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# Cloning, localization and focus formation at DNA damage sites of canine Ku70

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**ABSTRACT.** Understanding the molecular mechanisms of DNA double-strand break (DSB) repair machinery, specifically non-homologous DNA-end joining (NHEJ), is crucial for developing next-generation radiotherapies and common chemotherapeutics for human and animal cancers. The localization, protein-protein interactions and post-translational modifications of core NHEJ factors, might play vital roles for regulation of NHEJ activity. The human Ku heterodimer (Ku70/Ku80) is a core NHEJ factor in the NHEJ pathway and is involved in sensing of DSBs. Companion animals, such as canines, have been proposed to be an excellent model for cancer research, including development of chemotherapeutics. However, the post-translational modifications, localization and complex formation of canine Ku70 have not been clarified. Here, we show that canine Ku70 localizes in the nuclei of interphase cells and that it is recruited quickly at laser-microirradiated DSB sites. Structurally, two DNA-PK phosphorylation sites (S6 and S51), an ubiquitination site (K114), two canonical sumoylation consensus motifs, a CDK phosphorylation motif, and a nuclear localization signal (NLS) in the human Ku70 are evolutionarily conserved in canine and mouse species, while the acetylation sites in human Ku70 are partially conserved. Intriguingly, the primary candidate nucleophile (K31) required for 5′dRP/AP lyase activity of human and mouse Ku70 is not conserved in canines, suggesting that canine Ku does not possess this activity. Our findings provide insights into the molecular mechanisms of Ku-dependent NHEJ in a canine model and form a platform for the development of next-generation common chemotherapeutics for human and animal cancers.

**KEY WORDS:** canine, companion animal, DNA double-strand break, Ku80, nonhomologous DNA-end joining

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Resistance to chemotherapy and radiotherapy is a common problem in the treatment of cancer. Of all the forms of DNA damage, double-strand breaks (DSBs) are the most toxic. Non-homologous DNA-end joining (NHEJ) is one of the critical pathways by which a cell repairs such DSBs [6, 8, 28]. Indeed, cells knocked out of one or more factors involved in NHEJ, exhibit hypersensitivity to DSB inducers, such as X-ray, heavy-ion radiation and chemotherapeutic agents. [8, 13, 14, 27]. Thus, uncovering the detailed molecular mechanisms underlying NHEJ is critical for the development of next-generation radiotherapies and chemotherapeutics [8, 11, 13].

The recruitment of core NHEJ factors, protein-protein interactions and post-translational modifications (PTMs) of such factors are known to play essential roles in the modulation of NHEJ activity. Acting as DSB sensors, Ku70 and Ku80 play a central role in the initiation of the NHEJ repair pathway in humans [6, 14, 28]. Ku is a heterodimer of two proteins, Ku70 and Ku80, highly abundant in human cells, but not in rodent cells [14]. Previous studies in our lab have shown that the localization of the Ku protein is regulated through the cell cycle. It is mainly localized in the nucleus during interphase, while most of the Ku protein is found diffused in the cytoplasm during the mitotic phase in human culture cells, including both normal and cancerous [14,24]. Ku70 possesses a nuclear localization signal, which, at least in part, regulates its nuclear localization [14, 23]. Human Ku70 is phosphorylated by multiple kinases, including DNA-PK [1, 7, 26, 28, 29]. It is also acetylated *in vivo* at K539 and K542, and these modifications play an important role in regulating Ku70-Bax interaction, thereby promoting Bax-mediated apoptosis [4]. Moreover, it is reported that acetylation of K282, K338, K539 or K542 in the DNA-binding domain of human Ku70 reduces the ability of Ku70 to bind to DNA [3]. These findings suggest that PTMs of Ku70 are important for the regulation of its function and determination of cell fate.

Canines are known to naturally develop cancers that share many characteristics with human malignancies. Therefore, compared to other animals, canines have been proposed to be a good model for cancer research, and development of therapeutic drugs

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and next-generation radiotherapies [10, 25, 30]. Previous studies in human and rodent cells have shown that the localization and function of Ku70 at DSB sites might play a crucial role in modulating the NHEJ repair pathway [3, 14, 20, 26, 28]. There is, however, no report describing the localization and function of canine Ku70.

In this study, we have cloned *Ku70* cDNA from a beagle dog testis library and conducted comparative analysis to understand the mechanisms underlying Ku70 regulation and function. Additionally, we examined its expression and localization. We also asked whether canine Ku70 accumulates at DSB sites after laser-microirradiation.

## MATERIALS AND METHODS

### *Cloning of canine Ku70*

Oligonucleotide primers used to amplify canine *Ku70* cDNA from a male beagle dog cDNA library (Biochain, Newark, CA, U.S.A.) were designed based on the predicted *Ku70* genomic sequence of a female boxer dog, *Canis lupus familiaris* (XM_531714.3). PCR amplification with sense (F: 5′-CTCAAGCTTCGAATTCGATGTCAGGCTGGGAGTCTTA-3′) and antisense (R: 5′-TAGATCCGGTGGATCCTCAGTTCTTCTGGAAG-3′) primers was performed in a Thermal Cycler using *Premix LA Taq,* Version 2.0 (Takara Bio Inc., Otsu, Japan). Pre-denaturation was carried out for 1 min at 94°C. This was followed by 40 cycles of PCR amplification. Each cycle consisted of a denaturation step at 94°C for 0.5 min, annealing at 60°C for 0.5 min and extension at 72°C for 2 min. PCR products were subcloned into the *Eco*RI and *Apa*I sites of pEYFP-C1 (pEYFP-canine *Ku70*) using the In-Fusion HD cloning kit (Takara Bio Inc.), and the inserts were validated by sequencing. Other PCR primers used in this study were as follows: dX6 Nseq F1: 5′-GCACAAGGGTGAGCAATAAACAATATG-3′, dX6 Nseq R1: 5′-TGGATACACTGGATGCTCATGTCAAAAG-3′, dX6 Cseq F2: 5′-GGTGGGTTGAAGAAGCAGGAG-3′ and dX6 Cseq R2: 5-AAAGCACAGCAAAGTGGGAGG-3′.

### *Cell lines, cultures and transfections*

A Madin-Darby canine kidney cell line (MDCK) (HSRRB, Osaka, Japan), a canine lung adenocarcinoma cell line (CLAC) (HSRRB), a human cervical carcinoma cell line (HeLa) (Riken Cell Bank, Tsukuba, Japan) and a human colon cancer cell line (HCT116) (Riken Cell Bank) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). pEYFP-canine *Ku70* or pEYFP-C1 was transiently transfected into cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, U.S.A.). Post-transfection, cells were cultured for 2 days and then monitored under an FV300 confocal laser-scanning microscope (CLSM) (Olympus, Tokyo, Japan) as previously described [16, 17, 22].

### *Immunocytochemistry*

Immunocytochemistry was performed using a mouse anti-γH2AX monoclonal antibody (JBW301) (Upstate Biotechnology Inc., Charlottesville, VA, U.S.A.) and an Alexa Fluor 568-conjugated secondary antibody (Molecular Probes, Eugene, OR, U.S.A.) as previously described [20, 21].

### *Immunoblotting*

The extraction of total cell lysates and Western blot analysis were performed as described previously [20, 21] with the following modifications. The molecular weight marker was used 3-Color prestained XL-ladder (APRO science, Tokushima, Japan). The membranes were blocked in Blocking One (Nacalai Tesque, Kyoto, Japan) or ECL Prime Blocking reagent (GE Healthcare Bio-Sci. Corp., Piscataway, NJ, U.S.A.) for 30 min at room temperature. The following antibodies were used: rabbit anti-Ku70 polyclonal antibody (347V), rabbit anti-Ku70 polyclonal antibody (AHP316) (Serotec, Oxford, UK), rabbit anti-GFP polyclonal antibody (FL) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and anti-β-actin monoclonal antibody (Sigma, St. Louis, MO, U.S.A.). The anti-Ku70 antibody (347V) was raised for this study against corresponding to the N-terminal amino acids: 175–188 of canine Ku70, characterized and affinity purified. The amino acids sequence is perfectly conserved between canine and human. The anti-GFP antibody cross-reacts with GFP mutants, such as EYFP [20, 21]. The anti-Ku70 and anti-GFP antibodies were diluted in Signal Enhancer HIKARI (Nacalai Tesque). In accordance with the manufacturer's instructions, the binding to each protein was detected using a Select Western blotting detection system (GE Healthcare Bio-Sci. Corp.) and visualized using the ChemiDoc XRS system (Bio-Rad, Hercules, CA, U.S.A.).

### *Local DNA damage induction using laser and cell imaging*

Local DNA damage induction using laser and subsequent cell imaging was performed as described previously [15, 17]. Briefly, local DSBs were induced using a 3% power scan (for 1 sec) from a 405-nm laser. Images of living or fixed cells expressing EYFP-canine Ku70 or EYFP alone were obtained using an FV300 CLSM system (Olympus).

## RESULTS

### *Sequence analysis of canine Ku70*

Canine *Ku70* cDNA was cloned from a beagle dog testis library and sequenced. We isolated an 1,827-nucleotide open reading frame encoding a protein of 608 amino acids (Fig. 1). The cDNA sequence of Ku70 obtained from the beagle dog was identical to its predicted sequence from a female boxer dog genomic sequence (XM_531714.3). The obtained canine sequence has been

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ATG TCA GGC TGG GAG TCT TAT TAC AAA AAC GAG GGC GAT GAA GAA GGA GAG CAA GAA GAC GGC CTT GAA GCC GGT GGA GAA TAT ACA TAT   90
 M   S   G   W   E   S   Y   Y   K   N   E   G   D   E   E   G   E   Q   E   D   G   L   E   A   G   G   E   Y   T   Y   30

TCA GGA AGA GAT AGT TTG ATT TTT TTG GTT GAT GGT TCT AGG GCC ATG TTT GAA TCT CAG GGT GAA GTT GAA CTG ACT CCT TTT GAC ATG  180
 S   G   R   D   S   L   I   F   L   V   D   G   S   R   A   M   F   E   S   Q   G   E   V   E   L   T   P   F   D   M   60

AGC ATC CAG TGT ATC CAG AGT GTG TAT ACC AAT AAG ATC ATA AGC AGT AAT CAA GAT CTC TTG GCA GTG GTG TTC TAT GGT ACT GAG AAG  240
 S   I   Q   C   I   Q   S   V   Y   T   N   K   I   I   S   S   N   Q   D   L   L   A   V   V   F   Y   G   T   E   K   90

GAT AAA AAT TCA GTG AAT TTC AAA AAT ATT TAC GTC TTA CAG GAG TTG GAT AAT CCA GGT GCT AAA CGA GTG CTT GAG CTT GAC CAG TTT  360
 D   K   N   S   V   N   F   K   N   I   Y   V   L   Q   E   L   D   N   P   G   A   K   R   V   L   E   L   D   Q   F  120

AAG GGG GAG CGC GGG AAA AAA CAT TTC CAA GAC CTA ATT GGC CAT GGA TCT GAC TAC TCA CTG AGT GAG GTG CTG TGG GTC TGT GCC AAC  450
 K   G   E   R   G   K   K   H   F   Q   D   L   I   G   H   G   S   D   Y   S   L   S   E   V   L   W   V   C   A   N  150

CTC TTT AGC GAT GTC CAG GTT AAG ATG AGC CAT AAG AGG ATC ATG CTG TTC ACC AAT GAA GAT GAC CCC CAT GGC AAT GAC AGT GCC AAA  540
 L   F   S   D   V   Q   V   K   M   S   H   K   R   I   M   L   F   T   N   E   D   D   P   H   G   N   D   S   A   K  180

GCT AGC AGG GCC AGG ACC AAA GCT GGG GAT CTC CGT GAC ACA GGT ATC TTC TTG GAC TTG ATG CAC TTG AAG AAA CGT GGG GGT TTT GAC  630
 A   S   R   A   R   T   K   A   G   D   L   R   D   T   G   I   F   L   D   L   M   H   L   K   K   R   G   G   F   D  210

ATA TCC TTG TTC TAC CGA GAT ATC ATC AGC ATA GCA GAG GAT GAG GAC CTA GGG GTT CAC TTT GAG GAA TCG AGC AAG CTA GAA GAC CTG  720
 I   S   L   F   Y   R   D   I   I   S   I   A   E   D   E   D   L   G   V   H   F   E   E   S   S   K   L   E   D   L  240

TTG AGG AAG GTT CGT GCC AAG GAG ACC CGG AAG CGT GTC CTC TGC AGG TTG AAG CTT AAG CTC AGC AAA GAT ACT GCG CTC ACT GTC GGC  810
 L   R   K   V   R   A   K   E   T   R   K   R   V   L   C   R   L   K   L   K   L   S   K   D   T   A   L   T   V   G  270

ATT TAT AAT ATG GTC CAG AAG GCT GTC AGA CCT GCT CCG GTG AGG CTT TAT CGG GAG ACC AAT GAA CCA GTG AAA TCC AAA ACC CGG ACA  900
 I   Y   N   M   V   Q   K   A   V   R   P   A   P   V   R   L   Y   R   E   T   N   E   P   V   K   S   K   T   R   T  300

TTT AAT GTA AAT ACG GGC AGT TTG CTT CTG CCT AGT GAC ACC AAG AGA TCT CAG AAC TAT GGG AAT CGT CAG ATT GTA CTA GAG AAA GAG  990
 F   N   V   N   T   G   S   L   L   L   P   S   D   T   K   R   S   Q   N   Y   G   N   R   Q   I   V   L   E   K   E  330

GAA ACA GAA GAG CTG AAA CGC TTT GAT GAA CCA GGT TTG ATT CTC ATC GGT TTC AAG CCC TTG ATA ATG CTG AAG AAG CAC CAT TAC CTG 1080
 E   T   E   E   L   K   R   F   D   E   P   G   L   I   L   I   G   F   K   P   L   I   M   L   K   K   H   H   Y   L  360

AGG CCC TCC CTG TTT GTG TAC CCT GAA GAG TCC TTG GTG AAT GGG AGC TCA ACC CTG TTT ATT GCT CTG CTC ACC AAG TGT CTG GAG AAG 1170
 R   P   S   L   F   V   Y   P   E   E   S   L   V   N   G   S   S   T   L   F   I   A   L   L   T   K   C   L   E   K  390

GAG GTC ATG GCA GTG TGC AGA TAC ACC CCC CGT CGG AAC ATC CCT CCT TAT TTT GTG GCC TTG TTG CCA CAA GAA GAG GAG CTA GAT GAC 1260
 E   V   M   A   V   C   R   Y   T   P   R   R   N   I   P   P   Y   F   V   A   L   L   P   Q   E   E   E   L   D   D  420

CAG AAA ATT CAA GTG ACA CCC CCA GGC TTT CAG CTT GTC TTC TTA CCC TAT GCT GAT GAT AAA CGC AAG GTG CCC TTT ACT GAA AAA GTC 1350
 Q   K   I   Q   V   T   P   P   G   F   Q   L   V   F   L   P   Y   A   D   D   K   R   K   V   P   F   T   E   K   V  450

ATG GCA AAC CCA GAG CAG ATA GAC AAG ATG AAG GCT ATT GTT CAG AAG CTC CGA TTC AAT TAC AGA AGT GAC AGC TTT GAG AAC CCA GTG 1440
 M   A   N   P   E   Q   I   D   K   M   K   A   I   V   Q   K   L   R   F   N   Y   R   S   D   S   F   E   N   P   V  480

CTA CAG CAA CAC TTC AGG AAC CTG GAG GTG CTG GCT TTG GAT TTG ATG GAA CCC GAG CAG GCG GAG GAT CTG ACA CTG CCT AAG ATT GAA 1530
 L   Q   Q   H   F   R   N   L   E   V   L   A   L   D   L   M   E   P   E   Q   A   E   D   L   T   L   P   K   I   E  510

GCA ATA GAT AAA AGA CTG GGC TCC TTG GTG AAT GAG TTT AAG GAG CTT GTC TAC CCA CCA GAT TAC AGT CCT GAA GGA AAA GCT CCC AAG 1620
 A   I   D   K   R   L   G   S   L   V   N   E   F   K   E   L   V   Y   P   P   D   Y   S   P   E   G   K   A   P   K  540

CGG AGA CAA GAT GAT GAA GGT CTT GGA AGC AAA AGG CCC AAG ATG GAG TTA TCT GAA GAG GAG CTG AGG GCC CAT GTC AAC AAG GGC ACG 1710
 R   R   Q   D   D   E   G   L   G   S   K   R   P   K   M   E   L   S   E   E   E   L   R   A   H   V   N   K   G   T  570

CTG GGT AAG CTC ACT GTG CCC ATG CTG AAG GAA GCC TGC AGG GTG TGT GGG CTG AAA GGT GGG TTG AAG AAG CAG GAG CTG CTG GAC ATA 1800
 L   G   K   L   T   V   P   M   L   K   E   A   C   R   V   C   G   L   K   G   G   L   K   K   Q   E   L   L   D   I  600

CTC ACT AAG CAC TTC CAG AAG AAC TGA 1827
 L   T   K   H   F   Q   K   N   *   608
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**Fig. 1.** Nucleotide and deduced amino acid sequences of Ku70 cDNA from canine (*Canis lupus familiaris*, GenBank accession number: LC195221) species. The CDS of canine Ku70 is composed of 1,827 bp encoding 608 amino acids residues. Numbers on the right refer to nucleotides (top) and amino acids (bottom). The asterisk after the amino acid sequence shows the position of the termination codon.

deposited to the DDBJ/ENA/NCBI database [accession number LC195221]. Comparative analysis of Ku70 sequences showed that canine Ku70 had 87.1% and 81.8% amino acid identity with human and mouse, respectively. As shown in Fig. 2, it has been reported that human Ku70 is modified by some PTMs, including acetylation, phosphorylation, sumoylation and ubiquitination [1, 2, 4, 7, 9, 12, 26, 29]. In addition, it is shown that human Ku70 has a nuclear localization signal (NLS) sequence spanning amino acids 539–556, two putative sumoylation consensus motifs (ψ-K-X-E: $_{509}$PKVE$_{512}$ and $_{555}$PKVE$_{558}$) and a CDK phosphorylation motif ([S/T]Px[K/R]: $_{401}$TPRR$_{404}$) [9, 23, 29]. We found that the sequences of these three motifs are conserved in canine and mouse Ku70 (Fig. 2). The ubiquitination site (K114) in human was also found to be evolutionarily conserved in canine and mouse species. Furthermore, we found that the DNA-PK phosphorylation sites (S6 and S51), the cyclin B1/CDK1 phosphorylation sites (T401 and T428) and the putative cyclin E1/CDK2 phosphorylation site (T58) in human Ku70 are similarly conserved in canine and mouse species. On the other hand, the DNA damage-induced phosphorylation sites (S27, S33 and S155), the putative phosphorylation sites required for Ku70/Ku80 dissociation from DSBs (T305, S306, T307, S314 and T316), and the cyclin A2/CDK2 phosphorylation sites (T401, T428 and T455) in human Ku70 are partially conserved in canine and mouse species. In addition, seven (K317, K331, K338, K539, K542, K553 and K556) of the eight acetylation sites in humans are perfectly conserved in canine and mouse, whereas the acetylation site K544 is not conserved in canine species. Recently, it has been reported that human Ku has a 5′dRP/AP lyase activity [31, 32]. Interestingly, the primary candidate nucleophile (K31 in the Ku70) required for 5′dRP/AP lyase activity is not conserved in canine, although it is conserved in mouse species (Fig. 2).

### *Expression and subcellular localization of canine Ku70*

We wanted to examine the expression and subcellular localization of Ku70 in canine cells. First, we evaluated at the expression of Ku70 in the canine cell lines, MDCK and CLAC, and the human cell lines, HeLa and HCT116. Signals of canine Ku70 were detected in both MDCK and CLAC cells, although Ku70 exhibited lower expression levels in the two canine cell lines compared to in the human cell lines (Fig. 3A). Next, to examine subcellular localization of Ku70 in live canine cells, we generated MDCK cells transiently expressing EYFP-canine Ku70. To this end, the expression vector pEYFP-C1 containing canine *Ku70* (pEYFP-canine *Ku70*) was transfected into MDCK cells (Fig. 3B). Western blotting using anti-Ku70 and anti-GFP antibodies showed that EYFP-canine Ku70 was expressed in the transfected cells (Fig. 3C). Confocal laser microscopy showed that EYFP-canine Ku70 was localized in the nuclei of the EYFP-canine Ku70 transfected cells during interphase (Fig. 3D). EYFP, used as a control, was distributed throughout the cell excluding the nucleolus in EYFP transfectant cells (Fig. 3D), consistent with our previously reports [20, 21]. These data indicate that the canine Ku70 is expressed and localized in the nuclei of canine cells.

### *EYFP-canine Ku70 accumulates instantaneously at laser-microirradiation-induced DSBs*

We next examined whether EYFP-canine Ku70 accumulates immediately at 405 nm laser-induced DSB sites in canine cells (Fig. 4A). Microirradiation led to the accumulation of EYFP-canine Ku70 in live MDCK cells (Fig. 4B). To examine if EYFP-canine Ku70 accumulated at 405 nm laser-induced DSB sites, we immunostained cells with an antibody that detects γH2AX, a gold-standard marker of DSBs. EYFP-canine Ku70 was found to accumulate and colocalize with γH2AX at microirradiated sites in MDCK cells (Fig. 4C). To investigate the temporal dynamics of Ku70 localization, we performed time-lapse imaging of EYFP-canine Ku70 transfected MDCK cells. We observed EYFP-canine Ku70 accumulation at the microirradiated sites 5 sec after irradiation (Fig. 4D). These results reveal that after irradiation, EYFP-canine Ku70 accumulates immediately and forms foci at laser-induced DSBs in live canine cells.

## DISCUSSION

It is important to elucidate the underlying molecular mechanisms of DNA repair processes, such as NHEJ, for the development of new chemoradiotherapies and targeted drugs for cancer. Human Ku is the most important DSB sensor protein, and NHEJ is considered to initiate with the binding of Ku to the ends of the DSBs [14, 28]. Hence, Ku70 and Ku80 might be potential target molecules for the development of next-generation radiosensitizers to not only human cancers, but also canine cancers. In this study, we demonstrate that EYFP-canine Ku70 localizes in the nuclei and is recruited to micro-laser induced DSB sites soon after irradiation. Furthermore, sequence alignment revealed that the PTM sites and the protein-protein interaction motifs of Ku70 are not completely conserved in human and canine species. This study, amongst others, contributes to the understanding of the Ku-dependent NHEJ pathway and the development of a combination of radiotherapy and targeted therapy with DNA repair inhibitors.

It is well known that molecular mechanisms underlying protein-protein interactions and PTMs of DNA repair proteins play critical roles in the regulation of various DNA repair pathways [5, 28]. In this study, we found that almost all of these target-motifs and -sites in human Ku70 are conserved in canine Ku70. For example, sequence alignment revealed a CDK phosphorylation motif ([S/T]Px[K/R]: $_{401}$TPRR$_{404}$), the cyclin B1/CDK1 phosphorylation sites (T401 and T428) and the putative cyclin E1/CDK2 phosphorylation site (T58) in human Ku70 are conserved in canine and mouse Ku70. In addition, we found that two of the three cyclin A2/CDK2 phosphorylation sites (T401 and T428) in human Ku70 are conserved in canine and mouse species. There are several reports describing cell cycle-dependent Ku70-localization and -function in human and rodent cells [7, 14, 24, 29]. Altogether, we speculate that Ku70-localization and -function in canine cells might be regulated by cell cycle dependent-phosphorylation, which will be validated in the future. Previously, our findings using immunocytochemistry and GFP-technology revealed that Ku70 mainly localizes in the nuclei of various human and murine adhesion cells during interphase [16–19]. In our present study, the data showed that canine Ku70 mainly localizes in the nuclei of canine adhesion cells during

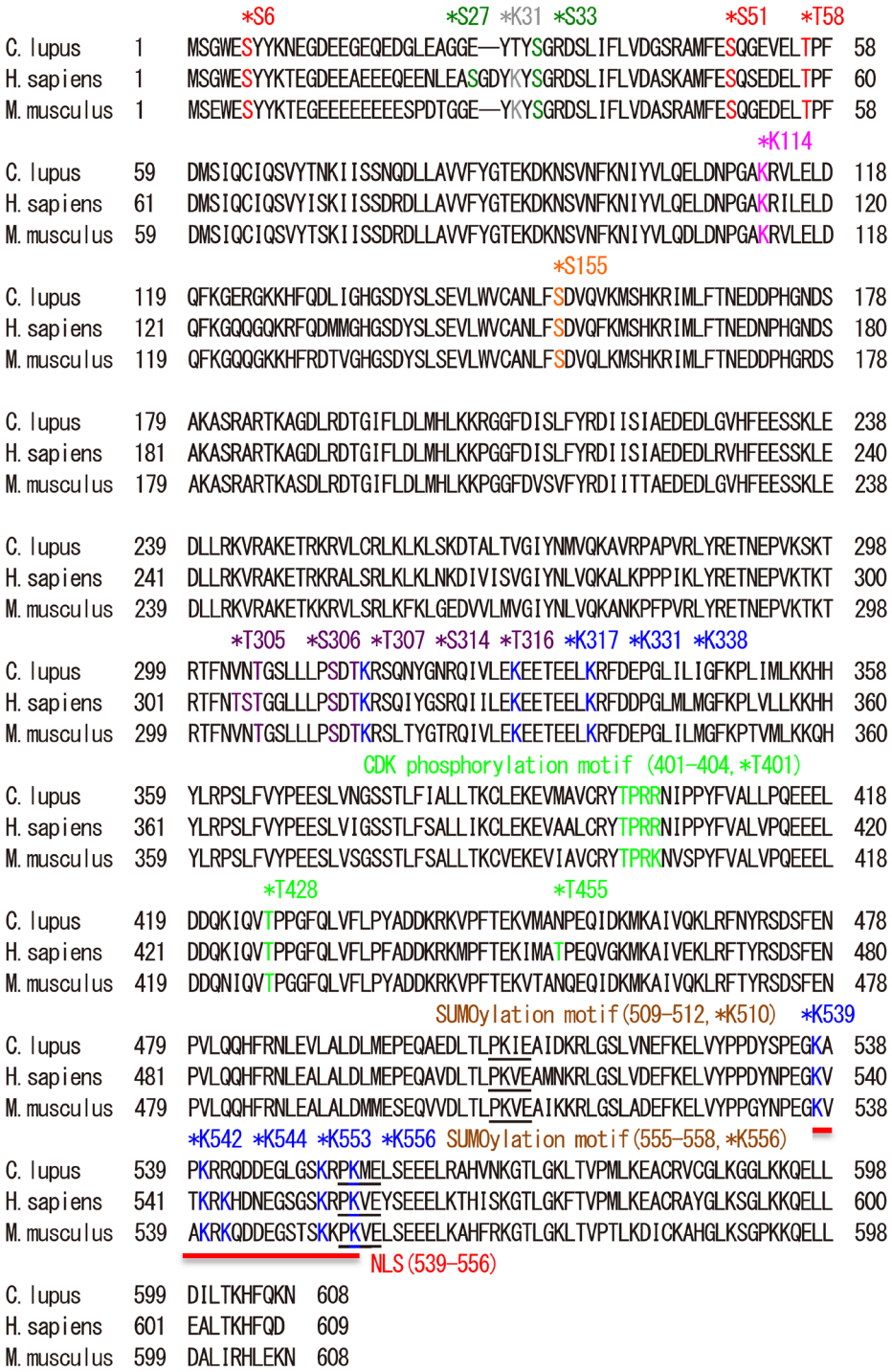


**Fig. 2.** Amino acid sequences of Ku70 from canine (*Canis lupus familiaris*, GenBank accession number: LC195221), human (*Homo sapiens,* GenBank accession number: NP_001460.1) and mouse (*Mus musculus*, GenBank accession number: NP_034377.2) species. The location of the nuclear localization signal (NLS) sequence (NLS: 539-556), the two putative canonical sumoylation consensus motifs (ψ-K-X-E: $_{509}$PKVE$_{512}$ and $_{555}$PKVE$_{558}$) and the CDK phosphorylation motif ([S/T]Px[K/R]: $_{401}$TPRR$_{404}$) in human Ku70 are shown [9, 18, 29]. The location of the primary candidate nucleophile required for 5′dRP/AP lyase activity (K31), the DNA-PK phosphorylation sites (S6 and S51), the DNA damage inducible phosphorylation sites (S27, S33 and S155), the putative phosphorylation sites required for Ku's dissociation from DSB (T305, S306, T307, S314 and T316), the cyclin B1/CDK1 phosphorylation sites (T401 and T428), the cyclin A2/CDK2 phosphorylation sites (T401, T428 and T455), the putative cyclin E1/CDK2 phosphorylation site (T58), the ubiquitination site (K114), the acetylation sites (K317, K331, K338, K539, K542, K544, K553 and K556) and the two putative sumoylation sites (K510 and K556) in human Ku70 [1, 2, 4, 7, 9, 12, 26, 28, 29] are marked with asterisks.

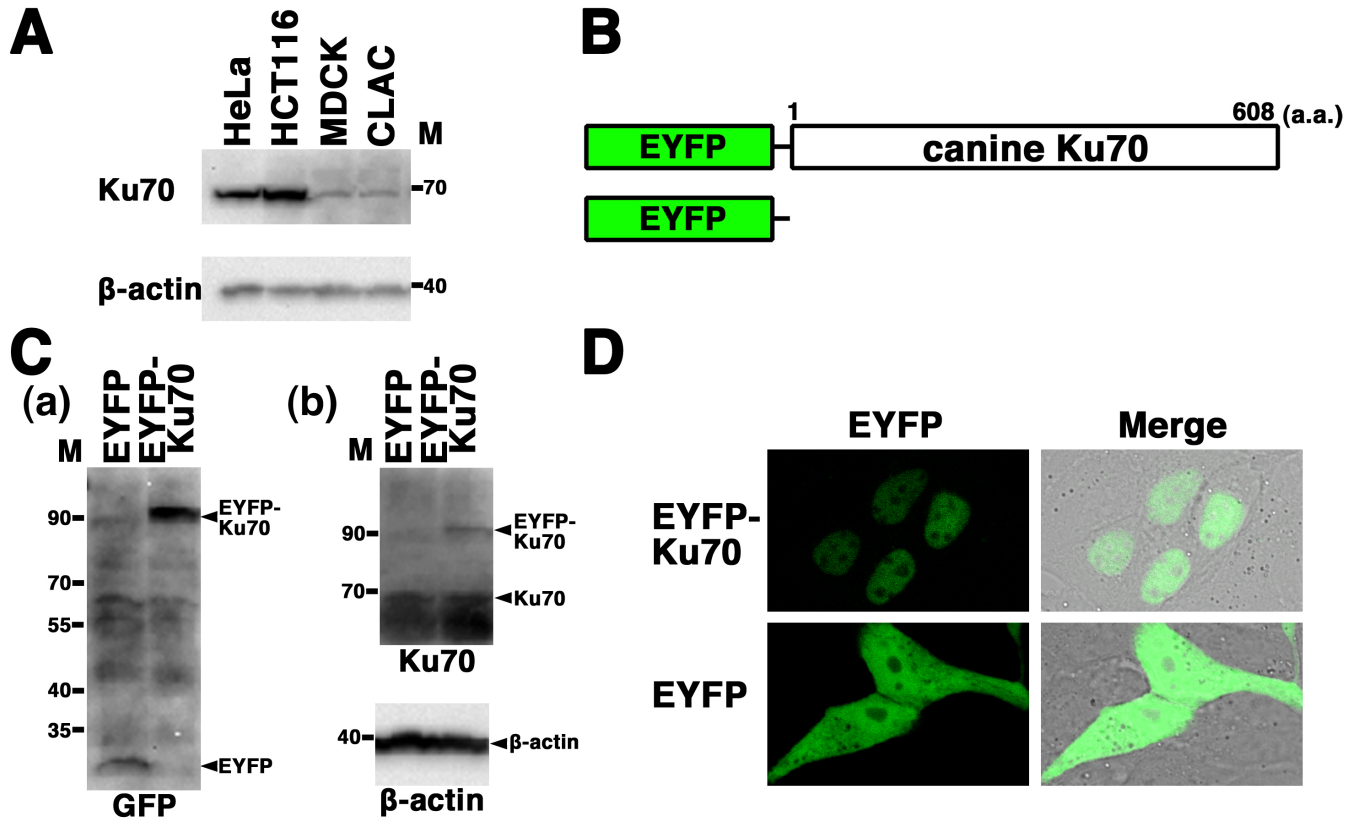


**Fig. 3.** Expression and subcellular localization of Ku70 in canine cells. (A) Total cell lysates from the two canine cell lines (MDCK and CLAC) and two human cell lines (HeLa and HCT116) were analyzed by Western blotting using an anti-Ku70 antibody (347V) or an anti-β-actin antibody. M, molecular weight marker. (B) Schematics of EYFP-canine Ku70 chimeric protein (EYFP-canine Ku70, top) and control protein (EYFP, bottom). (C) Extracts from MDCK cells transiently expressing the EYFP-canine Ku70 or EYFP were prepared and subjected to Western blotting using anti-GFP (a), anti-Ku70 (AHP316) (b) or anti-β-actin (b) antibody. M, molecular weight marker. (D) Imaging of live EYFP-canine Ku70-transfected cells. Live MDCK cells transiently expressing EYFP-canine Ku70 or EYFP were analyzed by confocal laser microscopy. EYFP images for the same cells are shown alone (left panel) or merged (right panel) with differential interference contrast images.

interphase. Previously, we identified the NLS motif in human Ku70 and showed that its structure is conserved among various species, including eight rodents, a chicken and a frog [14, 18, 19]. In this study, we found that this NLS motif is conserved in canine Ku70. On the other hand, accumulating evidence suggests the possibility that human Ku70 is a multifunctional protein possessing functions in the cell membrane and cytoplasm as well as the nucleus [4, 14]. Subcellular localization of human Ku70 dynamically changes during the cell cycle [14, 23, 24]. The nuclear localization of human Ku70 starts during the late telophase / early G1 phase after the nuclear envelope is formed [14, 23]. Previously, we clarified that human Ku70 translocates to the nucleus via interaction between its own NLS and classical NLS receptors [23]. Our sequence alignment showed that the two putative sumoylation consensus motifs in human Ku70 are conserved in canine and mouse Ku70, and one target site lies within the NLS motif. Moreover, we found that seven of the eight acetylation sites in humans are perfectly conserved in canine and mouse species, and four target sites lie within the NLS of three species. These findings suggest that the sumoylation and/or acetylation of lysine residues within the NLS control the nuclear localization of Ku70. Altogether, we speculate that the NLS is critical for the function and regulation of Ku70 not only in humans, but also in other species including canines, although further studies are needed to validate this.

In this study, we demonstrate that canine Ku70 is recruited to micro-laser induced DSB sites soon after irradiation. Binding to the DSB ends of Ku is essential for Ku-dependent NHEJ activity; Ku plays a key role in the recognition and protection of DSB ends and is important for the recruitment of other core NHEJ factors, including DNA-PKcs, XRCC4 and XLF, and other factors that process the ends [6, 8, 14, 17, 28]. In the present study, we found that the DNA-PK phosphorylation sites of Ku70 are conserved in human, mouse and canine species. Moreover, the DNA damage inducible phosphorylation sites and the putative phosphorylation sites required for Ku's dissociation from DSBs in human Ku70 are partially conserved in canine and mouse species. These findings suggest that canine Ku70 might, at least in part, be regulated by some kinases at DSB sites.

We are interested in understanding whether these PTMs affect the functions of canine Ku70 at DSB sites. Interestingly, it has been reported that human Ku has a 5′dRP/AP lyase activity [31]. This activity is important for excising abasic sites from DSB ends, suggesting that Ku has a novel and direct role in end-processing. Strande *et al*. identified K31 in Ku70 as the primary candidate

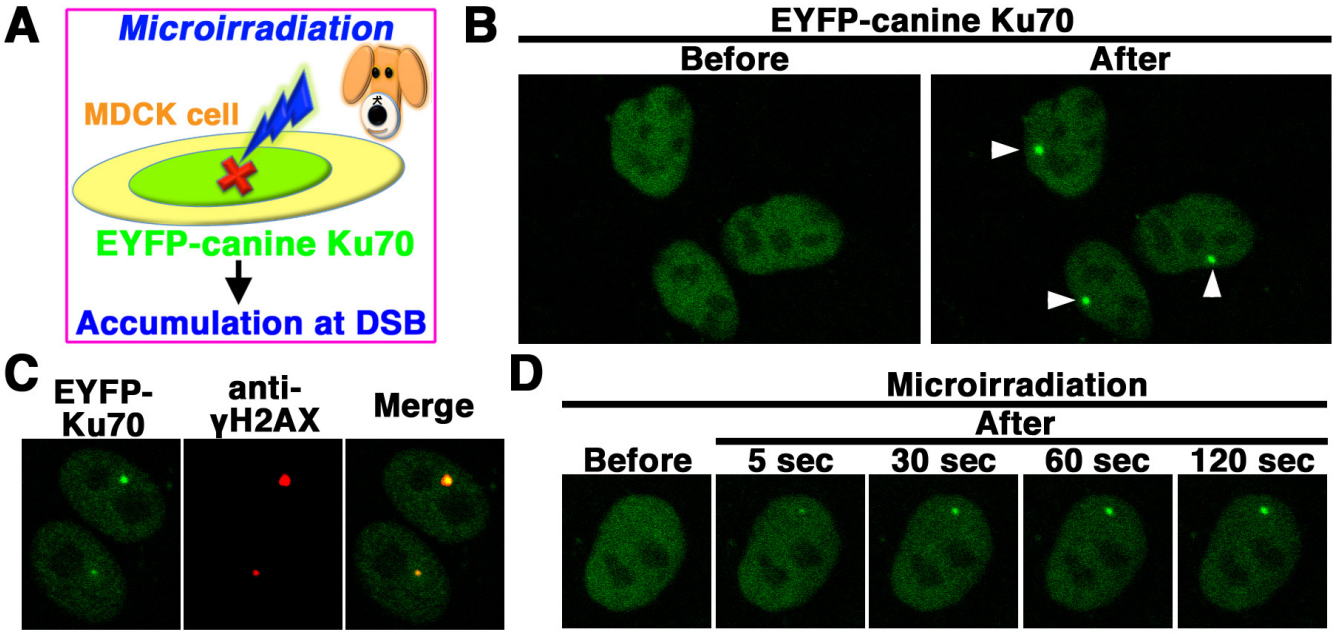


**Fig. 4.** EYFP-canine Ku70 accumulated immediately at DSBs induced by laser microirradiation. (A) The localization and accumulation of EYFP-canine Ku70 at DSBs induced by 405-nm laser irradiation were examined in MDCK cells. (B) Imaging of EYFP-canine Ku70-transfected live MDCK cells before (left panel) and after (right panel) microirradiation. Arrowheads show the microirradiated sites. (C) Immunostaining of microirradiated EYFP-canine Ku70-transfected cells with anti-γH2AX antibody. Cells were fixed and stained with anti-γH2AX antibody 5 min postirradiation. EYFP-canine Ku70 (left); γH2AX (center panel); merged image (right panel). (D) Time-dependent EYFP-canine Ku70 accumulation in live cells 5–120 sec after irradiation.

nucleophile required for catalysis [32]. In addition, they showed that the lysine is not conserved in Xenopus laevis Ku70 and that Ku70 in this species has negligible activity. Moreover, when 5′dRP/AP lyase activity in human Ku70 was inactivated, the inclusion of DNA-PKcs provided some compensating activity. In the present study, our data revealed that the lysine (K31) is not conserved in canine Ku70, suggesting that canine Ku70 does not possess any 5′dRP/AP lyase activity. Further studies may shed light on the functional differences between human and canine Ku70 at DSB sites.

In this study, we cloned and characterized canine *Ku70.* Sequence alignment provied valuable information for clarifying the regulation mechanism of Ku70 function in canine cells. Moreover, some of the motifs and PTM target sites, at least in part, might play a key role in the regulation of Ku70 localization and its functions in canine cells. Most recently, we cloned two other core NHEJ factors, *i.e.*, canine XLF (GenBank accession number: LC176889) and canine XRCC4 (GenBank accession number: LC168634), and characterized them [20, 21]. Taken together, these basic findings describing the core NHEJ factors contribute to the understanding of molecular mechanisms of underlying NHEJ and provide valuable information for the development of potential targets for next-generation chemotherapeutics in the treatment of human and canine cancers.

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