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The novel endonuclease Ankle1 requires its LEM and GIY-YIG motifs for DNA cleavage in vivo

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Summary

The Lamina-associated polypeptide, Emerin, MAN1 - (LEM) domain defines a group of nuclear proteins, which bind chromatin through interaction of the LEM motif with the conserved DNA cross-linking protein, Barrier-to-Auto-Integration factor (BAF). Here, we describe a novel LEM protein, annotated in databases as “Ankyrin and LEM domain containing protein 1” (*ANKLE1*). We show that Ankle1 is conserved in metazoans and contains a unique C-terminal GIY-YIG motif that confers endonuclease activity in vitro and in vivo. In mammals, Ankle1 is predominantly expressed in hematopoietic tissues. While most characterized LEM proteins are components of the inner nuclear membrane, ectopic Ankle1 shuttles between cytoplasm and nucleus, and Ankle1 enriched in the nucleoplasm induces DNA cleavage and DNA damage response. This activity requires both the catalytic C-terminal GIY-YIG domain and the LEM motif, which binds chromatin via BAF. Hence, Ankle1 represents a novel LEM-protein with a GIY-YIG type endonuclease activity in higher eukaryotes.

Keywords

chromatin; DNA damage; GIY-YIG endonuclease; LEM-domain; nuclear envelope

Introduction

Polypeptides containing a Lamina-associated polypeptide, Emerin, MAN1 (LEM) domain (Lin et al., 2000) comprise a protein family with important and essential functions in nuclear architecture, mitosis, cell signaling and gene expression (Gruenbaum et al., 2005; Schirmer and Foisner, 2007; Wagner and Krohne, 2007; Anderson and Hetzer, 2008). The LEM domain is a structural motif of 45 amino acids that folds as two α -helices (Laguri et al., 2001) and binds to Barrier-to-Autointegration Factor (BAF) (Furukawa, 1999; Cai et al.,

2001; Shumaker et al., 2001), an essential DNA cross-linking protein in metazoans (Umland et al., 2000; Zheng et al., 2000; Margalit et al., 2007).

The LEM protein family in mammals comprises the well characterized proteins Lamina-associated polypeptide 2 (LAP2), emerin and MAN1 (Schirmer and Foisner, 2007; Wagner and Krohne, 2007), the MAN1-related protein LEM2 (Brachner et al., 2005; Chen et al., 2006; Ulbert et al., 2006; Huber et al., 2009) and a partially characterized testis-specific protein, LEM domain-containing 1 (LEMD1, also LEM5) (Lee and Wilson, 2004; Yuki et al., 2004). Two of these proteins are conserved in *Caenorhabditis elegans*: emerin, and LEM2 (Lee et al., 2000; Gruenbaum et al., 2002; Liu et al., 2003). Most of the characterized LEM proteins are transmembrane proteins of the inner nuclear membrane where they interact with the nuclear lamina (Schirmer and Foisner, 2007; Wagner and Krohne, 2007). LEM protein - BAF complexes are involved in post-mitotic nuclear assembly and in chromatin organization in *C. elegans* (Liu et al., 2003; Margalit et al., 2005) and in mammalian cells (Haraguchi et al., 2001; Dechat et al., 2004; Shimi et al., 2004). In addition, several LEM domain proteins bind to and regulate transcription factors and signaling molecules such as β -catenin (Markiewicz et al., 2006), Smads (Lin et al., 2005; Pan et al., 2005; Jiang et al., 2008), germ-cell-less (gcl) (Nili et al., 2001; Holaska et al., 2003) and retinoblastoma protein (Markiewicz et al., 2002; Dorner et al., 2006). Mutations in genes encoding for emerin, MAN1 and LAP2 α were linked to a number of human pathologies (Vlcek and Foisner, 2007; Wagner and Krohne, 2007; Worman and Bonne, 2007; Chi et al., 2009), reflecting their multiple and diverse functions.

In silico analyses of mammalian genomes identified novel genes predicted to be members of the LEM family, originally termed LEM3 and LEM4 (Lee et al., 2000; Lee and Wilson, 2004) and annotated in databases as “Ankyrin and LEM domain containing protein” (ANKLE) 1 and 2, respectively. In this study, we report the first biochemical and cell biological characterization of Ankle1 (formerly LEM3, ANKRD41 or FLJ39369), an evolutionary conserved non-membrane bound LEM protein that shuttles between nucleus and cytoplasm and is predominantly expressed in hematopoietic tissues and cells. Intriguingly, the Ankle1 C-terminus contains an enzymatically active GIY-YIG endonuclease domain, previously described as the characteristic feature of a subgroup of the homing endonuclease superfamily. Homing endonucleases are encoded within “selfish” group I introns or inteins. They catalyze their lateral transfer and integration into intron-less homologous alleles in the host genome (Stoddard, 2005; Edgell, 2009; Stoddard, 2011). A large number of these enzymes were identified in all three biological kingdoms - archae, bacteria and eukaryotes (Belfort and Roberts, 1997; Sokolowska et al., 2011). The GIY-YIG homing endonucleases that have been characterized in detail include I-TevI in T4 phage (Bell-Pedersen et al., 1991; Van Roey et al., 2002) and I-SceI in the yeast mitochondrial genome (Perrin et al., 1993; Moure et al., 2003). The GIY-YIG motif has also been found in enzymes other than homing endonucleases, such as UvrC, a protein involved in nucleotide excision repair in bacteria (Yoakum and Grossman, 1981; Truglio et al., 2005). Only few genes in higher eukaryotes are known to contain a GIY-YIG motif: the recently characterized human endonuclease SLX1 which is involved in structure-specific DNA repair (Fekairi et al., 2009; Svendsen et al., 2009), the transpositionally active Penelope-like

elements in *Drosophila virilis* (Pyatkov et al., 2004; Evgen'ev and Arkhipova, 2005; Schostak et al., 2008) and the yet uncharacterized human gene encoding Ankle1 (Dunin-Horkawicz et al., 2006)

Results

Ankle1 is highly conserved in metazoans

ANKLE1 (Ankle and LEM domain containing 1) – also termed *ANKRD41* (Ankyrin repeat domain protein 41), *FLJ39369* or *LEM3* – was annotated in databases based on different computational screens for genes that contain either a LEM domain, Ankyrin repeats, or a GIY-YIG motif (Lee et al., 2000; Lee and Wilson, 2004; Dunin-Horkawicz et al., 2006). Assembly and alignment of Ankle1 sequences from numerous species obtained from the ENSEMBL and NCBI databases (Suppl. Fig. 1A) revealed a conserved domain organization of the protein: two to four (depending on the species) N-terminal Ankyrin repeats (Fig. 1, green boxes), a central putative LEM domain (red box), and a predicted C-terminal GIY-YIG motif (black hatched box) within a highly conserved C-terminal sequence stretch (violet box) termed “PB00913” in the Pfam database (www.pfam.sanger.ac.uk). This latter C-terminal domain including the GIY-YIG motif showed the highest homologies ranging from ~70% conserved residues among vertebrates to ~40% conservation between nematodes and mammals (Fig. 1 and Suppl. Fig. 1B). Unlike the domain architecture, the predicted molecular weights of Ankle1 vary considerably ranging from 58 kDa in mouse to 102 kDa in zebra fish (Fig. 1). Human Ankle1 (hAnkle1) has 615 residues and a predicted molecular weight of 65 kDa. The variability in size is particularly evident in the region between the Ankyrin repeats and the LEM-domain, indicating that the distance between these domains may be less important for its activity. In contrast, the spacing between the LEM-domain and the GIY-YIG motif was preserved and may thus be crucial for Ankle1's function(s).

Human Ankle1 is expressed in a tissue-restricted manner

We analyzed the expression levels of Ankle1 in human tissues and cell lines by semiquantitative RT-PCR using primers for the 3' region of Ankle1 cDNA. Analysis of mRNA samples from a collection of adult human tissues revealed predominant Ankle1 mRNA expression in bone marrow. Expression was also high in fetal liver, fetal spleen and fetal thymus, the primary organs of hematopoiesis during intrauterine development (Fig. 2A), suggesting that Ankle1 may be involved in hematopoiesis-specific processes. In addition, we observed expression in a panel of lymphoma- and leukemia-derived tumor cell lines (ARH77, CCRF, DAUDI, K562, RAJI, RAMOS, REH), while sarcoma- and carcinoma-derived lines (HACAT, HeLa, LSWW, MCF-7, SW-480, T-98-G, U2OS) expressed low or undetectable levels of Ankle1 mRNA (Fig. 2A). RT-PCR analysis of human bone marrow mRNA samples using a further upstream forward primer (Fig. 2B) identified a minor, slightly smaller isoform of Ankle1, originating from alternative splicing within exon 5 (see also Q8NAG6-1 in Uniprot database). Interestingly, the splice variant, which we termed Ankle1b, lacks half (aa 375-400) of the putative LEM domain (Fig. 2B).

Next, we tested Ankle1 protein expression, using rabbit polyclonal antibodies raised against the N-terminal recombinant mouse Ankle1 fragment (aa 95-260, antiserum '136'). Although

the antibodies readily detected ectopically expressed Ankle1 in Western blots of HeLa cell lysates (Fig. 2C, hAnkle1-V5, mAnkle1-V5) as well as in immunofluorescence microscopy (see Fig. 4B), endogenous Ankle1 was not detectable in various human or mouse lymphoma cell lines which contain abundant Ankle1 mRNA levels (Fig. 2C). We obtained similar results with antibodies raised against a highly conserved peptide within the C-terminal region (aa 388-402) of mouse Ankle1 (data not shown). Thus, the antisera clearly detected ectopic Ankle1 protein in mammalian cells in both the unfolded and native state (Western blotting and immunofluorescence microscopy, respectively), but we could not detect endogenous Ankle1. Therefore, we assumed that endogenous Ankle1 protein levels may be strictly controlled and expressed at levels below the detection limit of our assays.

Ankle1 binds to BAF

A characteristic feature of LEM-proteins is their interaction with BAF via the LEM-motif. In order to test whether Ankle1 also binds BAF, we performed in vitro pull down assays using recombinant 6xhis-tagged BAF (BAF-his). Bacterial lysates containing BAF-his were mixed with E. coli cell lysates expressing recombinant full length Ankle1, an Ankle1 fragment, Ankle1 CT, which lacks the C-terminus including the predicted LEM domain, and, as a positive control, the N-terminal part of the LEM-protein emerin (EmerinTM), which was shown to bind BAF (Lee et al., 2001). BAF-his was precipitated using magnetic Ni particles and supernatant and pellet fractions were analyzed by Western blotting. BAF-his efficiently co-precipitated full-length Ankle1 and emerin, while Ankle1 CT remained in the unbound fraction (Fig. 3A), indicating that the C-terminal half of Ankle1 binds BAF.

To test whether the LEM domain mediates Ankle1-BAF interaction we performed pull down assays with bacterially expressed full length 6xhis-V5-tagged Ankle1 (Ankle1-his-V5) and the corresponding Ankle1 splice variant lacking a part of its LEM domain (Ankle1b-his-V5). Precipitation of Ankle1-his-V5 with Ni beads brought down a significant fraction of recombinant, untagged BAF from bacterial cell lysates (Fig. 3B). Predominantly dimeric BAF was detected in the Ankle1 pellet fraction, while monomeric and oligomeric BAF mainly remained in the supernatant fraction. Similar results were obtained in the positive control using the LEM-protein LAP2 α , which efficiently pelleted BAF dimers. Ankle1b-his-V5, in contrast, lacking a functional LEM domain precipitated only trace amounts of BAF, and very little dimeric BAF was detected in both the supernatant and pellet fractions. Overall, these findings show that Ankle1 interacts directly with BAF via its LEM-domain, and indicate that binding of BAF to the LEM domain may favor BAF dimerization.

Ankle1 shuttles between cytoplasm and nucleus

Since antisera failed to detect endogenous Ankle1 protein (see Fig. 2C) we expressed GFP-tagged human Ankle1 in a lymphoma and a carcinoma-derived cell line, expressing or lacking endogenous Ankle1 mRNA, respectively (see Fig. 1A). In both, lymphoma-derived RAMOS cells and in HeLa cells we observed predominant cytoplasmic localization of GFP-Ankle1 by fluorescence microscopy (Fig. 4A). Identical results were obtained using a C-terminal GFP tag (data not shown) or a small tag, such as V5 (Fig. 4B). Since *in silico* analyses of Ankle1 primary sequence predicted potential nuclear export and nuclear localization signals (NES and NLS, Fig. 4A, scheme), we hypothesized that Ankle1 shuttles

between nucleus and cytoplasm. To test this hypothesis, we blocked Crm-dependent nuclear export using the drug Leptomycin B (Kudo et al., 1998). After one hour Leptomycin B treatment the majority of tagged Ankle1 accumulated in the nucleus in both HeLa and RAMOS cells (Fig. 4). Thus, Ankle1 is actively transported in and out of the nucleus and shows a predominant cytoplasmic localization at steady state.

Ankle1 has nuclease activity eliciting DNA damage signaling in the nucleus

Given that Ankle1 contains a putative GIY-YIG type endonuclease domain, we performed a series of in vitro and in vivo assays to test for endonuclease activity. Recombinant Ankle1 purified from HEK cells cleaved supercoiled plasmid DNA into relaxed circular (nicked) and linearized DNA molecules (Fig. 5A), suggesting that Ankle1 possesses in vitro nuclease activity. Several conserved residues in the GIY-YIG motif (Y453, G488, E551 in human Ankle1, see Suppl. Fig. 3A) were previously identified to be crucial for the formation of the catalytic surface of the nuclease (Van Roey et al., 2002; Truglio et al., 2005; Lagerback and Carlson, 2008). Point mutations of either of these residues abrogated Ankle1's in vitro DNA cleavage activity (Fig. 5A). Thus, we concluded that Ankle1 possesses intrinsic endonuclease activity mediated by its C-terminal canonical GIY-YIG motif.

In order to test whether Ankle1 also cleaves DNA in cells in vivo, we performed COMET (i.e. single cell gel electrophoresis) assays, an established method to detect genomic DNA fragmentation in vivo at single cell level. Expression of GFP-Ankle1 in HeLa cells for 24 hours caused little DNA damage similar to the negative control, cells expressing GFP only (Fig. 5B). In contrast, treatment of cells with Leptomycin, causing GFP-Ankle1 accumulation in the nucleus, induced DNA cleavage to a similar extent as cells treated with the topoisomerase II inhibitor Etoposide, a drug known to induce massive DNA double strand breaks (Sullivan et al., 1986). Leptomycin treatment of cells expressing GFP or a truncated Ankle1 lacking the C-terminal GIY-YIG motif did not induce DNA breaks (Fig. 5B).

In order to find out whether Ankle1-mediated DNA cleavage activates DNA damage signaling, we analyzed the expression and localization of components of the DNA damage response pathway in GFP-Ankle1-expressing cells before and after Leptomycin treatment. In more than 90% of cells (n>100) nuclear accumulation of GFP-Ankle1 following Leptomycin treatment caused relocalization of 53BP1 to intranuclear foci (Schultz et al., 2000), phosphorylation of Histone 2A.X (γ H2A.X) and activation of the downstream effector kinase Chk2 (pChk2) (Fig. 6A), similar as in Etoposide-treated cells (Fig. 6B). Identical results were obtained in cells expressing Ankle1-V5 (Suppl. Fig. 2). Leptomycin treatment of GFP expressing cells did not induce these markers (Fig. 6C), indicating that accumulation of Ankle1 in the nucleus, but not Leptomycin treatment and/or transfection, activated the DNA damage signaling pathway. Nuclear accumulation of a C-terminally truncated Ankle1 protein missing the GIY-YIG motif (GFP-Ankle1 CT) did not activate the DNA damage response pathway (Fig. 6D). These observations indicate that accumulation of Ankle1 in the nucleus causes DNA cleavage thereby activating the DNA damage response pathway.

The LEM domain of Ankle1 is required for DNA cleavage in vivo

Since defined DNA binding/targeting motifs described in homing endonucleases, such as zinc-finger domains, helix-turn-helix motifs and DNA minor groove-binding α -helical structures (Derbyshire et al., 1997; Kowalski et al., 1999; Liu et al., 2006; Carter et al., 2007), are not known in Ankle1, we hypothesized that Ankle1's LEM motif might be involved in correct targeting of the protein. In order to test whether the LEM domain is involved in Ankle1-mediated DNA cleavage we measured γ H2A.X staining intensity in mixed cultures of cells expressing either full length Ankle1 or the LEM-domain deficient variant Ankle1b. To correlate the extent of DNA damage (γ H2A.X staining intensity) with Ankle1 expression levels in individual cells we also measured the intensity of the V5 staining. Unlike Ankle1b, full-length Ankle1 was expressed from a vector also expressing a GFP-marker, allowing us to discriminate between cells expressing full-length Ankle1 or Ankle1b in the mixed culture (Fig. 7). Immunofluorescence analyses of mixed cell populations were performed before and after Leptomycin treatment (Fig. 7). The fluorescence intensity of the V5-specific signal in the nucleus of Leptomycin treated cells was measured in GFP positive cells (i.e. cells expressing full length Ankle1-V5) and GFP-negative cells (expressing Ankle1b-V5) and plotted over the intensity of γ H2A.X-specific fluorescence (Fig. 7, graph). DNA damage (measured via γ H2A.X intensity) increased with increasing levels of full length Ankle1, while only low levels of γ H2A.X were detectable in Ankle1b-expressing cells independent of the expression level (Fig. 7, graph). Thus, the presence of a functional LEM motif is required for the Ankle1-mediated activation of the DNA response pathway, suggesting that this domain might be involved in targeting Ankle1 to chromatin in vivo.

Discussion

In this study we performed biochemical and cell biological analyses of a novel human LEM protein, annotated as Ankle1 in databases. The LEM domain of Ankle1 interacts with BAF, confirming that Ankle1 is a *bona fide* LEM protein. Apart from its interaction with BAF, Ankle1 also showed unexpected novel and unique properties among the characterized LEM protein family members: (1) Ankle1 expression is largely restricted to hematopoietic tissues, while most other LEM proteins in mammals are widely expressed (Theodor et al., 1997; Ellis et al., 1998; Lin et al., 2000; Brachner et al., 2005). Only the LEM-domain-containing 1 gene (LEMD1, also LEM5) (Lee and Wilson, 2004) was reported to be expressed in a tissue-restricted manner (testis) so far (Yuki et al., 2004). (2) While all analyzed LEM proteins contain a LEM motif close to their nucleoplasmic N-terminus (Wagner and Krohne, 2007), Ankle1's LEM domain is located in the middle of the polypeptide. (3) Ankle1 lacks transmembrane domains and shuttles in and out of the nucleus. Non-membrane bound localization among characterized LEM proteins has only been reported for two isoforms of the LAP2 gene, LAP2 α (Dechat et al., 1998; Dechat et al., 2004) and LAP2 ξ (Shaklai et al., 2008). All other studied LEM proteins contain one or two transmembrane domains and localize to the inner nuclear membrane. (4) The most intriguing and exceptional feature of Ankle1, however, is an enzymatically active GIY-YIG motif which was previously described in homing endonucleases and a few other proteins, including the bacterial nucleotide excision repair protein UvrC and the DNA structure specific repair enzyme SLX1

in humans (Perrin et al., 1993; Stoddard, 2005; Truglio et al., 2005; Dunin-Horkawicz et al., 2006; Edgell, 2009; Fekairi et al., 2009; Svendsen et al., 2009). The comparison of 3D models generated from the canonical GIY-YIG homing endonuclease I-TevI and from human, *C. elegans* and hydra Ankle1 revealed striking similarities (Suppl. Fig. 3), indicating a strong structural conservation between I-TevI and Ankle1 proteins despite low primary sequence identity. Thus, Ankle1 represents a novel member of the LEM protein family and is, along with SLX1, the only active GIY-YIG type endonuclease described in higher eukaryotes so far.

Besides the catalytically active GIY-YIG domain, homing endonucleases also contain DNA-binding motifs, such as zinc-finger, α -helix and helix-turn-helix domains that position the catalytic core at the DNA. It is tempting to speculate that Ankle1 is targeted to DNA through the interaction between its LEM domain and BAF. In addition a DNA-binding α -helix might be present within Ankle1's C-terminus according to in silico modeling (Suppl. Fig. 3B), but so far we have no experimental evidence for additional DNA binding motifs in Ankle1. Our hypothesis that a Ankle1-BAF complex is involved in DNA cleavage is supported by the observation that the LEM-deficient splice isoform Ankle1b, did not induce DNA damage response.

What is the physiological role of Ankle1?

When forced to the nucleus by transient inhibition of nuclear export, Ankle1 caused DNA cleavage and induced DNA damage response. In all organisms, endonucleases are instrumental in diverse DNA repair pathways (Nishino and Morikawa, 2002; Marti and Fleck, 2004). Endonucleases are also essential for the processing of double strand breaks introduced during meiotic recombination (Borde, 2007; Mimitou and Symington, 2009) and for somatic genomic rearrangements during lymphocyte development in higher eukaryotes (Lieber et al., 2004; Rooney et al., 2004; Rivera-Munoz et al., 2007). Taking into account the predominant expression of human Ankle1 in hematopoietic tissues and in lymphoma-derived cell lines we postulate a role of Ankle1 in genomic rearrangement or associated DNA repair processes during the development of lymphocytes. In addition, Ankle1 may have a more general function in DNA repair pathways, which could explain its conservation in lower metazoan species. Consistent with this hypothesis, Dittrich et al. demonstrated that loss of function of the *C. elegans* ortholog of mammalian Ankle1, *lem-3*, causes a radiation hypersensitivity (Rad) phenotype (personal communication). Worms carrying an L to F point mutation in LEM-3 at position 659 (indicated in Suppl. Fig. 3C) are hypersensitive to DNA damage caused by chemicals and irradiation. This leucine residue is invariably conserved in all identified Ankle1 proteins at the respective positions. Interestingly, this site resides within a predicted C-terminal α -helical structure (Suppl. Fig. 3B,C) and could thus be important for Ankle1-DNA coordination. Interestingly, human Ankle1 mutated at the respective residue (L590F) did not trigger the DNA damage response pathway upon accumulation in the nucleus (Suppl. Fig. 4).

Altogether, Ankle1 is an evolutionary conserved GIY-YIG type endonuclease that is predicted to have functions in DNA damage repair pathways in lower metazoan organisms (Dittrich et al., personal communication), whereas it seems to have acquired more

specialized functions during evolution in mammals, in particular in the human lymphocyte development.

Material and Methods

Cell culture and reagents

HeLa, HEK, SW-480, T-98-G and U2-OS cells were routinely cultivated in DMEM, ARH-77, CCRF, DAUDI, K-562, VL-6, RAJI, RAMOS and REH cells in RPMI, both supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, USA), 100 U/mL Penicillin/Streptomycin and 2 mM L-Glutamine at 37°C in a humidified atmosphere containing 8,5% CO₂ and 5% CO₂ respectively. Transient transfections were performed using Nanofectin according to the manufacturer's instructions (PAA, Pasching, Austria). Crm-dependent nuclear export was inhibited with 10 ng/ml Leptomycin B (Enzo Life Sciences, Lausen, Switzerland) in complete growth medium for 1 to 3 hours.

PCR analyses

Poly(A⁺) RNA purified from cell lines using the mRNA isolation kit (Roche, Mannheim, Germany), or purchased human tissue total RNA samples from Agilent Technologies (Santa Clara, USA) and Biochain (Hayward, USA) were reverse transcribed using the first-strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Aliquots of the resulting products were used as templates for PCR-amplification of Ankle1, Actin and GAPDH using the Go-Taq PCR Master Mix (Promega, Germany) and the following primer pairs: Ankle1-forw(1) 5'-TGCCTGTGGGAGCACCAGACATC-3', Ankle1-forw(2) 5'-GCCCTGCGGACGGGCTGTATTC-3', Ankle1-rev 5'-GCTCGCCTTCAGCCAGGAAGAC-3', Actin-forw 5'-ATCTGGCACACACCTTCTAC-3', Actin-rev 5'-CAGCCAGGTCCAGACGCAGG-3', GAPDH-forw 5'-CATCACCATCTTCCAGGAGCGA-3' and GAPDH-rev 5'-CCTGCTTCACCACCTTCTTGAT-3'.

Antibodies

Mouse anti-V5 antibodies were purchased from Invitrogen, rabbit anti-Actin from Sigma Aldrich, rabbit anti-53BP1 and mouse anti-BAF both from Novus Biologicals (Littleton, USA), and rabbit anti-phosphorylated Chk2 (Thr68) from Cell Signaling Technology (Boston, USA). The MANEM5 anti-Emerin antibody was a kind gift from Glenn Morris (Manilal et al., 1996). Polyclonal rabbit anti-murine Ankle1 antibodies were raised against an N-terminal fragment encompassing aa 95-260, following the Austrian and European regulations on animal experimentation. Antibodies against Ankle1 were affinity purified from serum '136' using a purified recombinant human Ankle1 fragment corresponding to mouse Ankle1 aa 95-260. His-tagged proteins were detected using the India His probe-HRP (Pierce, Rockford, USA).

Plasmids and cloning strategy

Human Ankle1 and the splice variant Ankle1b were amplified from bone marrow cDNA using the Platinum Pfx PCR Kit (Invitrogen, Carlsbad, USA). Oligos used were (all in 5'-3' sequence direction): Ankle1-CACC-forw

CACCGCTAGCATGTGCTCGGAGGCCCGCCTGG and Ankle1-woSTOP-rev GTATCTAGAGCCCCGGGCCTGGATGTC or Ankle1-STOP-rev TCAGCCCCGGGCCTGGATG. PCR products were cloned into pENTR/D-TOPO or pET102-TOPO by topoisomerase based cloning (Invitrogen). GFP-Ankle1 fusion constructs were created by PCR-amplification using primers Ankle1-SalI-forw 5'-AGCGTCGACATGTGCTCGGAGGCCCGCCTGG-3' and Ankle1-STOP-rev. The PCR product was cut with SalI and ligated into SalI and SmaI sites of the pGFP-C1 vector (Clontech, Palo Alto, USA). The deletion construct hAnkle1 CT (aa 1-420) was constructed by cloning the PCR-generated fragment into pET102-TOPO and pGFP-C1 using the same cloning strategies as described above, using the respective forward primers and primer hAnkle1-1062-stop-rev 5'-CATTCTAGACCGGCTACAAGGGCCGACAG-3'. V5-tagged Ankle1 was generated by shuttling Ankle1 via LR-recombination reaction (Invitrogen) from pENTR plasmids into a Gateway®-compatible pTRACER plasmid (pTB) (Brachner et al., 2005) or pDEST-51 (Invitrogen). Quick-change site directed mutagenesis was performed according to the manufacturer's protocol (Stratagene, Santa Clara, USA) by amplification of pEntry-hAnkle1 with primers containing the desired mutations. Introduced point mutations were verified by sequencing and Ankle1 mutants were shuttled into pTB or pDEST-51. The HA-Strep-Ankle1 plasmid was generated by cloning the PCR-amplified human Ankle1 cDNA into the pENTR11 vector (Invitrogen) followed by Gateway-mediated shuttling into the pTO_HA_StrepIII_GW_FRT vector (obtained from Matthias Gstaiger, ETH, Switzerland). PCR generated human BAF cDNA was cloned via topoisomerase reaction into pET102-D-Topo for bacterial expression with a C-terminal His-tag or with a downstream stop codon into pEntry-D-Topo. The pEntry-hBAF-stop was then used for Gateway cloning into the bacterial expression vector pDEST42.

Preparation of cell lysates, gel electrophoresis and immunoblotting

Cells were harvested, washed in cold PBS and resuspended in cold high-salt RIPA buffer (25 mM Tris-HCl pH 7.4, 500 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, "Complete" protease inhibitor mix (Roche, Mannheim, Germany), 1 mM PMSF, 1 mM DTT). Following incubation on ice for 10 min, cell lysates were sonicated for 5 seconds and insoluble material was pelleted. Supernatants were mixed with Laemmli sample buffer, boiled for 3 minutes and resolved via polyacrylamid gel electrophoresis. Proteins were blotted onto nitrocellulose membranes and probed with antibodies. After incubation with HRP-conjugated secondary antibodies, the Supersignal West Pico chemiluminescent substrate and CL-XPosure films (Thermo Fisher Scientific, Rockford, IL, USA) were used to detect signals.

Pull down assay

Bacterial expression vectors containing hAnkle1a (pET102-hAnkle1a-stop or pET102-hAnkle1a-6xhis-V5), hAnkle1b (pET102-hAnkle1b-6xhis-V5), hAnkle1 CT (pET102-hAnkle1 CT-stop), hBAF (pET102-hBAF-6xhis or pDEST42-hBAF-stop), hLAP2α (pDEST42-hLAP2α-6xhis-V5) or EmerinTM (pET11c-EmerinTM, kindly provided by Kathy Wilson) were transformed into BL21-star (Invitrogen). After induction of recombinant protein expression with 1 mM IPTG for 3 hours at 37°C, bacteria were lysed in RIPA buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate,

0.1% SDS, “Complete” protease inhibitor mix, 1 mM PMSF, 1 mM DTT) and sonicated for 1 minute on ice. Insoluble material was pelleted and 20 μ L of soluble fractions containing hAnkle1-stop, hAnkle1 CT-stop or Emerin TM were mixed with 10 μ g of human BAF-6xhis and incubated for 1 hour at 37°C in 100 μ L pull down buffer (20 mM Tris pH7.4, 150 mM NaCl, 10 mM Imidazole, 0.5% NP40, 1 mM EDTA, protease inhibitor mix). BAF complexes were separated with magnetic Ni²⁺beads (Promega), washed 3 times with pull down buffer, eluted by incubation with HEPES elution buffer (100 mM HEPES pH7.4, 500 mM Imidazole, 1 mM DTT) for 10 minutes and analyzed by Western blotting. Pull down of untagged human BAF-stop was done accordingly: 20 μ L of lysate containing BAF-stop were mixed with 20 μ L of lysate of BL21 bacteria expressing hAnkle1a-6xhis-V5, hAnkle1b-6xhis-V5 or hLAP2 α -6xhis-V5) in 100 μ L pulldown buffer and incubated for 1 hour at 37°C. Complexes were precipitated with magnetic Ni particles, washed 3 times with pull down buffer and analyzed by Western Blotting.

Immunofluorescence

Cells were grown on poly-L-lysine-coated glass coverslips or seeded into ibidi-treat® microscopy slides for live cell imaging (see Fig. 4A) (Ibidi, Munich, Germany). Cells on coverslips were fixed in 4% paraformaldehyde / PBS for 10 minutes and permeabilized in PBS / 0.5% Triton X-100 for 5 minutes. Cells were blocked in PBS / 0.5% gelatine for 15 minutes, incubated with primary antibodies for 45 minutes, three times washed with PBS and re-probed with the appropriate secondary antibodies conjugated to either TexasRed or Cy-5 (Jackson Immuno Research, West-Grove, USA) for 45 minutes. After three washes with PBS, DNA was counterstained with 100 ng/mL DAPI (Sigma, Munich, Germany) for 5 minutes and samples were mounted in Mowiol (Fluka, Buchs, Switzerland). Images were taken using a confocal laser scanning microscope (LSM-Meta, Zeiss, Jena, Germany) using a Plan-Apochromat 63x oil immersion objective (NA=1.40). Live-cell imaging was performed on the same microscope with transfected cells seeded. Digital images were analyzed, adjusted for brightness and contrast and mounted using the LSM Image-Browser (Zeiss, Jena, Germany) and Adobe Illustrator (Adobe Systems Inc, San Jose, USA).

Computer-assisted analysis

Sequence alignments and database searches were performed by NCBI-BLAST (<http://www.ncbi.nlm.nih.gov/blast/>), GraphAlign (http://darwin.nmsu.edu/cgi-bin/graph_align.cgi) (Spalding and Lammers, 2004) and ClustalW2 (<http://www.ebi.ac.uk/clustalw2/>) (Thompson et al., 1994). Genomic analysis was done using the ENSEMBL Genome Browser (<http://www.ensembl.org/>) (Sanger Institute). Additional hAnkle1 orthologs were predicted using the GENSCAN software (<http://genes.mit.edu/genscan.html>) (Burge and Karlin, 1997). Protein motifs and pattern searches were performed using SMART (<http://smart.embl-heidelberg.de/>) (Schultz et al., 1998; Letunic et al., 2004) and PSORT-II (<http://psort.nibb.ac.jp/form2.html>). Transmembrane domains were calculated using the TMHMM 2.0 prediction software (<http://www.cbs.dtu.dk/services/TMHMM/>) (Krogh et al., 2001) and the DAS-TMfilter algorithm (Cserzo et al., 2004). Phylogenetic tree predictions were visualized using the PhyloDendron and PhyloDraw software (Choi et al., 2000).

3D-modelling was performed employing the CPHmodels 3.0 Server (Nielsen et al., 2002) and visualized using the Discovery Visualizer Studio 2.5 (Accelrys Software Inc., San Diego, USA).

COMET assay

Alkaline single cell gel electrophoresis (COMET assays) were performed as described previously (Ehrlich et al., 2008). In brief, GFP positive HeLa cells were sorted by FACS, pelleted and embedded in a thin layer of low-melting agarose (Invitrogen) on microscopy slides. In order to prevent DNA damage caused by UV light, lysis and all subsequent steps were conducted under red light. After solidification of the agarose, slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 1% Triton X, 10% dimethyl sulfoxide, pH 10.0) at 4°C for at least 1 hour. DNA was unwound by incubation of slides in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH 12.5) for 20 min followed by electrophoresis for 20 minutes at 25 V and 300 mA. Then, the slides were neutralized by 2 times 10 minutes incubations in neutralization buffer (0.4 M Trizma base, pH 7.5) at 4°C. Slides were air dried and stained with ethidiumbromide. Formation of 'comets' was measured in randomly chosen nuclei (n>150) for each condition in three independent samples using the Comet Assay IV software (Perceptive Instruments, Haverhill, UK), quantified and visualized in Graphpad Prism.

Endonuclease assays with HA-Strep Ankle1

HEK293 cells transiently transfected with a vector expressing HA-Strep-Ankle1 were grown on 15 cm dishes to 80 % confluency. 24 hours post transfection, the cells were pelleted by centrifugation, washed in PBS, and snap-frozen. The pellets were lysed (20 mM HEPES-KOH pH 8.0, 200 mM NaCl, 1% Triton, 1 mM EDTA, 10% Glycerol and protease inhibitors) and incubated on ice for 20 minutes. The lysate was centrifuged and incubated with Streptactin beads (IBA biotechnology, Göttingen, Germany) for 2 hours at 4°C on a rotating wheel (300 rpm). Subsequently, the beads were washed three times with 1 ml lysis buffer and twice with 1 ml wash buffer (20 mM HEPES-KOH pH 7.5, 2 mM MnCl₂, 0.05 mg/ml BSA and 50 mM KCl), each wash for 5 minutes at 4°C. HA-Strep Ankle1 was finally eluted in 100 µL of PBS containing 2.5 mM Desthiobiotin (IBA biotechnology) for 30 min at 4 °C on a shaker (600 rpm). Aliquots were immediately snap-frozen.

For the non-specific endonuclease assays, 80 ng of supercoiled pFastBac1 plasmid was incubated with wild-type Ankle1 or its variants in endonuclease buffer (20 mM HEPES-KOH pH 7.4, 2 mM MnCl₂, 45 mM KCl, 50 µg/mL BSA). After incubation at 37°C for 1 hour, the reaction was terminated by the addition of 0.1% SDS, 14 mM EDTA and 0.1 mg/mL Proteinase K and incubation at 55°C for 15 minutes. 10% glycerol was added and the samples were separated on a 0.8% ethidium bromide agarose gel for 45 min at 80 V.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Ankle1 is a novel LEM protein conserved among metazoans

Comparison of the predicted domain organization of Ankle1 orthologs from various species representing major metazoan clades: *Homo sapiens* and *Mus musculus* (mammals), *Danio rerio* (vertebrates), *Strongylocentrotus purpuratus* (echinodermata), *Drosophila melanogaster* (arthropods) and *Caenorhabditis elegans* (nematodes). Ankyrin repeats are shown as green boxes, the LEM motif as red boxes, the C-terminal PB000913 sequence as violet box. The predicted GIY-YIG motif is marked as black-hatched box. Number of amino acids (aa) of full length proteins, database accession numbers, and sequence homologies of various domains to human Ankle1 are shown on the right.

Fig. 2. Ankle1 is predominantly expressed in human hematopoietic tissues and cell lines
 (A) Representative agarose gels showing semiquantitative RT-PCR products amplified from human tissue and cell line samples. Actin and GAPDH were used as a control for equal loading. (B) Resolution of human Ankle1 PCR products from human bone marrow on polyacrylamid gels revealed a low abundant smaller Ankle1 isoform. Position of PCR primers are indicated (primer 1 was used for Ankle1 splice variant identification; primer 2 for expression analyses). The identity of the isoform was verified sequencing. (C) Representative Western blot detecting Ankle1 protein in total lysates of HeLa cells expressing ectopic human or mouse Ankle1-V5. Tubulin was probed as a loading control.

Fig. 3. Co-precipitates of BAF and Ankle1 in vitro

(A) BAF-6xHis was mixed with E.coli lysates containing recombinant Ankle1, Ankle1 CT or an N-terminal Emerin fragment Emerin TM and precipitated using Ni-beads. BAF-bound proteins were detected by Western blotting using antiserum '136' (Ankle1), India His-probe (BAF) and monoclonal antibody 'MANEM5' to emerlin (gift of Glenn Morris). S, supernatant fraction (10% of input); P, precipitated fraction (50% of precipitated proteins). Molecular weights are indicated in kDa. The scheme shows the Ankle1 constructs used in the assays. (B) Recombinant 6xHis-V5 tagged Ankle1, Ankle1b or LAP2 α were incubated with bacterial lysates containing untagged human BAF. His-tagged prey proteins were precipitated using Ni-beads and bound BAF was detected by Western blotting using antibodies to V5 tag and BAF. S, supernatant fraction (10% of input); P, precipitated fraction (50% of precipitated proteins). Note: BAF forms various homomeric complexes even under denaturing conditions (arrows indicating monomeric, dimeric and oligomeric states).

Fig. 4. Human Ankle1 shuttles between nucleus and cytoplasm

(A) GFP-tagged Ankle1 was expressed in HeLa or RAMOS cells and imaged by live cell microscopy (GFP) and differential interference contrast (DIC) microscopy before (-) and after (+) treatment with Leptomycin for one hour. Nuclear export and nuclear localization signals identified by *in silico* prediction software are indicated in the cartoon (NES, NLS). (B) HeLa cells were transfected with hAnkle1-V5, and processed for immunofluorescence microscopy before and after three hours of Leptomycin treatment. Bars, 10 μ m.

Fig. 5. Ankle1 cleaves DNA in vitro and in vivo

(A) Purified wildtype and mutated HASTrep-Ankle1 expressed in HEK cells was incubated with supercoiled pFastBac1 plasmid and DNA species were separated by agarose gel electrophoresis. Arrows indicate different intermediate steps of plasmid degradation (o.circ=open circle, lin=linear/nicked, sp.coil=supercoiled, deg=degraded). (B) Representative images of ethidiumbromide stained nuclei in a Comet assay. Bars indicate 10 μ m. Comets were measured using the Comet Assay IV software (Perceptive Instruments, Haverhill, UK), quantified and visualized in Graphpad Prism. Median values are indicated as horizontal lines. *** indicate statistically significant differences in median values between populations (p<0.001). Comet formation (i.e. DNA fragmentation) was evaluated in single cells of three independent cell populations (n>50 each).

Fig. 6. Accumulation of ectopic Ankle1 in the nucleus causes DNA damage

HeLa cells were transiently transfected with GFP-Ankle1 (A), GFP (C) or GFP-Ankle1 CT (D) or incubated with Etoposide (B), and treated with Leptomycin as indicated. All samples were processed in parallel for confocal immunofluorescence microscopy. Fixed cells were stained for 53BP1, γ H2A.X or phosphorylated Chk2 using specific antibodies (all red), DNA was stained with DAPI (blue), GFP and GFP-fusion proteins are shown in green. Bars indicate 10 μ m; arrows, cells shown in the insets. Representative images out of >3 independent experiments.

Fig. 7. The LEM domain in Ankle1 is required for inducing DNA damage response

HeLa cells were transfected separately with constructs expressing Ankle1-V5 or the LEM domain-deficient Ankle1b-V5 and seeded as a mixed culture onto cover slips after 24 hours. The plasmid expressing Ankle1-V5 also expresses a CMV-driven GFP-Blasticidin marker gene (GFP-Bsd), the plasmid encoding Ankle1b-V5 contains a Blasticidin gene without GFP fusion (Bsd) (plasmid maps). After 48 hours cells were either treated with Leptomycin or ethanol for three hours, fixed, stained for V5 (red) and γH2A.X (yellow) and imaged on a confocal fluorescence microscope. DNA was stained with DAPI (blue). GFP-Blasticidin is shown in green. Cells marked with asterisks lack GFP signal and are thus expressing Ankle1b; cells expressing Ankle1 (expressing GFP) are marked with arrows. Relative fluorescence intensities of V5 and γH2A.X signals within the nucleus of untransfected, GFP-positive and GFP-negative transfected and Leptomycin-treated cells (n>30 each) were measured and plotted using Graphpad Prism software. Representative results out of three independent experiments are shown.