

# Stress-induced recruitment of epiplakin to keratin networks increases their resistance to hyperphosphorylation-induced disruption

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## Summary

Epiplakin is a large (>725 kDa) cytoskeletal protein exclusively expressed in epithelial tissues. It has a unique structure, consisting entirely of plakin repeat domains (PRDs), one of the hallmarks of spectraplakin protein family members. Previous studies, including the phenotypic analyses of knockout mice, failed to reveal the biological function of epiplakin. Using *in vitro* binding assays, we show here that all but one of the 16 PRDs of mouse epiplakin bind to keratins of basal keratinocytes. Nevertheless, in primary keratinocyte cell cultures, epiplakin only partially colocalized with keratin intermediate filament networks. However, upon application of cellular stress in the form of keratin hyperphosphorylation, osmotic shock or UV irradiation, the entire cytoplasmic epiplakin pool became associated with keratin. In response to

such types of stress, epiplakin initially translocated to the still-intact keratin filament network and remained associated with keratin after its disruption and transformation into granular aggregates. Time-course experiments revealed that serine/threonine (okadaic acid) and tyrosine (orthovanadate) phosphatase inhibitor-induced filament disruption in differentiated keratinocytes proceeded faster in epiplakin-deficient cells compared with wild-type cells. Our data suggest that epiplakin plays a role in keratin filament reorganization in response to stress, probably by protecting keratin filaments against disruption in a chaperone-like fashion.

**Key words:** Epiplakin, Hyperphosphorylation, Keratin, Spectraplakin, Stress response

## Introduction

Epiplakin was originally isolated as an autoantigen with immunoreactivity toward the serum from a patient who suffered from a subepidermal blistering disease (Fujiwara et al., 1994; Fujiwara et al., 1996). The protein is expressed in epithelial tissues, with especially high levels found in skin (Spazierer et al., 2003). Its function is still unknown, because mice deficient in epiplakin did not show any discernible phenotype (Spazierer et al., 2006), except for a slight acceleration of keratinocyte migration, as reported by Goto et al. (Goto et al., 2006). Surprisingly, not even when the skin of knockout mice was challenged by tape stripping or wounding, were differences to wild-type mice observed (Spazierer et al., 2006).

Based on the sequence analysis of human and mouse cDNAs, epiplakin was classified as a member of the spectraplakin protein family of cytolinkers (Fujiwara et al., 2001; Spazierer et al., 2003). Its gene encodes a large protein consisting entirely of plakin-repeat domains (PRDs), of which there are 16 in mouse epiplakin and 13 in the human protein (Fujiwara et al., 2001). The PRD contains a highly conserved ~20 kDa core region, called the module (Sonnhammer et al., 1997; Janda et al., 2001) and a less conserved linker region of variable length. The lack of other domains typically present in spectraplakins makes epiplakin an unusual member of this protein family, and brings its functional relationship to the other members into question. In general, cytolinker proteins connect cytoskeletal filaments with one another and attach them to plasma-membrane-associated adhesive junctions (for a review, see Röper et al., 2002; Jefferson et al., 2004). The ability to interact with intermediate filaments has been shown for several members of the

spectraplakin family. Plectin, for example, binds to intermediate filament proteins of various types via an intermediate-filament-binding site located between modules 5 and 6 of its six PRDs (Nikolic et al., 1996) and an additional vimentin-binding site has been identified in its N-terminal actin-binding domain (Sevcik et al., 2004). For desmoplakin, keratin and vimentin binding have been reported to involve sites within its three C-terminal PRDs (Stappenbeck and Green, 1992; Stappenbeck et al., 1993; Kouklis et al., 1994; Yang et al., 1996; Meng et al., 1997) and an interaction with keratins and neuronal intermediate filaments has been demonstrated for the PRDs of BPAG1n and BPAG1e (Yang et al., 1996). Association with keratin filaments has also been shown for envoplakin and periplakin (Karashima and Watt, 2002; Kazerounian et al., 2002). In the case of epiplakin, binding of the most C-terminal PRD to keratins was shown in dot blot assays (Jang et al., 2005) and, in a recent study, keratin binding of module 8 has been demonstrated using slot blot assays (Wang et al., 2006). It is not known, however, whether all the PRDs in epiplakin can actually bind to keratin. Furthermore, colocalization of epiplakin with keratin filament networks has been reported so far only for human carcinoma (HeLa) and keratinocyte (HaCaT) cell lines, whereas it was not observed in primary keratinocytes (Jang et al., 2005). Thus, it remains an open question whether the interaction of epiplakin with keratins K5 and K14 observed *in vitro* is of biological significance.

Based on the available data and functions previously analyzed for other spectraplakin protein family members, one might expect epiplakin to play a role in the regulation of keratin filament dynamics and/or stabilization of the filaments. For example, it has been shown

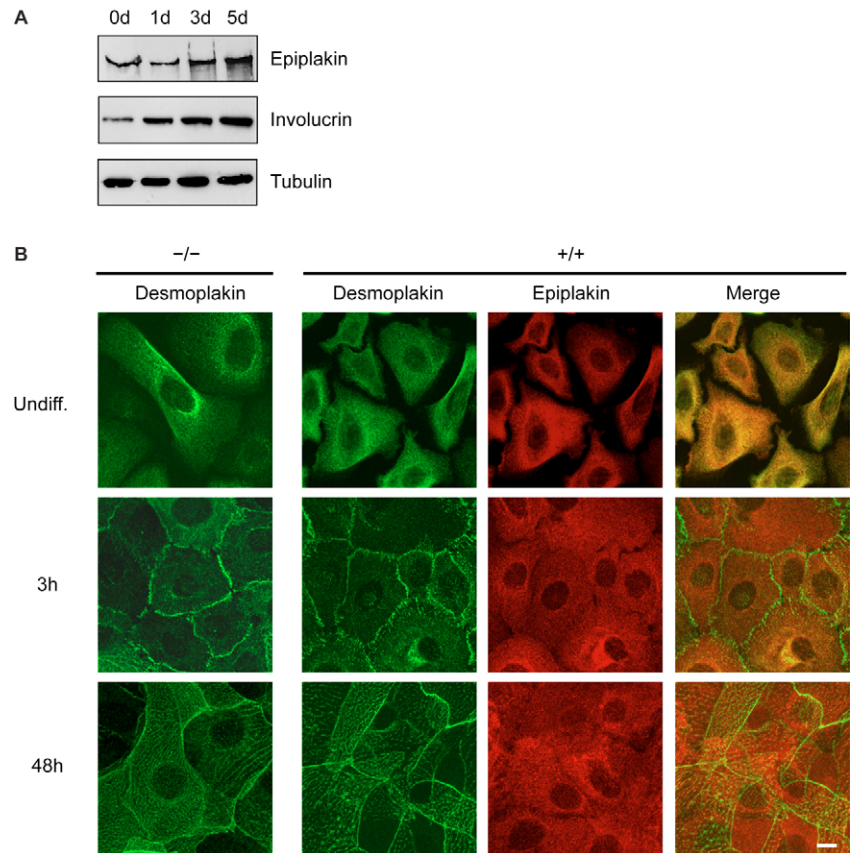


immortalized p53<sup>-/-</sup> keratinocytes. As shown in Fig. 1C, all epiplakin PRDs except PRD5 were found to bind to K5. Interestingly, PRD9, which, because of virtually identical primary structures (Spazierer et al., 2003), is representative of PRD9-15, additionally showed binding to K14. GST alone (negative control) showed no keratin binding. In addition, we investigated which of the PRD9 subdomains, the module and/or the linker, showed K5/K14 binding, and we similarly examined the linker subdomain of PRD8, which accounts for the structural difference between PRD8 and PRD9 (modules 8 and 9 are identical), and the linker-less module of PRD16 (see Fig. 1B), which differs from module 9 in just the last 16 C-terminal amino acid residues. Neither linker 8 nor linker 9 showed binding to keratins in blot overlay assay, whereas modules 9 and 16 both bound to K5, but not to K14 (Fig. 1D). From this it appeared that linker 9 and module 9 were capable of binding to K14 only when combined. In an alternative assay, GST-fusion proteins of PRD8 and PRD9 were covalently linked to Sepharose 4B beads to pull down keratins from keratinocyte cell extracts. As shown in Fig. 1E, K5 as well as K14 co-sedimented with PRD8- and PRD9-coupled beads, whereas beads coupled to GST alone were ineffective. Thus, whereas pull-down and blot overlay results were in full agreement for PRD9, K14 binding of PRD8 was detectable only by pull down assay. This difference can be explained, however, by heterodimer formation of K5 and K14 in cell-lysates, leading to the additional pull down of K14 with PRD8-bound K5.

#### Epiplakin deficiency has no obvious effect on Ca<sup>2+</sup>-mediated keratinocyte differentiation

The prominent expression of epiplakin in keratinocytes combined with its partial association with the keratin filament network prompted us to examine whether epiplakin plays any role in keratinocyte differentiation. In fact, Jang et al. (Jang et al., 2005) reported a substantial upregulation of epiplakin during Ca<sup>2+</sup>-mediated differentiation of normal human epidermal keratinocytes (NHEKs). However, for NHEKs, only 10% of the original (undifferentiated) cell population was found to be epiplakin-positive (Jang et al., 2005), whereas in undifferentiated primary mouse keratinocytes we observed that 100% of the cell population expressed epiplakin (Fig. 1A, and data not shown). Nevertheless, immunoblotting of cell lysates from primary mouse keratinocytes that had been cultivated in medium containing 1.3 mM calcium for up to 5 days revealed a significantly elevated expression of epiplakin. Using tubulin as a loading control (Fig. 2A), a ~1.2- and ~1.8-fold upregulation of epiplakin compared with basal levels (day 0) was observed after 3 and 5 days of differentiation, whereas corresponding levels of involucrin, which served as differentiation marker, were up ~2.4- and ~3-fold.

To examine whether the increasing epiplakin protein levels had any effect on the time course of differentiation, wild-type and epiplakin-deficient primary keratinocytes grown in 1.3 mM Ca<sup>2+</sup>-containing medium were subjected to immunofluorescence microscopy using desmoplakin as a differentiation marker. At 3

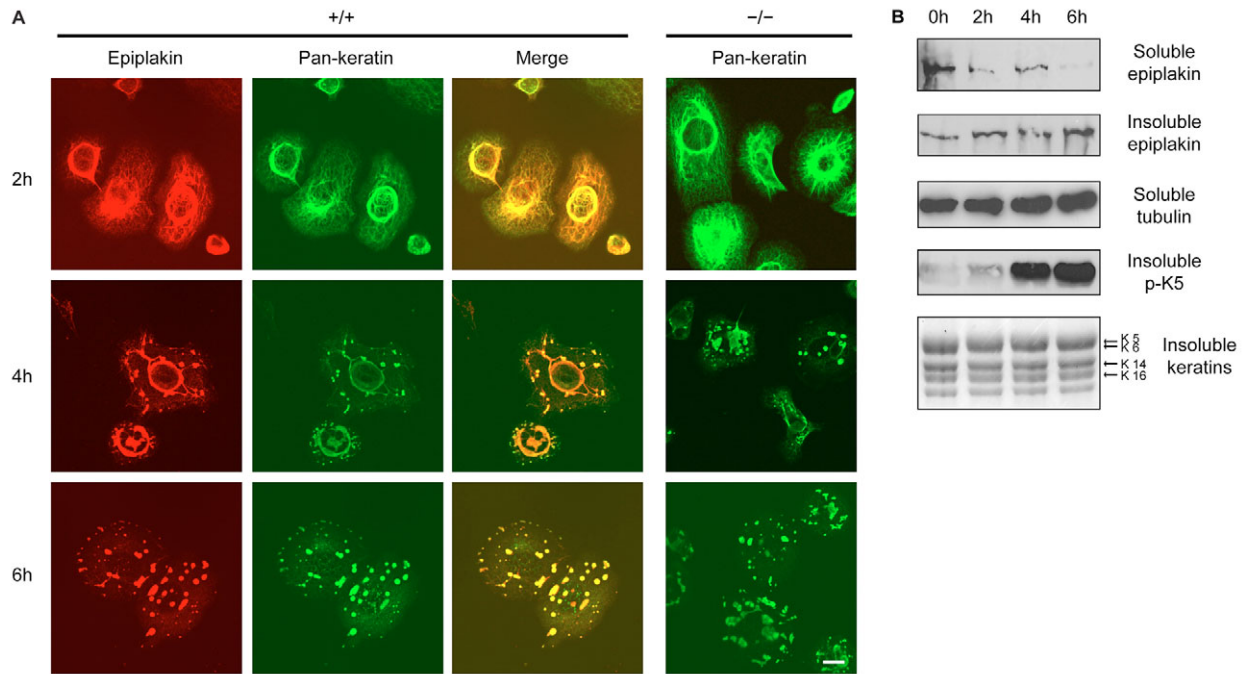


**Fig. 2.** Ca<sup>2+</sup>-mediated differentiation of primary mouse keratinocytes is independent of epiplakin. (A) Immunoblotting of protein extracts from keratinocytes grown in the presence of 1.3 mM Ca<sup>2+</sup> for 1, 3 or 5 days, using antibodies as indicated. Note the upregulation of epiplakin and involucrin (differentiation marker) compared with tubulin (loading control). (B) Primary keratinocytes isolated from wild-type (+/+) and epiplakin-deficient (-/-) mice were grown in the presence of 1.3 mM Ca<sup>2+</sup> for the times indicated and then subjected to immunofluorescence microscopy using antibodies as indicated. Scale bar: 10  $\mu$ m.

hours after initiating differentiation in both cell types, desmoplakin showed its typical association with cell-cell junctions, and no differences in the staining pattern became evident thereafter (Fig. 2B). Epiplakin showed few changes in its distribution during differentiation (Fig. 2B). Furthermore, we could not detect any differences between differentiated wild-type and knockout keratinocytes regarding the size or shape of cells, nor did we find any differences in keratin expression or filament organization (Fig. 2B, and data not shown).

#### Hyperphosphorylation, osmotic stress and UV irradiation stimulate epiplakin-keratin association

To investigate whether the phosphorylation status of keratins influenced their binding to epiplakin, primary keratinocytes were treated with the phosphatase inhibitor okadaic acid (OA) to specifically induce serine/threonine hyperphosphorylation. Immunofluorescence microscopy of cells exposed to OA for 2 hours revealed a predominantly filamentous staining pattern of epiplakin and a complete colocalization of the protein with keratin filaments (Fig. 3A). After 4 hours of OA exposure, keratin filaments were partially disrupted, with epiplakin being present in both the residual thick perinuclear filament bundles and the keratin granules, which appeared mainly at the cell periphery (Fig. 3A). After 6 hours, keratin



**Fig. 3.** Colocalization and subcellular co-distribution of epiplakin with keratin aggregates after OA-induced filament disruption in wild-type (+/+) and epiplakin-deficient (-/-) keratinocytes. (A) Primary mouse keratinocytes, treated with OA for 2, 4 and 6 hours were immunolabeled using antibodies as indicated. Merged images show complete colocalization of epiplakin with keratin. Compare with untreated cells (negative control) shown in Fig. 1A. Scale bar: 10  $\mu$ m. (B) Immunoblotting (upper four panels) of soluble and insoluble cell fractions obtained from 2, 4 and 6 hour OA-treated keratinocytes using antibodies to epiplakin, tubulin, and phospho (p)-K5. Coomassie Blue staining of high salt-extracted keratins is shown in bottom panel.

filaments had disappeared, and keratin as well as epiplakin was found exclusively in form of granules spread all over the cytoplasm (Fig. 3A). Next, we monitored OA-induced epiplakin-keratin association by immunoblotting and protein staining of soluble and insoluble cellular subfractions during OA treatment (Fig. 3B). In untreated keratinocytes (lanes 0h) epiplakin was found in both the insoluble and the soluble fractions (upper two panels), whereas keratins, as expected, were mostly insoluble (lower panel; and data not shown). The proportion of epiplakin in the soluble fraction decreased upon incubation with OA, and after 6 hours, almost all epiplakin was found associated with the insoluble keratin-containing fraction. Increased association of epiplakin with insoluble keratins correlated with increased phosphorylation of keratins, as demonstrated by immunoblotting using phospho-epitope-specific antibodies to K5. The phosphorylation status of epiplakin was found to be unchanged during OA incubation (data not shown).

As OA-induced serine/threonine hyperphosphorylation of keratins led to their association with epiplakin, it was of interest to examine whether a tyrosine phosphatase inhibitor such as orthovanadate (OV) would have similar effects. Strikingly, we observed an entirely filamentous distribution of epiplakin and full colocalization with keratins after a 2 minute incubation of cells with OV (Fig. 4). After a 10 minute incubation with OV, filamentous structures were no longer visible. The entire keratin networks had collapsed, forming numerous aggregates of various sizes, with undistinguishable staining patterns of both keratin and epiplakin (Fig. 4).

Osmotic shock assays, such as treatment with 150 mM urea, serve as a useful tool for monitoring keratin network properties and their alterations in epidermolysis bullosa simplex (EBS) caused by keratin mutations (Morley et al., 1995; D'Alessandro et al., 2002). To

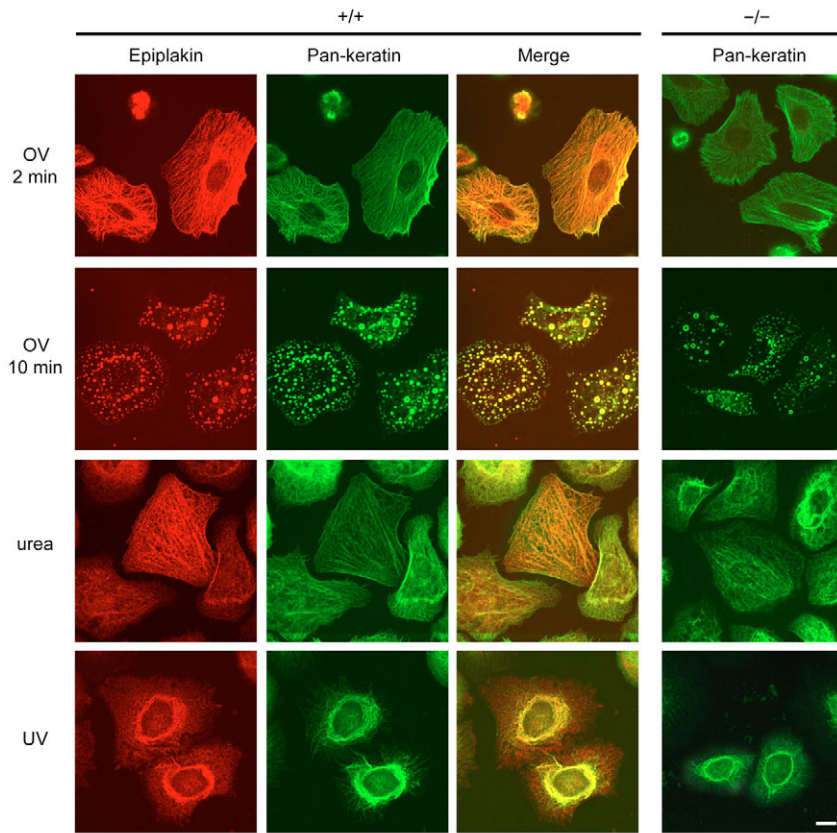
examine whether this type of stress, in a similar manner to hyperphosphorylation, leads to an association of epiplakin with keratins, primary mouse keratinocytes incubated with urea for 5 minutes were double labeled for pan-keratin and for epiplakin (Fig. 4). Indeed, compared with untreated cells, the staining pattern of epiplakin appeared more filamentous under these conditions (compare Fig. 1A with Fig. 4), indicating increased epiplakin-keratin association.

UV irradiation of keratinocytes has been reported to cause a redistribution of keratin networks into ring-like structures around the nucleus (Zamansky et al., 1992). As shown in Fig. 4, UV irradiation of primary keratinocytes at 400 J/m<sup>2</sup> led to a conspicuous colocalization of epiplakin with such perinuclear structures.

#### OA-induced keratin filament disruption in differentiated keratinocytes proceeds faster in the absence of epiplakin

The tendency of epiplakin to associate with keratin structures under conditions leading to filament reorganization suggested that the protein might play a role in the cellular stress response. To examine whether epiplakin had any effect on the rate of OA-induced filament disruption, we subjected primary cultures of undifferentiated epiplakin-deficient keratinocytes to a time-course experiment. Immunofluorescence microscopy carried out 2, 4 and 6 hours after OA addition revealed no differences in keratin network disruption patterns in these cells compared with the wild type (Fig. 3; compare keratin staining of +/+ and -/- cells). Similarly, no detectable differences in keratin filament disruption or rearrangement were observed between the two cell types when treated with OV or urea, or with UV irradiation (Fig. 4).

A different situation was observed when epiplakin-deficient and wild-type keratinocytes that had undergone Ca<sup>2+</sup>-induced



**Fig. 4.** Recruitment of epiplakin to keratin filaments and aggregates after application of various types of stress. Primary mouse keratinocytes isolated from wild-type (+/+) or epiplakin-deficient (-/-) mice were treated with orthovanadate (OV) for 2 minutes and 10 minutes, or were exposed to 150 mM urea or UV (400 J/m<sup>2</sup>) prior to immunolabeling using antibodies as indicated. Merged images of wild-type cells show colocalization of epiplakin and keratin. Scale bar: 10  $\mu$ m.

differentiation were subjected to a similar experiment. Under these conditions, OA-induced keratin network disruption in wild-type cells proceeded more slowly than in undifferentiated cells (Fig. 5A). In undifferentiated wild-type cells, keratin was found largely in the form of aggregates at the periphery of cells at the 4 hour time point, with just a filamentous ring (eventually also becoming fragmented within the next 2 hours) persisting around the nucleus (Fig. 3A). By contrast, in differentiated cells, keratin aggregates were rarely observed before the 6 hour time point, and even then, the majority of cells displayed just partially disrupted filament networks (Fig. 5A). Interestingly, however, in differentiated epiplakin-deficient cells, the keratin filament system seemed to be more susceptible to hyperphosphorylation. Partially disrupted filament systems became visible in these cells after 2 hours, and the formation of aggregates appeared to progress faster (Fig. 5A). For a statistical analysis, we counted wild-type and epiplakin-deficient cells exhibiting filamentous, partially disrupted (as exemplified by cells indicated with arrows in Fig. 5A) and totally disrupted (Fig. 5A, cells indicated with arrowheads) keratin filaments at 1 hour intervals over a period of 6 hours. As the time courses – including the onsets of the intermediate filament rearrangements – varied between different experiments, we calculated the percentages of cells with changed filament network organization during the first 2 hours after the onset of filament breakdown (Fig. 5B). In almost half (~46%) of epiplakin-deficient cells, keratin filaments were at least partially disrupted at

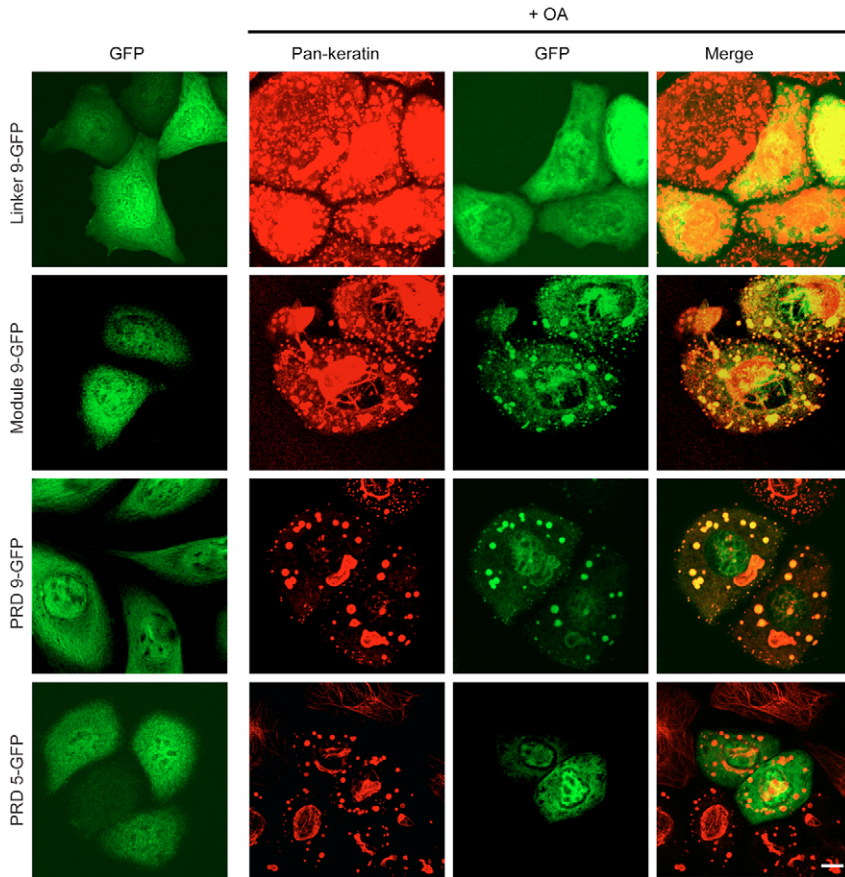
this time point, whereas only ~10% of wild-type cells were in this state. Similarly, the percentage of cells with totally disrupted filament networks (displaying exclusively aggregated keratins), calculated over a 2 hour period (starting from the first time point when cells of that type were appearing) was significantly higher for epiplakin-deficient cells than for wild-type cells (Fig. 5B). The faster disruption of filamentous intermediate filament networks, paired with an accelerated appearance of aggregated keratins, was noticeable in four independent experiments. The entire time course for one of these experiments is shown in Fig. 5C. To shed more light on the mechanism underlying increased filament disruption, we next investigated whether it was coupled with accelerated phosphorylation of keratins by western blot analysis using antibodies to phospho-K5. However, epiplakin-deficient and wild-type cells showed no significant differences in the extent or rate of keratin phosphorylation (data not shown). Since MAP-kinases, especially p38, are thought to play a pivotal role in the stress response, we also measured the activation of p38, ERK and JNK MAP kinases using phospho-specific antibodies to each of these proteins. Again, we could not detect any differences in the activation patterns of these kinases over the time course of OA-induced filament breakdown between differentiated wild-type and epiplakin-deficient cells (data not shown).

As the tyrosine phosphatase inhibitor OV led to a similar breakdown of keratin filaments (paired with the recruitment of epiplakin to the resulting aggregates) as serine/threonine phosphatase inhibitors, it was of interest to examine whether epiplakin deficiency had an accelerating effect on keratin filament disruption in this situation. Because of the faster and more distinct disruption pattern observed, only two types of cells could be distinguished: those with an intact keratin filament system and those with a disrupted network. When OV-treated epiplakin<sup>-/-</sup> and wild-type cells were statistically analyzed at 3-minute intervals over a period of 9 minutes, an acceleration of filament disruption was again found in epiplakin-deficient compared with wild-type cells. After 3 minutes of OV incubation, there was already a significant difference, which then continued over time (Fig. 5D).

#### Individual PRDs expressed by force mimic endogenous epiplakin without affecting keratin cytoarchitecture

To investigate whether fragments of epiplakin had any influence on keratin filament organization when overexpressed in keratinocytes, module 9, linker 9, PRD9 and PRD5 were expressed in keratinocytes as GFP-fusion proteins. Immunofluorescence microscopy of transfected cells revealed a partially filamentous staining pattern resembling that of endogenous (full length) epiplakin only for PRD9, whereas a diffuse distribution throughout the cells was observed in all other cases (Fig. 6, left column). In no case did the overexpression of epiplakin fragments lead to a change in keratin filament organization (data not shown). Furthermore, we examined whether OA-induced association of cellular epiplakin with keratin structures could be mimicked by





**Fig. 6.** Forced expression of epiplakin fragments in immortalized wild-type mouse keratinocytes. Cells were transfected with indicated epiplakin cDNA constructs, and subjected to immunofluorescence microscopy either directly (left column) or after incubation with OA (+OA) for 5 hours. Epiplakin-GFP-fusion proteins expressed were visualized using antibodies as indicated. Merged images show colocalization of overexpressed fragments after OA incubation only in the cases of module 9 and PRD9. Scale bar: 10  $\mu$ m.

appear to be rather immobile (Strnad et al., 2001) because of their anchorage into the cytoskeleton via plectin. It has been proposed that such anchorage might be a prerequisite for the quick and successful reintegration of keratin into a filamentous network (Strnad et al., 2002). By contrast, the OA-induced filament breakdown into 14-3-3 protein-positive granules and the recovery after drug removal occurred much more slowly, with each taking several hours to be completed (Strnad et al., 2001; Strnad et al., 2002). It is thought that 14-3-3 protein association with keratin granules keeps them soluble (Liao and Omary, 1996; Liao et al., 1997), and might abolish their capacity to quickly re-establish a fully spread filament system (Strnad et al., 2002). As epiplakin was found in both types of granules, it might have function(s) that are independent of the type of aggregate. One model could be that epiplakin, with its 15 keratin-binding modules, serves as a chaperone-like protein during filament remodeling. This would be consistent with the more rapid OA- and OV-induced filament breakdown in differentiated epiplakin-deficient keratinocytes compared with wild-type cells, especially as we could not detect any faster or stronger activation of MAP-kinase-based stress-response pathways in the absence of epiplakin. Furthermore, the phosphorylation status of K5 was not affected in epiplakin<sup>-/-</sup> cells,

making a mechanical role of epiplakin during the stress response more likely. Therefore, we suggest that epiplakin plays a protective function in keratin filament networks. It is conceivable that epiplakin spans a number of keratin subunit proteins within the filaments, serving as a stabilizer for the filament system when cells are subjected to stress, although a more indirect mechanism of stabilization cannot be ruled out. That this protective effect was noticed only in cells that had undergone differentiation, but not under basal conditions prompts us to speculate that epiplakin executes this function mainly in cell layers where filament systems span more cells, such as in the upper epidermis. Nevertheless, the initial analysis of mice deficient in epiplakin did not show any skin abnormalities, and even challenging the skin by tape-stripping or wounding did not reveal any discernible phenotype (Goto et al., 2006; Spazierer et al., 2006). However, this kind of mechanical stress might not have affected the keratin filament network system in the same way that phosphatase inhibitors do. The use of other (organ-specific) injury models with epiplakin-deficient mice might therefore shed more light on the putative filament-stabilizing function of epiplakin. Furthermore, with its 15 keratin-binding repeat domains, epiplakin might not only play a role in conserving the integrity of filaments by stabilizing them against disruption, but also in the recovery of filaments after drug removal. This issue could be addressed in the future by examining the dynamic properties of keratin during filament remodeling in epiplakin-deficient keratinocytes.

The mechanism underlying the rapid transition from a dotted and partially filamentous epiplakin staining pattern to one of complete colocalization of epiplakin with the keratin network, as observed after OA and OV treatment of keratinocytes, is still unknown. Our data suggest that hyperphosphorylation of keratins triggers the relocalization, because in resting keratinocytes, only part of the epiplakin pool showed association with keratin filaments, whereas all of it was recruited to the keratin network and developing aggregates upon stress exposure. Interestingly, in contrast to primary keratinocytes, the epiplakin staining pattern in primary hepatocytes was completely filamentous, showing a full overlap with the K8/18 intermediate filament network. Epiplakin was also found to colocalize with K8/18 in the human colon carcinoma cell line HT29 (our unpublished results) and in HeLa cells (Jang et al., 2005). Furthermore, the distribution of epiplakin in liver matches that of keratins (Spazierer et al., 2003; Spazierer et al., 2006). Therefore, the question arises, whether the subcellular localization of epiplakin is dependent on the type of cell or on the type of keratin involved. As epiplakin has been shown to colocalize with K5/K14 filaments in HaCaT cells almost perfectly (Jang et al., 2005), it seems likely that the degree of colocalization of epiplakin with keratin filaments is dependent on the cell type.

Unlike other spectraplakins, such as plectin and envoplakin (Wiche et al., 1993; DiColandrea et al., 2000), overexpression of



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