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Chromatin-bound bacterial effector AnkA recruits HDAC1 and modifies host gene expression

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Abstract

Control of host epigenetics is becoming evident as a mechanism by which symbionts and pathogens survive. *Anaplasma phagocytophilum*, an obligate intracellular bacterium, downregulates multiple host defense genes where histone deacetylase 1 (HDAC1) binds and histone 3 is deacetylated at their promoters, including the NADPH oxidase component, *CYBB*. How HDAC1 is targeted to defense gene promoters is unknown. Ankyrin A (AnkA), an *A. phagocytophilum* T4SS effector, enters the granulocyte nucleus, binds stretches of AT-rich DNA and alters transcription of antimicrobial defense genes, including downregulation of *CYBB*. Here we found AnkA binds to a predicted matrix attachment region in the proximal *CYBB* promoter. Using the *CYBB* promoter as a model of *cis*-gene silencing, we interrogated the mechanism of AnkA-mediated *CYBB* repression. The N-terminus of AnkA was critical for nuclear localization, the central ANK repeats and C-terminus were important for DNA binding, and most promoter activity localized to the central ANK repeats. Furthermore, a direct interaction between AnkA and HDAC1 was detected at the *CYBB* promoter, and was critical for AnkA-mediated *CYBB* repression. This novel microbial manipulation of host chromatin and gene expression provides important evidence of the direct effects that prokaryotic nuclear effectors can exert over host transcription and function.

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Author Contributions

K.E.R.-B. designed and did most of the experiments and prepared the manuscript. J.C.G.-G. demonstrated AnkA binding to MARS. S.H.S. tested AnkA for intrinsic histone deacetylase activity. J.S.D. helped analyze data and interpret results and supervised all studies. All authors contributed to discussions and manuscript preparation.

Introduction

Global transcriptome analyses of mammalian cells in response to intracellular bacteria have led to the identification of major pathways affected during infection (Bryant *et al.*, 2004). Targeting of individual cellular pathways by single effectors to alter global gene expression is very inefficient for microbes with limited genetic and metabolic resources. Eukaryotic cellular function is governed largely by transcriptional programs that are well-coordinated and tightly regulated, as evident with the reprogramming of differentiated cells toward a pluripotent state with Oct3/4, Sox2, Klf4 and c-Myc (Takahashi *et al.*, 2006, Takahashi *et al.*, 2007). The overarching mechanism of control over transcriptional and functional programs in eukaryotes occurs via the epigenome, including chromatin and associated histone and non-histone elements that regulate transcription both in “*cis*” from individual genes and in “*trans*” by modulating and coordinating activity over entire chromosomal domains. Chromatin modification by bacterial nucleomodulins is becoming more evident as a mechanism of host manipulation to allow for pathogen survival (Bierne *et al.*, 2012).

The obligate intracellular bacterial pathogen *Anaplasma phagocytophilum* survives and propagates within neutrophils, abundant, short-lived, and aggressive phagocytes whose main function is microbial killing. With infection, *A. phagocytophilum* abrogates key neutrophil antimicrobial functions including oxidative burst, apoptosis, margination, emigration, and phagocytosis while activating proinflammatory responses including degranulation and cytokine/chemokine production (Akkoyunlu *et al.*, 2000, Kim *et al.*, 2000, Mott *et al.*, 2002, Scaife *et al.*, 2003, Choi *et al.*, 2004, Garyu *et al.*, 2005). Changes in these neutrophil functions are in part attributed to *A. phagocytophilum*-modulated host cell transcription (Borjesson *et al.*, 2005, de la Fuente *et al.*, 2005, Pedra *et al.*, 2005, Lee *et al.*, 2008). Upon *A. phagocytophilum* infection, downregulation of many host defense genes occurs along with histone deacetylase 1 (HDAC1) binding and histone H3 (H3) deacetylation at their promoters (Garcia-Garcia *et al.*, 2009a). HDAC1 activity and expression are increased with infection and are critical for *A. phagocytophilum* survival (Garcia-Garcia *et al.*, 2009a). Yet, how HDAC1 is targeted to defense gene promoters with infection is not clear.

One gene target of HDAC1 with *A. phagocytophilum* infection is *CYBB*, which encodes the gp91^{phox} component (NOX2) of the NADPH oxidase. Downregulation of *CYBB* results in decreased superoxide anion production by the NADPH oxidase, a key mechanism of pathogen killing for neutrophils. Ankyrin A (AnkA), an *A. phagocytophilum* type IV secretion system (T4SS) effector localizes to the host nucleus and binds to the *CYBB* promoter, along with other defense genes, to decrease transcription. The mechanism of AnkA repression has yet to be determined. We previously showed AnkA binds DNA in a sequence-independent manner, but to regions that are rich in AT nucleotides. AT-richness is a common feature of unstable regions that become base unpaired (BURs) under negative superhelical stress. BURs are found within matrix attachment regions (MARs), specialized DNA structures that serve as attachment sites for nuclear matrix proteins such as lamins, scaffold attachment factor-1, and the special AT binding protein-1 (SATB1) that organize nuclear chromatin for tissue-specific gene expression, chromatin accessibility, and long-range chromatin modifications (Wang *et al.*, 2010). Here we show that AnkA binds known and predicted MARs, and interrogate domains in AnkA that control nuclear localization and

DNA binding. Further, we show that Anka mimics eukaryotic MAR-binding proteins to anchor to DNA, recruits HDAC1 to its binding site in *cis* to the *CYBB* promoter, and decreases its transcription, which ultimately provides enhanced microbial survival.

Results

Nuclear localization of Anka is determined by ANK repeats 1–6

Decreased transcription from the *CYBB* promoter requires that Anka enter the nucleus. Suppression of gene transcription by Anka in *cis* at *CYBB* requires the coordinated events of nuclear translocation, DNA binding, and suppressor activity. To investigate this, we constructed Anka 5' and 3' deletion mutants designed to interrogate the roles of predicted eukaryotic domains and motifs, including multiple (8 to 15) ankyrin (ANK) repeats (33-residue motifs that can mediate protein-protein interactions), a putative mammalian nuclear localization sequence (NLS), and a putative high mobility group N protein chromosomal unfolding domain (HMGN-CHUD) (Figure 1A); none have established functions in Anka. When transfected into HEK293T cells, we found that Anka- N and Anka- N2 truncations localized exclusively to the cytoplasm (Figure 1B; Figure S1), whereas Anka- C and - NLS truncations localized to the nucleus. These results contradicted the prediction of an NLS in the C-terminus and suggested that the NLS was in the N-terminal region of Anka, probably within the first 4–6 ANK repeats. Eukaryotic ANK repeats can act as nuclear localization signals via RanGDP binding at 2 consecutive ANK domains where the 13th residues in each are hydrophobic (Lu *et al.*, 2014). An alignment of Anka protein sequences among 31 selected from *A. phagocytophilum* consistently identified 15 ANK repeats. Conserved hydrophobic residues at the 13th positions in ANK repeat regions 1, 2, 3, 4, 7, 8, 9, 11, 12, and 15 were identified in all 31 Anka proteins (Fig. S2), despite overall amino acid sequence identity that ranged from 68% to 100% for the 31 AnkAs examined.

Anka binds a predicted MAR to decrease *CYBB* transcription

Once within the granulocyte nucleus, Anka accumulates to surround heterochromatin and bind AT-rich DNA, a common characteristic of MARs (Caturegli *et al.*, 2000, Lin *et al.*, 2007, Garcia-Garcia *et al.*, 2009b). To determine if Anka can bind to MARs we used electrophoretic mobility shift assays (EMSAs) to investigate a known SATB1-binding BUR/MAR within the immunoglobulin heavy chain gene promoter. Anka and SATB1 bound the native MAR, but not when specific AT nucleotides were mutated to GC nucleotides (Figure 2A). Multiple bands appear in the Anka lane as a result of partial degradation during the purification process (Figure S3).

Since SATB1 also binds and represses transcription from the proximal *CYBB* promoter (Hawkins *et al.*, 2001, Fujii *et al.*, 2003), we used MARFinder MAR-Wiz 1.5 to identify MARs within the *CYBB* promoter region (Singh *et al.*, 1997). Chromatin immunoprecipitation (ChIP) identified Anka binding within a predicted MAR located at the proximal *CYBB* promoter (Figure 2B). Anka was previously shown to bind within the +12 to -48 and -109 to -138 regions of the *CYBB* promoter which overlap the predicted MAR (Figure 3A) and to downregulate transcription (Garcia-Garcia *et al.*, 2009b). To interrogate the importance of specific AT nucleotides for Anka binding at the *CYBB* promoter, probes

containing GC nucleotide substitutions for AT nucleotides were used for EMSA (Figure 3B and 3C). AT to GC nucleotide changes at four positions in the *CYBB* promoter decreased AnkA binding (indicated by arrows in Figure 3C). To confirm the functional importance of AnkA binding AT nucleotides at these positions, a *CYBB* promoter containing all 4 mutations (A-mut2, A-mut3, B-mut2, E-mut1) that decreased AnkA binding via EMSA cloned into the pBlueTOPO vector (*pmutCYBB-LacZ*) and co-transfected into HL-60 cells with the *pEYFP-ankA* vector. While AnkA significantly decreased expression from the wild-type *CYBB* promoter, expression from the mutated *CYBB* promoter did not differ from controls (Figure 3D).

The central ANK repeats of AnkA are critical for binding to the *CYBB* proximal promoter

To identify the DNA-binding domain(s) within AnkA, the C- and N-terminal truncation mutants were used (Figure 1A). Several recombinant mutants were impaired in binding to the *CYBB* +12 to -18 promoter probe (Figure 4A). Binding was most dramatically reduced to as low as 5% of wild type AnkA with AnkA- N2 (that lacked 10 ANK repeats); binding was also reduced with AnkA- NLS (ranging from 8 to 23% of AnkA) and AnkA- C (ranging from 23% to 88% of AnkA) (Figure 4A; Supplemental Figure 3). In contrast, binding with AnkA- N was modestly reduced (56%) compared to AnkA. A competitive inhibition EMSA assay using an equimolar quantity of unlabeled *CYBB* promoter probe was used to confirm specificity of AnkA-*CYBB* promoter interaction-induced gel shifts (Figure 5). Because AnkA- C localized to the nucleus and bound to DNA, we further examined it for classical DNA-binding motifs; none were identified. As all constructs possessed at least 5 ANK repeats, and binding was most impaired with deletion of ANK repeats 7–10 (AnkA- N2) and to a lesser extent with loss of the flanking ANK repeats (1–6 in AnkA- N and 11–15 in AnkA- C), it is likely that DNA binding is predominantly mediated by the central ANK repeats. The additional reduction in DNA binding observed with AnkA- NLS indicated that the carboxy-terminus of AnkA also contributes to DNA binding. Despite the moderate to significant reduction in DNA binding of AnkA- NLS, it easily translocated into the nucleus and modestly suppressed reporter activity (Figures 4A, 4B, 5). To determine whether the deleted carboxy end of AnkA- NLS possessed other classical DNA binding motifs that could account for these changes, we identified an AT hook-like domain at the C-terminus that was highly conserved among more than 93 AnkA sequences obtained through a NCBI BLAST search of its C-terminal 39 amino acid residues (Supplemental Figure 4). Compared with alignments of known AT hooks in other Alphaproteobacteria, there was strong similarity with non-palindromic type II AT hooks at the core RGR sequence (Figure S4), but lacked other amino acid residues because of its position at the carboxy terminus.

Expression silencing of *CYBB* by AnkA is mediated by the central ANK repeats

Because AnkA- N2 was least able to bind the *CYBB* promoter, we sought to confirm that it and the other mutants were unable to decrease *CYBB* transcription. A *CYBB* – β -galactosidase reporter construct (*pCYBB-LacZ*) was co-transfected with each of the AnkA truncations. Neither AnkA- N nor - N2 decreased reporter activity, while AnkA- NLS and AnkA- C modestly decreased reporter activity (76% and 64%, respectively). As both DNA binding and nuclear translocation functions localized to the ANK repeat regions in AnkA, to

better interrogate and dissociate DNA binding and transcriptional repression, a surrogate NLS, the SV40 large T-antigen NLS, was fused to the carboxy termini of Anka- N and - N2 constructs to reestablish nuclear translocation (Figure 1C). As anticipated, Anka reduced expression from the *CYBB* promoter by approximately 70%, as we observed previously (Garcia-Garcia *et al.*, 2009b), and probably results from transfection efficiencies of about 50% into HL-60 cells. When the SV40 NLS was fused to the Anka- N truncation, it readily entered the nucleus and expression from the *CYBB* promoter decreased to levels similar to wild type Anka. Similarly, the Anka- N2-SV40 NLS fusion also readily entered the nucleus, but it remained unable to suppress *CYBB* promoter expression, presumably because it was unable to bind DNA or lacked a distinct domain for recruitment of auxiliary proteins such as HDACs and associated complexes (Figure 4B). These results coupled with the modest reduction in reporter activity with Anka- C provide evidence that most of the promoter suppressive activity, presumably related to reduced binding and/or auxiliary protein recruitment, resides in ANK repeats 7–10. Since binding is also reduced substantially with loss of flanking ANK repeats and the C-terminal AT hook-like domain, expression silencing best localizes to ANK repeats 7–10.

Anka interacts with host HDAC1

Hypothetically, Anka binds the *CYBB* promoter to decrease transcription by recruiting transcriptional repressors, inhibiting transcriptional activator binding, and/or altering chromatin structure. During the course of *A. phagocytophilum* infection, H3 is deacetylated at the *CYBB* promoter and HDAC1 binding is increased at defense gene promoters, including *CYBB* (Garcia-Garcia *et al.*, 2009a). We sought to determine if Anka plays a direct role in H3 deacetylation. Since recombinant Anka (rAnka) lacks HDAC activity (Figure S5), we tested the alternative hypothesis that DNA-bound Anka recruits histone modifying complexes. Using the proximal 200 bp of the *CYBB* promoter for DNA pull-down assays, we identified Anka and HDAC1 co-binding in *A. phagocytophilum*-infected HL-60 cell lysates (Figure 6A). A direct interaction was implied when binding of both Anka and HDAC1 was diminished (Figure 6A) using the *CYBB* promoter containing all four AT nucleotide mutants that abrogate Anka binding (Figure 3B–C). These results could be interpreted in a way that does not require a direct interaction between Anka and HDAC1. Therefore, a direct interaction was confirmed using a protein pull down assay where rFLAG-HDAC1 captured from transfected HEK293T cell lysates was used as bait on magnetic beads for purified rAnka (Figure 6B–D). Recombinant Anka had limited non-specific binding to the magnetic FLAG beads as bait; however, rAnka binding greatly increased when rHDAC1 was captured on magnetic beads and used as the bait (Figure 6A–C). Coomassie staining of the eluate from the pull down assay shows abundant Anka, and minimal contaminating proteins (Figure 6B) demonstrating the direct interaction between Anka and HDAC1. ChIP further confirmed that HDAC1 binding is increased at *CYBB* when Anka is overexpressed in HEK293T cells, while RNA polymerase II binding decreased (Figure 6E), demonstrating that Anka is sufficient to increase HDAC1 binding at the *CYBB* promoter and to exclude RNA polymerase from active transcription.

To determine the importance of HDAC1 for Anka-mediated repression of *CYBB*, HDAC activity was pharmacologically inhibited with sodium butyrate or mocetinostat and the

CYBB- β -galactosidase reporter assay was used to measure transcription from the *CYBB* promoter. AnkA significantly decreased transcription of *CYBB* when HDAC activity was uninhibited, but was unable to repress transcription when HDAC activity was inhibited with 5 mM sodium butyrate, a general HDAC inhibitor, or with 1 μ M mocetinostat, an HDAC1-specific inhibitor (Figure 6F). Together these data show that AnkA recruits HDAC1 to the *CYBB* promoter to decrease transcription.

Discussion

In part, as an agent of an emerging infection with an unusual intracellular niche, but more as a model of how an intracellular microbe manipulates its hosts' gene expression to improve fitness, it is important to understand how *A. phagocytophilum* survives and propagates within the neutrophil. What makes it capable of differentially regulating transcription of critical genes such as *CYBB* or those involved with proinflammatory or anti-apoptosis programs? Due to the large number of genes that are differentially regulated with infection and the relatively limited genomic capacity of the bacterium, we predicted that *A. phagocytophilum* influences global granulocyte transcription through chromatin/histone remodeling to broadly impact transcriptional programs. AnkA, an *A. phagocytophilum* effector protein that translocates to the neutrophil nucleus and binds DNA to interact with nuclear proteins, was predicted to play a role in host transcriptional reprogramming since over-expression resulted in host defense gene downregulation (Garcia-Garcia *et al.*, 2009b). Among many genomic targets, AnkA binds to the *CYBB* promoter in close proximity to regions of decreased histone H3 acetylation (Garcia-Garcia *et al.*, 2009a). Using the *CYBB* promoter as a model of AnkA-mediated *cis* transcriptional control we identified a unique mechanism by which a prokaryotic protein alters host transcription. The multiple ankyrin repeats in AnkA were identified to be critical for AnkA nuclear localization and transcriptional regulation, and to a great extent, its binding to *CYBB*, whereas deletion of both the putative HGMN-CHUD domain and the tandem repeats did not substantially effect nuclear localization, DNA binding or transcriptional activity, although these were not examined in detail.

Ankyrin repeats are commonly occurring motifs among proteins with diverse biological functions, usually mediating protein-protein interactions. In addition to its previous morphologic distribution around heterochromatin in transfected cells, here AnkA was shown to have additional properties of a MAR binding protein. SATB1, a known MAR binding protein that binds to the *CYBB* promoter and represses transcription early during myeloid differentiation, recruits the histone deacetylase contained in the NURD chromatin remodeling complex to deacetylate histones and repress gene expression (Hawkins *et al.*, 2001, McKinsey *et al.*, 2006). Similar to SATB1, here we showed AnkA recruited a histone deacetylase (HDAC1) to alter histone H3 acetylation and was critical for AnkA-mediated *CYBB* repression. Precedent for proteins with ANK repeat domains and HDAC recruitment include the eukaryotic protein, RFXANK, which interacts with HDAC4 and 5 through its ANK repeats to repress major histocompatibility complex II gene expression (McKinsey *et al.*, 2006), and ANKRA2, a paralog of RFXANK, which binds HDAC4 and 5 to decrease expression from xenobiotic responsive elements as a complex with the aryl hydrocarbon receptor repressor (Oshima *et al.*, 2007).

How Anka binds to DNA to recruit HDAC1 is of great interest, as most MAR-binding proteins do so to areas that easily uncoil. There are currently no reports of ankyrin repeat-containing MAR-binding proteins or any ankyrin repeat containing proteins that directly bind DNA. In fact, most examples of ankyrin-containing proteins that localize to DNA, such as GABP α/β , involve heterodimers for which one component binds DNA (GABP α) and the other recruits interacting proteins (GABP β) (Rosmarin *et al.*, 2004). Of interest here was that decreasing the repertoire of Anka ankyrin repeats with increasing truncations from the N-terminus was associated with diminished DNA binding, an observation congruent with contemporary understanding of ankyrin proteins as highly stable nanosprings (Itzhaki *et al.*, 2012) for which stability diminishes as the number of ankyrin repeats is reduced (Hagai *et al.*, 2012), as observed for Anka- N2. Of further interest was the reduced DNA binding observed with the minimal truncation in Anka- NLS at the carboxy end that deleted a putative bipartite NLS. While nuclear localization was not changed by this deletion, inspection of the sequence identified a potential AT hook-like region at the Anka C-terminus that was deleted in this mutant. Whether the identified AT hook that has similarities to other known Alphaproteobacteria AT hooks functions in DNA binding will require further experimental validation.

Likewise, ankyrin proteins are not well recognized to serve as nuclear localization signals. Yet even here precedent exists since, the second ankyrin repeat of I κ B α is critical for its nuclear import (Sachdev *et al.*, 1998), and the data for Anka are consistent with those observations. More recent investigations of eukaryotic ANK repeats in proteins that translocate into the nucleus, such as I κ B α , GABP β , mutated p16^{ink4a}, and ASPP2, do so in part via the presence of hydrophobic amino acid residues at the 13th positions in 2 consecutive ANK repeats (Lu *et al.*, 2014). An analysis of multiple Anka ANK repeats demonstrates that the first four N-terminal ANK repeats possess highly conserved hydrophobic residues in these positions, and the deletion of all 4 of these ANK repeats (Anka- N) is sufficient to inhibit nuclear localization. Whether this relates specifically to those hydrophobic positions will also need to be determined experimentally.

The specific domains or regions to which HDAC1 is recruited are still not established. Deletion of the 4 N-terminal ANK repeats inhibited nuclear localization but not DNA binding or promoter activity. Deletion of the C-terminus did not change nuclear localization or significantly reduce promoter activity, but moderately abrogated DNA binding. Similarly, the Anka- C mutant that lacked both the C-terminus and ANK repeats 11–15 entered the nucleus unimpaired, had a modest defect in DNA binding, and a 25% reduction in promoter activity. The most dramatic effect on promoter activity, and perhaps the ability to recruit HDAC1, was found with deletion of the first 10 ANK repeats for which nuclear localization, DNA binding, and promoter activity were all significantly impaired. While nuclear localization could be easily restored by an exogenous NLS in both Anka- N and Anka- N2, promoter activity was still significantly abrogated only in Anka- N2 that lacked the central ANK repeats. Thus, it could be that the central ANK repeats, in part shared with the deletion in Anka- C, provide most function toward suppression of promoter activity (Figure 7). Thus, this region is a prime candidate to examine for recruitment of HDAC1, and perhaps other proteins with which it complexes to achieve histone deacetylation and *cis* regulation of gene expression.

A prokaryotic protein that binds host DNA and recruits chromatin modifying complexes to alter the epigenetic state is fundamentally distinct from bacterial effectors that modify signaling pathways, even in the nucleus, to alter host transcription. Currently, only a few bacterial pathogens are known to translocate effectors to the host cell nucleus and alter chromatin; the mechanism(s) of gene and transcriptional program targeting and chromatin modifications are poorly understood. *Shigella flexneri* OspF (Arbibe *et al.*, 2007), *Listeria monocytogenes* LntA (Lebreton *et al.*, 2014), *Chlamydia trachomatis* Nue (Pennini *et al.*, 2010), and *Legionella pneumophila* RomA (Rolando *et al.*, 2013) are nuclear-translocated proteins (nucleomodulins) that influence host cell transcription by modifying histone structure.

A. phagocytophilum Anka is the first for which a mechanism that directly involves DNA binding and *cis*-recruitment of histone modifying enzymes to chromatin at an individual gene. The influence Anka exerts over *CYBB* and neutrophil respiratory burst provides the pathogen a significant fitness advantage, as expression from *CYBB* under the control of distinct constitutive promoter reverses pathogen growth (Carlyon *et al.*, 2002). While *CYBB* expression occurs dominantly during neutrophil maturation, Anka is bound to host DNA genome-wide (Park *et al.*, 2004) and histone deacetylation or methylation is frequently observed at promoters of host defense genes in *A. phagocytophilum*-infected cells (Garcia-Garcia *et al.*, 2009a, Garcia-Garcia *et al.*, 2009b), suggesting a much broader and robust effect on transcription and cellular functions. Lastly, microbial survival depends on upregulation of HDAC expression (Garcia-Garcia *et al.*, 2009b). Considering these observations and the need for economy in reduced-genome intracellular prokaryotes, more investigation is needed to determine if Anka and/or similar effectors in other prokaryotes behave similarly, but also act in “*trans*” to modify the epigenome and chromosomal territory architecture as a broad regulatory mechanism over global transcriptional and functional programs (Sinclair *et al.*, 2014). Prokaryote-induced host epigenetic alteration is an emerging topic that will lead to a better understanding of both microbes and hosts. How small genome intracellular prokaryotes broadly manipulate eukaryotic cell functions via transcriptional regulation is of significant interest to explain microbial fitness, and pathogenesis, as well as reprogrammed cellular functions.

Experimental Procedures

Cell Lines and Cell Culture

Promyeloblast HL-60 cells (ATCC CCL-240) and the Webster strain of *A. phagocytophilum* were maintained in HL-60 cells as previously described (Garcia-Garcia *et al.*, 2009b). HEK293T cells were cultured in DMEM media supplemented with 10% FBS and Glutamax (Life Technologies, Carlsbad, CA) in a humidified incubator at 37°C with 5% CO₂.

Expression of Anka and SATB1 in *E. coli* and mammalian cells

Full-length and truncated *anka* coding sequences were amplified via PCR (Table S1) using Webster strain genomic DNA and cloned into pT7-FLAG-MAT-Tag-2 (Sigma-Aldrich, St. Louis, MO) or pCAL-n-EK (Agilent Technologies, Santa Clara CA) expression vectors for expression in *E. coli*. Recombinant protein was purified using a HisTrap HP column (GE

Healthcare, Buckinghamshire, United Kingdom) or calmodulin sepharose (GE Healthcare Bio-Sciences, Pittsburg, PA) following the manufacturer's recommendations. Plasmid pGEX2T-*SATB1*, donated by Terumi Kohwi-Shigematsu, Lawrence Berkeley National Laboratory, Berkeley, CA was used to overexpress SATB1 in *E. coli*, purified as described previously (Dickinson *et al.*, 1997). The *ankA* coding sequences were also cloned the pCMV-Tag2b vector for expression in HL-60 and HEK293T cells (Table S2). pEYFP-*ankA* plasmid was constructed as described previously (Garcia-Garcia *et al.*, 2009b). Plasmids were transfected into HL-60 cells using a Lonza 96-well Nucleofector, solution SF, program EN-138 (Lonza, Basel, Switzerland) or into HEK293T cells using Xfect transfection reagent (Clontech Laboratories, Mountain View, CA).

Electrophoretic Mobility Shift Assay

Purified full-length or truncated rAnkAs and rSATB1 were assayed by EMSA for their ability to bind to the wild-type or mutated immunoglobulin heavy chain MAR or specific *CYBB* DNA probes that included a putative MAR. Oligonucleotides were biotinylated by the supplier (Figure 1A and 2B) (Integrated DNA Technologies, Coralville, IA). EMSA reactions were performed as previously described using 20 fmoles of *CYBB* probe and 25 ng/ μ L protein (Garcia-Garcia *et al.*, 2009b). To prove interaction specificity, an N-terminal amine-modified *CYBB* probe was fluorescently labeled with AlexaFluor 790 (Life Technologies) and 1280 to 5000 fmoles was competed against equimolar unlabeled probe for binding to 20 μ M protein, separated in TBE Criterion (Bio-Rad Laboratories, Hercules, CA) 4–20% polyacrylamide gels for in-gel infrared imaging (LI-COR Odyssey, LI-COR Biosciences, Lincoln, NE). The pixel density from scanned blots or LI-COR digital images was analyzed by densitometry using ImageJ 1.48v, comparing pixel density of shifted (Figure 5 above dark line) to total lane density.

Reporter Assay

Plasmid p*CYBB-LacZ*, kindly donated by Erol Fikrig, Yale University, New Haven, CT was used to express the *lacZ* gene under the control of the –209 to +12 region of the *CYBB* promoter (Thomas *et al.*, 2005). A mutant *CYBB* promoter (p*mutCYBB-LacZ*) was synthesized in pIDT Blue vector (Integrated DNA Technologies, Coralville, IA) to contain mutations A-mut2, A-mut3, B-mut2 and E-mut1 (Figure 2B). The mutated promoter was amplified by PCR and cloned into the pBlueTOPO vector (Life Technologies, Coralville, IA) (Table S3). HL-60 cells were transfected as described above with indicated plasmids. For HDAC inhibition reporter assays, 18 h post transfection cells were treated for 6 h with 5 mM sodium butyrate (Sigma-Aldrich) and 1 μ M mocetinostat. β -galactosidase activity was measured using the Galacto-Star System (Life Technologies, Coralville, IA). Luminescence was read every 2 min for 2 h. For analysis, relative light units between 54 and 64 min were averaged.

Immunofluorescence

pCMV-Tag2b-*ankA* or *ankA* truncation plasmids were transfected into HEK293T cells. 24 h post-transfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X in PBS. Rinsed cells were incubated with anti-FLAG antibody (Sigma-Aldrich, St. Louis, MO) for 1 h, washed 4 times with 0.2% Tween-20 in PBS, and incubated with

AlexaFluor488 goat anti-mouse secondary antibody (Life Technologies, Coralville, IA) for 1 h. Nuclei were stained with 1:5000 dilution of Hoechst 33342, trihydrochloride, trihydrate (Life Technologies, Coralville, IA) and washed again 4 times with 0.2% Tween-20 in PBS. For confocal microscopy, cells were viewed using a Zeiss LSM 510 Meta Confocal Microscope (University of Maryland, Baltimore Confocal Core Facility).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was completed using the ChIP-IT Express Chromatin Immunoprecipitation Kit (Active Motif, Carlsbad, CA) or as previously reported (Garcia-Garcia *et al.*, 2009b). DNA was immunoprecipitated with the AnkA monoclonal antibody 5C2 (prepared against a recombinant AnkA devoid of most ANK repeats in the N-terminus), RNA polymerase II and HDAC1 antibodies (Active Motif, Carlsbad, CA). ChIP using goat anti-mouse IgG was also included as negative control in experiments. Immunoprecipitation of the proximal *CYBB* promoter was analyzed via qPCR using USB Veriquest SYBR Green (Affymetrix, Santa Clara, CA) or regions along the X-chromosome using iQSYBR Green Supermix (Bio-Rad, Hercules, CA) (Tables S3 and S4). Percent of input chromatin was calculated and normalized to ChIP with non-transfected HEK293T cells or uninfected HL-60 cells.

HDAC activity assay

rAnkA was tested for HDAC activity using the fluorescent HDAC assay kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions. 5 μ g rAnkA was diluted in assay buffer and activity expressed as pmol of fluorescent product formed after incubation at 37°C for 1 h. Fluorescence intensity was measured using a Perkin Elmer Victor II plate reader with an excitation wavelength at 355 nm and emission at 460 nm. HeLa cell extract was used as a positive control.

DNA Pull Down Assay

DNA pull down assays were performed using biotinylated 5' wild-type or mutated -209 to +12 *CYBB* promoter probes. The mutated probe contained all four mutations found to decrease AnkA binding via EMSA (Figure 2B and C). Probes used for DNA pull down were synthesized using primers in Table S3 and the mutated probes were amplified from the pmut*CYBB-LacZ* plasmid. Uninfected and *A. phagocytophilum*-infected HL-60 cells were lysed with lysis buffer (20 mM HEPES pH7.5 with KOH, 150 mM KCl, 0.5 mM DTT, 10% glycerol) for 15 min on ice. NP-40 was added to a concentration of 0.5%. Cells were sonicated 3 times for 10 sec with a Branson Sonifier 250 (Branson Ultrasonics, Danbury, CT) at output 4 and centrifuged at 13,000 rpm for 10 min. Lysates were pre-cleared for 1 h at 4°C with 1 μ g/mL salmon sperm DNA and 25 μ L streptavidin paramagnetic beads (Promega, Madison, WI) in DNA pull-down buffer (20 mM Tris, 15% glycerol, 150 mM KCl, 1 mM EDTA, 0.5 mg/mL BSA, 0.05% NP-40). Approximately 500 μ g of pre-cleared lysates were then incubated with 500 ng biotinylated *CYBB* probe, 1 μ g/mL salmon sperm DNA, and 25 μ L fresh streptavidin paramagnetic beads (Promega, Madison, WI) for 1 h at 4°C. As controls, lysates were also incubated with non-biotinylated probes. Proteins bound to the *CYBB* promoter were eluted in SDS-PAGE loading buffer and protein immunoblot analysis was performed to detect AnkA and HDAC1.

Protein Pull Down Assay

HEK293T cells were transfected with pFLAG-HDAC1 plasmid (Addgene plasmid 13820 (Emiliani *et al.*, 1998)) using Xfect transfection reagent (Clontech Laboratories, Mountain View, CA). 48 h post transfection, cells were lysed as described in the DNA pull down assay procedure above. HDAC1-FLAG was incubated with mouse monoclonal IgG₁ anti-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO) overnight at 4°C and washed with 1X Tris-buffered saline with 1 M NaCl and 5% Tween-20. rCBP-AnkA was purified as described above. Approximately 5 µg of rCBP-AnkA was incubated with HDAC1-FLAG beads overnight at 4°C and washed with 1X PBS with 0.02% Tween-20. rCBP-AnkA was also incubated with Anti-FLAG M2 affinity gel incubated with non-transfected HEK293T cellular lysate as a negative control. Proteins were eluted with Laemmli sample buffer at 95°C for 5 min. Samples were analyzed by SDS-PAGE with Coomassie blue stain and protein immunoblots using anti-mouse IgG-HRP (Cell Signaling Technology, Danvers MA). Images were obtained with a Kodak Image Station 4000R and band intensities measured using ImageJ (<http://rsb.info.nih.gov/ij/>). The AnkA band intensities were normalized to immunoglobulin heavy chain band intensity and the average for the negative control was set to one.

Bioinformatics

Full-length AnkA was examined for DNA binding motifs using GYM 2.0 (for helix-turn-helix motifs; <http://users.cis.fiu.edu/~giri/bioinf/GYM2/prog.html>), 2ZIP (for leucine zippers; <http://2zip.molgen.mpg.de/index.html>), and DP-Bind (for evolutionarily-conserved binding sequences; <http://lcg.rit.albany.edu/dp-bind/>). To assess the potential for an AT hook at the carboxy terminus of AnkA, previously identified AT hook regions among 48 Alphaproteobacteria were downloaded from the Simple Modular Architecture Research Tool (SMART; http://smart.embl.de/smart/do_annotation.pl?DOMAIN=SM00384; Table S6) and aligned using CLUSTALX. Similarly, the C-terminal 39 amino acid residues of *A. phagocytophilum* Webster strain AnkA were used as template in a BLAST (Altschul *et al.*, 1990) search of the NCBI Non-redundant protein sequences database to obtain those most similar (Table S7). The CLUSTALX (Thompson *et al.*, 1997) aligned sequences were used as a template for sequence logo creation (Crooks *et al.*, 2004) and examination of similar linear features. To assess the diversity of hydrophobic residues in the 13th positions of the ANK repeats of AnkA, 31 AnkA protein sequences representing diverse strains from human and animals, and from several continents were obtained (Table S5), aligned by CLUSTALX, and ANK repeats were characterized by screening the consensus sequence using the Eukaryotic Linear Motif resource for Functional Sites in Proteins (ELM; <http://elm.eu.org/>).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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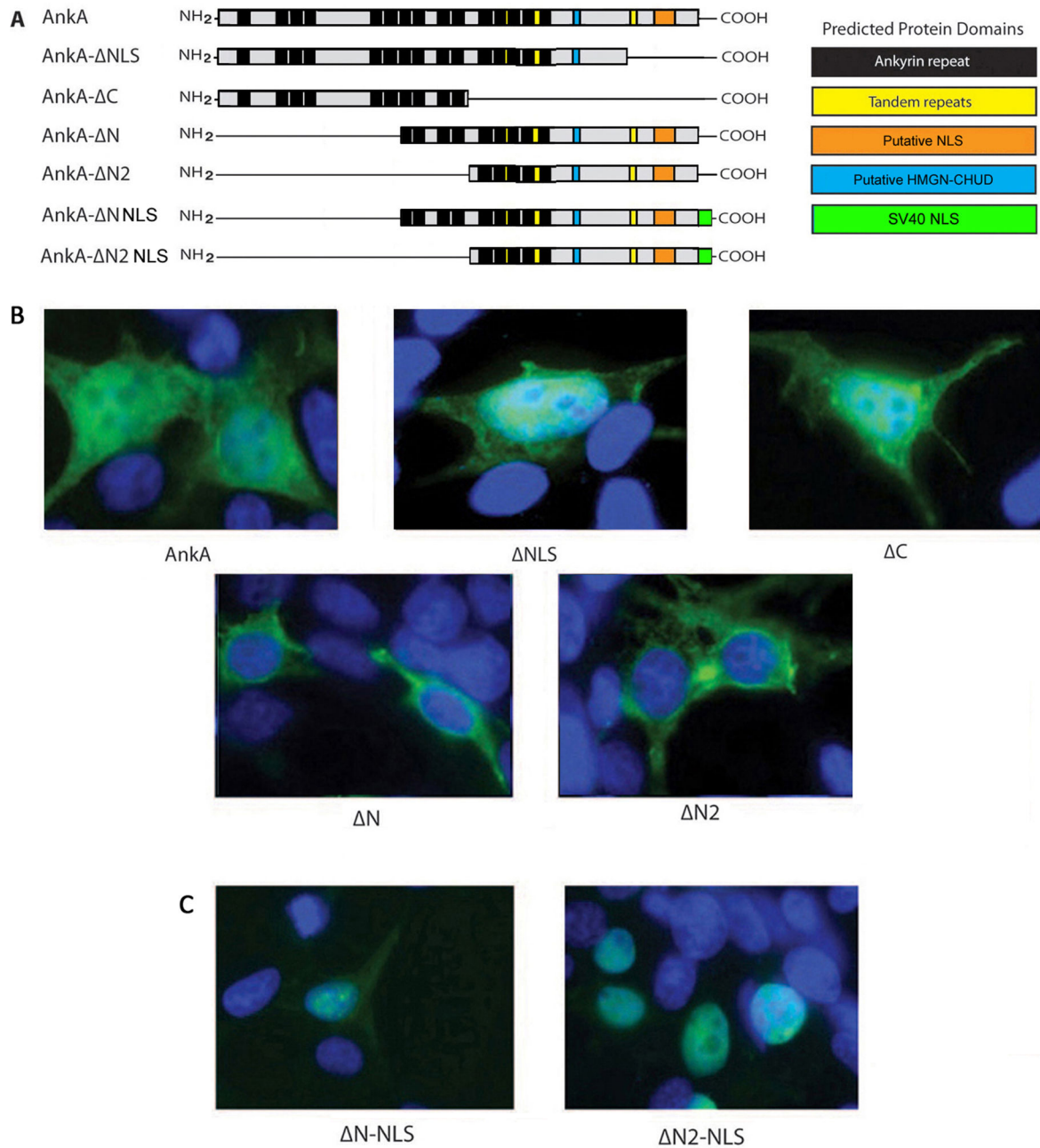


Figure 1. NLS activity in Anka resides within the first 4 ANK repeat regions

(A) Schematic of Anka truncated mutants encoded by the pCMV-tag2b and pT7-FLAG-MAT-Tag-2 vectors and predicted locations of protein domains. (B) Merged confocal immunofluorescence microscopy of Anka truncations at 24 h post-transfection in HEK293T. (C) Merged confocal immunofluorescence microscopy at 24 h post-transfection in HEK293T with Anka Δ N and Anka Δ N2 truncations engineered with the SV40 large T-antigen NLS to re-establish nuclear localization.

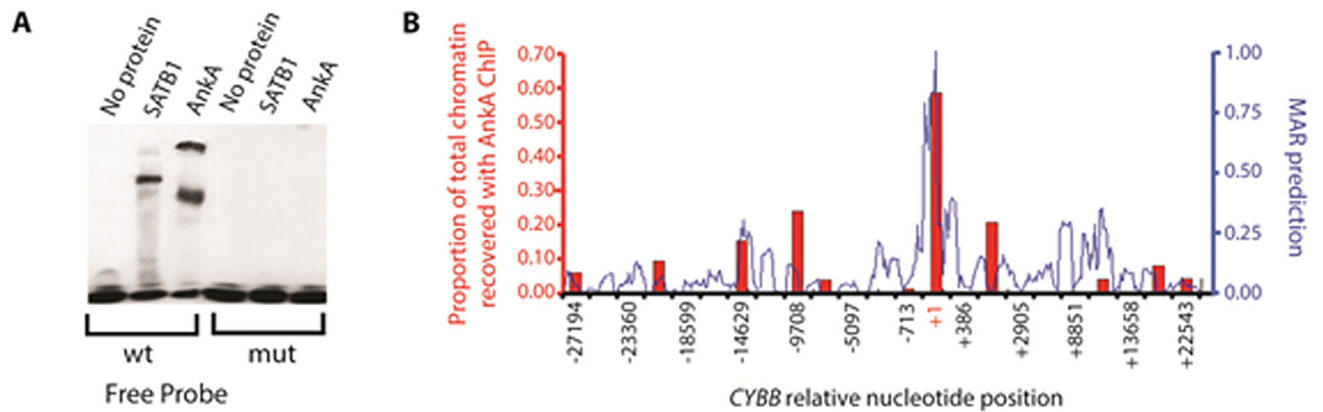


Figure 2. Anka binds known and predicted MARs

(A) EMSA with rAnkA and rSATB1 incubated with the matrix attachment region (MAR) of *IGH* (wt = 5'-TCTTTAATTTCTAATATATTTAGAAAttc-3') known to bind SATB1, and with a mutated *IGH*MAR (mut = 5'-TCTTTAATTTCTACTGCTTTAGAAAttc-3'). The underscored nucleotides indicate the areas where AT changes were made. Because AnkA is degraded during purification (Figure S1), several bands are present in EMSA. (B) MARs within the *CYBB* promoter were predicted with MARFinder MAR-Wiz 1.5 (blue line) using default values; a high likelihood was assigned with a value greater than the 0.6 cut-off threshold. Anka binding along the entire *CYBB* promoter and downstream positions was assessed by chromatin immunoprecipitation-quantitative PCR (ChIP-qPCR) (red bars).

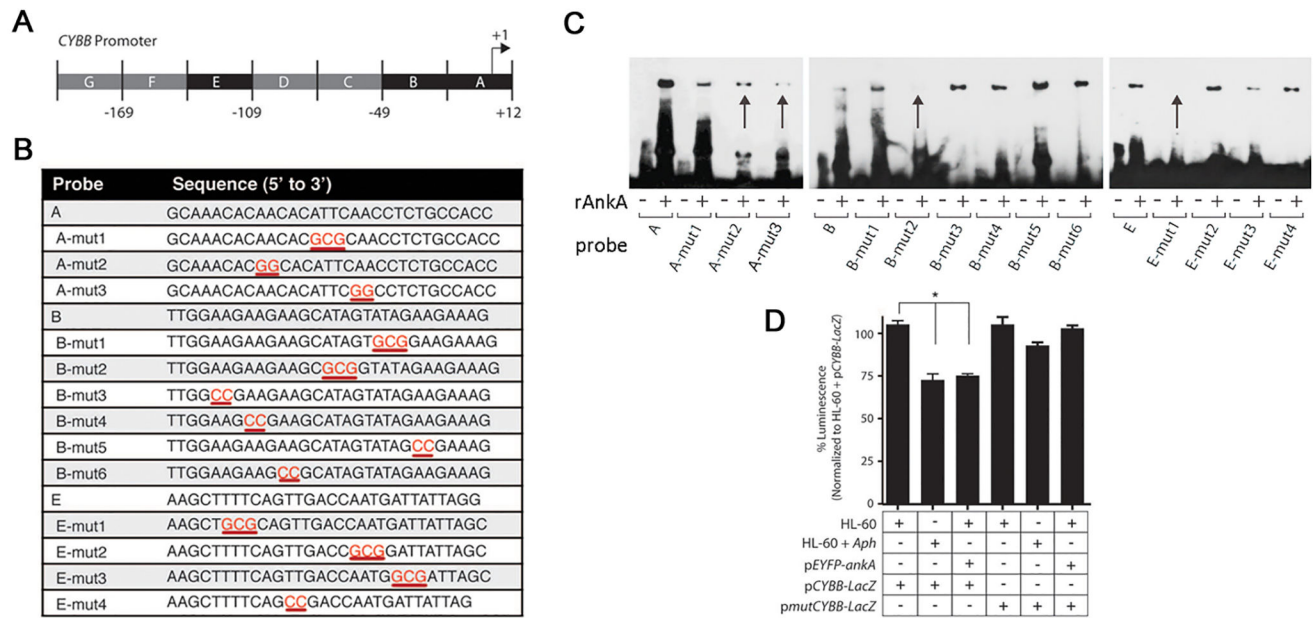


Figure 3. AnkA binds to AT-rich regions in the *CYBB* promoter that are critical for transcriptional repression

(A) Schematic of proximal *CYBB* promoter and regions where AnkA binds (shown in black boxes) (Garcia-Garcia *et al.*, 2009b). (B) Double stranded wild-type and mutated probes used for EMSAs. The mutated bases are shown in red and are underlined. (C) EMSA with rAnkA and mutated *CYBB* probes. Arrows indicate probes with decreased AnkA binding. (D) Reporter β -galactosidase activity in uninfected HL-60 and *A. phagocytophilum*-infected HL-60 cells transfected with the indicated plasmids (n=3). * $P < 0.001$ (Student's t-test, $\alpha = 0.05$). Data are represented as mean \pm SEM.

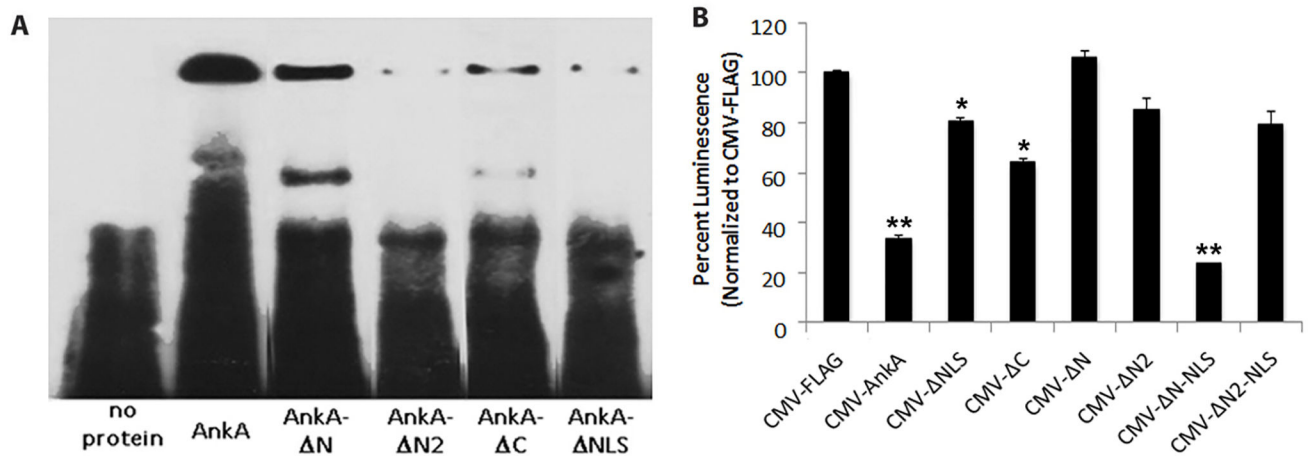


Figure 4. DNA-binding by the N-terminus of AnkA is required to suppress transcription from the *CYBB* promoter

(A) Wild-type *CYBB* probe A (from Figure 3B) EMSA assays with truncated AnkA mutants and labeled proximal *CYBB* promoter probe. (B) β -galactosidase reporter assay in HL-60 cells co-transfected with p*CYBB-LacZ* and plasmids expressing the truncated AnkA mutants (n=3); * P <0.05, ** P <0.001 vs. FLAG control (Student's t-test, two-sided α =0.05). Data are represented as mean \pm s.d.

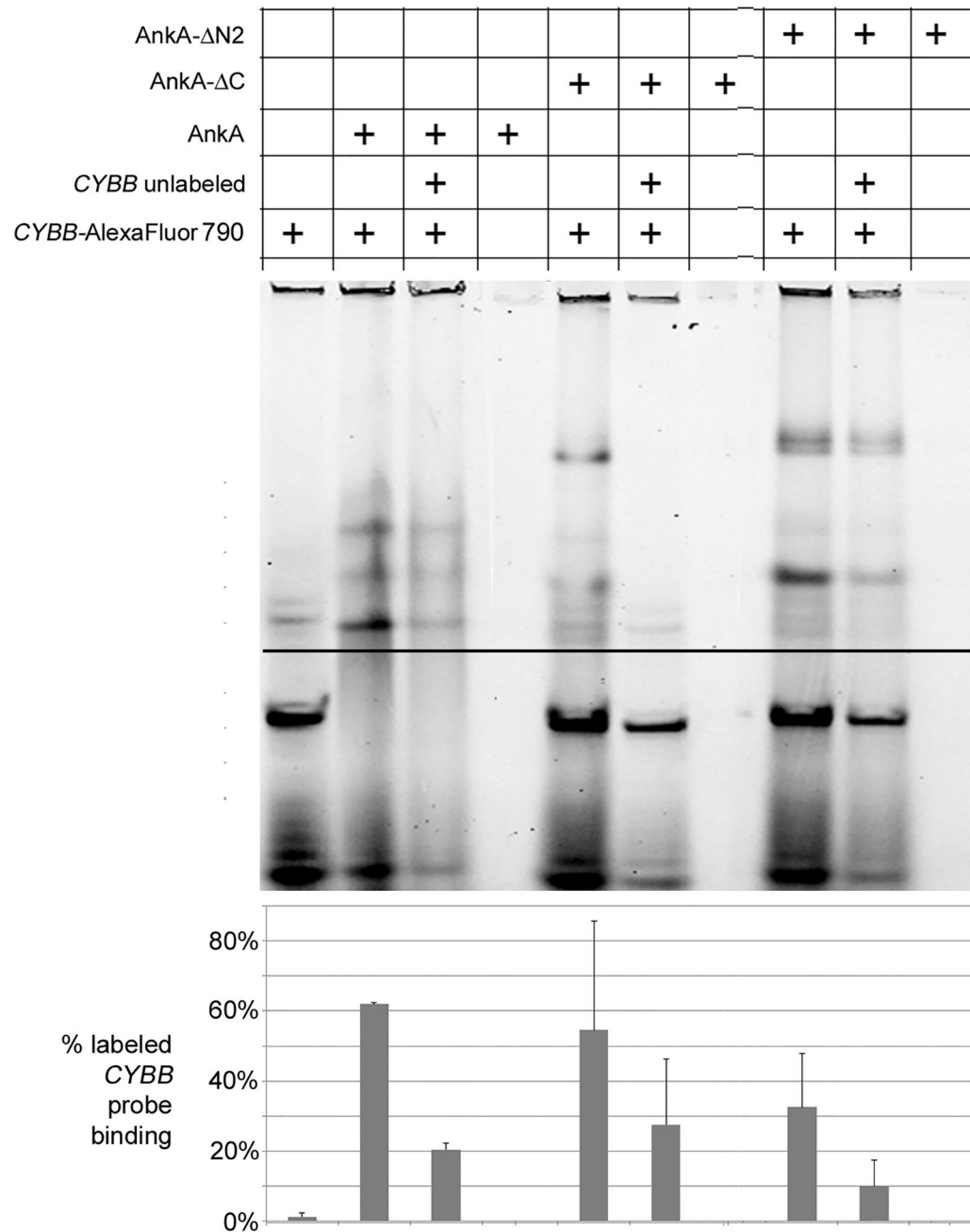


Figure 5. Anka, Anka- N2, and Anka- C binding to the *CYBB* promoter is specific rAnkA, rAnka- N2, and rAnka- C binding to the AlexaFluor 790-labeled *CYBB* promoter probe is antagonized by equimolar unlabeled probe by 67, 69 and 49%, respectively. Recombinant proteins were assayed by EMSA in 4–20% polyacrylamide non-denaturing gels. The pixel density from the LI-COR digital images was analyzed by densitometry using ImageJ 1.48v, comparing pixel density of shifted (above dark line) to total lane density, shown quantified in the graph below the gel image.

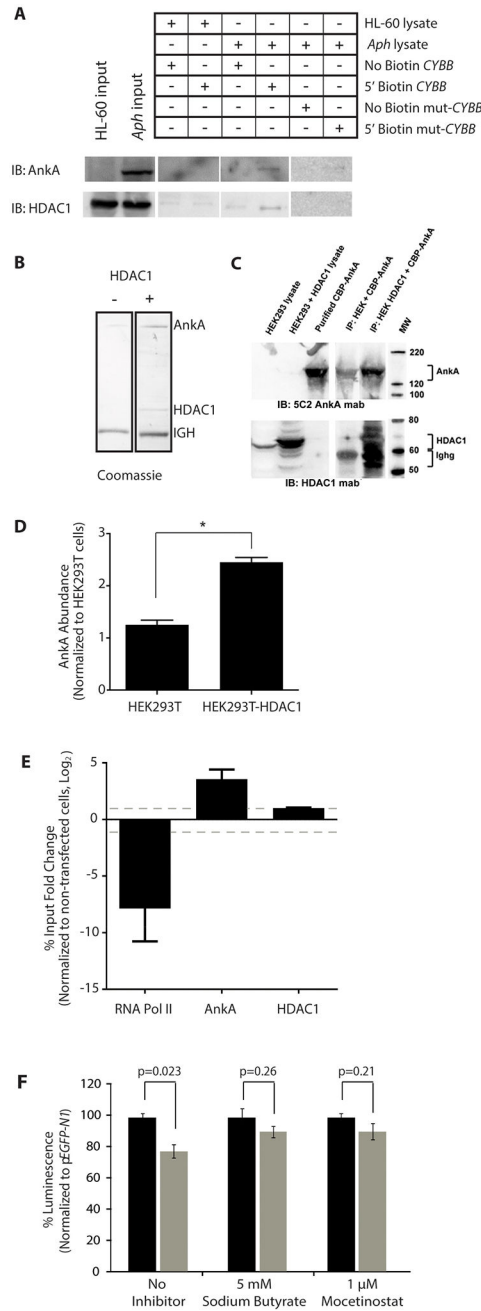


Figure 6. Anka and HDAC1 directly interact

(A) DNA pull-down assay with uninfected (HL-60) or *A. phagocytophilum*-infected HL-60 cell (*Aph*) lysates and wild-type or mutated *CYBB* promoters. The first two lanes are controls that demonstrate the presence of Anka and/or HDAC1 in samples before reaction with the *CYBB* or mutated probes. The remaining lanes show the results of interactions in the absence of Anka (HL-60), with Anka (*Aph*), with unlabeled promoter or mutated promoter probe (No Biotin *CYBB* or No Biotin mut-*CYBB*) that will bind the magnetic beads, and 5' biotinylated promoter or mutated probe (5' Biotin *CYBB* or Biotin mut-*CYBB*) that will not bind the magnetic beads. (B) Coomassie blue stain and (C) protein

immunoblot analysis of eluted proteins from protein pull-down assay with HDAC1-FLAG overexpressed in HEK293T cells mixed with purified rAnkA. The overexpressed HDAC1 (60–65 kDa) readily bound to the FLAG mab matrix and was detected in proximity to large quantities of mouse immunoglobulin gamma heavy chains (Ighg; 55 kDa) dissociated from the matrix in the Laemmli buffer. HDAC1 detection increased simultaneously with a marked increase in AnkA binding compared to cells lacking HDAC1 overexpression. (D) Densitometry analysis of relative AnkA abundance in protein pull-down assay. (E) ChIP analysis of AnkA-, HDAC1- and RNA polymerase II-binding at the proximal *CYBB* promoter in AnkA-transfected HEK293T cells (n=3). Dashed line indicates 2-fold change in binding. (F) β -galactosidase reporter assay in HL-60 cells co-transfected with p*CYBB-LacZ* and p*EYFP-N1* (black bars) or p*EYFP-ankA* (gray bars) and treated with 5 mM sodium butyrate HDAC inhibitor or 1 μ M mocetinostat HDAC1 inhibitor (n=3). Data normalized to empty vector, p*EYFP-N1* control. * $P < 0.001$ (Student's t-test, $\alpha = 0.05$). Data are represented as mean \pm SEM.

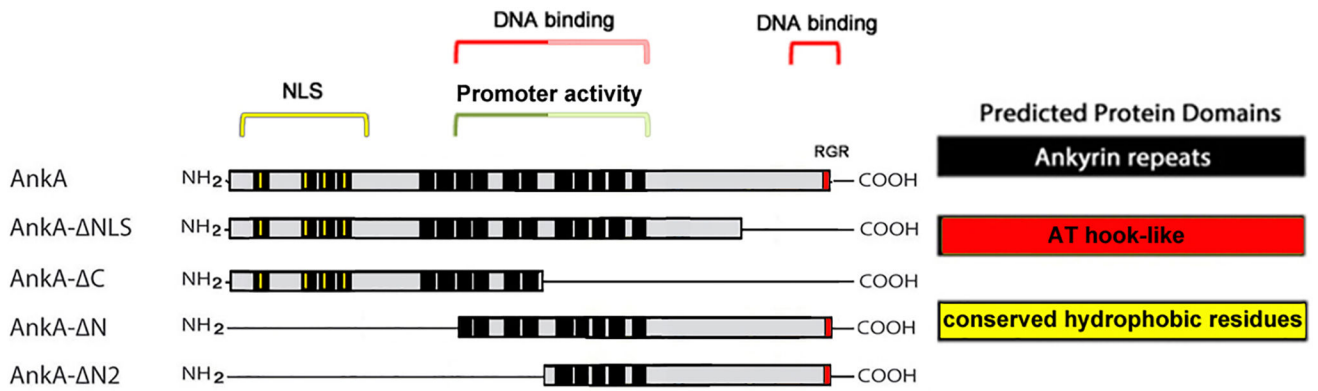


Figure 7. Schematic of Anka regions for nuclear localization, DNA binding, and promoter activity

The diagram depicts the specific regions in Anka that mediate nuclear localization (red lines in first 4 ANK repeats show conserved hydrophobic domains at the 13th residues), regions most associated with DNA binding, specifically localized to the central ANK repeats deleted in Anka- N2 or to the AT hook-like C-terminal residues (RGR), and regions most strongly associated with promoter activity deleted in Anka- N2 and less in Anka- C.