Nucleosome-based screening in the context of epigenetic diversity enables improved methods to study histone modifications as disease biomarkers

Natalia L.H. Phillips[1], Nathan Hall[1], Anup Vaidya[1], Zachary B. Gillespie[1], Rachel L. Watson[1], Matthew R. Marunde[1], Matthew J. Meiners[1], Marcus A. Cheek[1], Sarah A. Howard[1], Shruti Saxena-Beem[2], Susan L. Gloor[1], Zu-Wen Sun[1], Keith Maier[1], Martis Cowles[1], Saira Z. Sheikh[2], Jonathan M. Burg[1], Michael-Christopher Keogh[1] & Andrea L. Johnstone[1]

[1] EpiCypher, Inc., Research Triangle Park, NC 27709, USA.

[2] Department of Medicine, Division of Rheumatology, Allergy and Immunology, The University of North Carolina, Chapel Hill, NC, 27517, USA.

Background: Accurate & reliable assays are needed to quantify histone citrullination (H3Cit)

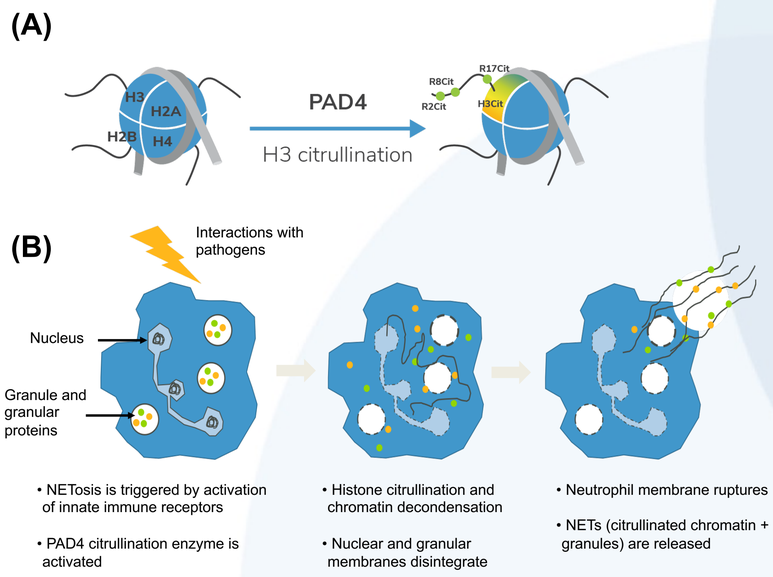


Figure 1. (A) Peptidylarginine deiminase 4 (PAD4) converts arginine to citrulline on nucleosome-associated histones **[6]**. This post-translational modification (PTM) underlies many pathophysiologies, including vascular disease **[6]**, autoimmunity **[3]**, viral infections **[1]**, and cancer **[7]**. **(B)** Histone hypercitrullination is a hallmark of PAD4-dependent neutrophil extracellular traps (NETs), where chromatin is decondensed and expelled into plasma **[6]**. Thus, citrullinated histones are a promising blood biomarker for PAD4-dependent diseases where NETs are a contributing factor (*e.g.* thrombosis **[6]**, rheumatoid arthritis **[4]**, coronavirus **[1]**, septic shock **[3]**).

Identification of an optimal citrulline antibody for capture and detection of citrullinated nucleosomes

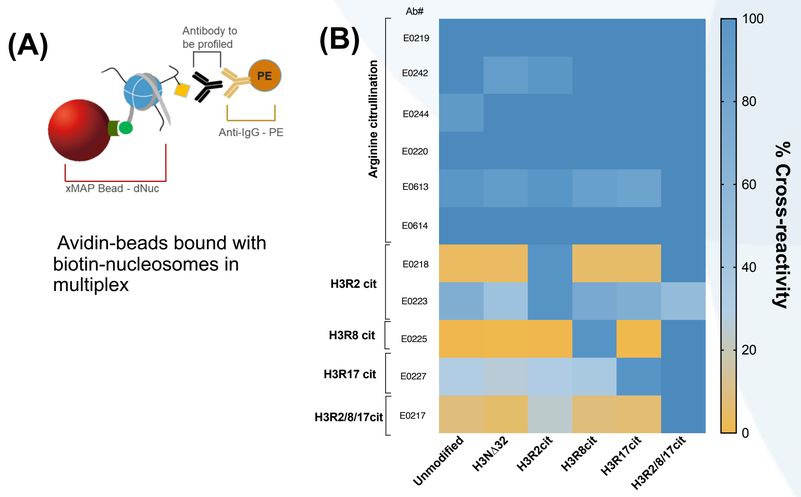


Figure 2. The specificity of commercial 'anti-citrulline' antibodies was determined in multiplex against a panel of PTM-defined nucleosomes **[6]**. **(A)** Assay setup with Luminex xMap® technology. **(B)** Unmodified (WT), H3 truncated (H3NΔ32), or PTM-defined nucleosomes (H3R2cit, H3R8cit, H3R17cit, and H3R2/8/17cit) were created by EpiCypher dNuc™ and versaNuc™ approaches. Three antibodies [E0218, E0225, E0217] recognize their target with <10% cross-reactivity (relative signal for each reagent normalized to its reported target). However, most antibodies fail to differentiate their reported target within the test panel (including unmodified nucleosome).

Best-in-class anti-H3R8Cit fails to detect disease-associated changes when paired with DNA detection

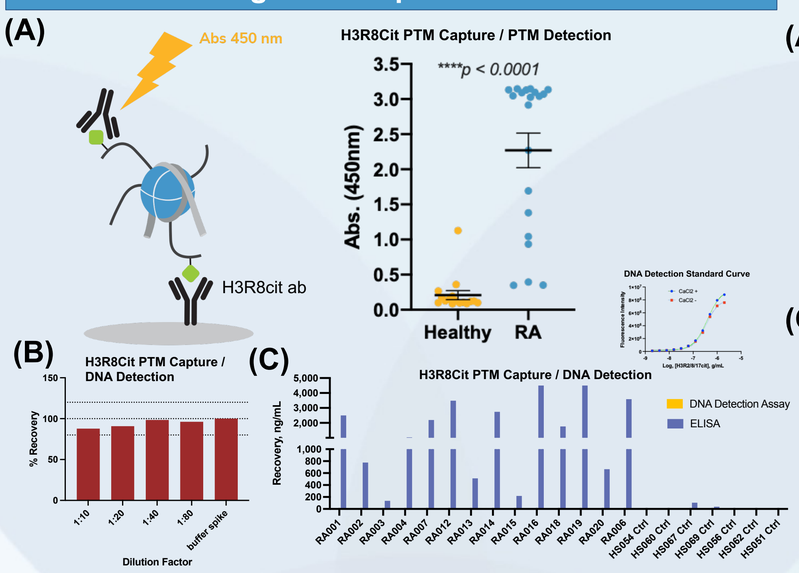


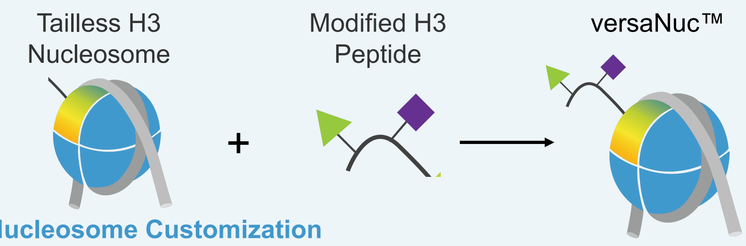
Figure 3. (A) Using the best commercially available anti-citrullinated Histone [E0225; anti-H3R8cit; Abcam ab232939], we developed an ELISA to capture / detect citrullinated nucleosomes in clinical samples (human plasma). This confirmed significantly more target recovery in rheumatoid arthritis (RA) *vs.* healthy controls. **(B)** Spike-in and recovery of semi-synthetic H3R2,8,17cit nucleosomes quantified by DNA detection confirms assay functionality (plasma dilution factor [to explore potential inhibition] is noted). **(C)** Follow-on studies suggested minimal recovery of the DNA that would be expected to accompany nucleosomes. This appears due to anti-H3R8cit capturing additional citrullinated proteins (not shown), and prompted a full assay re-design, including the development / characterization of more anti-citrulline reagents.

Generation of combinatorial PTM versaNuc™ panel to assess antibody blind spots

Example PTMs on H3 tail (aa1-18) :

A R T K Q T A R K S T G G K A P R K

cit, ac, me1, me2, me3 | cit, ac, me1, me2, me3 | phos | ac | cit, ac



Nucleosome Customization

- DNA template: 147x601 (± biotin)
- Histone: H3.1 NΔ32

Figure 4., **(A)** Endogenous histones can contain multiple PTMs, which could impact the ability of an antibody to detect / recover its PTM target. **(B)** A PTM-defined versaNuc™ panel was assembled to assess the impact of neighboring modifications on antibody binding to H3 citrullination in the nucleosome context. This panel was used to characterize new monoclonal antibodies (mAbs) raised against a peptide immunogen (H3 [aa1-25] R2cit, R8cit, R17cit).

Novel H3Cit mAbs have improved binding profiles for combinatorial PTM context

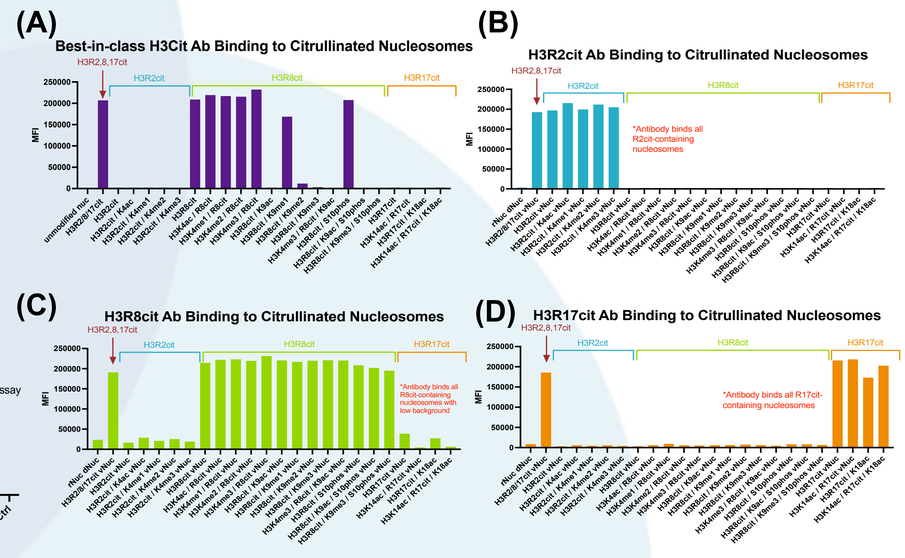


Figure 5. (A) Commercial anti-H3R8cit [Abcam ab232939] is blind to multiple PTM contexts (*e.g.* H3R8cit with H3K9me2 or H3K9me3: these Kmes respectively comprise 40% and 20% of all endogenous nucleosomes **[5]**). Consequently, this reagent fails to comprehensively detect H3R8cit in clinical samples. **(B-D)** A range of improved binding profiles on PTM-defined nucleosomes were identified within 88 newly developed H3Cit mAbs. Note that antibody linear paratopes can vary in size but typically extend for 6 to 8 amino acids **[2]**. **(B)** anti-H3R2cit; **(C)** anti-H3R8cit **(D)** anti-H3R17cit. Collectively, these antibodies (and others not shown) could be used to comprehensively detect endogenous H3cit.

Conclusions

- **Most commercial anti-citrulline Abs have major specificity issues**
- **The best commercial H3R8cit is primarily binding a non-nucleosome target in Rheumatoid Arthritis plasma** (and is also blind to many potential endogenous PTM contexts)
- **The dNuc™ / versaNuc™ platforms are invaluable** for screening antibody capability in the context of epigenetic diversity
- **Evaluating antibody blind spots is critical** if these reagents are to have use with endogenous samples
- **EpiCypher's anti-citrulline mAbs are perfectly poised to study the involvement of PAD4 in human disease** (see below)

References

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Nucleosome screened anti-citrulline mAbs are now being used to study the involvement of PAD4 in human disease

- Using physiological nucleosome substrates, PAD4 citrullinates effectively at H3R8 and H3R17 / poorly at H3R2
- PAD4-mediated histone citrullination is largely independent of flanking PTMs (so H3Cit antibodies have to be multi-capable)
- Novel H3Cit mAbs developed with rigorous nucleosome-based screening are agnostic to combinatorial PTMs (as single or pooled reagents)
- This work showcases the importance of physiological screening tools and will enable the reliable quantification of H3Cit in clinical samples

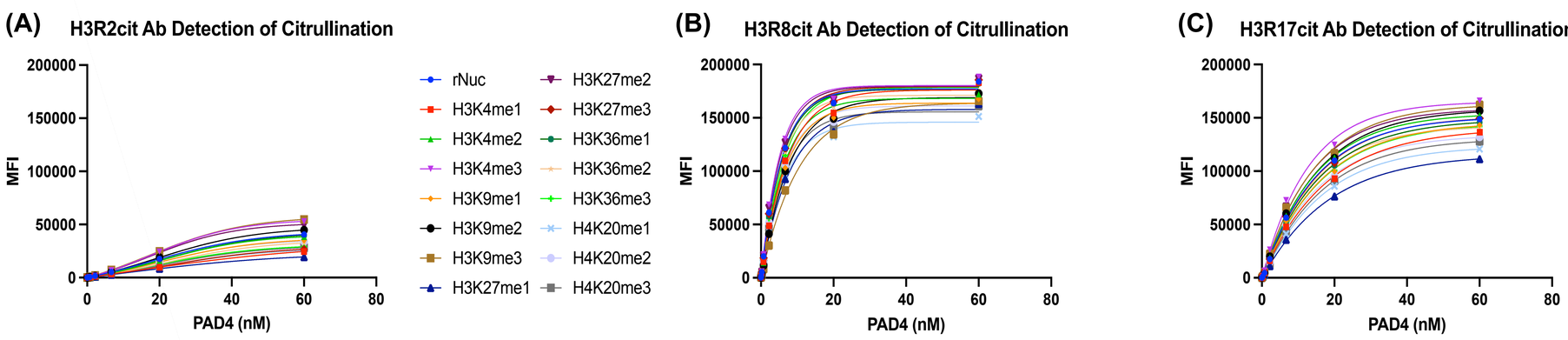

Figure 6. Pre-exiting lysine methylations have a minor impact on PAD4 H3 tail citrullination. However H3R2cit may not be as physiologically relevant as H3R8cit and H3R17cit (H3R2 is certainly a less effective substrate). PAD4 activity was assessed by Luminex xMap technology (as in **Figure 2a**) with new mAbs (e.g. those from **Figure 5**). Nucleosomes on Luminex beads were incubated with PAD4 at 37ºC for 30 minutes and detected with anti-citrulline mAb and secondary anti-IgG*PE. PAD4 citrullination at H3R2 **(A)** is less efficient than at H3R8 **(B)** and H3R17 **(C)**.