Keratins Are Going Nuclear

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Previously thought to reside exclusively in the cytoplasm, the cytoskeletal protein keratin 17 (K17) has been recently identified inside the nucleus of tumor epithelial cells with a direct impact on cell proliferation and gene expression. We comment on fundamental questions raised by this new finding and the associated significance.

Everything should be made as simple as possible, but not simpler.

—Albert Einstein

Intermediate filaments (IFs) are 10-nm-wide fibrous polymers that, alongside microtubules, F-actin, and associated proteins, form the cytoskeleton in multicellular organisms. Remarkably, IFs are formed by a large and diverse group of >70 proteins (mass range 20–240 kilodaltons) that are expressed and regulated in a tissue-, differentiation-, and context-specific fashion (Pan et al., 2013; Schweizer et al., 2006). IFs are abundant intracellular elements that partake in all basic cellular functions, including growth, death, and virtually everything in between, and are necessary to maintain cellular integrity and function in the face of mechanical and other forms of stress (Pallari and Eriksson, 2006; Pan et al., 2013; Toivola et al., 2010). Genetically determined mutations in IF proteins account for >100 diseases to date (Omary et al., 2004; Szeverenyi et al., 2008; see the Human Intermediate Filament Database at http://www.interfil.org for an up-to-date account), rendering their study immensely relevant to medicine.

With the notable exception of the nuclear lamins, all other IFs (n = 68) are believed to reside and function exclusively in the cytoplasm. With this in mind, how could one account for the puzzling observation, independently made by two laboratories, that the type I IF protein keratin 17 (K17) impacts the nuclear localization and function of the transcriptional regulator autoimmune regulator (AIRE) (Hobbs et al., 2015) or the cell-cycle inhibitor p27KIP1 (CDKN1B) (Escobar-Hoyos et al., 2015)? Whereas a number of intricate mechanisms could account for this finding, a simple explanation is that K17 would itself be present and function inside the nucleus, a possibility that had not been considered until recently. The two aforementioned studies (Hobbs et al., 2015; Escobar-Hoyos et al., 2015), in addition to an unbiased screen to identify nucleocytoplasmic shuttling proteins (Kumeta et al., 2013), have separately converged in demonstrating the presence of endogenous K17 and other keratin proteins inside the nucleus of tumor epithelial cells. This unexpected finding raises several questions and calls for new ideas regarding the rationale underlying the context-dependent regulation and significance of K17 and other IF proteins for cells, tissues, and organs, in both health and disease.

The latest developments regarding nuclear keratins are as follows. Kumeta et al. (Kumeta et al., 2013) identified several cytoskeletal proteins, including the actin-binding protein α-actinin, the versatile cellular cytolinker plectin, and several keratins (e.g., K7, K8, K17, and K18), in the context of a screen for nuclear matrix components that dynamically shuttle in and out of the nucleus in cultured HeLa cells. Hobbs et al. (Hobbs et al., 2015) found K17 to occur in the nucleus of human and mouse tumor epithelia as part of a quest to decipher the mechanisms underlying the ability of this keratin to regulate the induction and levels of mRNAs that code for inflammatory and immune cytokines in skin and cervical tumor paradigms. A key observation in the study by Hobbs et al. was that manipulating K17 expression had a marked impact on the distribution of the AIRE protein inside the nucleus, i.e., punctate versus diffuse, in response to relevant stimuli. Escobar-Hoyos et al. (Escobar-Hoyos et al., 2015) found K17 to occur in the nucleus of cervical tumor epithelial cells, following up on the observation of a slower proliferation rate and increased incidence of G1 cell-cycle arrest when K17 expression is silenced via RNAi. In all three studies, uncovering the presence of keratin proteins inside the nucleus of cultured tumor cells necessitated the use of a potent inhibitor of CRM1/Exportin 1-mediated nuclear export, namely Leptomycin B (LMB), so as to “trap” the protein of interest inside the nucleus and thus facilitate its detection. This same strategy had been used before to uncover the presence of other cytoskeletal-associated proteins, e.g., LPP (lipoma-preferred partner; Petit et al., 2000), Zyxin (Nix et al., 2001), ZO-2 (zona occludens protein 2; Islas et al., 2002) and KEAP1 (Velichkova and Hasson, 2005), inside the nucleus. No such inhibitory treatment is needed, however, to detect nuclear K17 in BT-20-cultured cells (Figure 1A) and in biopsy samples of human BCC skin tumors by microscopy (Hobbs et al., 2015) (Figure 1B), or to biochemically detect the presence of K17 in subcellular fractions enriched for nuclear proteins (Escobar-Hoyos et al., 2015; Hobbs et al., 2015; Kumeta et al., 2013), which altogether
Figure 1. Nuclear Localization and Nuclear Export and Import Sequences for K17 and All IF Proteins

(A and B) Indirect immunostaining for K17 (green) in representative single-plane confocal micrographs of (A) cultured human epithelial tumor cells derived from three tissue types (breast, BT-20 [left]; cervix, HeLa [middle]; vulva, A431 [right]), treated with 40 nM LMB (bottom row) or vehicle (Veh, 70% methanol) (top row), treated with 40 nM LMB (bottom row) or vehicle (Veh, 70% methanol) (top row),

(legend continued on next page)
indicate that nuclear keratins occur in the natural context of tumor cells in culture and tissues in situ.

The idea of nuclear-localized keratins may not be so far-fetched. Is it not the case, for instance, that other cytoskeletal proteins previously thought to reside and function exclusively in the cytoplasm (e.g., β-catenin, actin, tubulin, and even myosin motors) have later on been found to both occur and fulfill important roles in the nucleus (McCrea and Gottardi, 2016; Pellegrini and Budman, 2005; Philimonenko et al., 2004)? Certainly, the notion of IF proteins localizing in the nucleus is not unprecedented, because the A-, B-, and C-type lamins are bona fide nuclear proteins responsible for the formation of a dense meshwork of 10-nm filaments covering the inner surface of the nuclear envelope (Aebi et al., 1986), as well as a diffuse, less well-defined structure in the nucleoplasm (Brider et al., 1993). In fact, IF proteins are believed to have initially appeared as nuclear-localized elements in more “primitive” organisms (Peter and Stick, 2015), as inferred from the notion that the two IF-encoding genes found in Oroschela melanogaster and the lone IF-encoding gene in Dictyostelium discoideum encode lamin-like proteins that localize to the nucleus (Erber et al., 1998; Krüger et al., 2012). The loss of a small exon coding for a classical nuclear localization signal (NLS) along with a membrane-targeting CAAX motif likely facilitated the appearance of lamin-like IFs in the cytoplasm at some point during metazoan evolution (Peter and Stick, 2015). The element of intrigue here is that at least some, and perhaps many, of the remaining 68 non-lamin polypeptides forming the modern IF superfamily may not exhibit an exclusively cytoplasmic localization in interphase cells, as previously thought.

As part of early efforts geared toward understanding the basic properties of IF proteins and the significance of their tissue- and context-specific expression, non-lamin IF proteins have been shown to associate with or localize to the nucleus in various experimental settings. Early studies found that nuclear elements (e.g., nuclear envelope, matrix, DNA) could physically associate with non-lamin IF proteins added to cells or nuclear extracts as purified products in vitro, or after subjecting cells or tissues to harsh challenges (e.g., Bastos et al., 1992; Djabali et al., 1991; Georgatos and Blobel, 1987; Tolstogn et al., 2002; Ward et al., 1984). Nuclear-localized keratin proteins have also been seen in intact culture cell models, e.g., when transiently expressing tail-less truncation mutants of human K8, K18, or K19 (the latter being a natural tail-less keratin) in murine fibroblasts (Bader et al., 1991), when transiently expressing the epidermal-specific K1 in HeLa cells or murine fibroblasts (Blessing et al., 1993), or when human K18 or the temperature-sensitive Xenopus vimentin were modified to include a “strong” NLS and were transiently expressed in SW13 cells (Herrmann et al., 1993; Reichenzeller et al., 2000). Finally, nuclear-localized keratins have been observed in intact tissues of transgenic mice engineered to ectopically express human K1 and/or K10 in pancreatic islet cells (Blessing et al., 1993) or to overexpress human K16 in skin (Takahashi et al., 1994). A key distinction between these early studies and the most recent findings regarding nuclear K17 resides in the latter’s assessment of endogenous protein and the assignment of a functional role for a nuclear-localized keratin.

In the case of K17 protein, current-day in silico sequence analysis algorithms readily identify a classical bipartite NLS beginning at Glutamate 380 and ending at Isoleucine 411 (Figure 1C). Based on available crystal structure data for the K5/K14 heterodimer (Lee et al., 2012) and the vimentin homodimer (Chernyatina et al., 2012), the central rod domain ends with either the “LLEG” or “LLEE” sequence, a highly conserved helix-terminating segment that corresponds to amino acid residue Glycine 390 or Glutamate 391 in human K17. Interestingly, therefore, the predicted NLS for K17 spans the boundary between the α-helical, coiled-coil-forming rod domain, which represents the main driving force toward assembly into 10-nm filaments (Herrmann and Aebi, 2004), and the non-helical tail domain located at the C terminus of K17 (Figure 1C) (Hobbs et al., 2015). Such a location for a subcellular localization signal within the IF protein backbone is very intriguing. On one hand, the surface accessibility and functionality of the bipartite NLS, the amino-terminal moiety of which is embedded in a coiled-coil-forming region of the rod, may well depend upon the polymerization status of the protein. It is easy to envision how either a cryptic or intrinsically “weak” NLS may be masked or ineffective in the setting of 10-nm filaments (or smaller-sized IF subunits) but be kept accessible when a given IF protein takes on an alternative conformation. On the other hand, the carboxy-terminal moiety of the K17 NLS occurs in its tail domain, a preferred site of regulation of IF proteins via either post-translational modifications (PTM) or protein-protein interactions (Snider and Omary, 2014). Large-scale proteomics-based surveys have identified K17 to harbor PTMs on Tyrrosine 398 (phosphorylation), Lysine 399 (acetylation, ubiquitination), Lysine 400 (ubiquitination), and Tyrosine 410 (phosphorylation) (see http://www.phosphosite.org; Hornbeck et al., 2015). As is the case for the conventional polymeric state of K17 or any other IF protein (Snider and Omary, 2014), the functionality of the NLS is likely subject to tight regulation by modifications and/or interactions with key partners in vivo. The predicted bipartite NLS for human K17 is highly conserved (given >50% sequence identity) in primates, rodents, amphibians, and fish (Figure 1C). Of
note, the di-lysine motif located in the tail domain moiety of K17’s predicted NLS is required for the nuclear localization and function of both the human (Lysine 399, Lysine 400) and mouse (Lysine 399, Lysine 401) orthologs (Escobar-Hoyos et al., 2015; Hobbs et al., 2015). This motif appears to be specific to mammals, the significance of which is unclear. In addition, in silico analyses using ValidNESs (Fu et al., 2013) or NetNES1.1 (la Cour et al., 2004) reveal that K17 orthologs feature a nuclear export signal (NES; see Figure 1C) that is conserved in many other type I keratins (unpublished data). This motif comprises three leucine residues—Leucine 194, Leucine 197, and Leucine 199—that were shown, via mutagenesis, to markedly impact the nuclear localization of K17 (Escobar-Hoyos et al., 2015). For K17 protein, therefore, cis-acting determinants affecting nuclear import and export have been identified in silico and experimentally verified, though the partners, pathways, and regulatory modalities involved await further investigation. The presence of a classical NLS does not exclude the possibility that K17 could also enter the nucleus via a non-classical mechanism. K17 interacts with a growing list of proteins that shuttle in and out of the nucleus, including AIRE (Hobbs et al., 2015), 14-3-3σ (Kim et al., 2006), hnRNP K (Chung et al., 2015), and CDKN1B/p27kip1 (Escobar-Hoyos et al., 2015). Together with a classical NLS-based, importin-mediated mechanism, this potential for alternative nuclear import as a piggyback element may allow for greater adaptability and/or broader regulation, depending on physiological circumstances.

In silico analyses of all other human IF sequences reveal that a sizable number of non-lamin IFs exhibit one or even two monopartite or bipartite predicted NLS motifs. In fact, 37 out of the 68 non-lamin human IF sequences exhibit a predicted NLS (Figure 1D) from the outcome of an in silico survey using NCBI consensus coding sequences together with the cNLS Mapper freeware (Kosugi et al., 2009). The vast majority of these NLSs occur either in the non-helical head domain (e.g., K10, K6, desmin, GFAP, CP49) or in the tail domain (e.g., K25, K78, GFAP, vimentin, NF-L, NF-H, NF-M). Akin to the lamin IFs, a few cyttoplasmic IF proteins feature two predicted NLS motifs, including the type I IFs K23, K32, and K35, the type II K4, the type III GFAP, peripherin, syncoilin, and, finally, the type IV α-internexin, NF-H, and NF-L. A recent study demonstrated the presence of desmin in the nucleus of cardiac stem cells, where it occurs at the promoter of actively transcribed genes (just as is the case for K17; see Fuchs et al., 2016). Also, nestin has been observed in the nucleus of neurogenic tumor cell lines (Krupkova et al., 2011). The type II IF K8 represents an example of a “cytoplasmic” IF protein known to occur in the nucleus without the apparent benefit of a recognizable NLS (Kumeta et al., 2013). In this case—and likely others—it could be that the remarkable propensity of IF proteins to multimerize with compatible IF partners and/or interact with proteins that shuttle in and out of the nucleus plays a role in their nuclear import. In all but a few of these cases, the nuclear occurrence of the said IF protein and functionality of predicted NLS motifs have yet to be experimentally validated.

What form do keratins take while in the nucleus? Prior studies showed that mutated forms of IF proteins, such as tail-less human K8/K18 and K8/K19 and Xenopus NLS-vimentin, can occur as conventional 10-nm filaments in addition to punctae while inside the nucleus (Bader et al., 1991; Herrmann et al., 1993; Reichenzeller et al., 2000), suggesting that conventional polymerization inside the nucleus is possible under specific circumstances. However, the three recent reports discussed here are in full agreement in finding that endogenous K17 occurs as discrete punctae and/or in a diffuse pattern while in the nucleus of tumor epithelial cells (Kumeta et al., 2013; Hobbs et al., 2015; Escobar-Hoyos et al., 2015). Neither study found nuclear K17 to exist in conventional 10-nm filaments, despite the observations that nuclear K17 can co-localize with the type II IF keratin 5 (K5), one of its natural assembly partners, in intra-nuclear punctae (Hobbs et al., 2015) and can occur in its full-length version, as can be inferred from the use of GFP fusion proteins and of antibodies to known epitopes (Hobbs et al., 2015; Kumeta et al., 2013). At this time, one cannot comment on the additional possibility of cleaved K17 (or other keratins) inside the nucleus, though K17 (and other keratins) is (are) subject to caspase-mediated cleavage, e.g., in breast epithelial cells in culture (Badock et al., 2005). Additionally, the number and size of K17-containing nuclear punctae per cell, as well as the fraction of cells showing intra-nuclear keratins, vary depending on the tumor cell line being cultured. For instance, we find that following exposure to a relevant stimulus, significantly fewer A431 cells show nuclear K17 relative to HeLa and BT-20 cells, and the latter shows a diffuse rather than punctate pattern for intra-nuclear K17 (Figure 1A). Thus, it follows that various factors likely specify endogenous keratins against polymerization inside the nucleus. Kumeta et al. (2013) elegantly established that nuclear- and cytoplasmically localized K8 in HeLa cells show a differential reactivity with various monoclonal antibodies that recognize conformation-specific epitopes, along with a differential sensitivity to detergent extraction. From this they inferred that the form adopted by keratin inside the nucleus is fundamentally distinct from that occurring in the cytoplasm (10-nm IFs). Other cytoskeletal proteins, including actin (Schoenenberger et al., 2005) and tubulin (Akoumianaki et al., 2009), have been shown to adopt a distinct conformation while inside the nucleus, correlating with their association with a distinct set of protein partners. Going forward, identifying these regulatory factors (i.e., post-translational modifications and/or unique interacting protein partners) represents a fundamentally important though technically challenging aspect of understanding the significance of nuclear-localized keratins.

What is the functional significance of nuclear-localized keratins? The limited data available originate from two studies focused on K17 in cervical (Escobar-Hoyos et al., 2015) and skin (Hobbs et al., 2015) tumors and already hint at the prospect that this keratin may exert multiple roles in the nucleus, as it does in the cytoplasm (Kim et al., 2006; McGowan et al., 2002; Tong and Coulombe, 2006). Though functionally important in both settings, expression of K17 impacts cervical and skin tumor epithelia in distinct ways. In skin tumor keratinocytes, K17 plays a key role in promoting the cell-autonomous expression of genes encoding inflammatory and immune cytokine effectors (e.g., Cxcl5, Cxcl10, Cxcl11,
Ccl2, Ccl19, Ifng, Mmp9). Nuclear K17 has been found to associate with promoter regions of cytokine genes, as well as the transcriptional regulator AIRE and the p65 subunit of NF-κB, altogether indicating roles in chromatin binding and transcription (Hobbs et al., 2015). Additionally, K17 acts with the ribonucleoprotein hnRNP K to stabilize its target cytokine transcripts in skin tumor keratinocytes, suggesting a possible role for K17 in RNA export and/or processing (Chung et al., 2015). In cervical tumor epithelia, K17 has been shown to promote the nuclear export and deactivation of CDKN1B/p27KIP1, a key negative regulator of the G1 to S transition (Escobar-Hoyos et al., 2015). Furthermore, in cervical lesions in situ, K17 acts to suppress inflammation and immune cell recruitment, in addition to impacting p63 expression and the balance between epithelial cell proliferation and differentiation (Hobbs et al., 2016), a notion that could be related to nuclear K17’s role in cell-cycle progression (Escobar-Hoyos et al., 2015). Importantly, K17’s ability to regulate immune and inflammatory cytokine gene expression in skin tumor keratinocytes, and CDKN1B/p27KIP1 activity and cell-cycle progression in cervical tumor keratinocytes, depends in part on its presence in the nucleus (Escobar-Hoyos et al., 2015; Hobbs et al., 2015). The differential significance of nuclear K17 in skin versus cervical tumor keratinocytes likely reflects distinct sites of expression for K17 in these two tissues in relation to the origin of tumor cells in the paradigms exploited (Hobbs et al., 2016). High-throughput screens focused on the K17-associated genome, transcriptome, and nuclear proteome are examples of next steps likely to shed light on the ultimate significance of nuclear-localized K17 protein.

A model that incorporates what we have learned so far regarding the import and export of K17 to and from the nucleus and its significance as a nuclear protein is presented in Figure 2. Its main points are as follows. First, we posit that IF subunits destined for nuclear import are likely to be small, and, whether they are newly synthesized elements or derived from the existing (e.g., polymer-bound) pool of IFs, they have to be specified for this fate via post-translational modifications (e.g., phosphorylation, sumoylation, acetylation) and/or interactions with accessory proteins. Second, import of non-lamin IF proteins into the nucleus can occur via classical NLS/importin-mediated and/or non-canonical pathways (Wagstaff and Jans, 2009). Third, nuclear-localized non-lamin IF proteins likely partake in multiple processes in the nucleus: so far, there is evidence for participation of keratins in transcription and cell-cycle regulation, depending on the tissue context and tumor paradigm. Fourth, export of non-lamin IF proteins from
the nucleus can occur via a canonical, NES-mediated path via direct association with exportins or indirectly via piggy-backing, and/or that loss could also entail degradation in the nucleus (von Mikecz, 2006).

In conclusion, endogenous keratins have now been found to occur and function inside the nucleus. The available evidence suggests that the nuclear pool of keratin proteins is dynamically regulated and can exist in conformations that are distinct from canonical cytoplasmic 10-nm IFs. In the case of K17, classically defined cis-acting determinants mediating nuclear import and export have been elucidated, and specific leads regarding functional significance secured from studies focused on skin and cervical tumors indicate active roles in regulating gene expression and cell cycle. These developments add to the notion that cytoskeletal proteins play a fundamental role in facilitating dynamic and tightly regulated signaling processes and create a new dimension to the challenge of understanding the diversity and context-dependent regulation of keratins and all other IF proteins, along with the significance of their dysregulation in chronic diseases such as cancer.

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