

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).

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*), tala-poin (*Miopithecus talapoin*), white-cheeked gibbon (*Nomascus leucogenys*), olive baboon (*Papio anubis*), black mangabey (*Ophoebus 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 dT₂₃ 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 ± 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 µl of first-strand cDNA as template. In the second cycle, 0.5 µl 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 µl 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 (NH₃)SO₄, 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 µg 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 confluence 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 confluence 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 retroseudogenes. NUKU3, located on chromosome 10, has acquired two Alu insertions (AluSp and AluSq elements) and a 251 bp insertion of nonKU70 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).

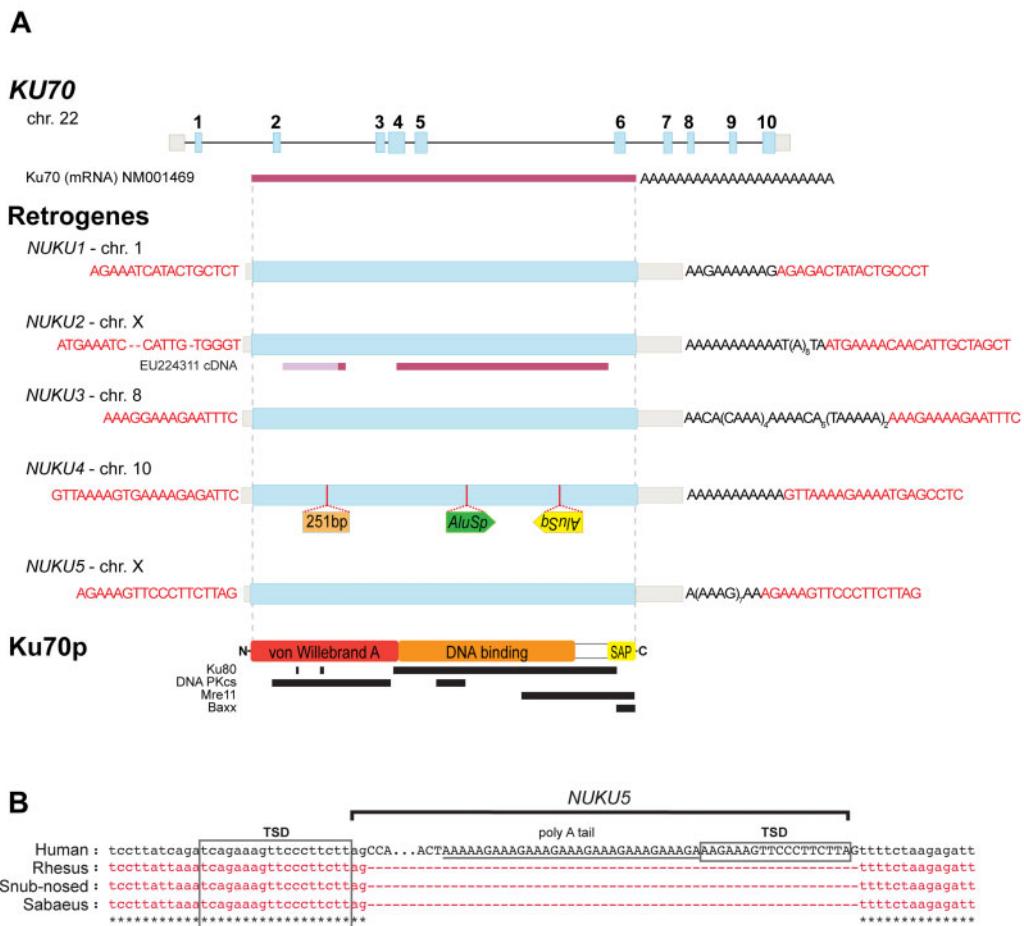


Figure 1 Identification of Five KU70 Retrocopies in the Human Genome (A) A diagram of the KU70 parent gene locus and the loci of its five retrocopies. Exons are shown in thick blue boxes and introns appear as black lines. 3' and 5' UTR structures are shown in light gray. TSD sequences are highlighted in red text. (B) Insertion of NUKU5 in the human X chromosome compared to the syntenic locus of other Old World primates and evidence of LINE-1 mediated TSD.

NUKU2 has evolved under positive selection

There are three fates for any duplicated gene. A newly copied gene may be preserved by purifying selection if there is an adaptive advantage to having a second copy of the original gene. If the new gene copy is not expressed or confers no selective advantage, it will undergo neutral decay and accumulate point mutations and stop codons. Finally, if one of the duplicated genes is selected to evolve a novel function, this will occur through positive selection for advantageous mutations that arise and result in a period of relatively rapid sequence evolution in one of the copies. Each of these three fates can be read within the DNA sequence of duplicated genes after they have diverged. Looking at the evolutionary signatures recorded may offer clues as to the potential function of the retrocopy and how it may relate to the parent gene's function. Specifically, patterns of accumulation of nonsynonymous versus synonymous mutational accumulation can be analyzed. Conserved genes like KU70 would be expected to accumulate fewer nonsynonymous changes than synonymous changes ($dN/dS < 1$). If a retrocopy does not contribute to the fitness of the organism, it will accumulate these two types of changes at an equal rate ($dN/dS = 1$). However, if a retrocopy acquires a new function and is selected for optimization of this function, it would bear the signature of increased nonsynonymous mutation accumulation ($dN/dS > 1$).

The increased number of NUKU retrocopies is likely due in part to high expression of KU70, but their retention could be rationalized if there is positive selection. The codeml program in the PAML package (Yang 2007) was used to analyze the selective pressures that have acted on each of the NUKU retrocopies since they were formed. A tree of the human KU70 and NUKU retrocopies was analyzed by the branch-sites model (Figure 4A). The analysis of patterns of nonsynonymous and synonymous mutational accumulation can only be performed in ORFs, so a region at the C-terminal end of the retrocopies was analyzed because it is an ORF in all of the retrocopies except for NUKU4, which has experienced an Alu insertion in this region. The free-ratio model uses maximum likelihood to estimate a dN/dS ratio for each branch on the tree. As would be expected, the branch leading to KU70 has a value of $dN/dS = 0.45$, indicating that nonsynonymous changes have accumulated at a rate less than half of the rate of synonymous changes (Figure 4A). Three of the pseudogenes, NUKU1, NUKU3, and NUKU5, have a dN/dS signature not statistically different from 1, indicating neutral evolution of these genes. However, the branch along which NUKU2 has been evolving shows a dN/dS value of 2.3. We retrieved the predicted ancestral sequence from the node marked "Anc," which is the prediction of the NUKU2 sequence as it looked at the time of retrotransposition (Figure 4A). Comparing this to the extant NUKU2 sequence (Figure 4B) allowed us to determine that 17

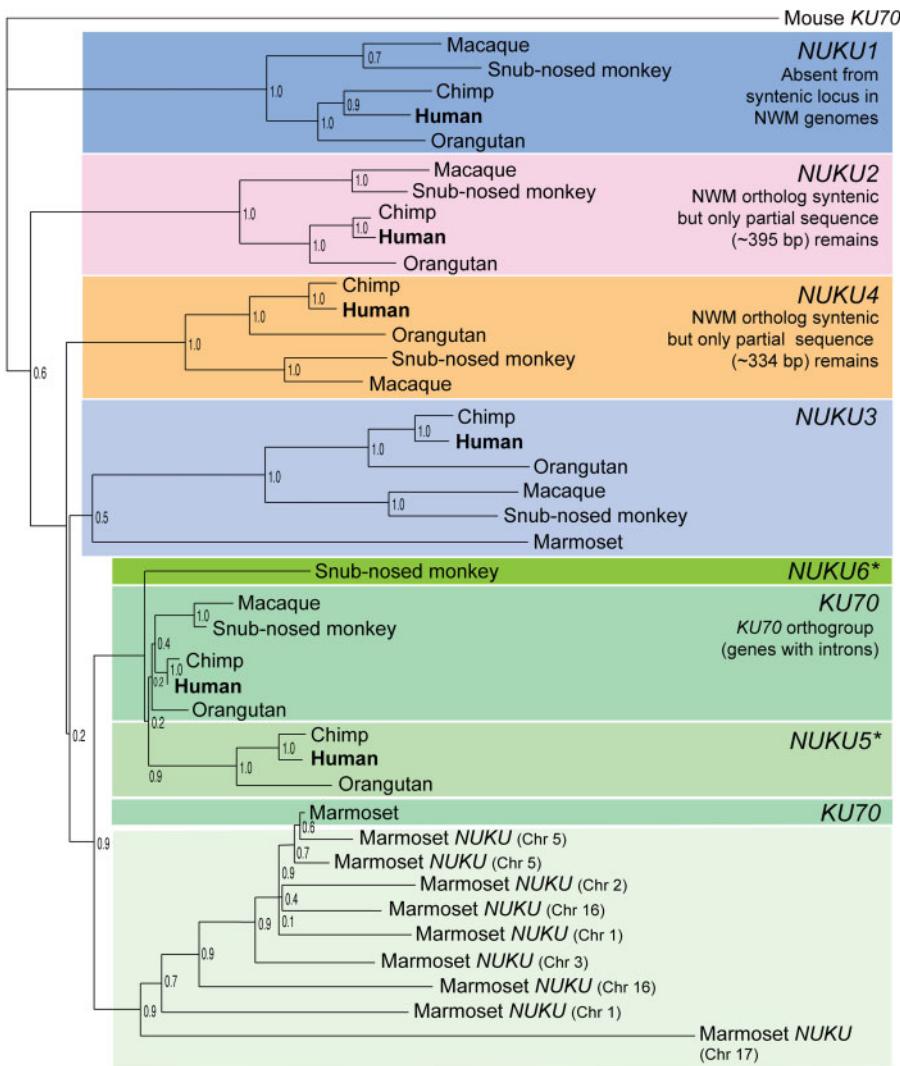
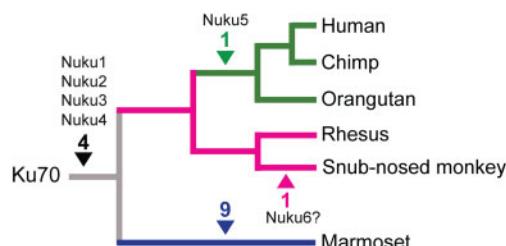
A**B**

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

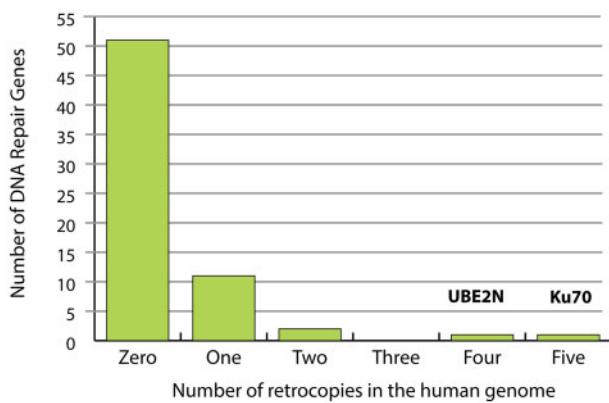


Figure 3 Prevalence of human retrocopies among double-strand break repair genes. The GO database was used to compile a list of 66 genes involved in DNA double-strand break repair. The human genome was searched for retrocopy copies of each of these. The number of repair genes with 0, 1, 2, 3, 4, or 5 retrocopy copies is shown. None of the 66 genes had more than 5 retrocopy copies.

amplified and sequenced from genomic DNA. These sequences were combined with those available from several primate species with sequenced genome projects (human, chimpanzee, orangutan, and rhesus macaque), and genes were also re-sequenced from these species where appropriate. Our analysis includes only Old World monkey and hominoid species as NUKU2 has been largely deleted in the marmoset and squirrel monkey genomes (Supplementary Figure S2). Interestingly, the predicted ORF in human NUKU2 (Figures 1A and 4C) was conserved in all hominoid species. In Old World monkeys, there was also a conserved NUKU2 ORF, but it was shorter due to an upstream stop codon leading to the potential use of an alternative ATG codon further downstream (Figure 4C). Since NUKU2 ORFs were predicted to be under positive selection and not KU70, we used the branch-sites model and specified all of the NUKU2 branches as the foreground clade (Zhang et al. 2005). This allowed us to look for positive selection of codons specifically in these species. Two analyses were performed, one with all KU70 sequences and only the hominoid species where the longer

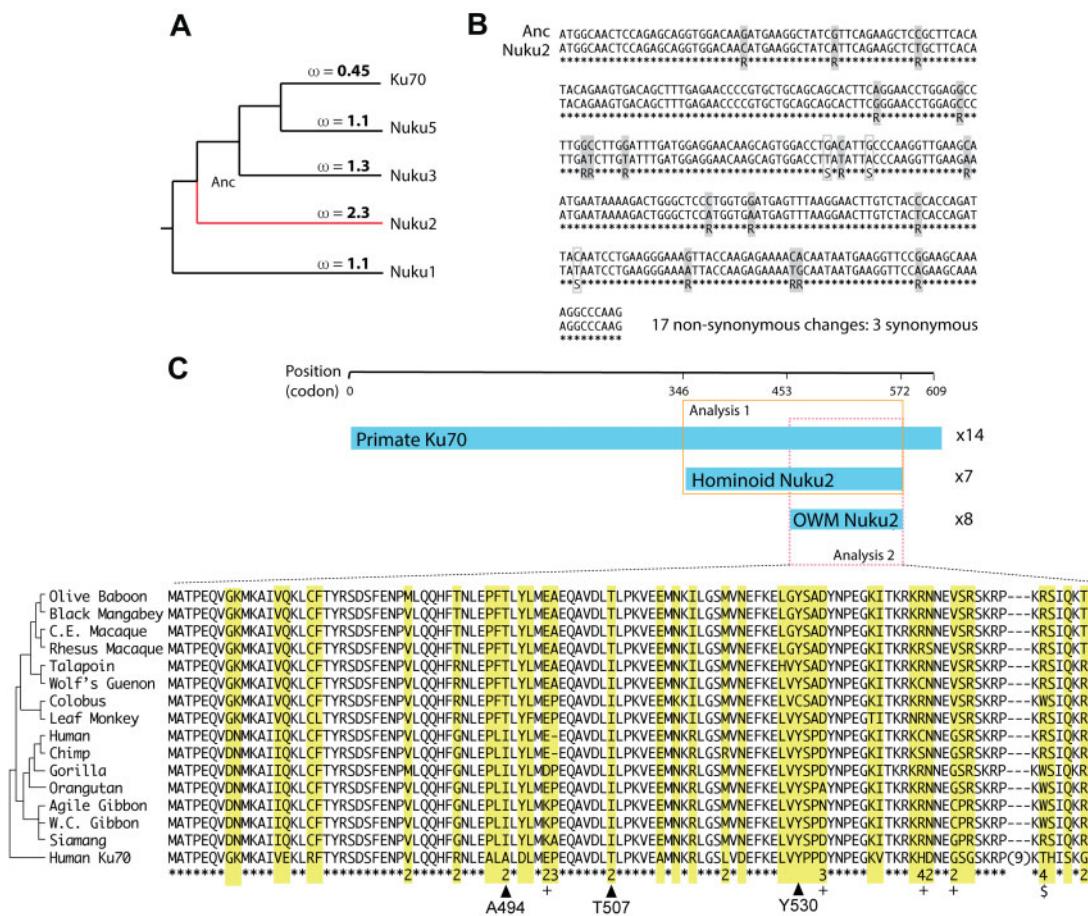


Figure 4 Molecular evolution of KU70 retrocopies. (A) Human KU70 and four of the human NUKU orthologs were aligned in the region of a common ORF. The branch-sites model assigned dN/dS values to each branch on the tree. These values summarize the evolution that has occurred since each retrocopy was formed. “Anc” refers to the node representing the formation of NUKU2, and the predicted sequence at this node was generated by codeml. (B) NUKU2 is aligned to the “Anc” ancestral sequence in the region of the ORF which was analyzed in the analysis in panel A. Nonsynonymous changes and synonymous changes are illustrated by gray and white boxes, respectively, in the alignment. (C) KU70 sequences were gathered for a total of 14 simian primate species, and NUKU2 sequences were gathered from 15 species. All NUKU2 sequences contain an ORF that is shorter than the KU70 ORF, and it is even shorter in Old World monkeys than it is in hominoids. Two analyses of codon evolution were performed, one containing the sequences in the orange box (Analysis 1; longer ORF, KU70 sequences plus 7 hominoid NUKU2 sequence), and one containing the sequences in the pink box (Analysis 2; shorter ORF, KU70 sequences plus all NUKU2 sequences). The alignment shows the region that is an ORF in all genes. All NUKU2 sequences are shown, with human KU70 as an outgroup. In yellow are diverged sites, and numbers at the bottom indicate how many amino acid changes have occurred at those positions during NUKU2 evolution (only indicated where dN/dS is greater than 1). The ‘\$’ indicates a site that has changes from R to W three different times during NUKU2 evolution. The ‘+’ sign indicates sites found to be under positive selection in the Analysis 1 branch-sites calculation (posterior probability > 0.5).

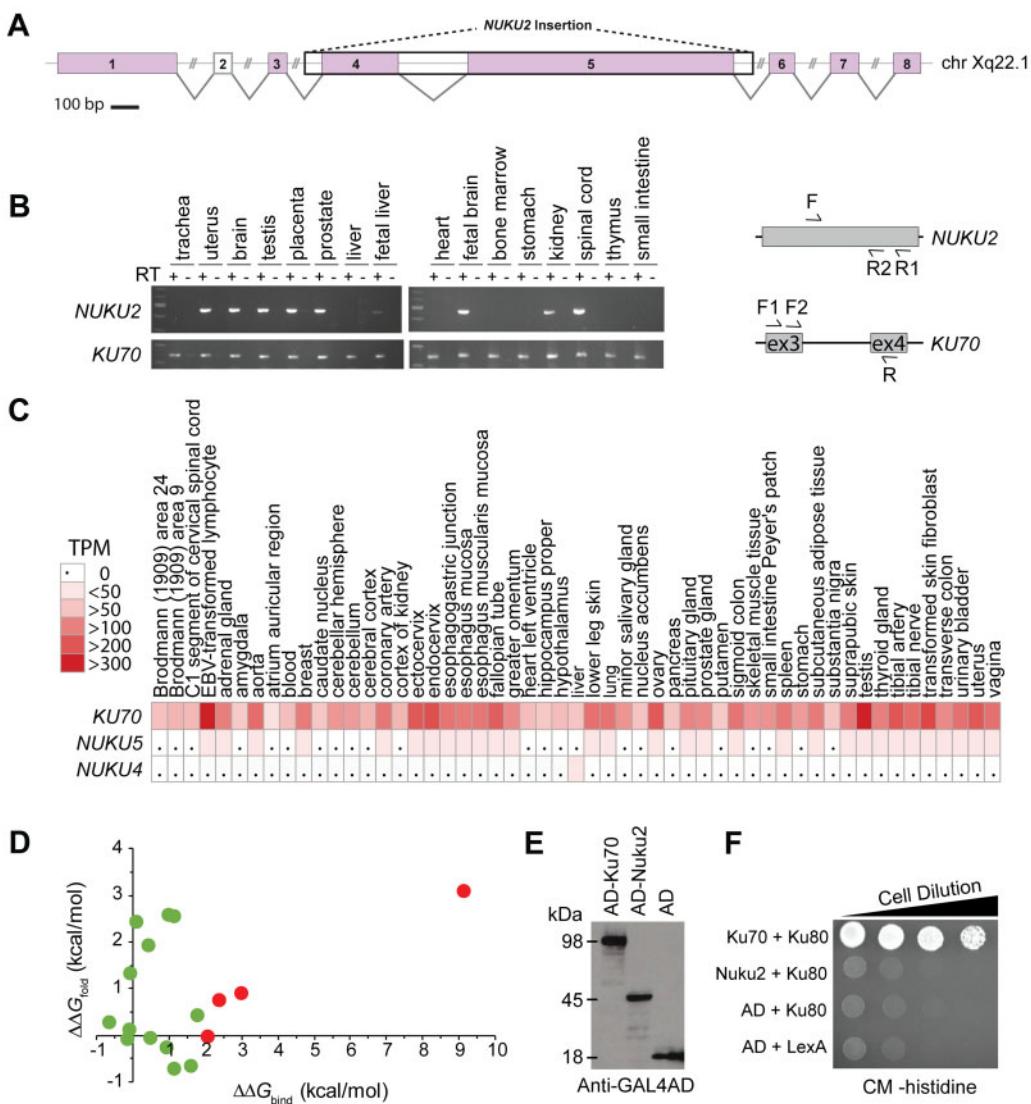


Figure 5 NUKU retrocopies are functionally distinct from KU70. (A) A lymphocyte-specific processed mRNA (EU224311) mapped to the human X chromosome with the insertion site of NUKU2 boxed in black. Predicted splice sites are indicated between exons with 100% identity to the X chromosome (pink boxes). 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 Nuk2p mutation within 5 Å of Ku80p, ΔG_{bind} was plotted on the x-axis, whereas ΔG_{fold} was plotted on the y-axis. Mutations are shown in green with x-axis values $\Delta G_{bind} < 2$ kcal/mol and y-axis values $-3 < \Delta G_{fold} < 3$ 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 $\Delta G_{bind} > 2$ kcal/mol and y-axis values $-3 < \Delta G_{fold} < 3$ 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 Nuk2p with Ku80p. The Gal4 AD is either fused to Ku70p (top row), Nuk2p (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. wolffii*, *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 ($\Delta\Delta G_{bind}$). The 11 mutations present in Nuku2p that were more than 5 Å from the Ku80p interface had an average $\Delta\Delta G_{bind}$ 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 ($\Delta\Delta G_{bind} < 2$ 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 $\Delta\Delta G_{bind} > 2$ 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; $\Delta\Delta G_{bind} > 2$) (Figure 4). Finally, nonsynonymous mutations in NUKU2 at sites under positive selection in primates have average $\Delta\Delta G_{bind}$ and $\Delta\Delta G_{fold}$ 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 ~70 kDa, 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 (~50 kDa), 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

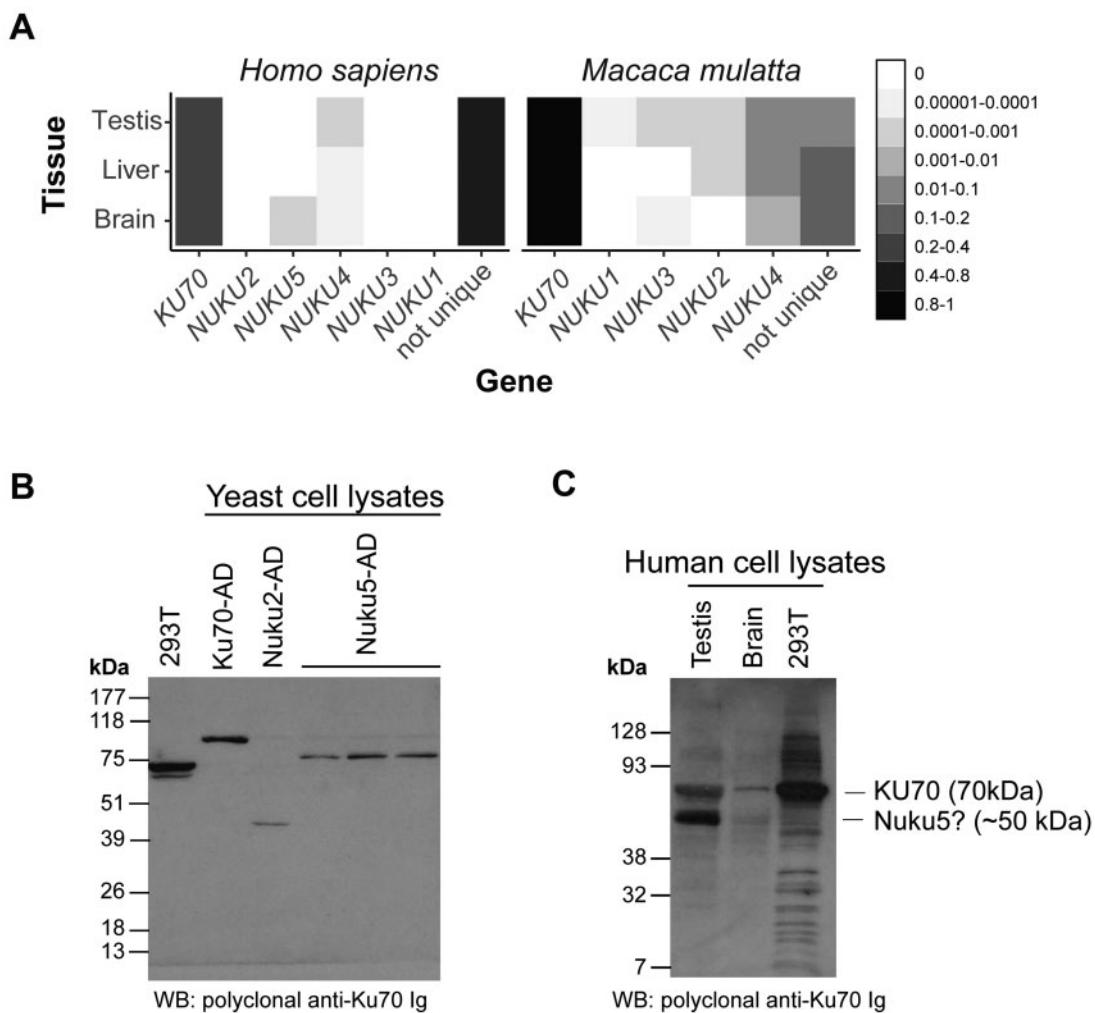


Figure 6 Detection of a putative KU70 retrocopy-encoded protein (A) A heat map representation of Ribo-seq data reveals probable translation of several NUKU retrocopies. Many of the short (~30bp) Ribo-seq reads identically match more than one gene and are classified as “not unique.” The remaining reads 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-PKcs 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-PKcs 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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