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REVIEW

The Ku complex: recent advances and emerging roles outside of non-homologous end-joining

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Abstract

Since its discovery in 1981, the Ku complex has been extensively studied under multiple cellular contexts, with most work focusing on Ku in terms of its essential role in non-homologous end-joining (NHEJ). In this process, Ku is well-known as the DNA-binding subunit for DNA-PK, which is central to the NHEJ repair process. However, in addition to the extensive study of Ku's role in DNA repair, Ku has also been implicated in various other cellular processes including transcription, the DNA damage response, DNA replication, telomere maintenance, and has since been studied in multiple contexts, growing into a multidisciplinary point of research across various fields. Some advances have been driven by clarification of Ku's structure, including the original Ku crystal structure and the more recent Ku–DNA-PK$_{cs}$ crystallography, cryogenic electron microscopy (cryoEM) studies, and the identification of various post-translational modifications. Here, we focus on the advances made in understanding the Ku heterodimer outside of non-homologous end-joining, and across a variety of model organisms. We explore unique structural and functional aspects, detail Ku expression, conservation, and essentiality in different species, discuss the evidence for its involvement in a diverse range of cellular functions, highlight Ku protein interactions and recent work concerning Ku-binding motifs, and finally, we summarize the clinical Ku-related research to date.

Keywords Ku heterodimer · Ku-binding motifs · Telomere maintenance · Genome stability · Non-homologous end-joining · Cancer

Introduction

Ku has been implicated in a diverse range of functions including transcription, DNA replication, innate immunity, the DNA damage response, and telomere maintenance, many of which are mediated through Ku-protein and Ku–RNA interactions [1]. Ku has also been researched in less spotlighted functions such as nucleolar transcript regulation [2], Bax-mediated apoptosis [3], and viral double-stranded DNA detection [4]. Our understanding of Ku is constantly expanding, with new research advances suggesting the heterodimer is doing more than what we currently understand. Incidentally, Ku is highly abundant in human cells, having multiple processed pseudogenes and a Ku80 isoform, all of which imply that Ku functions in processes or regulatory capacities we have yet to identify.

While most proteins are first identified in model organisms prior to the identification of human homologs, Ku was first identified as an autoantigen in the serum of a Japanese patient [5]. Many of the advances made in Ku understanding followed the characterization of Ku70/80 knockout mice, which were radiosensitive, immunocompromised, and proportional dwarves [6]. Curiously, while Ku70/80 knockouts are viable in some species, the genes appear to be indispensable in humans and human cell lines [7, 8]. The essentiality of Ku in some species over others is still unclear.

Acquisition of the Ku crystal structure represented a significant milestone in our mechanistic understanding of Ku [9], including our knowledge on Ku–protein interactions. Structural studies also led to the recent identification of the Ku-binding motif (KBM), which allows proteins to interact directly with Ku [10, 11]. Related to the identification of Ku protein interactors, our lab recently used the new, high-throughput proximity-dependent biotin identification

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(BioID) technique [12] to comprehensively identify in vivo Ku protein interactors in human cells [13].

In this review, we provide an interdisciplinary discourse on the Ku heterodimer outside of its traditional role as a DNA repair protein involved in non-homologous end-joining (NHEJ) and V(D)J recombination. We focus on unifying the different aspects of Ku research that will be critical to further understand the heterodimer including its structure, function, cellular localization, expression levels, conservation, and essentiality.

Ku composition: structure, domains, and function

Ku, originally named after a Japanese patient with an autoimmune disease that recognized Ku as an autoantigen, is a protein heterodimer [5]. In humans, Ku is composed of two subunits, Ku70 (~ 70 kDa, 609 amino acids) and Ku80 (~ 80 kDa, 732 amino acids), which are encoded by the *XRCC6* (X-ray repair cross-complementing protein 6, located on chromosome 22) and *XRCC5* (chromosome 2) genes, respectively. Eukaryotic subunits Ku70 and Ku80 undergo domain swapping to form the stable, Ku heterodimer complex, resistant to separation unless subjected to high salt (i.e. 1.5 M NaCl, 1 M KCl) or strong, ionic detergents (i.e. 0.5% SDS, 1% sodium deoxycholate) [14, 15]. Dimerization appears to be important for the stability of both Ku70 and Ku80. In mice, the deletion of one subunit leads to severely reduced expression of the other subunit, implying that most Ku exists as an obligate heterodimer [6, 16]. Heterodimerization also seems to be a necessary prerequisite for successful Ku80 purification in bacteria [17]. However, researchers have been able to isolate Ku70, independent of Ku80 [17, 18], supporting the notion that Ku70 can form a homodimer. Indeed, Tadi et al. (2016) were able to produce a stable Ku70 homodimer in vitro using purified protein from both insect and bacterial cells [18]. However, while a fraction of cellular Ku70 may exist as a homodimer, to date, the Ku70 homodimer has yet to be identified or studied in an intracellular context.

The Ku heterodimer has been implicated in binding double-stranded DNA (dsDNA) [19] and facilitating the repair of double-stranded breaks (DSBs) through the NHEJ repair pathway [reviewed in [20]]. In 2001, the X-ray crystallography structures of Ku unbound and bound to DNA were obtained [9], representing a major milestone that enhanced our understanding of Ku70/80 dimerization and DNA association (Fig. 1). Ku70 and Ku80 share structural homology and dimerize to form a pseudo-symmetrical basket structure that encircles the DNA duplex [9]. Both subunits show a common topology consisting of three domains: a N-terminal α/β domain, a central β-barrel domain, and a subunit-specific helical C-terminal domain (CTD).

The N-terminal α/β domain of each Ku subunit is a divergent member of the von Willebrand factor A (vWA) family of domains [21], composed of a six-stranded β-sheet in a Rossman fold [9]. Disrupting the vWA domain in yeast Ku70/80 has been found to impair DNA repair and telomere regulation [22]. Although the amino edge of the vWA domain lies proximal to the DNA-binding groove, the vWA domain is not required for DNA binding. Instead, consistent with vWA domains in other proteins, the Ku vWA domains may mediate protein–protein interactions. For example, the Ku80 vWA domain has been shown to interact with APLF, a NHEJ repair protein essential for the recruitment of other repair factors [23].

Meanwhile, the central DNA-binding domain, composed of a seven-stranded antiparallel β-barrel, is essential for DNA binding and heterodimerization [9]. Heterodimerization

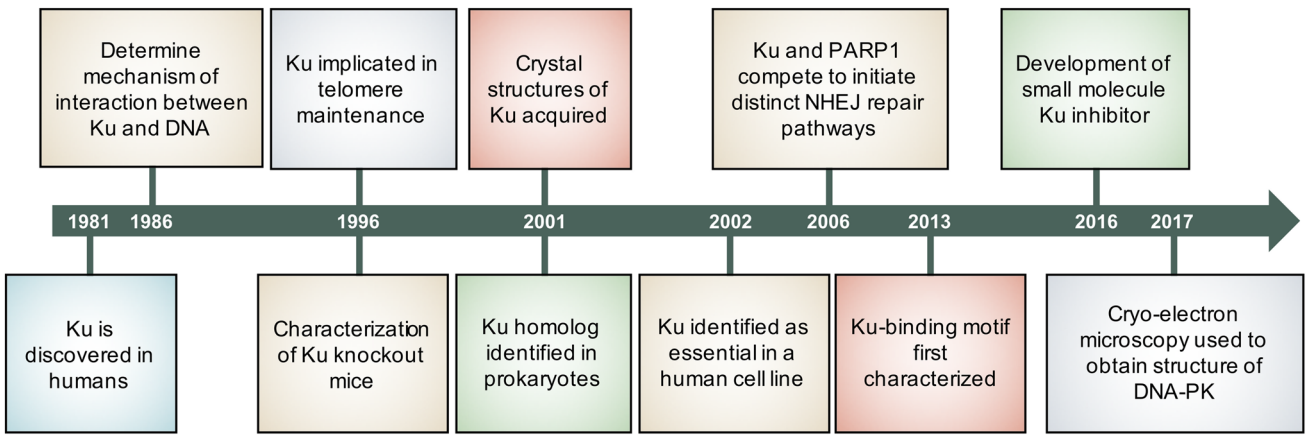

Fig. 1 Timeline of Ku research milestones. Notable developments over the last 40 years pertaining to various research aspects about the Ku70/80 heterodimer

results in a positively charged DNA-binding ring which fits sterically around the minor and major DNA grooves. Ku threads inwards on DNA similar to a nut threaded onto a bolt, with Ku70 positioned proximal and Ku80 distal to the DNA end [21, 24]. Ku slides onto DNA ends using an energy-free mechanism that is still poorly understood. No direct base contacts are made with DNA, implying that Ku associates with DNA in a sequence-independent manner [9]. Ku has a high affinity ($K_d = 10^{-9}$ M) for dsDNA ends, 5′ and 3′ overhangs or blunt ends, and a significantly lower affinity for circular DNA and single-stranded DNA ends [1].

Finally, the C-terminal Ku regions are unique to each subunit, however, both contain a flexible linker and a globular structural domain (Fig. 2). The Ku70 C-terminal region contains a putative DNA-binding domain called the SAP (SAF-A/B, Acinus, and PIAS) domain (5 kDa, residues 559–609) [9]. The C-terminal structure was determined both in isolation using NMR [25] and as part of the unbound Ku70/80 crystal structure [9], and these studies discovered a common helix-extended loop-helix structural motif. There is evidence that SAP domains can bind DNA from studies on other SAP domain proteins [26, 27]. In the context of Ku, some studies suggest there could be interactions between Ku70-SAP and DNA [28, 29]. During DNA binding, the domain undergoes displacement that positions it in close proximity to the DNA-binding region of the Ku heterodimer [30, 31]. It is conceivable that Ku70-SAP may stabilize the interaction between Ku and DNA, though its exact function has yet to be conclusively elucidated.

Initial crystallography studies were conducted with truncated Ku80 that was missing the CTD [9]. The Ku80-CTD structure (19 kDa, residues 542–732) was later determined using NMR by two different groups [32, 33]. The Ku80-CTD contains a globular domain composed of six α-helices (residues 592–709) that is flanked by disordered N- and C-termini. Single-particle electron microscopy and small angle X-ray scattering data show that in the presence of DNA, the Ku80-CTD undergoes displacement to form a flexible arm that extends from the DNA-binding core to interact with the DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) [30, 34].

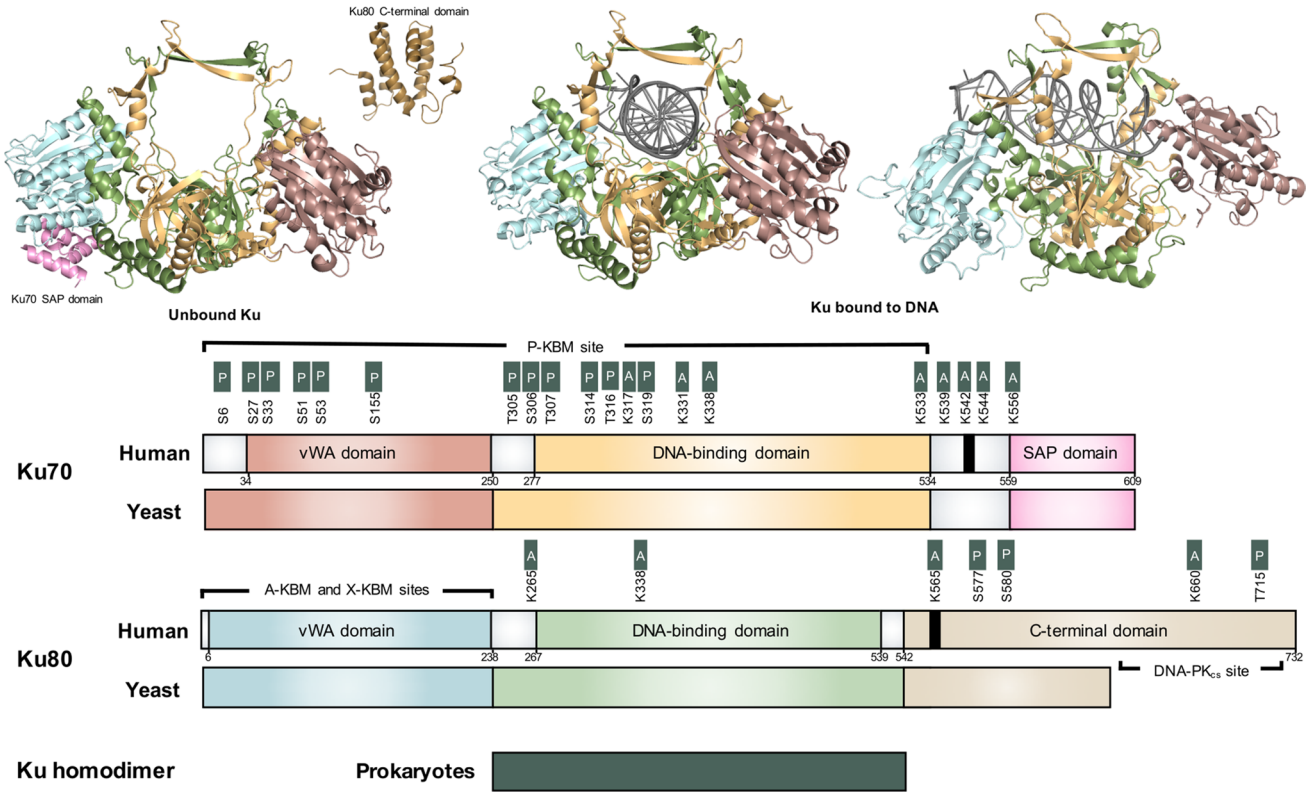

Fig. 2 Ribbon diagram and corresponding schematic of Ku heterodimer domains. Figure adapted from Fell and Schild-Poulter (2015) review [1]. Ku ribbon diagram bound and unbound by DNA. Crystal structure data of human Ku (PDB: IJEY) is colored in a domain-specific manner. Ku80 C-terminus domain is portrayed in isolation using NMR structural data (PDB: IQ2Z). In the Ku schematic, human, yeast, and prokaryote Ku subunits are compared while the Ku von Willebrand A (vWA) domain, DNA-binding domain, and SAF-A/B, Acinus and PIAS (SAP) or C-terminal domain, are color-matched to the ribbon diagram. Nuclear Localization Signal (NLS) is indicated as a thick black line. DNA-PKcs and Ku-binding motif (KBM) interaction sites are indicated, as are key post-translational modification (PTM) sites for phosphorylation and acetylation

Although the Ku crystal structure was obtained in 2001, important regions of each subunit were not mapped out due to experimental difficulties. These regions have since been resolved in the context of the crystal structure of DNA-PK$_{cs}$ bound to the Ku80-CTD [35], and in two cryogenic electron microscopy (cryoEM) studies of DNA-PK containing full length Ku70/80 [36, 37]. CryoEM studies show DNA-PK$_{cs}$ and Ku both recognize and bind DNA separately and together result in a 30° kink in the DNA duplex in contrast to previous models that assumed the DNA is unbent [35, 38]. The role of DNA distortion in NHEJ remains unclear. CryoEM data also indicates that DNA-PK$_{cs}$ is activated allosterically by conformational changes initiated by DNA-PK holoenzyme (dsDNA, Ku70/80, and DNA-PK$_{cs}$) formation [36, 37, 39]. CryoEM structures of Ku in isolation have not yet been reported but may be useful to further study Ku structure, protein interactions, and its role in NHEJ [39].

Ku conservation and essentiality

Conservation among organisms

Ku, an essential component of NHEJ, was first identified in humans [5], though functional homologs have since been identified in almost all eukaryotes including vertebrates, insects, fungi, and in some prokaryotic lineages [40–42]. Sequence homology analysis revealed that yeast and other lower eukaryotic Ku subunits may have arisen from a gene duplication event of a common ancestral gene [41, 43]. The eukaryotic Ku70 and Ku80 subunits have diverged in primary sequence but share similar secondary and tertiary structure [9]. Ku homologs in different organisms also have little sequence similarity but universally share similar structure and function [41].

In eukaryotes, Ku and DNA ligase IV form the core components of the NHEJ repair complex [41]. Many eukaryotes also have additional NHEJ factors such as DNA-PK$_{cs}$. It was previously thought that DNA-PK$_{cs}$ was only found in higher eukaryotes, however, recently DNA-PK$_{cs}$ has been identified in invertebrates, fungi, plants, and protists [44]. In organisms that have functional DNA-PK$_{cs}$, the Ku80 CTD binds with DNA-PK$_{cs}$, and the subsequently formed DNA-PK holoenzyme recruits downstream NHEJ factors [45]. Notably, DNA-PK$_{cs}$ is absent in yeast and *Caenorhabditis elegans* [45, 46], organisms that rely primarily on homologous recombination (HR) as their main mechanism of DSB repair [47, 48]. Accordingly, yeast Ku80 contains a unique CTD, distinct from higher eukaryotes, capable of binding DNA ligase IV (Dnl4) instead of DNA-PK$_{cs}$ [48].

Prokaryotes were initially thought to rely solely on HR, however, Ku homologs have been found in multiple bacterial genera, including Mycobacterium, Pseudomonas, Bacillus, and Agrobacterium [40]. The core bacterial NHEJ complex is composed of homodimeric Ku and LigD, a multifunctional ligase with multiple catalytic domains [49]. Bacterial Ku subunits are smaller than eukaryotic Ku (approximately 30–40 kDa) yet share related structural homology. The β-barrel ring domain responsible for heterodimerization and binding dsDNA in eukaryotes is structurally and functionally conserved in bacterial Ku, but the N-terminal vWA domain and the CTD are not [50].

Homodimeric Ku is also present in a limited number of Archaean species, implying that NHEJ repair is rare in these organisms [51]. Only one species has been found with NHEJ complex components, and these components are closely related to their bacterial homologs [52]. Ku homologs are widespread across the kingdom of life. The Gam protein, found in bacteriophage Mu, is a homodimer capable of binding dsDNA ends. Gam has considerable sequence homology with prokaryotic and eukaryotic Ku and appears to be an ortholog [53]. However, there are notable examples of organisms missing Ku and NHEJ capability. For example, NHEJ machinery (Ku and LigD) is absent in *E. coli* [40]. Interestingly, Gam, in conjunction with native *E. coli* LigA, has been shown to activate NHEJ in *E. coli* [54]. Multiple other NHEJ factors have also been independently lost in several species of parasitic protists [55].

Essentiality among organisms

To date, there is no published viable Ku homozygous knockout human cell line, although notable knockdown and knockout attempts have been made in various mammalian cell lines (Table 1). In human HCT116 cells, homozygous Ku80 knockouts were reportedly not viable after a limited number of cell doublings [7]. Similarly, for Ku70, a genome-wide knockout study found that deletion was lethal in near-haploid human cell lines, HAP1 and KBM7 [8]. Ku has been proposed to be essential in humans due to its role in telomere maintenance [7, 56]. Ku knockout was also lethal in *Ustilago maydis*, a fungal species, due to unprotected telomeres triggering cell cycle arrest [57].

Despite the inability to produce a viable total knockout of Ku in human cells, non-lethal Ku knockdowns through heterozygous gene knockout or RNA-based silencing have been achieved in different human cell lines [72–74]. Heterozygous Ku70 and Ku80 knockouts in HCT116 displayed slower cell proliferation, shortened telomeres, and hypersensitivity to ionizing radiation (IR), which indicates decreased NHEJ repair function [7, 73, 74]. Several human disorders are linked to NHEJ factor mutations, though no genetic disease has thus far been associated with a mutation in Ku. This combined data suggest that Ku is likely essential in humans. Contrarily, in most species, Ku appears to be dispensable (Table 2).

Table 1 Mammalian cell lines with Ku70 or Ku80 deficiencies

Subunit	Species	Cell line/tissue	Type of cell line	Type of deficiency	Viability	Reference(s)
Ku70	Human	RERF-LC-A1	Immortal	Knockdown	Viable	[58]
		Nalm-6	Immortal	Heterozygous	Viable	[59]
		HCT116	Immortal	Heterozygous	Viable	[60]
				Knockout	Inviable	
		HAP1	Immortal	Knockout	Inviable	[8]
		KBM7				
	Mouse	Mouse Embryonic Fibroblasts (MEFs)	Immortal	Knockout	Viable	[61]
		Embryonic Stem (ES) cells	Primary	Heterozygous	Viable	[16]
				Knockout		
		Fibroblasts	Primary	Heterozygous	Viable	[62]
				Knockout		
		B and T lymphocytes, thymocytes, MEFs, spleen, bone marrow	Primary	Knockout	Viable	[63]
Ku80	Hamster	*sxi*-2, *sxi*-3	Immortal	Knockout	Viable	[64]
		CHO-xrs-6 or xrs6[a]	Immortal	Knockout	Viable	[65, 66]
		CHO-xrs-4 or xrs4[a]				
		CHO-xrs-5 or xrs5[a]	Immortal	Knockdown	Viable	[66]
		XR-V15B	Immortal	Knockout	Viable	[67]
		XR-V9B	Immortal	Knockout	Viable	[68]
	Human	MRC5V1	Immortal	Knockdown	Viable	[69]
		HCT116	Immortal	Heterozygous	Viable	[7]
				Knockout	Inviable	
		HeLa	Immortal	Knockdown	Reduced viability	[70]
		U2OS				
		SAOS				
		Nalm-6	Immortal	Heterozygous	Viable	[59]
	Mouse	MEFs	Immortal	Knockout	Viable	[6]
		Bone marrow, B and T lymphocytes, MEFs, spleen	Primary	Knockout	Viable	[6, 71]
		ES cells, fibroblasts, thymus	Primary	Knockout	Viable	[6]
		Thymocytes	Primary	Knockout	Viable	[71]

[a]xrs hamster cell lines can be reverted to wild-type levels of Ku80 with azacytidine treatment suggesting they may not be true knockouts, but instead contain one silenced, but functional allele [66]

Unlike their human cell line counterparts, Ku knockout mice are viable and have been extensively studied [6, 75]. Despite Ku knockout mice being viable and capable of reproduction, Ku deficiency in mice is linked to a host of symptoms [71, 75–81] (Table 3). Ku70 or Ku80 knockout mice share many characteristic phenotypes, but there are a few notable differences including early aging for Ku80 deficient mice and early incidence of cancer in Ku70 knockouts.

Ku localization and expression

Cellular localization

Ku is predominantly observed in the nucleus [90]. Following transcription and translation, Ku subunits can translocate into the nucleus together, as a heterodimer [90], or independently, as each subunit contains its own nuclear localization signal [91]. In the nucleus, Ku has been shown

Table 2 Viable species in which Ku was knocked out

Kingdom	Species	Reference(s)
Animalia	*Bombyx mori*	[82]
	Caenorhabditis elegans	[83]
	Chicken cell line DT40	[84]
	Chinese hamster ovary (CHO) mutant cell lines	[85]
	Mus musculus	[16]
Fungi	*Claviceps purpurea*	[86]
	Penicillium chrysogenum	[86]
	Penicillium decumbens	[86]
	Penicillium marneffei	[86]
	Trichoderma virens	[86]
	Saccharomyces cerevisiae	[48]
	Schizosaccharomyces pombe	[87]
Plantae	*Arabidopsis thaliana*	[88]
Protozoa	*Toxoplasma gondii*	[89]

Species or cell lines, grouped by Kingdom, that were viable after *XRCC5* (Ku80) and/or *XRCC6* (Ku70) genes were successfully knocked out

to bind DNA DSBs with a high affinity [41, 92]. Ku's key roles associated with DNA repair [reviewed in [1]], telomere function [93], DNA replication [94, 95], and transcription [96, 97] validate its purpose as a nuclear protein.

Further investigations of the nuclear distribution pattern of Ku revealed its localization in both the nucleoplasm and nucleolus [98–100]. Ku is a dynamic, highly mobile protein complex and its nuclear mobility was shown to be regulated by inositol hexakisphosphate ($InsP_6$), an enzyme cofactor for DNA-PK and a direct Ku interactor [101–103]. In the absence of DNA damage, both Ku subunits are localized to the nucleolus [98, 104, 105], where Ku has been shown to associate with ribosomal RNA and RNA-binding proteins [98, 100]. In response to cellular stress signals, certain nucleolar proteins are found to be relocated to initiate countermeasures. Ku is one definitive example of this phenomenon as nucleolar Ku shuttles back to the nucleoplasm to initiate DNA break repair upon UV- or IR-induced DNA damage [98, 99]. These findings speculate that a portion of the Ku population exists in the nucleolus in the absence of DNA damage.

Although Ku has been well established as a nuclear protein, there are several studies which have observed its presence in the cytoplasm [reviewed in [90]]. Bax, a cytoplasmic protein, has been found to interact with Ku70, and this interaction was suggested to inhibit Bax-mediated apoptosis [3]. In addition, cytosolic Ku has been shown to act as a pattern recognition receptor that recognizes viral DNA in human cells [4]. Many of the studies that claimed to detect Ku in the cytoplasm relied on subcellular fractionation techniques where Ku may have "leaked out" of the nucleus to contaminate the cytoplasmic fractions, so the presence of Ku in the cytoplasm remains controversial.

Mitochondria contain their own genetic information in the form of a circular plasmid of DNA, which warrants mitochondria as a possible destination for Ku as a NHEJ repair factor. An earlier study reported that mitochondrial extracts of Ku-deficient hamster cells showed DNA end-binding activity, implying that some other factor associates with broken DNA ends and that Ku is not needed in the mitochondria to repair DSBs [106]. This speculation was recently confirmed as another group found that NHEJ was undetectable from the mitochondrial extracts of rat tissue and human cells [107]. Instead, the study identified factors involved in microhomology-mediated end-joining, an alternative DSB repair pathway to NHEJ, and suggest this pathway may be predominantly responsible for maintaining genomic integrity in the mitochondria [107].

Table 3 Ku70 and Ku80 knockout phenotypes in mice

Ku70 Knockout Mice	Ku80 Knockout Mice
Viable	Viable
Can reproduce	Can reproduce
Ku70 knockout mother unable to sustain pups	Ku80 knockout mother unable to sustain pups
K/O mice smaller than control mice	K/O mice smaller than control mice
Fewer proliferating cells	Early loss of proliferating cells for fibroblasts
Premature senescence of cells	Fibroblasts show prolonged doubling time
Cells sensitive to ionizing radiation	Cells are radiation sensitive
Lack mature B cells or serum immunoglobulin	Arrest in T and B lymphocyte development
V(D)J rearrangement deficiency	V(D)J rearrangement deficiency
Increased incidence of thymic tumors	Smaller spleen and lymph nodes
Chromosomal instability	Chromosomal instability
	Early aging/shorter lifespan

Protein expression

Ku70 and Ku80 are abundant proteins with approximately 400,000 units of Ku per cell in established human cell lines [108], and Ku levels appear to be ubiquitous and abundant regardless of the cell type or cell cycle phase. Some in vivo human tissues (i.e. skin, muscle, nerve, lung, ovary, kidney, etc.) were found to show heterogeneity in Ku70 expression [109]. Furthermore, tissue-specific overexpression of Ku70 and Ku80 homologs has been demonstrated in the ovary and testes of *Xenopus laevis* [110]. In *Drosophila melanogaster*, expression levels are constant through all developmental stages except oogenesis and early embryogenesis when they increase 25-fold [111]. These findings suggest a role for Ku in development. In *Arabidopsis thaliana*, expression was constant, albeit low, across all plant tissues examined [112]. Although Ku is conserved in almost all eukaryotic and a number of prokaryotic lineages, Ku expression patterns appear to be highly variable.

Highly expressed genes, like *XRCC6* (Ku70), are prone to producing processed pseudogenes (PP). PPs are created in germline cells through reverse transcription and random integration of mRNA into the genome. PPs are often non-functional and have lost the ability to produce proteins [113]. Ku70 has five PPs (*XRCC6P*1-5) in the human genome. While *XRCC6* is located on chromosome 22, three PPs are found on chromosomes 1 (*XRCC6P*3), 8 (*XRCC6P*4), and 10 (*XRCC6P*1) and two are found on chromosome X (*XRCC6P*2 and *XRCC6P*5). It is unclear, at present, whether any of these pseudogenes can or have been reactivated in human cells. Notably, no Ku80 pseudogenes have been reported.

The human Ku80 locus produces two protein isoforms: Ku80 and an alternative splice variant named Ku86 autoantigen related protein-1 (KARP-1), that is transcribed from a different upstream promoter [114]. KARP-1 shares certain biochemical properties with Ku80. KARP-1 is able to bind, colocalize, and stabilize Ku70 in vivo, strongly indicating that KARP-1 can heterodimerize with Ku70 [115]. KARP-1 has been shown to positively regulate DNA-PK activity [114] and partially complement the radiosensitivity of Ku80-deficient cells [115]. Notably KARP-1, not Ku80, expression is strongly induced by DNA damage in a p53- and ATM-dependent manner [116]. Whether KARP-1 has a distinct role or functions in mammalian DNA repair is still unclear, though KARP-1 was reported to have a protective role against cell damage caused by oxidative stress in rats [117].

Identifying Ku protein interactors

Ku70, Ku80, and DNA-PK$_{cs}$

Ku is often referred to as the DNA-binding subunit of the DNA-PK complex, which assembles in response to DSBs [1]. The extreme Ku80 C-terminus (residues 439–592) interacts with DNA-PK$_{cs}$ [43, 118, 119] and promotes DNA-PK$_{cs}$ autophosphorylation at DNA breaks. It is well recognized that Ku is important for recruiting and activating DNA-PK$_{cs}$ at DSBs, although one study observed DNA-PK$_{cs}$ could still be activated in Ku-deficient hamster cells [120], though this remains to be verified. Recently, another study found that the DNA sequence and end structure of the break may also play a role in DNA-PK assembly, specifically impacting the interaction between DNA-PK$_{cs}$ and the Ku80 C-terminus [121]. Aside from the broken DNA end composition, it is also possible that DNA-PK$_{cs}$ may interact differently with Ku depending on the NHEJ processing factors recruited to the break. DNA-PK acts as a hub for the rest of the DNA repair factors during NHEJ [reviewed in [122]]. Independent of Ku, DNA-PK$_{cs}$ function in other cellular processes has been reported [123].

Ku interactome studies

As highly abundant proteins in human cells, Ku70 and Ku80 are frequently identified in affinity purification and mass spectrometry datasets. In this sense, Ku is sometimes seen as a "contaminating" protein, as it is often observed outside of a logical biological context. However, at the same time, many diverse factors have been shown to interact with Ku [1, 13, 124], indicative of the fact that Ku has been implicated in multiple cellular processes: NHEJ [11, 18, 119, 125–127], telomere maintenance [128–131], DNA damage response [132–134], RNA biology [135–137], transcription [15, 138, 139], DNA replication [140, 141], V(D)J recombination [142, 143], and apoptosis [3, 144–146] (Fig. 3).

Originally, many studies identified candidate Ku interactors using high-throughput yeast-two-hybrid (Y2H) screens before validating interactions with low-throughput co-immunoprecipitation experiments and/or in vitro binding assays [10, 128, 148, 149]. Colocalization [150] and proximity ligation assays [151] are additional low-throughput techniques that can detect in vivo associations and have also been used previously to verify Ku interactors [132, 148].

We recently used proximity-dependent biotin identification (BioID) [12] to identify in vivo protein interaction

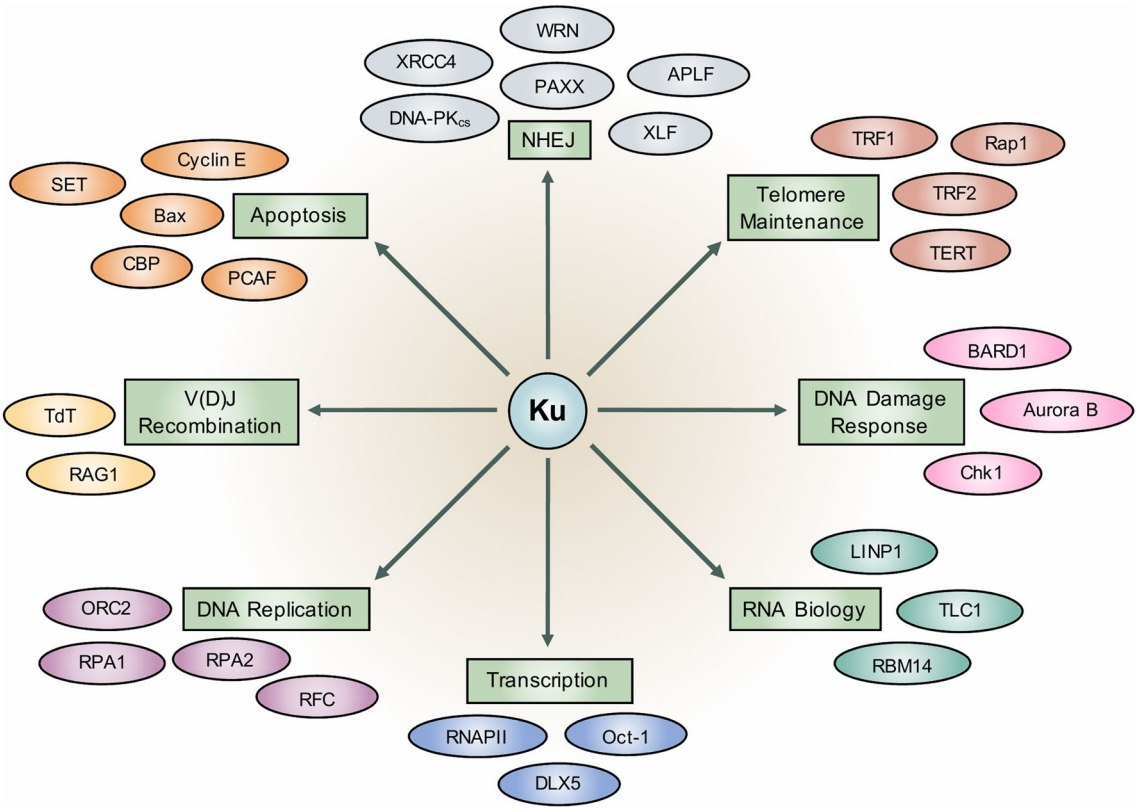

Fig. 3 The Ku heterodimer functions in various cellular processes and interacts with multiple proteins. Figure adapted from Downs and Jackson (2004) [147] review. A selection of well-characterized cellular functions that have been shown to involve Ku, including key human Ku protein/RNA interactors

candidates for Ku in human cells [13]. Using this technique with a second high-throughput proteomic technique known as affinity purification coupled to mass spectrometry (AP-MS), we were able to identify both known and novel Ku interactor candidates and create a comprehensive map of the Ku protein interactome [13]. Prior to our study, another group used tandem affinity purification tagging in human cells to identify Ku interactors and found 22 candidate proteins, many of which were shared with our study [124]. As technology continues to advance, more candidates can be identified using additional high-throughput techniques,

Fig. 4 The human Ku-Binding Motifs (KBMs). Figure adapted from Frit et al. (2019) [152]. Different classes of KBM with known proteins containing verified KBMs, amino acid sequences, and approximate sequence location. Note that there is no clear consensus on the exact lengths and boundaries of the KBMs. Bolded amino acids are conserved. Each KBM was initially discovered in and named after the respective underlined protein

allowing us to visualize the greater landscape and context of Ku functions throughout the cell.

The Ku-binding motif

Within the last decade, many studies identified a specific, 9-15 amino acid sequence referred to as a Ku-binding motif (KBM). The KBM is a conserved motif that mediates protein interactions with Ku [reviewed in [152]]. Thus far, only a handful of proteins have been found to contain a verified KBM, including Aprataxin PNKP-like factor (APLF) [125], Werner syndrome protein (WRN) [10], Modulator of retrovirus infection homologue (MRI, also known as CYREN) [10], XRCC4-like factor (XLF; also known as Cernunnos) [11], and Paralog of XRCC4 and XLF (PAXX) [11] (Fig. 4).

APLF, an intrinsically disordered protein that acts as an accessory factor for NHEJ, has been found to interact directly with Ku80, by binding to a hydrophobic pocket in the Ku80 vWA domain [11, 23]. Despite being predominantly nuclear, APLF does not contain its own nuclear localization signal [125]. Disrupting the interaction between Ku and APLF by mutating the APLF KBM (referred to as A-KBM in future studies) resulted in APLF becoming more cytoplasmic, suggesting that the A-KBM, and the interaction it mediates with Ku, is essential for retaining APLF in the nucleus [125].

Meanwhile, WRN was found to contain two KBMs: one at its N-terminus and another at its C-terminus [10]. While not a core NHEJ factor, WRN enhanced DSB repair using both KBMs [10]. The C-terminal KBM in WRN is adjacent to a novel, structurally similar but functionally distinct motif called the XLF-like motif [10]. This new motif was found at the extreme C-terminus of WRN, XLF (after which it was named), MRI/CYREN [11, 153], and PAXX [10].

Nemoz et al. (2018) proposed that this related XLF-like motif is also a true KBM and referred to it as X-KBM. Using crystallography, they showed that X-KBM also interacts with Ku80, albeit in a different region from the A-KBM. X-KBM seems to bind specific sites within the Ku80 vWA domain; specifically, X-KBM occupied an internal pocket formed when the Ku80 α/β domain underwent an outward rotation [11]. This unique interaction with the Ku80 vWA was also supported by a study conducted in *X. laevis*, where researchers suggested that the availability of this Ku80 vWA binding site may be conditional [154].

Nemoz et al. (2018) also identified a third sub-category of KBM, which they called P-KBM for the motif found in the C-terminus of PAXX. While PAXX was initially suspected to contain an X-KBM [10], implying an interaction with Ku80 in the same manner as XLF, the C-terminus of PAXX did not compete with XLF for Ku80 binding and instead bound Ku70 [11]. Another group had also previously deduced a PAXX-Ku70 interaction; they found that the C-terminus of PAXX was essential for Ku interaction, while the C-termini of both Ku70 and Ku80 were not essential for PAXX interaction [18]. Although the exact Ku70 binding site for P-KBM is unknown, the researchers noted that Ku binding to DNA results in a protrusion of the W148 residue in the Ku70 vWA, which they proposed could be an important contact for PAXX interaction [18].

Thus far, all the verified proteins containing KBMs are known or suspected to be involved in NHEJ [10]. At a double-stranded DNA break, Ku is threaded onto the ends of broken DNA and interaction with KBM-containing factors XLF or PAXX is only detectable in this specific context, with the inclusion of DNA [18]. In the future, it will be of interest to see if KBMs are identified in proteins outside of the NHEJ context. To this end, using the minimal KBM sequence (R-X-X-P-X-W), Grundy et al. (2016) identified over 600 putative KBM proteins [10]. Overall, KBMs represent a key motif that allows proteins to interact with either Ku80 or Ku70. Based on our current understanding, KBMs can be divided further into three sub-categories: A-KBMs, which dictate interaction with the Ku80 vWA hydrophobic pocket; X-KBMs, which also interact with the Ku80 vWA albeit distinct from A-KBMs; and finally P-KBMs, a third class that mediates interaction with Ku70 (Fig. 4).

Post-translational modifications

Ku functionality is modulated by several post-translational modifications (PTMs) including phosphorylation, acetylation, ubiquitination, and sumoylation. Some PTMs are associated with Ku function in NHEJ [155], DNA repair pathway selection [156], DNA damage response (DDR) [132], DNA replication [94], and Ku degradation [157], though many PTMs remain functionally uncharacterized.

Phosphorylation

Due to the close association of Ku with DNA-PK$_{cs}$ during NHEJ, initial studies focused on identifying potential phosphorylation sites of Ku by DNA-PK$_{cs}$ [158, 159]. Early in vitro studies identified several Ku80 residues (S577, S580, T715) that were phosphorylated by DNA-PK [158, 160, 161]. Similarly, Ku70 phosphorylation sites (S51, S53, S319) were also identified, and most were found as part of a DNA-PK consensus motif (S/T-Q) [159], with the exception of Ku70 S6 [158]. Phosphoablative mutation analysis of Ku80 S577, S580, T715 and Ku70 S6 demonstrated that phosphorylation of these sites was not needed for NHEJ repair function [160], raising the question of what functional role these phosphorylation events may play. A later study demonstrated that Ku70 phosphorylation by DNA-PK$_{cs}$ at other sites (T305, S306, T307, S314 and T316) displaced Ku

from DNA ends, thereby preventing NHEJ and promoting HR repair during the S phase of the cell cycle [156]. Taken together, these results suggest some Ku phosphorylation may be involved in DNA repair pathway selection while the function of other sites is still unclear.

Aside from DNA-PK$_{cs}$, cyclin-dependent kinases (CDKs) also phosphorylate Ku and other Ku70 phosphorylation sites have been identified using proteomic studies [162]. Cyclin A1-bound CDK2 was found to phosphorylate both Ku70 and Ku80 [163] and showed phosphorylation-mediated regulation of Ku70's heterodimer DNA-binding ability in vitro [164]. One group demonstrated that DNA replication was regulated through Ku70 phosphorylation by cyclin/CDKs. Various cyclin/CDK proteins were suggested to phosphorylate Ku70 and inhibit its interaction with replication origins during S, G2, and M phases, while dephosphorylation of Ku70 during the G1 phase facilitated the assembly of the origin recognition complex [94]. These early results imply that the balance between phosphorylated and dephosphorylated Ku70 may function to prevent untimely replication initiation.

Previous research in our lab identified Ku70 S155 as a novel phosphorylation site that is phosphorylated in response to severe DNA damage, although the kinase protein responsible is unknown [132, 165]. The proposed functions of Ku70 S155 phosphorylation in cell cycle arrest and the DDR are discussed later in this review (see "DNA damage response"). Finally, proteomic screens have identified other potential Ku70 phosphorylation sites phosphorylated by checkpoint kinase 1 (Chk1) and polo-like kinase, though the functions mediated by these phosphorylation events remain elusive [166, 167]. In some human cancer cell lines, Ku phosphorylation has been shown to confer mechanisms for radiation-resistance and survival [155, 168]. For example, in certain chemoresistant leukemia cell lines, a novel form of Ku70 was identified in which residues S27 and S33 were phosphorylated, and this phosphorylation seemed to lead to a faster, but more unfaithful NHEJ repair process [155].

Acetylation

Both Ku70 and Ku80 have multiple lysine residues that have been identified as acetylation sites. Ku80 sites include K265, K338, K565, and K660 [169] while numerous Ku70 sites have also been identified (Fig. 2) [3, 170, 171]. Ku70 acetylation has been shown to impair NHEJ by modifying the Ku70 lysine residues needed for binding dsDNA ends: K539 and K542 [171, 172] and K317, K331, K338 [170]. Two histone acetyltransferase enzymes, CBP and PCAF, have been shown to be responsible for Ku acetylation [3]. CBP was capable of acetylating both Ku70 [171] and Ku80 [173] at various lysine residues. Meanwhile, histone deacetylases, such as SIRT1 [174] and SIRT6 [175], deacetylate Ku.

Current research suggests that Ku70 acetylation negatively affects NHEJ-mediated DNA repair. Knocking down CBP in neuroblastoma cells decreased the levels of acetylated Ku70 while showing increased DNA repair activity and cell survival [171]. Acetylated Ku70 (K539, K542) has also been implicated in the initiation of Bax-mediated apoptosis [3, 172], providing a possible explanation for why increased cell survival is observed in CBP knockdown neuroblastoma cells. Interestingly, there are cellular mechanisms preventing Ku70 acetylation. Certain Ku70 interactors (i.e. SET and SMAR1) have been shown to safeguard Ku70 from acetylation [144, 176]. Inhibition of Ku70 acetylation by INHAT subunit SET/TAF-1β regulates Ku70-mediated DNA damage response [144], while SMAR1 was shown to coordinate HDAC6-induced deacetylation of Ku70 and dictate cell fate upon irradiation [176].

Ubiquitination and sumoylation

Ubiquitination and addition of ubiquitin-like proteins (UBLs) (i.e. NEDD8, SUMO) play a vital role in regulating cellular responses to DNA repair pathways [reviewed in [177]]. Ubiquitination by E3 ligases RNF138 and RNF8 has been associated with freeing Ku80 from repaired dsDNA by targeting the subunit for degradation [177, 178]. However, little is known about the impact of these modifications on Ku70. Neddylation-dependent Ku70 ubiquitination has been shown to promote Ku70 removal from DNA damage sites after repair [157]. In yeast Ku70, sumoylation at the C-terminal lysine residues were found to be favorable for Ku70 and broken DNA end association [179]. Ubiquitination is one of the more understudied Ku PTMs and an area in need of further investigation.

Diversity of Ku function

Ku is best characterized for its indispensable role in NHEJ, one of the available pathways for repairing DNA DSBs. Ku is the first protein of the NHEJ repair complex to recognize and bind broken DNA ends, binding within 10 s of a DSB forming [180]. Ku threads onto both broken ends in a sequence-independent manner, with most models depicting a single heterodimer binding each end [1, 20]. Once bound, Ku recruits DNA-PK$_{cs}$, which protects the DNA ends from degradation [181]. After autophosphorylation, DNA-PK$_{cs}$ undergoes a conformational change allowing other repair factors access to the ends [182]. Proteins involved in bridging and processing DSBs are also recruited, although the exact order of recruitment is still uncertain [183]. XRCC4 and XLF are recruited to DSBs and have been shown to form

an alternating filament that resembles a mobile, "molecular sleeve" to bridge the broken ends [184]. Other factors recruited include DNA polymerases and endonucleases that may be needed to process the DNA ends to be compatible for rejoining. DNA end processing without a homologous template can introduce errors, hence NHEJ is known for being an error-prone process [185]. The extent of the DNA damage may dictate which processivity factors are recruited and their abundance. Finally, DNA ligase IV is responsible for ligating the DNA backbone and completing repair [186]. Ku in NHEJ has been studied and reviewed extensively by others in various contexts: DSB repair pathway choice [20, 156, 187], function and kinetics of NHEJ [1, 188], and Ku removal from repaired dsDNA [189].

Related to its function in repairing DSBs, Ku is also essential for the repair of intentional, programmed DNA breaks formed during V(D)J recombination and class switch recombination, the cellular lymphocyte-specific processes that generate antigen diversity and immunoglobulin types, respectively, in human immune cells [190, 191]. V(D)J recombination relies on many of the same factors needed in NHEJ, discussed in the previous paragraph (for more information, see review by [192]). Ku's role in V(D)J recombination is further detailed later on in the review (see "Immune dysfunction"). Class switch recombination changes the type of immunoglobulin produced by a B cell through recombination of only the immunoglobulin constant region, modifying the function of the immunoglobin while leaving antigen specificity unchanged. In class switch recombination, intentional DSBs are introduced within the antibody gene locus and are typically repaired by Ku through the NHEJ pathway. However, unlike V(D)J recombination, if NHEJ factors are knocked out, class switch recombination breaks can also be repaired by the backup or alternative NHEJ repair process, microhomology-mediated end-joining [193].

Aside from NHEJ and immune system-related processes, Ku has been implicated in other cellular processes (Fig. 3). Here, we review research conducted about Ku function in the DNA damage response, DNA replication, transcription, telomere maintenance, and RNA biology.

DNA damage response

The DNA damage response (DDR) is a complex network of signaling pathways initiated upon detection of DNA damage [194]. DNA damage can arise internally from endogenous insults (DNA replication errors, reactive oxygen species, etc.) or externally from exogenous sources (ionizing radiation, chemicals, etc.) [195]. Numerous processes have been implicated in the DDR including cell cycle regulation, senescence, apoptosis, and DNA repair pathways including NHEJ [195, 196]. The DDR can be divided into three processes: scanning and detection, signal transduction and amplification, and repair and final appraisal. Here, we review the role of Ku in these processes in response to a DSB.

Various factors are responsible for scanning DNA for damage including the MRN (Mre11, Rad50, Nibrin/NBS1) [195, 197] and BASC (BRCA1-associated genome surveillance complex) [198] complexes. Some initially hypothesized that there was a specific "sensor" for each type of DNA lesion [199]. Conceivably, many suspected that Ku, an early responder to DSBs, played such a role in the DDR [197, 199]. However, Ku is not the only known sensor of DSBs, as MRN [200] and PARP1 [201] are also capable of recognizing and binding DSBs. By nature, not all DSBs are identical so there could be subtle differences governing the recognition of each DSB.

Once the damage has been detected, a "transducer" is needed to amplify the signal of damage and activate downstream proteins. Kinases such as ATM, ATR, and DNA-PK$_{cs}$ are the most commonly known transducer proteins responsible for signal amplification during the DDR [194, 202]. In response to DSBs, the ATM kinase phosphorylates multiple proteins including p53, 53BP1, BRCA1, CHK2, and histone H2AX at serine 139 [202, 203]. Aside from kinase proteins, our lab found that the S155 residue of Ku70, upon phosphorylation, appears to inhibit the Aurora B kinase and may be involved in promoting cell cycle arrest and the DDR [132]. Whether and how Ku interaction with Aurora B leads to cell cycle arrest is still unknown. Using irradiated murine Ku- and Ku70/ATM-knockout cell lines, another group demonstrated that Ku is needed for ATM-dependent ATR activation [204], providing further evidence that Ku may function in this aspect of the DDR.

In response to DSBs, phosphorylation of p53 leads to cell cycle checkpoint arrest, allowing the cell time to assess and repair the damage or trigger senescence/apoptosis if the damage is too great [202]. Ku is well established as being involved in the NHEJ repair aspect of the DDR. As Ku has been implicated in both DNA repair and apoptosis [3], it is tempting to speculate that Ku could also be appraising the repair attempt, ready to signal apoptosis if necessary [165]. The mechanism for deeming DNA damage beyond repair is still unclear though there are several possibilities (PTMs, cellular localization changes, retention time or occupancy levels at DSBs, etc.).

DNA replication

One of the less defined roles played by Ku is its function in DNA replication. In yeast, Ku was initially identified as being involved in the timing of the DNA replication cycle [205], and this was further supported by studies that established Ku as a modulator of activation time of replication origins in regions proximal to telomeric or sub-telomeric sequences [206]. Yeast Ku has also been shown to function

at DNA replication forks. Upon replication fork arrest, Ku has been implicated in the cell recovery process, leading to the restart of replication [207]. The function of Ku at fork arrests may be a stabilizing action in yeast [208]. Ku may also function to limit DSB resection during DNA replication-associated damage, which could inhibit the initial steps leading to the HR repair pathway [209]. In support of Ku's action as a regulator of DNA replication, one study characterized the removal of yeast Ku at arrested replication forks as integral to fork resection and replication restart [210].

Ku has also been implicated in regulating DNA replication in human cells. Early data suggested that there was a changing relationship between DNA-PK_{cs} forming a complex with replication protein A during replication, to a complex of DNA-PK_{cs} and Ku in response to DNA damage [140]. Through quantification of Ku binding in vivo at different cell cycle stages, Ku was identified as a protein that binds to mammalian sequences containing replication origins [211, 212]. Specifically, independent of DNA-PK_{cs}, Ku has been found to directly bind to the A3/4 sequence found in mammalian replication origins [95]. Ku's association with proteins involved in DNA repair may indicate a mechanism through which Ku is recruited to replication origins [141]. In the event of replication-induced DNA damage, Ku has been found to act as a protector of DNA replication by binding breaks and preventing the unloading of replication machinery from chromatin [213]. In advanced human metastatic breast cancer, higher levels of Ku were associated with chromatin, and Ku was found in the replication complexes formed at certain, more active origins of replication, implicating Ku's role in DNA replication in breast tumorigenesis [211]. Phosphorylation of human Ku70 has also been linked to replication-related functions and was described earlier in this review (see "Post-translational modifications"). Finally, Ku80 knockdown in HeLa cells was directly associated with a decrease in the total rate of DNA replication [214].

Transcription

Ku is well-known for its sequence-independent association with broken DNA ends [9, 41, 108]. However, in the late 1990s, many groups reported Ku-DNA site-specific interaction [215–218], implying that Ku acts as a transcription factor to regulate gene expression. In support of this, the Ku70 SAP domain has been shown to have some DNA-binding capability independent of Ku80 [25, 28, 29], although it is currently unclear if this DNA-binding activity is sequence-specific. Ku was also found to associate with other DNA structures like hairpins or bubbles [reviewed in [41]], implying that rather than recognizing DNA by sequence, Ku may instead recognize and bind in vivo DNA structures that form at certain sites [147].

Several studies have suggested that Ku binds directly to specific DNA elements, such as the heat-shock element in the promoter of the *hsp70* gene [219] and negative regulatory element 1 in vitro [216, 217], implicating Ku in the regulation of transcription. Using an in vitro transcription system with linear DNA, the displacement of transcription factors by Ku was observed [220]. However, Ku is not universally recognized as a bona fide transcription factor as its mode of DNA sequence-specific binding is not understood and the mechanisms through which it may regulate transcription remain unclear.

Ku has also been proposed to function more globally in transcription by modulating RNA polymerase II (RNAPII) expression or through protein–protein interactions with transcription-related proteins and factors. For instance, it has been reported that DSBs and DDR proteins may be a necessary component in regulating the release of paused RNAPII into active transcriptional elongation within human cells [221]. DNA-PK has been shown to phosphorylate the C-terminal domain of RNAPII [222] and Ku has been shown to interact with RNAPII [97, 138]. One group reported that Ku associates indirectly with RNAPII elongation sites via direct interaction between Ku80 and elongation proteins [97]. Recently, Ku was shown to bind the RNA hairpin structure of 7SK short nuclear RNA (snRNA), implicating Ku as a potential member of the 7SK short nuclear ribonucleoprotein (snRNP) complex that regulates transcriptional elongation [2]. Meanwhile, DNA-PK was shown to phosphorylate numerous transcription factors in vitro [reviewed in [223]]. Ku has also been found to work in tandem with other transcriptional proteins to initiate glucocorticoid receptor-dependent transcription [224].

While the connection between Ku and transcription is viable, Ku appears to regulate transcription under multiple contexts, making it a challenging and controversial area of study. Ku has also been implicated in the transcriptional regulation of gene expression in various diseases including breast cancer [225], Wilson disease [226], and HIV [227]. It is clear that a lot remains to be elucidated regarding Ku's precise role in transcriptional processes relating to gene expression and implications of Ku in disease states, as well as identifying specific mechanisms of action.

Telomere maintenance

Telomeres are repetitive DNA sequences found at the ends of chromosomes that maintain genomic integrity. In mammals, telomeres protect chromosomal ends through the formation of a t-loop. In a t-loop, single-stranded DNA overhangs invade double-stranded telomeric TTAGGG repeats and associate with a six-member complex of proteins that are collectively known as the shelterin complex [228]. Both the shelterin complex and the t-loop form a cap that prevents

DNA repair factors from recognizing the chromosome ends as potential DNA damage [228]. Telomeric DNA and associated protein complexes are involved in regulating gene expression through telomere position effects [229] and the three-dimensional genomic landscape [230], in which the proximity of genes to telomeric sequences and the conformation of chromatin can directly influence gene expression. Telomere biology, as well as telomeric dysregulation, have been extensively reviewed recently [229, 231], but despite this, some aspects of telomere regulation are still poorly understood.

One understudied area of research involves elucidating the network of proteins involved in telomere regulation. In the late 1990′s, researchers began to identify that Ku, which was classically recognized for its key role in DSB repair, was implicated in telomere regulation [129, 232–236]. Studies of mutant strains of yeast ku70 (yku70) and ku80 (yku80) revealed an essential role of Ku in telomere regulation, where a reduction or complete ablation of Ku function in yeast resulted in decreased telomere length [235, 236]. In contrast, in *Drosophila*, Ku deficiency results in abnormally long telomeres [237]. Ku-deficient mice displayed abnormal telomere lengths with telomeric fusions, indicating that Ku likely plays a role in preventing the occurrence of telomeric fusions [238, 239]. Depletion of a fungal form of Ku was found to cause cell cycle arrest, likely due to DNA damage signals from unprotected telomeres, although this needs further validation [57]. In mammalian cell lines, Ku depletion resulted in shortened telomeres and increased apoptosis [7, 74, 240]. These studies suggest Ku is required for proper telomere maintenance across different species, though the exact regulation mechanism is unclear. Ku has been shown to interact indirectly with telomeres through telomere-associated proteins in mice [238]. In mammals, Ku interacts with multiple members of the shelterin complex to protect chromosome ends [228]. In lower eukaryotes like yeast, Ku also functions in concert with other proteins to produce telomere position effects [241], but Ku's function in telomere position effects of mammals still needs to be elucidated.

While Ku function in telomere biology has been reviewed [56, 242, 243], the precise mechanism of Ku at telomeres has yet to be fully elucidated. Human cells with a knockdown of Ku80 were reported to have shortened telomeres and increased cell lethality, though the timeline of telomere shortening upon Ku depletion was not characterized [74]. In human cells, it was further demonstrated that loss of Ku80 resulted in telomere loss through the formation of t-circles and subsequent cell death [244]. These findings suggest that Ku is essential in humans due to its role in telomere maintenance, but future studies will be needed to fully characterize Ku's mechanism of action at telomeres that results in cell death.

RNA biology

Traditionally, RNA was understood for its central role as the template for protein synthesis, however, contemporary research has demonstrated that the majority of the human genome produces non-protein coding RNA species that can regulate gene expression or even impact disease progression [245]. Collectively, the interplay between RNA, proteins, and small molecules can also play an important role in the regulation of gene expression and protein function [246]. Recently, multiple DDR proteins have been found to bind RNA to regulate gene expression and modulate DDR signaling and repair [247]. Specifically, Ku has been found to associate with RNA structures to participate in multiple cellular functions.

Ku association with nuclear RNAs was identified in the 1990s as a means to modulate enzyme activity and potentially gene expression [248, 249]. In yeast, Ku associates with TLC1, the RNA component of telomerase, and acts to modulate telomerase activity [250, 251]. Yeast Ku recognizes and binds to a specific stem-loop hairpin structure of TLC1 [250, 252], and Ku binding to RNA and DNA was found to be mutually exclusive [253]. In human cells, a similar stem-loop structure within the RNA component of human telomerase was recognized and bound by Ku, suggesting that Ku interaction with telomerase RNA is conserved amongst species [254].

Ku's association with RNA may also function to regulate transcription. Ku has been found to bind TAR RNA at the 5′ end of mRNA transcripts of the HIV-1-gene [248, 249]. Ku may directly affect HIV-1 expression at the transcriptional level and its latency in infected cells through RNA interactions [227]. As reviewed earlier, Ku has been implicated in the regulation of transcriptional elongation through its interaction with the RNA hairpin structure of 7SK snRNA, which acts as a scaffold for the formation of the 7SK snRNP complex [2].

Ku has been shown to interact with a long noncoding RNA known as LINP1, which acts as a scaffold for the Ku heterodimer and DNA-PK_{cs} to function in response to damaged DNA [135, 255]. The inherent dsDNA repair function of Ku may play a role in resistance to cancer radiation therapies. The Ku-LINP1 interaction was shown to increase NHEJ repair efficiency. As this particular long noncoding RNA is overexpressed in triple-negative breast cancer, it was suggested that this Ku-RNA interaction may promote a radiation-resistance mechanism in tumor cells by enhancing NHEJ repair of DSBs [255]. Thapar et al. (2020) delve further into how LINP1 structurally functions in NHEJ and how it can effectively replace the NHEJ factor PAXX [135].

RNA:DNA hybrids, created during the formation of R-loops, appear to have varied and contrasting physiological effects that have been reviewed recently [256, 257]. While

RNA:DNA hybrids are known to be a source of genomic instability, more recent research suggests that R-loops may play beneficial roles such as regulating gene expression and facilitating DNA double-stranded break repair [257, 258]. Evidence for this restorative action in DNA repair has been demonstrated in yeast [259], as well as in mammalian cells [260]. The removal of R-loops in human cells has been shown to reduce the efficiency of both HR and NHEJ, lending support that the RNA:DNA hybrids are critical for DNA repair, though mechanistic details have yet to be fully elucidated [260]. Recent data indicate that R-loops are generated at break sites and play a role in the establishment of DNA repair and the DDR [260, 261]. A number of DNA repair factors have been found to interact with R-loops, notably PARP1, DHX9, DDX5, and DNA-PK [258, 262, 263]. Specifically, DHX9 has been implicated in suppressing R-loops and maintaining genomic integrity in vivo [263]. Recently, PARP1, DHX9, and DDX5 were also identified as potential Ku interactors using both BioID and affinity purification coupled to mass spectrometry [13]. Ku70 and Ku80 were also found to have an affinity for RNA:DNA hybrid structures using pull-down assays followed by mass spectrometry [262]. Given Ku's association with DNA repair factors that interact with RNA:DNA hybrids, as well as emerging evidence that Ku itself may interact with RNA:DNA hybrids, it will be interesting to explore and identify Ku's role in maintaining genomic stability through R-loop interactions.

One study identified a Ku-mediated link between DNA repair and mRNA translation through Ku's association with p53 mRNA [264]. The stem-loop structure in the untranslated region of the p53 transcript when bound by Ku was found to repress translation, and this repression was released as Ku relocated from the p53 mRNA to broken DNA ends in response to genotoxic stress [264]. In another study, Ku was found to move out of the nucleolus in response to sheared DNA that was added to live cells, suggesting that its association with RNA switches to DNA in the event of DSBs [98]. It was reported that in the nucleolus of mouse cells, Ku may affect rRNA processing, as the assembly of a catalytically defective or inactive DNA-PK by Ku resulted in an accumulation of unprocessed pre-rRNA precursors [100]. Overall, the context of Ku interaction with mammalian RNA within the nucleolus is still unclear. As a relatively new field, there is much to be learned regarding Ku and RNA biology.

Study of Ku in human diseases

Aging and telomere defects

Aging is the time-sensitive deterioration of physiological properties occurring at the cellular and organismal level. Accumulation of genetic defects over time and less efficient repair are contributing factors to aging [265].

Knocking out any of the DNA-PK subunits in mice caused premature aging [265, 266]. Yet the early understanding was that only deletions of Ku80 and/or DNA PK_{cs} were responsible for aging phenotypes in mice whereas deletions of Ku70 correlated with a high incidence of cancer [265]. Later, these findings were found to be inaccurate as deletions of Ku70 and Ku80 subunits lead to identical aging phenotypes, highlighting the essentiality of both subunits [266]. Other than its substantial role in DSB repair, DNA-PK is found to be involved in metabolic dysregulation during aging. In aging smooth muscle cells, increasing DSBs caused constitutive DNA-PK activation leading to down regulation of 5′-AMP-activated protein kinase (AMPK) activity [267]. DNA-PK phosphorylation of HSP90 impaired chaperone functionality on AMPK leading to misfolding. The depleted AMPK levels caused reduced fitness and declined mitochondrial function in aging mice. These findings accentuate a novel role for DNA-PK in mediating DNA damage-induced metabolic imbalance during aging [267].

Ku function in telomere maintenance can also contribute to the aging phenotype. Telomere shortening leads to replicative senescence of somatic cells [265]. Loss of t-loop structure and/or the protein cap protection (shelterin complex proteins) due to telomere shortening triggers the DDR [265] leading to permanent cell cycle arrest and/or apoptosis, depending on the cell type [268]. Ku plays a protective role at telomeres by binding the shelterin complex [231]. As discussed earlier, yeast [232, 235], human cancer cells [74, 240] and certain mouse models deficient in Ku [238] have shown reduced telomere length which emphasizes Ku's protective role against replicative senescence. Increased telomere associations and fusions were observed in aging Ku knockout mice [239, 266], and could be the result of defects in telomere cap protection.

A direct relationship between Ku and human aging disorders is uncertain, however, mutations to some potential Ku protein interactors may lead to premature aging. Hutchinson–Gilford progeria (HGPS) is a premature aging syndrome in humans caused by a single point mutation in the *LMNA* gene that codes for progerin, a truncated splice variant of lamin A [126, 269]. Ectopically-expressed DNA-PK_{cs} was shown to co-immunoprecipitate with progerin in HGPS fibroblasts, implying the two may interact [269]. Compared to normal fibroblasts, HGPS fibroblasts showed reduced expression of Ku70, Ku80, and DNA-PK_{cs} upon the accumulation of progerin and the loss of DNA-PK was found to contribute to the phenotypes observed in HGPS fibroblasts [269]. Another Ku protein interactor, WRN, enhances NHEJ repair [10, 270, 271], and loss of function mutations in WRN can result in Werner syndrome, an autosomal recessive disorder characterized by premature aging [271, 272]. Loss of

function mutations in WRN impair its localization to the nucleus [272], and may negatively affect WRN and Ku interaction and NHEJ repair during aging.

Immune dysfunction: the Ku autoantigen

Ku was initially discovered as an autoantigen after the detection of anti-Ku antibodies in the sera of scleroderma–polymyositis overlap syndrome patients [5]. Since then, anti-Ku antibodies have been identified with varying prevalence (< 1–27%) in a wide spectrum of connective tissue diseases including systemic lupus erythematosus, Sjögren's syndrome, rheumatoid arthritis, and systemic sclerosis (SSc) [273–276]. The prevalence of anti-Ku autoantibodies in connective tissue diseases varies due to the detection immunoassay, type of autoimmune disorder, and the genetic and geographical background of the subjects studied [5, 273, 274, 277–280]. Anti-Ku antibody profiles have been associated with some clinical manifestations. A significant association was reported between anti-Ku antibodies and musculoskeletal manifestations of disease (myositis, arthritis, and joint contractures) in SSc patients [276]. Anti-Ku positive patients who also display elevated serum creatine kinase levels appear to be at significantly higher risk of developing interstitial lung disease [281, 282]. Antibodies against other NHEJ factors have also been found in the serum of connective tissue disease patients suggesting a concerted autoimmune response to DDR proteins and that DNA damage may be a factor in the development of connective tissue diseases [273, 283, 284].

Ku in innate immunity

The innate immune response relies on the detection of bacterial and viral pathogens by pattern-recognition receptors (PRR) that induce the production of cytokines and chemokines. Membrane-bound and cytoplasmic PRR detect foreign DNA using conserved, essential molecules of pathogens [285]. DNA-PK has been identified as a cytosolic PRR for DNA viruses in murine fibroblasts and mice. After infection from the vaccinia virus (VV), which has a linear dsDNA genome, DNA-PK was shown to co-localize with sites of viral DNA replication in the cytoplasm and was involved in the activation of the innate immune system. Accordingly, the innate immune response is impaired in mice lacking DNA-PK components [286]. Ku alone has also been shown to detect viruses in human cells. Ku70 specifically functions as a cytosolic PRR that recognizes dsDNA and induced interferon activation in HEK293 cells [4]. In several human cell lines, Ku70 overexpression inhibited expression of human T lymphotropic virus type 1 (HTLV-1), a retrovirus that can cause leukemia, whereas Ku70 knockdown promoted HTLV-1 expression [287]. Cytoplasmic Ku was able to promote hepatitis-associated chemokine secretion after the detection of cytosolic hepatitis B viral DNA in human liver-derived cells [288]. Ku70 also mediated the innate immune response in human macrophages to a viral infection by herpes simplex virus-2 [289]. Interestingly, and speaking to the importance of DNA-PK in restraining viral infection, viruses appear to have developed subversion mechanisms to counter DNA-PK-mediated host detection. Two VV proteins, C4 and C16, are able to bind Ku and block its DNA-binding capabilities, attenuating the host innate immune response [290, 291].

Impairment in V(D)J recombination

The immune system relies on T- and B-lymphocytes producing an adaptive immune response using antigen-specific T-cell receptors (TCRs) and immunoglobulin (Ig) antibodies, respectively. V(D)J recombination generates diversity in the variable regions of TCRs and Ig antibodies by the controlled creation and NHEJ-driven repair of DSBs [292, 293]. Animals lacking NHEJ are severely immunodeficient as evidenced by Ku70/80 knockout mice. Immunological defects include smaller immune system organs and arrested T- and B-lymphocyte development associated with impaired V(D)J recombination [6, 75]. Although mutations in Ku have not been identified in humans, mutations in other NHEJ factors have been associated with severe combined immunodeficiency (SCID), a group of genetic disorders that results in persistent and recurrent infections [294, 295].

Cancer

In healthy cells, cell division is carefully regulated and adheres to a strict process; cells that bypass these regulations and divide unchecked are considered cancerous. DNA replication stress or repair errors can lead to chromosomal aberrations and genomic instability, a hallmark of cancer [296]. In many cancers, the expression of DNA repair proteins is dysregulated [296]. Aside from expression, the localization, function, protein interactions, and PTMs of these proteins can also be dysregulated in cancer cells.

In the early 2000s, a prevailing hypothesis was that Ku expression could correlate with cancer treatment outcomes [297, 298]. Many treatments target cancerous cells by creating intentional DSBs using radiation or chemotherapy. Since Ku is essential for NHEJ, it was reasonable to hypothesize that lower Ku levels indicated increased sensitivity to DNA breaks. In support of this, Ku knockout mice demonstrate increased sensitivity to IR [63]. Similarly, siRNA down-regulation of Ku70 in two cancerous cell lines also led to increased sensitivity to radiation and etoposide, a chemotherapy drug [299].

Ku levels were assessed in tumor biopsies using immunohistochemistry and were correlated to tumor radiosensitivity and the disease-free survival of patients. In two separate studies, Ku levels in colon cancer tumors were significantly reduced [298, 300]. Another group observed that in advanced rectal carcinoma tumors, tumors with a greater percentage of increased Ku-level cells were more likely to be radioresistant, while patients with a lower percentage tended to have better disease-free survival [297]. Another study assessed Ku levels in cervical cancer patients, though somewhat mixed results were observed as only some of the tumors showed correlation with Ku expression [301]. Contrary to the original hypothesis, some radiosensitive tumors actually had a higher percentage of cells showing increased Ku levels [301]. A second hypothesis proposed that Ku levels could be elevated in cancers, thus leading to an increased incidence of error-prone NHEJ and greater genomic instability. There was some evidence in support of this hypothesis too as some studies observed elevated Ku levels in various cancers [302, 303]. Elevated Ku expression has been correlated with increased resistance to both radiation [297] and cisplatin chemotherapy [304].

To a lesser degree, other studies have investigated possible Ku dysregulations at the functional level. For example, one study observed altered Ku DNA-binding activity in both breast and bladder tumors [305]. Another study found that EAF2, an androgen-responsive tumor suppressor, regulates NHEJ by recruiting and retaining Ku at sites of DNA damage in prostate cancer cells [306]. Finally, using chemotherapy-resistant lymphocytic leukemia cells, one group identified a novel phosphorylated form of Ku70 (residues S27 and S33) that conferred faster, more error-prone NHEJ [155]. Intriguingly, this study implies that some cancer cells adopt a Ku70-dependent chemotherapy resistance mechanism where NHEJ is enhanced.

Ku expression and NHEJ activity must be carefully regulated and balanced in human cells to preserve genomic stability, acting as an essential defense against oncogenesis. Directly connecting Ku to cancer in terms of diagnosis and prognosis has been challenging. In spite of some studies providing support for Ku levels as a prognostic tool, Ku expression does not appear to be a consistent parameter, possibly due to intra-tumor diversity, cancer progression, or variability among cancer types. However, Ku does merit further investigation as a direct therapeutic target. Recently, a small molecule Ku inhibitor capable of sensitizing human cells to radiation was published (Fig. 1). While some iteration of this molecule could be used in cancer treatments, problems such as tumor-specificity and toxicity still require resolution [307].

Conclusions

Recent research advances suggest Ku's cellular role is far more wide-reaching than its long-established DNA repair function. The study of Ku is expanding to encompass numerous fields of research, many of them involving regulatory processes. Significant structural knowledge has been gained from the recently acquired cryoEM structures of DNA-PK [36, 37], while the function of other protein domains such as Ku70′s SAP domain remains under investigation. Another active field of Ku research is the identification of Ku protein interactors, particularly those containing the recently identified KBMs. Identifying and validating Ku protein interactors, including KBM proteins, will be a useful tool in the future to infer new cellular processes that implicate Ku function. Recent promising research investigating Ku's role in innate immunity [289, 291] and the development of a small molecule Ku inhibitor [307] indicate that Ku's clinical relevance should not be overlooked. Studies have shown that Ku appears to function as a sensor for viral dsDNA, a trait that could be exploited to enhance human immunity to dsDNA viruses. Already, there is evidence that viruses can develop strategies to overcome DNA-PK's sensory capabilities as viral proteins capable of blocking DNA-PK's recognition of viral dsDNA have been identified. Additionally, while Ku's outlook as a tool for predicting cancer diagnosis and prognosis has yielded mixed results, increased cellular Ku levels seem to correlate with enhanced resistance to radiation, making Ku a worthy chemotherapy target to improve cancer treatment. The creation of viable Ku knockout cell lines in humans and a variety of other species would greatly assist in disease studies and elucidation of protein function. The multifunctional Ku protein has become a point of interdisciplinary research for many fields and further investigation could lead to more discoveries, at both the molecular and clinical level.

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