SATB1 targets chromatin remodeling to regulate genes over long distances

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How eukaryotic chromosomes are organized inside the nucleus allowing only a subset of the genome to be expressed in any given cell type is largely unknown [1-3]. SATB1, a protein found predominantly in thymocytes[4] regulates genes by folding chromatin into loop domains, tethering specialized DNA elements to a SATB1 network structure [5]. SATB1 ablation by gene targeting results in temporal and spatial miss-expression of numerous genes and arrested T cell development,

suggesting that SATB1 is a cell-type specific global gene regulator [6]. Here we show that SATB1 targets chromatin remodeling to the *IL-2Rα* gene, which is ectopically transcribed in SATB1 null thymocytes. SATB1 recruits the histone deacetylase contained in the NURD chromatin remodeling complex to a SATB1-bound site in the *IL-2Rα* locus and mediates the specific deacetylation of histones in a large domain within the locus. SATB1 also targets ACF1 and ISWI, subunits of CHRAC and ACF nucleosome mobilizing complexes, to the specific site and regulates nucleosome positioning over seven kilobases. SATB1 defines a new class of transcriptional regulators that function as a landing platform for several chromatin remodeling enzymes to regulate large chromatin domains.

SATB1 forms a three-dimensional network structure resembling chicken wire in mouse thymocyte nuclei [5] and specifically binds to double-stranded DNA with a specialized ATC sequence context that readily becomes base unpaired under negative superhelical strain [4,7,8]. Such base unpairing regions (BURs) are key elements of matrix attachment regions (MARs)[7,8]. SATB1 regulates genes located as much as ~50kb away from the attached sites. In SATB1 null thymocytes, which arrest at the $CD4^+CD8^+$ double positive (DP) stage, the normal SATB1-bound genomic loci are detached and associated genes are dysregulated [5]. What is the mechanism that connects SATB1 function in nuclear organization and transcription? We tested a hypothesis that the SATB1 network provides assembly sites for chromatin remodeling and modifying factors involved in gene regulation and other nuclear functions[9]. ATP-dependent chromatin remodeling factors use the energy gained by ATP hydrolysis to alter nucleosome array structures [2,10,11]. Histone modifying enzymes are involved both in transcriptional activation (e.g. histone acetylation) and repression (e.g. histone deacetylation)[9].

Using DNA affinity chromatography designed to purify SATB1, we asked whether chromatin remodeling and modifying factors specifically co-purify with SATB1. We compared elution profiles of extracts prepared from wild type and SATB1 null thymocytes. A BUR containing a core unwinding element found in a MAR 3' of the immunoglobulin heavy chain (IgH) enhancer was concatermerized and used to make a DNA affinity column to purify SATB1. In order to trap non-specific A+T-rich and other DNA binding proteins, thymocyte extracts were first loaded onto a mutated (MUT) BUR column that has lost the unwinding propensity, but retains the A+T rich feature. The flow-through of this column was applied onto the wild-type (WT) BUR column. As shown in Fig. 1a (middle panel, lanes, SM, MUT, WT), SATB1 flows through the mutant (MUT) BUR affinity

column and is almost completely retained in the wild-type (WT) BUR column [12]. SATB1 was eluted from this column by 0.4-0.8M [Na^+]. We asked whether the NURD (nucleosome re-modeling and histone de-acetylase) complex co-purifies with SATB1. The NURD complex has been implicated in transcriptional repression of several genes [13] and contains ATP-dependent remodeling enzyme Mi-2 (240kDa), histone deacetylase 1 (HDAC1; 62kDa) and 2 (HDAC2; 55kDa), histone deacetylase associated co-repressor mSin3A (160kDa) and MTA-2 (70kDa) [14-16]. In addition we monitored the presence of nucleosome-dependent ISWI-ATPase (140kDa), and ACF1 (180kDa), both subunits of the ACF and CHRAC complexes (reviewed in [17]). These complexes are known to mobilize nucleosomes to reorganize nucleosomal arrays over large distances *in vitro* [17]. However, nothing is known about their *in vivo* function and whether they are targeted to specific sites. Interestingly, in the fractions that contained SATB1, the NURD complex subunits Mi-2, mSin3A, MTA-2, HDAC1 and 2 and CHRAC/ACF complex subunits ACF1 and ISWI, were also detected (Fig. 1a, top panel). These chromatin remodeling factors were retained on the BUR column via SATB1, since none of these proteins were retained when SATB1 deficient extracts were applied (Fig. 1b, top panel). Mi-2, ACF1, mSin3A, ISWI, MTA-2, HDAC1 and HDAC2 proteins were present at roughly the same abundance in a SATB1 deficient thymic extract as the wild-type extract (Fig. 1a and 1b SM, Mut and WT lanes). Apparently, chromatin remodeling factors that co-purified with SATB1 represent a subfraction of each of these factors present in thymocytes since these proteins were also found in the flow through fraction of the BUR affinity column (Fig. 1a, lane WT). Poly (ADP-ribose) polymerase (PARP), another BUR-binding protein [18] found in both wild type and SATB1 null thymocyte extracts, was retained in the BUR affinity column (Fig. 1a, b, bottom panels), indicating that the affinity column used for either extracts was active. The data also indicate that unlike SATB1, a BUR-binding protein such as PARP is not sufficient to recruit chromatin remodeling factors to sites of the specialized DNA context. The elution profile of chromatin remodeling factors did not match exactly that of SATB1. One explanation could be that these factors associate with SATB1 less strongly than SATB1 to DNA. Co-elution of chromatin remodeling factors along with SATB1 is not an artifact of SATB1 potentially being a "sticky" protein, because other factors such as retinoblastoma protein (Rb) were absent in the eluted fractions (data not shown).

Do chromatin remodeling factors form protein complexes with SATB1 in the absence of DNA? Anti-SATB1 antibody co-immunoprecipitated ISWI, Mi-2, MTA-2, HDAC1 along with SATB1 from wild-type but not SATB1 null thymic extracts that lack any genomic DNA (Fig. 2a, lane 1 and 2). PARP, Rb and NFkappaB proteins were not detected in the anti-SATB1 immunoprecipitate from the wild-type extracts (Fig. 2a, lane 1 and data not shown). Control antibody (monoclonal anti-p27 antibody) fails to immunprecipitate any of these proteins (Fig. 2a, lane 3). In a separate experiment, anti-HDAC1 antibody immunoprecipitated SATB1 only from

wild-type extract, in addition to mSin3A, MTA-1 and HDAC1 itself (Fig. 2b, lanes 1 and 2). These data show SATB1 assembles protein complexes with factors found in CHRAC/ACF and NURD complexes including ATP-dependent remodeling enzymes, histone deacetylases and their associating factors in the absence of DNA.

We examined whether SATB1 directly interacts with the components of CHRAC/ACF using purified recombinant ISWI isoform human SNF2H, human ACF1, the CHRAC-specific histone-fold proteins p15 and p17 [19], and SATB1 (amino acid 90-763). By immunoprecipitation with antibodies against each protein, we show that SATB1, hACF1 and hSNF2H make a protein complex *in vitro* (Fig. 2c, lanes 1-4). Histone-fold proteins were dispensable for the formation of this complex. SATB1 directly interacted separately with both hSNF2H and hACF1 (Fig. 2c, lanes 5-7 and 8-10). Truncated SATB1 (amino acid 365-763) failed to interact with either ISWI or hACF1 (Fig. 2c, lanes 11-14), suggesting that the SATB1 domain of amino acid 90-365 contain an hSNF2H binding site(s). Consistent with the data from BUR DNA affinity chromatography, PARP did not interact with CHRAC/ACF (Fig. 2c, lanes 15-18). SATB1 represents the first example of a targeting factor of CHRAC/ACF.

We next examined whether SATB1 directs chromatin remodeling complexes to the SATB1 binding loci *in vivo*. We first studied HDAC1, a component of the NURD complex. This was done by chromatin immunoprecipitation (ChIP) assays. Briefly, thymocyte chromatin crosslinked with formaldehyde, purified with a urea gradient, and digested with a restriction enzymes was immunoprecipitated with either anti-SATB1 or anti-HDAC1 antibody [20,21]. We focused on the *IL-2Rα* locus because the *IL-2Rα* gene is ectopically transcribed in SATB1 null DP thymocytes [6]. The DP subset represents a majority (>84%) the total thymocyte population. Within the *IL-2Rα* locus, we identified a 700bp fragment that exhibits very high affinity (Kd=0.1-0.5nM) to SATB1 *in vitro* (SATB1-bound sequence 700; SBS700, data not shown) which mapped to the first intron, 6.8 to 7.5 kb downstream of the transcriptional start site (Fig. 3c). There is no additional SATB1-bound site identified within ~16kb upstream of SBS700. We found that SBS700 is indeed bound to SATB1 as well as to HDAC1 *in vivo* in wild type thymocytes (Fig. 3a, upper row, anti-SATB1 and anti-HDAC1; lane 3). In contrast, SBS700 was not bound to HDAC1 in SATB1 null thymocytes (Fig. 3a, lower row, anti-HDAC1; lane 3). This was revealed by PCR amplification with a specific primer set designed for SBS700 and regardless of the precise number of cycles used in the range of 25 to 30 cycle. An intronic sequence 2.8 kb downstream of exon 1, which was not bound to SATB1 in wild type thymocytes (Fig. 3a, upper row, anti-SATB1, lane 2) was also not bound to HDAC1 in both wild type and null thymocytes (Fig. 3a, upper and lower rows, anti-HDAC1; lane 2). As a positive control, we used SBS336, which was cloned as a SATB1-bound sequence [5] from total *in vivo* crosslinked genomic DNA (Fig. 3a, upper and lower rows, anti-SATB1; lane 4). Similar to SBS700, SBS336 was also found present in anti-HDAC1 antibody immunoprecipitates from wild type but not

SATB1 null thymocytes (Fig. 3a, upper and lower rows, anti-HDAC1, lane 4). Using a specific primer set that amplifies the *IL-2Rα* promoter region, a faint PCR product was seen in wild type thymocytes (Fig. 3a upper row, anti-HDAC1; lane 1), even though the first 3kb promoter totally lack SATB1 binding sequences. Such band was absent in null thymocytes (Fig. 3a, lower row, anti-HDAC1; lane 1). The non-immune serum precipitated DNA pool never gave rise to any PCR amplified signal under the same conditions (Fig. 3a, upper and lower rows, non-immune serum; lanes 1-4). These data show that SATB1 is required for recruitment of HDAC1 onto the SATB1's target sequence and the promoter within the *IL-2Ra* locus as well as other loci.

Acetylation of histones in nucleosomes indicates transcriptionally active chromatin [22,23]. HDAC1 specifically recruited to the SATB1-binding site in the *IL-2Rα* intron in thymocytes is expected to play a role in controlling the histone acetylation status of that locus. Therefore, we immunoprecipitated *in vivo* crosslinked chromatin fragments with anti-acetylated histone H4 antibody. SBS700 is absent in this immunoprecipitated chromatin from wild-type thymocytes. In contrast, in SATB1 null thymocytes where SBS700 is not bound to HDAC1 *in vivo,* SBS700 is acetylated (Fig. 3a, upper and lower rows; anti-H4ace; lane 3), consistent with a transcriptionally active state. Furthermore, in SATB1 null thymocytes, the SBS700 distal promoter region (Fig. 3a, lower row, anti-H4 ace; lane 1) and the remote intronic sequence (Fig. 3a, lower row, anti-H4 ace; lane 2) are also hyperacetylated, which is consistent with ectopic transcription of *IL-2Rα* in the absence of SATB1. Identical results on histone acetylation were obtained with the SBS336 locus. Thererfore, SATB1 affects chromatin structure over distance in the *IL-2Rα* gene locus by recruiting HDAC1 to specific DNA sequences, maintains a hypoacetylated nucleosome status, and packages chromatin into a transcriptionally inactive state.

Similar to HDAC1, we also found by ChIP assay that both ISWI and ACF1 were specifically bound *in vivo* to SBS700 of the *IL-2Rα* locus as well as to SBS336 in wild type thymocytes but not in SATB1 null thymocytes (Fig. 3b, upper and lower rows, anti-ACF1 and anti-ISWI; lanes 3 and 4). Furthermore, both factors were also found associated with the *IL-2Rα* promoter only when SATB1 is present (Fig. 3b, anti-ACF1 and anti-ISWI; lane 1), but never to the intronic sequence regardless of the presence of SATB1 (Fig. 3b, upper row, anti-ACF1 and anti-ISWI; lane 2). In budding yeast, a related ISWI-complex acts in transcriptional repression by creating an inaccessible local chromatin structure at several promoters (reviewed in [9]). Our data show that SATB1 is necessary for both the proper repression of the *IL-2Rα* gene in DP thymocytes[6] and the recruitment of ISWI and ACF1 to a specific site within the gene locus, implicating ISWI and ACF1 in repression of *IL-2Rα*.

The biological consequence of recruiting ATP-dependent chromatin remodeling enzymes such as ISWI to the *IL2-Rα* locus may be reflected by changes in nucleosome positioning in this locus. Therefore, we performed micrococcal nuclease digestion in isolated thymocyte nuclei followed by Southern hybridization and compared results between wild-type and SATB1 null mice. In each

set of regions examined, we also performed micrococcal nuclease digestion directly on purified genomic DNA to distinguish the cleavage sites of this enzyme at its preferred DNA sequences from its cleavages at nucleosomal linker sites. In the region 0.8 to 1.1 kb upstream of the *Pst 1* site, overlapping the 5' half of the SBS700 region which contains one of the two SATB1-binding sequences (~100bp each), we found a clear difference in the pattern of cleavages by micrococcal nuclease between wild-type and SATB1 null nuclei, reflecting changes in nucleosomal distribution (Fig. 4b, arrows). We also examined nucleosome positioning in the *IL-2Rα* promoter/first exon region which is ~8kb upstream of SBS700. The promoter region is known to contain two major nuclease hypersensitive regions (DH1 and DH2)[24]. Although DH1, which is proximal to the promoter, is retained even in the absence of SATB1 (data not shown), nucleosome positioning toward exon 1 exhibits changes. A major alteration in nucleosome positioning between wild-type and SATB1 null nuclei was revealed in intronic sequences immediately downstream of exon 1, located 0.2- 1kbp downstream of the *Pst1* site using probe 2 (Fig. 4a). Because this region as well as the remaining 7kbp intronic region upstream of SBS700 lack any ATC sequence cluster and fail to bind SATB1 *in vitro*, any change in nucleosome positioning close to exon 1 is not due to the potential alteration in chromatin structure caused by SATB1 binding. As summarized in Fig. 4c, these results show that changes in nucleosomal positioning due to SATB1 ablation can occur as far as 7 kb away from the actual SATB1-binding sequences.

The results shown here provide mechanistic insights for previously reported activities of matrix attachment regions surrounding the intronic immunoglobulin μ enhancer, which confer enhanced chromatin accessibility to distal positions and modulate chromatin structure [25-29]. SATB1 links higher order packaging of chromatin to gene regulation by targeting chromatin remodeling to the bases of chromatin loop domains, where DNA is anchored to the SATB1 network, to regulate chromatin structure and gene expression over long distances. We propose that the action of SATB1 represents a general guiding process by which multiple chromatin remodeling factors find entry sites into the chromatin **(at regions of high base-unpairing propensity) or (at the specialized ATC sequence context)**, providing a new mechanism for global gene regulation in higher eukaryotes.

Methods

BUR Affinity Chromatography

Ligated, double stranded oligos bearing either wild-type (upper strand , 5'-TCTTTAATTTCTAATATATTTAGAAttc-3') or mutant DNA (upper strand, 5'-TCTTTAATTTCTACTGCTTTAGAAttc-3') were coupled to Sepharose CL-4B beads (Amersham Pharmacia). Proteins were extracted from $5x10^8$ thymocytes in 0.4M NaCl buffer containing Complete Mini (Roche) protease inhibitor tablets according to Dignam. Extracts were diluted to

0.1M NaCl, incubated with 10 μg/ml salmon sperm DNA and passed first through the mutant BUR column then twice through the wild-type BUR column. The wild-type BUR column was washed with 0.1M NaCl buffer prior to elution with buffers of increasing NaCl concentration.

Immunoprecipitation

50 μg of protein in Dignam extract (100μl) was diluted 1 to 1 with 0.2% NP-40/PBS, pre-cleared with rabbit IgG (Santa Cruz) and protein A/G beads (Pierce), then incubated with either anti-SATB1 monoclonal antibody (1:25, Transduction Labs), anti-HDAC1 (1: 20, Novus) or an equivalent amount of control antibody (1:25, anti-p27 antibody, Santa Cruz). For *in vitro* co-immunoprecipitation, recombinant flag-tagged human SNF2H and hACF1, expressed and purified as described [30] and recombinant human CHRAC15/17 [19] was used. His-tagged SATB1 (90-763), SATB1 (365-763) and full length PARP were expressed and purified using standard protocols (Qiagen). 15 ng of each protein was added to each reaction and incubated for 30 min at 4□C in IP-150 buffer (20 mM HEPES, 150 mM KCl, 1 mM $MgCl_2$, 0.05% NP-40, 10% glycerol, 0.2 mg ovalbumin, 25 U benzonase), with either anti-ACF1 (1:100), anti-ISWI (1:100)[19], rabbit anti-SATB1[4,7,8] (1:100), anti-PARP (1:100, Santa Cruz) or pre-immune serum. The rabbit polyclonal antibody against mouse ACF1 was raised against the peptide SPSTVDQVSTPLAAKKSRI at the carboxy-terminus of mACF1. Protein-antibody complexes were recovered with protein A/G beads, washed in PBS containing 0.1% NP-40. Soluble proteins were obtained in Laemmli sample buffer at 95□C, 5 min. and identified by Western analysis with anti-Flag (M2, Sigma), anti-MTA-2 and anti-Mi-2 (provided by Dr. Yi Zhang, University of North Carolina at Chaepl Hill)) and antibodies used in immunoprecipitation.

Chromatin Immunoprecipitation (ChIP)

Crosslinked chromatin was prepared from thymocytes and purified by urea gradient centrifugation as described [4,20]. For each experiment, 150-200 μg of chromatin was digested with Sau3AI (New England Biolab) adjusted to 1.0% NP-40 and pre-cleared first by incubation with protein A/G beads alone, then non-immune rabbit serum and protein A/G beads. Pre-cleared chromatin was divided (~30μg/tube) and incubated overnight with 5 μl of either rabbit anti-SATB1, anti-HDAC1 (Santa Cruz), anti-acetylated histone H4 (Upstate Biotech), anti-ACF1, affinity-purified anti-ISWI or pre-immune serum. Complexes on beads were washed, then digested with 250μg/ml proteinase K and subjected to phenol/chloroform extraction prior to ethanol precipitation with glycogen. One twentieth of DNA from each pool were PCR amplified in reactions containing 1.0 unit of Amplitaq Gold (Roche), 10 mM Tris-HCl, 1.5 mM $MgCl_2$ and 1 μM of either: SBS700 MAR 5'-GCTTACTGGTCTACCAGCCAAAT-3' and 5'-CATGCAAAGTCTTATGCCTGAGC-3', *IL-2Rα* intron 5'- TACTGCCCACACTAGAAGTTCCG -3' and 5'- CAGGCATAAGTTCCATATCAGGC -

3', sbs336 5'-TCCTTACACACAAAGTGGGCTG-3' and 5'-GGAGTCTTAGATGAGTTGGCATTGC-3' or *IL-2Rα* promoter 5'-GACACTATGAGAGAAGGCAAAGGG-3' and 5'-ACACCTCGGTATTGGTTCACTCC-3' using 1 cycle of 95□C for 10 minutes, 25-30 cycles of 95□C for 45 sec, 60□C for 60 sec and 72□C for 90 sec. PCR products were resolved by acrylamide gel electrophoresis, stained with Sybr Gold (Molecular Probes) and imaged on a Storm scanner (Molecular Dynamics).

MNase digestion of nuclei and Southern hybridization.

Thymic nuclei were prepared in 3.75mM Tris-HCl, pH 7.6, 0.5 mM EDTA, 20mM KCl, 1mM $NaHCO_3$, 0.05mM spermine, 0.125mM spermidine, 0.5mM DTT, 1 tablet of protease inhibitor cocktail /10ml, 0.1% IGEPAL CA-630. Nuclei were suspended to 4.5 X 10^7nuclei /ml in 20mM HEPES, pH7.6, 20mM KCl, 0.5mM EDTA, 1mM $NaHCO_3$, 3mM $CaCl_2$. 4.5 x 10^6 nuclei in the 100 µl buffer were digested with MNAse at 4, 8 and 16 and 25U/ml concentration (Roche) for 4 min at 20^{o}C. Nuclease digestion was stopped by the addition of 1/10 volume of (0.5% SDS, 60mM EDTA, 3mg / ml Proteinase K). MNase-treated genomic DNA was purified and digested with *Bgl*II or *Pst*I and separated on a 1.3% agarose gel, alkali blotted onto Hybond-N^+ membrane (Amersham) and UV cross-linked(Stratalinker; Stratagene). To prepare DNA fragments for hybridization probes, PCR reactions was performed with genomic DNA with the following sets of primers: For probe 1; 5'-CTCTCCAGTTCCAGAAGAAAC-3' and 5'-CTGCAGTGTTAGGTTGAGAAC-3', for probe 2; 5'-CAAAACAGTCAGCAACTAGGC-3' and 5'- GAAGACTCAACAAAGACTGAG-3', for probe 3; 5'- GAGAATTTCATCCAGTTCCC-3' and 5'- AGAACCCATGATTCAACTCC-3'. Blotted DNA was hybridized at 43^{o}C overnight with probes labeled with α^{32}P-dCTP by Klenow large fragment.

Fig. 1. Chromatin remodeling factors co-purify with SATB1 in DNA affinity chromatography. a) Proteins in SATB1 (+/+) thymic extract (SM), mutant BUR column flow through (MUT), wild-type BUR column flow through (WT) and proteins eluted with increasing concentrations of NaCl were identified by Western analysis. Mi-2, ACF1, mSin3A, ISWI, MTA-2, HDAC1 and HDAC2 signals are shown in the top panel. SATB1 signal is shown in the middle panel and PARP, another BUR binding protein is shown in the lower panel. Molecular mass marker position is to the right of each panel. b) When BUR affinity chromatography was performed with SATB1 (-/-) extract only PARP was eluted (lower panel).

Fig. 2. SATB1 directly interacts with several chromatin remodeling factors. Proteins immunoprecipitated with a) anti-SATB1 antibody or b) anti-HDAC1 from SATB1 (+/+) (lane 1) and SATB1 (-/-) thymi (lane 2) were identified by western with antibodies as indicated. In both a and b control antibody (anti-p27 antibody) immunoprecipitation of SATB1 (+/+) extract, lane 3 and

proteins in starting material are shown in lane 4. c) Protein immunoprecipitated with anti-ISWI, anti-ACF1, anti-SATB1, and anti-PARP antibodies from mixtures of purified FLAG-fused recombinant ISWI (hSNF2H), ACF1 (hACF1), his-tag-fused SATB1 (amino acid 90-763), truncated SATB1 (amino acid 365-763), and full-length PARP were identified with anti-FLAG, anti-SATB1 and anti-PARP antibodies, P.I.: preimmune serum, IP: immunoprecipitation, IB: immunoblot.

Fig. 3. SATB1 targets HDAC1, ISWI, and ACF1 to the *IL-2Rα* locus *in vivo*. PCR amplification of chromatin immunoprecipitated DNA using antibodies to a) SATB1, HDAC1, acetylated histone H4 (H4ace), control non-immune serum and b) to ACF1, ISWI, or genomic DNA. Each DNA sample was amplified with four primer pairs; *IL-2Rα* promoter (lanes 1), *IL-2Rα* intron (lanes 2), *IL-2Rα* SBS700 (lanes 3), or SBS336 (lanes 4). DNA size markers are shown to the left of the panel. Samples prepared from SATB1 wild-type (+/+) chromatin (upper row) and SATB1 (-/-) chromatin (lower row) are depicted. c) A map of *IL-2Rα* with positions of the primers used for promoter, intron, and SBS700 sequences.

Fig. 4. Alteration of nucleosomal positioning in the *IL-2Rα* locus in SATB1 null thymocytes.
a, MNase cleavage sites downstream of the first exon region in wild type (WT), SATB1 null (KO) thymocyte nuclei and naked DNA determined by Southern hybridization of *Pst 1*-digested DNA with probe 1. b, MNase cleavage sites at the intronic SBS700 determined by Southern hybridization of *Pst 1*-digested DNA with probe 2. c, Summary map of the *IL-2Rα* gene locus. Location of SBS700 region (hatched boxes) and ATC sequences that binds SATB1(wavy lines) are indicated. For a-c, MNase cleavage sites restricted to WT (sold arrowheads) or KO nuclei (open arrowheads), enhanced cleavages for WT (solid circle) or KO nuclei (open circles) are shown.

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