Exploring Antibodies and Rationally Designed Readers for Genomic Mapping of Histone Monoubiquitin

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Background

Chromatin function is regulated by reversible histone post-translational modifications (PTMs) including lysine monoubiquitination (Kub1). Multiple Kub1 sites are involved in gene silencing (e.g., H2AK119) or activation (e.g., H2BK120), and marking DNA double-strand breaks (e.g., H2AK13/15). Misregulation of histone Kub1 has been linked to the pathogenesis of diverse diseases. However, the PTM class is a challenging study target due to a lack of physiologically relevant substrates and reliable, well-characterized detection reagents. Here we describe the characterization of diverse tools to overcome these issues and enable the accurate genomic mapping of histone Kub1. We set out to identify truly-capable genomic mapping reagents from two classes: **1)** ubiquitin and ubiquitinated-histone antibodies; and **2)** recombinant Kub1-nucleosome readers from a rational structure-guided approach. We used a high throughput, multiplexed platform (dCypher™ Luminex) to assess antibody / nucleosome reader binding to Kub1-peptides or PTM defined nucleosomes (Kub1-dNucs™) under conditions that mimic genomic mapping with CUT&RUN (Cleavage Under Targets and Release Using Nuclease). Most antibodies failed to bind Kub1-dNucs or lacked specificity for their purported target; exceptions being an **anti-H2AK119ub1** and a pan-Kub1. However, we also identified rationally designed Kub1-dNuc readers of **H2AK15ub1**, **H2BK120ub1** and **pan-Kub1**. Unsurprisingly, histone Kub1-peptides did not predict antibody or Kub1-nucleosome reader behavior towards Kub1-dNuc substrates. We next explored using antibodies / nucleosome readers to study the genomic enrichment of Kub1 PTMs by CUT&RUN, including a spike-in panel of fully defined dNuc standards to provide an *in situ* metric of reagent capability and assay performance. We showed that reagent behavior with dCypher Luminex was recapitulated by CUT&RUN and are able to effectively map Kub1 associated with gene repression (H2AK119ub1), gene activation (H2BK120ub1) and DNA DSB repair (H2AK15ub1, pan-Ub1). Such findings demonstrate the power of PTM-defined physiological substrates to characterize histone-PTM antibodies / readers and perform truly insightful genomic studies.

Survey of Ub and ubiquitinated-histone antibodies identifies Kub1-nucleosome specific antibodies

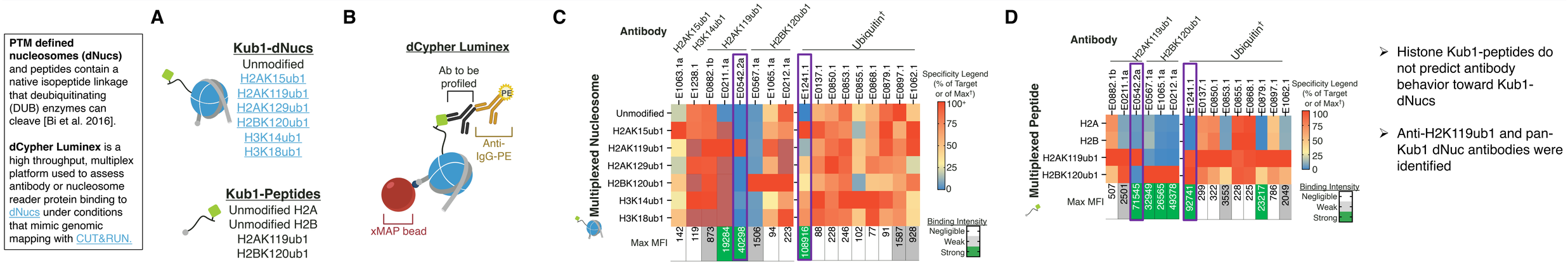

Figure 1. Survey of antibodies targeting ubiquitin and ubiquitinated-histones using PTM-defined Kub1-nucleosomes and -peptides under conditions that mimic genomic mapping with CUT&RUN. **A)** Schematic of PTM-defined nucleosome and peptide panels with a biotin group attached to the 5'-end of nucleosomal DNA or N-terminal of peptides. **B)** DCypher Luminex assay platform where biotinylated nucleosomes or peptides are bound to streptavidin coated, uniquely identifiable xMAP beads. Antibody binding to Kub1- nucleosome or -peptides is assayed in multiplex and non-specific binding is mitigated through a series of wash steps. **C)** Anti-histone Kub1 and Ub antibodies were screened for specificity by dCypher Luminex with the Kub1-nucleosome panel. Each antibody was tested at three dilutions (1:250, 1:1000, 1:4000) and that with the greatest percent specificity to the reported target is shown. Max MFI (median fluorescence intensity) indicates binding strength. MFIs were normalized to on-target MFI (Kub1 Abs) or Max MFI (Ub Abs) to determine percent specificity. **D)** Antibodies to H2AK119ub1, H2BK120ub1 or Ub were tested with the Kub1-peptide panel at three dilutions (1:16000, 1:64000, 1:256000). The optimal dilution and specificity are as described in (C).

Novel Kub1-nucleosome readers engage the nucleosome acidic patch and monoubiquitin

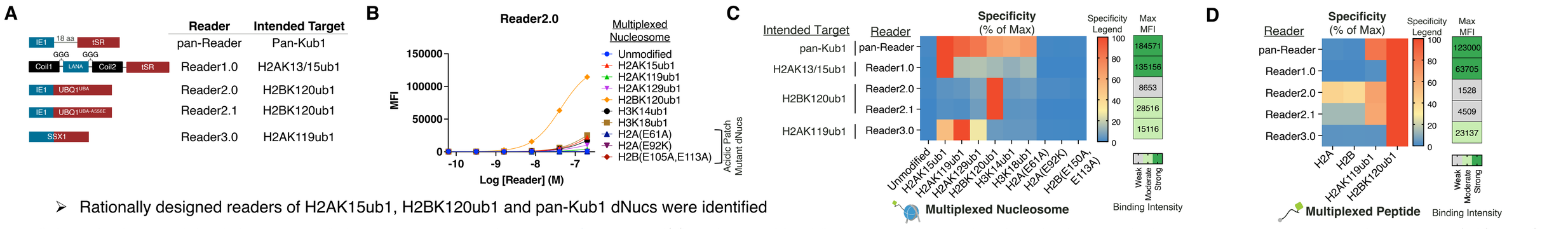

Figure 2. Binding strength and specificities of rationally designed Kub1-nucleosome readers were determined using dCypher Luminex **A)** Schematic representation of Kub1-nucleosome readers and their intended targets. Acidic patch *Anchor* domains: blue, *Linkers*: black, ubiquitin binding domain (*UBD*): red. **B)** The binding strength and specificity of all readers was tested by dCypher Luminex (**Figure 1B**) including three additional acidic patch mutant dNucs. Characterization of Reader2.0 shown under conditions that mimic genomic mapping with CUT&RUN. Binding to the unmodified nucleosome is disrupted by acid-patch mutations (as would be expected given the mode of engagement). **C)** The binding strength and specificity of each Kub1-nucleosome reader compared under optimized conditions as determined in (B): pan-Reader, 200 nM; Reader1.0, 8 nM; Reader2.0 / 2.1, 40 nM; Reader3.0, 200 nM. MFI were normalized to max MFI to determine percent specificity. **D)** The binding strength and specificity of each Kub1-nucleosome reader toward Kub1-histone peptides was similarly tested as in (C): (pan-Reader; 40 nM, Reader1.0; 8 nM, Reader2.0 / 2.1 / 3.0; 200 nM). Excluding the pan-Reader, Kub1-peptide binding profiles lack a clear link to their target nucleosome, underscoring the importance of both the acidic patch anchor and the UBD for Kub-dNuc readers' binging to the Kub1 nucleosomal target.

Conclusions

- Kub1-dNucs are an invaluable tool for studying histone lysine mono-ubiquitin.
- A survey of Ub and histone Kub1 antibodies identified an H2AK119ub1 and pan-Kub1 dNuc antibody while the majority failed to bind to Kub1-dNucs or were not specific for their target *in vitro.*
- Rationally designed readers of H2AK15ub1, H2BK120ub1, and pan-Kub1 dNucs were identified that can enable CUT&RUN studies where antibodies are unavailable.
- Kub1-histone peptides do not predict antibody or reader behavior toward Kub1-dNucs.
- CUTANA CUT&RUN genomic mapping with *in situ* SNAP-CUTANA spike-in controls using Luminex triaged reagents provides a powerful platform for studying Kub1-histone-PTMs.

References

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Kub1-dNuc standards support insightful genomic mapping of Kub1 PTMs

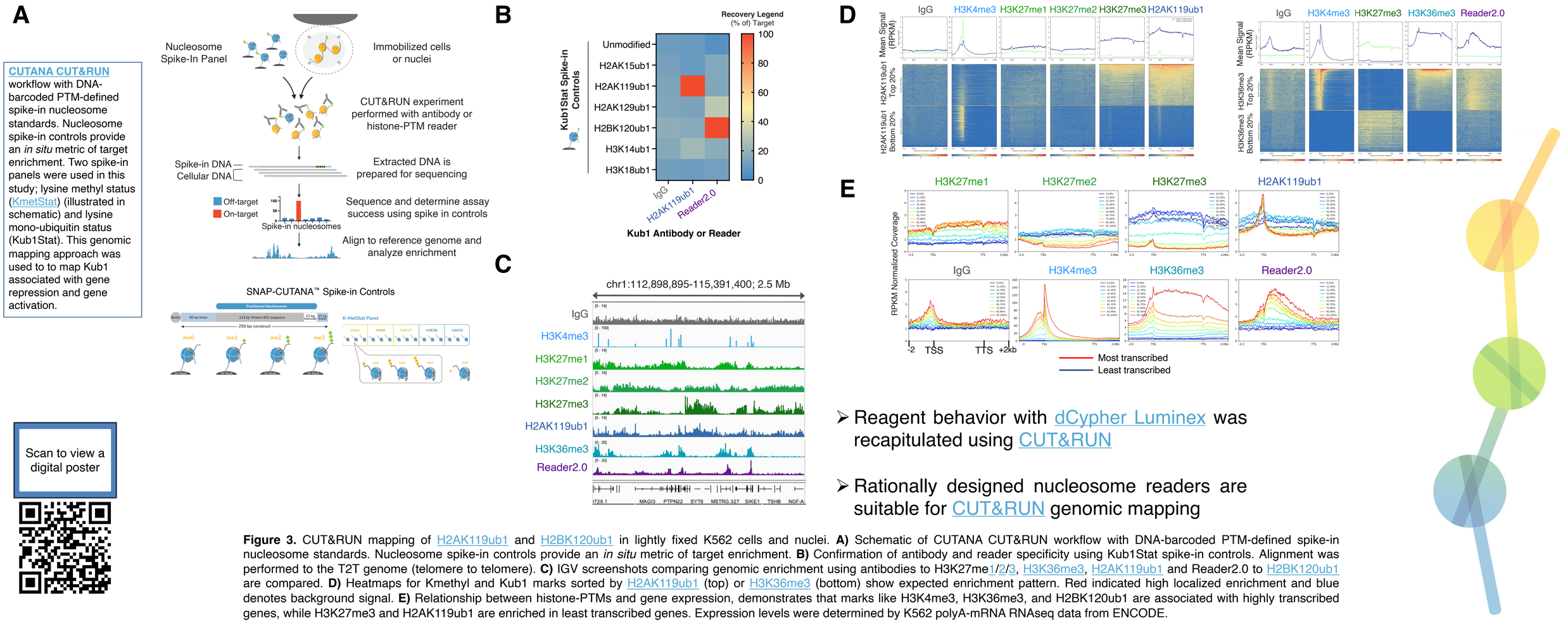

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Figure 3. CUT&RUN mapping of H2AK119ub1 and H2BK120ub1 in lightly fixed K562 cells and nuclei. **A)** Schematic of CUTANA CUT&RUN workflow with DNA-barcoded PTM-defined spike-in nucleosome standards. Nucleosome spike-in controls provide an *in situ* metric of target enrichment. **B)** Confirmation of antibody and reader specificity using Kub1Stat spike-in controls. Alignment was performed to the T2T genome (telomere to telomere). **C)** IGV screenshots comparing genomic enrichment using antibodies to H3K27me1/2/3, H3K36me3, H2AK119ub1 and Reader2.0 to H2BK120ub1 are compared. **D)** Heatmaps for Kmethyl and Kub1 marks sorted by H2AK119ub1 (top) or H3K36me3 (bottom) show expected enrichment pattern. Red indicated high localized enrichment and blue denotes background signal. **E)** Relationship between histone-PTMs and gene expression, demonstrates that marks like H3K4me3, H3K36me3, and H2BK120ub1 are associated with highly transcribed genes, while H3K27me3 and H2AK119ub1 are enriched in least transcribed genes. Expression levels were determined by K562 polyA-mRNA RNAseq data from ENCODE.