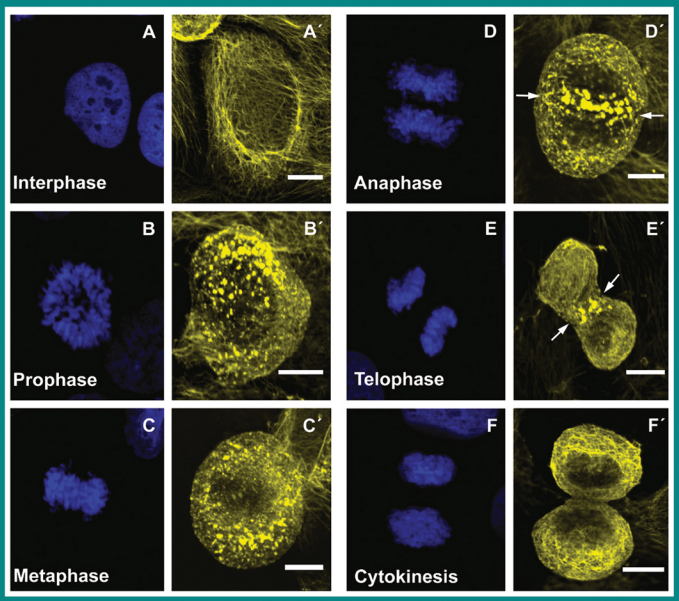
INTERNATIONAL REVIEW OF CELL AND MOLECULAR BIOLOGY

Edited by
Lorenzo Galluzzi



Volume 330

CHAPTER FIVE

Consequences of Keratin Phosphorylation for Cytoskeletal Organization and Epithelial Functions

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International Review of Cell and Molecular Biology, Volume 330
ISSN 1937-6448
http://dx.doi.org/10.1016/bs.ircmb.2016.09.005

Abstract

Intermediate filaments are major phosphoproteins. The complex patterns of intermediate filament phosphorylation make up a poorly understood code reflecting cytoskeletal properties and cellular function through an intense crosstalk with multiple signaling pathways. This review focuses on the epithelial keratin intermediate filaments highlighting the tight-knit relationship of keratin phosphorylation and network organization during cell division and apoptosis, and the importance of keratin phosphorylation during epithelial stress responses. The occurrence of keratin phosphorylation in genetic skin diseases and acquired diseases of simple epithelial tissues in liver, pancreas, and colon will be discussed. Finally, we will review the role of keratin phosphorylation in cancer with an emphasis on migration.

1. INTRODUCTION

Intermediate filament proteins are abundant cellular phosphoproteins. Individual polypeptides carry multiple phosphorylation sites that are regulated by numerous kinase and phosphatase activities resulting in complex context-dependent phosphorylation patterns that reflect cellular differentiation and function (Binukumar et al., 2013; Omary and Ku, 2006; Sihag et al., 2007; Snider and Omary, 2014). Clarifying how the interplay of different phosphorylation sites affects the structure and function of intermediate filaments is a challenging task because properties of the intermediate filament cytoskeleton may be altered only incrementally in certain situations and different phosphorylation sites may have opposing effects. To further complicate matters, the effects of phosphorylation may occur locally or globally and may manifest rapidly or only after some lag period.

Given the complexity of intermediate filament phosphorylation, we limit the review to the epithelial keratin intermediate filaments. Keratins are among the most abundant cellular proteins in epithelial cells and therefore provide a large buffer reservoir to cope with physical, chemical, and microbial stresses imposed by the hostile exterior environment (Pan et al., 2013; Pekny and Lane, 2007; Toivola et al., 2010, 2015). Keratins are obligatory heteropolymers that are held together by hydrophobic interactions through their α-helical rod domain forming stable coiled-coils (Herrmann et al., 2003, 2009). Filamentous keratin is composed of

equimolar amounts of types I and II keratin polypeptides, which are encoded by 28 keratin type I (KRT9, 10, 12–20, 23–28, 31, 32, 33a, 33b, 34–40) and 26 keratin type II genes (KRT1–5, 6a–c, 7, 8, 71–86) (www.interfil.org). Since the expression of specific keratin pairs is a reliable indicator of epithelial differentiation and function, detection of keratins has found wide application in tumor and histodiagnosis (Bragulla and Homberger, 2009; Moll et al., 2008). Our understanding of the contribution of individual keratins to particular epithelial properties, however, is still limited. Yet, it is generally accepted that these functions involve posttranslational modifications including keratin phosphorylation (Kim et al., 2015; Omary et al., 2006; Snider and Omary, 2014). The precise details remain to be elucidated.

We first review current knowledge on the modes of keratin phosphorylation and its consequences for the structure and function of the keratin cytoskeleton. We especially emphasize the importance of keratin phosphorylation during cellular stress response. We then summarize the role of keratin phosphorylation in diseases that are caused by toxins, gene mutations, and malignant transformation. All of this is based on our firm belief that the broad range of keratin phosphorylation and its multiple modes of regulation are ideally suited to modulate cytoskeletal plasticity in the context of complex cellular functions and thereby contribute to epithelial physiology and pathology.

2. CHARACTERISTICS OF KERATIN PHOSPHORYLATION

The complex patterns of keratin phosphorylation can be considered as a code, which not only reflects the specific status of cellular differentiation and function in a given context but also determines structural and functional properties of the keratin cytoskeleton itself. To understand the basis of these properties, it is important to elucidate the underlying rules governing keratin phosphorylation, some of which will be described in the following paragraphs.

2.1 Keratin Phosphorylation Is Complex, Fast, and Linked to Other Posttranslational Modifications

The difficulty in understanding and characterizing the consequences of keratin phosphorylation is that it involves multiple sites, which are recognized by different kinases (Table 1). Some of these sites may even be targeted by multiple kinases depending on the cell type and its functional state (see also Steinert, 1988). These activities are counteracted by phosphatases with a

Table 1 Different Kinases Known to Phosphorylate Keratins

Kinase	Keratin/Residue and Tissue/Cell Type	References
cAMP-dependent protein kinase	49–69 kD keratins from calf snout epidermis	Ikai and McGuire (1983)
	K8/K18 from rat liver	Yano et al. (1991) and Velasco et al. (1998)
	60 kD keratin from guinea pig epidermis	Inohara and Sagami (1983)
	K8-S12/S23/S36/S50 (major) K8-S8/S33/S42/S416/S423/S425 (minor) from rat liver	Ando et al. (1996)
Ca^{2+}/calmodulin-dependent protein kinase	K8/K18 from rat liver	Yano et al. (1991)
Cdc2 kinase	K18-S33 in HT29[a] cells	Ku et al. (1998a)
ERK1/2	K8-S431 in Panc-1 cells[b] and AGS cells[c]	Ku and Omary (1997)
Herpes simplex virus2-US3 kinase	K17 in A431 cells[d]	Murata et al. (2002)
JNK	K8-S431 in Panc-1[b] cells	Park et al. (2011)
	K8-S73 in HT29[a] cells	He et al. (2002) and Ku et al. (2002a)
MAPK-activated protein kinase (MK2)	K18-S52 in HT29[a] cells	Menon et al. (2010)
	K20-S13 in HT29[a] cells	
p38 kinase	K8-S73 in HT29[a] cells and A431[d] cells	Ku et al. (2002a), Menon et al. (2010), and Woll et al. (2007)
p42 kinase	K8-S73 in HT29 cells[a]	Ku et al. (2002a)
p90 ribosomal protein S6 kinase 1	K17-S44 in murine keratinocytes and HeLa cells[e]	Pan et al. (2011)
PKC	K20-S13 in HT29 cells[a]	Zhou et al. (2006)

Table 1 Different Kinases Known to Phosphorylate Keratins—cont'd

Kinase	Keratin/Residue and Tissue/Cell Type	References
PKCδ	K8-S73 in A549[f] and rat alveolar epithelial cells	Ridge et al. (2005)
PKCε	K8/K18 in HT29 cells[a]	Omary et al. (1992)
	K8-S8/S23 in GH_4C_1 cells[g]	Akita et al. (2007)
	K18-S52 (recombinant peptide)	Tao et al. (2006a)
PKCζ	K18-S33 in A549 cells[f]	Sivaramakrishnan et al. (2009)
Raf kinase	K18-S52 (major)	Ku et al. (2004)
	K18-S33 (minor) in BHK cells[h]	
Src	K19-Y391 in HT29[a] and NIH3T3 cells[i]	Zhou et al. (2010)

[a]Human colorectal adenocarcinoma-derived cell line.
[b]Human pancreatic carcinoma-derived cell line.
[c]Human gastric cancer-derived cell line.
[d]Human vulva squamous cell carcinoma-derived cell line.
[e]Human cervix adenocarcinoma-derived cell line.
[f]Human lung adenocarcinoma-derived cell line.
[g]Rat pituitary tumor-derived cell line.
[h]Syrian golden hamster kidney fibroblast-derived cell line.
[i]Murine embryonic fibroblast-derived cell line.

different spectrum of recognition sites and cell-type specificity (Toivola et al., 1997). Another layer of complexity is added by the different kinetics of the various enzymes. With this highly specialized and adjustable toolbox, slow and fast alterations can be accomplished in defined subcellular locations and in different functional contexts. This is also evident from the variable effects of a large number of reagents on keratin phosphorylation (Table 2).

The remarkable speed and efficiency of phosphorylation-dependent processes are exemplified in the observation that disassembly of the keratin cytoskeleton could be induced within less than a minute by an overall inhibition of tyrosine phosphatase activity (Strnad et al., 2002). Even more, short exposure to normal room light sufficed to efficiently prevent keratin network disassembly in this situation (Strnad et al., 2003; Woll et al., 2007).

The linkage of phosphorylation to other modifications such as acetylation, glycosylation, ubiquitination, and sumoylation adds another degree of complexity. For example, K8 phosphorylation regulates K8 transamidation

Table 2 Different Reagents Known to Induce Keratin Phosphorylation

Reagent	Keratin/Residue and Tissue/Cell Type	References
Acetaminophen	K8-S73 and K8-S431 in liver of K8-G62C and K8-R431C transgenic mice	Guldiken et al. (2015)
Acetone extract of *Bupleurum scorzonerifolium*	K8-S73 from A549[a] cells	Chen et al. (2005)
Acrylamide	PtK1 cells[b]	Eckert (1985) and Eckert and Yeagle (1980)
AICAR (5-aminoimidazole-4-carboxamide ribonucleoside)	K8/K18 in rat hepatocytes	Velasco et al. (1998)
Calyculin A	Keratins from rat parotid acinar cells	Takuma et al. (1993)
cAMP	Types I and II keratins in canine thyroid epithelial cells	Deery (1993)
EGF	K8-S431 in HT29 cells[c]	Ku and Omary (1997)
	K8-S73 in A431 cells[d]	Moch et al. (2013)
Estradiol-17 beta	Keratins in rat vaginal epithelium	Gupta et al. (1990)
Ethanol	K8/K18 from rat liver	Sanhai et al. (1999)
	MH_1C_1 cells[e]	Negron and Eckert (2000)
Forskolin	K8/K18 in CaCo-2 cells[c]	Baricault et al. (1994)
Griseofulvin	K8-S79/S436 and K18-S33 in Mallory–Denk bodies of murine liver	Fortier et al. (2010)
Pervanadate	K8/K19 tyrosine in HT29 cells[c] and normal mouse colon epithelium K8-S73 from HT29 cells[c]	Feng et al. (1999)
	K19-Y391 in colon epithelium of human K19-overexpressing mice	Zhou et al. (2010)

Table 2 Different Reagents Known to Induce Keratin Phosphorylation—cont'd

Reagent	Keratin/Residue and Tissue/Cell Type	References
	K19-Y394 in colon epithelium of nontransgenic mice K19-Y391 in HT29[c] and BHK-21 cells[f]	
	K8-S73 in A431 cells[d]	Woll et al. (2007)
Phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA)	K8/K18 in HT29 cells[c]	Chou and Omary (1991)
	K8-S431 in Panc-1 cells[b]	Lee et al. (2014)
Prostaglandins	K20-S13 in HT29-MTX cells[g]	Menon et al. (2010)
Sorbitol, H_2O_2, UV light, phorbol ester TPA	K17-S44 in murine keratinocytes, basaloid skin tumors and HeLa cells[h]	Pan et al. (2011)
Sorbitol, heat, urea	K8-S73 in A431 cells[d]	Woll et al. (2007)
Sphingosylphosphorylcholine	K8-S73 and K8-S431 in Panc-1 cells[i]	Park et al. (2011)
Thioacetamide	K8-S431 and K18-S33 in mice liver	Strnad et al. (2008)
Thyrotropin releasing hormone	K8-S8/S23 in GH_4C_1 cells[j]	Akita et al. (2007)

[a]Human lung adenocarcinoma-derived cell line.
[b]Rat kangaroo kidney epithelium.
[c]Human colorectal adenocarcinoma-derived cell line.
[d]Human vulva squamous cell carcinoma-derived cell line.
[e]Rat hepatoma-derived cell line.
[f]Syrian golden hamster kidney fibroblast-derived cell line.
[g]Goblet cells differentiated from HT29 cell line using methotrexate.
[h]Human cervix adenocarcinoma-derived cell line.
[i]Human pancreatic carcinoma-derived cell line.
[j]Rat pituitary tumor-derived cell line.

(Kwan et al., 2012), favors K8 sumoylation (Ku et al., 2010), coincides with changes in K8/K18 glycosylation (Budnar et al., 2010), and also has a counter-regulatory relationship with the neighboring glycosylation residues (Tao et al., 2006a). Conversely, K8 acetylation modulates K8 phosphorylation (Snider et al., 2013).

2.2 Phosphorylation Occurs Preferentially in the Keratin End Domains

Keratin polypeptides share basic structural features with all other cytoplasmic intermediate filament polypeptides: A conserved α-helical rod domain of ~310 amino acids is flanked by highly variable end domains (Steinert et al., 1983). The rod domain is involved in heterodimerization of a type I and a type II keratin polypeptide forming the very stable parallel coiled-coil structure through strong hydrophobic interactions (Coulombe and Fuchs, 1990; Hatzfeld and Weber, 1990). The vast majority of phosphorylation sites have been identified in the end domains which may be most accessible to kinases and phosphatases in the cellular environment (Steinert et al., 1982; Steinert, 1978, 1988). As an example, the distribution of phosphorylation sites is depicted for K8 in Fig. 1.

At the structural level, NMR studies revealed a conformational change in bovine hoof keratin that was phosphorylated by cAMP-dependent protein kinase (Yeagle et al., 1990). An increase in the rigidity of the otherwise flexible keratin head and tail domains may be responsible for this effect. Furthermore, phosphorylation-induced increased conformational motility as observed in the helical rod domains of ethanol-fed rat liver keratins as determined by circular dichroism and NMR analyses (Sanhai et al., 1999).

The end domains of the type II keratins contain the highly conserved subdomain H1 in the head region and the conserved subdomain H2 in

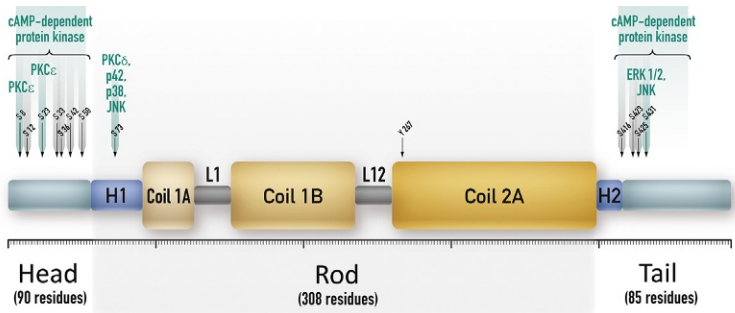

Fig. 1 Scheme depicting identified phosphorylation sites in the human type II keratin K8. Kinases that have been shown to target the respective sites are provided alongside. The scheme highlights the domain structure of K8 consisting of (i) the 90 amino acid-long head domain encompassing the H1 subdomain, (ii) the 308 amino acid-long central rod domain that is subdivided into coils 1A, 1B, and 2 with connecting linkers L1 and L12, and (iii) the 85 amino acid-long tail domain including the H2 subdomain. Note that most phosphorylation sites are located in the head and tail domains.

the tail region (Fig. 1; Steinert et al., 1985). K5 and K8 mutants lacking the H1 subdomain showed severe filament assembly defects (Hatzfeld and Burba, 1994; Wilson et al., 1992). Not only the protein sequence of the H1 domain but also its secondary conformation consisting of turn, β-strand, Ω-loop, and α-helical segments is highly conserved (Chipev et al., 1992). Thus, phosphorylation within the H1 subdomain may disrupt its structural arrangement. Furthermore, surface lattice models show that the H1 subdomain is critically involved in the alignment of neighboring molecules in keratin filament assembly (Parry and Steinert, 1992; Steinert, 1991a,b).

2.3 Phosphorylation Increases Keratin Solubility

Since keratins assemble rapidly and efficiently into higher order structures in vitro, mechanisms must exist to disassemble keratin filaments in vivo during keratin network remodeling. A prime mechanism appears to be phosphorylation. Phosphorylation is expected to prevent assembly of tetramers, which are the major soluble keratin species in cultured cells (Chou et al., 1993). They are composed of two keratin heterodimers that align in an antiparallel and partially staggered fashion and are held together through ionic interactions (Coulombe and Fuchs, 1990; Herrmann et al., 2003, 2009). Phosphorylation presumably prevents the lateral alignment of the nonpolar tetramers into the ~60 nm unit length filament (ULF) (Herrmann et al., 2009). In addition, phosphorylation may also interfere with the next assembly stage, i.e., the longitudinal annealing of ULFs. It is assumed that both assembly stages are regulated by the head and tail domains (Hatzfeld and Burba, 1994; Wilson et al., 1992), although a precise molecular understanding is still lacking. Besides involving keratin modification, it may also rely on accessory factors that are recruited or released.

It has been known for a long time that the equilibrium between the soluble and filamentous keratin pool is determined by phosphorylation (Feng et al., 1999; Strnad et al., 2002; Woll et al., 2007; Zhou et al., 1999). It is assumed that phosphorylation induces release of soluble tetrameric subunits from keratin filaments. An extreme example was observed in Xenopus oocyte maturation, where phosphorylation of type II keratins led to disassembly of cortical keratin filaments and the generation of keratin oligomers (Klymkowsky et al., 1991). However, another possibility is that phosphorylation preferably targets depolymerized keratin subunits which form the soluble pool of keratins (He et al., 2002). The adjustability in the equilibrium between the soluble and the filamentous state of keratins

plays an important role in maintaining cytoskeletal pliability. It is rapid, reversible, and can be temporally and spatially regulated. The phosphorylation-dependent dynamic properties of the keratin cytoskeleton may thus contribute to cellular plasticity.

2.4 Common Guidelines Determine Keratin Phosphorylation

Common principles determine keratin phosphorylation. They act at different levels and are influenced by multiple factors:

- Phosphate is mainly incorporated in serine residues of the nonhelical end domains of the keratin molecule (Fig. 1; Gilmartin et al., 1980; Ikai and McGuire, 1983; Steinert, 1988; Sun and Green, 1978).
- Increased keratin phosphorylation is the result of more keratin molecules being phosphorylated (Steinert, 1988).
- Phosphorylated keratin species are heterogeneous consisting of a mixture of molecules with single site and multiple site phosphorylations (Liao and Omary, 1996).
- Individual phosphorylation sites can be phosphorylated by more than one kinase (Fig. 1; Steinert, 1988).
- Each kinase phosphorylates keratins in a specific manner. For example, cAMP-dependent protein kinases phosphorylate K8/K18 exclusively at serine residues, whereas Ca^{2+}/calmodulin-dependent protein kinase II targets serine as well as threonine residues (Yano et al., 1991).
- Phosphorylation of type II keratins is preferred over phosphorylation of type I keratins (Liao et al., 1995a; Yano et al., 1991). For example, on average four phosphate molecules are incorporated into K8 and two phosphate molecules are incorporated into K18 by cAMP-dependent protein kinase whereas 1 and 0.8 phosphate molecules are incorporated into K8 and K18, respectively, by Ca^{2+}/calmodulin-dependent protein kinase II (Yano et al., 1991). Also, the stoichiometry of phosphorylation is more for K8 than K18 as determined by pulse-chase experiments using radiolabeled phosphate (Liao et al., 1995a).
- Although phosphorylation of type I and type II keratins often increases in similar proportion (e.g., phosphorylation of K8/K18 in heat stress or G2/M arrest), it is not directly linked to each other (Chou and Omary, 1993; Liao et al., 1995a).
- Keratin phosphorylation is highly dynamic. For example, the increase of K18-S52 phosphorylation by a factor of three in S-phase and four in the G2/M-phase, occurs in a reversible fashion (Liao et al., 1995a).

3. REGULATION OF PROTEIN BINDING TO KERATINS BY PHOSPHORYLATION

Intermediate filament-associated proteins regulate the state of keratin assembly in multiple ways. Thus, they sequester nonfilamentous keratin in the soluble cytoplasmic pool, they increase filament bundling and aggregation, they affect transport along microtubules and they cross-link keratin filaments to the other filament systems and to cell junctions (Listwan and Rothnagel, 2004; Snider and Omary, 2014; Sonnenberg and Liem, 2007). In addition, keratins bind to signaling molecules. The following sections present examples which support the notion that the interactions between keratins and these partners are regulated by phosphorylation.

3.1 Phosphorylation-Dependent Keratin-14-3-3 Protein Association Enhances Keratin Solubility and Affects mTOR Signaling

Among the proteins associating with keratins in a phosphorylation-dependent manner 14-3-3 proteins have received most attention. They are a ubiquitous family of proteins that play an important role in signal transduction and cell-cycle progression (Aitken et al., 1995; Burbelo and Hall, 1995; Gardino and Yaffe, 2011). 14-3-3 proteins associate preferentially with the hyperphosphorylated cytosolic but not with the less phosphorylated cytoskeletal K8/K18 fraction during the S/G2/M phase of the cell cycle (Liao et al., 1996). It was therefore suggested that 14-3-3 proteins act as solubility cofactors for keratins. It was further shown that 14-3-3 proteins bind to the phosphorylated K18-S33 residue and that the K18-S33–14-3-3 interaction contributes to normal mitotic progression (Liao et al., 1996).

A complex of solubilized K5/K17/actin was recently shown to be stabilized by 14-3-3σ, enhancing its "bioavailibility" and contributing to polarized assembly during migration, thus, making it highly relevant for breast tumor invasion (Boudreau et al., 2013). Complex formation was shown to be promoted by PKCζ, known to phosphorylate K18 at a 14-3-3 binding site. It was antagonized by phosphatase PP2A, which has been linked to keratin dephosphorylation (Park et al., 2011; Tao et al., 2006b). Hence, keratin phosphorylation likely contributes to regulating the switch between the filamentous and soluble keratin pool.

In case of K17, whose expression is upregulated in stratified epithelia upon wounding, phosphorylation of K17-T9/S44 is essential for its interaction with 14-3-3σ (Kim et al., 2006). The K17–14-3-3σ interaction ensures the cytosolic retention of 14-3-3σ, which concomitantly stimulates Akt/mTOR activity to support proper cell growth and size. Thus, keratin phosphorylation-regulated 14-3-3 association may affect tissue repair and homeostasis in skin development (Kim et al., 2006).

3.2 Sequestration of Raf Kinase Is Regulated by Keratin Phosphorylation

K8 directly associates with Raf kinase, sequestering it during basal conditions to the K8/K18 complex (Ku et al., 2004). During oxidative or chemical stress, however, phosphorylation of K18-S33 and of Raf S338 and S621 occured which led to the release of Raf from the keratin-Raf complex and induced Raf-kinase activation. Moreover, the K18-S52 residue served as a major physiologic substrate of the activated Raf-kinase. The released and activated Raf kinase bound preferentially to 14-3-3 instead of K8 (Ku et al., 2004). This example illustrates how keratin phosphorylation modulates kinase function by sequestration and activation and how the released and activated kinase reciprocally affects keratin phosphorylation.

3.3 Association of Cytolinkers With Keratin Is Influenced by Phosphorylation

It was observed that keratin aggregates associate with different plakin protein family members upon phosphatase inhibition. This became evident in studies demonstrating that K13 granules, which were formed by treatment with the tyrosine phosphatase inhibitor orthovanadate in A431 cells, contained the large plakin domain-containing cytolinker plectin (Strnad et al., 2002). In contrast, treatment of A431 cells with the threonine–serine phosphatase inhibitor okadaic acid resulted in keratin granules that were devoid of plectin immunoreactivity. Interestingly, the plectin-positive orthovanadate-induced keratin granules are reversible giving rise to a novel keratin filament network, whereas okadaic acid-induced keratin granules lack this capacity (Strnad et al., 2002). This suggests that plectin may serve as an anchoring or chaperone-like remodeling factor for the keratin network.

An association of K8 granules with periplakin, another plakin domain-containing cytolinker, was observed in the scratch-wound edges of MCF-7 monolayers that were treated with okadaic acid (Long et al., 2006). It was further demonstrated that the periplakin–K8 interaction was involved in

efficient collective epithelial sheet migration which went along with K8 reorganization and hyperphosphorylation of K8-S431 (Long et al., 2006).

In addition, keratin granules induced by either orthovanadate or okadaic acid in murine keratinocytes contain the cytolinker epiplakin (Spazierer et al., 2008). Remarkably, epiplakin only partially colocalized with the keratin filament network in untreated primary mouse keratinocytes but showed a near perfect colocalization after keratin hyperphosphorylation and in stressful conditions such as UV irradiation and osmotic shock, both of which were also linked to increased keratin phosphorylation (Long et al., 2006; Spazierer et al., 2008).

Taken together, phosphorylation of keratins appears to regulate their interaction with the different cytolinker proteins.

3.4 Phosphorylation-Dependent Association of the Ubiquitin Ligase Pirh2 With Keratin Affects Cell Survival

Phosphorylation-mediated granular aggregate formation of the keratin filament network is known to be a consequence of an altered association of keratin with Pirh2, a p53-induced RING-H2 type-ubiquitin E3 ligase. This was demonstrated in the human lung cancer cell lines H1299 and A549 (Duan et al., 2009). JNK- and p38-mediated phosphorylation of the K8/K18 filamentous network in response to UV irradiation resulted in dissociation of the Pirh2-K8/K18 complex and led to keratin aggregation accompanied by microtubule-dependent mitochondrial redistribution. This resulted in an increased sensitivity to apoptosis (Duan et al., 2009).

4. ROLE OF KERATIN PHOSPHORYLATION IN CELL PHYSIOLOGY

Stress–response, mitosis, and apoptosis are major events regulating epithelial cell fate. Each of these events involves climactic situations threatening cell and tissue homeostasis. Phosphorylation-mediated keratin reorganization is common to all these processes and serves diverse purposes in each of these situations as detailed below.

4.1 Mitosis Is Linked to Keratin Phosphorylation and Keratin Network Remodeling

Mitosis involves substantial rearrangements of all major structural elements leading to a reversible rounding of the cell. Given the role of phosphorylation in modifying the arrangement of keratin filaments, an elevated level of

keratin phosphorylation is to be expected during mitosis. This notion was fully supported by experimental observations that are summarized in Table 3 and exemplified in Figs. 2 and 3. Specifically, of the type II keratins K4, K5, K6, and K8 are phosphorylated by mitogen-regulated kinases in proliferating epithelial cells (Table 3) at the highly conserved LLS/TPL motif in the H1 head domain suggesting an evolutionary conserved function of keratin phosphorylation in mitosis (Liao et al., 1997; Toivola et al., 2002). The higher level of phosphorylated keratins in the soluble fraction as compared to the insoluble fraction during mitosis further indicated a role of phosphorylation in shifting the solubility equilibrium of the keratin pool (Baribault et al., 1989; Celis et al., 1983; Chou and Omary, 1993; Fey et al., 1983; Lane et al., 1982). Additionally, keratin filament reorganization into granules is often observed during mitosis (Figs. 2 and 3; Aubin et al., 1980; Baribault et al., 1989; Fey et al., 1983; Horwitz et al., 1981; Jones

Table 3 Keratin/Keratin Residues Known to Undergo Phosphorylation During Mitosis

Keratin/Keratin Residue	Source	References
K55/K49	Rat hepatocytes	Baribault et al. (1989)
K8S431	HT29 cells[a]	Liao et al. (1997)
	BHK cells[b]	Ku and Omary (1997)
K18S52	HT29 cells[a]	Liao et al. (1995a)
K18S33	HT29 cells[a]	Ku et al. (1998b) and Ku et al. (2002b)
K4T133[c]	Human esophageal epithelium	Toivola et al. (2002)
K5T150[c]	KC cells[d]	
K6T145[c]	Human epidermis	
K8S73[c]	HT29 cells[a]	Liao et al. (1997)
	Murine mitotic basal crypt cells of the intestine	Toivola et al. (2002)
	Murine regenerating hepatocytes	
	A431 cells[e]	Woll et al. (2007)

[a]Human colorectal adenocarcinoma-derived cell line.
[b]Syrian golden hamster kidney fibroblast-derived cell line.
[c]These residues are located in the conserved LLS/TPL motif of the type II keratins.
[d]Human foreskin primary keratinocytes.
[e]Human vulva squamous cell carcinoma-derived cell line.

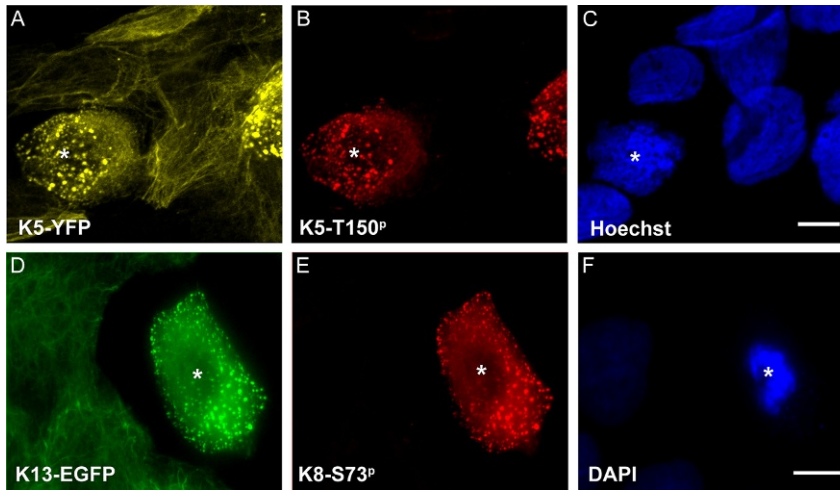

Fig. 2 Detection of keratin phosphorylation in mitosis. (A–C) show fluorescence micrographs of human keratin 5-enhanced yellow fluorescent protein chimeras K5-YFP in methanol-acetone fixed human epidermal keratinocyte-derived HaCaT B10 cells (Moch et al., 2013) and a corresponding indirect immunofluorescence micrograph using antibody LJ4 (Toivola et al., 2002) detecting phosphorylated T150 of keratin 5 together with the corresponding nuclear Hoechst staining. (D–F) The fluorescence micrographs depict the distribution of human keratin 13 chimera HK13-enhanced green fluorescent protein (K13-EGFP) in methanol-acetone fixed vulva carcinoma-derived AK13-1 cells (Windoffer and Leube, 1999) and the corresponding indirect immunofluorescence of antibody LJ4 reacting with phosphorylated S73 of K8 (Liao et al., 1997) together with the corresponding nuclear DAPI staining. Note the appearance of the respective keratin phosphoepitopes in granules that are detected in the mitotic cells (labeled by *) of both cell lines. The images D, E and F were kindly provided by Dr. Stefan Wöll. Scale bars = 10 μm.

et al., 1985; Lane et al., 1982; Schwarz et al., 2015; Windoffer and Leube, 2001). Mitotic human vulva carcinoma A431 and cervix carcinoma HeLa present both a diffuse and granular cytoplasmic keratin pattern, whereas the keratin network of rat kangaroo kidney-derived PtK2 cells does not disassemble into granules but is only redistributed (Horwitz et al., 1981). Another report showed that the percentage of transformed human epithelial amnion cells containing mitotic keratin granules increased from prophase onwards, reaching a peak during late anaphase/early telophase and plummeting during late telophase. In contrast, normal mitotic amnion cells did not show this characteristic keratin reorganization during mitosis, in spite of similar keratin phosphorylation levels as the transformed cells (Celis et al., 1983). This suggests that additional factors such as mitosis duration

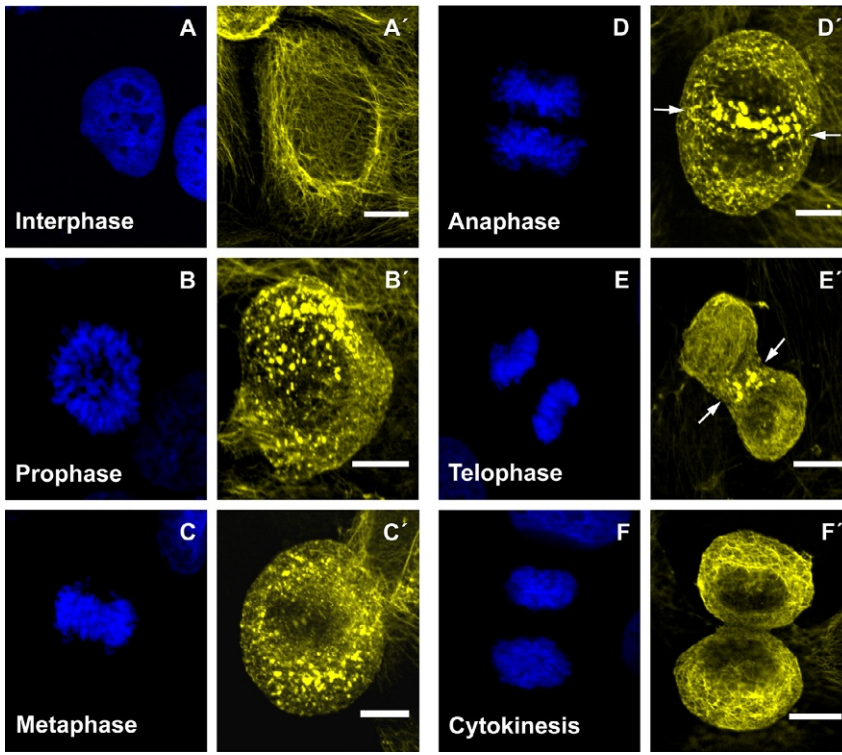

Fig. 3 Keratin filament network disassembly and reassembly during mitosis. The images in (A–F) show Hoechst stains and corresponding fluorescence of human keratin 5-enhanced yellow fluorescent protein chimeras in methanol-acetone fixed keratinocyte-derived cell line HaCaT B10 (Moch et al., 2013) during different phases of mitosis (A′–F′). Note the reversible keratin granule formation that is induced at late prophase. Accumulation of keratin granules is typically seen in the cleavage furrow (arrows in D′ and E′). The structural reorganization is coupled to keratin phosphorylation (see Fig. 2). Scale bars = 10 μm.

or overall kinase activity levels influence keratin reorganization during mitosis. Moreover, preferential phosphorylation of K8 over K18 was observed during mitosis, in rat hepatocytes. However, in this case, phosphorylation of both K8 and K18 upon triton X-100 permeabilization suggested that keratin phosphorylation during mitosis may also depend on the accessibility of their phosphorylation sites (Baribault et al., 1989). Mitotic keratin restructuring may influence the kinetics and efficiency of cell division as has been suggested for vimentin phosphorylation (Ikawa et al., 2014). Moreover, in vivo monitoring of K8 restructuring during the trophoectodermal cell divisions in blastocysts of K8-YFP knockin mice provided direct proof

for the significance of mitotic keratin reorganization in the native tissue context (Schwarz et al., 2015).

Interestingly, A431 and HeLa cells show a concentration of keratin granules in the cleavage furrow during mitosis. Fig. 3D–E′ highlights the enrichment of keratin granules in this region of dividing immortalized human HaCaT keratinocytes. The cleavage furrow harbors several kinases such as Rho-kinase and Aurora B which phosphorylate and thus regulate intermediate filament reorganization, which is essential for efficient cytokinesis (Horwitz et al., 1981; Kawajiri et al., 2003; Kosako et al., 1999; Yasui et al., 1998). Furthermore, p38 mitogen-activated protein kinase, which plays an important role in cell proliferation, colocalizes with keratin granules during mitosis in A431 cells (Woll et al., 2007). This indicates the necessity of a kinase-enriched environment for keratin reorganization during mitosis. It is likely also relevant for other cell types that do not form keratin granules during mitosis. In these instances, the locally increased kinase activities could sever the keratin network at the cleavage furrow for distribution of filamentous keratin into both daughter cells. Moreover, keratins are substrates of mitotic kinases as shown by in vitro phosphorylation of K8-S431 through mitogen-activated protein kinase and cdc2 kinase (Ku and Omary, 1997).

The hyperphosphorylation of keratins during mitosis also modulates their binding to associated proteins. This is well demonstrated by the requirement of K18-S33 phosphorylation for an interaction between K18 and 14-3-3 family of proteins, facilitating normal mitotic progression (Liao et al., 1996). Although K18-S33A mice did not have defects in liver regeneration, they displayed abnormal mitotic arrest-related figures such as tripolar and angular mitotic bodies and anomalous proportions of mitotic stages. Furthermore, retention of 14-3-3-ζ in the nucleus of K18-S33A hepatocytes suggested a role of keratin phosphorylation in modulating 14-3-3 distribution, which is an important sequestering and compartmentalizing factor for multiple proteins (Ku et al., 1998a, 2002b; Liao et al., 1996). Thus, keratin phosphorylation during mitosis may not only contribute to cytoskeletal reordering but may also impact the function of keratin-associated proteins.

4.2 The Epithelial Stress Response Is Coupled to Altered Keratin Phosphorylation

Stress-activated protein kinase signaling is a hallmark feature of the cellular stress response. Common phosphorylation targets are proteins known for

their abundance and cytoprotective functions. Because of these characteristics, keratins have been classified as stress proteins (Toivola et al., 2010). Keratins buffer the impact of the activated kinase signaling by acting as a "phosphate sponge," and actively contribute to molecular defense mechanisms against diverse forms of stress as discussed in the following sections.

4.2.1 Chemical Stress and Keratin Phosphorylation Contribute to Mallory–Denk Body Formation

Alcohol, heavy metals, *N*,*N*′-dicyclohexylcarbodiimide (DDC), microcystin-LR (MC-LR) and griseofulvin are hepatotoxic. They induce the formation of Mallory–Denk bodies (MDBs) that are keratin-rich cytoplasmic granular inclusions in hepatocytes (Denk et al., 1979; Franke et al., 1979; Zatloukal et al., 2007). MDBs are also observed in several chronic liver diseases such as alcoholic and nonalcoholic steatohepatitis, chronic cholestasis, metabolic disorders, and hepatocellular neoplasms (Denk et al., 2000; Fausther et al., 2004; Fickert et al., 2003; Stumptner et al., 2000; Zatloukal et al., 2004). MDBs comprise misfolded hyperphosphorylated K8/K18 with phospho-serines at positions 73 and 431 in K8 and at position 33 in K18.

Several experimental observations provided evidence that keratin phosphorylation is tightly linked to MDB formation. Thus, treatment of primary cultured rat hepatocytes with the MDB-inducing hepatotoxin, MC-LR, led to a dose-dependent increase in K8/K18 phosphorylation (Guzman and Solter, 2002; Ohta et al., 1992). Similarly, the phosphatase inhibitor okadaic acid increased keratin phosphorylation and induced inclusion body formation in murine liver within 15 min. These inclusions contained keratin aggregates, which stained positive for phosphothreonine and ubiquitin as is the case for typical MDBs (Ohta et al., 1988; Yuan et al., 1998). This observation indicated that keratin hyperphosphorylation plays a role during the early phase of MDB formation. In support, p38MAPK, which phosphorylates K8-S73, was a prerequisite for MDB formation in DDC-fed mice (Nan et al., 2006). Furthermore, autophagy, which leads to clearance of inclusion bodies, was inversely related to K8-S73 phosphorylation (Harada et al., 2008; Kongara et al., 2010), thus, explaining the role of keratin phosphorylation not only in the initiation but also in the continued maintenance of MDBs. Prevalence of MDBs in hepatocyte injury is associated with better tolerance to toxic stress. In accordance, the lack of MDBs in K8 null mice led to increased sensitivity to DDC toxicity (Zatloukal et al., 2000). Furthermore, mice overexpressing the phosphodeficient K18-S52A

mutant were more susceptible to griseofulvin- and MC-LR-induced liver lesions (Ku et al., 1998b). Hence, keratin hyperphosphorylation is not only a consequence of hepatotoxicity but is part of a cellular defense mechanism during liver intoxication.

4.2.2 Chemical Stress and Phosphorylation Alter the Assembly State of Keratins

Another consequence of increased levels of hyperphosphorylated keratins in MDB-containing hepatocytes is an elevation in keratin solubility (Fortier et al., 2010). Although keratin filaments isolated from MDBs assembled in the same manner as normal hepatocellular keratins, the soluble pool of keratins from griseofulvin-treated mouse livers was polymerization incompetent. This was linked to a rise in the acidic isoelectric variants of keratins in the soluble pool, presumably due to increased phosphorylation (Pollanen et al., 1994; Salmhofer et al., 1994; Toivola et al., 1998).

A prominent feature of MDB persistence is transglutaminase-mediated covalent crosslinking of K8 (Strnad et al., 2007). The consensus recognition sequence for transglutaminases is QXXφDP (Sugimura et al., 2006). The preferred transamidation site in K8 is Q69, which lies in proximity to the phosphorylation site K8-S73 ($_{69}$QSLLSP$_{74}$). The phosphorylation of K8-S73 may mimic an aspartic residue and thereby creates a near-perfect transglutaminase recognition site. This is supported by the observed reduced K8 crosslinking in human colon carcinoma-derived HT29 cells expressing phosphodeficient K8-S73A when treated with the phosphatase inhibitor okadaic acid. Moreover, K8-S73A mice have a reduced ability to form MDBs (Kwan et al., 2012). Since K8 is a better substrate for transamidation than K18, phosphorylation-regulated K8 crosslinking may be an important mechanism to maintain the high K8–K18 ratio observed in Mallory bodies (Nakamichi et al., 2005). It is assumed that phosphorylation ensures the maintenance of the aberrantly folded keratins in the inactive state. Moreover, the association of phosphorylated K8-S79 (corresponding murine residue to human K8-S73) with activated p38MAPK suggested that keratin phosphorylation plays a role in sequestering excessive kinase activity (Fortier et al., 2010). Interestingly, p38MAPK activity has been directly implicated in keratin aggregate formation in cultured cells (Fig. 4; Woll et al., 2007).

It will be interesting to find out why keratin phosphorylation has two fundamentally different effects on keratin organization, namely increasing keratin solubility on one hand and increasing crosslinking, on the other hand, and how these opposing effects are regulated.

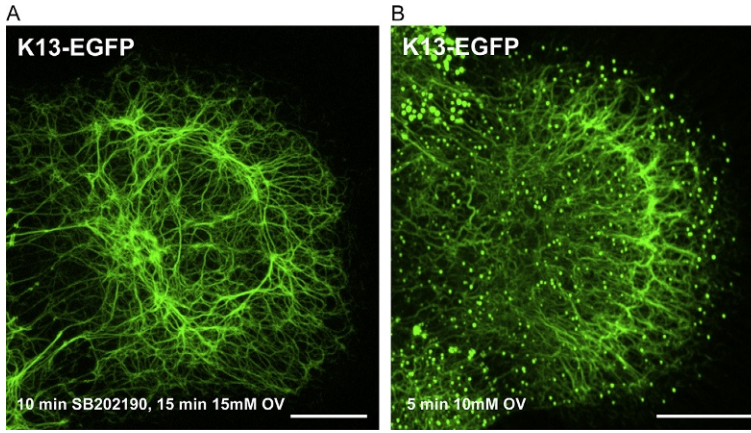

Fig. 4 Inhibition of p38 MAPK prevents vanadate-induced keratin granule formation. Confocal laser scanning microscopy of methanol/acetone-fixed AK13-1 cells producing fluorescent human keratin 13-enhanced green fluorescent protein chimera HK13-EGFP (Windoffer and Leube, 1999). Cells were treated with orthovanadate (OV) at 15 mM for 15 min after a preincubation with the p38 inhibitor SB202190 in (A) and at 10 mM for 5 min in the absence of SB202190 in (B). Note that the typical interphase morphology of the keratin network is preserved in (A) but not in (B) despite the lower vanadate concentration and shorter treatment period. The images were kindly provided by Dr. Stefan Wöll. Scale bars = 10 μm.

4.2.3 Heat Stress Leads to Recruitment of Chaperones to Phosphorylated Keratin Resulting in Keratin Aggregate Formation

Administration of heat stress to cell lines producing mutant keratins that are known to cause the skin blistering disease epidermolysis bullosa simplex (EBS) led to an increased stimulation of the stress-activated protein kinases p38, JNK1/2, and ERK activity (Chamcheu et al., 2011a). This heat-induced kinase overload is accompanied by increased K5 phosphoepitopes, which colocalise with HSP70 (Chamcheu et al., 2011a). The heat-shock response went along with an elevation in keratin aggregation, the extent of which depends on disease severity (Chamcheu et al., 2011a). Likewise, a heat stress of 42°C for 16 h in HT29 cells or of 60°C for 5 min in A431 cells induced the formation of hyperphosphorylated K8-S73 in granular aggregates (Liao et al., 1995b, 1997; Woll et al., 2007). This provided further evidence for the role of hyperphosphorylated keratins in cellular stress responses. Moreover, the hyperphosphorylated keratins associated with heat-shock proteins and up to 50% of the cells contained clumped keratin filaments (Liao et al., 1995b). But purified K8/18 proteins from heat-stressed cells were able to form normal filaments in vitro (Liao et al., 1995b)

indicating that cellular factors are responsible for the altered keratin organization in heat-shocked cells.

It was reported that HSP70 acts as a cochaperone for K8/K18 filaments along with HSP40 (Izawa et al., 2000). Moreover, HSP27 has the potential to modulate K8/K18 assembly dynamics (Kayser et al., 2013). In addition to their innate ability of supervising protein folding, the heat-shock proteins also have a potential role in regulating keratin (K5, K14, and K10) ubiquitination (Yamazaki et al., 2012) thereby affecting the half life of keratins during heat stress. This also appears to be relevant for the clearance of cytoplasmic aggregates containing EBS-mutant keratins (Loffek et al., 2010). Furthermore, the heat-induced architectural rearrangement of the keratin cytoskeleton also coincided with translational inhibition, polysome disruption, and organelle translocation in immortalized mouse mammary epithelial cells (Shyy et al., 1989) suggesting functions of keratins beyond providing a phosphate sink during stress.

4.2.4 Mechanical Stress Is Linked to Keratin Phosphorylation and Keratin Network Reorganization

Phosphorylation of keratins displayed a direct relationship with mechanical stress. Upsurge in K8-S73 and K18-S33 phosphorylation due to PKCδ and PKCζ activation, respectively, was shown to be an outcome of shear stress in human and rat alveolar epithelial cells (AECs) (Sivaramakrishnan et al., 2008). Moreover, the extent of phosphorylation in these residues showed a shear force-dependent increase. The resistance to shear stress increased from the perinuclear to the peripheral region in AECs as determined by storage modulus measurements using particle tracking microrheology. This correlated with decreasing keratin phosphorylation and increasing mesh size (Flitney et al., 2009; Sivaramakrishnan et al., 2008). Phosphorylation of keratins in shear stress resulted in increased bundling of the keratin network. Moreover, shear stress of 7.5–30 dynes/cm^2 for 0–24 h induced the formation of ubiquitin-positive aggregates in the perinuclear space of the AECs. Furthermore, an increase of the triton X-100 soluble keratins was observed. This was accompanied by phosphorylation of K8-S73 which served as a recognition factor for the ubiquitin-mediated keratin degradation that occurred under shear stress (Ridge et al., 2005; Sivaramakrishnan et al., 2009). Thus, keratin phosphorylation during shear stress may not only act as a keratin reorganizing factor, but also as a mechanism to prevent the subsequent accumulation of ubiquitinated aggregates (Johnston et al., 1998; Kopito, 2000; Zatloukal et al., 2007). Furthermore, K18-S33 phosphorylation was shown

to be required for association of K18 with 14-3-3ζ which led to keratin network rearrangement during shear stress. The reduced resistance of K18-S33A-containing AECs to shear force further confirmed the need for keratin phosphorylation in providing a resilient cytoskeletal defense against mechanical force (Flitney et al., 2009; Sivaramakrishnan et al., 2008, 2009).

A 10% increase in the cell surface area (CSA) by mechanical stretch was shown to lead to considerable straightening of the keratin filaments in primary cultures of pulmonary alveolar type II (AII) cells. This was accompanied by a widening of the extracellular gap of desmosomes (Felder et al., 2008). A 20% increase in CSA, however, restored keratin filament waviness and desmosomal gap width. This paradox was explained by increased keratin phosphorylation, notably of K8-S431, and the observed decrease in keratin bundles with concurrent increase in thinner filaments as determined by transmission electron microscopy. It remains to be shown whether this is a general keratin-dependent mechanoprotective mechanism.

4.2.5 Osmotic Stress Induces Kinase-Dependent Alterations of the Keratin Cytoskeleton

Similar to the various types of stress discussed so far, osmotic shock also induces hyperphosphorylation of keratins. As demonstrated in human colorectal carcinoma HRT18 and CaCo-2 cells, hyper- and hypoosmotic stress increased phosphorylation of K8-S73 and K8-S431 (Tao et al., 2006b). Furthermore, increased K8–73 phosphorylation was linked to formation of keratin aggregates that contain phosphorylated active p38 MAPK in osmotically challenged human vulva carcinoma A431 cells (Woll et al., 2007). A more complex situation was encountered in HT29 cells. While K8-S431 was hyperphosphorylated upon hyperosmosis, it was dephosphorylated in hypoosmosis. This may be explained by the association of the phosphorylated K8-S431 with protein phosphatase 2A (PP2A) which occurs uniquely under hypoosmosis in these cells (Tao et al., 2006b). Furthermore, 605 mM sorbitol induced K17-S44 phosphorylation in murine skin keratinocytes by p90 ribosomal S6 kinase1, which is downstream to the MAPK cascade (Pan et al., 2011).

4.2.6 Microbes Elicit Keratin Phosphorylation Leading to Keratin Network Reorganization

Formation of a kinase-rich environment is a common consequence of viral infection. Hepatitis C virus and coxsackievirus B4 infections in liver and pancreas, respectively, have been associated with K8/K18 hyperphosphorylation

(Toivola et al., 2004, 2009). Also, rotavirus infection in human colonic cell lines showed phosphorylation-mediated K8/K18 network alterations leading to a more hazy K8/K18 staining pattern and keratin network rearrangement in differentiating CaCo-2 cells (Brunet et al., 2000; Liao et al., 1995b). Besides, association of high viral titers with partially disrupted pancreatic keratin network was observed upon coxsackievirus B4 infection (Toivola et al., 2009). Similarly, Herpes simplex virus-2 US3 (HSV-2 US3) protein kinase expression resulted in a decrease in filamentous K17 along with an increase in K17 phosphorylation (Hertel, 2011; Murata et al., 2002). It still has to be sorted out in each case whether the phosphorylation-mediated keratin reorganization during viral infection is a cellular strategy to override the infectious attack or, contrariwise, a viral strategy to enhance pathogenicity.

The following reports portrayed another scenario of virus-induced keratin network reorganization. It was observed that human papilloma virus HPV-16 invasion led to a complete collapse of the keratin network in human HaCaT keratinocytes, in human SiHa cervical epithelial cells, and in low grade cervical neoplasia in vivo (Doorbar et al., 1991; McIntosh et al., 2010). The HPV16 E1–E4 proteins not only bind strongly to K18 and weakly to K8 through their YPLLKLL amino terminal peptide but also bind to each other through their carboxytermini (Wang et al., 2004). They therefore function as efficient keratin crosslinkers. Moreover, the filamentous pool of K18 in the HPV 16-infected SiHa cells showed a high degree of K18-S33 phosphorylation but was presumably unable to enter the soluble pool because of the E1–E4 crosslinking. In accordance, expression of HPV16 E1–E4 in SiHa cervical epithelial cells restricted K18 to the insoluble pool even in G2/M arrested and okadaic acid-treated cells both of which favor keratin solubilization and association of phosphorylated keratin with 14-3-3 protein (Wang et al., 2004). Similarly, it was reported for HaCaT cells that persistence of HPV16 E1–E4 protein induced a time-dependent increase of p38 MAPK and JNK activation as well as an elevation of keratin phosphorylation at K8-S73, K8-S431, K18-S33, and K18-S52 (McIntosh et al., 2010). Furthermore, time-lapse recordings of K13 in HaCaT cells showed a dramatic reduction in keratin dynamics in the presence of HPV16 E1–E4 (McIntosh et al., 2010).

Another situation of phosphorylation-dependent keratin restructuring has been described for the nerve parasite *Spraguea lophii* (Weidner and Halonen, 1993). Its outer envelope is stabilized by K4/K13. The keratin envelope undergoes phosphorylation and disassembly at the time of spore activation in a calmodulin- and calcium-dependent manner. When spore

activation was blocked, keratin phosphorylation and disassembly were also inhibited. Thus, keratin phosphorylation appears to enhance discharge of infective sporoplasm into the target cells.

4.3 Keratin Phosphorylation Protects Against Ubiquitin-Dependent Degradation and Reduces Sensitivity to Apoptosis

Keratin phosphorylation is an early event in apoptosis (Liao et al., 1997). This becomes evident in cells treated with the apoptosis-inducing drug anisomycin. Hyperphosphorylated K8-S73 was detected in A431 cells within 5 min of anisomycin treatment (Woll et al., 2007) and hyperphosphorylated K8-S73, K8-S431, and K18-S52 were identified in HT29 cells after 30 min of anisomycin addition (Ku et al., 1997). Interestingly, hyperphosphorylation of K8-S73 and K8-S431 was also observed in response to Fas- and TNF-induced apoptosis in mice (Ku et al., 2003). Furthermore, K20-S13 phosphorylation was noted during apoptosis as a result of chemically-induced mouse colitis (Ku et al., 1997; Zhou et al., 2006). Fig. 5 presents an example of spontaneously occurring apoptosis in cultured human HaCaT keratinocytes depicting the granule formation of a fluorescent keratin reporter. The newly formed keratin granules become positive for phosphorylated K5-T150.

The execution of apoptosis requires caspase-mediated cleavage of multiple cellular proteins. Keratins K14, K18, K19, and K20 harbor caspase target sites (VEV/MDA/S) in their rod domain. K18 contains the additional caspase cleavage sequence DALDS in its tail domain (Ku and Omary, 2001; Ku et al., 1997; Zhou et al., 2006). This explains why caspase activation leads to a collapse of the keratin filament network which is followed by the formation of large cytoplasmic inclusions. It was observed in TRAIL-induced apoptosis in the breast carcinoma MCF cell line that these cytoplamsic inclusions contain hyperphosphorylated keratins (K18-S52) together with caspase-cleaved keratin fragments and catalytic caspase subunits (MacFarlane et al., 2000). Although phosphorylation of keratins preceded caspase activation and phosphorylated keratins colocalized with caspase-containing apoptotic aggregates, keratin phosphorylation did not increase its propensity to be digested by caspases (Schutte et al., 2004). In agreement, induction of apoptosis in the absence of K8-S73 phosphorylation by simultaneous treatment with the apoptosis-inducing cyclin-dependent kinase inhibitor roscovitine and the broad-reactive protein kinase inhibitor staurosporine did not prevent the appearance of apoptotic inclusions

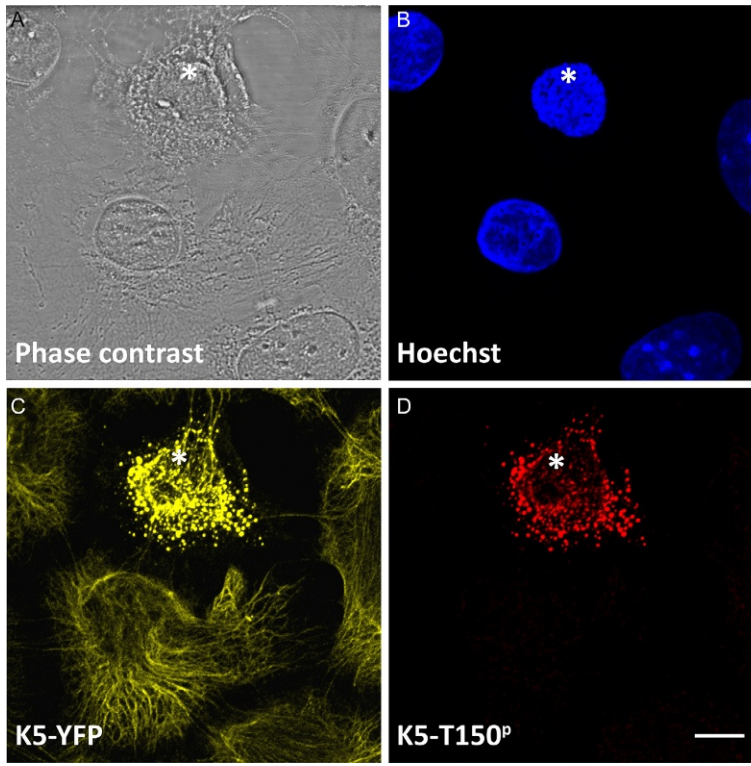

Fig. 5 Detection of keratin phosphorylation during apoptosis. (A–D) show corresponding phase contrast (A) and fluorescence images (B–D) of methanol-acetone fixed immortalized human epidermal keratinocyte-derived HaCaT B10 cells (Moch et al., 2013). (B) Depicts nuclear Hoechst staining, (C) the distribution of human keratin 5-enhanced fluorescent protein chimeras K5-YFP, and (D) the distribution of phosphorylated K5-T150 as detected by immunofluorescence using antibody LJ4 (Toivola et al., 2002). Note the altered morphology of the apoptotic cell marked by * along with nuclear condensation (B) and formation of keratin granules (C) that are enriched in keratin phosphoepitope K5-T150^{P} (D). Scale bar = 10 μm.

containing caspase-cleaved keratin fragments (Schutte et al., 2004). Yet, keratin phosphorylation may enhance the caspase-mediated disintegration of keratin filaments into aggregates.

Ubiquitination, which is closely related to apoptosis, is influenced by keratin phosphorylation. *N*-[*N*-(*N*-acetyl-L-leucyl)-L-leucyl]-L-norleucine (ALLN)-mediated proteasome inhibition in HT29 cells revealed the presence of low amounts of phosphorylated K8-S73, K8-S431, and K18-S52 in the ubiquitinated keratin fractions (Ku and Omary, 2000). The extremely low stoichiometric ratio of phosphorylated K8-S73/S431 to the total

ubiquitinated K8, however, indicated that keratin phosphorylation and ubiquitination are inversely related. This notion was supported by the observation that keratin degradation was increased in HT29 cells producing phosphodeficient K8-S73A mutants (Ku and Omary, 2000). K8-S73 is contained in the sequence motif $_{69}$QSLL**S**PL$_{75}$ that is conserved in type II keratins. Mutation of K8-P74 to K8-L74, which prevents phosphorylation of K8-S73 by the proline-directed kinase p38, elevated K8 ubiquitination. On the other hand, mutation of K8-L71 to K8-P71, which creates a new potential phosphorylation site at K8-S70, led to decreased K8 ubiquitination (Ku and Omary, 2000). Interestingly, the antiapoptotic protein P-element-induced wimpy testis (PIWI)-like 2-induced K8-S73 phosphorylation through p38MAPK and led to a decrease in ubiquitin-mediated K8 degradation (Jiang et al., 2014). Taken together, these observations demonstrated that keratin phosphorylation protects keratins from degradation during apoptosis. Furthermore, it was proposed that the maintenance of keratin levels reduces cellular sensitivity to Fas-mediated and TNF-induced apoptosis (Caulin et al., 2000; Gilbert et al., 2001; Lee et al., 2013).

5. DISEASE RELEVANCE OF KERATIN PHOSPHORYLATION

5.1 Keratin Phosphorylation Affects Keratin Network Organization and Function in Simple Epithelia

K8/K18 is the major keratin pair of the glandular epithelia in liver, exocrine pancreas, and intestine. Animal model systems expressing mutant versions of these simple epithelial keratins have been successful in defining the functional role of keratins in these organs (Ku et al., 1995, 2002b; Ku and Omary, 2006; Toivola et al., 2008). They also helped to elucidate aspects of keratin phosphorylation which will be described in the following sections. Besides, animal models of renal tubular epithelial cell injuries have also shown K8/K18 phosphorylation at K8-S73 and K18-S33 (Djudjaj et al., 2016).

5.1.1 Altered Keratin Phosphorylation Affects Keratin Network Organization and Stress Resilience in Hepatocytes

Induction of extensive liver damage by administration of serine/threonine phosphatase inhibitors serves as a paradigm for serious liver injury (Fujiki and Suganuma, 1993; Holmes and Boland, 1993). It has been demonstrated that the phosphatase inhibitor MC-LR increased hyperphosphorylation of

K8/K18 which resulted in perturbation of the keratin filament network in hepatocytes. It led to a time-dependent solubilization of keratin filaments. Furthermore, phosphopeptide maps of the phosphorylated keratins implicated Ca^{2+}/calmodulin-dependent kinase (Toivola et al., 1997). Similarly, K8-S73 and K8-S431 were hyperphosphorylated in acetaminophen-induced acute liver failure, which was accompanied by enhanced keratin solubility and perturbed keratin network formation (Guldiken et al., 2015).

K18-R89 is a highly conserved residue among type I keratins. Mutation of the corresponding residue in K14, K10, and K9 is associated with EBS, epidermolytic hyperkeratosis (EH), and epidermolytic palmoplantar keratoderma (EPPK), respectively (Fuchs, 1994; McLean and Lane, 1995). It is generally assumed that these mutations, which are located at the beginning of the helical rod domain within the helix initiation motif, directly interfere with proper keratin polymerization. Mutation of K18-R89 to K18-C89 alters the consensus sequence $_{89}$RXXS$_{92}$ for calmodulin-dependent protein kinase and protein kinase C to $_{89}$CXXS$_{92}$ thereby inactivating the potential phosphorylation site S92 (Pearson and Kemp, 1991). But so far there is no experimental evidence that K18-S92 is targeted by kinases (Ku et al., 1995). Yet, expression of K18-R89C led to altered phosphorylation at other sites of K18 and of other keratin polypeptides (Ku et al., 1995). Thus, transgenic mice expressing K18-R89C presented elevated phosphorylation of K18-S33 and K8-S73 with extensive alteration of the keratin filament network in hepatocytes (Ku et al., 1995). This was accompanied by hepatocyte fragility, inflammation, and necrosis whereas the pancreas showed no histopathological changes. Phosphoglycosylated K8/K18 was detected in the soluble fraction of the cultured cells and mouse tissues producing K18-R89C mutants but not in control cells and tissues expressing wild-type K8/K18. Moreover, K18-R89C mice exhibited a higher sensitivity to MC-LR-induced liver toxicity in comparison to mice overexpressing wild-type K18. The MC-LR treatment of K18-R89C mice was associated with a time-dependent increase of phosphorylated K18 in the soluble fraction. The keratin-solubilizing effect correlated with keratin phosphorylation and may play a major role in modifying the effects of K18-R89C by influencing its binding to associated proteins (Ku et al., 1995). Furthermore, hepatocytes of K18-R89C mice were more susceptible to Fas-mediated apoptosis than hepatocytes from mice overexpressing wild-type K18 (Ku et al., 2003). As compared to nontransgenic mice and mice overexpressing wild-type K18, the K18-R89C mice also showed increased susceptibility to thioacetamide-induced liver injury and fibrosis (Strnad et al., 2008).

5.1.2 The Pancreas Is Sensitive to Changes in Keratin Expression Levels Leading to Altered Keratin Phosphorylation

Similar to the situation in liver, cytoplasmic keratin filament reorganization was observed in the exocrine pancreas of K18-R89C mice. But no significant histopathological or functional alterations were detectable (Toivola et al., 2008). This may be due to the persistence of intact apicolateral keratin bundles in the pancreas of the K18-R89C mice in contrast to the network disruption in hepatocytes (Ku et al., 1995). Yet, pancreatic keratins also react to pharmacological stress by hyperphosphorylation as demonstrated in mice treated with caerulein, which induced pancreatic injury and increased phosphorylation of keratins 8 and 18 (Toivola et al., 2008).

Elevating K8 or K18 alone in the pancreas did not change the histology in comparison to the wild type. Yet, pancreatic K8 overexpression was accompanied by hyperphosphorylation of K18–S33, a 14-3-3 binding site that is crucial for mitotic progression (Liao and Omary, 1996). In addition, K8-overexpressing pancreas displayed elevation of K18, thus differing from pancreas overexpressing K18 which does not show any alterations in K8 levels (Liao and Omary, 1996). Furthermore, pancreatic overexpression of both K8 and K18 exhibited prominent pancreatic alterations with age-enhanced vacuolization and atrophy of the exocrine pancreas. In addition to phosphorylation of K18-S33 increased phosphorylation of K8-S73 was also noted in these mice (Liao and Omary, 1996). As described above, K8-S73 is phosphorylated in stress, mitosis, and apoptosis (Omary et al., 2006). Increased mitosis and apoptosis are unlikely reasons for the increase in K8-S73 phosphorylation in these mice, because no change in 14-3-3 distribution, in proliferating nuclear antigen expression or in the amount of caspase-cleaved K18 or K19 fragments was detected. This leaves stress as the most likely cause of keratin hyperphosphorylation in mice overexpressing K8 and K18 and suggests that elevated keratin is by itself a stressor.

In K8/K18-overexpressing mice K18-S33 phosphorylation is not restricted to the apicolateral keratin filaments of pancreatic acinar cells as is the case in the wild type but extends to cytoplasmic filaments (Ku et al., 2002b). Also, K18-S33A-overexpressing mice show more dispersed distribution of keratin filaments in pancreatic acini than in wild-type K18 transgenic mice (Ku et al., 2002b). Also, the K8/K18-overexpressing murine pancreata show a decrease in size but an increase in the number of zymogen granules. Similar pancreatic secretion defects were observed in mice lacking syncollin, a zymogen granule-interacting protein (Wasle et al., 2005). The function of keratin phosphorylation is not limited to

exocrine pancreas. Thus, phosphorylation of a 60 kDa keratin was described in hamster insulinoma cells during insulin release concurrent with depolarization-induced calcium influx (Schubart et al., 1980, 1982). This observation hints toward a contribution of Ca^{2+}-activated protein kinase-mediated keratin phosphorylation to regulated insulin release.

5.1.3 Keratin Phosphorylation Is Linked to Liver Disease Progression

The link between keratin phosphorylation and liver disease progression was investigated in transgenic mice expressing K8-G61C, a K8 variant that is associated with cryptogenic and noncryptogenic liver disease in humans (Ku et al., 2001, 2002a; Ku and Omary, 2006). K8-G61 lies in the highly conserved H1 subdomain of the K8 head region. The K8-G61 → K8-C61 mutation leads to more crosslinking of K8 upon oxidative challenge (Ku and Omary, 2006). The aberrant crosslinking of K8 reduces the accessibility of K8-S73 to p38MAPK, which is responsible for K8 hyperphosphorylation during apoptosis, stress, and mitosis (Ku and Omary, 2006; Liao et al., 1997). Therefore, K8-G61C transgenic mice exhibited increased predisposition to MC-LR-induced liver injury and Fas-mediated apoptosis as compared to mice expressing only wild-type K8 (Ku and Omary, 2006). Although the keratin filament collapse during Fas-mediated apoptosis in K8-G61C transgenic mice is comparable to that seen in wild-type K8 mice, the elevation of K8-S73 phosphorylation upon Fas stimulation was ~65% less than that of wild-type K8-expressing mice (Ku and Omary, 2006). This confirms the inhibitory effect of K8-G61C mutation on K8-S73 phosphorylation.

The attractive phosphate sponge concept put forward by the Omary Lab suggests that the keratin cytoskeleton neutralizes surplus kinase activity (Ku and Omary, 2006). Thus, inhibition of K8-S73 phosphorylation by p38 MAPK in K8-G61C mice may shunt the kinase activity to other targets such as cJun, cAMP response element-binding protein (CREB), or p90RSK which have been shown to arouse apoptosis upon phosphorylation (Baines and Molkentin, 2005; Deak et al., 1998; Roux and Blenis, 2004).

The significance of site-specific keratin phosphorylation for progression of liver disease was delineated in an investigation of hepatocytic events in chronic hepatitis patients (Shi et al., 2010). The analyses revealed elevated phosphorylation of K18-S33 and K18-S52 in cirrhotic and noncirrhotic hepatitis B correlating with increasing liver lesions (Shi et al., 2010). While K18-S52 phosphorylation increased parallel to liver injury progression, K18-S33 phosphorylation did not show such a tight correlation. Furthermore, K18-S52 phosphorylation increased in parallel to alanine aminotransferase (ALT)

activity, whereas K18-S33 phosphorylation upsurged irrespective of ALT activity. It was suggested that K18-S52 serves as a hepatocytic protective factor in hepatitis B whereas K18-S33 phosphorylation may serve as an early indicator of hepatitis B virus infection. Yet, both K18-S33 and K18-S52 phosphorylation are dependable markers of chronic hepatitis B progression (Shi et al., 2010).

5.1.4 Keratin Phosphorylation Affects Keratin Network Organization and Function of Intestinal Epithelial Cells

Treatment of human colon carcinoma-derived CaCo-2 cells with forskolin, which is known to increase cAMP levels, led to the redistribution of keratin filaments from the cell periphery to the cell interior (Baricault et al., 1994). This change in the spatial organization of the keratin network correlated with keratin hyperphosphorylation. 2D gel electrophoresis of the ^{32}P-labeled keratin extracts revealed increased phosphorylation of K8, K18, and K19 and appearance of additional isoelectric variants of K8. Morphologically, forskolin treatment resulted in reduction of intercellular spaces and shortening of microvilli. In addition, decreased expression of hydrolases was observed in the apical brush border of forskolin-treated confluent CaCo-2 monolayers (Baricault et al., 1994).

The following studies shed further light on the functional role of keratin phosphorylation in the intestine. K20-S13 phosphorylation, which positively responds to PKC activation, was shown to be essential for keratin filament reorganization. Thus, introduction of K20-S13A together with wild-type K8 into fibroblast-derived NIH-3T3 cells prevented okadaic-induced keratin filament network breakdown (Zhou et al., 2006). Additionally, the observed hyperphosphorylation of K20-S13 in apoptosis, starvation, and DSS-induced colitis can be taken as an indication of its physiological relevance. Besides, the preferential phosphorylation of K20-S13 in goblet cells of the murine small intestine makes it a unique intestinal goblet cell marker (Zhou et al., 2006). Furthermore, K20-S13 is also a physiological target of the MAPK-activated protein kinase (MAPKAP) MK2. Pharmacological blockade of MK2/3 or p38 MAPK inhibited prostaglandin-mediated mucin secretion in differentiated HT29-MTX cells, a mucus-secreting HT-29 subpopulation. Thus, p38/MK2/3-dependent K20 phosphorylation may be involved in mucin secretion of intestinal epithelia (Menon et al., 2010).

5.2 Keratin Phosphorylation Is Altered in Skin Diseases

Keratin gene mutations are linked to many skin diseases (Chamcheu et al., 2011b; Stevens et al., 2000). A hallmark feature of these diseases is the

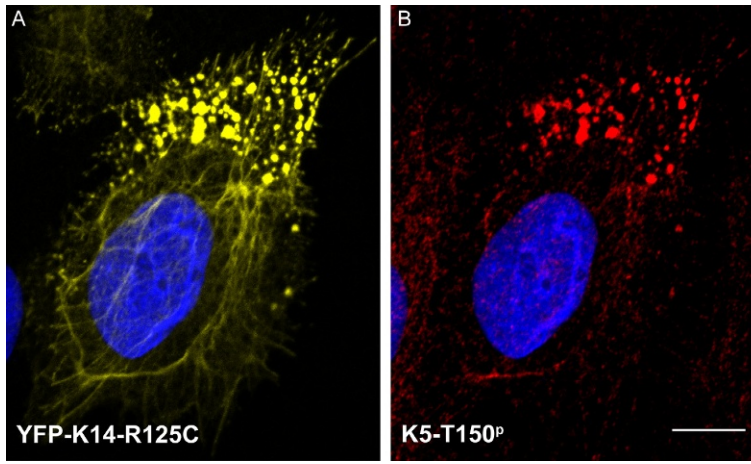

Fig. 6 Detection of keratin phosphorylation in aggregates containing mutant keratin. Human keratinocyte-derived cells of line HaCaT were transiently transfected with a cDNA construct coding for keratin mutant K14-R125C, which is known to cause the skin blistering disease epidermolysis bullosa simplex. The mutant keratin was tagged at its aminoterminus with enhanced yellow fluorescent protein. (A) The micrograph depicts the fluorescence elicited by the fluorescence-tagged mutant keratin (YFP-K14-R125C) together with a nuclear Hoechst stain. (B) Presents a corresponding fluorescence micrograph showing the distribution of phosphorylated K5 T150 as detected by primary antibody LJ4 (Toivola et al., 2002) together with the nuclear Hoechst stain. Note the preferential localization of the keratin phosphoepitope in granules and thickened filament bundles. Scale bar = 10 μm.

formation of keratin aggregates (e.g., Fig. 6), which aggravates upon mechanical stress. In addition, there is accumulating evidence that these structural alterations are linked to changes in keratin phosphorylation. We will describe this aspect in detail in the following sections.

5.2.1 *Increased Keratin Phosphorylation Is Related to Keratin Network Dynamics in Skin Disease*

EBS, an autosomal dominant skin disorder, manifests itself in the form of trauma-induced epidermal basal cell lysis leading to skin blisters (Coulombe et al., 1991b). Several studies hint toward the contribution of keratin phosphorylation in the pathogenesis and progression of EBS (Chamcheu et al., 2011a; Chan et al., 1993; Chipev et al., 1992).

Weber–Cockayne EBS (EBS-WC) is a mild form of EBS which has been linked to missense mutations in the H1 subdomain of the K5 head region and the nonhelical linker segments of the K5 coiled-coil domain (Albers and Fuchs, 1992). The K5-I161 to K5-S161 mutation in the K5 H1

subdomain of EBS-WC patients leads to subtle blistering of the palmar and plantar skin upon physical trauma (Chan et al., 1993). The substitution of isoleucine by serine creates the new potential PKC phosphorylation site S-Q-R (Woodgett et al., 1986). PKC phosphorylation-mediated keratin remodeling upon mechanical stretch has been discussed in Section 4.2.4. Furthermore, it has been suggested that structural alterations due to the additional phosphorylation site in the H1 subdomain may interfere with the lateral association of keratin dimers during filament assembly (Chipev et al., 1992; Parry and Steinert, 1992; Steinert, 1991a,b). Besides, K5-I161 is next to the helix initiation motif of the coiled-coil rod domain, which is crucial for proper filament formation (Stewart et al., 1989). In line with these theoretical assumptions, ultrastructural analyses of EBS-WC patient-derived keratinocytes revealed mild aggregation and waviness of keratin filaments in basal cells along with nuclear distortions (Chan et al., 1993). Taken together, these observations illustrate how a single keratin phosphorylation site impacts keratin network organization with consequences for cellular stability.

Dowling-Meara EBS (EBS-DM) is more severe than EBS-WC. It is known to be caused by missense mutations in either the helix initiation motif or the helix termination motif of the K5 or K14 rod domains. EBS-DM patient-derived epidermal keratinocytes display keratin aggregates and withdrawal of the keratin network from the plasma membrane (Coulombe et al., 1991a; Fine et al., 1991; Ishida-Yamamoto et al., 1991; Letai et al., 1993). Epithelial cell lines expressing the DM-EBS YFP-labeled mutant K14-R125C showed that a high percentage of the mutant K14 was in the soluble pool (Werner et al., 2004). The abundance of phosphoepitopes may safeguard the soluble mutant keratin against ubiquitin-mediated degradation, as discussed earlier (Jiang et al., 2014). In addition, K14-R125C-positive aggregates increased upon proteasome inhibition. Conversely, they were reduced by chaperone-associated ubiquitin ligase CHIP/STUB1 through an elevated degradation of the mutant K14 (Loffek et al., 2010).

Besides, K14-R125C aggregates (see Fig. 6 as a typical example) turned over rapidly with a half time of less than 15 min (Werner et al., 2004). This was interpreted as a consequence of perturbed keratin assembly (Windoffer et al., 2011). While the mutant keratin polypeptides initiate the assembly process in the cell periphery, in close proximity to focal adhesions as is the case for wild-type keratins (Windoffer et al., 2006), they do not elongate properly. Instead of forming filamentous structures, they generate growing

granules (Windoffer et al., 2004). Both, elongating filaments and growing granules are transported toward the cell interior with the help of actin filaments and microtubules (Kolsch et al., 2009; Werner et al., 2004). Yet, wild-type filaments are integrated into the peripheral keratin network to complete their prolonged turnover cycle (Windoffer et al., 2011), whereas the mutant keratin granules are incapable of such integration and, instead, dissolve to enter another futile cycle of assembly and disassembly (Kolsch et al., 2010; Moch et al., 2013; Windoffer et al., 2004).

5.2.2 Keratin Phosphorylation Is Increased in Stressed Keratinocytes Producing Mutant Keratins

Mutant keratin filaments in immortalized keratinocytes from patients with EBS-DM (K5 E475G and K14-R125P) and EBS-WC (K14 V270M) exhibit thermoinstability with progressive keratin filament disassembly and aggregate formation after 15 min exposure to 43°C unlike the keratin filaments in control cell lines derived from unaffected individuals (Morley et al., 2003). Furthermore, heat stress not only increased keratin aggregate formation in EBS-DM patient-derived keratinocyte cell lines carrying a K5-E475G mutation but also led to an increased level of K5 phosphoepitopes (Chamcheu et al., 2011a). Moreover, the phosphorylated EBS-DM keratins were associated with HSP70. Treatment of the mutant keratinocytes with the chemical chaperone trimethylamine-*N*-oxide (TMAO) reduced keratin granules as did kinase inhibition. This suggests a potential role of keratin phosphorylation in the misfolded protein response (Chamcheu et al., 2011a).

Furthermore, osmotic shock of the immortalized keratinocyte cell lines, that were derived from DM-EBS and WC-EBS patients, showed more pronounced keratin aggregation than the control cell line NEB-1, that was derived from a healthy individual (Beriault et al., 2012; D'Alessandro et al., 2002). Moreover, cell lines with clinically severe EBS mutations were more sensitive to osmotic stress and showed faster activation of the SAPK/JNK pathway (D'Alessandro et al., 2002). Also, gene expression profiles of the KEB-7 cell line derived from a DM-EBS patient with K14-R125P mutation revealed reduction in the levels of dual specificity MAP kinase-associated phosphatases along with higher and longer activation of the p38 and ERK pathways as compared to NEB-1 cells under hypoosmotic stress (Liovic et al., 2008). This suggests the presence of an intrinsic stress, thereby compromising the ability of EBS cells to deal with additional stress. It may in part explain the unexpected reduction of phosphorylated K5 in the triton-X100 soluble pool of osmotically stressed KEB-7 cells which is completely contrary to the increased phosphorylation

of K5 in the soluble pool of NEB-1 cells (Liovic et al., 2008). Also, rheological assays demonstrated a reduced resilience of EBS mutant K14-R125C filaments to large deformations (Ma et al., 2001).

5.2.3 Increased Keratin Phosphorylation Is Observed in Hyperkeratotic Skin Diseases

Links to keratin phosphorylation have also been found in EH, another autosomal dominant skin disorder which involves disruption of the structural integrity of the suprabasal layers of the epidermis. K1 L160P ($_{157}$NQS**L**LQPL$_{164}$ → $_{157}$NQS**P**LQPL$_{164}$) is a mutation in the H1 subdomain of K1 of patients with EH which leads to the generation of a new potential phosphorylation site in the adjacent serine (Chipev et al., 1992). In support, p38 MAPK mediated in vitro hyperphosphorylation of K8-L71P mutants, which also contain a novel phosphorylation site (K8-S70) in the H1 subdomain corresponding to K1-S159. Besides, K8-L71P-transfected cells showed increased keratin filament collapse in the presence of okadaic acid (Ku et al., 2002a). The significance of the K1-L160P mutation for keratin filament assembly is also evident from peptide inhibition experiments. While wild-type H1 peptide efficiently disassembled preformed K1/K10 filaments, a mutant H1 peptide encompassing the K1-L160P mutation was much less efficient (Chipev et al., 1992).

5.3 Keratin Phosphorylation Is Altered in Cancer

The biology of cancer is characterized by distinct hallmarks. These include increased cell migration needed for tissue invasion and metastasis, tumor progression that may be linked to dedifferentiation, and loss of growth control all of which have been linked in some way to altered keratin phosphorylation in epithelial carcinomas. We will describe some of these findings in the following paragraphs. They are also summarized in Fig. 7 highlighting the cell-type specificity of the observed phenomena. An explanation for the highly diverse and often opposing effects of keratin phosphorylation may be the complex nature of the phosphorylation patterns and the different overall cellular differentiation status in each instance.

5.3.1 Phosphorylation-Dependent Keratin Network Plasticity Correlates With Tumor Cell Migration

Both, cell invasion through a connective tissue and its directional migration require the presence of a flexible leading edge. Increased keratin phosphorylation speeds up keratin cycling and increases network plasticity inducing

cell shape changes that are advantageous for cell migration and invasiveness (Chung et al., 2013; Windoffer et al., 2011). This may also explain the unique mechanical properties of cancer cells such as softness and elasticity (Cross et al., 2007).

Sphingosylphosphorylcholine (SPC), a bioactive lipid that is elevated in blood and ascites of ovarian cancer patients, affects cell growth and cell migration (Boguslawski et al., 2000; Seufferlein and Rozengurt, 1995; Xiao et al., 2000). SPC causes keratin network reorganization into perinuclear, ring-like structures accompanied by an increase in phosphorylation of K8-S431 and K18-S52 in pancreatic cancer-derived Panc-1 cells (Beil et al., 2003). In vivo micromechanical assays and Boyden chamber assays revealed an increase in the viscoelastic and migratory behavior of Panc-1 cells upon SPC treatment. This suggested that the phosphorylation-induced keratin reorganization may be directly linked to decreased mechanical resilience and increased invasiveness of SPC-treated Panc-1 cells and may be relevant for other tumor types (Beil et al., 2003).

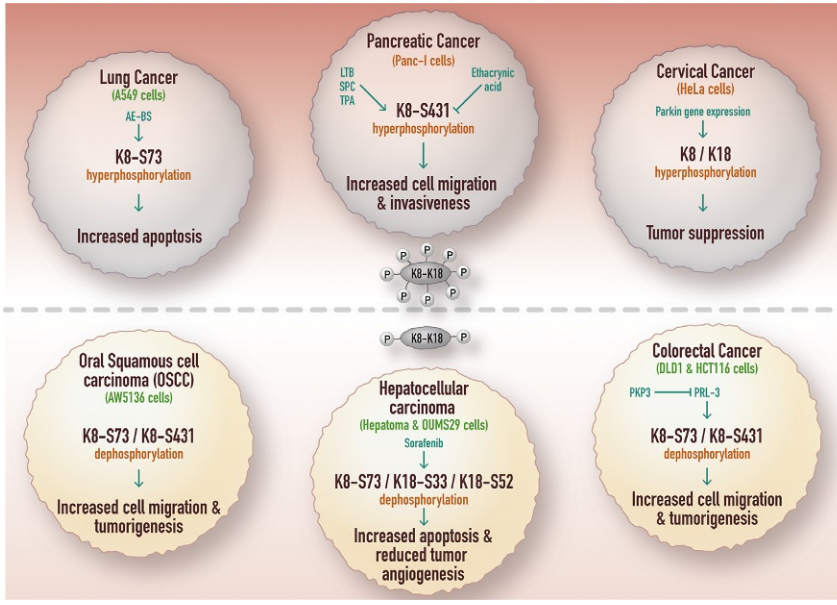

Fig. 7 Anomalous K8/K18 phosphorylation in human cancer and its consequences. The scheme shows hyperphosphorylation (*upper part*) and hypophosphorylation (*lower part*) of K8/K18 in the context of various cancer conditions. AE-BS: acetone extract of *Bupleurum scorzonerifolium*; LTB: leukotriene B4; SPC: sphingosylphosphorylcholine; PKP3: plakophilin3; PRL-3: phosphatase of regenerating liver-3; TPA: 12-*O*-tetradecanoylphorbol-13-acetate. Further details are in the text.

Additional investigation showed that SPC triggers ERK and JNK activity, phosphorylating K8-S431 (Park et al., 2011). SPC-induced keratin reorganization in human pancreatic Panc-1 and gastric cancer AGS cells was accompanied by ERK-mediated keratin phosphorylation (Beil et al., 2003; Mesecke et al., 2011). Considering the importance of ERK signaling for cell migration (Bove et al., 2008; Huang et al., 2004; Rajalingam et al., 2005), the above findings provide another mechanism for enhanced cell motility acting via increased keratin phosphorylation. The exocyst complex component Sec8 has been identified recently as upstream regulator of ERK- and p38-mediated phosphorylation of K8 in migrating oral squamous cell carcinoma (OSCC)-derived HSC3 cells (Tanaka and Iino, 2015). Furthermore, leukotriene B4 (LTB 4) is a component that is also elevated in pancreatic cancer. It induces keratin rearrangements in Panc-1 cells similar to those observed upon SPC treatment but acts via protein phosphatase 2A downregulation, which in turn induced ERK activation (Park et al., 2012).

SPC-mediated keratin phosphorylation and reorganization depends on transglutaminase-2 (Tg-2) whose cross-linking activity leads to the formation of a triple complex of K8, Tg-2, and p-JNK (Park et al., 2011). Remarkably, transglutaminase-2 is associated with cell survival, invasiveness, metastasis, and chemoresistance in pancreatic, lung, and ovarian tumors (Chhabra et al., 2009; Park et al., 2010a; Verma et al., 2006). Moreover, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent tumor promoter, also stimulates K8-S431 phosphorylation, keratin reorganization, and Panc-1 cell migration via transglutaminase-2 (Lee et al., 2014). Conversely, ethacrynic acid inhibits transglutaminase-2 and hence obstructs SPC-induced keratin phosphorylation and perinuclear keratin reorganization (Byun et al., 2013). Taken together, multiple players may be involved in keratin network reorganization via altered keratin phosphorylation to adjust viscoelastic and migratory properties of cancer cells.

5.3.2 Keratin Dephosphorylation Is Linked to Tumor Progression

In contrast to the above-mentioned observations, a gain of motility was observed in wound healing scratch assays of human OSCC-derived AW13516 cells, which produce either K8-S73A or K8-S431A phosphorylation-deficient keratins (Alam et al., 2011). Furthermore, subcutaneous injection of K8-S73A and K8-S431A OSCC cell clones into NOD-SCID mice demonstrated increased tumor formation as compared to the wild-type K8-producing OSCC cell clones (Alam et al., 2011). The relevance of these observations for human tumors is underscored by the

observed absence of phosphorylation at K8-S73 and K8-S431 in 46% and 58% of human OSCC tissue samples, respectively (Alam et al., 2011). A clinicopathological analysis of strongly K8-positive OSCC patient samples revealed a close correlation between loss of K8-S73 and K8-S431 phosphorylation and tumor size, tumor stage, and lymph node metastasis. Moreover, loss of K8-S73 phosphorylation correlated negatively with patient survival (Alam et al., 2011). Thus, keratin phosphorylation may serve as a prognostic marker in certain cancer types such as OSCC.

Keratin 8 is recognized as a physiological substrate of phosphatase of regenerating liver (PRL-3), which is consistently overexpressed in liver metastases derived from colorectal cancer (Saha et al., 2001). PRL-3 directly associates with K8 and dephosphorylates it at S73 and S431 residues. PRL-3 and K8 colocalization was observed in lamellipodia and ruffles of colorectal cancer-derived DLD-1 cells. Treatment of DLD-1 cells with PRL-3 inhibitor not only ablated the dephosphorylation at K8-S73 and K8-S431 but also ablated the colocalization of PRL-3 and K8 leading to redistribution of K8. In addition, the observed overexpression of PRL-3 and dephosphorylation of K8-S73 and K8-S431 at the invasive front of primary human colorectal carcinomas supports the role of PRL-3-mediated K8 dephosphorylation in invasion (Mizuuchi et al., 2009). Thus, PRL-3 activity, which has been shown to accelerate cancer cell motility, invasion, and metastasis, also modulates keratin phosphorylation, an important switch regulating keratin dynamics (Fiordalisi et al., 2006; Guo et al., 2004; Zeng et al., 2003).

Upregulation of PRL-3 is an essential factor for transformation and metastasis caused by loss of plakophilin 3 (PKP3) in human colon carcinoma-derived HCT116 cells (Khapare et al., 2012). PKP3 is a desmosomal plaque protein whose loss has been implicated in neoplastic progression and metastasis (Aigner et al., 2007; Kundu et al., 2008; Papagerakis et al., 2003; Schwarz et al., 2006). Moreover, PRL-3-dependent K8-S431 dephosphorylation leading to K8 overexpression was observed in PKP3 knockdown cells. In accordance, loss of K8 in PKP3 knockdown cells led to the reversal of the transformed phenotype (Khapare et al., 2012). Upregulation of K8 is a common observation associated with increased cell invasiveness, migration, tumorigenesis, and poor prognosis of many tumors (Fillies et al., 2006; Ku et al., 1996; Raul et al., 2004; Schaafsma et al., 1993).

Spheroid perinuclear inclusion bodies containing K8 aggregates are often observed in rhabdoid cells of malignant rhabdoid tumors (MRT) and other malignant neoplasms (Chetty and Asa, 2004; Shiratsuchi et al., 2001).

The presence of rhabdoid cells is an indication of high aggressive malignancy and poor prognosis of epithelial and mesenchymal tumors (Beckwith and Palmer, 1978; Oda et al., 1993; Oshiro et al., 2000; Tsuneyoshi et al., 1987). Sequence analyses of the entire human K8 gene in samples obtained from frozen MRT tissues and MRT-derived cell lines revealed several missense mutations. Of these, codon 89 (CGC to TGC [R to C]) and codon 290 (AGC to ATC [S to I]) may be of relevance for keratin phosphorylation (Shiratsuchi et al., 2001). K8-R89 is located in the H1 subdomain of the K8 head which is a target of phosphorylation. K8-S290 is a reported phosphorylation site of the K8 tail domain (Ku and Omary, 1997). In addition, both of these mutations may have significance for lateral interactions during keratin assembly. Thus, mutation-induced alterations in keratin phosphorylation may contribute to the aberrant accumulation of keratin in MRT (Chetty and Asa, 2004; Shiratsuchi et al., 2001).

The above reports suggested the association of K8 dephosphorylation with tumor progression and aggressiveness. A similar link was suggested from investigations of the proapoptotic effects of the crude acetone extract of *Bupleurum scorzonerifolium* (AE-BS) on human lung cancer-derived A549 cells (Chen et al., 2005). AE-BS, known for its anticancer properties, led to ERK1/2 activation causing K8-S73 hyperphosphorylation in A549 cells. This observation indicated that K8-S73 phosphorylation, which was also induced by stimulating the proapoptotic receptors Fas and TNF, was linked to diminished cancer cell viability (Gilbert et al., 2001; Ku et al., 2003). Similarly, overexpression of the tumor–suppressor parkin in human cervical cancer-derived HeLa cells was associated with increased phosphorylation of K8/K18 (Song et al., 2013). Loss or mutations of parkin have been linked to acute lymphoblastic leukemia, chronic myeloid leukemia, and colorectal carcinoma (Agirre et al., 2006; Cesari et al., 2003; Poulogiannis et al., 2010). In accordance with these studies, overexpressing parkin in HeLa cells caused growth-inhibitory effects, which were accompanied by phosphorylation of K8 and K18 (Song et al., 2013). However, there are contrary reports such as the occurrence of K8-Y267 phosphorylation in human cholangiocarcinoma tissues and the presence of phosphotyrosine peptides in lung cancer associated with high expression of the protooncogene tyrosine kinase ROS (Gu et al., 2011; Rikova et al., 2007; Snider et al., 2013). Furthermore, RSK-mediated phosphorylation of K17-S44 has been implicated in the interaction of K17 with hnRNPK, a partnership leading to CXCR3-dependent tumor growth and invasion as demonstrated in vulva carcinoma-derived A431 cells (Chung et al., 2015; Lo et al., 2010).

5.3.3 Keratin Phosphorylation Is Linked to Epithelial–Mesenchymal Transition

Carcinogenesis is often characterized by epithelial to mesenchymal transition (EMT), a critical mechanism leading to acquisition of invasiveness and malignancy in epithelial cancer cells (Brabletz et al., 2001; Fidler and Poste, 2008; Thiery, 2002). The EMT program is a biological process that converts the phenotype of an epithelial cell to that of a mesenchymal cell (Kalluri and Neilson, 2003). The oncogenic kinase Src, known to phosphorylate K19-Y391, is elevated in highly metastatic cancer cells resulting from loss of epithelial differentiation (Rikova et al., 2007; Zhou et al., 2010). Src-mediated phosphorylation of K19 at Y391 residue leads to its increased partitioning into the soluble fraction upon pervanadate treatment (Zhou et al., 2010). Additionally, the Src-K19 interaction is accompanied by facilitation of EGF-stimulated oncogenesis, which is critical for Src activation (Li et al., 2010).

5.3.4 Keratin Hyperphosphorylation Is a Consequence of Altered Growth Control in Cancer Cells

Activated growth signaling pathways in cancer cells have been shown to induce keratin hyperphosphorylation. Thus, increased phosphorylation of K8-S23, K8-S73, and K8-S431 was reported for colorectal cancer-derived CaCo-2 cells upon activation of the EGFR pathway (Arentz et al., 2011). Conversely, blockade of the EGFR pathway not only decreased keratin phosphorylation but also elevated apoptosis in CaCo-2 cells (Arentz et al., 2011). Similar mechanisms may be the reason for the high survival benefits of sorafenib in advanced hepatocellular carcinoma (HCC), which is a malignancy resistant to conventional therapies (Bruix et al., 2005; Cheng et al., 2009; Forner et al., 2012; Llovet et al., 2008; Schwartz et al., 2007). Sorafenib is an inhibitor of serine/threonine and receptor tyrosine kinases. The molecular action of sorafenib involves the generation of endoplasmic reticulum stress by decreasing protein ubiquitination and the unfolded protein response (Honma and Harada, 2013). Additionally, being a multikinase inhibitor, sorafenib blocks keratin phosphorylation at major phosphorylation sites such as K8-S73, K18-S33, and K18-S52 in hepatoma and human hepatocyte-derived OUMS29 cells (Honma and Harada, 2013; Wilhelm et al., 2004). In this way, sorafenib may prevent a defensive response against hepatocellular stress (Harada et al., 2007; Ku et al., 1998b; Ku and Omary, 2006; Kwan et al., 2012; Omary et al., 2004; Toivola et al., 2004; Zatloukal et al., 2007). The effects of sorafenib manifest

as an increase in autophagy and a decrease in proteasome-mediated MDB formation in HCC-derived Huh7 and OUMS29 cells (Bareford et al., 2011; Honma and Harada, 2013; Park et al., 2010b; Shimizu et al., 2012; Ullen et al., 2010). Both, induction of autophagy and loss of MDB formation are characterized by a reduction in phosphorylated keratin (Harada et al., 2007, 2008; Kongara et al., 2010; Kwan et al., 2012; Zatloukal et al., 2007). The cytotoxic action of sorafenib results in an antiproliferative effect in hepatoma cells, reduced tumor angiogenesis, and increased apoptosis (Honma and Harada, 2013; Liu et al., 2006; Wilhelm et al., 2004). Hence, administration of sorafenib reduces tumor cell viability through its pleiotropic antitumor effects, which exploit the role of phosphorylated keratins as stress proteins.

6. CONCLUDING REMARKS: SIGNIFICANCE OF KERATIN PHOSPHORYLATION

Based upon the various aspects discussed above, overall significance of keratin phosphorylation can be summarized as follows:

(1) Modulation of cellular plasticity. Phosphorylation affects keratin solubility and keratin filament turnover with consequences on network organization and mechanics during the cell cycle, in response to various types of stress and during carcinogenesis. (2) Regulation of keratin association with other proteins. Phosphorylation regulates binding of keratins to each other and other proteins affecting keratin assembly state, crosslinking to other cell components and signal transduction. (3) Role as a "phosphate sponge." Keratin phosphorylation sequesters kinase and phosphatase activities. (4) Protection of keratins from degradation. Phosphorylation prevents keratin ubiquitination with consequences for cell viability.

Similar to the posttranslational modifications in other intermediate filaments, phosphorylation endows the keratins with a wide gamut of functional properties. Due to its rapid kinetics, phosphorylation of the expansive keratin network serves as a suitable emergency system, mobilizing the well-connected keratin network to trigger appropriate switches for adaptation to the changing environment.

ACKNOWLEDGMENTS

The work was supported by the German Research Council (LE566/18-1 and LE566/22-1) and the IZKF and Boost Fund of RWTH Aachen University. We thank Drs. Nicole Schwarz and Reinhard Windoffer for helpful discussions throughout. We are also grateful to Dr. Stefan Wöll for providing the fluorescence micrographs in Figs. 2D–F and 4 and thank Adam Breitscheidel for graphic design of Figs. 1 and 7.

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INTERNATIONAL REVIEW OF CELL AND MOLECULAR BIOLOGY

Volume 330

Edited by

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ISBN 978-0-12-812467-3
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