidermal differentiation. Here, we identify kazrinE as a kazrin isoform that contains a liprin-homology domain (LHD) and forms complexes with kazrinA, kazrinB and kazrinC. As predicted from the presence of the LHD, kazrinE can associate with the leukocyte common antigen-related (LAR) protein tyrosine phosphatase in a phosphorylation-dependent manner. When overexpressed in epidermal keratinocytes, kazrinE induces changes in cell shape and stimulates terminal differentiation. Like the other kazrin isoforms, kazrinE localises to the nucleus and desmosomes. However, in addition, kazrinE associates with stabilised microtubules via its LHD. During terminal differentiation, the keratinocyte microtubule network undergoes extensive reorganisation; in differentiating keratinocytes, endogenous kazrinE colocalises with microtubules, but periplakin does not. We speculate that the kazrinE-microtubule interaction contributes to the mechanism by which kazrin regulates desmosome formation and epidermal differentiation.**

Key words: Plakins, Liprins, Epidermis, LAR, Microtubules

## Introduction

Kazrin was originally identified because it binds to the N-terminal domain of the cytolinker periplakin (Groot et al., 2004), which was previously shown to associate with cortical actin, desmosomes and the interdesmosomal plasma membrane (DiColandrea et al., 2000; Karashima and Watt, 2002; Kazerounian et al., 2002). Kazrin colocalises with periplakin at desmosomes and the interdesmosomal plasma membrane, although its subcellular distribution is independent of periplakin (Groot et al., 2004). Thus far, four transcripts encoding three protein isoforms of kazrin have been described, arising from alternative splicing of the gene. The three protein isoforms, kazrinA, kazrinB and kazrinC, have different N-termini but all contain the coiled-coil domain that is involved in periplakin binding and all have a putative nuclear-localisation signal (NLS) (Groot et al., 2004).

Kazrin not only regulates desmosome assembly but also embryonic development and epidermal terminal differentiation (Sevilla et al., 2008a; Sevilla et al., 2008b). In preimplantation mouse embryos, kazrin is found at the nuclear matrix, actin cytoskeleton and cell-cell junctions (Gallicano et al., 2005). Loss of kazrin in *Xenopus tropicalis* embryos causes defects in axial elongation, muscle and notochord differentiation, and epidermal morphogenesis (Sevilla et al., 2008b). Cultured human keratinocytes overexpressing kazrin exhibit a decrease in Rho GTPase activity and have abnormal cell shape, reduced F-actin content and impaired desmosome assembly (Sevilla et al., 2008a). The effects of kazrin overexpression on cell adhesion and the cytoskeleton can be rescued by expression of activated RhoA. In addition, kazrin overexpression induces keratinocyte differentiation, whereas kazrin knockdown inhibits terminal differentiation and stimulates proliferation. The effects of kazrin knockdown are independent of alteration in RhoA activity (Sevilla et al., 2008a).

In the present report we characterise a kazrin isoform (kazrinE) belonging to the liprin protein family (Serra-Pages et al., 1998; Spangler and Hoogenraad, 2007; Stryker and Johnson, 2007). Liprins were first identified because of their ability to bind to leukocyte common antigen-related transmembrane receptor protein tyrosine phosphatase (LAR) (Serra-Pages et al., 1998) and are implicated in a range of cellular processes, including synapse formation (Stryker and Johnson, 2007) and extracellular-matrix adhesion (Asperti et al., 2009). Our observations suggest new mechanisms by which kazrin can regulate cell adhesion, cytoskeletal organisation and epidermal differentiation.

## Results and Discussion

### Identification and detection of kazrinE, an alternatively spliced transcript of kazrin

In searching for the *Drosophila* orthologue of kazrin, we found CG11206, which encodes a longer protein than kazrinA, kazrinB or kazrinC. This led us to identify the corresponding human EST clone, kazrinE (GenBank accession number NM_201628). The transcript of kazrinE consists of seven additional exons inserted into a consensus splice donor-acceptor site in exon 8 (Fig. 1A). Exons 9 to 15 encode three sterile α motif (SAM) domains, which form a liprin-homology domain (LHD), the defining characteristic of liprins (Stryker and Johnson, 2007). As illustrated in the phylogenetic tree in Fig. 1B, the kazrinE, liprin-α and liprin-β genes arose from a common ancestor.

By quantitative reverse-transcriptase PCR (RT-PCR), kazrinE mRNA was detected in all human tissues tested, with highest

expression in the spleen and lowest in skeletal muscle (data not shown). To determine whether individual cell types coexpressed the different splice variants of kazrin, semi-quantitative RT-PCR was performed on cultured cells (Fig. 1C). Transcripts of all kazrin isoforms were expressed in all three cell types examined, with higher expression of kazrinE in A431 and EJ/28 cells than in primary human keratinocytes.

The predicted domains of kazrinE are illustrated schematically in Fig. 1D. It is possible that three forms of kazrinE, containing the N-terminus of kazrinA, kazrinB or kazrinC, exist; however, thus far only cDNA clones containing the kazrinA N-terminus have been identified in searches of public databases (data not shown). KazrinE and the shorter isoforms share the coiled-coil domain and putative nuclear-localisation signal (NLS; at amino acids 376 and 382 in kazrinA). However, whereas kazrinA, kazrinB and kazrinC share a common C-terminus, the kazrinE C-terminus contains the LHD with the three SAM domains located between amino acids 446 and 511, 524 and 588, and 612 and 679. KazrinE also has a PDZ-binding motif at the extreme C-terminus (amino acids 772-775) (Fig. 1D). Human kazrinE is highly homologous to the mouse and rat isoforms, having 90% amino acid identity (BLAST alignment) (Altschul et al., 1990) (data not shown).

### KazrinE interacts with the LAR-type receptor protein tyrosine phosphatase

Liprin α1 was originally identified by its ability to bind LAR, an interaction mediated by the SAM domains (Pulido et al., 1995; Serra-Pages et al., 1995). LAR is known to accumulate at adherens junctions and desmosomes, via binding to plakoglobin and β-catenin, and to regulate both junction assembly and β-catenin signalling (Kypta et al., 1996; Aicher et al., 1997; Muller et al., 1999). To probe the potential interaction between kazrinE and LAR, we performed co-immunoprecipitation experiments using total cell lysates from 293T cells co-transfected with N-terminally haemagglutinin (HA)-tagged kazrinE and green fluorescent protein (GFP)-LAR (Fig. 1E). HA-kazrinE and GFP-LAR could be co-immunoprecipitated from lysates prepared in the presence, but not in the absence, of phosphatase inhibitors (Fig. 1E), in agreement with previous work showing that liprin phosphorylation regulates binding to LAR (Serra-Pages et al., 2005).

To examine whether kazrinE can be tyrosine phosphorylated, we transfected A431 cells with HA-tagged kazrinE, serum starved the cells overnight and then treated them for 1 hour with epidermal growth factor (EGF) (Fig. 1F). Cell lysates were immunoprecipitated with anti-HA and then immunoblotted with anti-phosphotyrosine antibody or, as controls for transfection and loading, respectively, with anti-HA and anti-α-tubulin (Fig. 1F). On treatment with EGF, weak tyrosine phosphorylation of kazrinE was detected.

Although the ability of kazrinE to associate with LAR (Fig. 1E) is predicted from the existence of the LHD and could potentially be regulated by tyrosine phosphorylation (Fig. 1F), we were unable to co-immunoprecipitate endogenous LAR and kazrinE in the presence or absence of phosphatase inhibitors (data not shown). Furthermore, when the subcellular distribution of GFP-LAR and HA-kazrinE was compared in transfected primary human keratinocytes, HA-kazrinE and GFP-LAR showed only partial colocalisation (Fig. 1G). There was some colocalisation of LAR and kazrinE at the cell periphery (Fig. 1G). However, HA-kazrinE accumulated primarily in perinuclear filaments, whereas GFP-LAR had a more widespread and punctate distribution throughout the cytoplasm (Fig. 1G). We conclude that, although kazrinE, like liprin-α family members, can bind LAR, the majority of kazrinE is not associated with LAR and therefore kazrinE function is unlikely to be confined to its ability to interact with LAR.

### KazrinE expression in epidermis and cultured keratinocytes

In order to analyse endogenous kazrinE expression, we generated a series of affinity-purified rabbit anti-peptide antibodies that detect the different kazrin isoforms (Fig. 1D). We tested the specificity of the antibodies using lysates of 293T cells that had been transfected with HA-tagged kazrinA, HA-tagged kazrinE or the HA-tagged C-terminus of kazrinE containing the LHD and PDZ-binding motif (amino acids 408-775) (Fig. 2A).

Antiserum LS4 was generated against the C-terminal 20 amino acids common to kazrinA, kazrinB and kazrinC (Groot et al., 2004). The peptide immunogen thus has only four amino acids in common with kazrinE. When HA immunoprecipitates from transfected 293T lysates were immunoblotted with LS4, a single band of approximately 50 kD, corresponding to kazrinA, was detected (Fig. 2A). By contrast, the LS7 antiserum, raised against a peptide common to all kazrin isoforms (Fig. 1D), detected bands corresponding to both kazrinA and kazrinE (Fig. 2A). KazrinE had a mobility of approximately 105 kD, which is larger than the predicted molecular mass of 86 kD; this might reflect the conformation of the protein or post-translational modification, for example by phosphorylation. LS10, raised against a kazrinE-specific peptide (Fig. 1D), detected full-length kazrinE and the kazrinE LHD, but not kazrinA (Fig. 2A).

When extracts of A431 cells, EJ/28 cells and human keratinocytes were immunoblotted with LS7, bands corresponding to kazrinA and kazrinB (50 kD), kazrinC (37 kD) and kazrinE (approximately 105 kD) were observed (Fig. 2B). Endogenous kazrinE had the same mobility as HA-kazrinE (c.f. Fig. 2A,B). In addition, we detected a higher-molecular-mass protein of approximately 150 kD (Fig. 2B), which might correspond to complexes of the long and short forms of kazrin.

We previously observed that the abundance of the short kazrin isoforms increases as terminally differentiated cells accumulate during $Ca^{2+}$-induced stratification of primary human epidermal keratinocytes in culture (Sevilla et al., 2008a). To examine kazrinE expression, human keratinocytes grown under standard ('high calcium') conditions were harvested at 50, 80 or 100% confluence, or 3 days post-confluence (+3D) and immunoblotted with LS10. The abundance of kazrinE was reduced in post-confluent cultures (Fig. 2C), suggesting either that its expression decreases during the later stages of terminal differentiation or that, like the short kazrin isoforms, it becomes incorporated into the cornified envelope (Groot et al., 2004).

We next immunolabelled sections of human epidermis with antibodies to kazrinE and desmoplakin, a desmosome marker (Fig. 2D). Desmoplakin staining was observed in all the living suprabasal layers of human interfollicular epidermis (Mils et al., 1992). By contrast, kazrinE was expressed most highly in the basal to mid-spinous layers and decreased in the upper spinous layers (Fig. 2D). KazrinE was expressed both at cell-cell borders, where it showed partial colocalisation with desmoplakin, and in the nucleus (Fig. 2D).

The dual localisation of endogenous kazrinE at cell-cell borders and in nuclei was confirmed by immunostaining cultured primary human keratinocytes grown in standard 'high calcium' medium (Fig. 2E). As in the epidermis, partial colocalisation of kazrinE and desmoplakin was observed (Fig. 2E). In addition to the expected localisation of kazrinE in the nucleus and cell-cell borders, we also

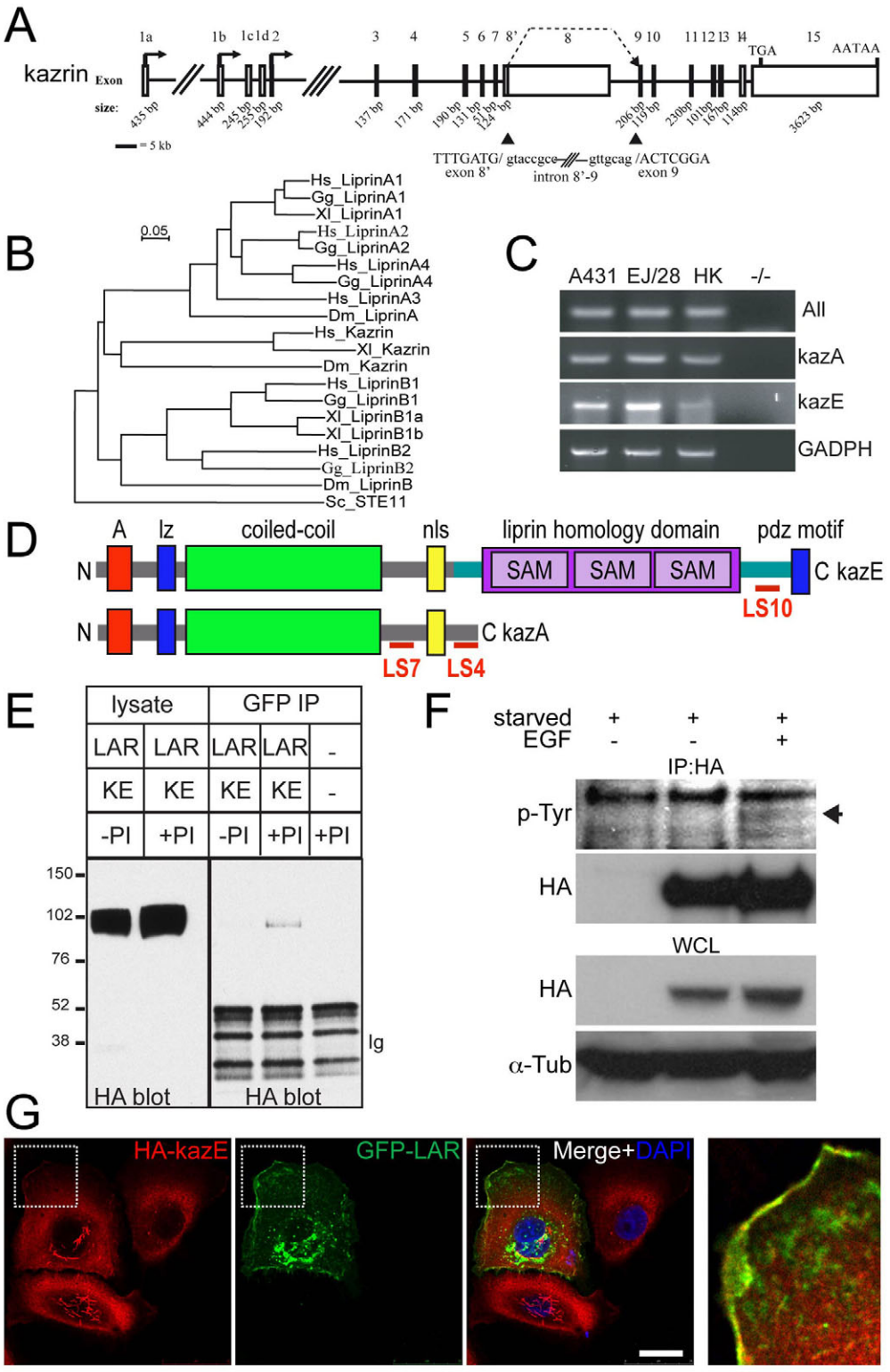


**Fig. 1.** KazrinE gene structure and interaction with LAR. (A) Intron-exon organisation of the human kazrin gene. Arrows, translation initiation sites; TGA, translation termination codon; AATAAA, polyadenylation signal. Dashed arrow shows generation of kazrinE by alternative splicing. (B) Liprin phylogenetic tree. Dm, *Drosophila melanogaster*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Sc, *Saccharomyces cerevisiae*; Xl, *Xenopus laevis*. Sc_STE11 is included as a SAM-domain-containing outlier. (C) RT-PCR using primers to detect the kazrin isoforms shown or GAPDH as an input-level control. HK, human keratinocytes; –/–, no RNA. (D) Schematic diagram of kazrinA and kazrinE. KazrinE is shown with the kazrinA N-terminus (A). lz, leucine-zipper-like domain; nls, putative nuclear-localisation sequence; SAM, sterile α motif. The location of peptide immunogens is shown. (E) 293T cells transiently co-transfected with HA-kazrinE (KE) and GFP-LAR (LAR) were lysed in the presence (+PI) or absence (–PI) of phosphatase inhibitors and either examined directly by anti-HA immunoblotting (lysate) or following immunoprecipitation with anti-GFP (GFP IP). As a control, anti-GFP alone was added to beads (–). (F) A431 cells were transiently transfected with GFP (left-hand lane) or HA-kazrinE, serum starved for 16 hours and either left untreated (–) or treated with EGF (+) for 1 hour prior to lysis in the presence of phosphatase inhibitors. Upper panels: HA-kazrinE was immunoprecipitated and immunoblotted with anti-phosphotyrosine (p-Tyr) or anti-HA. Arrow indicates phosphorylated kazrinE. Lower panels: western blots of total-cell lysates probed with antibodies to HA or α-tubulin (α-Tub) as controls for transfection and loading, respectively. (G) Keratinocytes retrovirally transduced with HA-kazrinE were transiently transfected with GFP or GFP-LAR (green), immunolabelled with anti-HA antibody (red) and counterstained with DAPI (blue). The boxed region is shown at higher magnification in the right-hand panel. Scale bar: 10 μm.

detected kazrinE in a filamentous network in the cytoplasm of some suprabasal cells that were undergoing terminal differentiation (Fig. 2F).

### KazrinE expression causes changes in keratinocyte shape and stimulates terminal differentiation

Overexpression of the short isoforms of kazrin causes profound changes in cell shape, reducing the amount of filamentous actin and decreasing Rho activity (Sevilla et al., 2008a). In addition, overexpression of kazrinA stimulates keratinocyte terminal differentiation (Sevilla et al., 2008a). To examine whether kazrinE had similar effects, primary human keratinocytes were transduced with a retroviral vector encoding HA-kazrinE or, as a control, GFP. Transduced cells were identified by GFP fluorescence or staining with an HA-specific antibody, and MetaMorph software was used to determine the perimeter and area of individual cells. As described

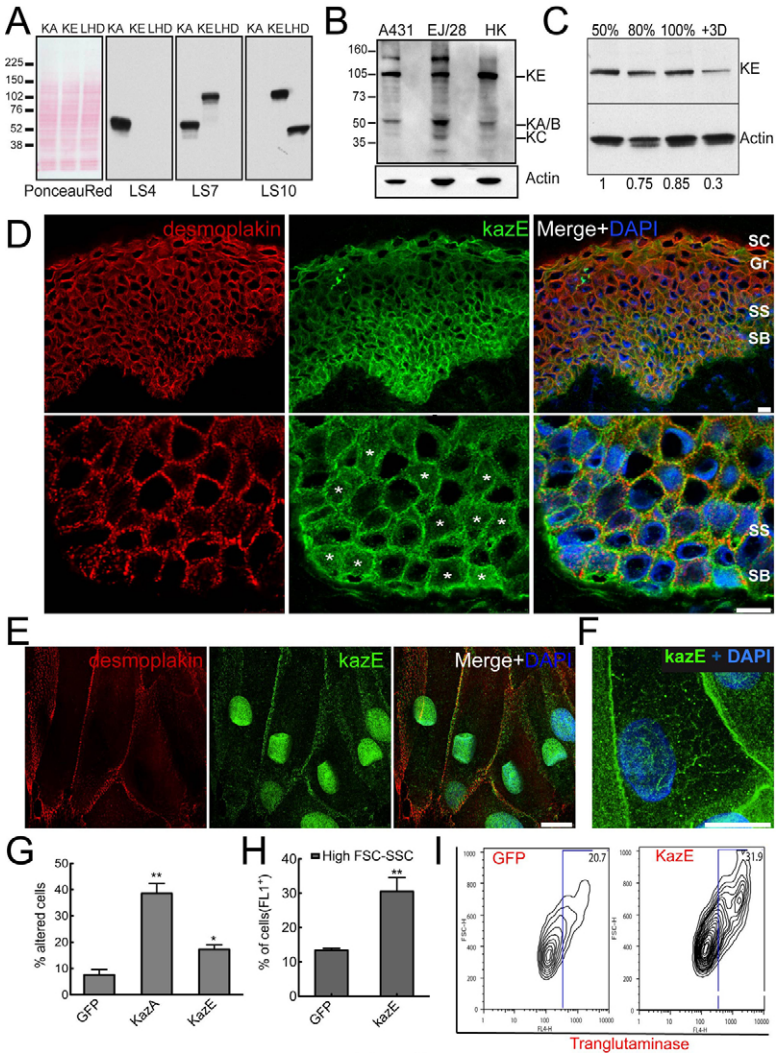


**Fig. 2.** KazrinE expression and function in epidermal keratinocytes. (A) Lysates of 293T cells transfected with HA-tagged kazrinA (KA), kazrinE (KE) or the kazrinE C-terminus (LHD) were immunoblotted with the anti-kazrin antibodies shown. Ponceau-Red staining shows equal protein inputs. (B) Immunoblot of A431, EJ/28 and human keratinocyte (HK) protein lysates probed with LS7 or anti-actin (loading control). KC, kazrinC; KA/B, kazrinA and B; KE, kazrinE. (A,B) Positions of molecular-mass markers (kD) are indicated. (C) Human keratinocytes harvested at 50, 80 or 100% confluence, or 3 days post-confluence (+3D) and immunoblotted with LS10 (KE) or anti-actin (loading control). Quantitation of kazrinE abundance relative to 50%-confluent cells is shown. (D) Immunofluorescence staining of human epidermis with LS10 (green) and desmoplakin (red) with DAPI nuclear counterstain (blue). Asterisks indicate nuclear staining. SB, basal epidermal layer; SS, spinous layer; Gr, granular layer; SC, cornified layer. (E,F) Keratinocytes grown in standard ('high calcium') medium were immunolabelled with antibodies to desmoplakin (red) or kazrinE (green). Nuclei were counterstained with DAPI (blue). (D-F) Scale bars: 10 μm. (G) Keratinocytes transiently expressing GFP, HA-kazrinA or HA-kazrinE were scored as 'altered' if they exhibited loss of the normal round or cuboidal shape characteristic of an undifferentiated keratinocyte, and/or loss of cortical actin. The total numbers of cells counted were: GFP, 1060; kazrinA, 1084; kazrinE, 985. Data are the means ± s.d. of three (kazrinE) or eight (GFP and kazrinA) independent experiments. Statistical significance relative to GFP control was assessed with the paired Student's *t*-test (***P*≤0.0005, **P*≤0.005). KazrinA and GFP data have been published previously (Sevilla et al., 2008a). (H) Quantitation of the percentage of keratinocytes with high forward (FSC) and side (SSC) scatter, an index of terminal differentiation. Cells were transduced with GFP or HA-kazrinE, gated on the GFP- or HA-positive populations and then analyzed for forward and side scatter. Data are mean ± s.d. of three independent experiments (unpaired Student's *t*-test; ***P*≤0.005). (I) Flow cytometry of keratinocytes transduced with GFP or HA-kazrinE and immunolabelled with anti-transglutaminase. Blue boxes show cells that are positively labelled compared with cells stained by secondary antibody alone. The percentage of cells in gated regions is indicated.

previously (Sevilla et al., 2008a), the shape of individual cells was scored as 'altered' if they exhibited loss of the normal cuboidal shape that is characteristic of basal keratinocytes. KazrinE induced a significant increase in cells with altered morphology; however, the effect was less pronounced than that of overexpressing kazrinA (Fig. 2G).

The effect of kazrinE overexpression on terminal differentiation was measured using three parameters: increased forward and side scatter, expression of involucrin or expression of transglutaminase (Sevilla et al., 2008a) (Fig. 2H,I and data not shown). By each criterion, there was a significant increase in the proportion of terminally differentiated cells when keratinocytes transduced with HA-kazrinE were compared with cells transduced with GFP. Although the effect of kazrinE on cell shape was not as marked as that of kazrinA, the stimulation of terminal differentiation was equivalent (Fig. 2G-I and data not shown) (Sevilla et al., 2008a).

### Homo- and heterotypic interactions between kazrin protein isoforms

All kazrin isoforms contain a coiled-coil domain, which could potentially mediate association of the different isoforms with one another (Burkhard et al., 2001). In addition, liprin-α protein family members are known to oligomerise via their coiled-coil domains (Serra-Pages et al., 1998). To examine potential kazrin-kazrin interactions, we performed co-immunoprecipitations using lysates from Cos cells co-transfected with N-terminally HA-tagged kazrinA, B, C or E (H-A, H-B, H-C, H-E) and C-terminally FLAG-tagged kazrinA, B or C (A-F, B-F, C-F) (Fig. 3A). Control cells were transfected with the HA-tagged construct together with pCIneo empty vector (V).

Proteins were immunoprecipitated with antibody against the FLAG-tag and immunoblotted with anti-HA or anti-FLAG (control for immunoprecipitation) antibodies (Fig. 3A). The higher-molecular-mass protein of approximately 150 kD observed in immunoblots of endogenous kazrin (Fig. 2B) was not evident in the co-immunoprecipitation experiments (Fig. 3A). FLAG-tagged kazrinA, B or C were co-immunoprecipitated with HA-tagged kazrinA, B and C. FLAG-tagged kazrinA was co-immunoprecipitated with HA-tagged kazrinE. We conclude that all the kazrin isoforms are capable of interacting with one another.

Endogenous kazrinA, B and C are localized to desmosomes, the nucleus and cortical actin-based structures, but not to perinuclear filaments (Groot et al., 2004). Because overexpressed kazrinE can accumulate in perinuclear filaments (Fig. 1G), we predicted that overexpression of kazrinE would recruit endogenous kazrinA into perinuclear filaments. Overexpression of HA-tagged kazrinE did

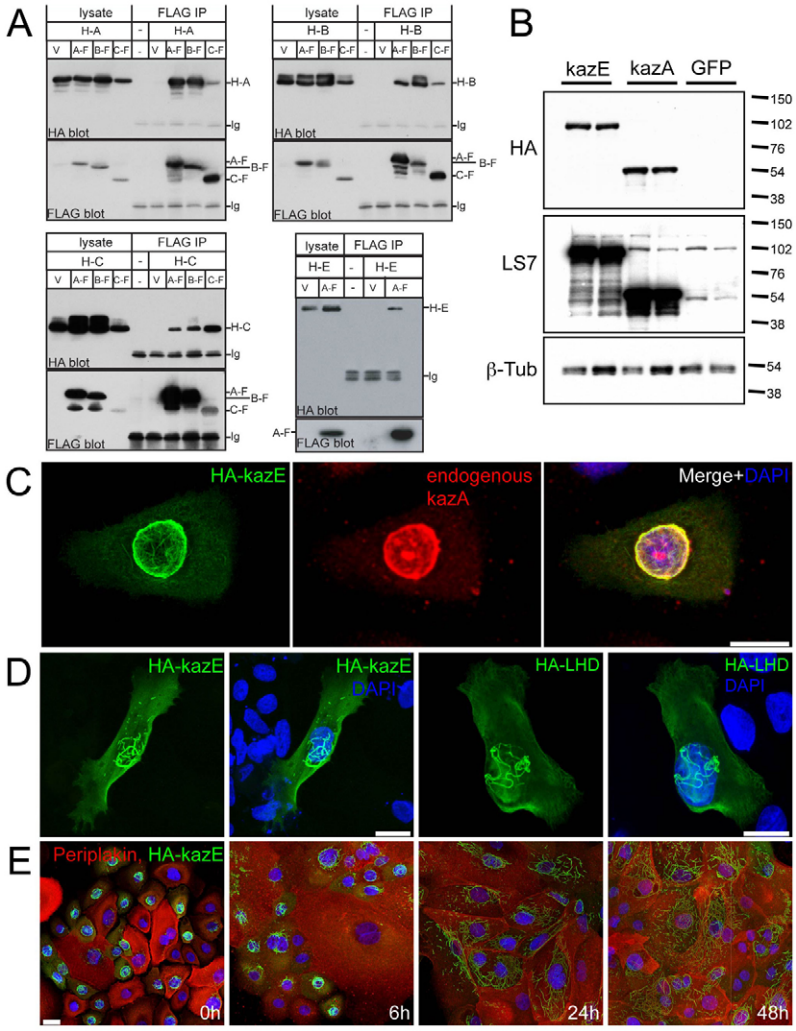


**Fig. 3.** Association of KazrinE and KazrinA isoforms and accumulation of kazrinE in cytoplasmic filaments. (A) Lysates of Cos-7 cells coexpressing different combinations of N-terminally HA-tagged and C-terminally FLAG-tagged kazrin isoforms were immunoprecipitated with anti-FLAG antibody (FLAG IP) and immunoblotted with anti-HA (upper panels) or anti-FLAG (lower panels). Cells were transfected with pCIneo empty vector (V), or with HA-tagged kazrinA (H-A), kazrinB (H-B), kazrinC (H-C) or kazrinE (H-E), in combination with FLAG-tagged kazrinA (A-F), kazrinB (B-F) or kazrinC (C-F). 5% of each lysate was loaded as a control for expression of the transfected constructs (lysate). –, antibody alone immunoprecipitation control. Positions of the kazrin isoforms and immunoglobulin light chain (Ig) are shown. (B) Lysates of human keratinocytes transduced with kazrinE, kazrinA or GFP retroviral vectors were immunoblotted with LS7, anti-HA or anti-β-tubulin (β-Tub) antibodies. Positions of molecular-mass markers (kD) are indicated. (C) Human keratinocytes transiently transfected with HA-kazrinE were immunolabelled with anti-HA (green) and LS4 (red; to detect endogenous short forms of kazrin), and counterstained with DAPI. (D) Human keratinocytes transiently transfected with HA-kazrinE or with HA-LHD (kazrinE liprin-homology domain and PDZ-binding motif) were immunolabelled with HA antibody (green) and counterstained with DAPI (blue). (E) Human keratinocytes transduced with HA-kazrinE were cultured in low-calcium medium (0h) and then transferred to standard calcium medium for the number of hours indicated. Cells were labelled with anti-HA (green) or periplakin (red) with DAPI nuclear counterstain (blue). Scale bars: 10 μm.

not affect the endogenous level of kazrinA in keratinocytes, nor did HA-kazrinA overexpression affect endogenous kazrinE levels (Fig. 3B). Nevertheless, HA-kazrinE recruited endogenous kazrinA into perinuclear filaments (Fig. 3C), consistent with the ability of the two proteins to associate with one another (Fig. 3A).

### Accumulation of kazrinE in perinuclear filaments is mediated by the LHD

In order to identify the nature of the kazrinE filaments, we transiently transfected or stably retrovirally infected low-calcium cultures of human keratinocytes with N-terminally HA-tagged kazrinE (Fig. 3D,E). To assess whether the C-terminus of kazrinE (LHD; containing the three putative SAM domains and the PDZ-binding motif) was responsible for the filamentous distribution of kazrinE, we transiently transfected keratinocytes with an N-terminally HA-tagged LHD construct. The LHD had a similar distribution to full-length kazrinE and was detected in perinuclear filaments (Fig. 3D). Because such filaments are not observed on overexpression of the short kazrin isoforms (Groot et al., 2004; Sevilla et al., 2008a), we conclude that the LHD is both necessary and sufficient for accumulation of kazrinE in perinuclear filaments.

Whereas the kazrinE-containing filaments were predominantly perinuclear in cells cultured in low-calcium medium, 6 hours after transfer to standard 'high calcium' medium (1.8 mM $Ca^{2+}$) the filaments had become finer and showed reduced nuclear association (Fig. 3E). Over time, the filaments elongated and extended through the cytoplasm towards the plasma membrane (Fig. 3E). The kinetics of the change in subcellular distribution were significantly slower than the kinetics of $Ca^{2+}$-induced desmosome assembly and accumulation of the short kazrin isoforms in desmosomes (Groot et al., 2004; Sevilla et al., 2008a).

### KazrinE associates with stabilised microtubules

KazrinE filaments did not colocalise with F-actin, keratins or with the majority of cytoplasmic microtubules (Fig. 4A). However, they did show complete colocalisation with stabilised, acetylated microtubules, both in untreated and nocodazole-treated cells (Fig. 4B and data not shown). In immunoblots, the total level of acetylated tubulin was the same in control cells and in cells retrovirally transduced with HA-tagged kazrinE (data not shown).

The microtubule cytoskeleton undergoes major reorganisation during keratinocyte terminal differentiation, coincident with a reduction in filamentous actin (Lewis et al., 1987; Lechler and Fuchs, 2007) (Fig. 4C-F). Whereas, in basal keratinocytes, microtubules are centrosome-associated (Fig. 4A; Fig. 4C, arrowheads), in differentiating, suprabasal cells the microtubules are organised into thick mats and distinctive coils (Fig. 4C, asterisks;

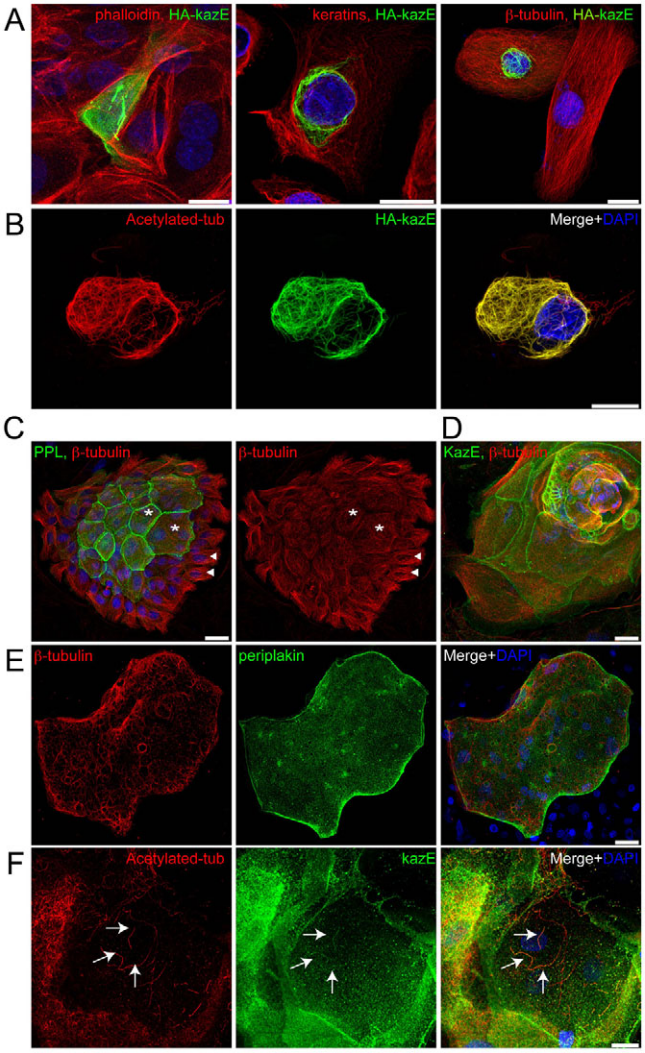


**Fig. 4.** Both endogenous and overexpressed kazrinE associate with acetylated microtubules. (A) Human keratinocytes transiently transfected with HA-kazrinE were immunolabelled with anti-HA antibody (green), anti-keratin, anti-β-tubulin or phalloidin (all red) and counterstained with DAPI (blue). (B) Human keratinocytes transiently transfected with HA-kazrinE were treated with 33 μM nocodazole for 3 hours and immunolabelled with anti-HA antibody (green) and anti-acetylated-tubulin antibody (red), with DAPI nuclear counterstain (blue). (C-E) Stratified colonies of human keratinocytes immunolabelled with anti-β-tubulin (red) and anti-periplakin (PPL; green; C,E) or kazrinE (green; D,F), with DAPI nuclear counterstain (blue). In C, both panels show the same colony. Asterisks indicate suprabasal, differentiated cells; arrowheads indicate basal cells. (F) Upper layers of a stratified colony immunolabelled for acetylated tubulin (red) and kazrinE (green) with DAPI nuclear counterstain (blue). Arrows indicate colocalisation of tubulin and kazrinE. Scale bars: 10 μm.

Fig. 4E) (Lewis et al., 1987; Lechler and Fuchs, 2007). The association of endogenous kazrinE with microtubules was observed in the uppermost layers of differentiating keratinocytes in culture (Fig. 4D,F; see also Fig. 2F). There was no colocalisation of periplakin with microtubules in differentiated cells (Fig. 4C,E). KazrinE was mainly desmosomal in the lower suprabasal layers, but in the more differentiated layers the microtubule association was evident (Fig. 4D,F). The colocalisation of endogenous kazrinE with microtubules was less pronounced than that of overexpressed kazrinE, which might explain why an association of the short kazrin isoforms with microtubules has yet to be observed (Groot et al., 2004) (data not shown).

We conclude that, in addition to its association with desmosomes and the nucleus, endogenous kazrinE can associate with microtubules. The microtubule association is mediated by the LHD and is a specific feature of differentiating cells. Our observations add to the emerging concept that the microtubule cytoskeleton contributes to the regulation of epidermal cell adhesion and cornified envelope assembly. Microtubule disruption in human keratinocytes can promote the assembly of adherens junctions (Kee and Steinert, 2001; Kee et al., 2002) and incorporation of S100A11 into the cornified envelope is thought to depend on microtubules (Broome and Eckert, 2004). Conversely, desmoplakin is required for differentiation-specific reorganisation of epidermal microtubules, and acts by recruiting ninein from centrosomes to desmosomes (Lechler and Fuchs, 2007). Our studies identify kazrinE as a novel interactor with desmosomes and microtubules, and suggest a potential role in coordinating the onset of differentiation with changes in keratinocyte adhesion and the cytoskeleton.

## Materials and Methods

### Sequence analysis

The BLAST 2.0 (NCBI, Bethesda, MD) and BLAT (Golden Path Genome Browser, Santa Cruz, CA) programs were used to search human and mouse EST databases and genome sequences. ClustalX software (Larkin et al., 2007) was used to generate the phylogenetic tree in Fig. 1B.

### DNA clones and constructs

The eGFP-LAR construct was kindly provided by Wiljan Hendriks (Nijmegen Centre for Molecular Life Sciences, The Netherlands). N-terminally HA-tagged kazrinA, kazrinB and kazrinC in EFplinkHA were described previously (Groot et al., 2004). C-terminally FLAG-tagged kazrin isoforms were generated using the forward primers 5′-GCGGAATTCGCCACCATGATGGAAGACAATAAGCAG-3′ (kazrinA), 5′-CGGGAATTCGCCACCATGCGGGCGGCCGACTCGGG-3′ (kazrinB) and 5′-GGCGAATTCGCCACCATGAAGGAGATGTTGGCGAAG-3′ (kazrinC), in combination with reverse primer 5′-GCGGTCGACTCACTTATCGTCGTCAT-CCTTGTAATCTCCTCCTCCCCAGTCCGCGTCCTCCTCTAT-3′. PCR products were digested with *Eco*RI and *Sal*I, and ligated into pCIneo (Invitrogen).

IMAGE clones encoding mouse kazrinA (no. 3667377) and human kazrinE (no. 5242289) in pCMV-Sport6 were obtained from the MRC UK HGMP Resource Centre and confirmed by sequencing. pCIneo HA-kazrinE was generated by PCR amplifying kazrinE using primers 5′-ATGATGGAAGACAATAAGCAG-3′ (forward) and 5′-GCGTCTAGACACGTTGGTGACCTCCAGGCT-3′ (reverse), then digesting with *Age*I and *Xba*I and ligating into similarly digested pCIneo HA-kazrinA (Sevilla et al., 2008a). pBABEpuro HA-kazrinE was generated by cloning the *Age*I-*Sal*I fragment from pCIneo HA-kazrinE into similarly digested pBABEpuro HA-kazrinA (Sevilla et al., 2008a).

The C-terminus of kazrinE (nucleotides 1222-2326) was amplified using primers 5′-GCGCTCGAGGCCACCATGTACCCCTACGACGTGCCCGACTATGCCGGA-GGAGGAGACTCGGACAGCCAGTGCA-3′ (forward) and 5′-GCGTCTAGA-TTACACGTTGGTGACCTCCA-3′ (reverse), digested with *Xho*I and *Xba*I, and ligated into pBluescript. The *Xho*I-*Not*I fragment of pBluescript HA-kazrinE-LHD was cloned into pCIneo.

### Cell culture

Primary human keratinocytes (strains kv and kt, passages 2-4) and mouse keratinocyte lines were cultured, transiently transfected and/or retrovirally infected as previously described (DiColandrea et al., 2000; Groot et al., 2004; Sevilla et al., 2008a). A431, Cos-7, EJ/28 and 293T cells were maintained in DMEM with 10% FCS. Transient transfection of Cos-7, A431 and 293T cells was performed using Superfect (Qiagen), Lipofectamine (Invitrogen) and FuGENE 6 Transfection Reagent (Roche), respectively.

Where indicated, cells were treated with 33 μM nocodazole (Sigma-Aldrich) or 50 ng/ml recombinant human EGF (Peprotech).

### RT-PCR

RNA was prepared using TriReagent (Molecular Research Center). The SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen) was used for semi-quantitative RT-PCR. Total kazrin mRNA (all isoforms) was amplified using a forward primer in exon 7 (5′-GGCGACAGTCCCGGCCCAGTTCAGAAG-3′) and a reverse primer in exon 8 before the kazrinE splice site (5′-CCCTCTGGCGAAGACG-

CGGGAGATGGA-3′). To selectively amplify the short isoforms, the forward primer was used in combination with a reverse primer after the kazrinE splice site in exon 8 (5′-CACCAGTCCGCGTCCTCCTCTATGTAAT-3′). To selectively amplify kazrinE, the forward primer was combined with a reverse primer in exon 9 (5′-CCAGCTCCACCTGCTGCAGCCGGTCCAT-3′). All PCR products were confirmed by sequencing. Primers specific for human *GAPDH* mRNA were used to control for input mRNA levels (Groot et al., 2004).

### Antibodies

Mouse monoclonal antibodies were used at the dilutions stated: HA.11 (1:1000; anti-HA, Covance Research Products), M2 (1:1000; anti-FLAG, Sigma Aldrich), AC40 (1:500; anti-actin, Sigma-Aldrich), LP34 (1:100; specific for keratin filaments) (Lane et al., 1985), clone 2-28-33 (1:1000; anti-β-tubulin, Sigma-Aldrich), clone 6-11B-1 (1:1000; anti-acetylated-tubulin, Sigma-Aldrich), SY5 (1:100; anti-human involucrin), BC.1 (1:100; anti-transglutaminase-1, a gift from Robert Rice, University of California, CA), p-Tyr-100 (1:1000; anti-phosphotyrosine, Cell Signaling) and T6199 (1:2500; anti-α-tubulin, Sigma-Aldrich). The following rabbit antisera were also used: Y11 (1:50; anti-HA, Santa Cruz Biotechnology), anti-FLAG (1:100; Sigma-Aldrich), LS4 (1:100; anti-kazrin) (Groot et al., 2004) and LS7 (1:500; anti-kazrin) (Sevilla et al., 2008a). For flow cytometry, SY5 and BC.1 were directly conjugated to Alexa Fluor 633 using the Protein Labeling kit from Invitrogen. HA.11 coupled to Alexa Fluor 488 was purchased from Covance Research Products.

Amino acids 1646-1693 of human periplakin (KREQRENHLRRSIVVIHPD-TGRELSPEEAHRAGLIDWNMFVKLRSQEC) were used to generate the TD2 rabbit antiserum (Määttä et al., 2001). Amino acids 733-750 (SKDPDFHDDYGSLQNEDC) of human kazrinE were used to generate LS10. Anti-kazrin antibodies were affinity purified on columns containing their respective immunogens coupled to Amino-Link Coupling Gel (Perbio).

Secondary antibodies were Alexa-Fluor-488- or -594-conjugated donkey anti-rabbit or mouse IgG and rabbit anti-goat IgG (Invitrogen; diluted 1:1000), or HRP-conjugated donkey anti-rabbit or anti-mouse IgG (Amersham Biosciences; diluted 1:4000).

### Immunofluorescence staining and microscopy

Immunofluorescence labelling was performed as described previously (DiColandrea et al., 2000). Samples were examined using a Leica Tandem SP5 laser scanning confocal microscope equipped with 405 nm, 488 nm and 594 nm lasers and immersion lenses HC PL APO 20× 0.7 IMM, HCX PL APO 40× 1.25 Oil and HCX PL APO 63× 1.4 Oil (Leica, Heidelberg, Germany). Three-dimensional reconstructions of *z*-stacks were made using the Leica Application Suite software. Digital images were prepared using Adobe Photoshop CS2 software and compiled using Adobe Illustrator CS2.

### Immunoprecipitation and immunoblotting

Cos-7 cells were lysed with ice-cold buffer (0.15 M NaCl, 0.05 M Tris, 5 mM EDTA, 0.5% NP-40) containing protease inhibitors (Roche). A431 cells transfected with pCINeo–HA-kazrinE or pEGFP-C2, and 293T cells were lysed with ice-cold buffer (0.15 M NaCl, 0.05 M Tris, 5 mM EDTA, 0.02% NP-40) in the presence of protease inhibitors (Roche) and in the presence or absence of phosphatase inhibitors as indicated (PhosphoSTOP tablets, Roche; or 100 mM NaF, 2 mM $Na_3VO_4$, 20 mM $Na_4P_2O_7$). Insoluble material was sedimented by centrifugation for 10 minutes at 4°C at high speed in a microcentrifuge. Immunoprecipitation and immunoblotting were performed as previously described (Groot et al., 2004; Sevilla et al., 2008a).

### Flow cytometry

Intracellular staining of keratinocytes for involucrin and transglutaminase 1 was performed essentially as described previously (Sevilla et al., 2008a). Flow cytometry was performed with the BD FACSCalibur System (BD Biosciences) using BD CellQuest software. FlowJo software (Tree Star) was used to further analyse the flow-cytometry data.

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## Note added in proof

A further kazrin isoform, kazrinF, has recently been reported (Wang et al., 2009).

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