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Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A–H2B acidic pocket

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Interleukin-33 (IL-33) is an IL-1-like ligand for the ST2 receptor that stimulates the production of Th2-associated cytokines. Recently, we showed that IL-33 is a chromatin-associated factor in the nucleus of endothelial cells *in vivo*. Here, we report the identification of a short IL-33 chromatin-binding peptide that shares striking similarities with a motif found in Kaposi sarcoma herpesvirus LANA (latency-associated nuclear antigen), which is responsible for the attachment of viral genomes to mitotic chromosomes. Similar to LANA, the IL-33 peptide docks into the acidic pocket formed by the H2A–H2B dimer at the nucleosomal surface and regulates chromatin compaction by promoting nucleosome–nucleosome interactions. Taken together, our data provide important new insights into the nuclear roles of IL-33, and show a unique example of molecular mimicry of a chromatin-associated cytokine by a DNA tumour virus. In addition, the data provide, to the best of our knowledge, the first demonstration of the existence of non-histone cellular factors that bind to the acidic pocket of the nucleosome.

Keywords: chromatin; cytokine; histone; IL-1; virus

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INTRODUCTION

Interleukin-33 (IL-33; previously known as NF-HEV) is the most recent addition to the IL-1 family (Baekkevold *et al*, 2003; Schmitz *et al*, 2005; Carriere *et al*, 2007). It has been shown to function as a ligand for the ST2 receptor that stimulates the production of Th2-associated cytokines in mast cells and Th2 lymphocytes (Schmitz *et al*, 2005; Ali *et al*, 2007; Allakhverdi *et al*, 2007), and protects the heart in response to cardiac stress and atherosclerosis (Sanada *et al*, 2007; Miller *et al*, 2008). Recently, we discovered that IL-33 is an abundant chromatin-associated factor in the nucleus of endothelial cells *in vivo* (Carriere *et al*, 2007). In addition, we showed that IL-33 has transcriptional regulatory properties (Carriere *et al*, 2007). This suggested that IL-33 is a dual-function protein that might act both as a cytokine and as an intracellular nuclear factor. As a chromatin-associated cytokine, IL-33 is similar to HMGB1 (high-mobility group box1), an architectural chromatin-binding nuclear factor that functions extracellularly as a cytokine when released by necrotic cells or when secreted by activated macrophages during inflammation (Wang *et al*, 1999; Scaffidi *et al*, 2002).

Although the association of IL-33 with chromatin has been shown previously, its nuclear partners and mechanisms of targeting have not yet been described. Here, we show that IL-33 tethers to chromatin by docking into the acidic pocket formed by the histone H2A–H2B dimer at the surface of the nucleosome. A similar mechanism has previously been shown to be used by Kaposi sarcoma herpesvirus (KSHV) for attachment of viral genomes to mitotic chromosomes (Barbera *et al*, 2006). Our results indicate that the virus pirated the chromatin-binding motif (CBM) of IL-33 for the establishment of latent infection in human cells.

RESULTS

Identification of a short IL-33 CBM

Previously, we identified a chromatin and mitotic chromosome association domain at the amino terminus of IL-33 (aa 1–65) that is not conserved in other IL-1 family members (Carriere *et al*, 2007). Further deletion mutagenesis showed that IL-33 residues 40–58 are sufficient for tethering N- or carboxy-terminal green fluorescent protein (GFP) to mitotic (Fig 1A) or interphase (Fig 1B) chromatin in living human embryonic kidney (HEK) 293T cells. The individual residues essential for chromosome association, within the IL-33 CBM (aa 40–58), were then identified by alanine scanning mutagenesis (Fig 1C). Six residues were required for binding to mitotic chromatin: human IL-33 residues M45, L47, R48, S49, G50 and I53. A triple alanine mutation within the IL-33 CBM, IL-$33_{40–58}$(47AAA49), also abrogated chromosome association (data not shown). The results were confirmed in the context of full-length IL-33 (IL-33FL). GFP-IL-33FL and GFP-IL-33FL(F44A) were found to associate with mitotic chromatin but not the GFP-IL-33FL(47AAA49) and GFP-IL-33FL(R48A) mutants (Fig 1D). We conclude that the panel of mutations introduced

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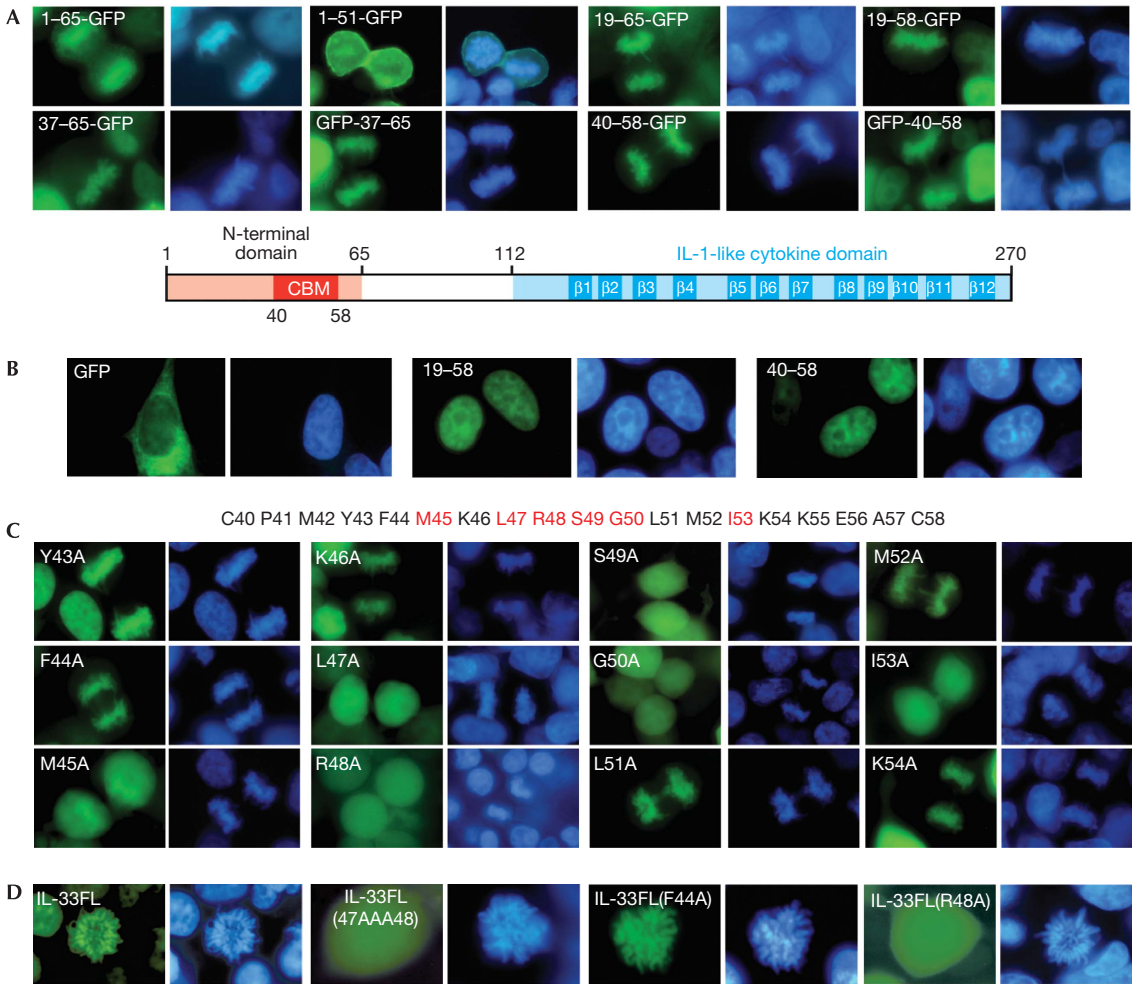

Fig 1 | Identification of the IL-33 chromatin-binding motif. (**A**) Deletion mapping of the IL-33 CBM. Interaction of the various IL-33-GFP fusion proteins (green) with mitotic chromosomes was analysed by fluorescence microscopy in living HEK293T cells; DNA was counterstained with Hoechst 33342 (blue). (**B**) Targeting of GFP fusion proteins to interphase chromatin (dense regions of Hoescht staining) was analysed in living HEK293T cells. (**C**) Human IL-33 residues 43–54 were individually mutated to alanine (A) in the context of GFP-IL-$33_{40–58}$. Individual residues essential for chromosome association in living cells are shown in red. (**D**) Chromosome association of GFP-IL-33FL, GFP-IL-33FL(47AAA49), GFP-IL-33FL(F44A) and GFP-IL-33FL(R48A) was analysed as described in (**A**). CBM, chromatin-binding motif; FL, full length; GFP, green fluorescent protein; HEK, human embryonic kidney; IL-33, interleukin-33.

within the IL-33 CBM has the same effects in the context of full-length IL-33 and IL-$33_{40–58}$ peptide.

Viral mimicry of the IL-33 CBM by KSHV

The IL-33 CBM was found to be evolutionarily conserved in murine and canine IL-33 orthologues (Fig 2A,B). Sequence alignment of the IL-33 CBM peptides (Fig 2C) showed striking homologies to the N-terminal CBM (aa 5–14) of KSHV LANA (latency-associated nuclear antigen; Piolot *et al*, 2001; Wong *et al*, 2004; Barbera *et al*, 2006), which also binds to mitotic chromosomes in human and mouse cells (Fig 2A,B). On the basis of the sequence alignment with LANA CBM, the minimal IL-33 CBM peptide was reduced further to amino acids 44–53 (Fig 2D). Interestingly, the conserved residues of the MXLRSG motif (Fig 2C) have been found to be individually required for mitotic chromosome association of both IL-33 (Fig 1C) and LANA (Wong *et al*, 2004; Barbera *et al*, 2006). This suggested that, similar to LANA, IL-33 might tether to chromatin through the histone H2A–H2B dimer. Thus we examined the ability of IL-33 CBM and single-point mutants to associate with H2A and H2B *in vivo*, using LANA CBM as a control. GFP-IL-$33_{40–58}$, GFP-$LANA_{5–22}$ and GFP-IL-$33_{40–58}$(F44A), which associate with chromosomes, precipitated H2A and H2B, whereas GFP and GFP-IL-$33_{40–58}$(R48A), which do not associate with chromosomes, did not (Fig 2E). Next, we analysed the interaction of IL-33 CBM with H2A and H2B *in vitro* by using acid-extracted histones from HeLa cells. Glutathione *S*-transferase (GST)-IL-$33_{40–58}$ precipitated H2A and H2B, similar to GST-$LANA_{5–22}$, whereas GST did not (Fig 2F). By contrast, H2A and H2B were not precipitated by GST-IL-$33_{40–58}$(47AAA49) or GST-IL-$33_{40–58}$(R48A) fusion protein (Fig 2G). Essentially, identical results were obtained with purified or recombinant H2A–H2B dimer *in vitro* (supplementary Fig S1 online)

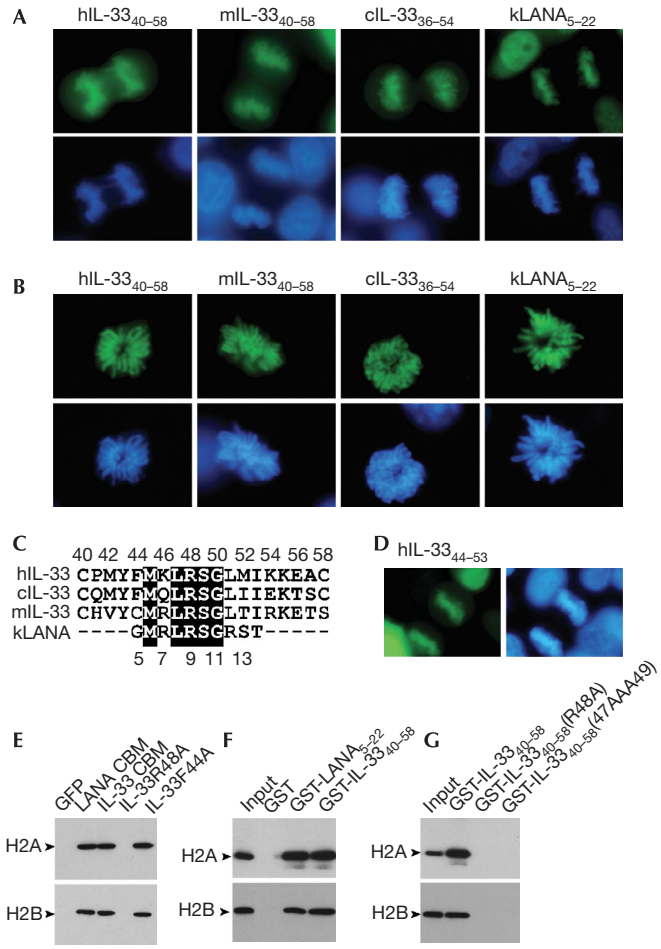

Fig 2 | Molecular mimicry between IL-33 and KSHV LANA for binding to chromatin through the histone H2A–H2B dimer. (**A**,**B**) Evolutionary conservation of the CBM in IL-33 orthologues (hIL-33, mIL-33, cIL-33: human, mouse and canine IL-33, respectively; kLANA: KSHV LANA). Chromosome association of GFP fusion proteins was analysed in living HEK293T cells (**A**) or mouse 3T3 fibroblasts (**B**). (**C**) Sequence alignment of IL-33 and LANA CBM sequences. (**D**) Human IL-33 residues 44–53 are sufficient for tethering GFP to mitotic chromatin. (**E**) The IL-33 CBM associates with H2A and H2B *in vivo*. GFP fusion proteins were immunoprecipitated from HEK293T cells and the H2A–H2B dimers were detected by immunoblot. (**F**,**G**) The IL-33 CBM binds to H2A and H2B *in vitro* (**F**) and mutations of IL-33 residues essential for chromosome association abrogate the interaction (**G**). GST fusion proteins were incubated with acid-extracted histones, and precipitated histones were detected by Western blot analysis with H2A and H2B antibodies. Input, 10%. CBM, chromatin-binding motif; GFP, green fluorescent protein; HEK, human embryonic kidney; IL-33, interleukin-33; KSHV, Kaposi sarcoma herpesvirus; LANA, latency-associated nuclear antigen.

and with full-length IL-33 *in vivo* (supplementary Fig S2 online). Taken together, these findings show a tight link between chromatin association of IL-33 CBM and its binding to H2A–H2B *in vitro* and *in vivo*, providing strong evidence that the H2A–H2B dimer mediates attachment of IL-33 to chromatin.

The IL-33 CBM recognizes the H2A–H2B acidic pocket

Next, we performed extensive molecular modelling studies of the IL-33–nucleosome complex. Modelling of IL-33 CBM on the basis of the X-ray structure of the LANA CBM complexed with the nucleosome core particle (Barbera *et al*, 2006) suggested that the IL-33 CBM can adopt a tight hairpin structure, stabilized by an array of intramolecular hydrogen bonds similar to that of the LANA CBM (Fig 3A,B). Remarkably, L51 and I53 in IL-33 replace LANA R12 and T14, respectively, which gives rise to a much denser network of intramolecular hydrophobic interactions (supplementary Fig S3 online). The strict conservation of the MXLRSG motif in the IL-33 and LANA CBM hairpins suggested that the IL-33 CBM might dock into the same cavity as the LANA CBM on the nucleosomal surface (Barbera *et al*, 2006). Molecular modelling showed excellent shape and charge complementarity between the IL-33 CBM and the nucleosomal surface (Fig 3C,D), and the presence of bulkier residues at the N and C termini of IL-33 CBM could be accommodated without steric clash. Similar to the LANA CBM, the IL-33 CBM is predicted to dock into the negatively charged acidic pocket formed by the H2A–H2B dimer on the nucleosomal surface (Fig 3E,F). The model suggests that hairpin binding is mediated by hydrogen bonds between IL-33 residues R48–S49 and negatively charged residues of the H2A–H2B acidic pocket (H2A residues E61, E64, D90; Fig 3G,H), and by stacking interactions involving the MXL submotif and H2A Y57 residue (supplementary Fig S3 online). To validate the molecular modelling results experimentally, we used histones carrying mutations in the putative interaction domain (Fig 4A). We found that IL-33 does not bind to H2A carrying mutations in the acidic pocket and binds only very poorly to histone variant H2A.Bbd (Burr body deficient), which has a divergent pocket sequence (Fig 4B). By contrast, IL-33 associated with histone variant H2A.Z, which has an extended acidic patch. Taken together, these data indicate that, similar to the LANA CBM, IL-33 CBM recognizes the acidic pocket formed by the H2A–H2B dimer at the surface of the nucleosome.

The IL-33 CBM regulates chromatin compaction

The recent demonstration of the crucial role of the H2A–H2B acidic pocket in chromatin compaction (Chodaparambil *et al*, 2007; Zhou *et al*, 2007) suggested that the IL-33 CBM might influence the higher order structure of chromatin. To test this hypothesis, we used a previously established *in vitro* chromatin model system based on the 208-12 DNA template (Simpson *et al*, 1985; Schwarz *et al*, 1996; Chodaparambil *et al*, 2007; Zhou *et al*, 2007), which consists of 12 repeats of a 208-bp nucleosome positioning sequence (Fig 5A). Nucleosomal arrays were assembled and chromatin reconstitution was verified by digestion with micrococcal nuclease (Fig 5B). Chromatin compaction was then assayed by measuring the abundance of monomeric arrays with increasing divalent cation ($MgCl_2$) concentrations known to promote chromatin condensation (Schwarz *et al*, 1996; Chodaparambil *et al*, 2007). The Mg_{50} value has previously been defined as the concentration of $MgCl_2$ at which 50% of nucleosomal arrays oligomerize into large, condensed higher order chromatin structures that are easily pelleted by microcentrifugation (Chodaparambil *et al*, 2007). We found that addition of the IL-33 CBM peptide lowered the Mg_{50} value needed for chromatin condensation ($Mg_{50} < 1$ mM), whereas a

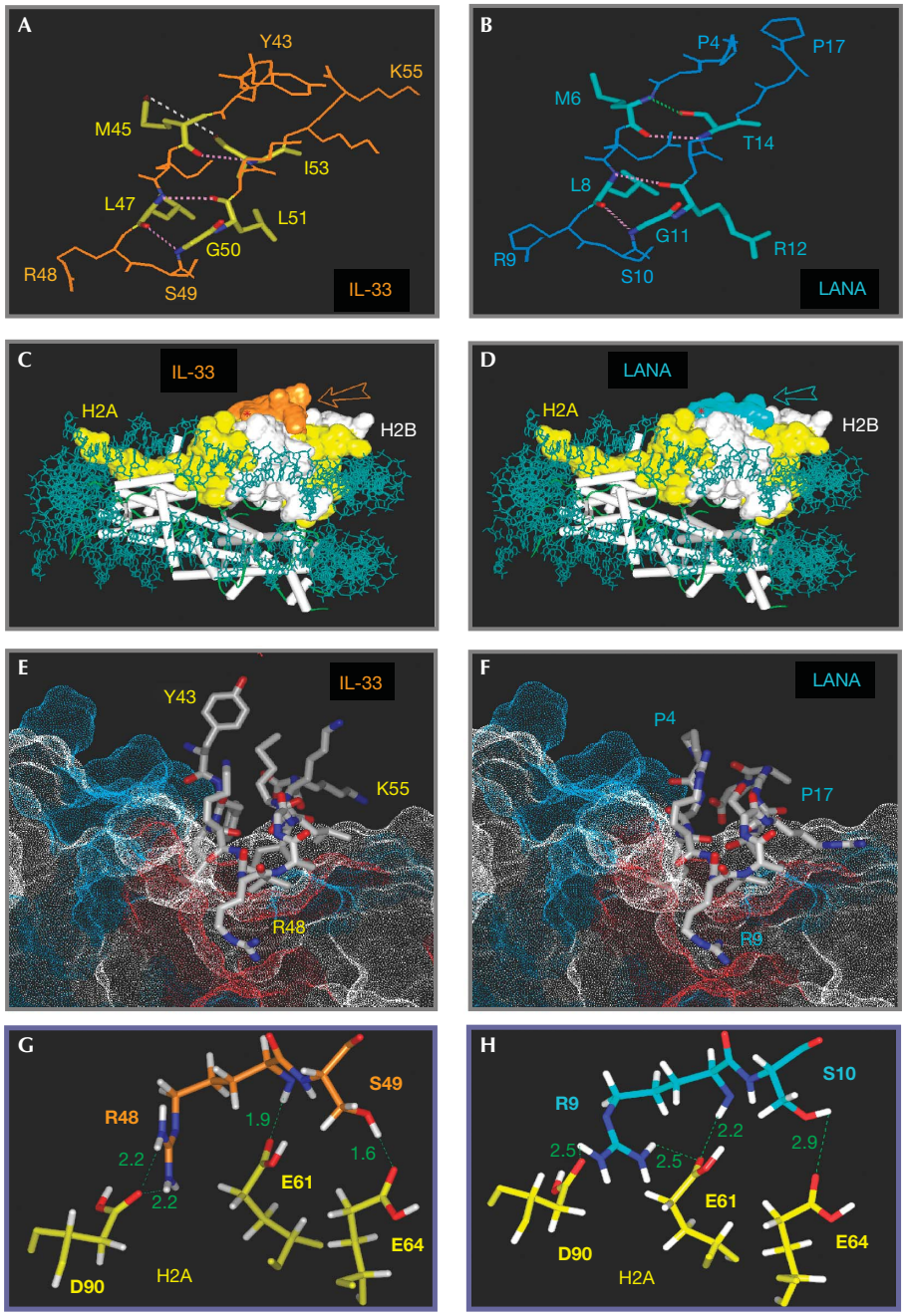

Fig 3 | Molecular mimicry between IL-33 and KSHV LANA for interaction with the acidic patch formed by the H2A–H2B dimer at the nucleosomal surface. (**A**,**B**) Modelling of the IL-33 CBM structure on the basis of the X-ray structure of the LANA CBM hairpin. Residues involved in intramolecular interactions are shown with thick lines. Equivalent arrays of hydrogen bonds between main-chain CO and NH are shown as dashed magenta lines. The white dashed line summarizes the network of hydrophobic interactions between IL-33 M45 and I53. O and N atoms are colour-coded in red and blue, respectively, and H atoms are hidden for clarity. (**C**–**H**) Molecular modelling of the IL-33 CBM–nucleosome complex on the basis of the X-ray structure of the LANA CBM complexed with the nucleosome core particle (PDB_1ZLA). Overview of the IL-33- and LANA CBM bound to the histone H2A–H2B dimer within the nucleosome core particle (**C**,**D**): Connolly surface mode for H2A (yellow), H2B (white) and the IL-33 (orange) or LANA CBM (turquoise); white cylinders for the second histone tetramer; full-atom mode for the core particle DNA (blue). The arrows point towards the N and C termini of each hairpin. (**E**,**F**) Top view of the IL-33 and LANA CBM fitted into the acidic pocket formed by H2A and H2B at the nucleosomal surface. (**G**,**H**) Details of the interaction between the IL-33 CBM or LANA CBM and the H2A–H2B acidic patch. Hydrogen bonds are shown as green-coded dashed lines. CBM, chromatin-binding motif; IL-33, interleukin-33; KSHV, Kaposi sarcoma herpesvirus; PDB, Protein Data Bank.

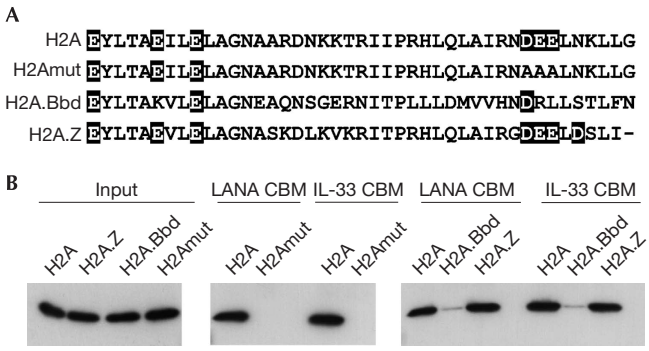

Fig 4 | The IL-33 CBM interacts with the acidic pocket of the H2A–H2B dimer. (**A**) Amino-acid sequences of the acidic patch regions of human H2A, H2A mutant (H2Amut), and histone variants H2A.Bbd and H2A.Z. Acidic residues that are part of the H2A–H2B acidic pocket are shown in black boxes. (**B**) The IL-33 CBM does not bind to an H2A mutant bearing a triple mutation within the acidic patch and binds only very poorly to H2A.Bbd. LANA-CBM or IL-33-CBM–GFP fusion proteins were immunoprecipitated from HEK293T cells expressing Flag-H2A, Flag-H2Amut, Flag-H2A.Bbd or Flag-H2A.Z, and precipitated proteins were detected by immunoblot with Flag antibodies. CBM, chromatin-binding motif; GFP, green fluorescent protein; IL-33, interleukin-33.

control peptide (IL-33 cont) had no effect compared with control arrays (Mg_{50} ~3 mM; Fig 5C; supplementary Fig S4 online). Interestingly, the LANA CBM had the same effect in this assay as that of the IL-33 CBM, as recently reported (Chodaparambil *et al*, 2007). On the basis of these findings, we conclude that the IL-33 CBM regulates chromatin compaction by promoting oligomerization of nucleosomal arrays into the higher order structures of chromatin.

The LANA CBM has been shown to alter nuclear architecture and to influence chromatin condensation *in vivo* (Chodaparambil *et al*, 2007). Interestingly, when tested in the same cellular assay in U2OS cells, the IL-33 CBM provoked similar changes in chromatin organization in the nucleus (Fig 5D,E). More than 85% of the cells expressing high levels of GFP-IL-$33_{40–58}$ or GFP-$LANA_{5–22}$ showed nuclei with large regions of Hoechst exclusion. These cellular effects were not observed with the IL-33 CBM mutant GFP-IL-$33_{40–58}$(47AAA49) or with GFP alone. Therefore, similar to the LANA CBM, the IL-33 CBM alters nuclear architecture *in vivo*.

Previously, we have shown that IL-33 has transcriptional repressor activity when tethered to a promoter by a heterologous Gal4-DNA-binding domain (Carriere *et al*, 2007). Interestingly, we found that a single-point or triple mutation within the IL-33 CBM greatly reduced the transcriptional repressor activity of IL-33 (Fig 5F,G). These results indicate that binding to the acidic pocket of H2A–H2B is important for the transcriptional regulatory function of IL-33.

Finally, we examined the possibility that IL-33 might share transcriptional targets with LANA. However, we found no change in the expression of principal LANA target genes after either IL-33 knockdown or overexpression (supplementary Fig S5 online), suggesting that IL-33 mimics certain functions of LANA (targeting to chromatin through H2A–H2B acidic patch and modulation of chromatin structure) but not all.

DISCUSSION

Here, we have identified the mechanism responsible for IL-33 attachment to chromatin. We showed that IL-33, similar to KSHV LANA, uses a short motif containing the crucial hexapeptide MXLRSG for docking into the acidic pocket formed by the H2A–H2B dimer at the surface of the nucleosome. Although many factors have been shown to recognize the flexible histone tails, IL-33 is the first cellular factor shown to bind to the acidic pocket of nucleosome, a region that is important for chromatin compaction (Chodaparambil *et al*, 2007; Zhou *et al*, 2007). This suggested that docking of IL-33 into the acidic pocket of H2A–H2B might modulate chromatin structure. Accordingly, we found that the IL-33 CBM alters nuclear architecture *in vivo* and regulates chromatin compaction *in vitro* by enhancing the self-association and oligomerization of the nucleosome into higher order chromatin structures. Similar effects on chromatin structure were recently reported for the LANA peptide (Chodaparambil *et al*, 2007). Interestingly, the acidic pocket of the nucleosome has been shown to couple chromatin compaction with transcriptional repression (Zhou *et al*, 2007), and this might explain our observation that a single-point or triple mutation within the IL-33 CBM that abrogates interaction with the H2A–H2B acidic patch greatly reduced the transcriptional repressor properties of IL-33.

Our data show that the IL-33 CBM is conserved in murine and canine IL-33 orthologues. By contrast, the motif is found in KSHV and RFHV (retroperitoneal fibromatosis herpesvirus) LANA but not in LANA equivalents from other primate and murine rhadinoviruses such as herpesvirus saimiri and MHV68 (murine herpesvirus 68). This suggests that the KSHV LANA CBM has been pirated from cellular IL-33. To the best of our knowledge, this is the first time a human DNA tumour virus has been shown to mimic a cellular factor for attachment to mitotic chromatin, an essential process for the maintenance of viral genomes in latently infected tumour cells. Although there are previous examples of viral mimicry of cellular cytokines (including mimicry of IL-6 and CC-chemokines by KSHV; Moore *et al*, 1996; Boshoff *et al*, 1997), the piracy by KSHV of the IL-33 CBM, rather than the IL-1-like domain, is unique. This surprising example of viral mimicry is likely to have been one of the crucial events during KSHV evolution for the establishment of latent viral infections in human cells. Further characterization of the IL-33/LANA CBM might lead to the identification of new therapeutic agents for the treatment of KSHV-associated diseases.

METHODS

Plasmid constructions. IL-33 deletion mutants were amplified by PCR using the human IL-33/NF-HEV cDNA (NM_033439) as a template. IL-33-CBM and LANA-CBM constructs, and IL-33-CBM alanine scanning point mutants were generated by cloning linker oligonucleotides into the *Eco*RI and *Bam*HI sites of plasmid pEGFP.C2 (Clontech, Mountain View, CA, USA).

Fluorescence microscopy. For live cell imaging, HEK293T cells were transfected with GFP fusion protein expression vectors using the calcium phosphate procedure. At 2 days after transfection, DNA was counterstained with Hoechst 33342, and living cells were observed by fluorescence microscopy on an inverted fluorescence microscope equipped with a digital camera (Eclipse TE300; Nikon, Tokyo, Japan).

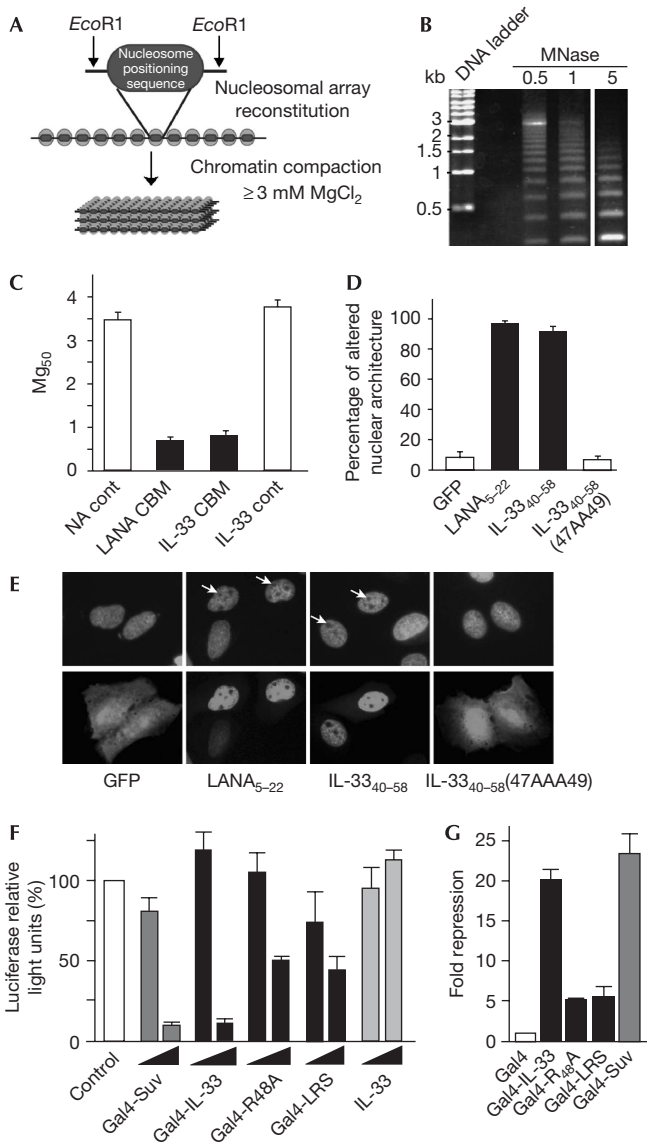

Fig 5 | The IL-33 CBM facilitates chromatin compaction, alters nuclear architecture and regulates the transcriptional repressor activity of IL-33. (**A–C**) The IL-33 CBM enhances oligomerization of nucleosomes into large condensed chromatin structures. Aliquots of reconstituted 208-12 nucleosomal arrays (**A**) were digested with MNase (0.5, 1 or 5 min at 37 °C) and run on a 1% agarose gel together with DNA size markers to show the nucleosome ladders (**B**). The Mg_{50} values were determined for control arrays (NA cont) or arrays incubated with the LANA CBM (aa 4–16), IL-33 CBM (aa 43–55) or IL-33 control (aa 1–15) peptides (molar ratio 1:20) (**C**). Each Mg_{50} value represents the average and standard deviation of three independent experiments. (**D**,**E**) The IL-33 CBM modifies chromatin organization within the cell nucleus. The percentage of U2OS cells expressing high levels of GFP fusion proteins, with altered nuclear architecture, was evaluated by counterstaining DNA with Hoechst 33342. Results in (**D**) are shown as means and standard deviations of three independent transfection experiments. (**E**) Large regions of Hoechst exclusion are indicated by white arrows. (**F**,**G**) A single-point mutation within the IL-33 CBM greatly reduces the transcriptional repressor activity of IL-33. Gal4-DB fusions (10 or 500 ng) were tested for their ability to repress a pLex-Gal4 luciferase reporter. In (**F**), normalized luciferase activities are shown as means and standard deviations of three independent transfection experiments. (**G**) Fold repression was calculated by dividing the normalized luciferase activity of cells expressing Gal4-DB alone (Gal4) by the activity of the Gal4-DB fusion proteins (500 ng expression vector). CBM, chromatin-binding motif; Gal4-DB, Gal4-DNA-binding domain; GFP, green fluorescent protein; IL-33, interleukin-33; MNase, micrococcal nuclease.

GST pull-down and immunoprecipitation assays. Details on the *in vitro* (GST pull-down) and *in vivo* (co-immunoprecipitation) binding assays used to analyse association of the IL-33 CBM with H2A–H2B are described in the supplementary information online.

Nucleosomal array reconstitution, analysis of oligomerization and transcriptional reporter assay. Nucleosomal arrays were prepared using the 208-12 DNA template, and their ability to oligomerize at various $MgCl_2$ concentrations was determined using a differential centrifugation assay. The effects of the IL-33 CBM mutations on the transcriptional repressor activity of Gal-4-IL-33 were determined using a Gal4-luciferase reporter. The details of the protocols are available in the supplementary information online.

Molecular modelling. Modelling was performed using the *Accelrys* modules InsightII, Homology, Discover, Docking and Delphi, run on a Silicon Graphics Fuel workstation. The structure of the IL-33 CBM and IL-33 CBM–nucleosome complex was modelled on the basis of the structure of LANA CBM in complex with the nucleosome (Protein Data Bank entry code 1ZLA).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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