Cloning of canine Ku80 and its localization and accumulation at DNA damage sites
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Keywords
canine; companion animal; DNA double-strand break; Ku70; nonhomologous DNA-end joining

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(Received 1 May 2017, revised 17 August 2017, accepted 25 August 2017)
doi:10.1002/2211-5463.12311

Molecularly targeted therapies have high specificity and significant cancer-killing effect. However, their antitumor effect might be greatly diminished by variation in even a single amino acid in the target site, as it occurs, for example, as a consequence of SNPs. Increasing evidence suggests that the DNA repair protein Ku80 is an attractive target molecule for the development of next-generation radiosensitizers for human cancers. However, the localization, post-translational modifications (PTMs), and complex formation of Ku80 have not been elucidated in canines. In this study, for the first time, we cloned, sequenced, and characterized canine Ku80 cDNA. Our data show that canine Ku80 localizes in the nuclei of interphase cells and is quickly recruited at laser-induced double-strand break sites. Comparative analysis shows that canine Ku80 had only 82.3% amino acid identity with the homologous human protein, while the nuclear localization signal (NLS) in human and canine Ku80 is evolutionarily conserved. Notably, some predicted PTM sites, including one acetylation site and one sumoylation site within the NLS, are conserved in the two species. These findings suggest that the spatial and temporal regulation of Ku80 might be conserved in humans and canines. However, our data indicate that the expression of Ku80 is considerably lower in the canine cell lines examined than in human cell lines. These important findings might be useful to better understand the mechanism of the Ku80-dependent DNA repair and for the development of potential next-generation radiosensitizers targeting common targets in human and canine cancers.

About 6 million dogs are diagnosed with cancer each year, and in USA, more than half of the dogs older than 10 years will develop cancers such as osteosarcoma, lymphoma, or melanoma [1]. Therefore, to develop new therapies and therapeutic drugs for canine cancer is a compelling problem. Generally, mouse models are used to develop novel cancer therapies and therapeutic drugs, but their success rates are very low: Only 11% of cancer drugs that appear to be promising in mouse models turn out to be safe and effective in humans [1]. Naturally developing cancers in human and canine share many characteristics [2]. Therefore, canines have been considered an excellent model for basic cancer research, and for the development of new therapeutic drugs and therapies including next-generation chemoradiotherapies for both humans and canines [1–3].

Resistance to chemotherapy and radiotherapy is a common problem in the treatment for cancers of human and companion animal such as canines. Indeed, the ability of cancer cells to repair therapeutically induced DNA damage affects the efficacy of

Abbreviations
APLF, aprataxin and polynucleotide kinase/phosphatase-like factor; DSBs, DNA double-strand breaks; HR, homologous recombination; NHEJ, nonhomologous DNA-end joining; NLS, nuclear localization signal; PTMs, post-translational modifications.
DNA double-strand breaks (DSBs) are the most harmful among all forms of DNA damage [4,5]. There are two major DSB repair pathways: nonhomologous DNA-end joining (NHEJ) and homologous recombination (HR) [4–6]. In human and rodent cells, the NHEJ pathway can be employed to repair DSBs throughout the cell cycle and is functional in both normal and cancer cells [4–6]. The repair of DSBs via NHEJ is initiated by the binding of the Ku70 and Ku80 heterodimer (called Ku) to the damaged ends. At the break sites, Ku works as a critical DNA repair protein, recognizing the DSBs, protecting them against nucleolytic degradation, and recruiting other core NHEJ factors [5,6].

The critical function of Ku in NHEJ raises the possibility that targeting of Ku might sensitize cancer cells to the effect of X-ray, heavy ion radiation, and traditional chemotherapeutics.

To develop novel molecularly targeted anticancer drugs for both humans and canines, it is essential to determine target amino acids (and their involvement in protein structure) in a specific molecule of the chosen pathway. The recruitment to DSBs, protein–protein interactions, and post-translational modifications (PTMs) of Ku80 might play critical roles in the regulation of NHEJ activity. Previously, we have shown that Ku80 has a nuclear localization signal (NLS) recognized by NLS receptors and is mainly localized in the nucleus during interphase in various human cell lines [6–8]. Furthermore, the localization of human Ku80 is regulated through the cell cycle [6,7]. Moreover, it was demonstrated that the human Ku80 is recruited to DSB sites immediately after laser irradiation [5,9]. On the other hand, it has not been clarified whether Ku80 of other species including canines is recruited to DSB sites immediately after DNA damage. Human Ku80 might be one charming target for the development of next-generation radiosensitizers [4,10,11]. Meanwhile, canine Ku80 cDNA has not been cloned until now. In addition, the sequence, localization, and control mechanisms of canine Ku80 have not been published.

In this study, we first cloned Ku80 cDNA from a canine testis library and performed comparative analysis to clarify the regulatory mechanisms of Ku80 functions. Furthermore, we investigated its expression, subcellular localization, and recruitment to DSB sites.

### Materials and methods

#### Cloning of canine Ku80

The primers used to amplify canine Ku80 cDNA from a male beagle dog cDNA library (Biochain, Newark, CA, USA) were designed based on the predicted Ku80 genomic sequence of female boxer dog, belonging to the species *Canis lupus familiaris* (XM_536061.3). The primers used for PCR and sequencing are listed in Table 1. PCR amplification with sense (F) and antisense (R) primers was performed in a thermal cycler using the Takara Tks Gflex DNA polymerase (Takara Bio Inc., Otsu, Japan). Predenaturation was carried out for 1 min at 94 °C, and it was followed by 30 cycles of PCR amplification. Each cycle consisted of denaturation at 98 °C for 10 s, annealing at 55 °C for 15 s, and extension at 68 °C for 1.5 min. PCR products were subcloned into the EcoRI and ApaI sites of the pEYFP-C1 plasmid (pEYFP-canine Ku80) using the Infusion HD cloning kit (Takara Bio Inc.). The inserts were validated by sequencing using sequencing primers, dX5 P1, dX5 P2, dX5 P6, and dX5 P7 (Table 1). PCR amplification with sense (dX5 Cseq F2 or dX5 Nseq F2) and antisense (dX5 Cseq R2 or dX5 Nseq R1) primers was carried out.

### Table 1. Primer sequences used for PCR and sequencing.

| Primer | Bases | Sequence (5′–3′) |
|--------|-------|-----------------|
| PCR forward primers | | |
| Forward: F | 37 bp | 5′-CTCAAGCTTCTGAATTCCGATGCCGGCGTTCCAGAGCAA-3′ |
| Reverse: R | 45 bp | 5′-ATCCGGTGGATCCGGGCCCTATATCAAGTCCAGTAAAATC-3′ |
| dX5 Cseq F2 | 27 bp | 5′-GAACCTCTCAATGAGCAACAGCAGCC-3′ |
| dX5 Cseq R2 | 27 bp | 5′-CACATAACCGACAGGACACCTTCCAG-3′ |
| dX5 Nseq F2 | 19 bp | 5′-ACTCCCCCCCGGACCTTGGC-3′ |
| dX5 Nseq R1 | 28 bp | 5′-TCTCTGATAGTCTCTTACCAGCAAGG-3′ |
| Sequencing primers | | |
| dX5 P1 | 21 bp | 5′-GCTGTAAGGATCTAGTACG-3′ |
| dX5 P2 | 21 bp | 5′-GACAGTGGCTGACGCTAGAC-3′ |
| dX5 P6 | 21 bp | 5′-CAGTGAACACTTCAATATGC-3′ |
| dX5 P7 | 21 bp | 5′-CAGAGATTCTTTTCCGTGTCG-3′ |
| T3 | 17 bp | 5′-ATTAACCCTCTAAG-3′ |
| T7 | 17 bp | 5′-AATACGACTCTATAG-3′ |
for 35 cycles in a Thermal Cycler Dice (Takara Bio Inc.) or a Thermal Cycler PC-700 (ASTEC, Fukuoka, Japan) using LA Taq polymerase (Takara Bio Inc.). After pre-denaturing (95 °C for 2 min or 94 °C for 5 min), each cycle consisted of denaturation at 94 °C for 1 min or 0.5 min, annealing at 60 °C for 1 min or 0.5 min and extension at 72 °C for 1 min or 0.5 min, followed by a final extension (4 min or 5 min). The PCR products were subcloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA, USA), and the nucleotide sequences were determined by sequencing using sequencing primers, T3 and T7 (Table 1).

**Cell lines, cultures, and transfections**

The Madin–Darby canine kidney (MDCK) cell line (HSRRB, Osaka, Japan), the canine lung adenocarcinoma (CLAC) cell line (HSRRB), a mouse embryonic fibroblast cell line (NIH3T3; Riken Cell Bank, Tsukuba, Japan), a murine lung epithelial (Ku70+/−/MLE) cell line, a human cervical carcinoma cell line (HeLa; Riken Cell Bank), and a human colon cancer cell line (HCT116; Riken Cell Bank) were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS [12]. Riken Cell Bank), and a human colon cancer cell line (HCT116; Riken Cell Bank) were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS [12]. The following modifications were applied: rabbit anti-Ku80 polyclonal antibody against human Ku80 (AHP317: Serotec, Oxford, UK), rabbit anti-Ku80 polyclonal antibody against canine Ku80 (583V AP), rabbit anti-GFP polyclonal antibody (FL, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-β-actin monoclonal antibody (Sigma, St. Louis, MO, USA). The anti-Ku80 antibody (583V AP) was raised for this study against corresponding to the amino acids: 277–290 of canine Ku80, characterized and affinity-purified. The two anti-Ku80 and anti-GFP antibodies were diluted in Signal Enhancer HIKARI (Nacalai Tesque, Kyoto, Japan).

**Western blot analysis**

The extraction of total cell proteins and western blot analysis were carried out as described previously [14,15,18] with the following modifications. The following antibodies were used: rabbit anti-Ku80 polyclonal antibody against human Ku80 (AHP317; Serotec, Oxford, UK), rabbit anti-Ku80 polyclonal antibody against canine Ku80 (583V AP), rabbit anti-GFP polyclonal antibody (FL, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and mouse anti-β-actin monoclonal antibody (Sigma, St. Louis, MO, USA). The anti-Ku80 antibody (583V AP) was raised for this study against corresponding to the amino acids: 277–290 of canine Ku80, characterized and affinity-purified. The two anti-Ku80 and anti-GFP antibodies were diluted in Signal Enhancer HIKARI (Nacalai Tesque, Kyoto, Japan).

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

Local DNA damage induction using laser and subsequent cell imaging was carried out as described previously [9,15,17]. Immunocytochemistry was carried out using a mouse anti-γH2AX monoclonal antibody (JBW301; Upstate Biotechnology Inc., Charlottesville, VA, USA) and an Alexa Fluor 568-conjugated secondary antibody (Molecular Probes, Eugene, OR, USA), as previously described [14,15,18].

**Results**

**Cloning and sequence analysis of canine Ku80**

Firstly, we cloned the canine Ku80 cDNA from a beagle dog testis library and sequenced it. For the first time, we isolated a 2202-nucleotide open reading frame encoding a protein of 733 amino acids (Fig. 1). The novel canine sequence has been deposited to the DDBJ/ENA/NCBI database [accession number LC195222]. Comparative analysis of Ku80 sequences from different species showed that canine Ku80 had 82.3% and 81.4% amino acid identity with the human and mouse proteins, respectively. As shown in Fig. 2, human Ku80 is modified through some PTMs, including phosphorylation, acetylation, and ubiquitination [9,19–24]. In addition, human Ku80 has two putative sumoylation consensus motifs [ψ-K-X-E: LKKΕ(284–287) and LΚΤΕ(567–570)] and the EEXXXDDL motif [EΕGDVDDL(720–729)], which is a PIKK interaction motif of human Ku80 [9,22,23]. In this study, we found that the two sumoylation motifs in human Ku80 are conserved in the canine protein, whereas one of them (the motif spanning amino acids 567–570) is not conserved in mouse Ku80 (Fig. 2), as described previously [9]. Contrarily, the EEXXXDDL motif in human Ku80 is conserved in mouse species and, surprisingly, not perfectly conserved in canine Ku80. We have previously demonstrated that human Ku80 has a NLS sequence spanning amino acids 561–569, which has been classified as a conventional single-basic type sequence [6,8]. We found that this NLS motif is conserved in canine and mouse Ku80 (Fig. 2). Additionally, the location of the DNA-PK phosphorylation sites (S577, S580, and T715), the aprataxin and polynucleotide kinase/phosphatase-like factor (APLF)-binding sites (S577, S580, and T715), the ubiquitination sites (K265, K265, and K481), and the acetylation sites (K265, K338, and K565) in human Ku80 was also found to be evolutionarily conserved in both canine and mouse Ku80 (Fig. 2) [19,24,26,27].

**Expression and localization of Ku80 in canine cells**

Next, we analyzed the expression and subcellular localization of canine Ku80. First, we examined the expression of Ku80 in canine (MDCK and CLAC), murine...
ATG GCC GGC TCC AGG AGC AAG GCA GCT GTT CTG TGT ATG GAT GTG GCC TCT GCC ATG GGT AAT TCC TTT CCT GGT GAA GAA GAC TCA CCA 90
M A S R S A A V V L C M D V G S A M G N S F P G E S P 80
TTT GTA CTA GCA AAG ACG TCT AAT ACC ATG TTT CTG CAG CGA CGC GTT TCT GCT GAC AAG AGC CAT GAA ATT GTA TGA TCT TTC TCT GCT 180
F E L A K K V I T M F V R Q V F A E S R D E I A L V L F 60
ACA GAT GCT ACT GAT AAG GCC TCT GCT GAT GAG CAT TAT GAC AAC ATC ACA GTG CAC ACA CAG CGA ATG CTA CCA GAA TTT GAC TTG 270
T D G T E N A L A G K B Q Y Q N I T V H R L M L P D F O L 90
CTG GAC GAC ATT GAA GCA AAA ATC CAA CCA GCT TCT CAA CAA CCT GAC TCC CTG GAT GCA GTC ATT GTA TGC GAT GTG ATT CAA CAA 360
L E D I E R K I Q P G S Q A D F D L D A L I V C M D V I Q Q 120
GAA ACT GTA GAA AAG TTG GAG AGA CAT ATT GAA GTC TCT ACT GAC CTG ACC AGG CCA TTC AGC CAA AAA GAC CAG TTG GAT GTT GTA 450
E T V G K K F E K R H I E V F T O L S S P F S K O Q L V I 58
ATC CAT ACG TTG AAA GTC GCC GCT CCT CCT GCT CCT CCT GCT ACG ACC ATT GCT CCT GG CAG GAG GTC GAT GTG ATG GCT GTT AAA 450
I H N L K K F G I S L Q F F F L P F P I G K E D G T G D P G D 180
GTC TCA GCC TCA GAC CAC GAG TCA GCC TCA TCT CCT CAA AAA GGA ATT ATT ACT CAG CAG CAA AAA GAA GGT ATT CGG ATT GTG AAA 630
G N S R S D H S F K P L K C I T E Q Q K E G I R M V K K 210
GTA AGG TCG TTA GTA GGT GAA GAT GGC CTG GAT GAA ATT CAT TCC TCC ACT GAT ATG GTC CAA AAA AGA TAT ACT TTC ATT GGA GAA 720
V M R S L E G D G L E I Y S F S E S L R Q L C V F K K K 240
GAA AGG GCC TCT CCT GCC CCC TCA ACA CTG GAT ACC GTC TCC ATT GCC TCC ACT TCT ATA AAT TCT GCT TAT AAA TCG ATT ATA CAC GGC 810
M E R S I P I W P C T G S S L S I K I V A Y S K I Q E 270
AAA GTC AAA AAA GTC TGA ACA CCT GTG GCC GCT AGG AGA CTA TGG AGG TGG GTA AAG CAA AAA AAA AAA ATT CAT GTC GTA AAA GAC 960
K V K K G N T V T L T E L K K E D L Q K E T V Y C L N D D 330
AAT GAA ACA GAG TTC GCC CGG AAG GAG GAC ACT ATT CAA GGG TCC TGG TAT GAA GAT ACT ATT GGT TCT AAC GTC GAT GAA GAC 990
N E T E V P K E D K G F R Y G R S G D I V F P S K V D E E H 330
AGT AAA TAT AAA TTG GAG GGG AAG CTG TTC GCC CCT TCC ATT GCC TCC ACT GAC AAG AGA AAA ATT ATG ATT GTG GAA AAA 1080
M K Y K L E G K F S V L G F C R S S Q S V H R Y F M G N Q 360
CTG TCA ACA GTC TTG GCA GCT AAA GAT GAT GCA GCA GTA GCT TCC TCC TCC ATT CAT GTT CCT GAT GAA TTA GAC ATG GTG 1270
V L K V F A A K D D E E A A A A V A A L S I L H A D E M 390
GCC GCT GTT GAA TAT GAT FTT GAT GCT ACC CCT AGG ACT TAT GAT GTG TAT GAA GTA AAC TAC CTC TTT AAA AAA TTT AAA GAC TAT 1350
A V Y R A V Y A Y D R R S H P Q V G M A F P F I K D V Y E C L I 420
TAT GTC CAG CTG CCT TTT ATT GAA GAC TTG CGA CAA TAC ATG GTC TTA TCA GAT TTA TAA AAT ATT AAG AAA TGC ACT CCC AGC GAG 1350
Y V Q L P F M E D L R Q Y M F S F K K N K K K C T P T E A Q 450
CTG AGT GGC GCT GAT GTC GAT CTG ATT GAC TCC ATG AGC TTG ATA AAG AAA GAA GAG GAC ACC ATT GAA GAC GTA TTA TCC CCC ACC ACC 3440
L S A V D A L I S M S L I K D E K D D T I E D L F P T S 480
AAA ATC ACA ATT CCT CAA TTG CAG AGA TTA TTT CCTAG TCT CTG CAG CTC GAT CCT GCT CAT CCC CAG GAG CCT CTA CCC CCA ATT CCC CCG 3530
K I P N P Q F Q R Q L C L H R A L H P Q E P L L P P Q 510
CAC ATT TGG ACT ATG CTG GAC CCC CCC ACT CAG GAT ACA ACT AAA TGG CAT GTT CTC TCT TCT AAA AAA AAG ACC ATT TCT CCG ACT 1520
H I L S M L D P P T E V T K T C Q V V P L S K I T I K F P L T 540
GCA GTC ACA AAC AAG GAT CAG CGT ACT GCT GAC ATT TCT CAA GAC AAC CAA CAG CGA CCC CAC TCT AAA AAA TTG GAC ACT 1710
E V I K K K D Q V T A Q D I F Q D O N H E E G P D S K K L K T 570
GAA GAC GAG GCC CCC TCT GCT ACG ACT TCC AGC ACT GCG ACC ACT GTC ACT GGA ATG GTC ATT AAT ACT GCT GAT GAA GAC TCG 3880
Q E G E P C F S I S L G A E S G V T S V G S V N P A E N F R 680
GTT GTA GAG CAG CAG AAA GCC ACC TCT GAA GAA GCA ATG GCA CAG ACT CAG CAT GAA GAA ATT CTT GCAG ATT CAG CTA CAC GAC AGC 1890
V L V R Q K K A T E F E A A S C Q L I S R I E Q F L D T N E T 630
CCG TAT TCT ATG AAG AGC ATG ACT GCC GTT CTG CCT CAG CAG GAA GAC CAG CCC ATT TCT TCT GTA GAA GAA GAC CTC TTT AAA CAA TCG 2590
P Y F M K S A M D C I C T V F R Q E A I Q F S E E Q R P R N D F L 660
AAA GCC GTC CAG AAA GAT ATT AAA AGA CAT ATT CAT TTC TGG GAA ATT GTC ACT CAG GAT ATT ACT ATG CTG ACC AAC GAT 2590
K A L R E K V W M K Q L N H F W E I V I Q D G I L T K D 690
GAA GCC ATG ACC TCT GCC ACG ACC AGG GAC AAA GAG AGC ATG GCC GTT CAA AAA AAA GAA CTT CCA AAT GAA GAC ACA GCC ATG TT 2160
E A P G S S V T A A E A K Q F L A P E K P N E D T A A I F 720
GAA GAC TCT GTC TCT GAT GTA AAC ACC ATG GTA TGG GCT GAT ATG AAG ACA 1890
A E G G D V D V D L L D L I * 734

Fig. 1. Nucleotide sequence and deduced amino acid sequences of canine Ku80 (Canis lupus familiaris, GenBank accession number: LC195222). The CDS of canine Ku80 is composed of 2202 bp encoding 733 amino acid residues. The asterisk after the amino acid sequence indicates the position of the termination codon. Numbers on the right correspond to nucleotides (top) and amino acid residues (bottom).
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Fig. 2. Comparison of the canine, human, and mouse Ku80 amino acid sequence. Canine (Canis lupus familiaris, GenBank accession number: LC195222), human (Homo sapiens, GenBank accession number: NP_066964.1) and mouse (Mus musculus, GenBank accession number: NP_033559.2) species. The location of the DNA-PK phosphorylation sites (S577, S580, and T715), the APLF-binding sites (L68, Y74, and I112), the ubiquitination sites (K195, K265, and K481), the acetylation sites (K265, K338, K565, and K660), and the two putative sumoylation sites (K285 and K568) in human Ku80 [9,19,22,24,26,27] are marked with asterisks. The location of the NLS (NLS: Y74, I112), the DNA-PK phosphorylation sites (S577, S580, and T715), the APLF-binding sites (L68, Y74, and I112), the ubiquitination sites (K195, K265, and K481), the acetylation sites (K265, K338, K565, and K660), and the two putative sumoylation sites (K285 and K568) in human Ku80 [9,19,22,24,26,27] are marked with asterisks. The location of the NLS (NLS: S561–569), the two putative canonical sumoylation consensus motifs [ω-K-X-E: LKKE(284–287) and LKTE(567–570)], and the EEXXXDDL motif [EEGDVDDL(720–729)] in human Ku80 are shown [8,9,22,25].

(95x1042) NIH3T3 (95x1042) and Ku70+/− (95x1042) cell lines. As shown in Fig. 3A, the expression of canine Ku80 was detected by the two antibodies (AHP317 and 583V AP) in the two canine cell lines examined; similar to what observed in the murine cell lines. Ku80 displayed lower expression levels in the two canine cell lines compared to the human cell lines. Next, to investigate subcellular localization of Ku80 in live canine cells, we generated MDCK cells transiently expressing EYFP-canine Ku80. For this purpose, the expression vector pEYFP-C1 containing canine Ku80 (pEYFP-canine Ku80) was transfected into MDCK cells (Fig. 3B). As shown in Fig. 3C, western blotting using anti-Ku80 and anti-GFP antibodies showed that the chimeric protein was expressed in the transfected cells. Confocal laser microscopy demonstrated that, during interphase, EYFP-canine Ku80 localized in the nuclei (with the exclusion of the nucleoli) of the cells transfected with pEYFP-canine Ku80 (Fig. 3D). Expectedly, EYFP, used as a control, was distributed throughout the cytoplasm of the transfected cells, but was not localized to mitotic chromosomes (Fig. 3E). These results showed that the localization of canine Ku80 dynamically changes during the cell cycle.

**EYFP-canine Ku80 accumulates immediately at laser-microirradiated DSB sites**

Previously, we and others have demonstrated that human Ku80 accumulates immediately at DSB sites after irradiation [5,9]. Meanwhile, it has not yet been investigated whether Ku80 of other species, including canine, accumulates at DSB sites immediately after DNA damage. Next, we investigated whether, in canine cells, EYFP-canine Ku80 accumulates immediately at DSB sites induced by a 405-nm laser (Fig. 4A). Local DSBs in canine cells were induced using a 3% power scan (for 1 s) from a 405-nm laser. Laser microirradiation resulted in the accumulation of EYFP-canine Ku80 at the microirradiated sites in live MDCK cells (Fig. 4B). To test whether EYFP-canine Ku80 actually accumulated at the DSB sites induced by the 405-nm laser, we immunostained cells with an antibody that detects γH2AX, a marker of DSBs. As shown in Fig. 4C, EYFP-canine Ku80, but not EYFP, was found to accumulate and colocalize with γH2AX at microirradiated sites in MDCK cells. To examine the temporal dynamic of Ku80 localization, we carried out time-lapse imaging in MDCK cells transfected with pEYFP-canine Ku80. We found that EYFP-canine Ku80 accumulates at the microirradiated sites 5 s after irradiation (Fig. 4D). These results indicate that after irradiation, EYFP-canine Ku80 accumulates immediately and forms foci at laser-induced DSBs in canine cells.

**Discussion**

Molecularly targeted therapies have high specificity and the significant cancer-killing effect, but their impact is known to decrease remarkably when there is a variation, such as an SNP in the target site. Comparative analysis of certain DNA repair proteins between humans and canines is useful in the attempt to develop more effective repair inhibitors and more efficient cancer-killing drugs for both human and canine cancers. However, there is no information regarding the function and the regulation of canine Ku80. In the present study, for the first time, we cloned, sequenced, and characterized canine Ku80 cDNA. Comparative analysis showed that canine Ku80 had only 82.3% amino acid identity with the homologue human protein. Sequence analysis demonstrated that the sites of PTMs and protein-binding motifs are not perfectly conserved between canine Ku80 and human Ku80. Additionally, we demonstrated that EYFP-canine Ku80 mainly localizes in the nuclei of interphase cells, and the localization of EYFP-canine Ku80 dynamically changes during the cell cycle. Moreover, we showed that canine Ku80 is recruited to DSB sites immediately after irradiation. These findings might be useful for the development of next-generation radiosensitizers targeting potential common targets in human and canine cancers.
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C. lupus 1  MAASRSAAVLVDGVSAMNSGSPFFPESLAKKVTMFQVRQVFQASRE幖IALVLF  60
H. sapiens 1  MVRSGNKAAVCLMDQFTMSNPSGPEIPESQFQAKVTMFQVRQVFQAFKDEigliaVLF  60
M. musculus 1  MAWNSKAAVCLMDQGAMNSGSPFESPIAKKVTMFQVRQVFQFSDEigliaVLF  60

*C195
C. lupus 181  GNSRSDHGSSFPFKGITEQKQEGIRMVKVKMSLEGDEGLIDEYISFSLRLQCVFKII  240
H. sapiens 181  GPFRFGLGHPSFPLXGITEQKQEGIRMVKMSLEGDEGLIDEYISFSLRLKCVFKII  240
M. musculus 181  LDGSDLHKLPSPSFKGLTQEQKQEGIRMVKMSLEGDEGLIDEYISFSLRLQCVFKII  240

*K205 *K265 Sumoylation motif(284–287, *K285)
C. lupus 241  ERHSHWPCTLRTITIONSISIVAYSVQKQIFKKSVMVVDARTLKKELEQKETYCVLCLND  300
H. sapiens 241  ERHSHWPCTLRTITIONSISIVAYSVQKQIFKKSVMVVDARTLKKELEQKETYCVLCLND  300
M. musculus 241  ERSMPWPCQTLTIGNLISIVAYSVQKQIFKKSVMVVDARTLKKELEQKETYCVLCLND  300

*K338
C. lupus 301  NETEVPKEDEITQQFRYGRSDIVPSKVDVDEMVKMYKLEGKCFSLGFCRSSQVHRYPDNGQ  360
H. sapiens 301  DETEVKEDETIQQFYGRSDIVPSKVDVDEMVKMYKLEGKCFSLGFCRSSQVHRYPDNGQ  360
M. musculus 301  DETEVKEDETIQQFYGRSDIVPSKVDVDEMVKMYKLEGKCFSLGFCRSSQVHRYPDNGQ  360

C. lupus 361  VLCVFAAKDEAAAAVALLSLILHDHELDMVXAVVAYDRAHSPHQVQVMAFPFIDFKDYECLI  420
H. sapiens 361  VLCVFAAKDEAAAAVALLSLILHDHELDMVXAVVAYDRAHSPHQVQVMAFPFIDFKDYECLI  420
M. musculus 361  VLCVFAAKDEAAAAVALLSLILHDHELDMVXAVVAYDRAHSPHQVQVMAFPFIDFKDYECLI  420

C. lupus 421  YVQLPMFEDLRQMSSFKNKKKCTPTEATLQSAVADILDSMLIKKDEKDDTIEDLFTPT  480
H. sapiens 421  YVQLPMFEDLRQMSSFKNKKKCTPTEATLQSAVADILDSMLIKKDEKDDTIEDLFTPT  480
M. musculus 421  YVQLPMFEDLRQMSSFKNKKKCTPTEATLQSAVADILDSMLIKKDEKDDTIEDLFTPT  480

*K481
C. lupus 481  KIPNPQQFQRLQFCILHLRHAIPQELQLPQIPHQHLSMIDLPETVITKCQVPLSKIKITFPLT  540
H. sapiens 481  KIPNPQRFLQRCILHLRHAIPQELQLPQIPHQHLSMIDLPETVITKCQVPLSKIKITFPLT  540
M. musculus 481  KIPNPQRFLQRCILHLRHAIPQELQLPQIPHQHLSMIDLPETVITKCQVPLSKIKITFPLT  540

*K565 *S577 *S580 Sumoylation motif(567–570, *K568)
C. lupus 541  EIKKIKIQVTAQIFQDHNEEGPDSKLLT*EGEECFUNSILSAEGTSVASVGPVAENFR  600
H. sapiens 541  EA–KKKIDIQVTAQIFQDHNEEGPDSKLLT*EGEECFUNSILSAEGTSVASVGPVAENFR  599
M. musculus 541  ETAIKKIKIQVTAQIFQDHNEEGPDSKLLT*EGEECFUNSILSAEGTSVASVGPVAENFR  599

*NLCS(561–569)
C. lupus 601  VLRQASKKAFGEASQCAIQLSIREDQFLNETPQFMSMDCITVFQREAIAQEEQFQPNDFEL  660
H. sapiens 600  VLRQASKKAFGEASQCAIQLSIREDQFLNETPQFMSMDCITVFQREAIAQEEQFQPNDFEL  659
M. musculus 601  FVRQASKKAFGEASQCAIQLSIREDQFLNETPQFMSMDCITVFQREAIAQEEQFQPNDFEL  660

*K660 *T175
C. lupus 661  KALRKEKVMQNLHNFWEIVIQDQGTLITKDEAPGGSVTAAEAKFQFLAPEENEDTAAIF  720
H. sapiens 660  KALRKEKVMQNLHNFWEIVIQDQGTLITKDEAPGGSVTAAEAKFQFLAPEENEDTAAIF  719
M. musculus 661  KALRKEKVMQNLHNFWEIVIQDQGTLITKDEAPGGSVTAAEAKFQFLAPEENEDTAAIF  719

EEXXDDL motif (720–729)
C. lupus 720  AEGGOQVLLDLI  733
H. sapiens 720  AEGGOQVLLDLI  732
M. musculus 720  AEGGOQVLLDLI  732
Molecular mechanisms underlying protein–protein interactions and PTMs of DNA repair proteins play key roles in the control of some DNA repair pathways [5,28]. Three DNA-PK phosphorylation sites (S577, S580, and T715) have been identified in human Ku80 [20], but the role of these modifications is still unclear. In humans, Ku80 phosphorylation at DNA-PK phosphorylation sites might not be required for its interaction with Ku70, its nuclear translocation, and DNA DSB repair [26,29]. In this study, we found that the DNA-PK phosphorylation sites might not be required for its interaction with Ku70, its nuclear translocation, and DNA DSB repair [26,29]. In this study, we found that the DNA-PK phosphorylation sites are perfectly conserved in human and canine Ku80. Further studies are necessary to clarify the significance of DNA-PK-mediated phosphorylation of canine Ku80, and shed light on its function and/or regulation of Ku80, not only in canines, but also in various species, including humans.

In this study, we showed that canine Ku80 accumulated at laser-induced DSB sites immediately after irradiation. Human Ku80 is required for the recruitment of core NHEJ factors such as DNA-PKcs, XRCC4, Ku70, and XLF [5,9]. Additionally, a study has reported that the Ku80-binding motif of APLF promotes APLF accumulation at DSBs in human cells [27]. In the same study, the authors also showed that APLF promotes the assembly and activity of core NHEJ protein complexes. In this study, our data showed that the APLF-binding sites (L68, Y74, and I112) in human Ku80 are perfectly conserved in canines, suggesting that the interaction between APLF and Ku80 is important for the Ku's function in both species. Falck et al. [25] reported that the PIKK interaction motif of human Ku80 [also called EEXXXDDL motif (720–729)] is required for the interaction with DNA-PKcs and for DNA-PK activation. Deletion of the last 14 residues of Ku80 (amino acids: 719–732) affects the efficient recruitment of DNA-PKcs to DNA ends in vitro, and point mutations (E720A/E721A) of Ku80 inhibited its interaction with DNA-PKcs. In addition, Gell and Jackson [30] reported that the final 12 amino acid residues (amino acids: 721–732) of human Ku80 are sufficient to bind DNA-PKcs. On the other hand, in this study,
we found that the EEXXXDDL motif (amino acids: 720–729) of human Ku80 is not perfectly conserved in canine Ku80 (amino acids: 721–730): Canine Ku80 has an alanine residue (A721) instead of the glutamic acid residue in human Ku80. Altogether, we speculate that as well as the first glutamic acid residue (E720) of the EEXXXDDL motif in human Ku80, the corresponding alanine residue (A721) in canine Ku80 is not essential for the recruitment of DNA-PKcs to DNA ends. Additional studies, however, are needed to clarify this issue.

The mechanism responsible for the nuclear localization of Ku70 and Ku80 appears to play, at least in part, a key role in the regulation and the physiological function of Ku in vivo [6]. Previously, we showed that subcellular localization of human Ku80 dynamically changes during cell cycle [7]. Additionally, we identified the NLS motif spanning amino acids 561–569 in human Ku80 [6,8]: Its structure is conserved among various species, including three rodent species and a frog [6,8]. We also clarified that human Ku80 translocates to the nucleus through the interaction between its own NLS and classical NLS receptors [8]. In the present study, our data showed that the localization of canine Ku80 dynamically changes during the cell cycle. Combining with our previous findings, our data indicate that the patterns of subcellular localization of human and canine Ku80 are similar throughout the cell cycle [7]. We found that the NLS motif is conserved in canine Ku80. In addition, sequence alignment analysis showed that the four acetylation sites present in human Ku80 are conserved in the canine protein, and one of these sites lies within the NLS of the three species examined. We also found that the two sumoylation consensus motifs in human Ku80 are conserved in canine Ku80, and one of these sites lies within the NLS motif as well. Collectively, these findings raise the possibility that the modifications of all or a part of these amino acids within the NLS determine the cell cycle phase-dependent subcellular localization of Ku80, and thereby control Ku80 function. We are interested in further studies investigating this possibility.

RNF8-, RNF138-, and NEDD8-dependent ubiquitin ligases have been reported to mediate human Ku80 ubiquitination [19,21,23]. Brown et al. [19] reported that neddylation promotes ubiquitination and release of Ku from DNA damage sites and showed that ubiquitination of K195, K265, and K481 of human Ku80 is increased after treatment with the DSB inducer phleomycin. In this study, our data showed that these three lysines are conserved among the three species examined. Most recently, Ishida et al. [31] reported that RNF126 is a novel regulator of NHEJ that promotes completion of DNA repair by ubiquitinating Ku80 and releasing Ku from damaged DNA. Using proteomics and structural analyses, the authors identified 19 lysine residues including several novel ubiquitination sites in Ku80: The mutation of all of these sites inhibited the dissociation of Ku from chromatin and DNA damage response. In this study, our data showed that these 19 lysine residues in human Ku80 sites are perfectly conserved in canine Ku80, whereas three of these 19 lysine residues in human Ku80 sites, K144, 

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**Fig. 4.** Accumulation of EYFP-canine Ku80 at the sites of DSBs induced by laser microirradiation. (A) The recruitment of EYFP-canine Ku80 to DSBs induced by 405-nm laser irradiation in MDCK cells. (B) Imaging of live MDCK cells transfected with pEYFP-canine Ku80 before (left panel) and after (right panel) microirradiation. Arrowheads indicate the microirradiated sites. (C) Immunostaining of microirradiated cells transfected with pEYFP-canine Ku80 using an anti-\(\gamma\)H2AX antibody. Cells were fixed and stained with an anti-\(\gamma\)H2AX antibody 5 min postirradiation. Left panel, EYFP-canine Ku80 (upper panel) or EYFP (lower panel); center panel, \(\gamma\)H2AX; right panel, merged images. (D) Time-dependent EYFP-canine Ku80 accumulation in live cells, from 5 to 90 s after irradiation.
K282, K469, correspond to different amino acids in mouse Ku80. Further studies need to clarify whether the ubiquitination of these three lysines has specific roles in human and canine cells. Interestingly, our findings showed that Ku80 exhibited lower expression levels in the canine cell lines compared to the human ones. The ubiquitin-proteasome pathway can regulate the levels of certain protein via ubiquitination [32]. We are interested in understanding whether the protein level of Ku80 is regulated via the ubiquitin-proteasome pathway in human and canine cells.

In this study, we cloned, sequenced, and analyzed canine Ku80. Our findings provide important information for clarifying the regulation mechanism of Ku80 and its functions in canine cells. In addition, these findings might contribute to clarify the molecular mechanism of the Ku80-dependent NHEJ pathway. Further comparative studies on Ku80 will provide valuable additional information in order to develop common molecularly targeted drugs having high specificity and significant cancer-killing effect in both humans and canines.

Acknowledgements

This work was supported in part by JSPS KAKENHI Grant Numbers JP26450438 and JP16K00554.

Data Accessibility

The sequence of canine Ku80 cloned in this study has been deposited to the DDBJ/ENA/NCBI database [accession number LC195222].

Author contributions

MK and AK conducted all experiments. MK wrote the manuscript. MK, AK, and YY performed experiments and analyzed data. All authors have read and approved the final manuscript.

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