





Nuku, a family of primate retrocopies derived from *KU70*

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Abstract

The gene encoding the ubiquitous DNA repair protein, Ku70p, has undergone extensive copy number expansion during primate evolution. Gene duplications of *KU70* have the hallmark of long interspersed element-1 mediated retrotransposition with evidence of target-site duplications, the poly-A tails, and the absence of introns. Evolutionary analysis of this expanded family of *KU70*-derived “*NUKU*” retrocopies reveals that these genes are both ancient and also actively being created in extant primate species. *NUKU* retrocopies show evidence of functional divergence away from *KU70*, as evinced by their altered pattern of tissue expression and possible tissue-specific translation. Molecular modeling predicted that amino acid changes in Nuku2p at the interaction interface with Ku80p would prevent the assembly of the Ku heterodimer. The lack of Nuku2p-Ku80p interaction was confirmed by yeast two-hybrid assay, which contrasts the robust interaction of Ku70p-Ku80p. While several *NUKU* retrocopies appear to have been degraded by mutation, *NUKU2* shows evidence of positive natural selection, suggesting that this retrocopy is undergoing neofunctionalization. Although Nuku proteins do not appear to antagonize retrovirus transduction in cell culture, the observed expansion and rapid evolution of *NUKUs* could be being driven by alternative selective pressures related to infectious disease or an undefined role in primate physiology.

Keywords: retrocopy; Ku70; primates; NHEJ

Introduction

Protecting the integrity of a cell’s genetic material is important for both survival as well as for ensuring the faithful transmission of genes to daughter cells. Thus, DNA repair genes are conserved throughout the evolutionary history of prokaryotes and eukaryotes, with homologs present in every major organismal clade. A prime example is the *KU70* gene, involved in DNA double-strand break repair mediated by nonhomologous end-joining (NHEJ). Human Ku70p and Ku80p together form the Ku heterodimer, a well-established initiator of the NHEJ pathway for DNA double-strand break repair (Roth et al. 1985; Milne et al. 1996; Gu et al. 1997; Jin and Weaver 1997). In addition to its well-documented role in the NHEJ pathway, Ku70p is also involved in V(D)J recombination (Nussenzweig et al. 1996; Gao et al. 1998), telomere maintenance (Gravel et al. 1998; Hsu et al. 1999), Bax-mediated apoptosis (Subramanian et al. 2005), innate immune signaling (Zhang et al. 2011; Ferguson et al. 2012; Sui et al. 2017), and even cell–cell adhesion and extracellular matrix remodeling at the cell membrane (Monferran et al. 2004; Martinez et al. 2005; Chan et al. 2009). The *KU70* and *KU80* genes are present in eukaryotic and archaeal genomes, while in bacteria the role of the heterodimer is

performed by a homodimer of the protein Ku (Aravind and Koonin 2001; Weller et al. 2002).

Gene duplication is an important mechanism by which new genes arise. After gene duplication, multiple possible fates await the new gene copy, depending on the selective forces at play: decay, purifying selection, subfunctionalization, or neofunctionalization (Long et al. 2003; Schlötterer 2015). Retrocopies (previously known as “processed pseudogenes”) are a type of gene duplication created when retrotransposons erroneously reverse transcribe a cellular mRNA and insert the cDNA copy of the gene back into the host genome (Kaessmann et al. 2009). As a result, retrocopies often lack introns (Long and Langley 1993; Schacherer et al. 2004; Benovoy and Drouin 2006). In addition, they can also be flanked by target-site duplications (TSDs), as is the case for mammalian LINE-1 mediated retrotransposition (Maestre et al. 1995; Esnault et al. 2000). Retrotransposition and the subsequent formation of retrocopies are cited as having had a singular effect on primate and human evolution, with a so-called “burst” in retrocopy formation during the last 63 million years have contributed to the emergence of many novel genes (Ohshima et al. 2003; Zhang et al. 2003).

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Approximately 3771–18,700 retrocopies of human genes exist in the human genome, with about 10% of these found to express mRNA transcripts (Harrison et al. 2005; Sisu et al. 2014; Casola and Betrán 2017).

The main *KU70*-related gene duplication that is known is the ancient duplication that gave rise to *KU70* and *KU80*, and thereby the eukaryotic Ku heterodimer. Here, we report the description of five *KU70* retrocopies in the human genome, which we have named *NUKU1*–*NUKU5*. Four of these retrocopies are present in all simian primate genomes, and therefore predate the split between Old World monkeys and New World monkeys over 30 million years ago (MYA). However, a newer retrocopy found on the human X chromosome, *NUKU5*, is specific to apes (human, gorilla, chimpanzee, and orangutan). *KU70* has spawned an unusual number of retrocopies, as it is the only one out of 66 genes linked to DNA double-strand break repair to have five retrocopies in the human genome. While the original open reading frames (ORFs) appear to be disrupted by mutations and indels, there is evidence for expression of *NUKU2*, *NUKU4*, and *NUKU5* and a spliced transcript that exists for *NUKU2*. *NUKU2* has also evolved under positive selection, and functional tests of *NUKU* genes and molecular modeling simulations reveal that it has functionally diverged from *KU70* in two ways. First, whereas *KU70* is expressed in all tissues, *NUKU2*, *NUKU4*, and *NUKU5* display a tissue-specific expression pattern and these transcripts are associated with ribosomes. Second, while Ku70p interacts with Ku80p, Nuku2p does not. Given the extensive functional characterizations of human *KU70* and *KU80* that have occurred over decades, it will now be of great interest to determine what potential role these additional *KU70*-like genes play in human biology.

Materials and methods

Identification and classification of retrocopies

The *KU70* coding sequence was used as a query in the UCSC genome browser against the human genome (<http://genome.ucsc.edu/>, March 2006 NCBI36/hg18 assembly, Last accessed 5.24.21). Six top hits of Blat scores were identified, the topmost of which matched with 100% sequence identity to the original *KU70* gene. The next five hits appeared as retrocopies upon closer inspection. *NUKU* orthologs from chimpanzee, orangutan, and rhesus macaque were also obtained using this method. For inspection of insertion sites in the marmoset genome, the *calJac1* and *calJac3* assemblies were used. All other insertion sites were interrogated using the current version of primate genomes found on the UCSC genome browser (<https://genome.ucsc.edu>). The phylogenetic trees of *KU70* and *NUKU* sequences were built with MEGA v.7 (maximum likelihood method) (Hall 2013) from DNA alignments built using MUSCLE (v3.8) and were manually curated to ensure accuracy.

The GO term “double-strand break repair” was queried in the GO database (GO term ID 0006302). Because not all genes have been fully annotated and assigned to appropriate GO categories (leading to exclusion of certain relevant genes from this list), we combined genes assigned to this GO category in either *Homo sapiens*, *Mus musculus*, and *Rat norvegicus*. This resulted in a list of 66 genes (Supplementary Table S1). cDNA coding sequences for all 66 hits were retrieved from NCBI. In the case of genes with multiple transcript variants or splicing variants, the longest transcript was used. To find retrocopies of each gene, cDNA sequences were used as queries in the UCSC human genome database (hg18). RetrogeneDB2 was also used in an attempt to identify *KU70* retrocopies in primates, but failed to identify *NUKU4* and was unable

to identify any *KU70* retrocopies in primates and so was not used further (Rosikiewicz et al. 2017). Retrocopies were defined as hits in the human genome that met the following two criteria: (1) they lack introns (RepeatMasker was used to differentiate introns from transposable element insertions), and (2) they match the parent gene in a reciprocal best hit analysis of the human genome. Reciprocal best hit analysis was performed by taking each putative retrocopy and using the BLAST server at NCBI to query the human RefSeq mRNA database.

Sequencing *KU70* and *NUKU* orthologs

KU70 orthologs and *NUKU2* ORF orthologs were sequenced from mRNA-derived cDNA for *KU70* and from genomic DNA for *NUKU2* from 12 primates: gorilla (*Gorilla gorilla*), agile gibbon (*Hylobates agilis*), colobus (*Colobus guereza*), crab-eating macaque (*Macaca fascicularis*), gibbon (*Pongidae Hylobates syndactylus*), leaf monkey (*Trachypithecus francoisi*), Borneo orangutan (*Pongo pygmaeus*), talapoin (*Miopithecus talapoin*), white-cheeked gibbon (*Nomascus leucogenys*), olive baboon (*Papio anubis*), black mangabey (*Ophocebus albigena*), and Wolf’s guenon (*Cercopithecus wolfi*) (Supplementary Table S2). Genes were PCR-amplified using the strategy described in Supplementary Table S3 and sequenced with primers shown in Supplementary Table S4. The full structure of the *NUKU2* transcript was determined with 5’ and 3’ RACE using the GeneRacer kit (Invitrogen), and testis total RNA (Ambion, catalog #7972). All nucleotide sequences are provided within Supplementary File S3.

Evolutionary analysis of *KU70* retrocopies

Sequences of the human *KU70/NUKU* paralogs were collected from the UCSC genome browser and aligned using ClustalX. Sequences were analyzed under the free-ratio model implemented in the *codeml* program of PAML 3.14. In order to determine whether $dN/dS > 1$ on the *NUKU2* branch, we made a pairwise comparison between the ancestral (Anc) sequence (generated by *codeml*) and *NUKU2*. K-estimator (Comeron 1999) was used to run Monte Carlo simulations of neutral evolution of these sequences, creating a null distribution from which a *P*-value could be derived.

The branch-site test allows identification of positive selection that might be limited to a subset of codons along only a subset of the branches being analyzed (Zhang et al. 2005). To implement this test, multiple alignments were fitted to the branch-sites models Model A [positive selection model, codon values of dN/dS along background branches are fit into two site classes, one (ω_0) between 0 and 1 and one (ω_1) equal to 1, on the foreground branches a third site class is allowed (ω_2) with $dN/dS > 1$], and Model A with fixed $\omega_2 = 1$ [null model, similar to Model A except the foreground ω_2 value is fixed at 1]. *NUKU2* branches (back to their last common ancestor) were defined as the “foreground” clade, with all other branches in the tree being defined as background branches. The likelihood of Model A is compared to the likelihood of the null model with a likelihood ratio test.

NUKU expression in human tissues

Total RNA from human tissues was purchased from Clontech (catalog number 636643). Most of these samples represent pooled RNA from multiple individuals (between 2 and 63 individuals). First-strand cDNA was produced with the NEB Protoscript II kit (E6400S), using a dT_{23} primer that anneals indiscriminately to poly-A tails on mRNA molecules. First-strand reactions were carried out twice in parallel for each tissue, one with reverse transcriptase (RT), and one with water added instead of RT (indicated by \pm RT on Figure 5). First-strand cDNA was then amplified with

KU70- and NUKU-specific primers using Invitrogen PCR Supermix HiFi (cat 10790020). In order to increase specificity, two successive PCRs were performed. In the first round of PCR, 20 cycles were performed using primers specific to that gene, along with 2 μ l of first-strand cDNA as template. In the second cycle, 0.5 μ l of the first round PCR reaction was used as template, and one of the gene-specific primers was substituted with a nested primer (F2 or R2 in Figure 5B). In this round, amplification was performed for 40 cycles, and 2 μ l of the final product was then run on a 2% agarose gel for separation. Primers used were: SS004 (Nuku F), SS011 (Nuku R1), SS009 (Nuku R2), SS030 (Ku70 F1), SS031 (Ku70 F2), and SS032 (Ku70 R) (Supplementary Table S4). The KU70-specific primers span an intron so that cDNA can be differentiated from the product that would be produced from genomic DNA. There are no introns in NUKU. Products were sequenced to confirm that they unambiguously represent KU70 or NUKU.

Molecular modeling of NUKU2 using FoldX

To understand the effect of single missense variation on Ku70p stability (i.e., folding) and its binding with Ku80p, we estimated both folding and binding $\Delta\Delta G$ values (difference of free energies between wild-type and the mutant) using FoldX software (Schymkowitz et al. 2005). To run FoldX calculations, the X-ray crystal structure of the human Ku heterodimer was first downloaded from Protein Data Bank (PDB id: 1JEQ) (Walker et al. 2001). The file was modified to remove all but the two chains of Ku70p and Ku80p. Several residues were missing in both the chains of the protein complex. These missing residues were not modeled to complete the structure of the complex before running FoldX calculations for the following two reasons: (1) Missing residues were either at the terminal ends or in the disordered region hence they are difficult to build using the molecular modeling software and (2) the gaps in the input X-ray structure does not affect the performance of the FoldX software as it relies on rotamer libraries to model any mutation at a particular site and semi-empirical scoring function to estimate $\Delta\Delta G$ values (Guerois et al. 2002). The clean starting structure of Ku70p-Ku80p complex was then used to create mutant models and subsequently estimate both binding $\Delta\Delta G$ and folding $\Delta\Delta G$ values. We started by performing 6 rounds of minimization of the protein complex using the RepairPDB command to obtain convergence of the potential energy. All 19 possible single amino acid mutations at each site on Ku70p (548 amino acid residues \times 19 possible substitutions) were then generated using BuildModel. Finally, folding $\Delta\Delta G$ values were estimated using Stability command on Ku70 and AnalyseComplex command was used to estimate the effect of each modeled mutation on Ku70p-Ku80p binding i.e., binding $\Delta\Delta G$ values.

Yeast two-hybrid assay

We used the LexA-Gal4 yeast two-hybrid system, which employs the LexA DNA-binding domain (DBD) and the Gal4-activation domain (Gal4-AD) with the yeast strain EAY1098 (*His3*, *Leu2*, *Trp1*, and genotype). If the candidate proteins interact, the DNA-binding domain and activation domain (AD) will be in close proximity and will be able to drive the transcription of a *HIS3* reporter gene downstream of the LexA promoter. The Clontech pGADT7 plasmid, which creates an N-terminal fusion protein between a gene of interest and the Gal4 AD, was engineered to carry the full 1830 bp coding sequence of human KU70. Another pGADT7 vector was engineered to carry the full 654 bp NUKU2 ORF. The full-length coding sequence of human KU80 (2199 bp) was cloned into the LexA expression vector pBTM116, which creates an N-terminal fusion protein between the inserted gene and the LexA DNA

binding domain. All cloning was done with TA-vectors and plasmids compatible with the Gateway system (Invitrogen). EAY1098 was transformed using the standard lithium-acetate PEG transformation protocol with the following plasmid pairs: pGADT7-Ku70 and pLexA-Ku80; pGADT7-Nuku and pLexA-Ku80; pGADT7 and pLexA-Ku80; and pGADT7 and pLexA. Transformants were selected on leucine and tryptophan drop-out media to select for and stimulate expression of plasmids. After 2 days growth at 30°C, saturated cultures at an OD₆₀₀ of 2.7–2.8 were diluted and plated onto media lacking histidine in addition to leucine and tryptophan to stimulate *HIS3* gene reporter expression. Growth was observed 3 days post-plating.

Western blots

Thirty micrograms of denatured protein lysate were loaded onto 10% Tris-HCl polyacrylamide gels and then transferred onto a nitrocellulose membrane. Membrane was blocked overnight in 5% milk-TBS + 1% Tween and incubated the next day with a primary antibody directed against the Gal4-AD (1:5000 dilution; Clontech, cat # 630402) or against human Ku70p (1:1000 dilution; GeneTex, cat # GTX101820). The secondary antibody for Gal4 probes was goat anti-mouse-HRP (1:1500; Fisher, cat #32430), and for Ku70p probes was goat anti-rabbit-HRP (1:1500 dilution; Fisher cat. #32460). Signal was detected using ECL plus reagents (VWR cat #95040-056). For analysis of two-hybrid constructs, total protein from yeast strains was prepared using the glass-bead disruption method. Fifty mL yeast cultures were grown to OD₆₀₀ 0.5–0.7 and were pelleted. This pellet was suspended in disruption buffer: 20 mM Tris-HCl, pH 7.9, 10 mM MgCl₂, 1 mM EDTA, 5% glycerol, 0.3 M (NH₄)₂SO₄, with 1 mM DTT, 1 mM PMSF, and Protease inhibitor cocktail (Roche). Acid-washed glass beads were added, and cells were vortexed for a total of 10 minutes.

Western Blot analysis of Ku70 retrocopies

Human brain and testis tissue total protein lysates were purchased from ProSci Incorporated (catalog numbers 1303 and 1313, respectively). HEK293T cells were grown in standard DMEM with 10% fetal bovine serum in 75 cm³ tissue culture flasks. Total protein was prepared using the reagents and protocol described in the Qiagen Mammalian Protein preparation kit. Protein was quantified using Pierce Coomassie Bradford Assay reagent. About 30 μ g of protein was separated using polyacrylamide gel electrophoresis on a Tris-HCl gel and transferred to a nitrocellulose membrane. Membranes were blotted with 1:1000 dilution of the Ku70p antibody raised in rabbit (GeneTex XRCC6 antibody, Cat.# GTX101820). Secondary antibody of Goat anti-rabbit conjugated to horseradish peroxidase at 1:1500 dilution was used (Cat. #32460 Thermo Scientific Pierce Goat anti-Rabbit IgG, Peroxidase Conjugated). Maltose binding protein/hemagglutinin-tagged Nuku proteins were detected using an anti-HA peroxidase-conjugated monoclonal rat antibody [3F10; 12013819001 (Roche)].

Virus infection assays

Human HEK293T (4×10^5) and HeLa (4×10^4) cells were seeded in 12-well dishes (DMEM growth medium with 10% fetal bovine serum) and grown at 37°C with 5% CO₂ for 24 hours until reaching a confluency of ~75%. Each well was transiently transfected with 800 ng of plasmid encoding either human NUKU5, NUKU2 or rhesus macaque NUKU2 in addition to a transfection control plasmid expressing RFP. After 24 hours incubation, each well was trypsinized and the HEK293T (2×10^5) and HeLa (4×10^4) cells used to seed three wells of a 24-well dish. After 24 hours of incubation at 37°C (5% CO₂) monolayers with a confluency of ~50% were

infected with VSV-G pseudotyped HIV, FIV, or MLV containing a GFP reporter gene as described previously (Stabell *et al.* 2016). After 48 hours, cells were trypsinized and fixed with 1% paraformaldehyde by incubating for 1 hour at 4°C. GFP and RFP positive transduced cells were detected by flow cytometry using appropriate compensation controls to account for spectral overlap of fluorophores.

Ribo-seq analysis

Raw sequencing data were downloaded from Wand *et al.* (2020). The reads were quality filtered and trimmed using fastp v0.20.0 using default parameters then mapped to *KU70* and retrocopies from the respective species using bwa mem v0.7.17 with default parameters. Alignments were processed using Samtools v1.10 (-F 4). Reads mapping to more than one reference sequence were further investigated and removed by parsing the "XA" tags from the alignment files using scanBam from the Rsamtools v2.4.0 package in R v4.03. Plotting was done in ggplot v3.3.3.

Data availability

The data underlying this article are available in the article and in its online supplementary material available at figshare: <https://doi.org/10.25387/g3.14267312>.

Results

Five *KU70* retrocopies in the human genome

Five ORFs with high similarity to *KU70* were identified on four different human chromosomes (Figure 1A). Unlike the human *KU70* gene locus, each of the five copies lack introns. TSDs characteristic of LINE-1 mediated insertion were identified flanking each of the retrocopies, as were 3' poly-A tails that are relics of the mRNA from which these genes arose (Figure 1, A and B). All human retrocopies are between 89 and 97% identical to the parent *KU70* processed mRNA transcript and have been named *NUKU1*–*NUKU5*. Each of the five TSDs is unique, confirming that these copies represent five independent retrotransposition events, and did not arise from segmental duplication of an existing retrocopy-containing region. Thus, the human genome contains one *KU70* gene and five LINE-1 mediated *NUKU* retrocopies (Supplementary Table S5).

We then analyzed several primate genomes for the presence of *KU70* retrocopies (Supplementary Table S1). Phylogenetic analysis (Figure 2A and Supplementary Figure S1) and inspection of pre-insertion target sites (as in Figure 1B and Supplementary Figure S2) define the order in which these retrocopies arose, and places them at distinct positions in the tree of primate speciation (Figure 2B). These data show that four of the *KU70* retrocopies arose before the split between Old World and New World monkeys, over 30 MYA, consistent with a burst of retrocopy formation that has been reported in this time frame (Ohshima *et al.* 2003; Zhang *et al.* 2003; Marques *et al.* 2005). Remnants of *NUKU2* and *NUKU3* are present in the marmoset and squirrel monkey genomes, although they have experienced large subsequent deletions (Figure 2A and Supplementary Figure S2). We were unable to identify *NUKU1* in either the marmoset or squirrel monkey genomes (Supplementary Figure S2). Comparing the syntenic location of *NUKU1* in both marmoset and squirrel monkeys to the human genome reveals large indels that prevent the reconstruction of the evolutionary history of the locus in New World monkeys (Supplementary Figure S2). Because *NUKU1* is the most basally branching retrocopy, we predict that it also predates the

last common ancestor of the species being analyzed. Interestingly, the genomes of both marmoset and squirrel monkeys have acquired many additional *KU70* retrocopies that are not found in any of the other primate genomes investigated, meaning that these arose after the last common ancestor of New World and Old World monkeys (30–40 MYA) (Figure 2 and Supplementary Figure S1). The human genome contains one new retrocopy, *NUKU5*, that is found in the genomes of chimpanzee and orangutan, but not in rhesus or marmoset. The pre-insertion site in the syntenic location in the rhesus macaque, snub-nosed monkey, and sabaeus monkey genomes are perfectly preserved (Figure 1B), confirming that this retrocopy post-dates the split between Old World monkeys and hominoids that occurred approximately 20 MYA. Analysis of the genome of golden snub-nosed monkey also revealed the birth of a new *KU70* retrocopy (*NUKU6*) with a TSD, remnants of a poly-A tail, which is absent from other Old World monkeys and humans (Supplementary Figure S3). This suggests that *NUKU6* is a unique retrocopy of *KU70*. Thus, *KU70* retrocopies have been consistently birthed over a period lasting more than 30 million years, with evidence of continued retrocopy birth in extant primate species (Supplementary Table S5).

None of the ORFs in any of the primate *NUKU* retrocopies have been conserved in their full-length form as compared to *KU70*, and at first glance they all appear to be retropseudogenes. *NUKU3*, located on chromosome 10, has acquired two Alu insertions (*AluSp* and *AluSq* elements) and a 251bp insertion of non*KU70* related sequence in the middle of the coding region (Figure 1A). The ORF in *NUKU5* is approximately 75% the length of *KU70*, although *NUKU* ORFs are smaller, and the putative start codon of all of them is downstream of the *KU70* start codon.

KU70 has an unusually large number of retrocopies

We were interested in determining whether the presence of five retrocopies of *KU70* in the human genome is typical for a gene involved in double-stranded break repair. Because some gene families might be more or less prone to retrocopy formation and retention than others, we compared the number of retrocopies formed from *KU70* to other genes involved in DNA double-strand break repair. A list of all genes in the "double-strand break repair" biological process category (GO: 0006302) was compiled using the Gene Ontology (GO) database. Each was used as a query to identify retrocopy copies elsewhere in the human genome. A retrocopy was defined as any sequence match that (1) contains no introns, and (2) returns the parent gene when it itself is used to query the human genome (*i.e.*, the gene and retrocopy are reciprocal "best hits"). No criteria for conservation of the ORF was included, and some retrocopies appear to be degraded by mutation. In total, 51 double-strand break repair genes had no discernable retrocopies. Eleven genes [*MRE11*, *RAD21*, *FEN1*, *TRIP13*, *UBE2V2*, *PIR51* (*RAD51AP1*), *SHFM1* (*SEM1*), *BRCC3*, *RNF168*, *OBFC2B* (*NABP2*), and *RTEL1*] had one retrocopy. Two genes (*SOD1* and *FAM175A*) had two retrocopies, and one gene (*UBE2N*) had four retrocopies. *KU70*, with five retrocopy copies, is the only one out of 66 with five retrocopy copies (Figure 3). Expression patterns of these genes in the ovary and testis indicate that the expression of *KU70* is the highest of all 66 genes in the testis and the second highest in the ovary (after *SOD1*) (Supplementary Figure S4). High expression of genes does not always lead to retrocopies as *CIB1*, *VCP*, and *KU80* all have high expression in the testis and ovary without the creation of retrocopies (Supplementary Figure S4).

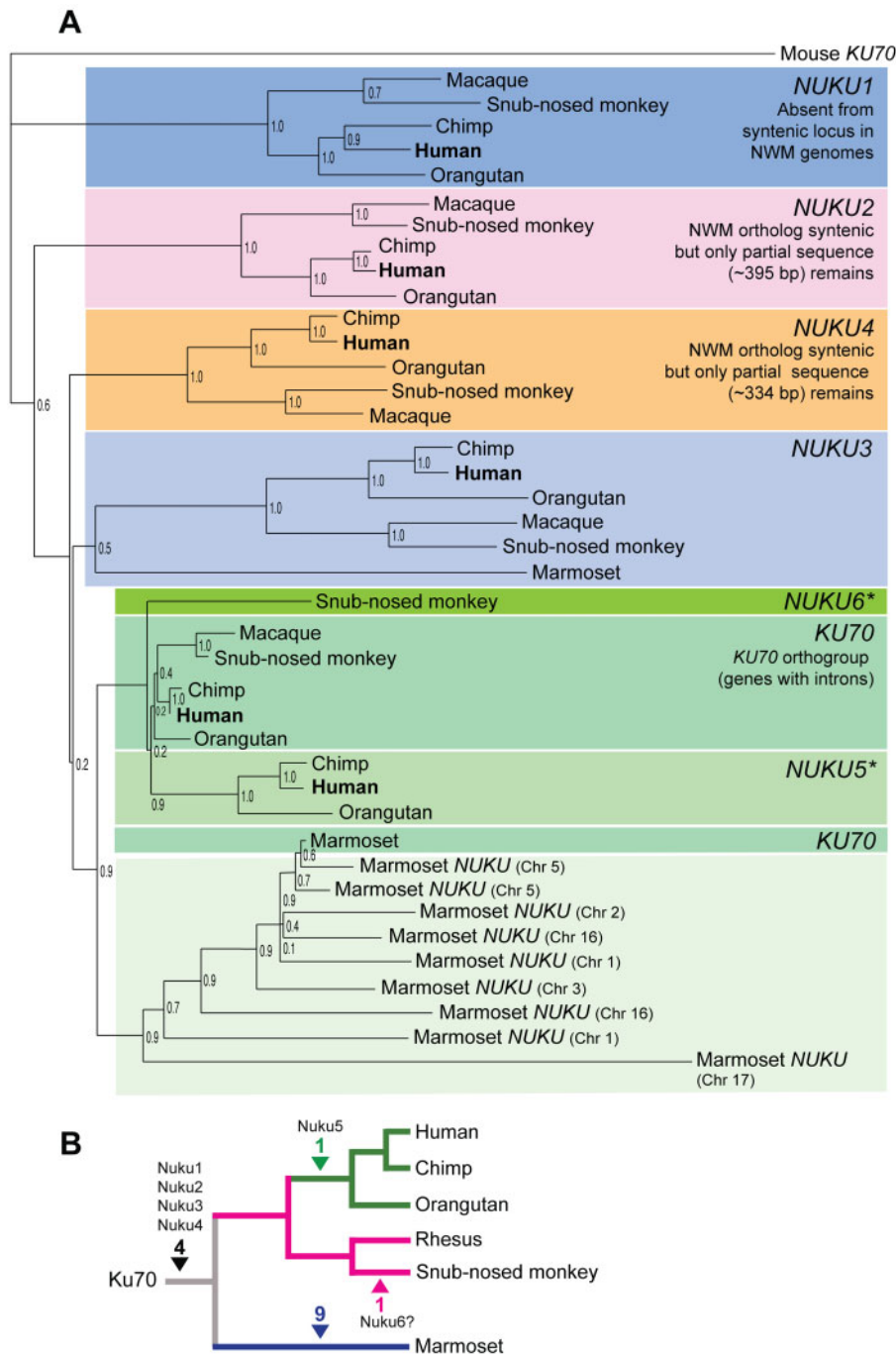


Figure 2 Phylogenetics and insertion sites of *KU70* derived retrocopies (A) Once the five *NUKU* retrocopies had been identified in the human genome, orthologous retrocopies were identified in other primate genome projects through inspection of the syntenic target sites. A tree of these sequences is shown. Unless indicated, none of the genes on the tree contain introns. Bootstrap values generated with the maximum likelihood method are shown. Marmoset *NUKU3* was verified to be orthologous to the other *NUKU3* sequences by target site analysis. *NUKU2* and *NUKU4* are apparent in the marmoset genome, but are almost completely deleted, and therefore they were not included in the alignment used to make the tree. We were unable to locate the syntenic region of *NUKU1* in the Marmoset genome, indicating that this region may have been deleted (Supplementary Figure S2). Marmoset-specific retrocopies were not named but are designated by the chromosome on which they are found. *target site of *NUKU5* and *NUKU6* insertion is empty in Old World monkeys. (B) Based on the phylogenetic analysis and target site inspection, *NUKU1*–*NUKU4* predate the split between Old World monkeys, New World monkeys, and hominoids. *NUKU5* is specific to the great ape genomes analyzed and *NUKU6* appears to be unique to the snub-nosed monkey. The time of acquisition of *NUKU6* is somewhat uncertain due to the low bootstrap support in the phylogenetic analysis. The marmoset genome has birthed 9 additional *KU70*-like retrocopies.

nonsynonymous mutations and three synonymous mutations have occurred in this region of the retrocopy since it was formed more than 30 MYA. We used Monte Carlo simulation to determine that this rate of evolution is significantly greater than the neutral expectation of $dN/dS = 1$ ($P = 0.007$) (Figure 4).

To further analyze the evolution of *NUKU2*, we determined the genetic sequence of *NUKU2* and *KU70* from 12 simian primate genomes (Supplementary Table S2). Because it is expressed in all tissue types and contains multiple introns, *KU70* was amplified and sequenced from mRNA, whereas *NUKU2* was

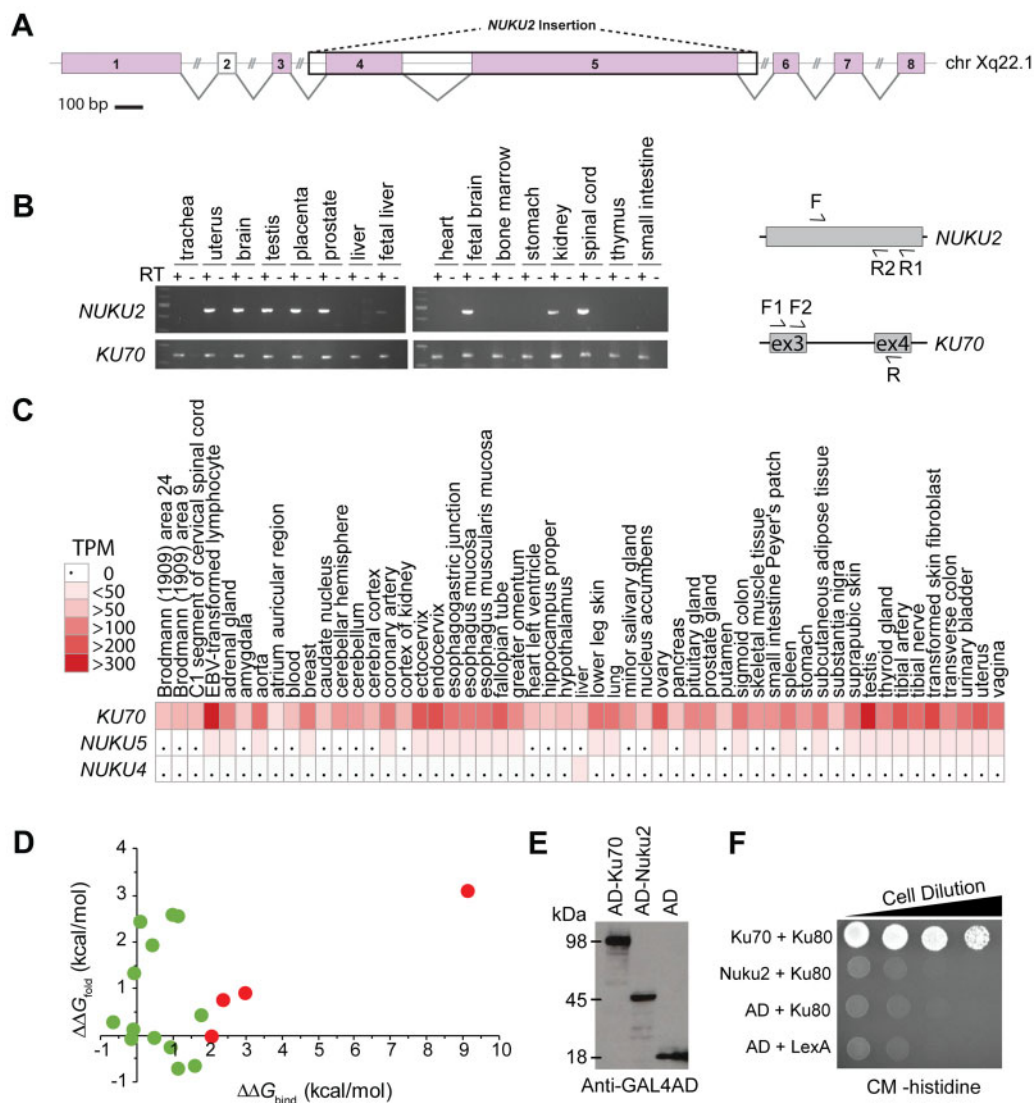


Figure 5 NUKU retrocopies are functionally distinct from KU70. (A) A lymphocyte-specific processed mRNA (EU224311) mapped to the human X chromosome with the insertion site of NUKU2 boxed in black. Predicted splice sites are indicated between exons with 100% identity to the X chromosome (pink boxes). A significant match to exon 2 was not identified within the X chromosome. (B) RT-PCR was used to analyze the expression of NUKU2 and KU70 from total mRNA harvested from different human tissues. Nested primer pairs are shown to the right. The product of a first-round RT-PCR reaction (primers F—R1) was then amplified with a second set of primers (F and R2), where R2 sits interior to R1. All three primers were designed to be specific to transcripts from NUKU2, as the ultimate base at the 3' end of the primer placed such that it pairs with a base that is unique to NUKU2 relative to the other five retrocopies. NUKU2 does not have introns, but the KU70 primers span an intron. Nested PCR with specific primers was also used to amplify the KU70 transcript, which is different in size from the product obtained from genomic DNA. (C) Relative tissue-specific expression patterns of KU70, NUKU4, and NUKU5 measured in transcripts per million (TPM) (GTEx Consortium *et al.* 2017). (D) For each Nuku2p mutation within 5 Å of Ku80p, $\Delta\Delta G_{\text{bind}}$ was plotted on the x-axis, whereas $\Delta\Delta G_{\text{fold}}$ was plotted on the y-axis. Mutations are shown in green with x-axis values $\Delta\Delta G_{\text{bind}} < 2$ kcal/mol and y-axis values $-3 < \Delta\Delta G_{\text{fold}} < 3$ kcal/mol are considered functional since they are likely to retain the ability to fold and bind. Mutations are shown in red with x-axis values $\Delta\Delta G_{\text{bind}} > 2$ kcal/mol and y-axis values $-3 < \Delta\Delta G_{\text{fold}} < 3$ kcal/mol are predicted to retain folding but disrupt Ku80p binding. (E) Western blot confirming protein expression of each AD fusion construct in the yeast strains used for two-hybrid analysis. (F) A yeast two-hybrid test assaying the interaction of Ku70p or Nuku2p with Ku80p. The Gal4 AD is either fused to Ku70p (top row), Nuku2p (second row), or expressed alone (third and fourth rows). LexA is a DNA binding domain and is either fused to Ku80p (top three rows) or expressed alone (bottom row). A positive interaction enables growth on complete media (CM) lacking histidine.

reading frame was analyzed (orange box in Figure 4C), and one with all species where the shorter ORF was analyzed (pink box in Figure 4C). When the larger ORF was analyzed in hominoids only, it was estimated that 9% of the codons in NUKU2 had a dN/dS of 7.05. Comparison to the null model shows the inference of positive selection to be statistically significant ($P = 0.029$; Table 1). Support is not as strong when the shorter ORF of NUKU2 was analyzed across all species ($P = 0.130$), likely due to analyzing a shorter sequence of aligned DNAs.

The tissue-specific expression and loss of Ku80 interaction by Nuku2

Two human mRNA transcripts (EU224311 and ENST00000435236.2) were identified in the GenBank and Ensembl databases that verify the transcription and splicing of NUKU2 on the X chromosome (Figure 5A). While we were unable to detect the spliced transcript of EU224311 by PCR, potentially because it is lymphocyte-specific, we performed 5' and 3' RACE and were successful in characterizing the structure of the unspliced

Table 1 Molecular evolution of NUKU2 in primates

Dataset ^a	Branch-site model	Estimate of parameters ^b	Test 2		
			2Δl ^c	P-value	
Analysis 1 hominoid NUKU2	Model A with ω_2 fixed at 1 Model A	$l = -1,421.47$ $l = -1,419.10$	$p_0 = 0.354$ $p_1 = 0.375$ $p_2 + p_3 = 0.271$ $\omega_0 = 0.000$ $\omega_1 = 1.000$ $\omega_2 = 1.000$ $p_0 = 0.455$ $p_1 = 0.456$ $p_2 + p_3 = 0.089$ $\omega_0 = 0.000$ $\omega_1 = 1.000$ $\omega_2 = 7.054$	4.75	$P = 0.029$
Analysis 2 OWM and hominoid NUKU2	Model A with ω_2 fixed at 1 Model A	$l = -853.17$ $l = -852.02$	$p_0 = 0.353$ $p_1 = 0.603$ $p_2 + p_3 = 0.044$ $\omega_0 = 0.000$ $\omega_1 = 1.000$ $\omega_2 = 1.000$ $p_0 = 0.353$ $p_1 = 0.516$ $p_2 + p_3 = 0.131$ $\omega_0 = 0.000$ $\omega_1 = 1.000$ $\omega_2 = 3.488$	2.29	$P = 0.130$

^aBoth datasets included the KU70 sequences from seven hominoids: *Homo sapiens*, *Gorilla gorilla*, *P. pygmaeus* (Sumatran orangutan), *P. pygmaeus* (Borneo orangutan), *Hylobates syndactylus*, *Hylobates leucogenys*, *H. agilis*, and from eight Old World monkeys: *Macaca mulatta*, *M. fascicularis*, *Lophocebus albigena*, *P. anubis*, *M. talapoin*, *C. wolff*, *C. guereza*, *T. francoisi*. Both datasets also included NUKU2 from the seven hominoids listed above as well as from chimpanzee (*Pan troglodytes*). Analysis 2 also included NUKU2 from the eight Old World monkey species. In both analyses, the NUKU2 clade was defined at the foreground clade and the KU70 clade was defined at the background clade.

^bModels were run using the f61 codon frequency model. $l = \ln$ of the likelihood.

^cTwice the difference in the natural logs of the likelihoods ($\Delta l \times 2$) of the two models being compared. This value is used in a likelihood ratio test along with the degrees of freedom (1 in this case). In Test 2, Model A, which allows positive selection on the foreground clade, is compared to a null model (Model A with ω_2 fixed at 1). The P-value indicates the confidence with which the null model can be rejected.

transcript of NUKU2 (ENST00000435236.2) from total RNA isolated from the human testis (Supplementary File S1). These transcripts are almost certainly derived from the transcription of the NUKU2 locus due to 100% identity to the nucleotide sequence and putative translation products from NUKU2 (Supplementary File S1 and Supplementary Table S1). To determine whether there were other NUKU2 transcripts within other human tissues we designed PCR primers to specifically detect transcripts of the NUKU2 retrocopy. We used nested PCR with NUKU2-specific primers, determined the genetic sequence of all products and confirmed that they were a perfect match only to the NUKU2 retrocopy. As shown, NUKU2 is expressed in uterus, brain, testis, placenta, prostate, fetal liver, fetal brain, kidney, and spinal cord (Figure 5B). We confirmed the absence of contaminating genomic DNA by performing RT-PCR reactions in which the RT had been omitted. We also amplified KU70 by a similar nested strategy, using primers located in two neighboring exons, to distinguish by size products of RT-PCR from PCR products that may be produced from contaminating genomic DNA. No genomic DNA was detected by this assay. This ubiquitous tissue expression pattern of KU70 reflects its function as an essential housekeeping gene and is shown in other published datasets (Figure 5C) (GTEx project version 7) (GTEx Consortium et al. 2017). We also found evidence for the tissue-specific expression of both NUKU4 and NUKU5 and a transcript from NUKU4 in the Ensembl database (ENST00000420392.1) (Figure 5C and Supplementary Table S1) (GTEx Consortium et al. 2017). These results confirm that NUKU2, NUKU4, and NUKU5 are expressed in humans, expression is tissue-specific, and tissue-specificity has diverged from that of KU70, likely due to new regulatory signals at their new genomic location.

Ku70p is known to interact with Ku80p, thereby forming the Ku heterodimer that associates with broken ends of double-stranded DNA. To explore the potential biochemical function of a putative Nuku2 protein, compared to Ku70p, we examined the functional consequences of more than 10,000 mutational

changes in Ku70p when bound to Ku80p using semi-empirical molecular modeling, as implemented by FoldX (Supplementary Figure S5 and Supplementary File S2) (Guerois et al. 2002; Schymkowitz et al. 2005). By comparing the amino acid changes between Ku70p and Nuku2p we individually modeled 27 nonsynonymous mutations that are present in NUKU2 onto the heterodimeric co-crystal of Ku70p-Ku80p [PDB: 1JEY (Walker et al. 2001)] and measured the change in free energy for binding ($\Delta\Delta G_{\text{bind}}$). The 11 mutations present in Nuku2p that were more than 5 Å from the Ku80p interface had an average $\Delta\Delta G_{\text{bind}}$ of 0.04 kcal/mol (SD \pm 0.17), indicating that these changes would not be expected to disrupt Ku80p binding (Supplementary Figure S5). The majority (81%) of the remaining 16 NUKU2-specific mutations that are within 5 Å of the Ku70p-Ku80p interface are also predicted to have little impact upon the interaction of these proteins ($\Delta\Delta G_{\text{bind}} < 2$ kcal/mol; average 0.60 kcal/mol, SD \pm 0.74) (Figure 5D; green data points). However, four mutations at this interface (G349V, F410L, A494I, and T507I) had a $\Delta\Delta G_{\text{bind}} > 2$ kcal/mol (Figure 5D; red data points). This indicates that these mutations alone would be predicted to disrupt the binding of Ku70p to Ku80p, and therefore, in combination are likely to prevent binding of Nuku2p to Ku80p. In addition, because Nuku2p is predicted to be truncated relative to Ku70p, there would be a 39% reduction in the surface area available for Ku80p binding from ~ 9500 to ~ 5800 Å², which would also reduce the likelihood of a Nuku2p-Ku80p interaction (PISA analysis, Krissinel 2015). Analysis of disruptive mutations in hominoid NUKU2 shows the presence of the same G349V, A494I, and T507I mutations that are found in human NUKU2. Only T507I appears within the NUKU2 gene of Old World monkeys, in addition to a single disruptive mutation unique to colobus monkey (Y530C; $\Delta\Delta G_{\text{bind}} > 2$) (Figure 4). Finally, nonsynonymous mutations in NUKU2 at sites under positive selection in primates have average $\Delta\Delta G_{\text{bind}}$ and $\Delta\Delta G_{\text{fold}}$ values of 0.33 (SD \pm 0.45) and 1.00 (SD \pm 1.46), respectively. This would suggest that these mutational changes were not driven by selection to disrupt Ku80p interaction or to alter Nuku2p folding.

Molecular modeling predicts that the truncation of *NUKU2* and several nonsynonymous mutations disrupt an interaction with Ku80p. To validate these *in silico* predictions we used the yeast two-hybrid *in vivo* protein interaction assay to test the interaction of either Ku70p or Nuku2p with Ku80p. Ku70p and Nuku2p were both fused to the Gal4 activation domain (AD), and each construct was co-transformed with a plasmid encoding the LexA-Ku80p fusion protein (Figure 5E). Co-transformants of AD-Ku70p and LexA-Ku80p were able to grow on media lacking histidine, signifying a positive interaction. AD-Nuku2p and LexA-Ku80p were unable to interact, and yeasts were unable to grow on histidine deficient plates. The LexA DNA binding-domain was also unable to interact with Ku80p or the AD (Figure 5F). Both the tissue-specific expression and inability to interact with Ku80p suggest that *NUKU2* has diverged from its parent gene *KU70* and potentially acquired new biological functions.

Expression of *NUKU2* and *NUKU5* does not impact retrovirus transduction

Ku is known to be important for the replication of many different viruses, including mammalian retroviruses and retrotransposons (Daniel *et al.* 1999; Downs and Jackson 1999; Li *et al.* 2001; Suzuki *et al.* 2009; Zheng *et al.* 2011). We considered that Nuku proteins could act to antagonize viral replication by mimicking Ku70p and evidence of positive selection might suggest host-virus antagonism (Figure 4). To test whether the expression of *NUKUs* might disrupt the early stages of the retroviral lifecycle, we first confirmed the transient expression of *NUKU2* (human and rhesus macaque) and *NUKU5* (human) within the human HEK293T and HeLa cell lines (Supplementary Figure S6). Twenty-four hours post-transfection these cell lines were transduced with GFP using a single-cycle VSV-G pseudotyped human immunodeficiency virus 1 (HIV-1), feline immunodeficiency virus (FIV), and murine leukemia virus (MLV). Forty-eight hours post-infection the percentage GFP-expressing cells was measured using flow cytometry, and we found that *NUKU* expression did not affect retroviral transduction, relative to a control cell line expressing maltose-binding protein (Supplementary Figure S6).

Evidence of Nuku gene translation and Ku70-like proteins

Since several *NUKUs* appear to be transcriptionally active, we wished to address if these retrocopies were capable of producing proteins. Ribosome profiling (Ribo-seq) data derived from large scale surveys of tissues from human and rhesus macaque were first assessed for unique *NUKU* transcripts that could be unambiguously identified by their sequence from *KU70* and other *NUKU* transcripts (Wang *et al.* 2020). There is evidence of transcripts from *NUKU4* and *NUKU5* associated with ribosomes in humans, and all *NUKU* transcripts in rhesus macaque (Figure 6A). In both species, *NUKU4* transcripts appeared to be more commonly associated with ribosomes in the three tissues surveyed.

We assume that the two retrocopies most likely to be expressed as proteins are Nuku2p and Nuku5p. The former has documented tissue-specific expression, a spliced transcript, and positive selection, and the latter, which is the youngest retrocopy, produces transcripts in many tissues, has the most intact ORF, and has evidence of translation in humans from Ribo-seq data. We screened several anti-Ku70 polyclonal antibodies for cross reactivity with Ku70p, Nuku2p, and Nuku5p. We used Gal4AD-Ku70 or Gal4AD-Nuku fusion proteins expressed in yeast to test this, and we identified an antibody that specifically recognized all three constructs (Figure 6B). The protein band in HEK293T cell

extracts shows the position of untagged Ku70p, and this antibody does not appear to cross-react with the endogenous copy of Ku70p in yeast. The tagged copy of human Ku70-AD is larger than the untagged version (Figure 6B, lane 2 vs lane 1). The tagged versions of Nuku2p and Nuku5p are shorter, due to the truncated ORFs in these two genes (Figure 1A).

We detected high levels of *NUKU2* transcription in brain and testis among other tissues (Figure 5C). Therefore, we probed protein lysates from human brain tissue, testis tissue, and HEK293T cells with our anti-Ku70p antibody (Figure 6C). Protein lysates from HEK293T cells show only a single strong band at ~70kDa, the size of human Ku70p. This band is also evident in the testis and brain cell lysates. We did not detect a prominent band at ~25 kDa in any of the samples, the predicted molecular weight of Nuku2p based on the transcript that we amplified by RACE (Supplementary File S1). However, cell lysates from brain and testis tissues, but not HEK293T cells, show a second band at the predicted size of human Nuku5p (~50kDa), with the band being more prominent in testis than in brain.

Discussion

KU70 is highly conserved across primates, which contrasts other genes that are required for DNA repair that have been found to be evolving rapidly within humans and yeasts, potentially in response to prior or current selective pressure from viruses and retrotransposons (Sawyer and Malik 2006; Demogines *et al.* 2010; Lou *et al.* 2014; Abdul *et al.* 2018). Despite the conservation of *KU70*, we describe the accumulation and diversification of *KU70*-derived retrocopies within humans and nonhuman primates (*NUKUs*). The contribution of retrocopies to *de novo* gene formation and the evolution of novel gene functions has been extensively documented in different organisms (Long and Langley 1993; Courseaux and Nahon 2001; Betrán *et al.* 2002; Wang *et al.* 2002). *KU70* appears to be unique regarding the number of retrocopies that it has birthed relative to other genes required for NHEJ in primates. In addition to the expansion of the *NUKUs* we have also detected the rapid evolution and functional divergence of these retrocopies during primate speciation. Evidence of transcription and the association of transcripts with ribosomes would suggest that these proteins are present within human and primate tissues.

NHEJ is an important mechanism for DNA double-strand break repair in cellular organisms and is also important for the replication of DNA viruses and retroviruses/retrotransposons that generate DNA intermediates during their lifecycles. There are examples of NHEJ DNA repair mechanisms helping or hindering viral replication (Weitzman *et al.* 2010). For example, lack of DNA-PK (DNA-dependent protein kinase holoenzyme, consisting of Ku70p, Ku80p, and DNA-PKcs) during HIV replication results in reduced viral integration and an increase in cellular apoptosis due to integrase-mediated DNA damage (Daniel *et al.* 1999; Li *et al.* 2001). Also, loss of Ku70p causes the proteasome-mediated degradation of the viral integrase (Zheng *et al.* 2011). Retrotransposons and adenovirus have also been shown to be sensitive to the loss of Ku (Downs and Jackson 1999; Suzuki *et al.* 2009; Frost *et al.* 2017). Bacteriophages encode Ku homologs that recruit other host DNA repair proteins and appear to protect phage DNA from degradation (Pitcher *et al.* 2006; Bhattacharyya *et al.* 2018). Furthermore, the hijack of NHEJ machinery is not specific to viruses as the bacterial pathogen *Rickettsia conorii* binds to cell surface-exposed Ku70p as its receptor for cell entry (Monferran *et al.* 2004; Martinez *et al.* 2005; Chan *et al.* 2009). In

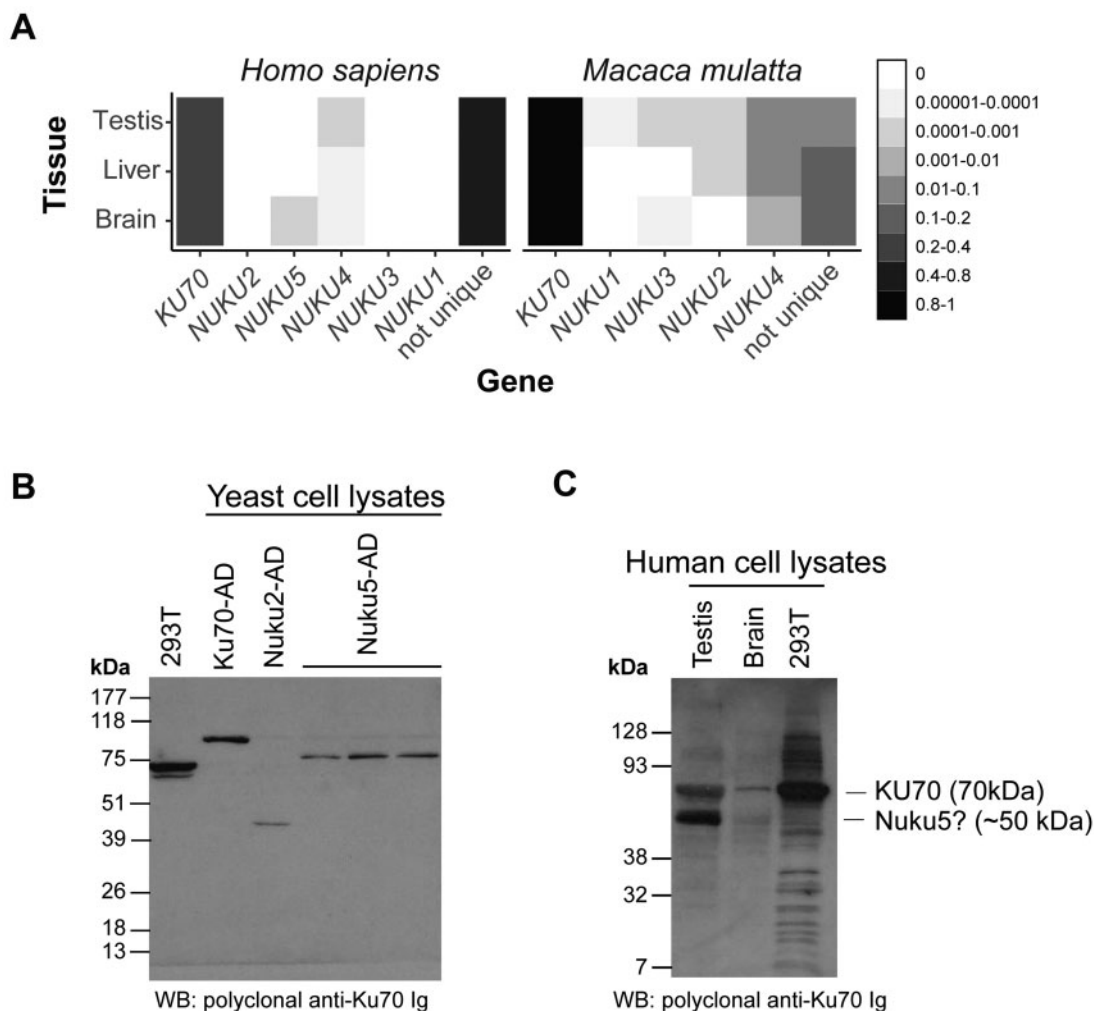


Figure 6 Detection of a putative *KU70* retrocopy-encoded protein (A) A heat map representation of Ribo-seq data reveals probable translation of several *NUKU* retrocopies. Many of the short (~30bp) Ribo-seq reads identically match more than one gene and are classified as “not unique.” The remaining reads align uniquely to either *KU70* or one retrocopy. The mean proportion of reads aligning to each gene in three tissues is shown ($n = 3$). (B) Identification of an anti-Ku70 antibody that recognizes Nuku2p, Nuku5p, and Ku70p. The ORFs of *KU70*, *NUKU2*, and *NUKU5* were fused to the *GAL4* AD and expressed in yeast. A Western blot of these proteins shows that a single polyclonal anti-Ku70 antibody recognizes all three Nuku fusion proteins (three independent transformants of Nuku5-AD are shown). (C) Whole-cell protein lysates from testis, brain, and HEK293T cells were purchased or cultured. The anti-Ku70 antibody characterized in panel A was used to probe these extracts.

these cases, it is apparent that NHEJ machinery (including Ku70p) is aiding the replication and survival of viruses and bacteria. Conversely, there are many examples of DNA viruses that encode protein effectors that actively disrupt the function of NHEJ. Specifically, adenoviruses prevent the concatenation of their genomes by NHEJ machinery by producing the proteins E4-34 kDa and E4-11 kDa that bind DNA-PK and inhibit NHEJ (Boyer et al. 1999). Human T-cell leukemia virus type-1 proteins Tax and HBZ and the agnoprotein of JC virus bind and interfere with the function of DNA-PK, impairing DNA repair and aiding cellular transformation (Darbinyan et al. 2004; Durkin et al. 2008; Rushing et al. 2018). Viral proteins also block the activity of DNA-PK as a pattern recognition receptor that binds cytoplasmic DNAs triggering innate immune signaling mechanisms mediated by IFN regulatory factor 3 (IRF-3), TANK-binding kinase 1 (TBK1), and stimulator of interferon genes (STING) (Zhang et al. 2011; Ferguson et al. 2012; Sui et al. 2017). DNA-PK has been shown to be directly targeted by the vaccinia virus effectors C4 and C16 by binding Ku and preventing interaction with DNAs and triggering of innate immune signaling pathways (Peters et al. 2013; Scutts et al. 2018).

The abundance of viruses and bacteria that subvert the function of DNA-PK suggests that the *NUKUs* could play a role as dominant-negative proteins that would bind viral effectors. It has already been shown in higher eukaryotes that a dominant negative Ku80p with an N-terminal extension [Ku80/Ku86-autoantigen-related protein-1 (KARP-1)] interferes with DNA-PKs activity causing X-ray hypersensitivity when expressed in cell lines (Myung et al. 1997). However, molecular modeling studies and empirical binding assays show that Nuku2p does not bind Ku80p and would therefore not be predicted to assemble as a component of DNA-PK. Furthermore, *NUKU2* appears to have only maintained coding capacity within the C-terminal domain, which is required for binding to DNA, Mre11p, and Bax, whereas the N-terminal domain binds to DNA-PKs and Ku80p (Figure 1; Goedecke et al. 1999; Subramanian et al. 2005). Therefore, we would expect that *NUKU2* would not influence DNA-PK function, V(D)J recombination, or telomere maintenance, but might still be competent as a transcription factor, or regulate apoptosis and NHEJ by binding Mre11p or Bax, respectively (Subramanian et al. 2005; Goedecke et al. 1999). The observed expansion and maintenance of *KU70*

retrocopies and the rapid evolution of *NUKU2* could have been driven by evolutionary conflict with viruses or other pathogenic microorganisms free from the constraints of maintaining DNA repair of innate signaling functions. Indeed, the retrotransposition of genes involved in innate immunity can create new host restriction factors to fight rapidly evolving viruses (Sayah et al. 2004; Brennan et al. 2008; Wilson et al. 2008; Yang et al. 2020). Although we do not observe any significant effect of *NUKU* expression upon retrovirus transduction in tissue culture, it remains plausible that other viruses known to interfere with DNA-PK or directly interact with Ku70p (i.e., JC virus agnoprotein or adenovirus E1A) might be sensitive to the presence of *NUKUs* (Darbinyan et al. 2004; Frost et al. 2017). Alternatively, as we have detected tissue-specific transcription from *NUKUs* it is also possible that they might have a function in the regulation of *KU70* expression as antisense transcripts (Tam et al. 2008). Altogether, these data suggest that primate-specific *NUKUs* are significantly altered compared to *KU70* in their expression and protein-coding capacity. Our analyses suggest that their structure and function differ from *KU70* and that they have evolved rapidly during primate speciation. However, it remains to be further investigated the biological function of these retrocopies, which is complicated by the multifaceted role of *Ku70* in the cell.

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Conflicts of interest

None declared.

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