Novel antibody development enables new insight into citrullination as a regulated modification

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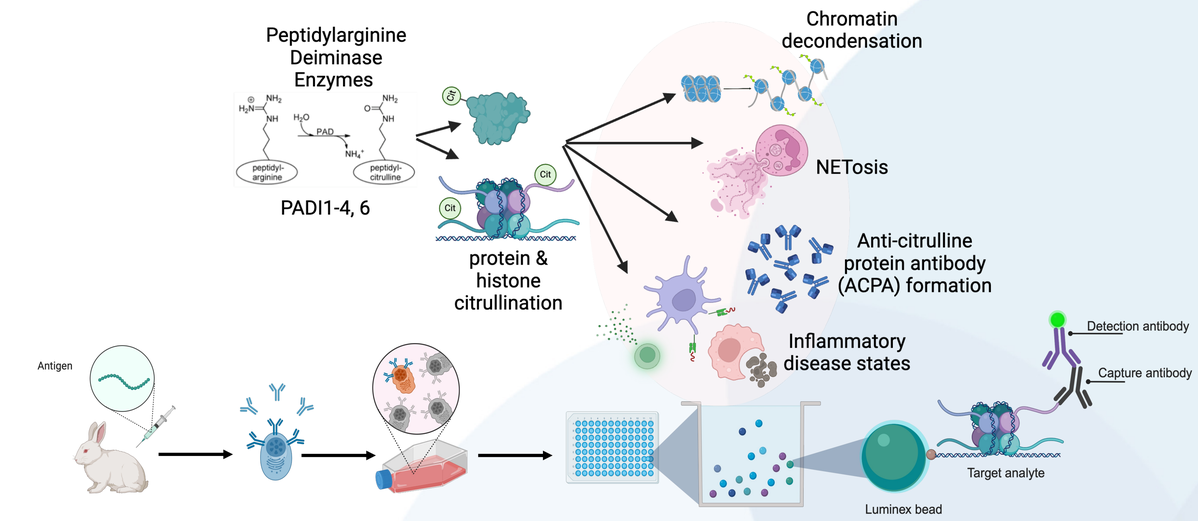
Citrullination is a potential biomarker for inflammatory disease states

PADI enzymes and elevated citrulline levels are strongly associated with inflammatory disease states, including rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, psoriasis, type I diabetes and cancer. A family of five calcium-dependent peptidylarginine deiminase enzymes, (PADI1, PADI2, PADI3, PADI4 and PADI6) with tissue and cellular specificity control the conversion of arginine residues to citrulline. This post-translational modification of histones and other cellular proteins can give rise to the formation of anti-citrulline protein antibodies and is important for NETosis, a neutrophil-specific immune response. Histones are a key target of PADI enzymes, converting the positively charged arginine residues to neutral citrulline residues on histone tails. This impacts the interaction of the histones with negatively charged DNA resulting in chromatin decondensation, a process necessary for NETosis and important to epigenetic regulation more generally. To date, the lack of an erasing enzyme for citrulline modifications has led to the idea that this mark is irreversible. Furthermore, in the absence of precise reagents the function of multiple citrulline modifications have been conflated despite evidence of target specificity amongst PADI enzymes. To investigate the role of citrulline in inflammatory pathways we embarked on a program to develop highly specific antibodies against histone citrullination marks. We created panels of nucleosomes with defined modifications against citrulline and neighboring histone PTMs to rigorously screen antibodies using a nucleosome-based multiplex screening system to identify highly specific clones that are minimally impacted by surrounding non-citrulline marks.



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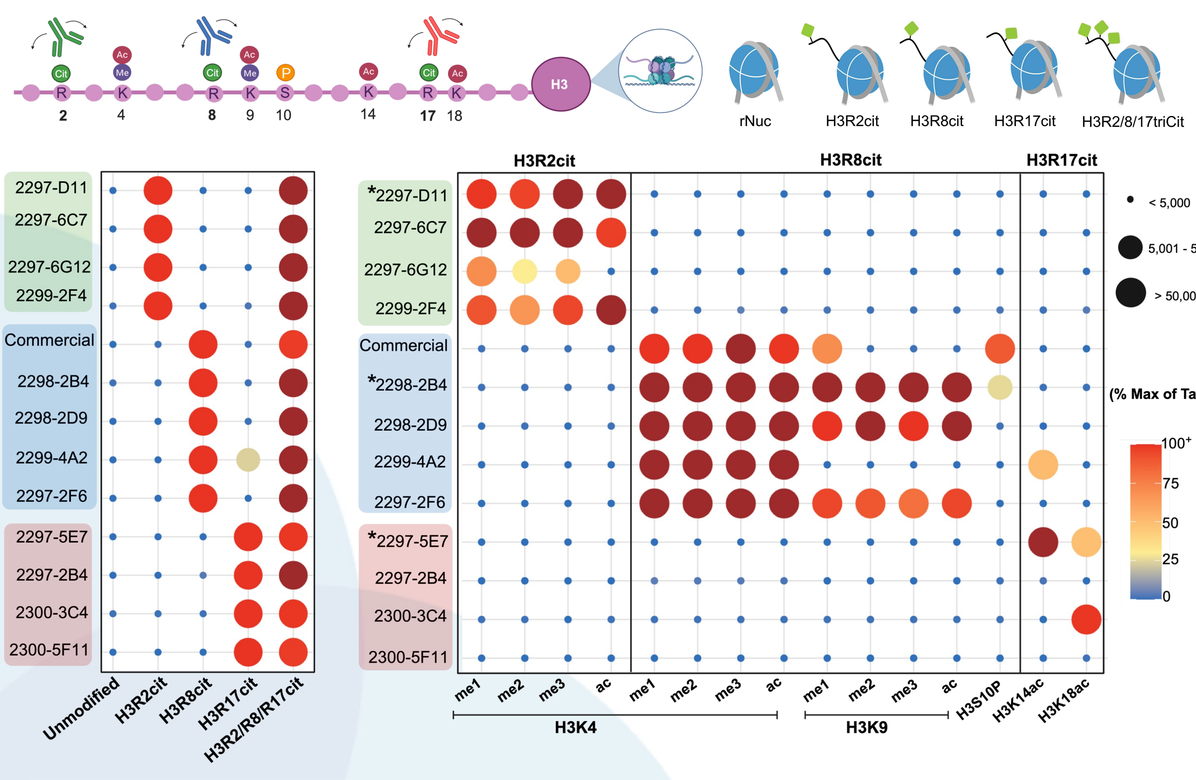
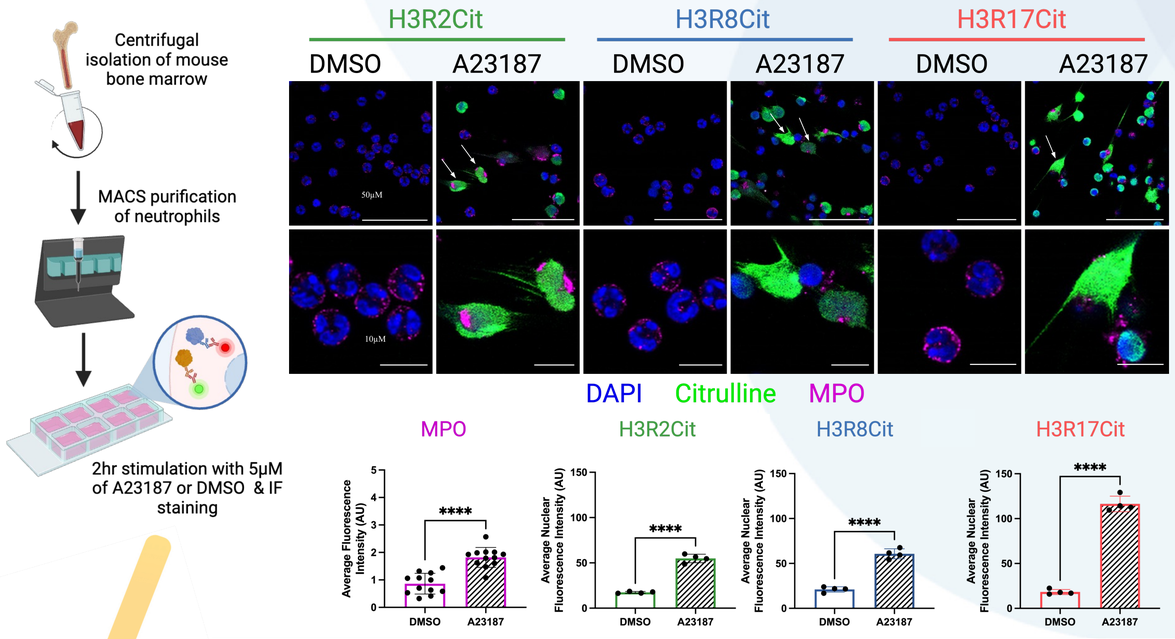
Nucleosome based screening for highly specific anti-H3RCit antibodies

Using precisely modified nucleosomes we screened antibody clones specific to H3R2Cit, H3R8Cit and H3R17Cit to identify antibodies that were both highly specific to their target residue and uninhibited by common neighboring PTMs using a Luminex-based system.



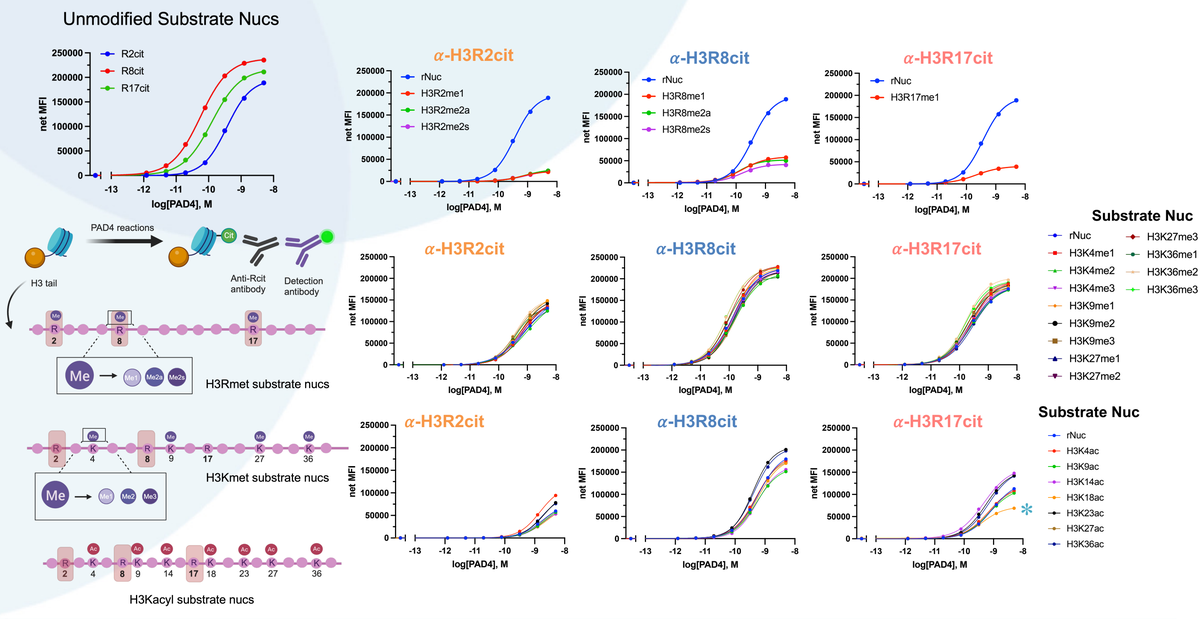
Antibodies specific to H3R2Cit, H3R8Cit and H3R17Cit mark neutrophils undergoing NETosis

To validate our antibodies in a cellular context, we isolated neutrophils from mouse bone marrow, induced NETosis with a calcium ionophore (A23187) and performed immunofluorescence in conjunction with the nuclear marker DAPI and myeloperoxidase (MPO). Strong signal was detected for H3R2Cit, H3R8Cit and H3R17Cit in stimulated conditions and correlated with increases in MPO and changes in nuclei morphology, consistent with NETosis.



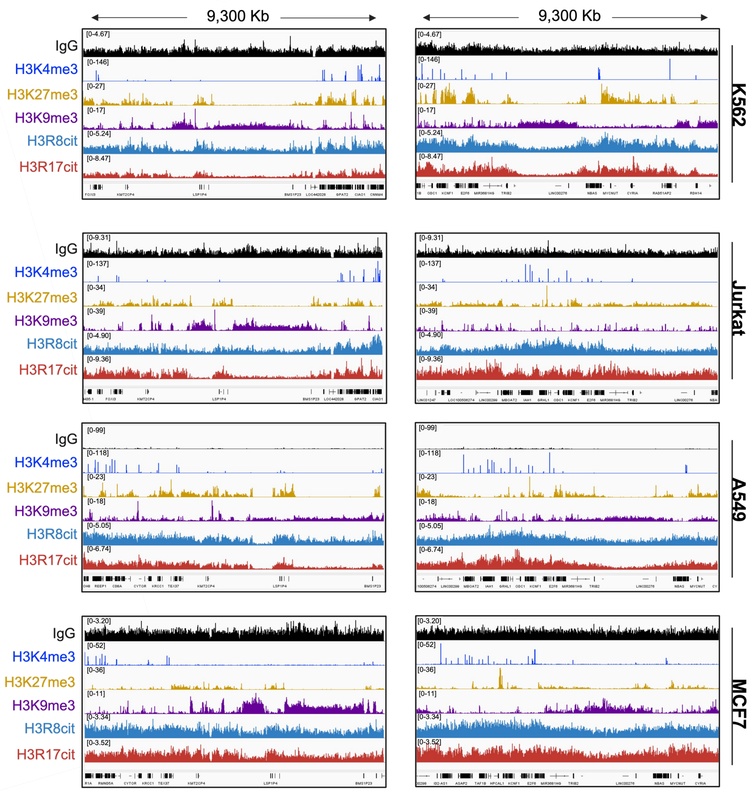
PADI4 is inhibited by pre-existing arginine methylation but unaffected by neighboring lysine modifications

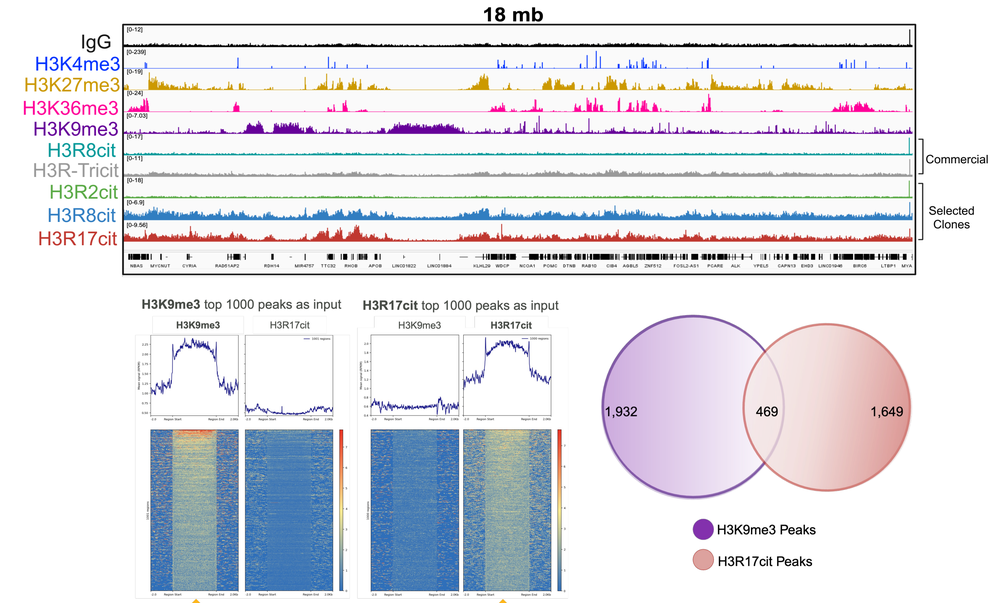
In vitro Luminex assays with unmodified nucleosomes showed preferred binding of PADI4 to R8 residues over R17 residues with R2 being its least preferred substrate. Using panels of nucleosomes with defined modifications we found that prior methylation of R2, R8, R17 severely impacted the ability of PADI4 to citrullinate these residues. However, methylation or acetylation of neighboring lysine residues had minimal impact on the activity of PADI4.



Novel H3R8Cit and H3R17Cit antibodies reveal defined peak structures across the genome of proliferating cancer cells

To further understand the capabilities of these novel antibodies we tested their compatibility with our CUT&RUN assay and surprisingly identified distinct peak structures for R8 and R17 across the genomes of different cancer cell lines. Further analysis found that these citrulline domains were anti-correlated across multiple cell types with the presence of H3K9me3, a marker of constitutive heterochromatin. The presence of wide-spread citrulline domains raises many questions about the function of citrulline as a regulated histone modification and its impact on chromatin and gene regulation. It calls into question its presumed irreversibility in the absence of an erasing enzyme and possible differences that may exist between specific citrulline residues.





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