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## TALE Proteins Bind to both Active and Inactive Chromatin

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### Abstract

Transcription activator-like effector (TALE) proteins can be tailored to bind to any DNA sequence of choice and thus are of immense utility for genome editing and the specific delivery of transcription activators. However, to perform these functions, they need to occupy their sites in chromatin. Here, we systematically assessed TALE binding to chromatin substrates and find that *in vitro* TALEs bind to their target site on nucleosomes at the more accessible entry/exit sites but not at the nucleosome dyad. We show further that *in vivo* TALEs bind to transcriptionally repressed chromatin and that transcription increases binding by only two-fold. These data therefore imply that TALEs are likely to bind to their target *in vivo* even at inactive loci.

**Keywords:** TALE proteins; Nucleosomes; Transcription; Chromatin; Genome engineering

**Running title:** Chromatin binding by TALE proteins

### Summary statement:

TALE proteins are of immense utility in genome engineering. We show here that they can occupy their sites in both active and repressed chromatin. These data therefore imply that, in principle, TALEs can function at most sites in the genome.

## Introduction

The packaging of eukaryotic DNA into chromatin provides a formidable obstacle to protein binding and thus to the initiation of essential nuclear processes such as transcription, recombination and repair. Even the first level of chromatin packaging, the nucleosome, restricts protein binding but the extent of this inhibition depends on the protein in question [1]. For example, the affinity of some transcription factors such as heat shock factor is reduced by >100 fold, essentially eliminating physiological binding [2]. In contrast, glucocorticoid receptor and Sp1 bind to their target sites on nucleosomes but with affinities reduced by between 2-5 fold and 10-20 fold, respectively compared to free DNA [3],[4],[5].

Complementary studies using restriction enzymes to probe nucleosome accessibility have shown that DNA at the nucleosome dyad is 2-3 orders of magnitude less accessible than at the more “breathable” entry/exit points [6]. Binding close to the nucleosome dyad can be achieved, however, by synergism between transcription factors where binding of the first factor aids subsequent factor binding [7]. In contrast to these detailed studies of nucleosomes, relatively little is known about the influence of higher order chromatin structure on protein accessibility, although one study suggested that the effect is relatively small [8].

The ability of proteins to target their sites in chromatin is particularly important for transcription activator-like effectors (TALE proteins). These proteins were identified in the plant pathogen *Xanthomonas* and are extremely powerful since they can be tailored to bind to any DNA sequence of choice. Moreover, provided the target is greater than 16 bp, TALEs can pinpoint unique locations within the mammalian genome. TALEs have already been used to great effect to regulate gene expression by linking them to activation or repression domains [9],[10]. In addition, TALEs linked to Fok I endonuclease are highly effective in genome editing [11]. However, their ability to perform these functions absolutely requires TALE binding to target sites in chromatin.

Initial studies showed that TALE binding to the repressed *Oct4* promoter in neuronal stem cells is enhanced by pre-treatment with either the histone deacetylase inhibitor, valproic acid or the DNA methyltransferase inhibitor, 5-azacytidine [12]. This implies that repressive chromatin modifications restrict TALE binding. Consistent with this, genome editing was found to occur with variable efficiency, possibly due to different chromatin environments influencing TALE binding [13, 14].

Whilst these studies imply that chromatin reduces TALE binding, they do not determine how it specifically mediates its inhibitory effects: One possibility is that packaging the TALE binding site into a nucleosome prevents binding. A second possibility is that repressive chromatin marks additionally, or alternatively, play a role. Here, we systematically examined the ability of TALEs to bind to chromatin templates and find that they cannot bind to nucleosomes *in vitro* when their binding site is close to the dyad axis; however, they can bind at more accessible sites closer to the entry/exit points. We complement these studies with an *in vivo* analysis of TALE binding and

show that TALEs bind strongly to an actively transcribed region. This is reduced by only about two-fold in cells where the gene is not transcribed. By measuring the nucleosome repeat length and the change in chromatin accessibility upon transcription, we propose that a key determinant of TALE binding is the availability of linker DNA between nucleosomes. Together, these data imply that TALEs are likely to bind and perform effective genome editing and gene regulation at most sites in the genome.

## Experimental

Further details are given in the Supplementary Methods

Cloning of TALE A2. This was cloned via Golden Gate ligation [13] into the pTALEN(HD) vector to generate the plasmid pTALEN-TALEA2. The DNA binding domain was sub-cloned into: (i) a mammalian expression vector containing a HA tag and (ii) an *E. coli* expression vector with a biotin tag.

Purification of TALE A2. The full length TALE protein was expressed in *E. coli* BL21(DE3) and biotinylated *in vivo* by co-expression of biotin ligase BirA, from the pBirAcm plasmid (Avidity). It was purified using Softlink Avidin Resin (Promega).

Cloning of the TALE A2 binding site. A 122 bp fragment from the *Jλ1* gene, containing the TALE binding site, was amplified from mouse genomic DNA using primers TALElamb\_F (5'-CAGCCCGGGATTTTCTGGAAAGACTTCCTATGAG-3'; primer 1; Supplementary Table 1) and TALElamb\_R (5'-CTGAAGCTTTGCATCTGTGATGTATGCAGCTC-3'; primer 2). The PCR product was cloned into Xma I and Hind III sites of pBluescriptSK to generate pBS-TALEA2bind.

Nucleosome reconstitution and binding assays. Two DNA fragments were excised from pBS-TALEA2bind: Fragment 1 (156 bp) using Acc 65I and Xma I and Fragment 2 (146 bp) using Not I and Hind III. These were end-labelled and reconstituted with recombinant *Xenopus* histone octamers via salt-dialysis [15]. Nucleosomes were gel purified and their positions mapped via micrococcal nuclease digestion as described [16]. Binding reactions contained 20 mM Tris pH 8.0, 100 mM NaCl, 10% glycerol, 0.05 µg/µl polydI-dC.

Preparation of RNA and Reverse Transcription. Total RNA was isolated using Trizol® (Invitrogen) and reverse transcribed with M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions. cDNA was amplified with primers for the *Hprt* and *Jλ1* genes (Supplementary Table 1).

Transfection and ChIP analysis. Cells were transfected with pmRuby2-TALEA2-HA and an expression vector for GFP (pmaxGFP, Lonza) via electroporation using the relevant Amaxa nucleofactor kit (Mouse B cell kit for 103/BCL-2 cells and nucleofactor R kit for NIH 3T3 cells). Transfection levels (of 25.3% and 35.1%, respectively) were determined 48 hours later using a LSRFortessa FACS analyser (Becton Dickinson). Chromatin immunoprecipitation was performed with 5 x 10<sup>6</sup> cells per ChIP as described [17].

Bandshift analysis of TALE A2 binding. Extract was made from transfected cells [18] and the level of functional TALE protein determined via gel mobility shift assay.

Mapping nucleosome positions *in vivo*. Nucleosome positioning at the *Igλ* locus was analysed by indirect end labelling according to [19].

Restriction enzyme accessibility Nuclei were prepared as described [20] and incubated at 37°C with or without 50 U of enzyme for 20 minutes. DNA was prepared and normalised by qPCR using the Int III primers (11 & 12, Supplementary Table 1); the level of accessibility was detected using the J1Sty primers (13 & 14; Supplementary Table 1).

## Results

### TALE binding to nucleosomes

To measure TALE binding to nucleosomes *in vitro*, a TALE protein was generated to bind to an 18 bp sequence within the J $\lambda$ 1 region of the murine immunoglobulin lambda (Ig $\lambda$ ) light chain locus (Figure 1A). This TALE (A2) was expressed in *E.coli* and purified using a C-terminal biotin tag (Supplementary Figure 1A). Next, the TALE binding site and surrounding DNA sequence, was amplified from mouse genomic DNA and cloned into pBluescript. A 156 bp DNA fragment was then excised and reconstituted into a nucleosome *in vitro* by salt dialysis. Notably, this fragment (Fragment 1) has the TALE binding site located centrally and following reconstitution, the binding site mapped close to the nucleosome dyad (Figure 1B, and Supplementary Figure 1B, left).

The ability of the purified TALE protein to bind to Fragment 1 was then examined, using both free DNA and nucleosome substrates. Whilst strong binding was observed to free DNA, no detectable binding was observed to the nucleosome (Figure 1B) or the hexasome (Supplementary Figure 2). However, the TALE/DNA complex has an almost identical mobility to the nucleosome and a band of increased intensity is observed at the position of the nucleosome following addition of TALE A2 to the nucleosome preparation. Free DNA is present in the nucleosome preparation and to verify that the band is due to the formation of a TALE/DNA complex, streptavidin was added. The biotinylated TALE/DNA complex was supershifted whilst the intensity of the nucleosome and hexasome bands remain unchanged (Figure 1C). Together, these data imply that the TALE has negligible binding to nucleosomes (or hexasomes) reconstituted onto Fragment 1.

Although the TALE is unable to bind when its target site is at the nucleosome dyad, it remained possible that it could bind to more accessible sites at the nucleosome entry/exit points. Therefore, a second DNA fragment was prepared where the TALE binding site is close to the 5' end. Importantly, following nucleosome reconstitution and mapping, the TALE binding site was located within the last 30-35 bp of nucleosomal DNA (Supplementary Figure 1B, right). Addition of the TALE protein to this nucleosome resulted in a marked shift (Figure 1D), consistent with the idea that the TALE can indeed bind to nucleosome 2, albeit with an affinity about 3-fold lower than to free DNA (which has a  $K_d$  of 21.7 nM; Supplementary Figure 4).

Together, these data imply that TALEs can bind to a nucleosome when the binding site is located close to the more accessible entry/exit points. However, if the site lies further towards the nucleosome dyad, TALE/nucleosome binding is impaired.

### TALE binding *in vivo*

Next, we examined how chromatin packaging affects TALE binding *in vivo*. To this end, two cell types were used: the fibroblast cell line, NIH 3T3, where Ig $\lambda$  is inactive and the pro/pre-B cell line, 103/BCL-2 [21]. In this latter cell line, Ig $\lambda$  is poised for activity when the cells are grown at 33°C; however, temperature shift to 39.6°C strongly induces Ig $\lambda$  transcription.

Firstly, we verified the transcription levels through the TALE binding site in the two cell types. Negligible expression is observed in NIH3T3 cells and in 103/BCL-2 cells grown at 33°C; in contrast, transcription of this region increases significantly in 103/BCL-2 cells following temperature shift to 39.6°C (Figure 2A), consistent with previous observations [22].

To determine the ability of the TALEs to bind *in vivo*, we transfected the two cell types with an expression construct for TALE A2. This has a C-terminal HA epitope and after determining the number of transfected cells by flow cytometry, the binding of TALE A2 was determined by chromatin immunoprecipitation (ChIP) using an anti-HA antibody. Remarkably, in 103/BCL-2 cells following temperature shift, binding was enriched over 100-fold (Figure 2B) compared to a non-transfected control. In contrast, in NIH3T3 cells and 103/BCL-2 cells grown at 33°C, binding was enriched by an average of 35 to 50-fold, respectively. This implies that TALEs can bind to their sites in non-expressed chromatin but that this is reduced compared to when the region is transcribed. Control experiments verified that the TALE is expressed in both cell types and that it is capable of binding to DNA (Figure 2C). Moreover, to eliminate differences due to transfection efficiency, 103/BCL-2 cells from the same transfection were used to compare binding at 33°C and 39.6°C.

Next, to investigate the molecular basis for increased TALE binding in the temperature shifted cells, we added the transcription inhibitor,  $\alpha$ -amanitin. Consistent with the idea that transcription modulates TALE binding,  $\alpha$ -amanitin reduces binding in temperature shifted 103/BCL-2 cells to close to the levels observed in the non-temperature shifted cells (Figure 2B, right).

### **Modulation of TALE binding *in vivo***

Transcription could increase TALE binding in a number of ways: It could alter histone modifications to increase accessibility of the TALE binding site; alternatively or additionally, it could unravel higher order chromatin structure. To begin to differentiate these possibilities, we firstly examined the level of the major active chromatin mark, histone acetylation. Remarkably, relatively little enrichment in acetylation is observed at the TALE binding site even in the presence of ongoing transcription (Figure 3). This suggests that changes in histone acetylation do not significantly influence TALE A2 binding.

Therefore, we next assessed how transcription alters higher order structure. Initially, we measured the accessibility of the TALE binding site directly using the restriction enzyme, Sph I, as a probe. This has a recognition site 30-40 bp from the TALE binding site and, using an amount of enzyme that had been previously shown to give maximum digestion [20], nuclei were digested, followed by analysis of the level of cutting via qPCR. When transcription is ongoing, the average accessibility of the site is 28%; this is reduced by about 50% when  $J\lambda 1$  is transcriptionally inactive in NIH 3T3 cells and 103/BCL-2 cells at 33°C (Figure 4A). This decrease correlates well with the observed reduction in TALE binding (Figure 2B).

Next, we determined the expected level of accessibility. To this end, the nucleosome repeat length was measured by digesting nuclei with micrococcal nuclease and separating the resulting DNA by electrophoresis. This showed that the average distance between nucleosomes is 195 and 205 +/- 5 bp, in NIH3T3 and 103/BCL-2 cells, respectively (Figure 4B), implying that approximately 25% of DNA lies within the linker.

If nucleosomes are randomly distributed, then the probability of the TALE binding site being within this linker DNA is the same as that predicted by the repeat length, i.e. 25%. If, however, nucleosomes are positioned, then the TALE binding site might always be accessible or always inaccessible. To assess nucleosome positioning at the  $J\lambda 1$  gene, nuclei were digested with micrococcal nuclease and used in an indirect end labelling experiment. A smear is observed in both NIH 3T3 and 103/BCL-2 cells (Figure 4C), implying that nucleosomes are not positioned, regardless of  $J\lambda 1$  transcription. Together with the results in Figure 4B, these data suggest that approximately 25% of TALE binding sites lie in the linker DNA.

Notably, in the absence of transcription the level of accessibility is lower (14%; Figure 4A) than predicted by the nucleosome repeat length (25%). To test if this is because transcription increases accessibility by disrupting higher order chromatin structure, we added  $\alpha$ -amanitin for 40 minutes to 103/BCL-2 cells grown at 39°C. This reduces accessibility to close to that seen in cells where this locus is not being transcribed (Figure 4A, right). This suggests that a key function of transcription is to increase the accessibility of linker and nucleosomal DNA, which then facilitates TALE binding. Accessibility does not fall completely to repressed levels upon inhibition of transcription, however; this might be because changes in chromatin modifications make a small contribution to the accessibility. Alternatively, it might be due to incomplete re-folding of higher order chromatin structure during the relatively short  $\alpha$ -amanitin treatment. It is also notable that the decrease in TALE binding upon  $\alpha$ -amanitin treatment is slightly greater than the decrease in Sph I accessibility. This might be because the TALE binding site (18 bp) is longer than the Sph I site (6 bp) and is thus more likely to be occluded.

## Discussion

TALE proteins are of immense utility in genome editing and gene regulation but their ability to carry out these functions relies on their effective binding to target sites in chromatin. Here we show that whilst the nucleosome partially inhibits binding, TALE binding is possible *in vivo* even at a transcriptionally repressed locus. Our data therefore contrast with early ideas that TALEs should be targeted to hypersensitive sites. Instead, we find that there is a relatively small difference in binding to active and repressed loci, implying that TALEs should function at inactive loci. Consistent with this, TALEs were recently shown to function at such loci [23].

We find that TALE A2 binds at the more accessible nucleosome entry/exit sites, albeit with about 3-fold lower affinity than to free DNA. This, together with the recent reports that TALE proteins synergise to activate genes [23, 24], begins to explain how TALEs can function on repressed chromatin substrates [23]: The binding of a TALE to the linker DNA or to the more accessible regions of the nucleosome could increase binding of a second TALE or another transcription factor closer to the nucleosome dyad, a phenomenon first described for GAL4 [7].

Transcription increases accessibility of the TALE binding site and this correlates well with the disruption of higher order chromatin structure and the release of linker DNA. Transcription is also known to dislodge histone H1, thereby removing a protein that competes with transcription factors for nucleosome binding [6]. Recently, a third way in which transcription increases accessibility was described: Transcription transiently evicts an H2A/H2B dimer from each nucleosome; this temporarily releases 30-40 bp of DNA from the nucleosome, providing a window of opportunity for protein binding [25]. It is likely that all three of these processes cooperate to aid TALE binding to transcriptionally active chromatin. However, the strong correlation between the level of accessibility and amount of linker DNA, suggests that the transcription-dependent release of linker DNA contributes significantly to increased protein binding.

The lack of TALE binding at the nucleosome dyad means that the target site for any given TALE is likely to be occluded in some cells in the population. Consistent with the idea that TALEs do not bind in all cells, genome editing was found to vary in efficiency between 2 and 100% [14]. Moreover, some TALE proteins were found not to function at all [13]. Whilst this could be due to weak interaction with the particular DNA sequence, it could also be due to the TALE binding site falling close to the dyad of a positioned nucleosome. Although nucleosome occlusion will prevent the binding of some TALEs, the data we present here suggest that in the absence of such occlusion, TALEs can bind to repressed chromatin at least in some cells in the population. Together with the recent report that TALEs synergise to perform their function, our data reinforce the idea that using a series of TALEs with adjacent binding sites [23, 24] is likely to increase the chances of TALE proteins being effective *in vivo*.

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## Author contribution

JNFS, APK, CMK and JB designed and performed the experiments, analysed the data and wrote the paper. RT analysed the data and wrote the paper.

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## Figure legends

### Figure 1: TALE binding *in vitro*.

A) Schematic of the TALE binding site in the J $\lambda$ 1 gene. A triangle indicates the J $\lambda$ 1 recombination signal sequence (RSS); the grey rectangle (T) indicates the TALE-A2 binding site; arrows indicate PCR primers (1 and 2; Supplementary Table 1). B) TALE A2 binding to DNA and nucleosomes reconstituted with Fragment 1. Increasing amounts of TALE A2 (11.5, 23 and 46 nM) are indicated. A TALE/DNA complex was formed with the free DNA that has the same mobility as the nucleosome. C) Supershift of the TALE/DNA complex on Fragment 1 with streptavidin. Increasing amounts of streptavidin are indicated. D) TALE A2 binding to DNA and nucleosomes reconstituted with Fragment 2. Increasing amounts of TALE A2 were added as for (B) except that the TALE binding site is at the 5' end of the DNA fragment. The mobility of the nucleosome reconstituted with Fragment 2 is similar to the hexasome reconstituted with Fragment 1 (Supplementary Figure 3).

### Figure 2: TALE binding *in vivo*.

A) Transcription of the J $\lambda$ 1 gene in NIH3T3 (N) and 103/BCL-2 (B) cells. cDNA levels were normalised to the *Hprt* gene. The relative levels of J $\lambda$ 1 transcripts are shown. B) ChIP analysis of TALE binding *in vivo*. Bound and input DNA were normalised by PicoGreen and verified by PCR of *Gapdh*. The relative fold increase of binding to the TALE A2 site is shown. Error bars show standard error. C) Expression of TALE A2 in 103/BCL-2 and NIH3T3 cells. Extracts from equivalent numbers of transfected cells were used in a band-shift assay to verify HA-tagged TALE expression and binding.

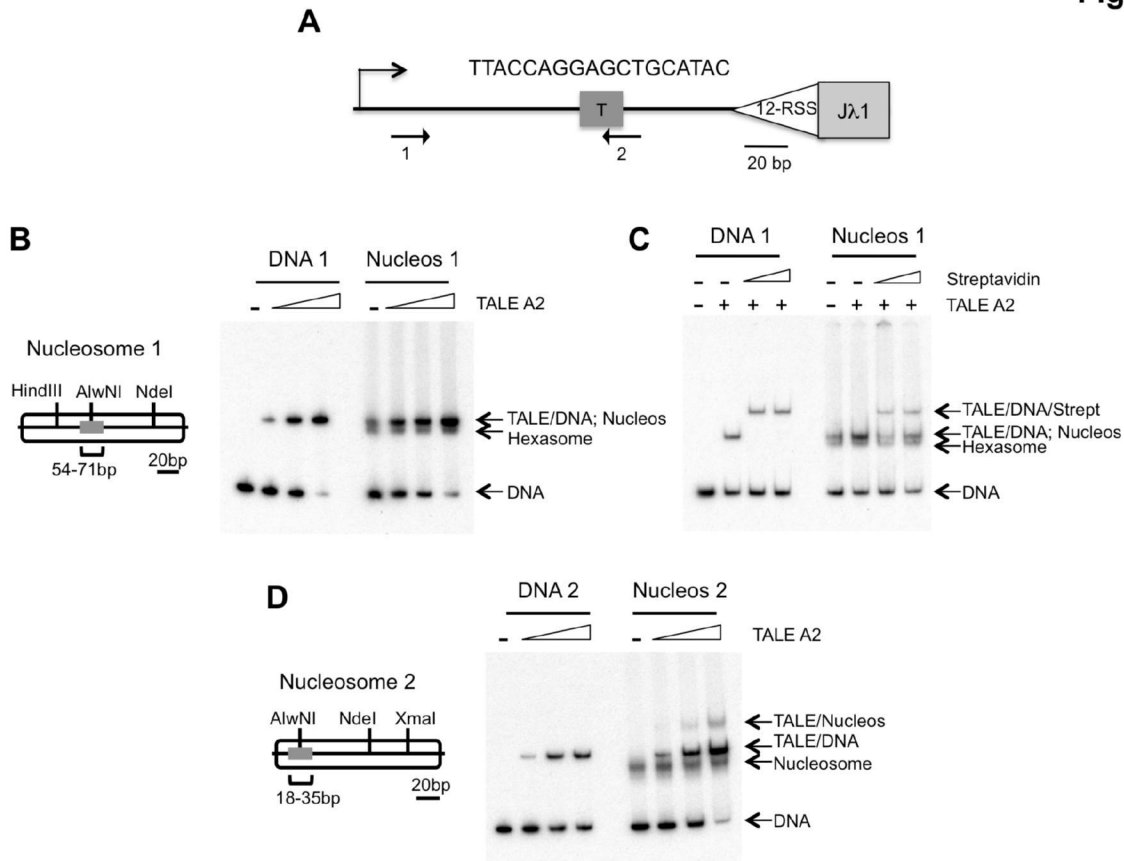
### Figure 3: Levels of histone H3 acetylation

Levels of histone H3 acetylation at the TALE binding site (J1Sty) and adjacent J $\lambda$ 1 RSS (J1RSS) compared to positive (CD19) and negative (Int III) controls. Black and grey bars indicate the absence and presence of  $\alpha$ -amanitin, respectively. Error bars show standard error. The schematic shows the positions of the Int III (11 & 12) and J1RSS (15 & 16) primers in the Ig $\lambda$  locus [25]. The data, excluding CD19 is shown in expanded form in Supplementary Figure 5.

### Figure 4: Accessibility of the TALE binding site *in vivo*.

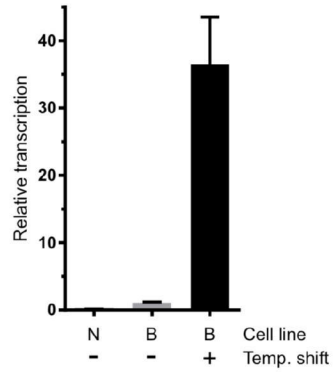
A) Accessibility of the TALE binding site. Left: Schematic showing the Sph I and TALE binding site. The triangle indicates the RSS; arrows indicate PCR primers (13, 14, 15 & 16; Supplementary Table 1). Right: Per cent accessibility of the Sph I site in the cell lines indicated; ts refers to temperature shift. B) Nucleosome repeat length in NIH3T3 and 103/BCL-2 cells determined by electrophoresis of micrococcal nuclease digests. C) Nucleosome positioning in NIH3T3 and 103/BCL-2 cells. Upper: Schematic of the J $\lambda$ 1 region showing the restriction enzymes and probe used. Lower: Southern blots to determine nucleosome positioning. The presence of signal in all lanes indicates that sample was not lost during the experiment. Nucleosome positioning was detected in an equivalent experiment using a probe adjacent to the *Ig $\kappa$*  3' enhancer [25].

**Figure 1**

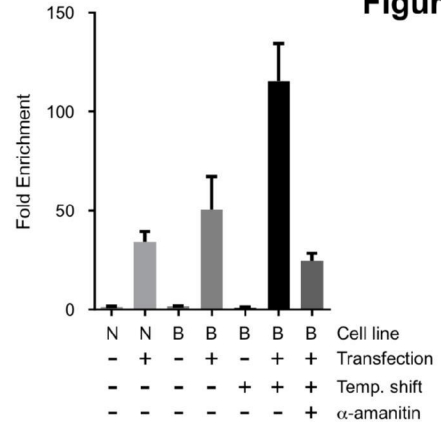


**Figure 2**

**A**



**B**



**C**

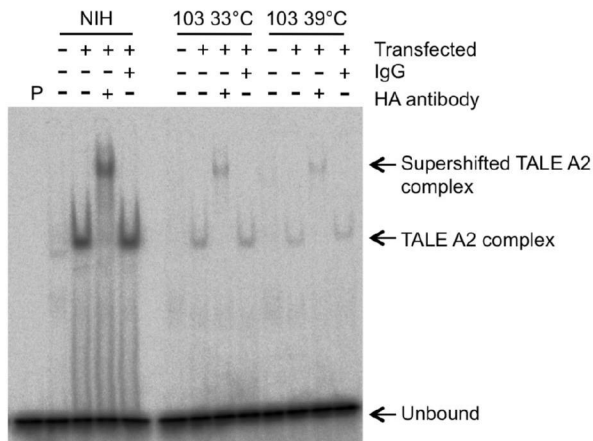
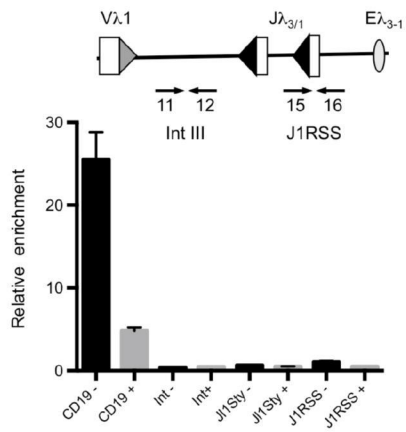


Figure 3



**Figure 4**

