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# Colorectal keratins: integrating nutrition, metabolism and colorectal health

**Caroline A. Evans[1] & Bernard M. Corfe[2]**

**1. ChELSI Institute, Department of Chemical and Biological Engineering, University of Sheffield, Mappin St, S1 3JD, Sheffield, United Kingdom**

**2. Population Health Sciences Institute, Human Nutrition Research Centre, Faculty of Medical Sciences, Newcastle University, Newcastle, NE2 4HH**

***Author for correspondence*: Prof Bernard Corfe, Population Health Sciences Institute, Human Nutrition Research Centre, Faculty of Medical Sciences, Newcastle University, Newcastle, NE2 4HH, email: bernard.corfe@newcastle.ac.uk**

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## 1. Abstract

The colon mucosa is lined with crypts of circa 300 cells, forming a continuous barrier whose roles include absorption of water, recovery of metabolic energy sources (notably short chain fatty acids), secretion of a protective mucus barrier, and physiological signaling. There is high turnover and replenishment of cells in the mucosa, disruption of this may lead to bowel pathologies including cancer and inflammatory bowel disease. Keratins have been implicated in the processes of cell death, epithelial integrity, response to inflammation and as a result are often described as guardians of the colonic epithelium. Keratin proteins carry extensive post-translational modifications, the cofactors for kinases, acetyl transferases and other modification-regulating enzymes are themselves products of metabolism. A cluster of studies has begun to reveal a bidirectional relationship between keratin form and function and metabolism. In this paper we hypothesize a mechanistic interaction between keratins and metabolism is governed through regulation of post-translational modifications and may contribute significantly to the normal functioning of the colon, placing keratins at the centre of a nutrition-metabolism-health triangle.

## 2. Architecture of the colon epithelium, expression of keratins

The human colon is lined with several million crypts with an estimated cellularity of 1,500-4,600 cells [1]. The entire colon surface, including the crypt walls, is covered in a simple epithelium conferring a physical barrier regulating and determining update of molecules and excluding

unwanted agents from the host system, and in secreting a mucus layer to protect against mechanical damage. Crypts have stem cells at the base, a proliferating compartment with dividing cells accounting for nearly half of the total cells of the crypt: dividing cells differentiate into enterocytes which fulfill both absorptive and barrier functions of the mucosa. There is a continuous and tightly regulated turnover of cells, from birth in the stem cell compartment to detachment and anoikis at the flat mucosa, which takes around 3-4 days in the human gut.

As with other epithelia, keratin expression is a characteristic of the colonic mucosa, the reader is referred to an exceptionally thorough review of research on intestinal keratins as the gold standard review [2]. In brief the critical keratins expressed in the colon mucosa in humans are keratin 8 (K8), keratin 18 (K18) , and keratin 19 (K19). Several reports, primarily based on mouse data, suggest each of these principal keratins are expressed equally at all regions of the mucosa. Our data in human colonic sections suggest a more variable distribution for K8, with expression consistently observed at the crypt mouth and flat mucosa, but with extent of expression in the crypt (depth) variable[3, 4], shown in **Figure 1**. The differences between mouse and human findings may partly be accounted for by species differences and differences in technical approach (our studies used formalin-fixed tissue and may have been at lower sensitivity than the cryostaining approach used in some studies), however the intra-crypt gradation of K8 expression and variability between individuals may offer insights into wider potential activities and impacts of keratin profile in the colon.

Emerging data are beginning to show that several factors, including nutritional and inflammatory state may influence the expression patterns observed for keratin in the gut mucosa. The interaction between keratin, inflammation, nutrition and metabolism is the focus of this review.

## 3. Nourishing the colonoctye: Glucose and SCFA uptake and metabolism, potential relationship to keratin expression

Colonocytes may access a number of different sources of metabolic energy: the mucosa is supported by the systemic circulation and can access glucose through basolateral GLUT-1 receptors. Additionally, microbiomic fermentation of fibres and other dietary residues yields high levels (1-50mmol/l) of short-chain fatty acids (SCFAs), principally acetate, propionate and butyrate. Of these butyrate has been shown to be a critically fuel source for colonocytes[5] which derive energy via uptake through MCT1 followed by β-oxidation[6]. Butyrate levels (or the ability to metabolize butyrate) associate with the length and cellularity of colon crypts [7] and have been inferred as contributing to the nutripreventive effect of dietary fibre [8].

Our research has used both *in vitro* and human studies to identify a relationship between butyrate and keratin level in colonocytes. We reported two *in vitro* studies[9, 10] showing that treatment of colon cancer cell lines with physiological levels of butyrate led to an elevation of K8.

In tissue samples with linked metadata on faecal butyrate, we saw an inverse relationship between butyrate and K8 staining in cancer tissue, but this relationship was not observed in paired samples from the same patients taken at the macroscopically normal mucosa. In contrast, in earlier stages of colorectal carcinogenesis, again in human samples, we observed a positive relationship between faecal butyrate and expression of K8 in macroscopically normal mucosa [4]. We also noted that following resection there was an increase in keratin expression, implicating interplay between disease state, possibly mediated via inflammation [11]. The elevations observed in keratin in these models could be hypothesized to be due to altered expression as butyrate is a potent regulator of transcription [12]. Turnover of keratin may be further mediated through change in post-translational modification: increased acetylation (e.g. in response to SCFA) will block ubiquitination-scheduled proteolysis of keratins (see section 5).

**Keratins, Intermediate filaments and the cellular metabolic state**

The colonocyte depends on butyrate, produced by luminal metabolism of fibres, for energy derived through β-oxidation [5, 6]. Stem cells, located at the base of the colonic crypt, are thought not to metabolise butyrate but rather to depend on glycolytic metabolism of glucose for energy [13]. An emerging literature implicates keratins as not only the functional guardians of the colonic epithelium, providing structural resilience [2] but as closely and functionally linked to the metabolic status of the cell.

Glucose is taken up via GLUT providing intracellular glucose to enter glycolysis, yielding energy and pyruvate In turn pyruvate can enter mitochondrial oxidative metabolism or be converted to lactate and exported via monocarboxylate transporter (MCT1). Butyrate (specifically, not propionate or acetate) has been shown to upregulate pyruvate kinase, the exit reaction from glycolysis[14]. Intriguingly, MCT1 is also the principal uptake route for butyrate (and other short-chain fatty acids) [5]. Butyrate enters the β-oxidation cycle yielding acetyl-coA (also the product of pyruvate dehydrogenase) which can be consumed by the tricarboxylic acid (TCA) cycle or converted to ketone bodies by HMGcoA Synthetase (HMGCS2). Mitochondria themselves are directly tethered to the cytoskeletal network, in the case of intermediate filaments (IFs) via trichoplein (TCHP1), which itself is a governor of mitochondrial motility[15]. These interrelationships are summarized in **Figure 2**.

Studies using keratin knockout models have revealed reciprocity between cellular architecture and metabolism. $K8^{-/-}$ mice exhibited features of metabolic perturbation in the colonic mucosa [16]: MCT1 expression was reduced, HMGCS2 response was blunted and levels of faecal SCFA were elevated. Taken together these data suggest that less butyrate is absorbed and metabolised through to ketone bodies, implying perturbation of normal mucosal energy harvesting. Using $K8^{-/-}$ in hepatic cells and hepatocellular carcinoma cells, Matthew [17] described elevated levels of

glycolysis . This was not explained by increase glucose uptake as glucose transporter (GLUT) GLUT1 expression was unchanged, however hexokinase activity was markedly increased. In accordance with Helenius' findings about impaired mitochondrial metabolism, elevated glycolysis was not linked to increases in oxidative metabolism, and there was an increase in lactate export. In a different cellular model, K17 knockdowns did elevate the level of GLUT family proteins [18], whereas $K8^{-/-}$ in β–cells [19] and global ablation of the keratin gene cluster in embryonic epithelia [20] each led led to cytoplasmic of GLUT accumulation arising from mislocalisation. A complementary approach to analysis of this bidirectional relationship comes from assessment of the impact of mitochrondrial knock-outs. Ohmeier showed that ρ0 cell exhibited collapsed IFs with perinuclear clustering of filament stumps [21]. Targeted analysis showed that inhibition of Complex IV and reduction in ΨM both led to IF collapse. Intriguingly microtubules also collapsed, but microfilaments remained intact.

In addition to impaired metabolism, several recent studies have highlighted a role for IF at the level of cytoplasmic organisation. Specifically analysing the effect of keratin mutations and impairement in epithelial cells, Schwarz & Leube [22] noted IF-impairing keratin mutation was associated with perinuclear localisation of mitochondria [23], and that $K8^{-/-}$ knockout lines exhibited malformed mitochondria [24]. Keratin depletion is notably associated with impaired lipid metabolism, potentially through reducing ER-mitochondrial interaction through TCHP1[25]. Schwarz and Leube concluded there is significant evidence for a role for IF in orchestrating metabolism through correct positioning and structuring of the mitochondria [22].

Taken together these data suggest that there is close linkage between keratins / IF structure and function and the metabolic state of the cell. IFs are essential for mitochondrial organization and stability, thereby supporting oxidative phosphorylation. In the specific context of the fuel economy of the colonocyte, the correct organisation of IFs supports β-oxidation of the primary energy source (butyrate) as well as management of the outputs of its oxidation through ketogenesis. In contrast, impairment of keratin structure and function may lead to greater dependence on glucose and glycolytic metabolism for energy generation. In the case of early carcinogenesis, the impact of lowered keratins on a shift to glycolytic metabolism is also characteristic of the Warburg-like state associated with the cancer phenotype in transformed cells [26]. Glucose flux is higher in colorectal cancer relative to normal tissues [27] requiring increased glucose transport and glycolytic rate [28]. A small proportion (2-5%) of glucose enters the hexosamine biosynthesis pathway (HBP), which shares the first two steps in common with glycolysis, diverging at fructose-6-phosphate (F6P). The rate-limiting enzyme of the HBP pathway is GFAT (glutamine: fructose-6-phosphate amidotransferase), which utilizes glutamine that enters the cell. The HBP regulates the pentose phosphate pathway, glutamine and glucose uptake and is proposed to function as bioenergetic and metabolic sensor [29]. Hyperglycaemia

has been linked to colon cancer malignancy [30], attracting interest as a novel therapeutic target [29, 31]. Critically, the small molecule products of these metabolic cascades (acetyl co-A, ATP, UDP-GlcNAc) are themselves co-factors in the post-translational modification of proteins including cytoskeletal components and metabolic enzymes.

## 4. Post-translational modifications – the link between nutrition, metabolism and function

Keratins are highly post-translationally modified proteins that are subject to a range of both PTM types (**Table 1**) and sequence variation (**Table 2**). Sequence and PTM variants are encompassed by the term proteoforms [32]. PTMs regulate fundamental cell processes such metabolism, transcription and cellular signaling pathways. These modifications are dynamic and reversible due to the action of regulatory classes of enzymes, which enable fine and tunable control (**Table 3**). Keratin PTMs include phosphorylation, O-glycosylation, acetylation, methylation, ubiquitination and SUMOylation as reviewed by [33, 34]. This set of PTMs is in general the most extensively studied in a range of proteins and cell systems, supported by technical developments directed to their analysis and reflective of technical challenges in this area including the low stoichiometry of PTM occurrence [35] . There are likely more proteoforms to be discovered as new methodologies are developed to analyse more challenging-to-study PTMs.

PTMs identified for K7, K8, K18, K19 and K20 are listed in **Table 1**. PTM data generated from low throughput (LTP) methods (classical biochemical methods such as amino acid sequencing, site-directed mutagenesis, immunoprecipation with keratin specific antibodies) identified phosphorylation and a specific form of O glycosylation, addition of O-GlcNAc at key regulatory serine residues located in the head and tail domains of K8 and K18. LTP approaches identified 6 lysine acetylation sites for K8 and 1 for K18 [36, 37]. This is high confidence site assignment with manual verification of peptide sequence and PTM site assignment. High throughput methods (HTP) – couple unbiased discovery-mode mass spectrometry to pre-enrichment of specific PTM subtypes. HTP has confirmed LTP PTM sites and revealed previously unknown PTM sites including phosphorylation of tyrosine residues, lysine methylation, ubiquitination and SUMOylation in a range of epithelial cell types including breast and squamous cancer as well as cells of the digestive system: liver, pancreas and colon. It is likely that different proteoforms (sequence and PTM variants) have cell type and context specific in addition to some common roles in regulation of keratin function, for example the formation of keratin-containing Mallory Denk hepatic inclusion bodies [38] .The literature on keratin PTM would benefit from mining to establish PTM determined in colorectal cells *in vitro* and *in viv*o to identify relevant (independently verified) PTM sites. This is challenging given that the data encompasses 100s of sites, many from HTP cataloging experiments with few from in depth analysis afforded by LTP approaches utilising in depth biochemical analysis [4, 11, 33, 36]. Known PTM sites and the enzymes associated with site

specific PTM regulation, that are curated in iPTMnet, are shown in **Table 4**. While not listed in iPTMnet, there is evidence for the citrullination, also known as deimination, PTM of K8 in the breast cancer MCF-7 epithelial cell line and for K7 in colorectal liver metastasis [39]. The putative demination sites in the region remains to be assigned in human K8 in colon tissues, but is of potential interest given the recent association of demination with inflammation and fibrosis in IBD [40] as well as colorectal cancer associated liver metastasis [41].

***4.i Associations between nutritional state and keratin PTM status***

Donor molecules for the major identified PTM sites phosphorylation, acetylation and O-glycosylation are key metabolites of central metabolism. They are generated by nutrient utilisation: Adenosine triphosphate (ATP), Uridine diphosphate N-Acetyl Glucosamine (UDP-GlcNAc) and acetyl CoA respectively. ATP is generated by mitochondrial fatty acid β-oxidation and also by oxidative phosphorylation. ATP is link between energy producing and energy requiring processes, trapping free energy in the form of a 'high energy bonds' in the terminal phosphate. Acetyl CoA can be derived from both the major energy sources in the colon; butyrate and glucose, via mitochondrial fatty acid β oxidation and glycolytic pathways respectively. Acetyl CoA is also generated during protein catabolism. UDP-GlcNAc is synthesized from fructose 6-phosphate, glutamine, acetyl-CoA, and UTP and is the end product of the HBP pathway. The levels of ATP, acetyl-coA and UDP-GlcNAc reflect the metabolic status of the cell. Both Acetyl-coA and UDPGlcNAc have been proposed as nutrient sensing mechanisms associated with K8/K18 IF function, consistent with the role of K8/18 in metabolic homeostasis [16] and interestingly both are associated with altered PTM status and PTM cross talk [42-44] . Assembly of IFs *in vitro* occurs spontaneously, but this finding shows that an ATP-dependent mechanism regulates the initial steps of assembly *in vivo*, proposed to occur at the level of PTM [45].

***4.ii The functional significance of keratin PTMs***

While some PTM are assigned biological functions in regulating the function of K8/K18, the biological functions of the majority of PTM sites remain to be determined. It is likely that K8/K18 function is regulated by alteration of PTM status and specific proteoforms described as the Histone code [46] , Tubulin code [47] and most recently the 'PTM switchboard' for Notch protein [48]. Notch protein is of particular interest since K8/K18 interact with Notch1 and regulate Notch1 signalling activity during differentiation of the colonic epithelium [49], in turn Notch signalling interplays with Wnt signalling [50] which is subject to regulation by butyrate [51]. The terms 'code' and 'switchboard' refer to specific proteoform(s) regulating protein function and protein-protein interaction.

What is known about K8/K18 PTMs? The key effects are on the dynamics of K8/K18 filament assembly and disassembly. IFs formed by keratins release non-filamentous subunits, which are

reused in the cell periphery for filament assembly in a process that recycles existing keratin pools independent of a requirement for protein synthesis. The data suggest that assembly for keratins occurs from a pre-existing keratin pool that is either of significant size or continuously replenished [52]. The process is complex (see [53] for review). The keratin-filament cycle of assembly and disassembly is a major mechanism of intermediate-filament network plasticity [52] and subject to regulation by PTM at specific PTM sites [33, 34] to effect solubilty, stability and filament assembly. PTMs include cleavage products and caspase mediated cleavage K18 [54] is associated with breakdown and reorganisation of IFs during apoptosis [55] (see **Table 3**).

### *4.iii Cross-talk between keratin PTMs*

More than one modification can also occur on the same amino acid residue, which enables additional control via PTM crosstalk. . The list of overlapping PTM sites and the types of PTM are listed for K8 and K18 in **Tables 5, 6** respectively. The presence of overlapping PTMs suggest potential for 'competitive cross talk', for example, ubiquitination, acetylation, methylation and SUMOylation mainly occur on lysine residues, as exemplified for mitochondrial enzymes. [56]. Such overlapping sites occur at lysine (Lys) for K7, K18, K19 but not K20. K8, K18, have overlapping O-glycosylation and phosphorylation sites on serine residues located in the N terminal head domains (K8: pSer13, pSer15; K18: pSer30, pS31, pSer49) with additional sites in the C terminal tail domain of K8 (pSer475). K19 lacks a tail domain but can form a functional heterodimer with K8 *in vitro* [57]. The list of overlapping PTM sites and the types of PTM are listed for K8 and K18 in **Tables 5, 6** respectively. There is evidence for reciprocal interactions of phosphorylation and O-GlcNAc exemplified by pSer52 and O-GlcNAc-Ser48 on K18 [58]. PTM cross talk can also occur, with K8 acetylation of Lys207 affecting phosphorylation at distal phosphorylated residues pSer74 [42]. It is interesting in this regard that K8/K18 can act as a "phosphorylation sponge" for stress-activated kinases [59] when perturbations in ATP level might impact on other catabolic pathways [60] or hypothetically alter the equilibrium for kinase and phosphatase reactions.

In terms of acetylation sites, our previous data identified 5 lysine acetylation sites present in the insoluble IF fraction. These correspond to Lys-11, Lys-101, Lys-393, Lys-472 and Lys-483 of the full length sequence, and were reported at the -1 position to reflect removal of the N terminal methionine. Acetylation of K8 is associated with depolymerization of filaments, and is associated acetylation of Lys-483 at the C-terminus in the tail domain (Drake et al. 2009). Of the four other K8 acetylation sites, Lys-11 and Lys-472 occur predominantly outside the coiled-coil domains, Lys-101 and Lys-393 are located near the ends of the coiled-coil domains. An analysis of the global acetylome identified six acetylation sites within the coiled coil domain, present in the soluble cell fraction [61]. Lys-117, Lys-207, Lys-295, Lys-325, and Lys-347), all located within the coiled-coil rod domain region of the protein [62]. Interestingly, all of these acetylated lysine sites (identified

in the insoluble IF fraction and soluble cell fractionation are subject to overlapping PTM: one of more of ubiquitination, SUMOylation and methylation PTM (**Table 5**). The specific location of the PTM and overlapping PTM sites is of significance given the structural features of keratins.

The N terminal "head" and C-terminal "tail" domains are each non α-helical, located on either side of a central α-helical, coiled-coil forming "rod" domain. The rod domain is highly conserved between keratins with a 7-residue (heptad), and 11-residue (hendecad) repeat pattern along the ~310 amino acid sequence. The rod domain has four regions, coil 1A, coil 1B, coil 2A, and coil 2B, separated by short unstructured linker regions - L1, L12, and L2 [63]. The head and tail regions are variable and distinct for each keratin. Structure function analysis using recombinant human K8 and K18 has been used to study their solubility and assembly properties *in vitro*; establishing that the elongation reaction of individual unit length filaments proceeds via head-to-tail association of the first three heptads of the coil 1A dimer with the three heptads of the coil 2B dimer [64]. Study of interactions between representative K8/K18 peptides by Hydrogen Deuterium Exchange (HDX) mass spectrometry, suggests there are localized unique structural elements that undergo stabilization in tetramer to filament formation: the N-terminus of coil 1A, coil 2B centre and the C-terminus of coil 2B [65]. These data represent information derived from unmodified keratins. It is interesting to note that overlapping PTM at lysine sites for K8 and K18 include (but are not exclusive to) residues in the regions identified of interest by the HDX analysis (**Tables 5,6**) Where competing PTMs have distinct regulatory roles, we speculate that modifiable residues in question may be pinch points for fine tuning organisational regulation or response to cellular metabolic state in the context of the colonic crypt.

## 5. Potential value of PTMS as Biomarkers

Circulating serum K18 levels is a biomarker of malignancy and levels are associated with adverse clinical outcome, with higher serum levels of the pro-inflammatory CXCL8 cytokine reflective of systematic inflammation in colorectal cancer [66, 67]. The body of literature suggests that it is not just alteration keratin levels that have biomarker potential: PTM status and proteoforms are also of value as biomarkers of nutritional status and of early disease stage. The *in vitro* studies cited above demonstrate that K8 expression is responsive to butyrate at cellular level. This was confirmed in a human cross-sectional study by comparing K8 expression using bottom-up proteomics in samples from subjects with high (~13mmol/l) versus low (~1.4mmol/l) butyrate[4]. The data additionally showed a prevalent higher $M_w$ form of K8 was present at higher butyrate concentrations only. An open label intervention with high fibre showed the potential to restore KRT8 levels following an 8 week intervention. Additionally, selenium status associates with elevated levels of other KRT family proteins (K9,10,19) in top-down proteomic analyses of the soluble proteome, reinforced with microarray analyses[68]. Taken together these data suggest that at least two nutripreventive nutrients (fibre, selenium) exert an effect via modulation of KRT

expression. Research has additionally show that alterations in keratin expression and proteoforms occur in the colorectal mucosa in lesion-prone tissue and potentially in protection against remission. Our analysis of the soluble proteome in the macroscopically normal rectal mucosa of patients with a cancer using top-down approaches (2DGE/MALDI) revealed 7 different mass/charge variants of K8 downregulated in cancer [69]. An independent series where we analysed the soluble and insoluble proteomes using bottom-up approaches (iTRAQ) confirmed global downregulation of KRT8 in both fractions, with orthogonal validation by western blot [4]. The western blot also confirmed the existence of multiple proteoforms with around 5 separable in 1D gel systems [*ibid*.] each of which showed differing degrees of ser431 phosphorylation according to both nutritional and disease status. In a separate study we noted multiple proteoforms in patients with IBD, and that patients with longstanding remission expressed higher levels of K8 than patients with relapsing, aggressive or recent onset IBD [11]. Taken together these data show that K8 exists in multiple proteoforms, the balance of which associates with health or disease state, however questions remain on the nature and function of these forms.

## 6. Conclusions and Future perspectives

Data emerging of the last decade imply a close relationship between the nutritional and metabolic status of the colonocyte and keratin IF structure and organisation with the effect being bi-directional. Keratin levels are altered in association with difference in nutritional and inflammatory status, and in turn alterations in keratin may impact upon the metabolic status of the cell, making it more or less Warburg-like. Post-translational modifications of the keratin protein backbone may form the mechanistic link between nutrition, metabolism, inflammation and IF organisation. As such future opportunities and priorities for research may be around characterising at biochemical and functional level key proteoforms in order to assess their impact of proteoform balance on function. Such work may yield new antigens to evaluate as potential biomarkers of gut health.

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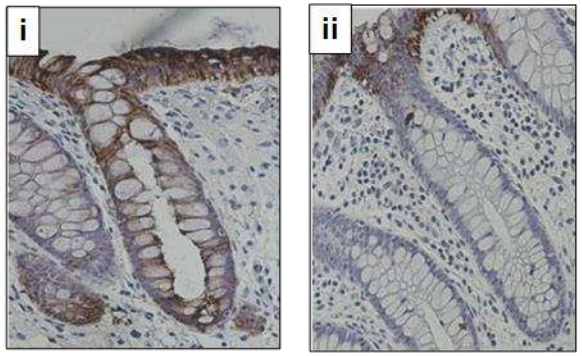
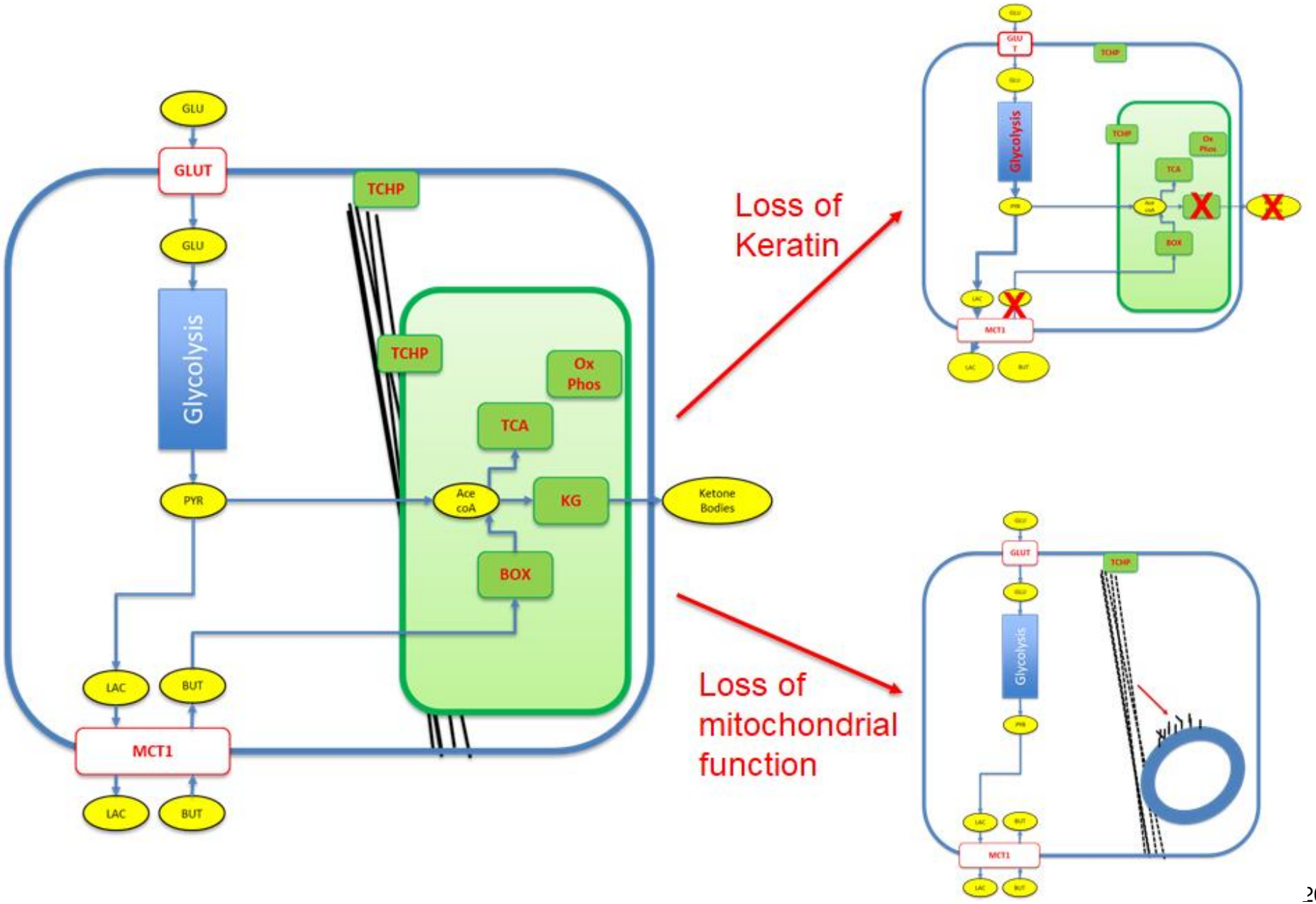



**Figure 1 Heterogeneity if K8 expression in human colon crypts**

Images of K8 staining in colon crypts from the same clinical study. Sections are from the macroscopically normal mucosa in patients with patients with an adenoma. There is inter-patient variation in staining intensity at the flat mucosa and within crypts, and in depth of staining in crypts: (i) shows positive staining for KRT8 to the bottom of the colon crypt; in contrast (ii) shows positive staining reaching only just below the crypt mouth.

Images reproduce from our own previous work [3,4].


GLU
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Glycolysis
PYR
LAC
BUT
MCT1
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BUT
TCHP
TCHP
Ox Phos
TCA
Ace coA
KG
BOX
Ketone Bodies
Loss of Keratin
Loss of mitochondrial function

**Figure 2 – Interplay of Intermediate Filament organisation and metabolic status**

*The usual status of colonocytes is shown in the left panel where mitochondria (in green) are tethered to the IF network (Black lines) via TCHP. Apical MCT1 imports butyrate which is transported to the mitochondria, metabolised via –oxidation yielding acetyl-coA, which may be exported to form ketone bodies. In cell models where keratin is lost, uptake of SCFA via MCT1 is reduced as is formation of ketone bodies, in compensation uptake of glucose and glycolysis are increased. In models where cells are cured of mitochondria, there is collapse of the IF network to residual perinuclear bodies.*

**Table 1. PTM sites identified on Keratins 7, 8, 18, 19, and 20 indicating the number of sites and the number of PTM**:

Some sites can be modified by multiple PTM at the same site. The data were derived from iPTMnet integrates information identified by at least one study from the UniProt, PhosphoSite, HPRD and Phospho.ELM curated databases.

| Keratin | Sites | Number of PTM | Overlapping PTM sites/ amino acid | N-acetyl (% number of PTM) | Acetyl K (% number of PTM) | Phosphorylation (% number of PTM) | 0-GlcNAc (% number of PTM) | Ubiquitination (% number of PTM) | Sumo (% number of PTM) | Methylation (% number of PTM) |
|---|---|---|---|---|---|---|---|---|---|---|
| K7 | 78 | 92 | 11; K | 0 | 12 | 50 | 0 | 20 | 4 | 14 |
| K8 | 127 | 163 | 24; K, S | 0 | 12 | 56 | 2 | 17 | 6 | 7 |
| K18 | 88 | 117 | 17; K, S | 2 | 11 | 51 | 3 | 15 | 9 | 10 |
| K19 | 79 | 86 | 5; K, S | 0 | 2 | 66 | 0 | 16 | 3 | 12 |
| K20 | 18 | 18 | None | 0 | 0 | 83 | 0 | 6 | 0 | 11 |

**Table 2. Amino acid sequence variants and Colorectal cancer (CRC) associated mutations**.

The data were retrieved from UniProt protein sequence database 27.1.21

| Keratin | Proteoforms (sequence variants) | Disease associated PTM mutations |
|---|---|---|
| KRT7 | P08729 | Methylation; R77 -> Q77 |
| KRT8 | P05787; P05787-1; P05787-2 | Phosphorylation: S31->Y31; S31->A31; T468-> A468 Biomuta; DOID:9256 / colorectal cancer G62C, I63V and K464N Inflammatory Bowel Disease [70] |
| KRT18 | P05783; P05783 Met-1 | Phosphorylation S34->R34; Methylation site R50->C50 Biomuta; DOID:9256 / colorectal cancer |
| KRT19 | P08727 | Methylation; R280 ->Q280 Biomuta; DOID:9256 / colorectal cancer |
| KRT20 | P35900 | None |

**Table 3. Major Keratin PTM, enzymes associated with addition and removal, details of the PTM.**

* denotes that the lysine acetylation sites were reported at the -1 position accounting for N terminal cleavage at the initiator methionine.

| PTM | Residue | Enzymes | Chemical modification | Function |
|---|---|---|---|---|
| Acetylation | K<br>N terminal amino acid | Lysine Acetylases/Acetylases | Addition of an acetyl group | Regulation of keratin stability. Acetylation at specific sites (Lys-11, Lys-472, and Lys483*) were increased in response to butyrate, associated with keratin filament breakdown [9]. |
| Phosphorylation | STY | Kinase/Phosphatase | Addition of a phosphate group | Phosphorylation on Ser and Thr residues (p-Ser/Thr) typically promotes disassembly of filaments into ULF and increases IF protein solubility and triggers triggers reorganization of the keratin filament K8, K18, K19 [71, 72] |
| O-GlcNAc | ST | O-GlcNAc transferase/O-GlcNAcase | Addition of beta linked N-Acetylglucosoamine (GlcNAc) | Keratin – O-GlcNac regulates the K8/K18 mediated signalling for example protein kinase C and Akt mediated cell survival signalling |

| | | | | |
|---|---|---|---|---|
| | | | | [73].Protects liver cell and pancreatic cells [74] .<br><br>Associated with keratin solubility, stability and filament organisation [75]. |
| | | | | |
| Ubiquitination | K | Ubiquitin-activating enzymes, ubiquitin conjugating enzymes, ubiquitin ligase/deubiquitinases | linkage of one molecule of ubiquitin to a protein, known as mono-ubiquitination. Additional molecules can be attached to any of the seven lysine residues or the N-terminus of the ubiquitin molecule to form ubiquitin chains, resulting in polyubiquitination. | Ubiquitylation and SUMOylation use similar enzymes for conjugation and deconjugation. Both have a similar 3D structure termed the beta -grasp fold [76] .<br><br>SUMO and Ubiquitin functions are unique - they cannot compensate for each other. They are not mutually exclusive for a protein [77]<br><br>Ubiquitination is associated with targeting for proteasomal degradation. SUMO is not generally associated with protein |
| SUMOylation | K | Ubiquitin-activating enzymes, ubiquitin conjugating enzymes, ubiquitin ligase/deubiquitinases | SUMO (small ubiquitin-related modifier) proteins are ∼10-kD polypeptides | |

| | | | | |
|---|---|---|---|---|
| | | | | degradation but is implicated in protein–protein interactions, protein activity, and protein subcellular localization.<br><br>Keratin solubility is regulated by sumoylation in liver epithelial cells [78]<br><br>A potential functional role for sumoylation of K8 is indicated by data from genetic deletion of SUMO E2 enzyme Ubc9 in mice; defects include stem cell depletion in the crypts of the small intestine and fragile enterocytes [79]. |
| Methylation | K,R | Methylases, demethylases | Addition of one or more methyl groups | Methylation of the liver disease-associated variants of K8/K18 regulate keratin protein stability. K8-Arg47 and K18-Arg55 as methylation sites. Keratin mutation (Arg- |

| | | | | |
|---|---|---|---|---|
| | | | | to-Lys/Ala) results in decreased stability [80] |

**Table 4: Post-translational modification identified on Keratins expressed in colonic epithelial cells and associated enzymes for which they have been identified as substrates**

The PTM information was retrieved using the iPTMnet web based tool [81].
tool, date of access 27 January 2021 https://research.bioinformatics.udel.edu/iptmnet/ and Leech et al., 2008. Note that HDAC 5 was identified as associated with the insoluble fraction of HCT116 colon cancer cells, in a study identifying acetylation sites in K8 and K18 (Leech et al., 2008).

| *Keratin* | *PTM residue* | *Specific PTM site and associated kinase (Residue, name and UniProt accession number)* |
|---|---|---|
| KRT7 | K, S, T, Y, R | None identified to date |
| KRT8 | K, S, T, Y, R | T6: Aurora kinase B (Q96GD4).<br><br>S9, S13, S24, S34, S37, S43, S417*: cAMP-dependent protein kinase catalytic subunit alpha (P17612)*;<br><br>S74: Dual specificity mitogen-activated protein kinase kinase 2 (P36507), Mitogen-activated protein kinase 13 (O15264), Protein kinase C delta type (Q05655), Mitogen-activated protein kinase 12 (P53778), Mitogen-activated protein kinase 14 (Q16539), Mitogen-activated protein kinase 11 (Q15759), Mitogen-activated protein kinase 8 (P45983), Dual specificity mitogen-activated protein kinase kinase 1 (Q02750);<br><br>T431: Mitogen-activated protein kinase 1 (P28482);<br><br>S432: Mitogen-activated protein kinase 3 (P27361), Cyclin-dependent kinase 1 (P06493), Mitogen-activated protein kinase 8 (P45983)<br><br>S432, S74: Mitogen-activated protein kinase 8 (P45983)<br><br>Lysine acetylation: HDAC 5 was identified as associated with the insoluble fraction of HCT116 cells, in a study identifying acetylation sites in K8 and K18 (Leech et al., 2008).<br><br>SIRT2 is an NAD+ (nicotinamide adenine dinucleotide)-dependent deacetylase regulating K8 acetylation [42] |

| | | |
|---|---|---|
| KRT18 | K, S, T, Y, R, and N terminal M | S34: Cyclin-dependent kinase 1 (P06493);<br><br>S53*: cAMP-dependent protein kinase catalytic subunit alpha (P17612)*, Ribosomal protein S6 kinase β-2 (Q9UBS0), MAP kinase-activated protein kinase 2 (P49137), Protein kinase C epsilon type (Q02156), Ribosomal protein S6 kinase alpha-3 (P51812), Calcium/calmodulin-dependent protein kinase type II subunit alpha (Q9UQM7), Calcium/calmodulin-dependent protein kinase type 1 (Q14012)<br><br>Lysine acetylation: HDAC 5 was identified as associated with the insoluble fraction of HCT116 cells, in a study identifying acetylation sites in K8 and K18 [36] |
| KRT19 | K, S, T, Y, R | S35: RAC-alpha serine/threonine-protein kinase AKT1 (P31749);<br><br>Y391: Proto-oncogene tyrosine-protein kinase Src (P12931) |
| KRT20 | K, S, T, Y, R | S13: Protein kinase C alpha type (P17252) (PRKCA), MAP kinase-activated protein kinase 2 (P49137) |

**Table 5. Overlapping PTM sites in Keratin 8.** The region that the PTM site is noted and the data source curated by iPTMnet.

| Keratin 8 PTM Site | PTM | Region | Source |
|---|---|---|---|
| K8 | Acetylation | Head | PhosphoSitePlus |
| K8 | Methylation | Head | PhosphoSitePlus |
| K8 | Ubiquitination | Head | PhosphoSitePlus |
| K11 | Acetylation | Head | PhosphoSitePlus |
| K11 | Methylation | Head | PhosphoSitePlus |
| K11 | Sumoylation | Head | PhosphoSitePlus |
| K11 | Ubiquitination | Head | PhosphoSitePlus |
| S13 | O-Glycosylation | Head | PhosphoSitePlus |
| S13 | Phosphorylation | Head | neXtProt PhosphoSitePlus UniProt |
| S15 | O-Glycosylation | Head | PhosphoSitePlus |
| S15 | Phosphorylation | Head | PhosphoSitePlus UniProt |

| | | | |
|---|---|---|---|
| K101 | Acetylation | Coil 1A | PhosphoSitePlus |
| K101 | Methylation | Coil 1A | PhosphoSitePlus |
| K101 | Sumoylation | Coil 1A | PhosphoSitePlus |
| K101 | Ubiquitination | Coil 1A | PhosphoSitePlus |
| K108 | Acetylation | Coil 1A | PhosphoSitePlus |
| K108 | Ubiquitination | Coil 1A | PhosphoSitePlus |
| K117 | Acetylation | Coil 1A | PhosphoSitePlus |
| K117 | Ubiquitination | Coil 1A | PhosphoSitePlus |
| K122 | Acetylation | Coil 1A | PhosphoSitePlus |
| K122 | Methylation | Coil 1A | PhosphoSitePlus |
| K122 | Ubiquitination | Coil 1A | PhosphoSitePlus |
| K130 | Acetylation | Linker 1 | PhosphoSitePlus |
| K130 | Sumoylation | Linker 1 | PhosphoSitePlus |
| K130 | Ubiquitination | Linker 1 | PhosphoSitePlus |
| K158 | Acetylation | Linker 1 | PhosphoSitePlus |
| K158 | Ubiquitination | Coil 1B | PhosphoSitePlus |
| K178 | Methylation | Coil 1B | PhosphoSitePlus |
| K178 | Ubiquitination | Coil 1B | PhosphoSitePlus |
| K185 | Methylation | Coil 1B | PhosphoSitePlus |
| K185 | Ubiquitination | Coil 1B | PhosphoSitePlus |
| K197 | Sumoylation | Coil 1B | PhosphoSitePlus |
| K197 | Ubiquitination | Coil 1B | PhosphoSitePlus |
| K207 | Acetylation | Coil 1B | PhosphoSitePlus UniProt |
| K207 | Ubiquitination | Coil 1B | PhosphoSitePlus |
| K285 | Acetylation | Coil 2 | PhosphoSitePlus |
| K285 | Methylation | Coil 2 | PhosphoSitePlus |
| K285 | Sumoylation | Coil 2 | PhosphoSitePlus |
| K285 | Ubiquitination | Coil 2 | PhosphoSitePlus |
| K295 | Acetylation | Coil 2 | PhosphoSitePlus UniProt |

| | | | |
|---|---|---|---|
| K295 | Ubiquitination | Coil 2 | PhosphoSitePlus |
| K304 | Sumoylation | Coil 2 | PhosphoSitePlus |
| K304 | Ubiquitination | Coil 2 | PhosphoSitePlus |
| K325 | Acetylation | Coil 2 | PhosphoSitePlus UniProt |
| K325 | Sumoylation | Coil 2 | PhosphoSitePlus |
| K325 | Ubiquitination | Coil 2 | PhosphoSitePlus |
| K347 | Acetylation | Coil 2 | PhosphoSitePlus |
| K347 | Ubiquitination | Coil 2 | PhosphoSitePlus |
| K352 | Acetylation | Coil 2 | PhosphoSitePlus |
| K352 | Ubiquitination | Coil 2 | PhosphoSitePlus |
| K393 | Acetylation | Coil 2 | PhosphoSitePlus |
| K393 | Sumoylation | Coil 2 | PhosphoSitePlus |
| K393 | Ubiquitination | Coil 2 | PhosphoSitePlus |
| K465 | Acetylation | Tail | PhosphoSitePlus |
| K465 | Ubiquitination | Tail | PhosphoSitePlus |
| K472 | Acetylation | Tail | PhosphoSitePlus |
| K472 | Sumoylation | Tail | PhosphoSitePlus |
| K472 | Ubiquitination | Tail | PhosphoSitePlus |
| S475 | O-Glycosylation | Tail | PhosphoSitePlus |
| S475 | Phosphorylation | Tail | phospho.ELM PhosphoSitePlus UniProt |

**Table 6.** Overlapping PTM sites in Keratin 18. The region that the PTM site is noted and the data source curated by iPTMnet.

| Keratin 18 PTM site | PTM | Region | Source |
|---|---|---|---|
| S2 | N-Acetyl | Head | UniProt |
| S2 | Phosphorylation | Head | PhosphoSitePlus |
| S30 | O-Glycosylation | Head | PhosphoSitePlus |
| S30 | Phosphorylation | Head | HPRD phospho.ELM PhosphoSitePlus RLIMS-P |
| S31 | O-Glycosylation | Head | PhosphoSitePlus |
| S31 | Phosphorylation | Head | HPRD PhosphoSitePlus |
| S49 | O-Glycosylation | Head | PhosphoSitePlus |
| S49 | Phosphorylation | Head | HPRD PhosphoSitePlus UniProt |
| K81 | Acetylation | Coil 1A | PhosphoSitePlus |
| K81 | Sumoylation | Coil 1A | PhosphoSitePlus |
| K81 | Ubiquitination | Coil 1A | PhosphoSitePlus |
| K118 | Methylation | Linker 1 | PhosphoSitePlus |
| K118 | Ubiquitination | Linker 1 | PhosphoSitePlus |
| K131 | Acetylation | Linker 1 | PhosphoSitePlus UniProt |
| K131 | Methylation | Linker 1 | PhosphoSitePlus |
| K131 | Ubiquitination | Linker 1 | PhosphoSitePlus |
| K167 | Acetylation | Coil 1B | PhosphoSitePlus |
| K167 | Methylation | Coil 1B | PhosphoSitePlus |
| K167 | Sumoylation | Coil 1B | PhosphoSitePlus |
| K167 | Ubiquitination | Coil 1B | PhosphoSitePlus |
| K187 | Acetylation | Coil 1B | PhosphoSitePlus |
| K187 | Methylation | Coil 1B | PhosphoSitePlus |
| K187 | Sumoylation | Coil 1B | PhosphoSitePlus |
| K187 | Ubiquitination | Coil 1B | PhosphoSitePlus |
| K207 | Sumoylation | Coil 1B | PhosphoSitePlus |

| | | | |
|---|---|---|---|
| K207 | Ubiquitination | Coil 1B | PhosphoSitePlus |
| K214 | Acetylation | Coil 1B | PhosphoSitePlus |
| K214 | Ubiquitination | Coil 1B | PhosphoSitePlus |
| K247 | Acetylation | Linker 12 | PhosphoSitePlus |
| K247 | Sumoylation | Linker 12 | PhosphoSitePlus |
| K247 | Ubiquitination | Linker 12 | PhosphoSitePlus |
| K317 | Acetylation | Coil 2 | PhosphoSitePlus |
| K317 | Sumoylation | Coil 2 | PhosphoSitePlus |
| K317 | Ubiquitination | Coil 2 | PhosphoSitePlus |
| K370 | Acetylation | Coil 2 | PhosphoSitePlus |
| K370 | Ubiquitination | Coil 2 | PhosphoSitePlus |
| K372 | Acetylation | Coil 2 | PhosphoSitePlus |
| K372 | Sumoylation | Coil 2 | PhosphoSitePlus |
| K372 | Ubiquitination | Coil 2 | PhosphoSitePlus |
| K417 | Acetylation | Tail | PhosphoSitePlus |
| K417 | Sumoylation | Tail | PhosphoSitePlus |
| K417 | Ubiquitination | Tail | PhosphoSitePlus |
| K426 | Acetylation | Tail | PhosphoSitePlus UniProt |
| K426 | Methylation | Tail | PhosphoSitePlus |
| K426 | Sumoylation | Tail | PhosphoSitePlus |
| K426 | Ubiquitination | Tail | PhosphoSitePlus |