



Metabolic imprint induces histone hypermethylation during *Chlamydia trachomatis* infection

Chloé Charendoff

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Sorbonne Université

École doctorale Complexité du Vivant

Unité de Biologie Cellulaire de l'Infection Microbienne

Metabolic imprint induces histone hypermethylation during *Chlamydia trachomatis* infection

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ABSTRACT

Chlamydia trachomatis is an obligate intracellular bacterium that proliferates exclusively in human hosts. Its genome has undergone intensive gene reduction and is missing many genes coding for essential metabolic pathways. As a consequence, the bacteria rely on their host cell to obtain several nutrients. Bacterial sequestration of metabolites shifts host cell metabolism which may impact many cellular processes. In this thesis, we sought to understand if a metabolic imbalance could account for epigenetic modifications in the host, and to understand its consequences.

During late *C. trachomatis* infection, we observed increased histone methylation at multiple histone lysine residues, despite the antagonistic nature of some of these modifications. Importantly, the level of hypermethylation was cell-dependent and all the histone marks tested were expressed at similar level for every individual cell, indicating co-regulation. We hypothesized that a decrease in the activity of histone demethylases, the enzymes responsible for removing methyl groups from the N-terminal tails of histones, might account for this hypermethylation phenotype. A large family of demethylases, the Jumonji domain - containing (JmjC) demethylases, require oxygen, iron and α -ketoglutarate as co-factors to carry out their function, all metabolites that are used by *C. trachomatis*. In support of our hypothesis, we observed that infected cells were sensitized to inhibition of this family of histone demethylases, compared to non-infected cells.

Our experiments suggest that iron availability is not limiting during infection. In contrast, supplying cell permeable dimethyl-ketoglutarate (DMKG) in the culture medium prevented hypermethylation of histones during late infection. This result indicates that histone hypermethylation is a consequence of a metabolic imbalance, likely caused by α -ketoglutarate consumption, or its precursors, during infection. At the transcriptional level, we observed that DMKG supplementation affected about one third of the genes that are differentially expressed late in infection. DMKG supplementation resulted in gene up-regulation and in gene down-regulation in similar proportion, indicating that histone hypermethylation can have opposite consequences on gene expression, that need to be analyzed at the individual gene level. Finally, histone hypermethylation correlated with the presence of indicators of DNA damage in the nuclei of infected cells. DMKG supplementation prevented DNA damage, confirming a link between these two phenotypes.

To conclude, we demonstrated that *C. trachomatis* infection generates a metabolic imbalance in the host, causing histone hypermethylation late in infection. This metabolic imprint of infection at the epigenetic level has an impact on the host transcriptomic response to infection and on the integrity of its DNA.

RÉSUMÉ

Chlamydia trachomatis est une bactérie intracellulaire obligatoire adaptée exclusivement à l'Homme. Son génome, très réduit, a perdu plusieurs gènes codant pour des voies métaboliques essentielles. En conséquence, les bactéries dépendent de l'hôte pour obtenir plusieurs nutriments. La séquestration de métabolites par les bactéries crée des déséquilibres, avec des conséquences potentielles sur plusieurs processus cellulaires de l'hôte. Dans cette thèse nous avons entrepris de déterminer si un déséquilibre métabolique pourrait être à l'origine de modifications de l'épigénome de l'hôte, et de comprendre ses conséquences.

Nous avons observé que l'infection s'accompagne, tardivement, d'une augmentation de marques de méthylation sur plusieurs lysines, et ce en dépit du caractère antagoniste de certaines de ces marques. Le niveau de méthylation dépend des cellules, et pour une cellule donnée, toutes les marques testées sont exprimées à des niveaux similaires, indiquant un phénomène de co-régulation. Nous avons émis l'hypothèse qu'il puisse découler de l'inhibition d'enzymes à activité déméthylase. La grande famille des déméthylases d'histones contenant un domaine Jumonji (déméthylases JmjC) utilisent l'oxygène, le fer et l' α -ketoglutarate, métabolites qui sont tous utilisés par *C. trachomatis*. L'observation que les cellules infectées sont sensibilisées à un inhibiteur de cette famille d'enzymes soutient l'hypothèse d'un fonctionnement réduit de ces enzymes dans les cellules infectées.

Nos expériences indiquent que la disponibilité en fer n'est pas limitante durant l'infection. En revanche, l'apport de diméthyl-ketoglutarate (DMKG), un substitut d' α -ketoglutarate, permet d'abolir l'hyperméthylation des histones. Ce résultat indique que ce phénotype est le résultat d'un déséquilibre métabolique, probablement causé par la consommation bactérienne d' α -ketoglutarate ou de ses précurseurs durant l'infection. L'apport en DMKG modifie le niveau de transcription d'environ un tiers des gènes qui montrent une expression différentielle tard dans l'infection. Les conséquences sont à la fois négatives et positives, et ce dans des proportions similaires, indiquant que l'hyperméthylation des histones peut avoir des conséquences opposées sur l'expression des gènes, qui doit être analysée au niveau individuel. Enfin, nous avons observé que l'hyperméthylation des histones corrélait avec la présence d'indicateurs de cassures de l'ADN dans les noyaux des cellules infectées. L'apport en DMKG résulte en une perte de ces cassures, confirmant le lien entre ces deux phénotypes.

En conclusion, nous avons démontré que l'infection par *C. trachomatis* crée un déséquilibre métabolique chez l'hôte, à l'origine d'hyperméthylation des histones. Cette empreinte métabolique de l'infection au niveau épigénétique a des conséquences sur la réponse génétique de l'hôte à l'infection et sur l'intégrité de son ADN.

RÉSUMÉ DÉTAILLÉ

Le phylum des *Chlamydiae* est constitué de bactéries intracellulaires obligatoires à Gram négatif. Ses membres se distinguent par un cycle de développement original, se déroulant à l'intérieur d'un hôte eucaryote, pouvant être un protozoaire (amibes par exemple) ou un métazoaire. Le phylum ne compte pour le moment qu'un seul Ordre et qu'une seule Classe, mais plusieurs Familles, telles que les *Chlamydiaceae*. Cette dernière regroupe des pathogènes d'animaux, dont plusieurs ont un impact en santé vétérinaire et humaine. Deux espèces de *Chlamydiaceae* sont couramment retrouvées dans des infections humaines : *Chlamydia trachomatis* et *Chlamydia pneumoniae*. *C. pneumoniae* est capable d'infecter des hôtes variés (koala, cheval, reptiles...) et cause des infections de l'appareil respiratoire chez l'Homme. En revanche, *C. trachomatis* utilise exclusivement l'espèce humaine pour se reproduire. Certaines souches (serovars A à C) infectent la conjonctive de l'œil. En absence de traitement, des infections répétées peuvent conduire à une altération de la cornée. Les séquelles de l'infection donnent lieu à ce qu'on appelle le trachome, première cause de cécité acquise au monde, essentiellement circonscrite à des régions ne bénéficiant pas de condition d'hygiène adéquates. Les autres sérovars sont retrouvés dans toutes les populations et infectent le tractus uro-génital. Les infections sont le plus souvent asymptomatiques, et, non détectées, ne sont pas toujours traitées. A l'origine d'inflammations pelviennes chroniques, de grossesse extra-utérines, elles peuvent être causes d'infertilité. Globalement, l'infection par *C. trachomatis* représente la première infection sexuellement transmissible d'origine bactérienne, et l'on recense plus de 100 millions de nouveaux cas par an.

Le cycle de développement des *Chlamydiae* est désigné par le terme « biphasique ». En effet, deux formes de la bactérie de morphologie et de métabolisme distincts alternent au cours de ce cycle. La forme infectieuse est le corps élémentaire (EB), qui est non répliatif et adapté à la survie en milieu extracellulaire. La forme répliatif est le corps réticulé (RB). Elle n'est pas infectieuse, et ne survit que dans l'environnement intracellulaire à laquelle elle est adaptée. Le cycle infectieux est initié par l'adhésion d'un EB à la surface d'une cellule-hôte, et son internalisation au sein d'une vacuole parasitophore appelée inclusion. L'internalisation de l'EB induit une décondensation progressive du chromosome bactérien, et un changement du programme d'expression de ses gènes, qui accompagne la transformation progressive vers la forme RB. La

première division bactérienne a lieu environ 6 h après l'initiation de l'infection (6 hpi), suivi d'une croissance exponentielle, jusqu'à atteindre plusieurs centaines de bactéries par inclusion. Dès 20 hpi, certains RBs changent de programme transcriptionnel et initient leur conversion en EB, de manière asynchrone, de sorte que 48 hpi, l'inclusion renferme très majoritairement des EBs. Les EBs sont relargués de la cellule-hôte soit parce que celle-ci est lysée, soit par un processus d'extrusion qui laisse l'inclusion intacte, extracellulaire. Les EB libérés commencent un nouveau cycle dans les cellules épithéliales avoisinantes.

Les *Chlamydiaceae* ont perdu de nombreux gènes codant pour des fonctions métaboliques essentielles. C'est un trait commun chez les bactéries intracellulaires, et poussé à l'extrême chez intracellulaires obligatoire telles que les *Chlamydiaceae* qui ne comptent plus qu'un petit millier de gènes. Le corolaire de cette situation est que les bactéries dépendent de l'hôte pour combler les « trous » dans des voies métaboliques essentielles, telles que la synthèse de nucléotides, ou de certains acide aminés. Les bactéries ont acquis un transporteur d'ATP, qui leur permet d'utiliser les ressources énergétiques de l'hôte. La croissance de l'inclusion nécessite elle un apport constant de lipides de l'hôte. Pour faire face à ces besoins, ainsi que pour contrecarrer les défenses innées que possèdent les cellules épithéliales pour éliminer les microbes qui les agressent, les bactéries utilisent des protéines qu'elles sécrètent dans l'inclusion ou dans le cytoplasme, appelées protéines effectrices. Le principal mécanisme de sécrétion des *Chlamydiaceae* est appelé système de sécrétion de type 3, il est commun à plusieurs bactéries à Gram négatif pathogènes. Les protéines effectrices permettent aux bactéries de manipuler l'hôte et de détourner certaines fonctions cellulaires à leur profit. Une des protéines effectrice de *C. trachomatis*, NUE, possède un domaine également trouvé dans des enzymes capables de méthyler des histones. NUE possède effectivement une activité histone méthyl transférase *in vitro*, suggérant que par son intermédiaire *C. trachomatis* puisse modifier l'épigénome des cellules qu'elle infecte.

L'information génétique des cellules hôtes est renfermée dans le noyau, au sein de la chromatine, terme qui désigne l'ensemble de l'ADN et des protéines associées. La lecture de cette information est conditionnée à son accessibilité, elle-même gouvernée par la composition de la chromatine. L'élément de base en est le nucléosome, constitué de 146 paires de bases d'ADN enroulées autour d'un assemblage de 2x4 molécules d'histones (H2A, H2B, H3 et H4). Les histones et l'ADN sont susceptibles de subir des modifications chimiques réversibles qui

influencent l'accessibilité de l'ADN, et donc l'expression génique. Certaines bases de l'ADN peuvent être modifiées par méthylation, tandis que les histones sont l'objet d'une variété de modifications, telles que la méthylation, l'acétylation, la phosphorylation, l'ubiquitylation, surtout concentrées dans la partie N-terminale des histones, appelées queues d'histones. La combinatoire des modifications dites « épigénétiques » affectant les histones constitue un deuxième code, dit « code histones », qui se superpose au code génétique. Cette combinatoire est complexe car plusieurs résidus peuvent être modifiés, avec plusieurs types de modification. Les résidus lysines sont abondants dans les queues d'histones, et sont sujets à un grand nombre de modifications différentes, surtout des acétylations et des méthylations. Ces dernières peuvent concerner un, deux, ou trois groupe méthyl, ajoutant à la complexité de la combinatoire. De ces modifications, ainsi que de l'association d'autres protéines à l'ADN, et des modifications de l'ADN lui-même, va dépendre le degré de condensation de la chromatine. Elle est traditionnellement décrite comme euchromatine, peu condensée, accessible, et hétérochromatine, plus dense, moins accessible, mais ces termes ne rendent pas compte de différences locales qui gouvernent l'expression des gènes concernés. Les modifications épigénétiques sont réversibles, elles sont effectuées par des enzymes qualifiées d'« écrivaines » pour celles qui inscrivent les marques épigénétiques (methyl transférase, acetyl transférase ...) et d'« effaceuses » pour celles qui les retirent (demethylase, deacetylase...). Les méthylations d'histones sont assurées par plusieurs familles d'enzymes écrivaines histone methyl transférase, qui toutes utilisent la S-adenosylmethionine comme co-facteur. Leur déméthylation est assurée par plusieurs histones demethylase, regroupées en deux familles. La plus vaste, appelée famille Jumonji C (JmJc), contient des deoxygénases dépendante de l' α -ketoglutarate, tandis que les deux enzymes connues de la deuxième famille utilisent la flavine adénine dinucléotide (FAD) comme co-facteur. Selon la lysine méthylée, le type de méthylation (mono, di, tri), et la position de l'octamère d'histone par rapport au gène (enhancer, promoteur...) la méthylation peut faciliter l'accès de la machinerie de transcription ou au contraire la rendre plus difficile, ayant donc des effets antagonistes sur l'expression génique.

Naturellement, l'infection par *C. trachomatis* entraîne une modification importante de l'expression des gènes de l'hôte, impliquant certainement des modifications épigénétiques. Une partie de cette réaction constitue une « réponse » de l'hôte à l'infection. Par exemple la reconnaissance de déterminants « étrangers » déclenche l'activation de voies de défenses, en

particulier inflammatoires, visant à l'élimination de l'intrus. En outre, la cohabitation avec les bactéries en division nécessite que l'hôte adapte son métabolisme pour faire face aux demandes accrues en nutriment. Une partie de ces modifications pourraient aussi être sous le contrôle des bactéries, par analogie à ce qui a été documenté dans le cas de la cohabitation microbiote/tissu, où le métabolisme du microbiote influence l'épigénome du tissu à son contact. De plus, la découverte d'une enzyme capable de méthyler les histones parmi les protéines sécrétées de *C. trachomatis* suggère que les bactéries aient la capacité de modifier directement les histones. Une étude précédente a montré que globalement plusieurs lysines des histones 3 et 4 sont méthylées au cours de l'infection. Nous démontrons ici que cette hyperméthylation est un phénomène tardif du cycle infectieux, et concerne tous les résidus lysines, de façon corrélée. Nous avons alors cherché à comprendre les raisons de cette hyperméthylation des histones, et ses conséquences.

Nous avons observé que toutes les marques de méthylation d'histones que nous avons testées étaient détectées de façon concomitante dans certaines cellules infectées, phénomène que nous désignons sous le terme d'hyperméthylation des histones. Il apparaît tardivement dans le cycle infectieux, après l'initiation de la conversion des RBs en EBs. Initialement observé dans la lignée de cellules épithéliales d'origine cancéreuse HeLa, cette observation a également été faite dans des cellules épithéliales primaires issues de l'ecto-col. Elle n'est pas limitée à *C. trachomatis*, puisque l'espèce *C. muridarum*, adaptée aux espèces murines, induit également une hyperméthylation des histones. Enfin, cette hyperméthylation n'est pas l'œuvre de la méthyl transférase bactérienne NUE, puisqu'elle est aussi observée lorsque les cellules sont infectées par une souche mutante n'exprimant pas NUE.

Nous avons ensuite démontré que l'hyperméthylation des histones était liée à un changement métabolique de l'hôte sous l'effet de l'infection. Les cellules infectées sont sensibilisées à l'action d'un inhibiteur des déméthylases de la famille JmjC. L'activité de ces enzymes est dépendante de l'oxygène, du fer et de l' α -ketoglutarate (α KG), un métabolite engagé dans plusieurs voies, en particulier le cycle de Krebs dans les mitochondries. Nos résultats suggèrent que la concentration en fer dans les cellules infectées reste suffisante pour assurer l'activité des déméthylases de la famille JmjC. En revanche, l' α KG semble limitant puisque l'addition d'un analogue qui traverse les membranes, le diméthyl- α KG (DMKG), dans le milieu de culture, prévient l'hyperméthylation des histones. De façon surprenante nous avons observé

que l'inhibition de l'enzyme qui convertit l' α KG en succinyl-coA dans le cycle de Krebs, l'oxoglutarate dehydrogénase (OGDH) n'empêche pas l'hyperméthylation des histones, indiquant qu'elle ne permet pas de rétablir la concentration normale d' α KG dans le cytoplasme et le noyau. Ce résultat indique que l'acteur principal de la déplétion d' α KG dans les cellules infectées est la consommation de ce métabolite, ou de ses précurseurs, par les bactéries. Plusieurs données de la littérature, et en particulier la consommation de glutamine, précurseur d' α KG, par les bactéries, soutiennent cette hypothèse.

Nous avons ensuite caractérisé les conséquences de l'addition de DMKG dans le milieu de culture sur la réponse transcriptionnelle de l'hôte à l'infection. Nous avons observé qu'un nombre restreint de gènes étaient transcrits différemment en présence et en absence de DMKG. Globalement, l'addition de DMKG stimule la transcription de certains gènes qui sont déjà activés ou inactivés par l'infection, et réduit la transcription d'autres. En d'autres termes, il n'y a pas une influence majoritaire, et l'effet du DMKG dépend du transcrit considéré. Ce résultat s'explique probablement par le fait que la méthylation des histones peut réprimer ou stimuler la transcription, selon la marque considérée et sa position par rapport au gène. Dans le cas d'une hyperméthylation, l'addition d'effets parfois contraires s'exprime. Une analyse des catégories de gènes particulièrement affectés par l'addition de DMKG a révélé que deux catégories sortent du lot : les gènes contrôlés par la protéine p53, et des gènes impliqués dans la cascade de signalisation contrôlé par la MAP kinase. La protéine p53 est dégradée lors de l'infection par *Chlamydia*, et il a été montré qu'en absence de p53 l'addition de DMKG permet de restaurer certaines voies transcriptionnelles sous contrôle de p53. Nos résultats sont donc cohérents avec ces travaux précédents. La voie de la MAPK est activée lors de l'infection par *C. trachomatis*, et est nécessaire au développement optimal des bactéries. Des efforts en cours cherchent à déterminer si l'hyperméthylation des histones joue un rôle dans le contrôle de cette voie.

Enfin, nous avons montré que l'hyperméthylation des histones corrélait avec l'abondance de marqueurs de dommages dans l'ADN dans les cellules infectées. De plus, l'addition de DMKG permet de prévenir l'apparition de cassures dans l'ADN. Ces observations suggèrent que l'hyperméthylation des histones favorise les cassures de l'ADN, et/ou empêche leur réparation, deux hypothèses mutuellement compatibles.

En conclusion, ce travail de doctorat a révélé que le métabolisme d'un microbe intracellulaire peut affecter la méthylation des histones de son hôte à un niveau global. Cette

influence épigénétique a des conséquences non seulement sur la capacité de réponse transcriptionnelle de l'hôte à l'infection, mais également sur l'intégrité de son génome. Ainsi, le poids métabolique de l'infection pourrait expliquer, au moins en partie, le caractère pro-tumoral des infections à *C. trachomatis*.

INTRODUCTION

1. *Chlamydia trachomatis*, an obligate intracellular pathogen

1.1 Phylogeny and Evolution

Chlamydia are intracellular bacteria that have long been shrouded in mystery. The name originally proposed for this pathogen, Chlamydozoa, is derived from the Greek word “Chlamys/khlamus” meaning “mantle” or “cloak” and referenced the mechanism in which the bacteria appear to hide themselves within an intracellular vacuole, that we now know as the inclusion (Nunes and Gomes, 2014). Descriptions of trachoma, the blinding disease caused by the ocular strains of *Chlamydia trachomatis*, have been described as early as 1550 BC in the Ebers Papyrus. Such archaic evidence of pathogenic *Chlamydiae* demonstrates its close link with early humans and suggests a co-evolution of the two (Bryan, 1930; Clarke, 2011). For such an ancient pathogen, it has not been well described until more recently. In fact, *Chlamydiae* were classified, incorrectly, as viruses until the 1960s due to their ability to pass through mesh filters, which were classically considered to retain bacteria, as well as their inability to grow outside of a host cell. It was characteristics such as: their replication by binary fission, their Gram-negative stain, their susceptibility to antibiotics, and the presence of both DNA and RNA in a membrane-less nucleoid that helped correctly categorize these pathogens as bacteria (Moulder, 1966).

Chlamydiae is a phylum united by obligate intracellular bacteria that undergo a unique biphasic developmental cycle. The phylum has been found to be genetically distinct and isolated from other free-living bacteria. Genomic analysis did not uncover any transposon homologs, bacteriophages, or any transformation or competence-related proteins similar to other bacteria. This finding, or lack thereof, supports the theory that the modern *Chlamydiae* did not undergo frequent genetic exchange with other free-living bacteria (Stephens *et al*, 1998). Based on 16S rRNA-based phylogenetic analysis, the phylum, *Chlamydiae*, emerged about 2 billion years ago, with present-day pathogenic *Chlamydiae* having diverged about 700 million years ago (Horn *et al*, 2004). The phylum counts a single class, *Chlamydiia*, which, in turn, houses the single order of *Chlamydiales*. Variety arises at the family level, since the *Chlamydiales* order counted 8 families in 2013, and this number is increasing with the metagenomic data obtained from samples collected in various environments (Fehr *et al*, 2013; Dharamshi *et al*, 2020). Genomic sequence analysis of pathogenic

Chlamydia and one of its *Parachlamydia* cousins, within the *Chlamydiales* order, revealed a high percentage of sequence similarity, despite a greater than two-fold difference in genome size. Many of the genes related to metabolism, invasion, and virulence have been shown to remain intact in both strains, suggesting that their last common ancestor, 700 million years ago, was already adapted for intracellular life (Horn *et al*, 2004). The *Chlamydiaceae* family is housed within the order, *Chlamydiales*, and contains a single genus, *Chlamydia*. Whether the genus should be split into two, *Chlamydia* and *Chlamydophila*, was proposed based on 16S and 23S rRNA phylogenetic analysis (Everett *et al*, 1999). However, the matter has since been hotly debated as the aforementioned study did not account for polymorphisms due to geographic isolation of the strains and ignored the otherwise high levels of gene homology and synteny across the strains. The community decided to come back to the use of the single genus, *Chlamydia* (Stephens *et al*, 2009).

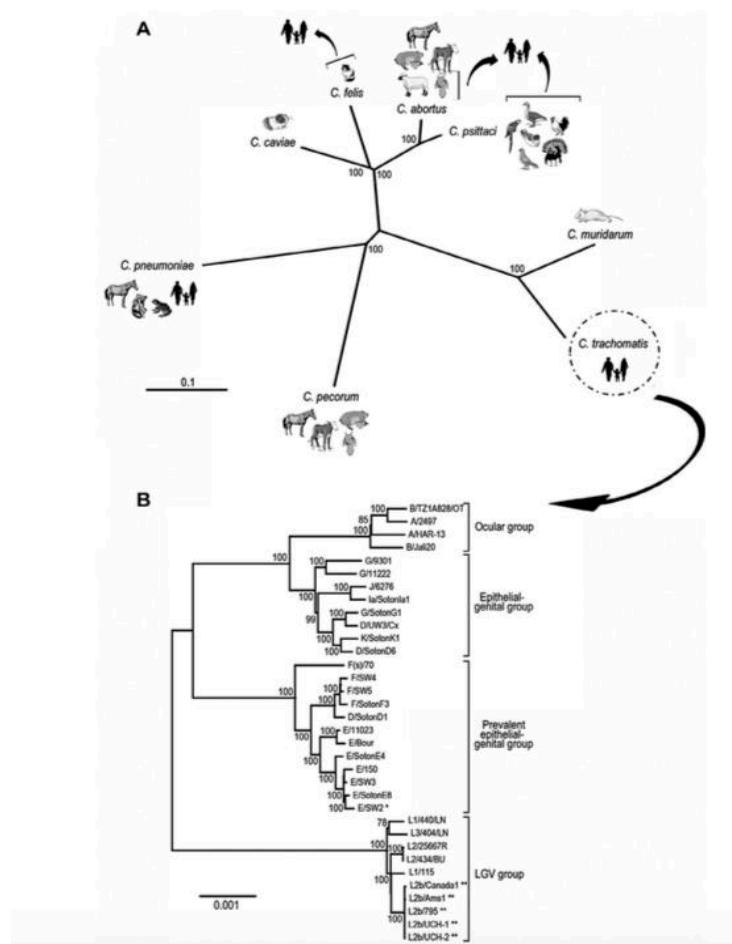


Figure 1 (A) Phylogenetic tree of *Chlamydia* genus with natural hosts shown for each species. (B) *C. trachomatis* strains segregated by tissue-tropism based on the analysis of 819 genes which were conserved across the serovars. (From Nunes and Gomes, 2014).

There are sixteen species within the *Chlamydia* genus: *C. pneumoniae*, *C. pecorum*, *C. suis*, *C. abortus*, *C. felis*, *C. caviae*, *C. psittaci*, *C. avium*, *C. ibidis*, *C. gallinacea*, *C. muridarum*, *C. serpentis*, *C. poikilothermis*, *C. corallus*, *C. sanzinia*, and the focus of this dissertation, *C. trachomatis* (Fig 1A) (Phillips *et al*, 2019). Three of these species, *C. psittaci*, *C. abortus*, and *C. felis*, can be transmitted to humans zoonotically. *C. pneumoniae* and *C. trachomatis* are human pathogens that infect the lungs, and the eyes or urogenital tract respectively. Unlike *C. trachomatis*, *C. pneumoniae* infects a number of other animals, including amphibians and marsupials.

Chlamydia trachomatis consists of 15 serovars in which there is a high degree of identity and synteny across the genomes (Fig 1B). In addition to the $\sim 10^6$ bp circular chromosome, the *Chlamydia trachomatis* genome also contains a ~ 7.5 kb plasmid (Stephens *et al*, 1998). It has been suggested that the plasmid plays a role in the virulence and tropism of a given strain. For example, the ocular strains of *C. trachomatis* (A-C) have a lower level of expression of the plasmid, than urogenital serovars (D-K, LGV L1 and L2) (Ferreira *et al*, 2013). Differential expression of various effectors, polymorphic membrane proteins (PMPs), and Inc (inclusion) proteins also play roles in virulence and tropism amongst the serovars, which we will now discuss in more detail (Nunes and Gomes, 2014).

1.2 Diseases, Treatments and Sequelae

1.2.A Trachoma (A, B, Ba, and C)

Ocular infection of *C. trachomatis* serovars A, B, Ba, and C are the leading infectious causes of blindness globally. The WHO estimates that active trachoma affects up to 21 million people, with about 2.2 million who are blind or visually impaired. At least 7.3 million individuals suffer from potentially blinding trichiasis, an inversion of the eyelashes such that they scratch and damage the cornea.

Trachoma is most prevalent in Africa, but also affects poor and remote areas in Asia, the Middle East, and Australia (Fig 2) (WHO).

Transmission occurs through contact between infected people, or via eye-seeking flies that have been in contact with infected fluids (Emerson *et al*, 2000; Miller *et al*, 2004). However, socioeconomic factors also play a role in endemic areas. Crowded communities combined with poor hygiene contribute to the spread of *C. trachomatis* in humans (Taylor *et al*, 2014).

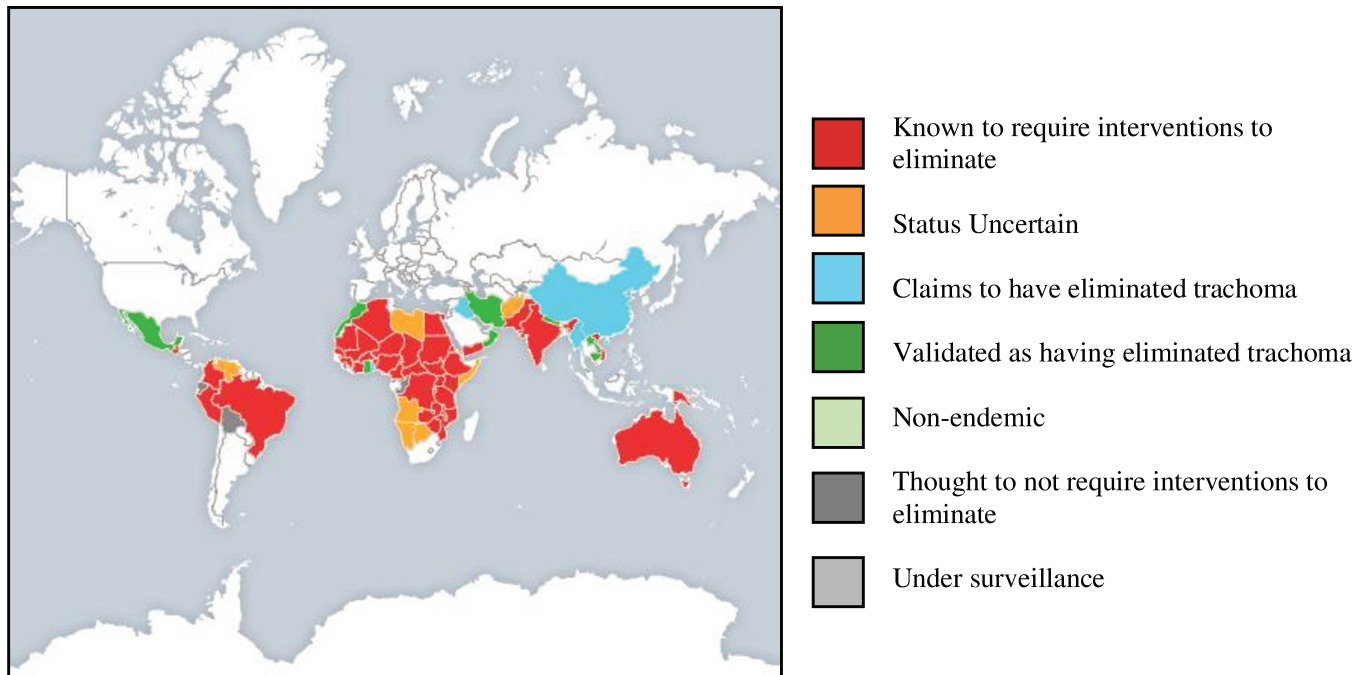


Figure 2 Status of Global Trachoma Endemicity of Trachoma. This map was generated by the International Trachoma Initiative using the most recent statuses reported in the Global Health Observatory. (From Trachoma Atlas, 2019).

Upon infection, the immune system elicits an innate immune response which triggers the release of pro-inflammatory cytokines that contribute to the severe inflammation that occurs during trachoma infection (Rasmussen *et al*, 1997; Natividad *et al*, 2010). Clearance of the infection is believed to be dependent on IFN γ mediated depletion of tryptophan and iron (Natividad *et al*, 2010). Persistent or reoccurring infections, in concert with severe inflammation, lead to a scarring of the upper tarsal conjunctiva which, in turn, can turn into trichiasis. The irritating, and often blinding effect of trichiasis can occur after bacterial clearance (Burton *et al*, 2011).

Treatments consists of oral azithromycin, (20 mg/kg up to 1g), given as a single dose, or a 6-week course of topical tetracycline. However, surgery is required to treat trichiasis and correct the inversion of the eyelashes (Taylor *et al*, 2014).

Multiple studies have shown that acquired immunity to reinfection is weak, short-lived, and often serovar specific, which makes vaccine development especially tricky (Hu *et al*, 2013).

Trachoma is classified, by the WHO, as a neglected tropical disease. To that end, in 1997 a resolution by the World Health Assembly, the governing body of the WHO, was established for the Global Alliance for the Elimination of Blinding Trachoma by the year 2020 (GET 2020). Public health initiatives using the “SAFE” strategy were implemented to help reduce the transmission of trachoma. SAFE stands for Surgery, Antibiotics, Facial cleanliness, and Environmental improvement. It involved offering surgery to individuals with trachomatous trichiasis to correct the inversion of the eyelid, and antibiotics to those with active trachoma. Facial cleanliness campaigns have been implemented to stress the importance of hygiene in the context of trachoma, as well as campaigns to ameliorate environmental factors that contribute to exposure to eye-seeking flies, such as improved water sources and waste management. In June of 2012, the Global Trachoma Mapping Programme was launched to map the global incidence of endemic trachoma using a population-based survey, as only about half of the at-risk areas were being covered by international trachoma control activities at the time (WHO). Despite the great strides made by both programs to eliminate trachoma, it is still endemic in 44 countries (Burkhi, 2019).

1.2.B Urogenital Serovars (D-K)

Urogenital infections of *Chlamydia trachomatis*, serovars D-K, are the leading bacterial cause of sexually transmitted infection worldwide. In 2016, there were 127 million new cases of urogenital *Chlamydia* infections globally, mostly in men and women ages 15-49 (Fig 3) (WHO). Serovars E and F cause up approximately 50 percent of all chlamydial urogenital infections, while serovars G, D, and J are more prevalent in rectal infections (Nunes et al, 2014).

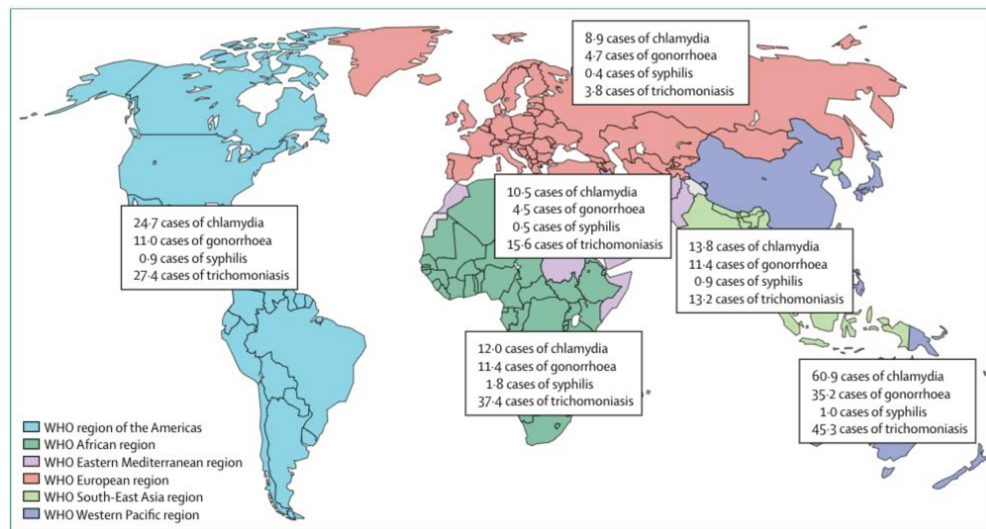


Figure 3 Regional estimates of new cases of the four curable STIs: chlamydia, gonorrhea, syphilis, and trichomoniasis. The data, gleaned from 2012, is estimated by the millions of new cases per year in WHO regions. (From Unemo *et al*, 2017).

Genital infections of *C. trachomatis* are often asymptomatic, which contributes greatly to their spread in the population. In men, *C. trachomatis* is the most common cause of nongonococcal urethritis and may have symptoms such as a mucoid urethral discharge, and painful urination. Should the bacteria travel through the urethra, the infection could then result in inflammation of the epididymides and testes. An estimated 1% of men will develop reactive arthritis, which is an extreme inflammatory response that can take up to 1 year to resolve with symptoms like conjunctivitis, arthritis, and urethritis (O’Connell and Farone, 2016; Wu and Schwartz, 2008).

Symptoms in women, when infections are not asymptomatic, may include changes in vaginal discharge, intermittent inter-menstrual bleeding, or post-coital bleeding. *C. trachomatis* can ascend to the upper reproductive tract and cause endometritis and salpingitis, inflammation of the endometrium and fallopian tubes, respectively. Like the ocular serovars, the urogenital serovars also induce an immune response that causes severe inflammation and tissue remodeling that leads to scarring of the reproductive tract (Darville and Hiltke, 2010). Chronic or repeated infections may result in pelvic inflammatory disease, which increases the risk of ectopic pregnancies and infertility (Brunham *et al*, 2015).

Treatment for serovars D-K is usually a single dose of azithromycin, (1g), or 100 mg of doxycycline twice daily for two weeks. As most infections are asymptomatic, the current recommendations are for regular sexual health screenings to test for infection in tandem with partner alert systems, to help drive down the incidences of infection. There have been no cases antibiotic-resistance to *Chlamydia* infection, however recurrence is quite common, so post-treatment screenings are also recommended (Workowski *et al*, 2015).

Women with repeated *C. trachomatis* infections have been associated with having a higher risk of developing ovarian and cervical cancers (Zhu *et al*, 2016). More studies have been conducted to determine by what mechanism infection may lead to cancer. *Chlamydia*-infected cells often display phenotypes associated with genomic instability, which makes them more vulnerable to becoming cancerous. Phenotypes include: multinucleation, dysregulation of centrosomes and spindle poles, and defects in chromosomal segregation (Grieshaber *et al*, 2006; Johnson *et al*, 2009; Andreaolas *et al*, 2011). Further studies have shown that, while DNA damage is induced, DNA repair mechanisms are suppressed, which is also leads to genomic instability (Chumduri *et al*, 2013).

1.2.C LGV invasive Serovars (LGV L1-L3)

LGV serovars are endemic to subtropical regions of Asia, Africa, South America, or the Caribbean (Herring *et al*, 2006). However, there has been a rise in the incidence of LGV infections in North America and Europe in the population of men who have sex with men (MSM) (de Vreize *et al*, 2014).

LGV serovars (L1, L2, L2a, and L3) are much more invasive and cause lymphoma granuloma venereum. Infection often first presents with an ulcer at the site of inoculation, followed later by swelling at the site and a fever. As the bacteria invade the lymphatic system, more serious symptoms like bubo formation, fistulae, fibrosis, and rectal stenosis arise (Herring *et al*, 2006; de Vreize *et al*, 2014). These serovars are usually treated by 100 mg of doxycycline administered twice daily for 3 weeks (Workowski *et al*, 2015). Tissue damage from the lymphatic invasion can

be permanent if the infection isn't treated in time and may result in edema at the site of infection (de Vrieze *et al*, 2014).

1.3 Developmental Cycle

1.3.A An Overview

One of the unifying features of the *Chlamydiae* is their unique biphasic developmental cycle. The bacteria cycle through two distinct forms: elementary bodies (EBs) and reticulate bodies (RBs). This nomenclature harkens back to when the *Chlamydiae* were classified as viruses. The first study to indicate that the *Chlamydiae* developmental cycle was biphasic was in 1932. Bedson and Bland observed in the spleens of mice infected with *C. psittaci*, that the bacteria differed greatly in size and shape at different time points during the course of infection (Fig 4). They correlated the larger bacterial forms, that we now know to be RBs (~1 μm), with early infection and found evidence of division. During late infection, they observed a return of the bodies to their smaller size (0.3 μm), that we now know are the infectious EBs (Bedson and Bland, 1932). Investigations of the chlamydial developmental cycle since then have uncovered even more of the inner workings of these bacteria and showcase their complexity.

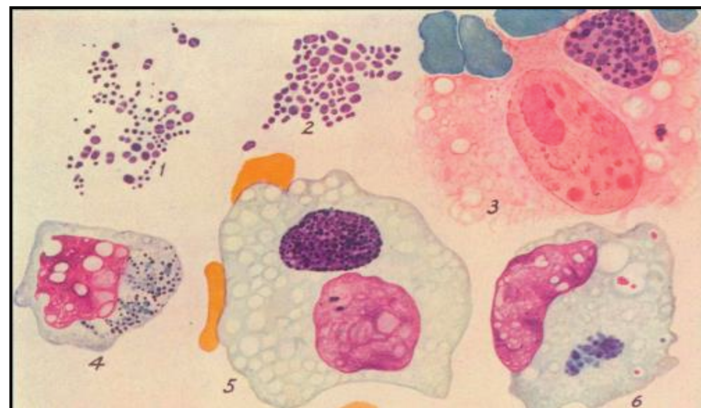


Figure 4 Evidence of biphasic development. Spleen smears of *C. psittaci*-infected mice were made at multiple times points post-infection and stained by Giemsa differentiated with orange G-tannin. A gradient in the staining was observed with EBs presenting as a deep blue purple, and the RBs appearing paler. (1) Free bacteria, like escaped from a lysed cell, at 24 hpi in which both EBs and RBs can be observed. (2) Free bacteria, like escaped from a lysed cell, at 15 hpi in which only the larger RBs can be observed. (3) Infected cell in which the RBs in the inclusion vacuole can be observed at 15 hpi. (4) Infected cell 24 hpi. (5-6) Infected cell at 24 hpi that appears to be in an intermediate stage with both EBs and RBs present in the inclusion. (Adapted from Bedson and Bland, 1932).

EBs are the extracellular, infectious form of the bacteria. They are non-replicative, and their spore-like cell wall enriched in cross-linked proteins helps them survive in the harsh extracellular environment. Upon adherence to the host cell, the bacteria create a specialized vacuole, referred to as the inclusion, where they remain throughout the course of their intracellular life. Once safely inside the inclusion, the EBs differentiate into RBs. RBs are the replicative form the bacteria which are larger and much more metabolically active. They divide until it is time to convert to EBs is initiated. RB to EB conversion is not well understood and will be discussed in more detail below. Once the RBs have differentiated into EBs, the new infectious bacteria exit the host cell via extrusion or host lysis. The whole cycle takes 48-72 hours for *C. trachomatis* and can be longer for other species (Fig 5).

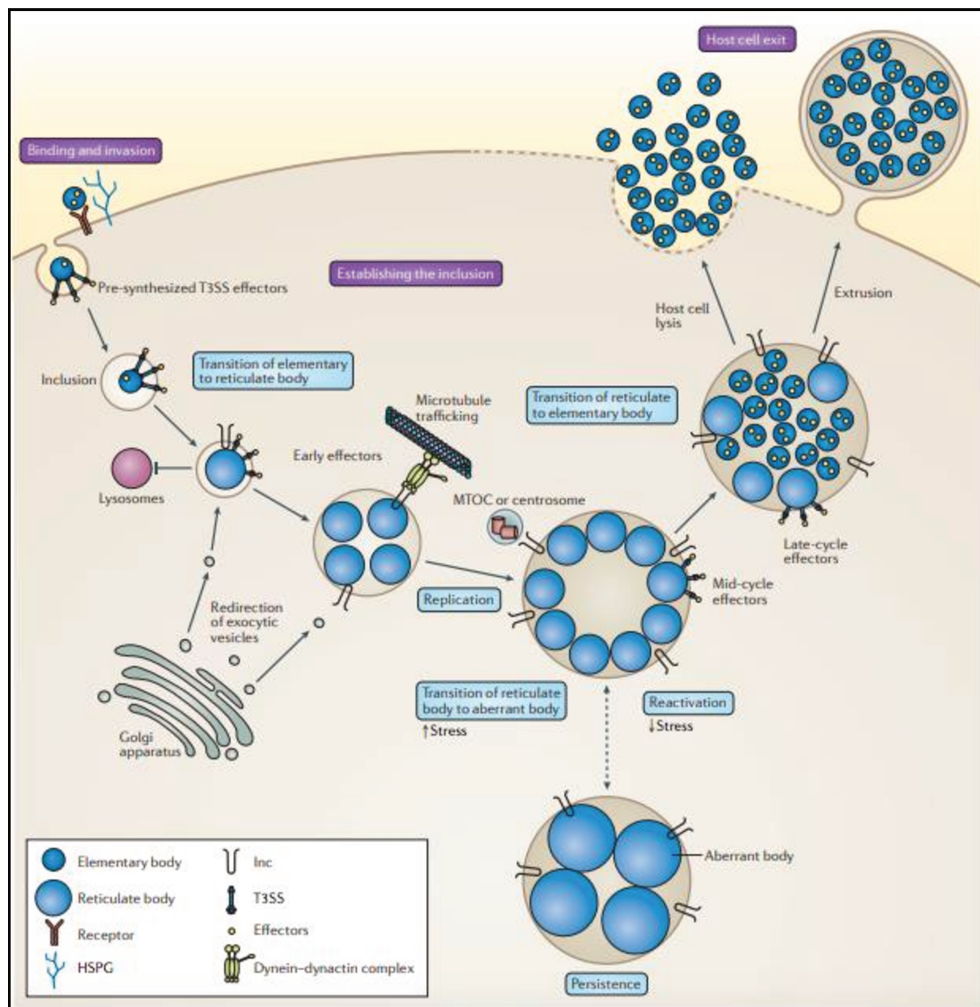


Figure 5 Schematic of the chlamydial developmental cycle. (From Elwell *et al*, 2016).

In the event of metabolic stress, RBs may convert to a persistent state, termed intermediate bodies (IBs), where the bacteria do not continue dividing, nor do they convert to EBs. This persistent state is reversed by removal of the stress stimuli, and then the IBs convert back to RBs, which in turn continue development until new EBs are ready to exit and infect new cells. Throughout development, the bacteria hijack and modulate host cell processes to their advantage, often through the secretion of effector proteins via a type three secretion system (Abdelrahman and Belland, 2005; Elwell *et al*, 2016).

1.3.B Knock-knock - May I come in? (Entry)

Multiple mechanisms by which *Chlamydiae* may bind to a host cell have been uncovered over the years. While so many different interactions may seem to muddy the waters, they support the idea that chlamydial replication cannot take place extracellularly, and thus gaining entry into a host cell is a requirement. Utilizing a variety of mechanisms ensures that the bacteria can carry out their biphasic development, but also may play a role in tissue tropism (Hegemann and Moelleken, 2012; Elwell *et al*, 2016).

The host proteins largely implicated in chlamydial entry are heparan sulfate proteoglycans (HSPGs). HSPGs are integral membrane proteins that are found in great numbers on host-cell membranes, and their extracellular domain consists of a negatively charged glycosaminoglycan (GAG). Though there has been variation between species and serovars, it has been shown that pretreatment of EBs with HS or heparin reduce their infectivity, indicating that there is a GAG-specific receptor on the EB surface that is necessary for attachment and subsequent invasion (Zhang and Stephens, 1992).

While the first interaction of EBs with the host cells is believed to be reversible electrostatic interactions with HSPGs, the second step is believed to be an irreversible binding stage with other host proteins, of which many have been implicated (Dautry-Varsat *et al*, 2005). Among the host proteins identified, cystic fibrosis transmembrane conductance regulator (CFTR) has been shown to bind chlamydial LPS, and CFTR KO mice are less susceptible to infection (Anjonuma *et al*, 2010). Platelet derived growth factor receptor β has also been implicated as a potential receptor

for chlamydial adhesion, and a potential bridge that could act with HSPGs (Elwell *et al*, 2008). Protein disulfide isomerase (PDI) has been shown to be important for adhesion and entry. Interestingly, EB adhesion via PDI does not require the protein to be catalytically active, while entry does (Abromaitis and Stephens, 2009). Receptor tyrosine kinases, such as EphrinA2 receptor, have also been shown to be important for binding, invasion, and subsequent downstream signaling (Subbarayal *et al*, 2015). While the identification of these host proteins that bind EBs have been exciting additions to the understanding of *Chlamydia* adherence, for most of them a bacterial binding partner has yet to be determined.

There are several chlamydial proteins that are associated with adhesion. Proteins of the outer membrane complex, such as MOMP, OmcA, and OmcB have been implicated in chlamydial EB attachment. These proteins create a rigid structure via disulfide bonds and are important for maintaining structural integrity and osmotic stability of the EB (Hegemann and Moelleken, 2012). It has shown by electron microscopy that MOMP aggregates on epithelial cell surfaces. It was then determined that MOMP binding is inhibited when cells are pretreated with heparinidase, which indicated that heparan sulfate proteoglycans (HSPGs) are important for *Chlamydia* adhesion by MOMP (Su *et al*, 1996).

OmcB was also shown to be capable of binding human epithelial cells via HSPGs. OmcB-expressing yeast were pre incubated with soluble GAG derivatives and found that adhesion was diminished in OmcB-expressing yeast that had been pre-incubated with heparin. Heparin is an analog of heparan sulphate, thus this set of experiments determined that OmcB is not only capable of binding human cells, but that it binds via HS-like structures on host-cell surfaces. Experiments similar to those described above for the pmps, in which host cells were pre-incubated recombinant OmcB proteins from different *Chlamydia* species, and the OmcB from opposite strains had no effect on the infectivity of one another (Moelleken *et al*, 2008).

Polymorphic membrane proteins (Pmps) are a family of chlamydial proteins that have also been implicated in adhesion. They represent 3-5% of the chlamydial genomes, which indicates that they must have an important functional role. Interestingly, the members of the pmp gene family show little similarity to one another, which points to roles in virulence and tropism (Stephens *et al*,

1998). While a specific host binding partner has yet to have been identified, Pmp6, Pmp20, and Pmp21 from *C. pneumoniae* have been shown to encode adhesion domains and are capable of binding to human epithelial cells. Furthermore, pretreatment of cells with recombinant pmps has been shown to reduce infectivity, thus indicating that pmps are important for entry (Moelleken *et al*, 2010). It was later shown that pre-incubating host cells with recombinant pmps of *C. trachomatis*, also reduced infectivity of *C. trachomatis*, but did not affect infectivity of *C. pneumoniae*, and vice versa. This indicated that pmps have species-specific functions that are likely important for tropism. (Becker and Hegemann, 2014).

1.3.C Teamwork makes the dream work (Entry with the help of T3SS effectors)

The entry of *C. trachomatis* into its host cell requires the cooperation or co-opting of host proteins classically implicated in endocytic events. The bacteria accomplish this through the secretion of bacterial proteins, termed effectors, that recruit and modulate the activity of host proteins. To that end, *C. trachomatis* employs the use of a type three secretion system (T3SS), commonly found in many pathogenic Gram-negative bacteria.

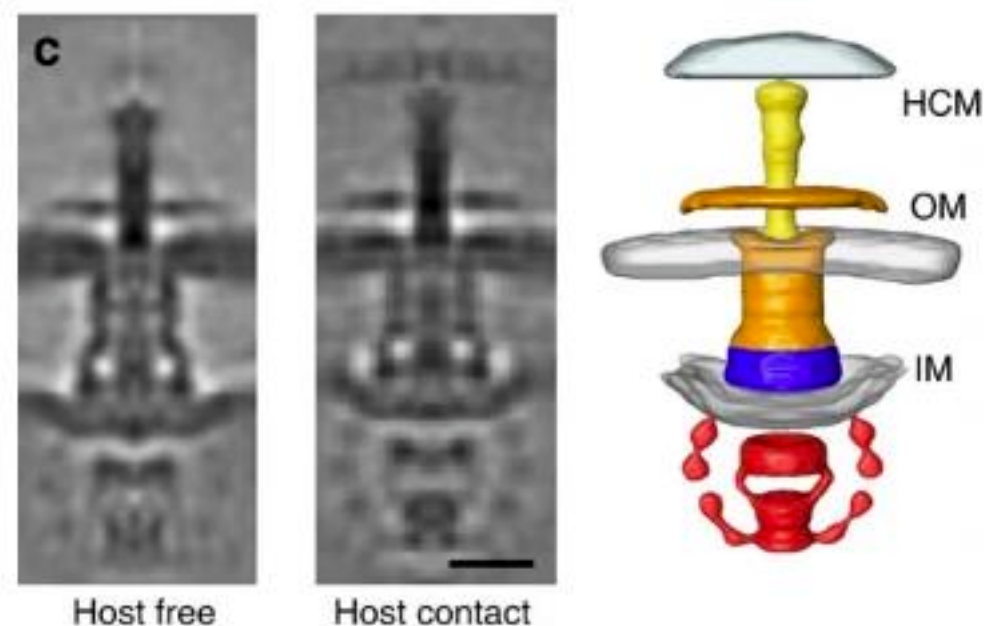


Figure 6 Cryo-electron tomography of chlamydial T3SS in contact with host cell membrane during early infection in which an EB has made contact with a HeLa cell. Schematic of T3SS structure in which the injectosome reaches through the inner membrane (IM), outer membrane (OM) of the bacteria and makes contact with the host cell membrane (HCM). (Adapted from Nans *et al*, 2015)

The chlamydial T3SS is responsible for translocating bacterial effectors from the bacterial cytosol into the host cytosol. It is a needled-like structure composed of a basal core, which links the inner and outer bacterial membranes, the needle complex, which protrudes from the outer membrane of the bacteria, and the translocon, which inserts into the host membrane upon contact (Fig 6). Initiation of injection occurs upon contact of the needle-tip complex with the host membrane (Mueller *et al*, 2014; Nans *et al*, 2015).

The chlamydial T3SS is utilized throughout its development in both EBs and RBs. Pre-made effectors are injected upon the EB's contact with the host membrane to help modulate the host cytoskeleton, dampen the immune response, subvert vesicular trafficking and block fusion of the new inclusion with the phagolysosome/acidic compartments (Bugalhão and Mota, 2019). *De novo* synthesis of effectors in RBs is used to block fusion of the inclusion with endosomal compartments, modify further the inclusion membrane, promote immune evasion, inhibit cell death, acquire nutrients, as well as to make effectors ready for the next round of infection (Mueller *et al*, 2014; Bugalhão and Mota, 2019). Overall, there are 80-100 of effectors secreted by *Chlamydia trachomatis*' T3SS, many of which have yet to be characterized.

One well studied example of an early effector is TarP (translocated actin-recruiting phosphoprotein). TarP is secreted upon the EB binding to the host cell and its interactions with host ARP2/3 and the GTPase Rac, modulate actin polymerization resulting in encapsulation of the EB (Carabeo *et al*, 2002; Carabeo *et al*, 2004; Lane *et al*, 2008; Jiwani *et al*, 2013; Cossé *et al*, 2018). It was recently shown to work in concert with a second effector targeting the actin cytoskeleton, TmeA. (Keb *et al*, 2021). We will mention a few other effectors as we move through the description of the developmental cycle.

1.3.D I think I'll make myself at home (Formation of the Inclusion Vacuole)

The EBs are initially engulfed in a plasma membrane-derived vesicle, termed the nascent inclusion. Nascent inclusions appear largely separated from endo-lysosomal traffic, and do not fuse with late endocytic compartments (Scidmore *et al*, 2003). Within a few hours they shuttle

towards the microtubule organization center (MTOC) propelled by the microtubule motor protein, dynein.

The developmental regulation of infectious EBs to replicative RBs, coincides with a timed cascade of temporally regulated genes. The transition from EB to RB marks several differences in the bacterial forms. The chromatin of the nucleoid becomes decondensed from histone-like proteins, Hc1 and Hc2, as the bacteria becomes transcriptionally active (Barry *et al*, 1992; Brickman *et al*, 1993). There is also a reduction in disulfide bonds that crosslink the outer membrane proteins on the exterior of the bacteria (Hackstadt *et al*, 1985; Hatch *et al*, 1986).

Among the early genes expressed by *C. trachomatis* (first detected ~1-2 hpi) are genes coding for proteins translocated into the inclusion membrane by the T3SS machinery, called inclusion proteins (Incs) (Belland *et al*, 2003). These proteins decorate the cytosolic surface of the inclusion and control its interactions with cellular compartments. For instance, several Inc proteins have been shown to interact RAB GTPases and SNARES proteins, two large families of proteins that play prominent roles in the regulation of membrane fusion (Elwell and Engel, 2016; Hackstadt, 2012). Soluble effector proteins are also implicated in redirecting host traffic to the inclusion, as recently illustrated by the role of TaiP in redirecting Rab6 positive vesicles towards the inclusion (Hamaoui *et al*, 2020).

As RBs replicate, with a doubling rate of ~2 h, the inclusion grows rapidly in size and by 24 hpi, its volume is usually bigger than that of the nucleus (Shaw *et al*, 2000). It continues to grow, although at a slower rate, for another 24 h (Lee *et al*, 2018). The inclusion is supported and stabilized by an “cage” of cytoskeletal components composed of actin and septins, which are further stabilized by microtubules and intermediate filaments (Al-Zeer *et al*, 2014; Kumar and Valdivia, 2008; Zuck *et al*, 2017; Tarbet *et al*, 2018).

1.3.D The more the merrier - EBs to RBs and back again

The RBs asynchronously begin converting to EBs between 24-72 hpi (Hackstadt 2012; Elwell and Engel, 2016). The trigger for this conversion remains a mystery, but it has recently been hypothesized that it could be size-dependent. Quantitative analysis of serial block-face scanning

electron microscopy (SBEM) suggests that once dividing RBs reach a lower size threshold, they convert to EBs (Lee *et al*, 2018). Late cycle genes encode outer membrane complex proteins, Hc1/2 to induce chromatin condensation, as well as effectors that will be prepackaged in the EBs to aid in entry and invasion of the next round of infection (Saka *et al*, 2011).

1.3.E Time to go! (Exit by Extrusion or Lysis)

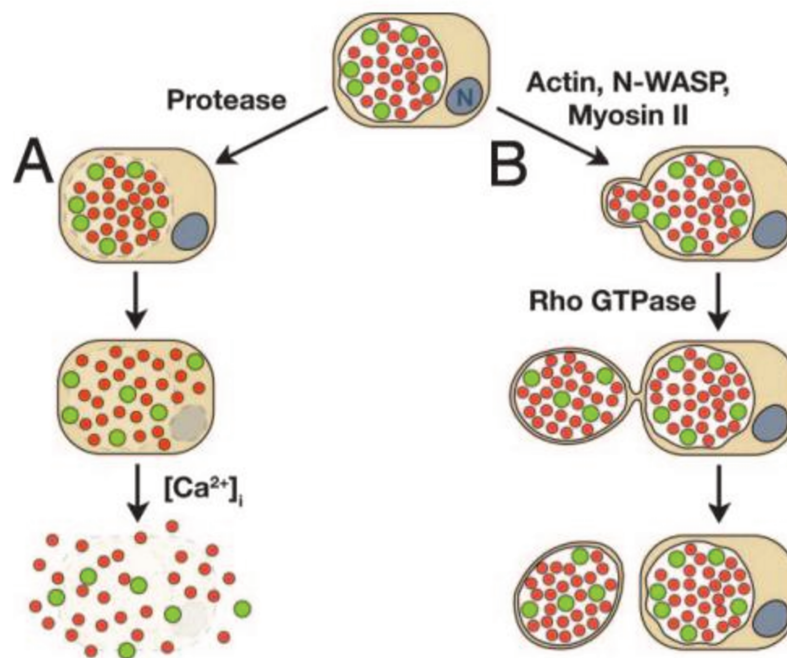


Figure 7 Schematic of mechanisms by which *Chlamydia* may exit the cell (A) Calcium-dependent lysis (B) Extrusion (From Hybiske and Stephens, 2007)

There are two mutually exclusive methods for exit of the newly formed EBs: lysis of the host cell or exocytic-like extrusion (Fig 7).

Lysis occurs in a stepwise fashion in which the inclusion ruptures, followed by the permeabilization and collapse of the host-cell's plasma membrane. Through the use of protease inhibitors and calcium starvation, respectively, inclusion rupture was shown to be mediated by

host proteases, while plasma membrane breakdown was demonstrated to be calcium dependent (Hybiske and Stephens, 2007).

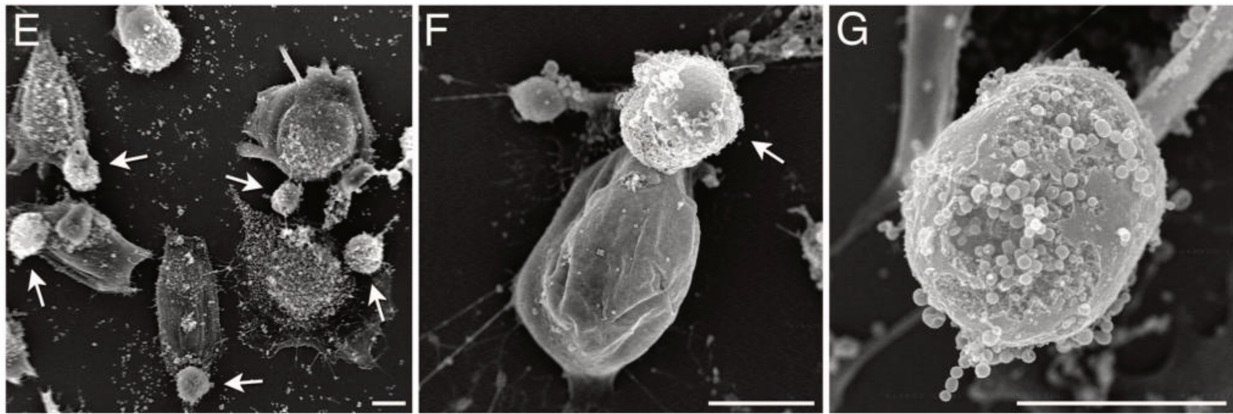


Figure 8 Scanning Electron Microscopy (SEM) of extrusion events (E) SEM of cells with extrusion (indicated with white arrows) (F) SEM of a single cell with an extrusion (G) SEM of a detached extrusion (From Hybiske and Stephens, 2007)

In vitro, extrusion occurs at the same frequency as lysis but does not kill the host cell in the process, since the inclusion protrudes from the cell and is subsequently pinched off into a separate compartment and set free (Fig 8). Two Inc proteins, MrcA and CT228, have been shown to regulate extrusion by manipulating the actin motor protein, non-muscle myosin II (Hybiske 2007; Nguyen *et al*, 2018).

1.4 *C. trachomatis* rely on the metabolic capacities of the host

During its coevolution toward an obligate intracellular existence, *Chlamydiae* lost many metabolism-related genes. The genomic reduction is even more drastic in *Chlamydiaceae*, whose genome only encodes about 1,000 genes compared to >2,000 genes encoded by the environmental species. Due to their reduced genomes, many of the necessary biosynthetic pathways are incomplete and lack crucial enzymes. Thus, the bacteria depend on the host cell for the synthesis of several amino acids, all nucleotides, and of some lipids. In addition, many basic metabolites and co-factors necessary for feeding the bacterial metabolic pathways such as glucose, glutamine, Nicotinamide adenine dinucleotide (NAD), S-adenosyl-L-methionine (SAM), and biotin are

scavenged from the host. Below we will discuss some of the major metabolites and pathways that are exploited by *C. trachomatis*.

Glucose and Glycogen

C. trachomatis requires glucose from its host for energy and the biosynthesis of various metabolites. It's been shown that *Chlamydia*-infected cells have increased glucose uptake, and host glucose transporters GLUT1 and GLUT3 have been shown to be upregulated during infection demonstrating that *C. trachomatis* infection rewires host glucose metabolism (Wang *et al*, 2017; Maffei *et al*, 2020). The *C. trachomatis* genome is missing hexokinase-2, the enzyme necessary to convert glucose to glucose-6-phosphate (G6P), so it must import directly G6P from its host through chlamydial protein, UhpC (Gehre *et al*, 2016). From there, it may be used to create glycogen, a glucose storage molecule, or as a substrate for other biosynthetic pathways.

Gehre *et al.*, also demonstrated that host UDP-glucose, a building block of glycogen, could be transported into the inclusion lumen via the exploitation SLC35D2, a host transporter that is normally found on the ER and Golgi. Once in the inclusion lumen, chlamydial effector proteins GlgA and GlgB use it to synthesize glycogen for glucose storage (Gehre *et al*, 2016).

TCA cycle and its intermediates

The Tricarboxylic Acid cycle (TCA) is coupled with oxidative phosphorylation to generate energy in the host cell. The TCA cycle of *C. trachomatis* is missing three key enzymes, citrate synthetase, aconitase, and isocitrate dehydrogenase (Fig 9) (Stephens *et al*, 1998). This suggests that *C. trachomatis* obtain certain TCA cycle intermediates from the host. It's been shown that bacteria can grow when the culture medium contained 20 mM α -ketoglutarate, oxaloacetate, or malate as their sole carbon source, although the progeny was reduced by 100-fold compared what was obtained with 1 mg/ml glucose (Iliffe-Lee and McClarty, 2000; Nicholson *et al*, 2004; Mehrlitz *et al*, 2017). The mechanisms by which these TCA cycle intermediates are taken up by the bacteria remain elusive.

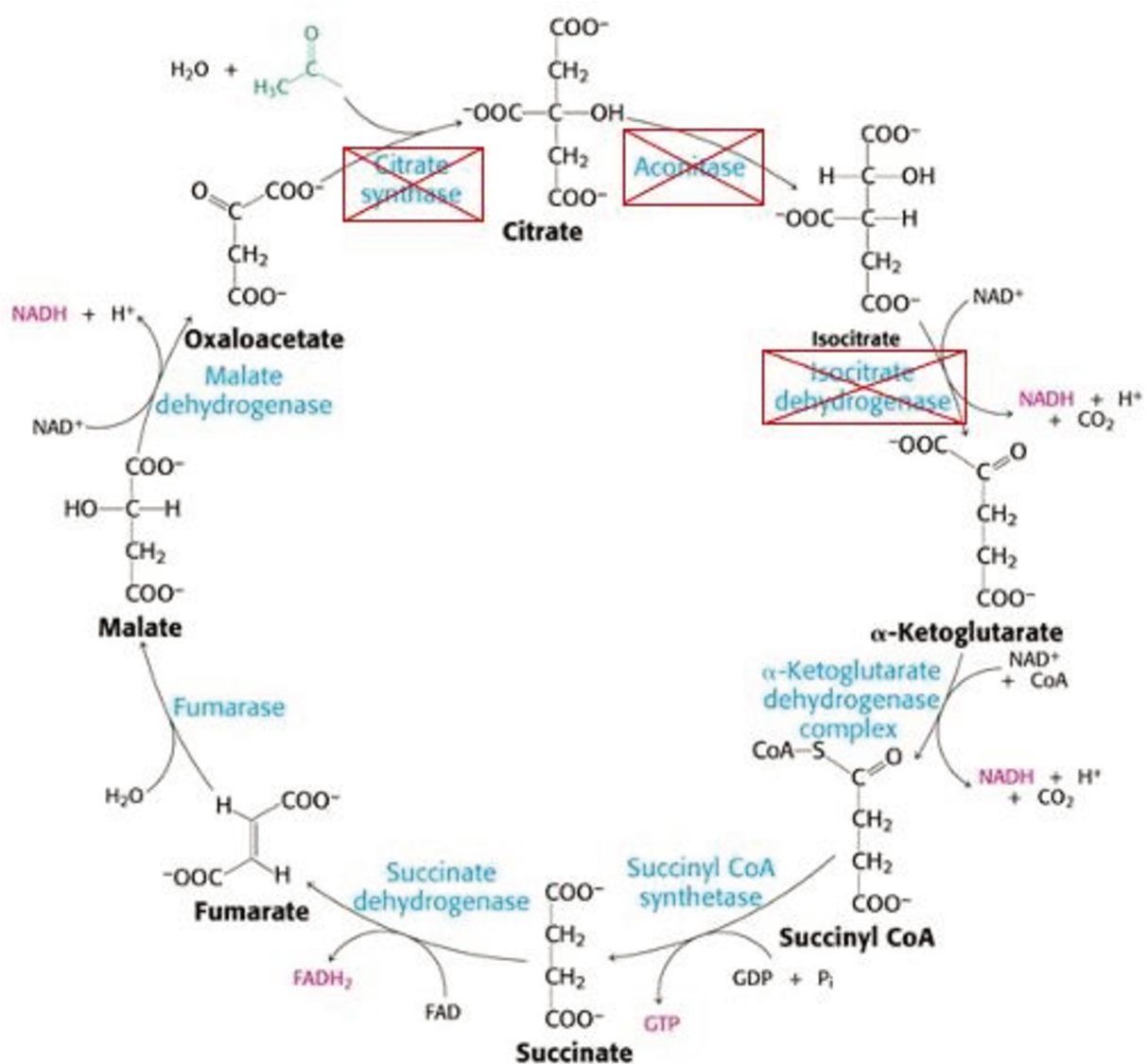


Figure 9 TCA cycle of *C. trachomatis*. Missing enzymes indicated with red boxes. (Adapted from https://teaching.ncl.ac.uk/bms/wiki/index.php/Krebs_cycle)

Nucleotides and Amino Acids

Chlamydia do not possess any machinery for nucleotide synthesis and rely entirely on the host cell for their biosynthesis. Blocking host purine synthesis inhibits *C. trachomatis* development *in vitro* and *in vivo* (Rother *et al*, 2018). Bacterial nucleotide transporters, Npt1 and Npt2, regulate the exchange of ATP against ADP, and the uptake of GTP, CTP, UTP, and ATP, respectively (Tjaden *et al*, 1999). Npt1 has also been shown to be capable of transporting NAD (Fisher *et al*, 2013).

While it appears that *C. trachomatis* can make some amino acids de novo from host-derived glucose, (alanine, aspartate, and glutamate), they do require import of others from the host (Karayiannis and Hobson, 1981; Mehltitz *et al*, 2017). It was recently published that EBs take up glutamine and that its funneled through their TCA cycle to make diaminopimelate, a precursor of peptidoglycan. The authors also demonstrated that the host glutamine metabolism is reprogrammed through increased transcription of host glutamine transporter, SLC1A5, in response to the increased demand for glutamine that occurs during infection (Rajeeve *et al*, 2020).

Lipids

Early studies suggested that *C. trachomatis* lipids are largely obtained from the host (Wylie *et al*, 1997). However, it was shown more recently, that the bacteria have the capacity to produce most of their lipids (Yao *et al*, 2015). They do import some eukaryotic lipids that are not normally produced in prokaryotes such as sphingomyelin (Hackstadt *et al*, 1995). Host membrane trafficking is subverted during infection to enable expansion of the inclusion (Elwell and Engel, 2012).

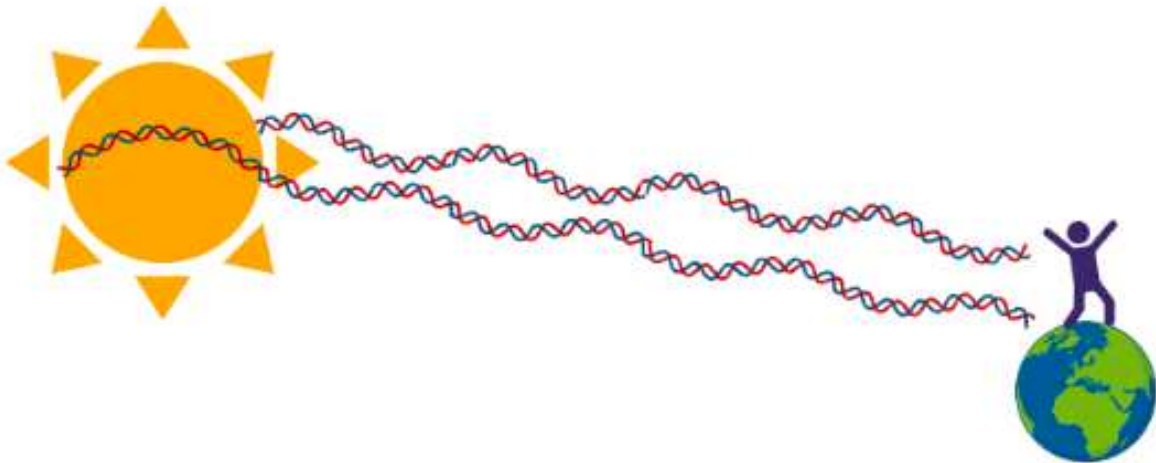
Other Metabolites

Several other small metabolites need to be obtained from the host. For instance, S-adenosyl-L-methionine (SAM), a methyl donor for transmethylation reactions common in chromatin modifications, may be relevant for chlamydial reactions as a SAM/SAH transporter has been identified and characterized. Binet *et al*, found that CTL843 was capable of importing S-adenosyl-L-methionine (SAM) and exporting the toxic byproduct of its methyl donation S-adenosylhomocysteine (SAH) (Binet *et al*, 2011). Another illustration of the import of metabolites by the bacteria is provided by biotin, a cofactor necessary for a multitude of cellular processes, lipid metabolism among them. Biotin has been shown to be imported into the inclusion via a host transporter, the sodium multivitamin transporter (SMVT). Once in the inclusion lumen, biotin may be transported into the bacteria through the bacterial transporter BioY (Fisher *et al*, 2012).

2. Chromatin remodeling and its influence on gene expression

2.1 Chromatin Structure and Function

2.1.A Overview of Chromatin Function: Heterochromatin vs. Euchromatin



A human diploid cell contains approximately 6 billion nucleotide base pairs, which adds up to about 2 meters worth of DNA per cell (Annunziato, 2008). The average human is made up of an estimated 3.72×10^{13} cells, which puts the total length of our combined DNA, were we to line it up end-to-end, at 7.44×10^{13} meters (Bianconi *et al*, 2013). That is long enough to travel to the sun and back just under 250 times. While the length is impressive, its sheer bulk would certainly impede other cellular processes if left loose and unorganized. How do cells organize their DNA in such a way that it is severely compacted, but also easily accessed for transcription when the need arises?

The cell's answer to this organizational dilemma is to first package its genome into an ultra-condensed fiber known as chromatin. Chromatin is comprised of an equal ratio of genomic DNA to specialized packing proteins. Its formation is not only the first step in the construction of

chromosomes during mitosis, but also maintains the organization of DNA throughout the cell cycle (Janssen *et al*, 2018).

Chromatin is highly dynamic and constantly undergoing sub diffusive movement and reorganization. It can be sub-categorized as euchromatin or heterochromatin. Euchromatin contains gene-rich regions in which DNA is readily accessible for transcription. It is often referred to as “open” or “active” chromatin. The counter part of euchromatin is heterochromatin. Commonly identified as “closed” or “inactive” chromatin, heterochromatin is comprised of DNA that is highly condensed, and is therefore less transcriptionally active (Hauer and Gasser, 2017). The concepts of heterochromatin and euchromatin were first coined by German scientist, Emil Heitz in 1928 (Heitz, 1928; Berger, 2019). He observed in *Pellia epiphylla*, a type of liverwort moss, that some regions of the chromosomes were not visible, via his improved staining protocol, after telophase. This non-stained chromatin, he dubbed “euchromatin”, and postulated correctly that it was associated with actively transcribed genes, and that the stained chromatin, which he termed ‘heterochromatin’, represented genetically inert regions (Fig 10) (Heitz, 1928; Passarge, 1979; Allshire and Mahandi, 2018).

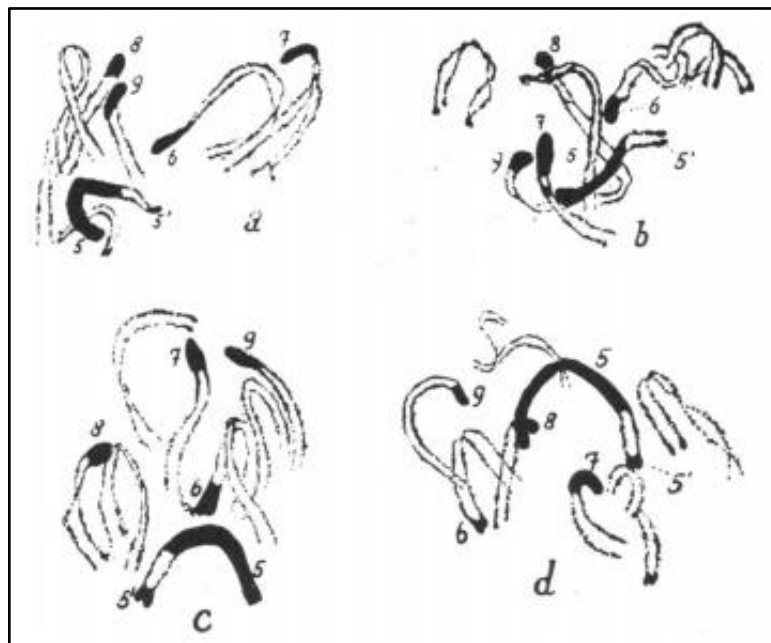


Figure 10 Illustration of Heitz’s chromatin staining of *Pellia epiphylla* chromosomes. Heterochromatin is identified by its dark staining, and euchromatin by light staining. From Heitz, 1928.

Since then, it has been discovered that heterochromatin contains repetitive genetic elements such as satellite repeats and transposons. An important function of heterochromatin compaction is to ensure genetic stability through suppression of such elements. Heterochromatin has also been shown to be responsible for maintaining centromeres and telomeres during cell division, as well as directing cell-type-specific transcription via its spread or recession throughout the genome (Allshire and Madhandi, 2018).

In this section we will go over the basics of chromatin formation and introduce some of the proteins responsible.

2.1.B The Basic Unit of Chromatin: The Nucleosome

The basic unit of chromatin is the nucleosome. It's comprised of approximately 145-147 nucleotide base pairs of DNA wrapped 1.65 turns around a histone octamer. This octamer is comprised of two of each of the canonical core histones: H2A, H2B, H3 and H4 (Klug *et al*, 1980). The core histones house a conserved hydrophobic histone fold domain that mediates the dimerization of H2A-H2B and H3-H4 (Arents *et al*, 1991; Arents and Moudrianakis, 1995). Exposed positive charges on the outside of the histone core help stabilize histone-DNA interactions via ionic interactions with negatively charged phosphate groups of DNA (Luger *et al*, 1997; Luger and Richmond, 1998). Histone H1, known as the “linker histone,” binds the linker DNA that stretches between nucleosomes where it enters and exits the nucleosome. It has been shown to stabilize the structure of the nucleosome and aid in the formation of the secondary structure of chromatin known as the 30-nm chromatin fiber (Woodcock *et al*, 2006; Kruithof *et al*, 2009; Kalashnikova *et al*, 2016; Korolev *et al*, 2018). Interactions between nucleosomes mediated by post-translational

modifications (PTMs) of the histones themselves, as well as non-histone chromatin associated proteins induce fiber-fiber interactions and looping that leads to further compaction (Fig 11).

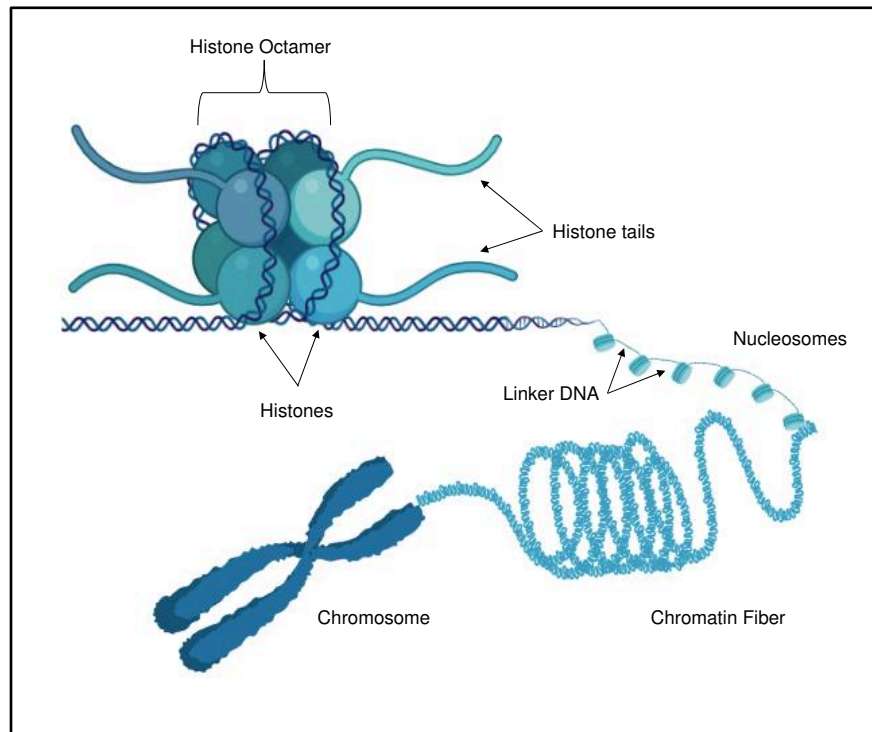


Figure 11 Schematic of chromosome formation. Free DNA associates with histone core to form nucleosomes that further compact into the chromatin fiber, which condenses even further into a chromosome.

Stability of the nucleosome core particle (NCP) determines DNA accessibility and can be influenced by several factors such as: nucleosome-DNA interaction, histone variants, DNA methylation and post-translational modifications of histone tails. It is important to note that DNA wrapped around histones is not necessarily unavailable for transcription. It has been shown that DNA can spontaneously, and reversibly, dissociate from its histones, giving transcription machinery accessibility (Li *et al*, 2005). DNA binding affinity of the histone core can also be dependent on sequence (Gottesfeld *et al*, 2001).

Variant swapping a major-type histone with its non-canonical variant can induce further condensation or relaxation of the chromatin. For example, H2A.Z, a variant of H2A, has been

shown to be enriched within euchromatin and acts to block the spread of heterochromatin into these regions undergoing active transcription (Meneghini et al, 2003; Chakravarthy *et al*, 2008). Structural studies utilizing fluorescence resonance energy transfer (FRET) revealed that nucleosomes containing H2A.Z-H2B variant dimers are more stable than the canonical H2A-H2B containing nucleosome. This increased stability lends to this variant's ability mechanistically contribute to blocking heterochromatin spread (Park et al, 2004; Chakravarthy *et al*, 2008).

Modifications to the histones themselves are a major mechanism for influencing chromatin structure and function. They occur on the amino acid side chains of the eight lysine-rich tails that extend from the NCP via the N-terminal domains of histones H3 and H4 (Fig 11). These histone tails are largely responsible for interacting with neighboring nucleosomes and provide a binding site for non-histone proteins. Common PTMs to histone tails include but are not limited to acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation, and SUMOylation. Histone PTMs recruit chromatin remodeling machinery that work to either condense chromatin, rendering DNA transcriptionally inactive, or alternatively, to decondense chromatin for transcription. The different types of histone PTMs and their effect on chromatin remodeling will be further detailed later in the chapter. (Luger *et al*, 2012; Hauer and Gasser, 2017).

2.1.C Chromatin Associated Proteins

While histones are key in the formation of nucleosomes, there are a number of other non-histone proteins that are important for influencing chromatin compaction.

The most abundant non-histone protein present in chromatin are the high mobility group proteins (HMG). They bind DNA and nucleosomes to modulate DNA accessibility for transcription machinery or other chromatin remodeling-complexes and can be subclassified into three subfamilies: HMGA, HMGB, and HMGN. Members of the HMG subfamilies have been shown to bind DNA in a structure-dependent manner and can influence chromatin state by competing with histone H1 to bind linker DNA, which then promotes loosening of the chromatin for

transcription (Stross, 2010). They are also capable of bending and looping DNA to bring enhancer elements closer to transcriptional start sites (Ozturk *et al*, 2014).

Heterochromatin protein 1 (HP1) has been well characterized for its architectural role in heterochromatin formation, chromosomal segregation, chromatid cohesion, DNA replication and repair, telomere maintenance, and transcriptional regulation. There are three isoforms in humans, HP1 α , HP1 β , and HP1 γ , which recognize di- and trimethylation of histone H3, at lysine 9 (H3K9me_{2/3}), and bind nucleosomes via their conserved chromodomains (Kumar and Kono, 2020). HP1 isoforms interact with themselves between nucleosomes to form higher-order chromatin structures. HP1 also interacts with histone methyltransferases, such as SUV39 as well as histone deacetylases to encourage heterochromatin spread (Janessen *et al*, 2018).

The dynamic activities of chromatin require rapid deposition and turnover of histones to keep up with transcriptional demands. A vast network of histone chaperones and co-chaperones are responsible for regulating the histone supply. Chaperones aid in the transport of histones to their new nucleosome, nucleosome assembly, and store of histones that are not DNA-bound by shielding them from non-DNA interactions. They also function to modulate histone turnover in which histones are evicted from the nucleosome at a given loci, and then rapidly replaced. Failure to maintain the balance of the histone pool could result in problems with centromere and telomere formation, DNA replication, and ultimately compromise genome integrity (Hammond *et al*, 2017).

2.1.D The Main Players in Chromatin Modulation: Writers, Readers, and Erasers

Whether a given gene is available for transcription depends on the state of the chromatin in which it is located. Transcription machinery is able to access the freely available, non-histone bound, euchromatin with ease.

There are two major mechanisms employed to regulate chromatin accessibility. One such mechanism involves chromatin remodeling complexes that can slide or evict nucleosomes from

the DNA, thus exposing DNA for transcription. The other mechanism for chromatin remodeling, and the focus of this thesis work, is the post-translational modification of histones. These PTMs recruit chromatin remodeling complexes capable of influencing the activity state of chromatin to make it more or less available for subsequent transcription (Längst and Manelyte, 2015).

Before we get into the mechanisms by which chromatin accessibility is modulated, we need to introduce the concept of readers, writers, and erasers in this context.

Proteins capable of recognizing chromatin landmarks are termed, “readers”. Readers can recognize a multitude of chromatin targeting signals, such as histone modifications, DNA methylation, as well as DNA sequence/structure, non-coding RNAs, histone variants or DNA-bound interacting proteins. They may act within large protein complexes to recruit chromatin remodelers, or they can be a domain of protein that also contains a chromatin-modifying domain within the same protein (Torres and Fujimori, 2015). For example, Clr4, the yeast homolog of human histone lysine methyltransferase, Suv39h1/2, contains a 'reader' chromodomain at its N-terminus capable of recognizing H3K9 (the 9th lysine residue the N-terminal tail of histone H3). Within the same peptide, Clr4 harbors a SET domain at its C-terminus with methyltransferase activity capable of adding methyl groups to H3K9 (Nakayama *et al*, 2001; Zhang *et al*, 2008). This is an example of a protein that is both a “reader” and a “writer”, which brings us to the ideas of “writers” and “erasers”.

“Writer” is the term used to describe proteins that add modifications to DNA or histone tails. Writers include enzymes like methyltransferases, acetyltransferases, kinases, and ubiquitinases. “Eraser” describes proteins capable of removing modifications made by the writers. The most pertinent of example of an eraser for the scope of this work would be histone demethylases; enzymes that remove methyl groups to histone tails. Other well characterized erasers include

deacetylases, phosphatases, and deubiquinases. (Fig 12) Readers identify chromatin landmarks, and in turn recruit writers or erasers to make modifications that influence chromatin accessibility.

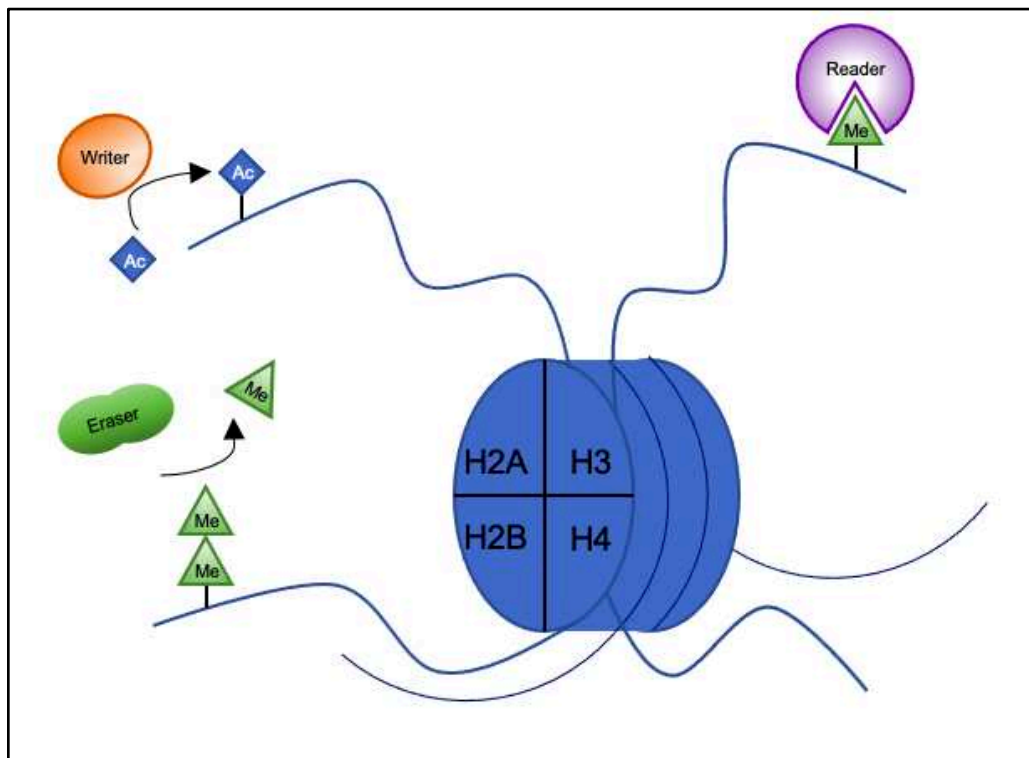


Figure 12 Readers recognize post-translational modifications made to histone tails of H3 and H4, among other chromatin associated landmarks. Writers add modifications like methyl, acetyl, and phosphate groups. Erasers are capable of removing modifications made by the writers.

Writers, readers, and erasers often work within larger chromatin remodeling complexes which also house chromatin remodeling enzymes to carry out more targeted activities. Such chromatin remodeling enzymes are ATPases capable of repositioning or evicting nucleosomes. For example, Chd3 and Chd4, from the CHD family of ATPase chromatin remodelers, work in concert with histone deacetylases, HDAC1 and HDAC2 in the NURD (nucleosome remodeling and deacetylase) complex to repress transcription during development (Murawska and Brehm, 2011). Thus, the activity of writers and erasers often depends on the PTM identified by a reader and the other enzymes with which they are complexed.

2.2 Histone Modification: An Overview

2.2.A Types of Histone Modification

There are a multitude of modifications that can be made to histones, and their mechanisms of influence on chromatin accessibility varies. In this section, we will give a brief overview of the following modifications: acetylation, phosphorylation, ubiquitination, and SUMOylation. Methylation will be discussed in its own section later.

Acetylation

Acetylation and deacetylation involve the addition or removal of an acetyl group to the lysine residues of histone tails. The writers of histone acetylation are a group of enzymes called histone acetyltransferases (HATs), and the erasers are referred to as histone deacetylases (HDACs).

HATs use acetyl-CoA as a co-factor transfer an acetyl group from acetyl-CoA to the E-amino group of lysine side chains (Fig 13). This neutralizes the lysine's positive charge, thus weakening the DNA-nucleosome interaction. HAT activity is often associated with transcriptional activation as the disruption of electrostatic interactions between the histone and the DNA loosens the DNA and provides access to transcriptional machinery.

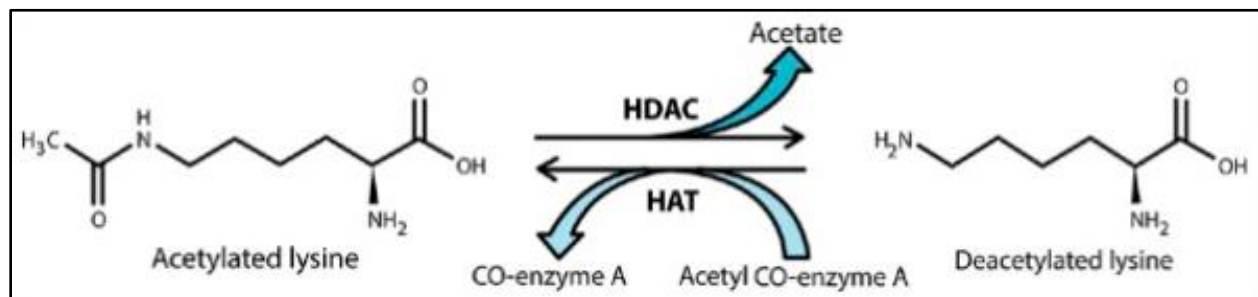


Figure 13 Schematic summarizing the activity of histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs utilize Acetyl-CoA as a co-factor to attach an acetyl group to lysine residues of histone tails. HDACs remove acetyl groups, forming acetate. (From Sanaei and Kavousi, 2019)

HDAC enzymes reverse the acetylation of lysine residues on histone tails, restoring their positive charge. Thus, they are associated with transcriptional repression. HDACs have low substrate specificity and are capable of deacetylating a variety of sites within histones. They are usually

active within larger protein complexes, which likely aids in directing substrate specificity. (Bannister and Kouzarides, 2011).

Phosphorylation

Histone phosphorylation is performed by kinases that transfer a phosphate group from ATP to the hydroxyl group of the side chains of the target amino acids: serine, threonine, or tyrosine. This adds a negative charge to the histone tail that influences chromatin structure, resulting in a loosening of DNA. Phosphorylation can be reversed by phosphatase activity, though the mechanisms by which phosphatases, as well as kinases, are recruited to histones are not well understood.

Histone phosphorylation near the DNA entry/exit regions of the nucleosome has been shown to destabilize DNA binding and results increased accessibility for transcription. This DNA-unwinding is further increased when phosphorylation works in tandem with histone acetylation (Brehove et al, 2015). In fact, histone phosphorylation is often associated with the recruitment of other histone modifying enzymes that encourage active transcription. For example, phosphorylation of H3T11 (histone H3, threonine 11) induced the activity of demethylase, JMJD2D, which is responsible for moving repressive methyl groups from H3K9 (Metzger, 2008).

Ubiquitination and SUMOylation

Addition of one or more ubiquitin polypeptides to histone tails is another major modification of histones that influences gene expression. While all histones may be affected by ubiquitination, this modification is best characterized by the study of mono-ubiquitination of H2A and H2B. Mono-ubiquitination of H2A (H2AK119ub1) is associated with transcriptional repression. Inversely, mono-ubiquitination of H2B (H2BK123ub1) is associated with gene activation. Both have been shown to be important in regulating the DNA damage response (Bannister and Kouzarides, 2011; Meas and Mao, 2015).

The addition of ubiquitin to its target, a lysine residue on histone tails, involves the sequential activity of three enzymes (Figure 14). Ubiquitin activating enzyme, E1, activates the ubiquitin residue in an ATP-dependent manner, and then loads it onto E2, a ubiquitin-conjugating enzyme. E3, the ubiquitin-ligase, covalently ligates the ubiquitin to its target, and is largely responsible for substrate specificity. Ubiquitin residues may be removed by deubiquitinating enzymes (DUBs) (Jeusset and McManus, 2019; Vaughan et al, 2021).

SUMOylation, the addition of small ubiquitin-like modifiers (SUMO) to histone tails, has been shown to influence transcription. Covalent attachment of SUMO to the lysine residue of its target sequence occurs via a similar mechanism to that of ubiquitination, described above with the consecutive activity of E1, E2, and E3 enzymes (Fig 14) (Boulanger *et al*, 2021; Vaughan *et al*, 2021). SUMOylation of histones has been classically associated with transcriptional repression, but recently it's been suggested that it may have roles in active transcription of some genes (Wotton

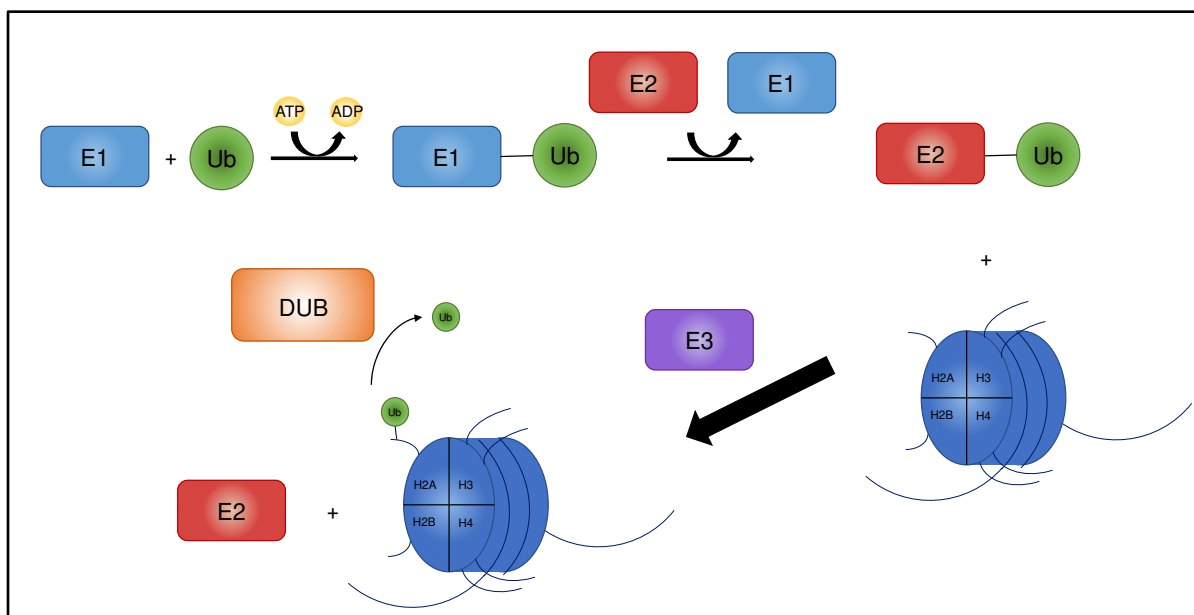


Figure 14 Scheme representing ubiquitination and deubiquitination process of H2A. Ubiquitin activating enzyme, E1, attaches ubiquitin to ubiquitin-conjugating enzyme, E2. Ubiquitin ligating enzyme, E3 then ligates ubiquitin from E2 to the target mark. Ubiquitin can be removed by deubiquitinating enzyme (DUB) activity.

et al, 2017).

Methylation

Histone methylation has many layers of complexity. It does not influence the charge of the nucleosome, thus its mechanism for influencing chromatin structure relies on the recruitment of other chromatin-modifying proteins. Lysines and arginines are the predominant amino acids targeted for methylation. Arginines can be mono-, and symmetrically or asymmetrically dimethylated, while lysines can be mono-, di-, or tri-methylated (Bannister and Kouzarides, 2011). For the purpose of this work, we will focus on methylation of lysine residues.

Histone methylation may induce transcriptional repression or activation depending on the position of the modification within the gene region, which histone residue is methylated, or even how many methyl groups are on a given residue. Some histone methylation marks, especially when co-expressed, can be indicative of cellular processes. For example, methylation at H3K9, H3K27, and H4K20 are generally implicated in heterochromatin formation and subsequent transcriptional repression. Methylations at H3K4, H3K36 and H3K79 are associated with active transcription. Dysregulated histone methylation, through unbalanced activity of histone lysine methyltransferases (HKMTs) and/or histone lysine demethylases (HKDMs) has been implicated in a number of cancers and neurodegenerative diseases, such as Alzheimer and Huntington diseases (Hyun *et al*, 2017). In the next section, we will introduce the enzymes responsible for methylation and demethylation of histone lysine residues and will discuss in more detail about the histone methylation marks most relevant for this work.

2.3. Writers and Erasers of Histone Lysine Methylation

2.3.A - HKMT Writers

As mentioned in the previous section, there are 24 different histone lysine methyltransferases. With the exception of DOTL1, whose methyltransferase activity is catalyzed by its 7 β S (seven - beta-strand) domain, the rest of the HKMTs contain SET domains (Husmann and Gozani, 2019). The SET domain catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to the ϵ -amino group of a lysine residue (Fig 15) (Koturbash *et al*, 2012). SAM is a metabolite synthesized from ATP and L-methionine and is a precursor for a multitude of cellular biochemical

reactions including, transmethylation, and transsulfuration reactions, as well as polyamine synthesis (Bottiglieri, 2002; Loenen, 2006). As the central player for so many metabolic processes, fluctuation in intracellular SAM levels can influence HKMT activity, thus linking metabolism to chromatin state. We will be discussing the association between metabolism and chromatin

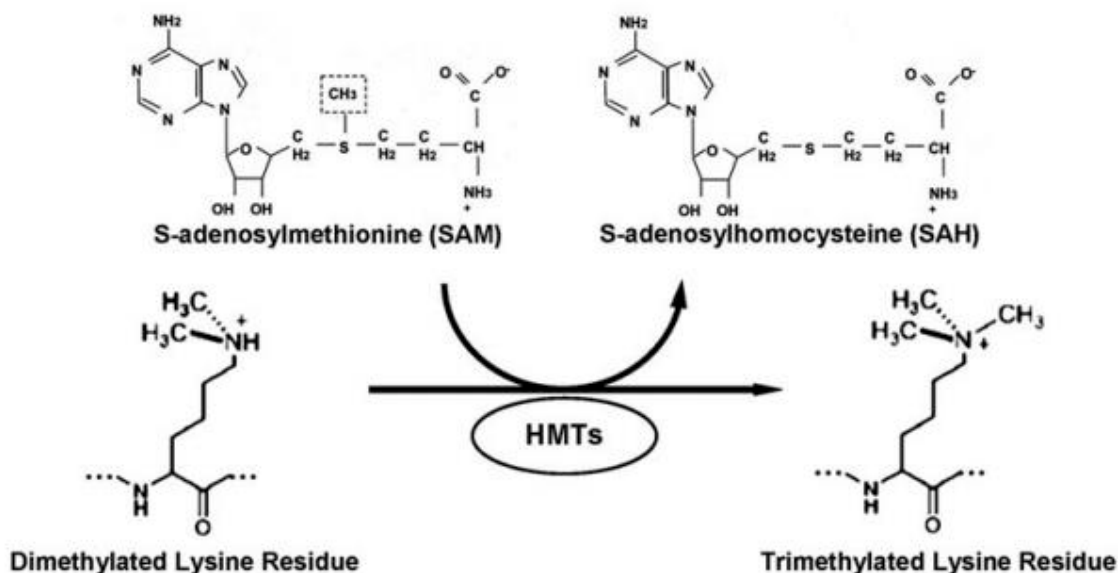


Figure 15 Schematic of SET domain – containing histone methyltransferase activity. HMTs remove a methyl group from S-adenosyl-L-methionine and transfer it to the ϵ -amino group of the target lysine residue (From Koturbash *et al*, 2012).

modification later on.

There is both redundancy and specificity of activity within these enzymes. Each HKMT is usually specific for methylating just one residue, though they may be able to influence more than one methylation state. However, there are often multiple HKMTs that can methylate at a given lysine residue. For example, SUV39H1 can only di- or tri- methylate H3K9, but H3K9 can also be dimethylated by G9a, GLP, and SETDB1, as well as di- and trimethylated by SUV39H2. While it may seem redundant, it is likely useful for targeting methylation to specific genomic regions in a context-dependent manner (Husmann and Gozani, 2019).

As discussed previously, HKMTs often work within larger protein complexes that modulate their target specificity. X-ray crystallography studies have shown that the specificity for multi-methylation by different HKMTs is also dependent on which aromatic amino acid is present in its catalytic binding pocket (Zhang *et al*, 2003). For example, if there is a phenylalanine in the

catalytic domain of G9a at position 1205 (Phe1205), the enzyme is capable of di- and trimethylation. Alternatively, a tyrosine at the same position (Y1205), blocks trimethylation, and G9a activity shifts to mono- and di-methylation. (Collins *et al*, 2005).

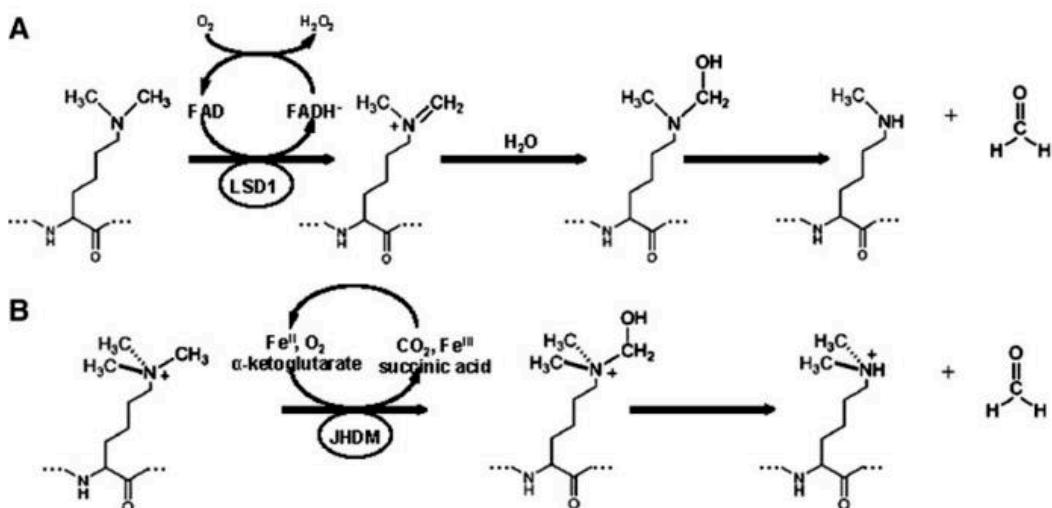


Figure 16 Schematic of histone demethylase mechanisms of action. A) LSD1 oxidizes the methyl group on the methylated lysine with FAD to form an imine intermediate. Hydrolysis of the imine intermediate results in formation of a demethylated lysine residue, and formaldehyde. B) JHDM hydroxylates the methyllysine group using O₂ and α-ketoglutarate which creates a hydroxyl-methyllysine intermediate, and byproducts of carbon dioxide and succinate. This hydroxyl-methyllysine intermediate decomposes to yield a demethylated lysine residue and formaldehyde. (From Koturbash *et al*, 2012)

2.3.B - HKDM Erasers

Lysine-specific demethylases (LSD1/2)

LSD1 was the first histone demethylase ever to be characterized (Shi *et al*, 2004). LSD1 and its homolog LSD2 are FAD-dependent amine oxidases that utilize cofactor, flavin adenine dinucleotide (FAD), to demethylate H3K3me1/2 or H3K9me1/2 via their amine-oxidase (AO) – like domains. As depicted in Figure 16A, LSD1 utilizes FAD to oxidize the methyl group on the methylated lysine which forms an imine intermediate. This intermediate is then hydrolyzed which results in a now demethylated lysine residue, and formaldehyde as a byproduct. Reduced FAD (FADH⁻) is reoxidized by molecular oxygen to replenish FAD and produces peroxide as a byproduct (Koturbash *et al*, 2012; Maiques-Diaz and Somervaille, 2016).

Similar to the HKMTs, the specificity of HKDMs is often determined by the complex in which it is associated. For instance, when LSD1 complexes with the Co-REST repressor complex, its demethylates H3K4me1/2. However, its specificity shifts to H3K9me1/2 demethylation when coupled with androgen receptor (Klose and Zhang, 2007).

Jumonji (JmjC) domain demethylases

Table 1 Histone Lysine Demethylases and their associated substrates (adapted from Accari and Fisher, 2015).

Enzyme Family	Group	Subgroups	Substrate(s)
LSD	KDM1 (Lysine (K) Demethylase -1)	LSD1	H3K4me1/2; H3K9me1/2
		LSD2	H3K4me1/2
JMJC	KDM2	JHDM1A/KDM2A	H3K36me1/2; H3K4me3
		JHDM1B/KDM2B	H3K36me1/2; H3K79me3
	KDM3	JHDM2A/JMJD1A/KDM3A	H3K9me1/2
		JHDM2B/JMJD1B/KDM3B	H3K9me1/2
		JHDM2C/JMJD1C/KDM3C	H3K9me1/2
	KDM4	JHDM3A/JMJD2A/KDM4A	H3K9me2/3; H3K36me2/3
		JMJD2B/KDM4B	H3K9me2/3; H3K36me2/3
		JMJD2D/KDM4C	H3K9me2/3; H3K36me2/3
		JMJD2D/KDM4D	H3K9me1/2/3; H3K36me2/3
	KDM5	JARID1A/KDM5A	H3K4me2/3
		JARID1B/KDM5B	H3K4me2/3
		JARID1C/SMCX/KDM5C	H3K4me2/3
		JARID1D/SMCY/KDM5D	H3K4me2/3
	KDM6	UTX/KDM6A	H3K27me2/3
		UTY	H3K27me2/3
		JMJD3/KDM6B	H3K27me2/3
	KDM7	KDM7A/KIAA1718	H3K27me1/2; H3K9me1/2
		PHF8/KDM7B	H3K9me2; H3K27me2; H4K20me1
		PHF2/KDM7C	H3K9me1; H4K20me3

The largest group of HKDMs are the jumonji domain-containing histone demethylases (JHDMs). First discovered in 2006, they are capable of demethylating trimethylated lysine residues, unlike LSD1/2. This discovery was important as it demonstrated that histone methylation could be completely reversed and was more flexible than initially proposed (Tsukada *et al*, 2006; Whetstone *et al*, 2006). The JHDMs belong to a superfamily of proteins called Fe²⁺-dependent dioxygenases (summarized in Table 1). These enzymes require ferrous iron (Fe²⁺), molecular oxygen (O₂), and α-ketoglutarate (also called 2-oxoglutarate) as co-factors to perform their function.

Their mechanism of action, as depicted in Figure 16B, begins with the hydroxylation of the methyllysine group which uses O₂ and α-ketoglutarate to create a hydroxyl-methyllysine intermediate, and byproducts of carbon dioxide and succinate. This hydroxyl-methyllysine intermediate is inherently unstable and will spontaneously decompose to yield a demethylated lysine residue with formaldehyde as a byproduct (Accari and Fisher, 2015). By virtue of their metabolite co-factors, JDHM activity may be influenced by fluctuations in metabolism. The TCA intermediates, fumarate and succinate, and 2-hydroxyglutarate, a derivative, are competitive inhibitors of multiple α-ketoglutarate - dependent dioxygenases which will be detailed in the next chapter (Xiao *et al*, 2012).

2.3.C Histone Lysine Methylation Modifications of Interest

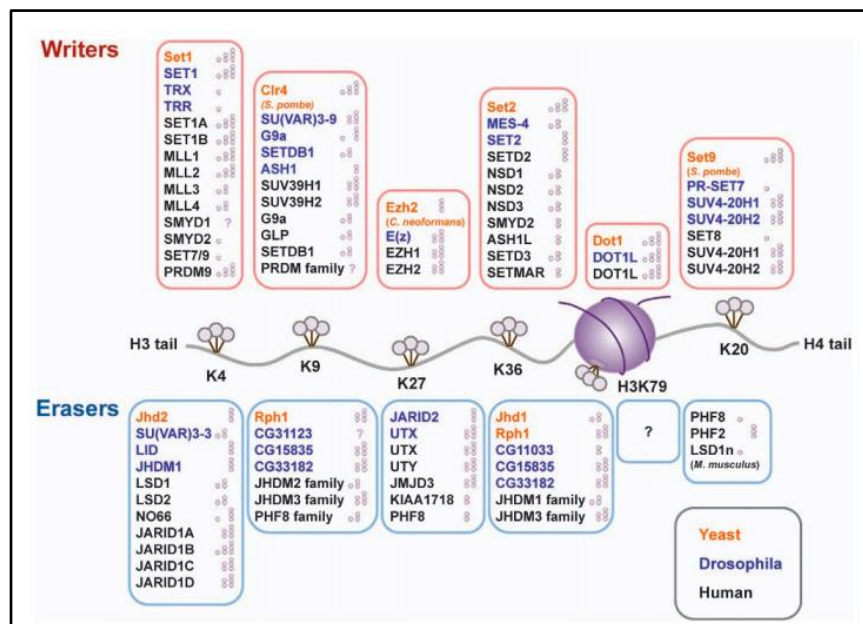


Figure 17 Schematic summarizing methyltransferases (writers) and demethylases (erasers) for the major histone lysine methylation sites. Level of methylation associated with each is displayed by a single circle (me1), double circles (me2), and triple circles (me3). (From Hyun *et al*, 2017)

Histone H3, Lysine 4 (H3K4)

H3K4 methylation is enriched near promoter regions and transcriptional start sites. It is associated with gene activation. H3K4 is methylated via protein complexes that contain a methyltransferase from the SET1 family. The target specificity of these methyltransferases is often determined by other subunits within the multi-protein complex. For example, the SET domain of MLL1 (mixed-lineage leukemia 1) alone is weakly capable of monomethylating H3K4. However, its specificity is enhanced when it works within the WRAD complex. The WRAD complex is composed of four common subunits: WD repeat containing protein 5 (WDR5), Retinoblastoma Binding Protein 5 (RbBP5), Absent-Small-Homeotic-2-Like protein (ASH2L) and Dumpy-30 protein (DPY30) (Patel *et al*, 2009).

Monomethylation (me1) of H3K4 is enriched at enhancer regions, and dimethylation (me2) occurs most frequently at the 5' end of transcribed genes. Trimethylation (me3) of H3K4 is associated with promoter regions of actively transcribed genes (Hyun *et al*, 2017). Methylation of H3K4 is also involved in poised chromatin of embryonic stem cells (ESCs). Poised chromatin is a term used to describe chromatin that is in transition between active and inactive states. It has been well characterized in embryonic stem cells (ESCs) and is important for cell-type differentiation. H4K3me3 and H3K27me3 have been shown to be important in regulating transcription at these bivalent promoters during development (Bae and Lesch, 2020).

H3K4 methylation is recognized by chromatin remodelers and transcription factors. For example, CHD1, an ATP-dependent chromatin remodeler, recognizes H3K4 methylation via its chromodomains, and then utilizes ATPase hydrolytic activity to reposition nucleosomes and promote transcriptional elongation (Sims *et al*, 2019).

H3K4 demethylase activity results in gene repression. There are a number of enzymes capable of demethylating H3K4, among them are FAD-dependent demethylases, LSD1 and LSD2 (KDM1A/B respectively) (Shi *et al*, 2004; Ciccone *et al*, 2009). Several jumonji domain-containing (JmjC) demethylases have also been implicated in demethylation activity, such as:

JARID1A-D/KDM5A-D, and NO66 (Højfeldt *et al*, 2013). The mechanism by which the JmjC demethylases work will be described in more detail later on in the chapter.

Histone H3, Lysine 9 (H3K9)

Methylation of H3K9 is most associated with heterochromatin and gene silencing. Subsequent interactions with HP1 isoforms HP1 α/β , who can read H3K9 methylation, result in heterochromatin spread between neighboring nucleosomes (Lachner *et al*, 2001; Canzio *et al*, 2014). It is especially important for the formation of pericentromeric heterochromatin and has been shown to play a role in alternative splicing (Hyun *et al*, 2017).

There are several HKMTs that methylate at this residue which include: SUV39H1, SUV39h2, G9a-GLP dimers, SETDB1, and the PRDM family of HKMTs. SETDB1 and SUV39H1/2 catalyze the formation of pericentromeric heterochromatin by trimethylation of H3K9me3 (Rea *et al*, 2000).

Methylation via G9a-GLP heterodimers is associated with repressing gene expression from within euchromatin. (Tachibana *et al*, 2002; Shinkai and Tachibana, 2011).

Histone demethylases that remove methyl groups from H3K9 include members of the JmjC demethylases - 2 (JHDM2/KDM3), the JHDM3/JMJD2/KDM4 family, and PHF8/KDM7. Interestingly, PHF8 preferentially demethylates H3K9 from histones that already contain H3K4me3, thus revealing a mechanism in which demethylation supports transcriptional activation (Horton *et al*, 2010).

Histone H3, Lysine 27 (H3K27)

Trimethylation of H3K27 is associated with transcriptional repression and plays an important role in the stabilization of gene silencing; the most well characterized example of which is the inactivation X-chromosomes in mammalian cells in which H3K27me3 is highly enriched (Silva *et al*, 2003). H3K27 methylation, and demethylation, also play a crucial role in directing cell differentiation in embryonic stem cells (ESCs) (Margueron and Reinberg, 2011).

Mono-, di-, and trimethylation of this residue are carried out by the polycomb repressive complex 2 (PRC2), which is a multi-protein complex. The methyltransferase activity is carried out by the EZH2 subunit of PRC2, while the SUZ12 and EED subunits are capable of recognizing and binding the N-terminal domain of H3 and H3K27me3 respectively. This allows for the initiation and subsequent spread of H3K27me3 across a gene (Margueron and Reinberg, 2011).

H3K27 demethylases, UTX/KDM6A, UTY/KDM5C, and JMJD3/KDM6B are all jumonji domain-containing (Hyun *et al*, 2017). They are all involved in gene derepression. JMJD3/KDM6B demethylates H3K27me2/3 to activate transcription of development-related genes during endoderm development (Jiang *et al*, 2013). Interestingly, UTX/KDM6A may be regulated by the H3K4 methyltransferase complex, MLL4 (Issaeva *et al*, 2007). This is an example of crosstalk between activating (H3K4) and repressive (H3K27) histone modifications.

Histone H3, Lysine 79 (H3K79)

H3K79 methylation has been associated with active transcription. H3K79me2/3 are enriched in the coding regions of actively transcribed genes. Methylation at this residue is also important in the DNA damage response and is necessary for the recruitment of 53BP1 to repair sites (Huyen *et al*, 2004; Jones *et al*, 2008).

DOT1L is the methyltransferase responsible for methylation at H3K79. Unlike the other HKMTs, DOT1L does not have a SET domain, and it has been shown to preferentially methylate nucleosomes that have been ubiquitinated at H2B. H3K79me2/3 is enriched in actively transcribed genes (Feng *et al*, 2002; McGinty *et al*, 2008). Its role in transcription is supported by the activity of its HKMT, DOT1L. DOT1L is known to associate with transcription elongation factors, like ENL, or in complexes that contain transcription elongation factors (Wood *et al*, 2018).

The identification of any H3K79 demethylase was unknown until recently. KDM2B was shown to interact with H3K79 via co-immunoprecipitation, and demethylase assays with recombinant H3K79me3 revealed that KDM2B is capable of demethylating H3K79 *in vitro* (Kang *et al*, 2018).

Histone H4, Lysine 20 (H4K20)

Methylation at H4K20 is associated with transcriptional repression and is implicated in DNA repair pathways, DNA replication, chromatin compaction, nucleosome turnover, and overall maintenance of genome integrity (Jørgensen *et al*, 2013; Shoaib *et al*, 2018).

Mono-, di-, and tri-methylation of H4K20 is catalyzed by different enzymes; SET8, SUV4-20H1, AND SUV4-20H2 respectively (Jørgensen *et al*, 2013; Hyun *et al*. 2017). Interestingly, H4K20 methylation may be regulated by H3K9 methylation via a trans-tail mechanism in which the C-terminus of SUV4-20H2 interacts with HP1, which in turn recognizes and binds H3K9me2/3. When H3K9 methylation is blocked, there is a global reduction of H4K20me3, suggesting that H4K20 methyltransferase activity is modulated by H3K9 methylation state (Schotta *et al*, 2004).

PHD8 and PHF2 are the jumonji domain-containing demethylases capable of demethylating H4K20me1 and H4K20me3 respectively (Jørgensen *et al*, 2013). The PHD domain of PDH8 also interacts with H3K4me2 and H3K4me3, implicating the role of H4K20 demethylation in the activation of transcription (Liu *et al*, 2010). An alternative splice variant of LSD1/KDM1A, termed LSD1n, has been shown to demethylate H4K20me1/2 *in vitro* (Wang *et al*, 2015).

2.4 Influence of Metabolism on Histone Methylation

As discussed previously, histone modifying enzymes rely on common co-factors to carry out their function. Many of these cofactors, like SAM, acetyl-CoA, FAD, and α -ketoglutarate are important metabolites or metabolic intermediates for multiple cellular processes, and their intracellular levels fluctuate with changes in metabolism (Fig 18). This is an important mechanism by which environmental changes can influence gene expression through chromatin modification (Dai *et al*, 2020). In this section, we will discuss relevant examples of how metabolism can influence chromatin structure and function through the co-factor requirements of histone methylases and demethylases.

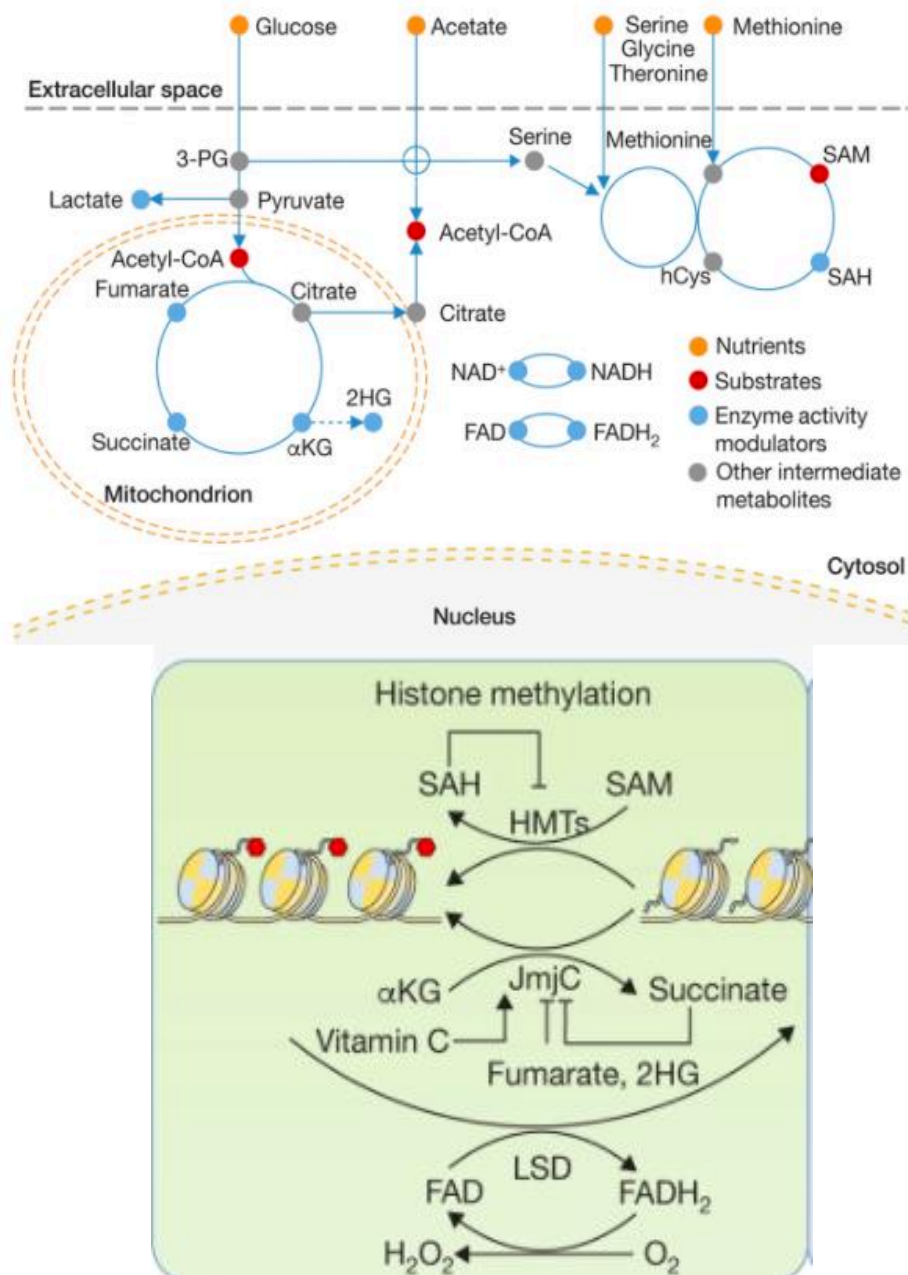


Figure 18 Schematic summarizing the metabolites involved in histone methylation and demethylation. SAM is required for histone methylation and is synthesized from methionine via the methionine and folate cycles. Once SAM donates its methyl group, it is converted to SAH. Restoration of SAM is also dependent on the methionine and folate cycles. LSD1/2 activity are dependent on FAD, generated in the cytosol, while the JmjC demethylase require α -ketoglutarate (α -KG) generated by the TCA cycle in the mitochondria. Succinate, fumarate, and 2-HG (2-hydroxyglutarate) are α -KG analogs that act as competitive inhibitors for the JmjC demethylases. (Adapted from Reid *et al*, 2017).

S-adenosyl-L-methionine (SAM) metabolism influences histone methylation

S-adenosyl-L-methionine (SAM) is an important precursor for a number of intracellular processes such as: synthesis of cysteine and glutathione via transsulfuration, synthesis of polyamines like spermine and spermidine, and transmethylation of a wide variety of substrates (Finkelstein, 2007; Koturbash *et al*, 2012).

Transmethylation reactions involving SAM, like the methylation of histone lysine residues, creates S-adenosyl-L-homocysteine (SAH) as a byproduct. SAH competitively inhibits histone methyltransferases and must thus be rapidly converted back into SAM else risk blocking methyltransferase activity. The balance of SAM and SAH is maintained by the methionine and folate cycles (Fig 19).

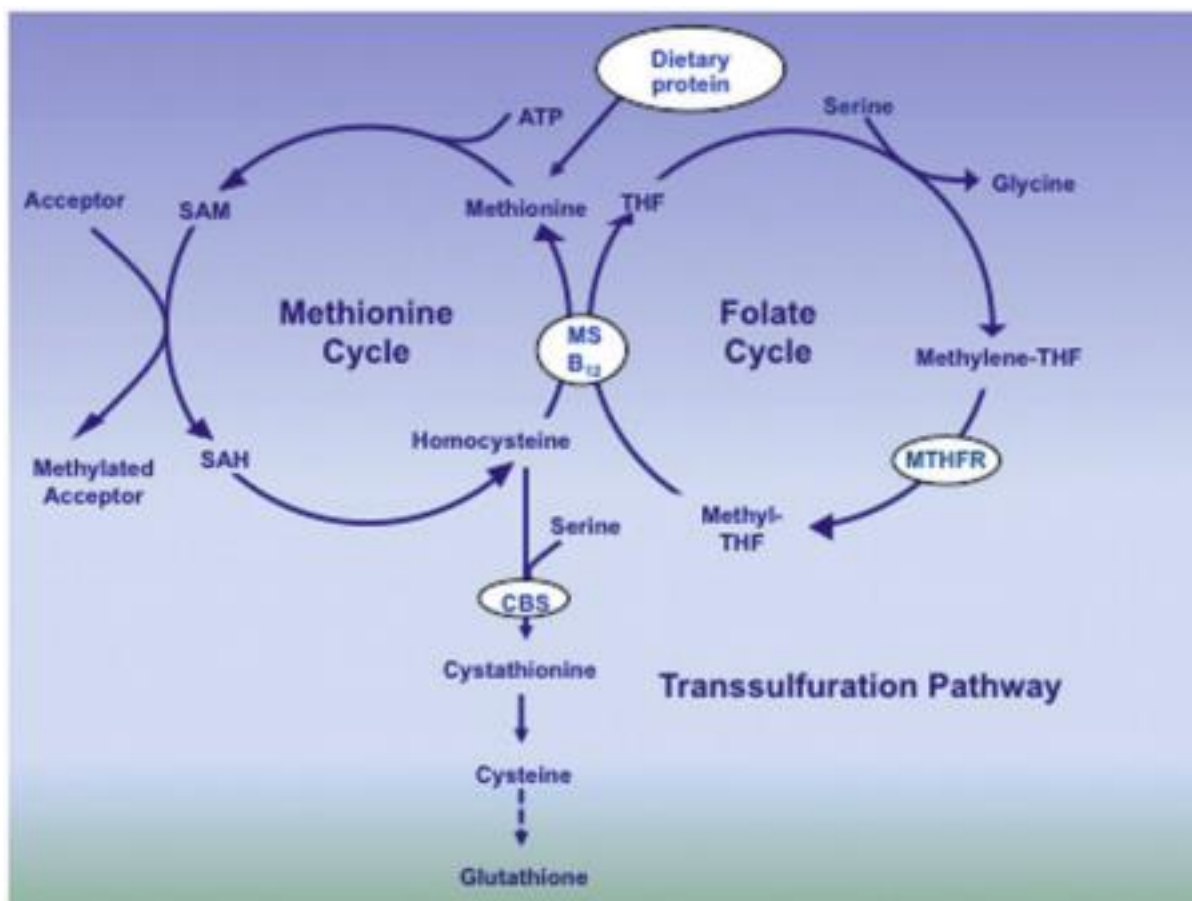


Figure 19 Schematic demonstrating how the methionine and folate cycles interact to restore methionine levels. Dietary methionine and ATP are necessary for the formation of SAM, which is converted to SAH after donating its methyl group. SAH is converted to homocysteine, which can either be further converted to glutathione, or back to methionine. Methionine synthetase (MS), which is B₁₂-dependent, catalyzes the formation of methionine by removing a methyl group from methyl-tetrahydrofolate to homocysteine (methyl-THF). (From McCaddon and Miller, 2015)

SAM is generated from dietary methionine in humans and fluctuations in amount of methionine consumed affect global histone methylation thus influencing gene expression (Dai *et al*, 2020). Dietary methionine restriction in mice, as well as human cancer cells lines, have been shown to reduce global methylation (Mentch *et al*, 2015; Dai *et al*, 2018). Subsequently, dietary methionine restriction can influence transcription in an anti-tumor capacity (Gao *et al.*, 2019).

Flavin adenine dinucleotide (FAD)

FAD(H₂), derived from riboflavin (vitamin B₂), is an important co-factor for TCA cycle reactions, as well as oxidative phosphorylation and fatty acid β -oxidation (Fan *et al*, 2015). While FAD is manufactured in the mitochondria and the cytosol, there is also a pool maintained in the nucleus where it serves as a co-factor for histone demethylases, LSD1/2 (Giancaspero, 2013). LSD1 is capable of demethylating H3K4, which acts to suppress transcription. It has been shown that LSD1-mediated transcriptional repression is dependent on FAD. When FAD is limited in adipocytes, LSD1 activity is inhibited, resulting in transcriptional activation of genes related to energy expenditure and mitochondrial metabolism via maintenance of H3K4me₃ at promoter regions (Hino *et al*, 2012).

TCA cycle intermediates: α -ketoglutarate, succinate, and fumarate

The largest family of histone lysine demethylases are the JmjC – containing α -ketoglutarate dependent dioxygenases. These enzymes require oxygen, iron, and α -ketoglutarate-KG as co-factors to carry out their function.

α -ketoglutarate is a TCA cycle intermediate produced from isocitrate via the activity of one of three isocitrate dehydrogenase (IDH) isoforms. IDH1 produces α -ketoglutarate in the cytosol, while IDH2/3 produce α -ketoglutarate in the mitochondria. α -ketoglutarate can also be produced from glutamine. Mutations of TCA cycle enzymes or dysregulation of glutaminolysis results in α -ketoglutarate depletion leading to inhibition of JmjC demethylases, and subsequent hypermethylation of histones, as reported in different pathological contexts described below.

Glutamine deficiency in a melanoma model showed decreased JMJD3/KDM6B activity, resulting in hypermethylation of H3K27 that induced cell dedifferentiation and rendered the cells less sensitive to chemotherapeutics (Pan *et al*, 2016). α -ketoglutarate analogs succinate, fumarate, and 2-hydroxyglutarate (2-HG), produced by succinate dehydrogenase (SDH), fumarate hydratase (FH), and mutated isoforms of IDH respectively, act as competitive inhibitor JmjC demethylases *in vitro* (Fan *et al*, 2015). Tumor-derived loss-of-function mutations in FH and SDH result in an accumulation of succinate and fumarate and a depletion of α -ketoglutarate, which results in inhibition of JmjC demethylases and increased histone methylation (Xiao *et al*, 2012; Letouzé *et al*, 2013). Mutations in IDH1/2 found in gliomas and acute myeloid leukemias produce 2-hydroxyglutarate (2-HG) from α -ketoglutarate and were shown to inhibit demethylase activity resulting in increased histone methylation at repressive residues and blocking of cell differentiation (Ward *et al*, 2010; Lu *et al*, 2012).

α -ketoglutarate has also been shown to have important roles in ESC differentiation and autophagy through its activity as a demethylase co-factor (Carey *et al*, 2015; TeSlaa *et al*, 2016). This finding supports the afore mentioned dedifferentiation phenotypes observed in cancers with mutations that deplete intracellular α -ketoglutarate.

Iron

Iron is an important metal for countless cellular processes and has been well characterized as a necessary co-factor for JmjC demethylases. However, there is little in the literature characterizing the effect of iron modulation on histone methylation and subsequent chromatin state. It's been demonstrated that iron chelation induces increased sensitivity of breast cancer cell line, MCF-7, to chemotherapy by inhibiting demethylase activity (Pogribny *et al*, 2013). Mitochondria biogenesis was also shown to be impacted by iron depletion via gene repression mediated by histone methylation (Rensvold *et al*, 2016). Recently, one study observed that iron-dependent demethylation of H3K9 is important for cyclin E1 mediated B-cell proliferation (Jiang *et al*, 2019).

Illustration of epigenetic modifications by metabolism: the influence of the microbiota

Fluctuations in metabolism can shift chromatin remodeling activity and subsequently, transcription. The influence of gut microbiota on metabolism has been well characterized as a means of influencing chromatin landscape through shifting metabolism. Microbes within the microbiome have their own metabolism that work to serve their own needs but are also tied in with that of the host's metabolism. Gut microbiota may influence host chromatin state through multiple avenues including: *de novo* synthesis of metabolites, conversion of host dietary nutrients into other active compounds, co-metabolism of shared dietary substrates, and direct competition between the host and microbiota for metabolic intermediates (Kratukramer *et al*, 2017).

De novo synthesis of B-vitamins by the microbes of the gut are an interesting example of how the microbiome are involved in chromatin remodeling. Humans do not efficiently make enough B-vitamins necessary for daily function, but readily absorb those made by their microbiota (Said, 2011). Gut bacteria are capable of *de novo* synthesis of B-vitamins which play important roles as co-factors for histone modifying enzymes as well as for the cycles that support their activity. Among them are riboflavin (B2), niacin (B3), panthothenic acid (B5), pyridoxine (B6), folate (B9), and cobalamine (B12) (LeBlanc *et al*, 2013). As mentioned previously, riboflavin is the precursor for the formation of FAD, the co-factor required for the activity of demethylases LSD1/2. Folate (B9), pyridoxine (B6), and cobalamine (B12) are all crucial to the methionine and folate cycles, which are necessary for restoration of SAM, the methyl donor for methyltransferase reactions (McCaddon and Miller, 2015). Vitamins B3 and B5 are important precursors for NAD and Acetyl-CoA, which are required for histone deacetylation and acetylation respectively (Kirkland, 2009; Leonardi and Jackowski, 2007).

The degradation of dietary substrates like polysaccharides, complex starches, proteins, and amino acids by the gut microbiome into products like short and branched chain fatty acids, and amines are another mechanism by which they may influence chromatin activity. In a colorectal cancer mouse model, the mice were colonized with a minimal microbiome in addition to high butyrate producer, *Butyrivibrio fibrisolvens*, and fed a high fiber diet. Donohoe *et al* observed that there was an increase in butyrate production from the microbiome's break-down of fiber, which resulted in HDAC inhibition and increased acetylation of Histone H3 subsequently influencing apoptosis and cell proliferation. These *in vivo* results were consistent with findings of higher butyrate and

histone acetylation levels in human colorectal cancer samples, suggesting that diet can influence the outcome of chromatin regulation and gene expression in the context of disease (Donohoe *et al*, 2014).

The idea that diet and the microbiome are linked to the chromatin landscape was further demonstrated by Kratkramer *et al* in which it was demonstrated that mice with different gut microbiome compositions resulted in differences in the epigenetic states of liver, colon, and adipose tissue. Germ-free mice fed on a diet that included SCFAs normally produced by gut bacteria, recapitulated the histone PTM profile of conventionally raised mice, suggesting that the metabolites produced by the gut microbiota are important for regulating chromatin remodeling and gene transcription (Krautkramer *et al*, 2016).

These data and other support the model that gut microbiota serve as environmental sensors that influence host chromatin through their metabolic response to external stimuli. Metabolites secreted by the microbes of the gut during environmental changes are absorbed by the host and act as effectors that can modulate chromatin state and ultimately influence host gene expression. Next, we will explore some examples in which bacteria can directly modulate chromatin remodeling.

2.5 Modulation of histone PTMs by bacteria

While the shifting of host metabolism and availability of metabolite pools can influence chromatin state through its chromatin modifying enzymes, pathogenic bacteria may do so through the secretion of virulence factors and toxins. Such effectors have been termed, “nucleomodulins,” and are capable of localizing to the host nucleus to manipulate nuclear activities. Nucleomodulins may manipulate transcription by directly transforming host chromatin, or through the modulation or mimicry of host chromatin regulatory factors (Bierne and Cossart 2012; Dong and Hamon, 2020; Hanford *et al*, 2021). Here we will discuss a few examples of nucleomodulins that influence histone modifications and subsequent transcription, that were all discovered at the Institut Pasteur in Paris.

An interesting example of a bacterial effector indirectly influencing histone modifications is the action of LntA (*Listeria* nuclear targeted protein A) in *Listeria monocytogenes*. LntA has been shown to encourage gene expression through interactions with its host binding partner, BAHD-1. BAHD-1 (bromo adjacent homology domain-containing-1) acts as a scaffold for a larger chromatin remodeling complex composed of both histone deacetylases (HDAC1/2), and histone methyltransferases (G9a and SETDB1). BAHD-1-mediated binding of its complex to interferon-stimulated gene (ISG) promoters induces heterochromatin formation and gene silencing via histone deacetylation, and subsequent methylation in those regions. LntA binding to BAHD-1 inhibits BAHD-1 binding to ISG promoters, thus maintaining transcription in those regions (Bierne and Cossart, 2012; Bierne and Hamon, 2020).

Contrasting to LntA, AnkA of *A. phagocytophilum* has been shown to bind DNA directly and recruit HDACs for directed deacetylation and promotes silencing of genes related to granulocyte response (Rennoll-Bankert *et al*, 2015). Recently, a non-invasive strain of *Streptococcus pneumoniae* has been shown to induce changes in methylation of H3K27me2 at the IL-11 promoter region through the recruitment of JmJC demethylase, KDM6B. The authors demonstrated that inhibition of KDM6B activity during infection with non-invasive *S. pneumoniae* induced disease *in vivo*. Conversely, the addition of exogenous IL-11 during infection with a normally invasive strain *S. pneumoniae* decreased bacterial burden and hampered bacterial invasion *in vivo*. While the mechanism by which KDM6B activity is induced by the bacteria under these conditions is not yet known, it is an interesting example of how commensal bacteria influence host chromatin remodeling (Connor *et al*, 2021).

There are also examples of bacterial derived effectors that are capable of directly modifying histones. Among them is NUE (Nuclear effector), a T3SS effector secreted by *Chlamydia trachomatis*. NUE localizes to the host-cell nucleus through a nuclear localization signal in its C-terminus. It contains a domain homologous to the eukaryotic SET domain, capable of methyltransferase activity, and has been shown to automethylate, as well as transfer methyl groups to H2B, H3, and H4 *in vitro* (Pennini *et al*, 2010). While the *in vivo* targets of NUE activity have yet to have been characterized, their potential for directly regulating host chromatin was an exciting discovery.

Legionella pneumophila, *Paris* and *Philadelphia* strains also secrete SET-domain containing effectors via their type 4 secretion system, termed RomA and LegAS4 respectively. After localizing to the host-cell nucleus, RomA has been shown to methylate H3K14, which has not been shown to be methylated in mammalian cells under normal conditions. Methylation of H3K14 by RomA inhibits transcription and blocks the innate immune response (Rolando *et al*, 2013). LegAS4 also methylates H3K14 and has also been shown to methylate H3K4 to promote transcription of rDNA through associations with HP1 (Li *et al*, 2013).

3. Consequences of *Chlamydia trachomatis* infection on host transcription and chromatin

3.1 Consequences of infection on gene expression

The first microarray study of the consequences of infection on host gene transcription was published 20 years ago (Hess *et al*, 2001). Since then, several microarray or RNA-seq data have been made available (Hess *et al*, 2003; Porcella *et al*, 2015; Zadora *et al*, 2019; Hayward *et al*, 2019). As expected, infection induces important changes in the transcriptional profile of the host, which evolves with the time of infection.

Early microarray studies by Hess *et al*. indicated that *Chlamydia* infection induces increased expression in antiapoptotic genes, glycoprotein 130 cytokines (IL-11, IL-8, and IL-6), and transcription factors associated with growth (EGR1, FRA1, JUN) (Hess *et al*, 2001). They demonstrated that these changes in host cell transcription were due to active infection that is *Chlamydia* specific (Hess *et al*, 2003).

Simultaneous RNA-seq of host cells and *Chlamydia trachomatis* by Humphreys *et al*. revealed transcriptional evidence of innate immune system dampening through modulation of expression of antimicrobial peptides, and mucins. Early host expression of collagen formation-related proteins, Tenascin C and Gremlin-1 are hypothesized to contribute to tissue damage and fibroid formation associated with persistent infection.

They also found that during immediate-early infection, that there was differential expression of linker histone, histone H1 variants. H1.0, the H1 variant most expressed in differentiated cells, was down-regulated compared to upregulated variants H1.4 and H1.5, which are expressed mostly during S-phase (Pan and Fan, 2016). The core histones H2A and H3 are also up regulated, suggesting that modulation of chromatin dynamics due to infection begin as early at 1 hpi (Humphreys *et al*, 2013).

In 2015, Porcella *et al*. performed a microarray study on two genomically isogenic strains of *Chlamydia trachomatis*, one with the chlamydial plasmid and one without, to determine if it contributes to virulence. At 24 and 48hpi time points, both strains induced increased expression of pro-inflammatory cytokines (GM-CSF, MCSF, IL-1, IL-6, and IL-8), apoptotic proteins (CASP1, CASP9, FAS, IL-24), the T-cell chemokine response, and immune suppression (PD-L1), as well as genes involved in cell growth, metabolism, and fibrosis. However, the strain containing the chlamydial plasmid had significantly increased levels of expression of these genes relative to the plasmid-free strain. These results indicated that the chlamydial plasmid acts as a virulence factor that mediates inflammatory response (Porcella *et al*, 2015).

A recent single cell RNA-Seq study of early infection (3, 6, and 12 hpi), supports previous studies indicating that infection induces upregulation of inflammatory cytokines, signal transduction, and fatty acid metabolism. Genes involved in cell cycle progression, centrosome maturation, mitotic and centrosomal proteins, and the p53-related DNA damage response were down regulated at these very early times of infection (Hayward *et al*, 2019).

Analysis of the phosphoproteome carried out by Zadora *et al*. indicated that the MAPK signaling cascade is increased during infection, which leads to the induction of genes implicated in the epithelial-to-mesenchymal transition. This study is interesting in that it provides a mechanism by which *C. trachomatis* infection may induce pro-carcinogenic transcription (Zadora *et al*, 2019).

3.2 Global consequences of *Chlamydia trachomatis* infection on host chromatin

The consequences of *Chlamydia* infection on host-cell chromatin had not been explored until more recently. The concept chlamydial effectors had the potential to directly modify host chromatin was introduced with the discovery of NUE in 2010 (Pennini *et al*, 2010). A global view of histone modifications during infection was published in 2013 by Chumduri *et al*. Their work revealed hypermethylation during infection at histone residues associated with heterochromatin formation (*i.e.*, H3K9me3, H3K27me3, H4K20me3) during acute and persistent infection *in vitro*. They also demonstrated that *Chlamydia* infection induces DNA double strand breaks (DSBs), likely due to reactive oxygen species, and that these DSBs do not go on to be repaired by the DNA DSB repair pathway. In fact, p53 and pATM are downregulated, despite clear DNA damage observed via phosphorylation of γ H2AX and comet assays (Fig 20). Infected cells that were treated with doxycycline continued to proliferate but did not go on to repair the damage inflicted by infection. These results suggested that *Chlamydia* inhibit DNA-repair mechanisms and leave cells genomically unstable and vulnerable to malignancy (Chumduri *et al*, 2013).

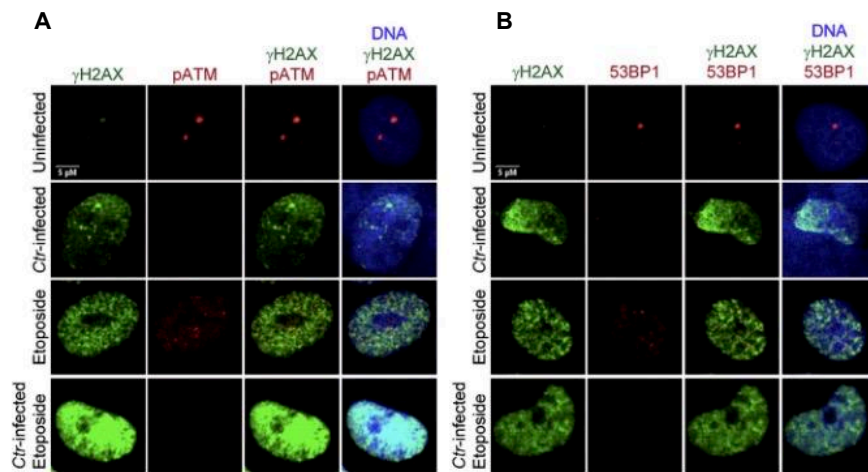


Figure 20 Infection with *C. trachomatis* (Ctr) induces DNA damage but blocks DNA repair mechanisms. (A) End1 E6/7 cells 36 hpi with or without etoposide treatment were stained for phospho- γ H2AX to indicate DSBs and phospho-ATM to indicate recruitment of DNA repair machinery (B) End1 E6/7 cells 36 hpi with or without etoposide treatment were stained for phospho- γ H2AX to indicate DSBs and 53BP1 to indicate recruitment of DNA repair machinery (From Chumduri *et al*, 2013)

Recently, Hayward *et al*. performed FAIRE-Seq (formaldehyde-assisted isolation of regulatory elements enrichment) at 1, 12, 24, and 48 hpi to identify differentially regulated regions of accessible chromatin during infection. They found that chromatin state was dynamic, and regions of accessible chromatin changed during the course of infection. Late infection displayed the

largest proportion of differential chromatin accessibility, with 3128 differentially accessible genomic regions, against 1358 ones at 24 hpi. Overall, the trend was towards a more open chromatin in intergenic and intragenic regions of late infected cells, compared to mock-infected controls. However, when focusing on promoter and enhancer regions, the inverse trend was observed, with more occurrence of decrease in accessibility (Hayward *et al*, 2020).

A recent study in a male model of infection demonstrated that *Chlamydia* infection induces DNA damage in testicular cell lines and that DNA was globally hypomethylated (Bryan *et al*, 2021).

4. Questions addressed by this thesis

During *C. trachomatis* infection, the host cell undergoes major changes to its transcriptome. Many of these changes are due to the break in homeostasis imposed by the needs of developing bacteria (i.e., metabolites, lipids, nucleotides etc.), as well as the innate response of the host to fight the infection. It is also likely that some of the transcriptional regulation is controlled by the bacteria, either directly (e.g., through the secretion of chromatin modifiers) or indirectly (e.g., by interfering with signaling cascades that ultimately result in gene transcription). In agreement with these major changes in the host transcriptional program there is evidence that chromatin is remodeled during *C. trachomatis* infection, which is unsurprising as chromatin state informs downstream transcription.

Since *C. trachomatis* has the capacity to directly control histone methylation through T3SS effector, NUE, and potentially by other effectors that have yet to have been characterized, I began this work by investigating the evolution of several histone marks over the course of infection. It was observed that all histone methylation marks tested were co-regulated during late infection, with an overall increase in said marks in infected cells.

In the following section, I will present the experimental work aimed at addressing two questions:

1. What is the mechanism responsible for hypermethylation of histones during *C. trachomatis* infection?

2. What are the consequences of this hypermethylation on the host response to infection at the transcriptomic level?

Data omitted, but not forgotten

Before we dive into the method section, I would like to acknowledge that this dissertation does not cover the entirety of the work done during my thesis. I began my work on a different project in which I was attempting to characterize a putative nuclear effector of *Chlamydia trachomatis*, named CT790.

The preliminary results performed by immunofluorescence analysis of transfection of CT790 with an amino-terminal FLAG tag had indicated that the effector localized to the nucleus in HeLa cells, a result which I confirmed. CT790 had also been observed in the host cell nucleus during infection with bacteria expressing CT790 with a carboxy-terminal FLAG tag, which suggested that it may be secreted. However, I could not reproduce this result. It is possible that the signal was just outside the limit of the sensitivity of this assay, but this was definitively not encouraging. Furthermore, secretion assays to determine if the putative effector was secreted using the bacterial type III secretion system were initially inconclusive. After some optimization, that assay determined that the effector was not secreted by the bacterial T3SS. This does not rule out the possibility that it is secreted by other means, but again this was a disappointing result.

In parallel to these experiments, a yeast Two-hybrid screen was performed using CT790 as bait, to try to identify a host binding partner. After many attempts to verify the host candidates from the screen by co-immunoprecipitation, I finally confirmed one. However, I was unable to confirm any interaction with the endogenous proteins or determine if either protein had an effect on the other *in vitro* during infection.

An antibody had been made, in rabbits, against CT790 and was used for much of the characterization. This was our most encouraging result, since the antibodies stained the nuclei of 10-20% of the infected cells at late time points of infection. However, based on additional control experiments we later came to the conclusion that, in addition to its ability to recognize CT790, the antibody was cross-reacting with an epitope that was only present in the nuclei of infected cells, not uninfected cells, but that was not CT790.

I also attempted to make a knock-out mutant strain of *C. trachomatis* for CT790 using the Targetron Gene Knock-out system that had been modified for use in *Chlamydia*. *Chlamydia* is notoriously difficult to manipulate genetically, and after much effort on my part, and the part of our collaborators who also attempted to make the KO strain, we were unable to obtain the mutant. While it might mean that the protein is essential for *C. trachomatis*, not having a mutant severely limited any other study we could do to characterize the role of this putative effector.

In view of the inability to obtain a CT790 KO mutant, and of the artefactual reaction of our antibody, we decided to shift our focus to the project detailed in this manuscript concerning increased histone methylation during *C. trachomatis* infection. While I was initially disappointed to set the effector project aside after all of time I had dedicated to it, it was ultimately the best choice we could have made. In the end, it was wiser to pursue another equally interesting scientific question, and I am looking forward to sharing those results with the reader in this dissertation. As the data regarding CT790 remain inconclusive at this point, we have decided to omit it from this manuscript.

MATERIALS AND METHODS

Cells and Bacteria

HeLa cells (ATCC) were grown in Dulbecco's modified Eagle's medium with Glutamax (DMEM, Invitrogen), supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (FBS) (D'Eletto *et al*, 2009).

Primary cells were isolated from endocervix biopsies of female patients and were cultivated in keratinocyte- SFM medium (Thermo Fisher Scientific) containing 50 mg.L⁻¹ of bovine pituitary extract (Thermo Fisher Scientific) and 5µg.L⁻¹ of epidermal growth factor (EGF) human recombinant (Thermo Fisher Scientific) (Wu *et al*, in preparation). All cell cultures were maintained at 37 °C, in 5 % CO₂ atmosphere.

C. trachomatis serovar LGV L2 strain 434, GFP-expressing L2 (L2^{IncD}GFP) and *C. muridarum* strain MoPn were propagated on HeLa cells, purified on density gradients as previously described and stored at -80 °C (Scidmore *et al*, 2005; Vromman *et al*, 2014).

Immunofluorescence

100,000 HeLa cells, primary cells, or MEFs were seeded per well in a 24 well-plate. The following day cells were infected as indicated. Cells were treated at either 2 hpi or 24 hpi with 2,2'-Byridyl (Sigma-Aldrich D216305), iron (III) citrate (Sigma-Aldrich F5879), or DMKG (Dimethyl α-ketoglutarate Sigma-Aldrich 349631). 48 hpi, cells were fixed in 4% PFA for 20-30 minutes, quenched in 50mM NH₄Cl, and then washed in 1XPBS. Coverslips were permeabilized with 0.15% Triton X in PBS for 10 minutes, and then blocked for 1 hour in blocking buffer (10% FBS, 3% BSA, and 0.1% Triton X in 1XPBS). Samples were incubated for 1 hour in primary antibodies diluted 1:500 in blocking buffer, washed 3X in 1XPBS, and then incubated for 1 hour in secondary antibodies and Hoechst diluted in blocking buffer. Antibodies used may be found in Table 2. Samples were washed 3X in 1XPBS and then mounted on slides in mowiol. Microscopy was performed on an Axio observer Z1 microscope equipped with an ApoTome module (Zeiss, Germany) and a 63X Apochromat lens. Images were taken with an ORCAflash4.OLT camera (Hamamatsu, Japan) using the ZEN software from Zeiss. Images were analyzed with ImageJ (Rasband, 1998-2018)

Table 2. Antibodies for Immunofluorescence

	Target	Species	Supplier	Catalog #
Primary Antibodies	H3K9me3	Rabbit	Abcam	ab8898
	H4K20me3	Mouse	Abcam	ab78517
	H3K27me3	Mouse	Bp biosciences	25243
	H3K4me3	Rabbit	Diagenode	C15410003
	H3K79me2	Rabbit	Abcam	ab3594
	Phospho- γ H2AX	Rabbit	Cell Signaling Technology	2577
	γ H2AX	Mouse	Merck Millipore	05-636
	Phospho-ATM Ser 1981	Rabbit	R&D	AF1655
	53BP1	Rabbit	Novus Biologics	NB100-304
	H3phosphoSer10	Rabbit	Abcam	ab5176
	H3phosphoSer28	Rabbit	Abcam	ab5169
Secondary Antibodies	Alexa Fluor TM 488 goat anti-rabbit IgG (H+L)	Goat	Invitrogen	A-11034
	Alexa Fluor TM 647 goat anti-rabbit IgG (H+L)	Goat	Invitrogen	A-21244
	Alexa Fluor TM 488 goat anti-mouse IgG (H+L)	Goat	Invitrogen	A-11029
	Alexa Fluor TM 647 goat anti-mouse IgG (H+L)	Goat	Invitrogen	A-21235

Progeny assay

Progeny assays were performed in HeLa cells in which 100,000 cells were seeded in each well of a 24-well plate and were infected with L2^{IncD}GFP bacteria at a MOI = 0.20 the following day. Either 2 or 24 hpi after the initial infection, cells were treated with 5 mM DMKG (Dimethyl α -ketoglutarate Sigma-Aldrich 349631, diluted to 500 mM stock in DMSO) or an equal volume of DMSO as a control.

Forty hpi, cells were detached and fixed in 2 % PFA in PBS prior to flow cytometry analysis in order to evaluate the first round of infection. In duplicate wells, cells were detached, lysed using glass beads and the supernatant was used to infect new untreated cells) plated the day before (100 000 cells/well in a 24-well plate), in serial dilution. The next day, 3 wells per condition with an

infection lower than 30 % (checked by microscopy) were detached and fixed as described above, before analysis by flow cytometry and determination of the bacterial titer (Käser T *et al*, 2016). Statistical analysis was performed in GraphPad Prism version 9.0.

Nuclear Isolation

For each condition, a 10 cm dish was seeded with 2.5 million HeLa cells. Cells were infected MOI 2 with *C. trachomatis* LGV L2. 24 hpi, cell culture media was exchanged, and cells were treated with either 5 mM DMKG or an equal volume of DMSO. 48 hpi, cells were collected fractionation and resuspended in 574.6 µl of Hypotonic Buffer (10 mM Tris pH 7.65, 1.5 mM MgCl₂, 10 mM KCl, 0.2% NP-40, 0.6mM PMSF, 1X Protease inhibitor cocktail (Sigma)). Cells were lysed using a Dounce homogenizer, and then 192 µl of Sucrose buffer (20 mM Tris-HCl pH 7.65, 15 mM KCl, 60 mM NaCl, 0.34 M sucrose, 150 µM Spermine, 50µM Spermidine, 0.6mM PMSF, 1X Protease inhibitor cocktail (Sigma)) was added to the samples. Sample were centrifuged 500xg for 10 minutes. The supernatant (cytosolic fraction) was reserved, and the pellet was resuspended in 5 ml of Wash buffer (1 mM Tris-HCl pH 7.5, 5 mM MgCl₂, 60 mM KCl, 0.5 mM DTT, 15 mM NaCl, 300 mM sucrose). Nuclei were centrifuged at 1700xg for 10 minutes and the nuclei was discarded. Nuclei were resuspended in 500 µl of 1XPBS to be used for other experiments.

Histone extraction

Nuclei from nuclear isolation were resuspended in RIPA buffer (50mM Tris pH 7.5, 150 mM NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, 1 mM DTT, 1X cOmplete EDTA-free protease inhibitor cocktail from Roche) and incubated on ice for 10 minutes. Samples were then digested with 0.0025 units/µl of MNase with 1 mM CaCl₂ at 37 C for 15 minutes, vortexing often. Next, samples were sonicated using Bioruptor Pico sonication device on HIGH, at 30 second intervals for 8 minutes. Finally, samples were centrifuged for 10 minutes at 13,200 rpm to pellet debris, and the supernatants were stored at -80 C.

Western blot

Nuclear samples from nuclear isolation were boiled for 10-15 minutes in 1X Lammeli buffer (300 mM Tris-HCl pH 6.8, 10% SDS, 50% Glycerol, 0.05% Bromophenol blue, 5% β -mercaptoethanol) Samples were then subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS- PAGE) followed by transfer to polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked in 1X phosphate buffered saline (PBS) + 5% milk for 45 minutes-1 hour. After blocking, the membrane underwent immunoblotting with primary antibodies diluted in 1XPBS + 5% milk for one hour. Between the primary and secondary antibody incubations, the membranes were washed in three-ten-minute intervals in 1XPBS + 0.1% Tween-20. Secondary antibodies, mouse-HRP (Amersham cat# NA 931) or rabbit-HRP (Amersham cat# NA 9340), were diluted in 1XPBS+5% milk and left to incubate for one hour. Incubation of the secondary antibody was followed by the wash cycle carried out previously. Blots were developed using Immobilon HRP Substrate (Merck), and the signal was detected using the PXi Touch (Syngene).

qRT-PCR

A 24 well-plate was seeded with 50,000 HeLa cells/well. After cells had adhered, they were transfected with Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's recommendation, using 10 nM of siRNA. The transfection incubated for 30 hours, and then the cells were retransfected again the same way and infected with LGLV2 at MOI = 1. 48hpi, RNA was extracted using RNeasy Mini Kit (Qiagen) with DNase treatment (DNase I, Roche). RNA concentrations were measured with a spectrophotometer NanoDrop (Thermo Fisher Scientific).

Reverse transcription (RT) was performed on 500 ng of each sample using the M-MLV Reverse Transcriptase (Promega) and quantitative PCR (qPCR) undertaken on the complementary DNA (cDNA) with LightCycler 480 system using LightCycler 480 SYBR Green Master I (Roche). Primers used are listed in Table 3, their specificity was ensured through the analysis of melting curves and their efficiency tested using serial dilutions of samples encoding the genes of interest. For cDNA, data were analyzed using the Delta-Delta Ct method with either *36B4* or *ACTIN* as a control (Schmittgen and Livak, 2008).

Table 3. siRNA and qRT-PCR primers

Experiment	Target Gene	Sequence (5' → 3')
siRNA	2-oxoglutarate dehydrogenase complex – E1 subunit #1	GCUAGGACAUUUCAACAGA
	2-oxoglutarate dehydrogenase complex – E1 subunit #2	GAUCUUCUGUCAAUUCGAU
	Control siRNA duplex negative control (Eurogentec cat# SR-CL000-005)	N/A
qRT-PCR	2-Oxoglutarate Dehydrogenase Complex - E1 subunit	GGCTTCCCAGACTGTTAAGAC
		GCAGAATAGCACCGAATCTGTTG
	36B4	TGCATCAGTACCCCATCTATCAT
		AAGGTGTAATCCGTCTCCACAGA
	GADD45A	GAGAGCAGAAGACCGAAAGGA
		CACAACACCACGTTATCGGG
	Noxa (PMAIP1)	GTGCCCTTGGAAACGGAAGA
		CCAGCCGCCAGTCTAATCA
	Actin	GGACTTCGAGCAAGAGATGG
		GCAGTGATCTCCTTCTGCATC

Microarray

A 6-well plate was seeded with 50,000 HeLa cells per well. The following day, cells were infected with *C. trachomatis* LGV L2. 24 hpi, the media was exchanged, and cells were treated with 5 mM DMKG or an equivalent volume of DMSO. 48 hpi RNA was harvested using the RNeasy kit (Qiagen), and their quality was analyzed using the BioAnalyzer (Agilent). The samples were processed on a Human Transcriptome Array (HTA) by the company Eurofin, using a Thermo Fisher GeneChip Scanner 3000 7G. The data was analyzed using Transcriptome Analysis Console Software 4.0.1 (Thermo Fisher).

RESULTS

I. *Chlamydia trachomatis* induces hypermethylation of histones during late infection

HeLa cells infected with *C. trachomatis* LGV L2 for 48 hours were immunostained using antibodies against various histone PTMs, with a focus on methylation. Trimethylation of H3K9, H4K20, H3K27, H3K4, as well as demethylation of H3K79, were only detected in infected cells (Fig 21A). Not all infected cells had observable increases in histone methylation, and the level of methylation varied between cells within the population. Interestingly, cells with increased methylation of one PTM usually had increased methylation of another PTM, indicating co-regulation of said modifications (Fig 21B).

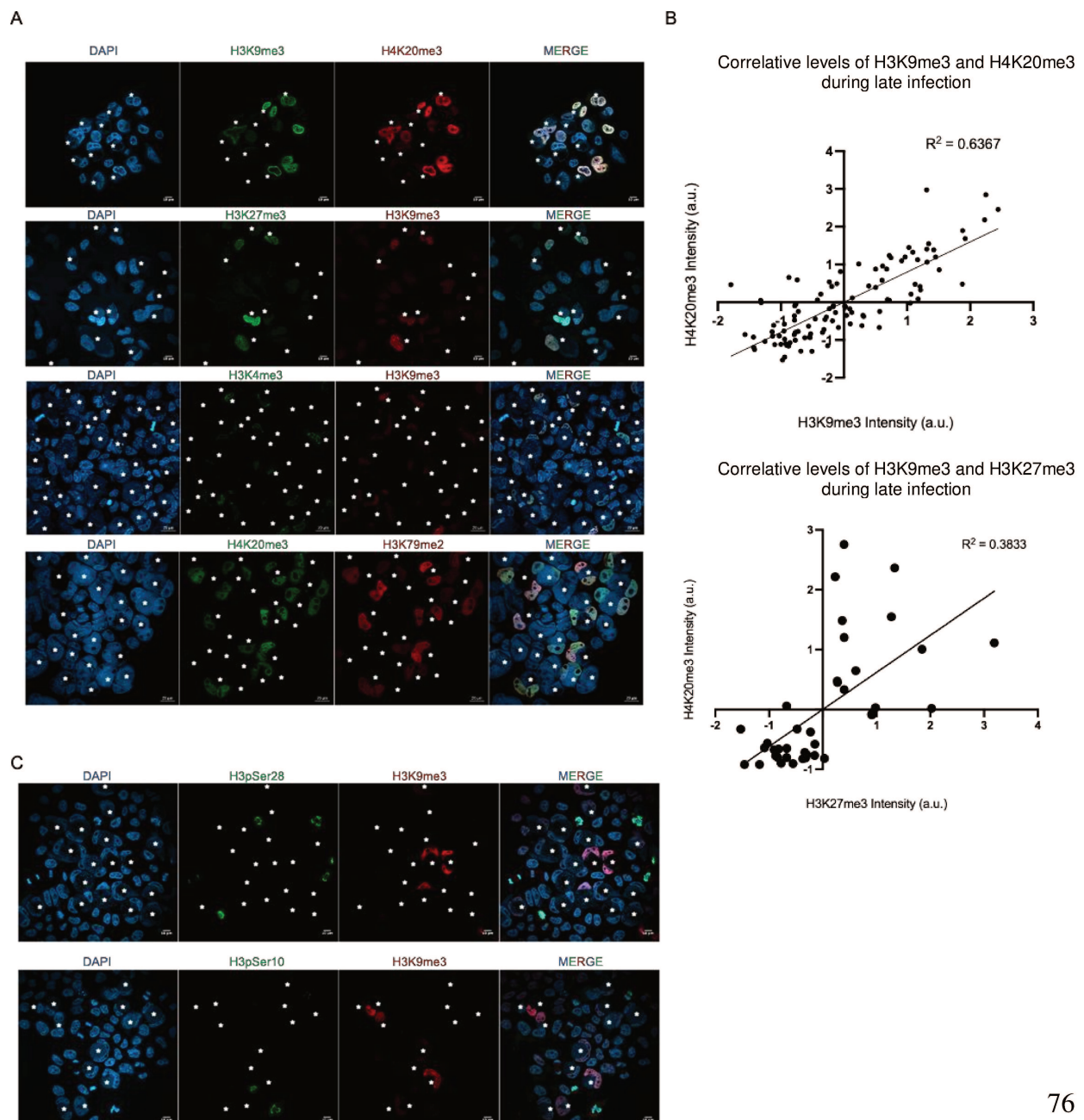


Figure 21 Immunostaining of histone modifications in HeLa cells infected with *C. trachomatis* for 48 hours. (A) Immunostaining of histone lysine methylation of H3K9, H4K20, H3K4, and H3K79. (B) Correlative levels of H3K9me3 vs.H4K20me3 and H3K27me3 vs. H4K20me3. Fluorescence intensity measurements were standardized and normalized using Z-normalization. GraphPad Prism 9.0 was used to plot the Z-scores and determine the R-squared value (C) Immunostaining of histone H3 phosphorylation. (*) Denotes an inclusion

To ensure that the effect we were observing was not due to increased permeability to antibodies in infected cells, we looked at other types of histone PTMs. Cells were immunostained for phosphorylation of H3Ser10 or H3Ser28 in parallel with H3K9me3 (Fig 21C). Cells with detectable methylation at H3K9 did not stain positive for phosphorylation at H3Ser10 or H3Ser28. These two marks were detected in cells undergoing division, as expected (Dong and Bode, 2006). These results indicate that the increased signal for histone methylation marks does not result from better access of the antibodies to chromatin in infected cells.

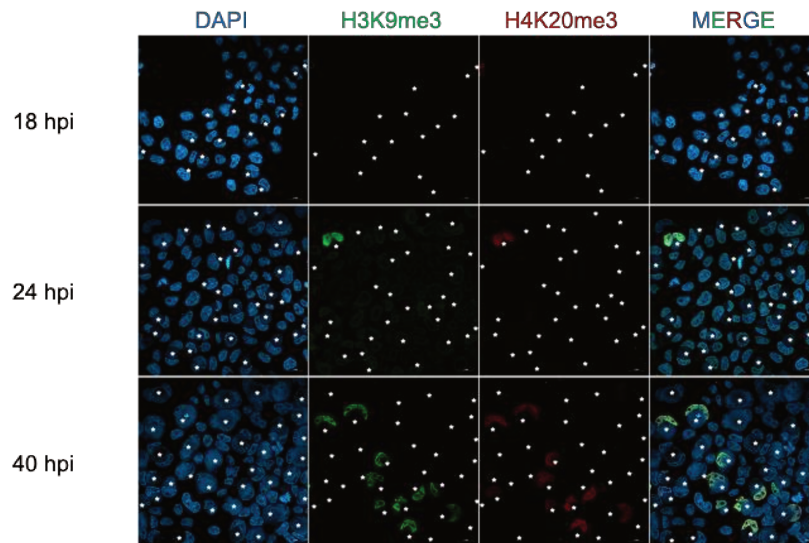


Figure 22 Immunostaining of histone modifications in HeLa cells infected with *C. trachomatis* at 18, 24, and 40 hpi.

To determine at what point during the course of infection histone hypermethylation occurs, a time course study was carried out (Fig 22). There was no detectable histone methylation 18 hpi, and little to no detectable histone methylation at mid-infection (24 hpi). Forty hours after the initiation of infection, histone hypermethylation was detected in about 25% of the infected cells. Thus, histone hypermethylation is a late event during the infectious cycle, which occurs after the initiation of RB to EB transition.

These data were obtained in HeLa cells, the epithelial cell line most commonly used in the *Chlamydia* field. However, their cancerous origin and passaging over multiple generations call for validation of the results in another cellular background. To that end, we looked at histone methylation in late-infected and non-infected primary epithelial cells isolated from the ectocervix (Fig 23A). Hypermethylation of H3K9 and H4K20 was observed in infected primary cells, compared to non-infected cells, confirming the observation made in the HeLa cell line.

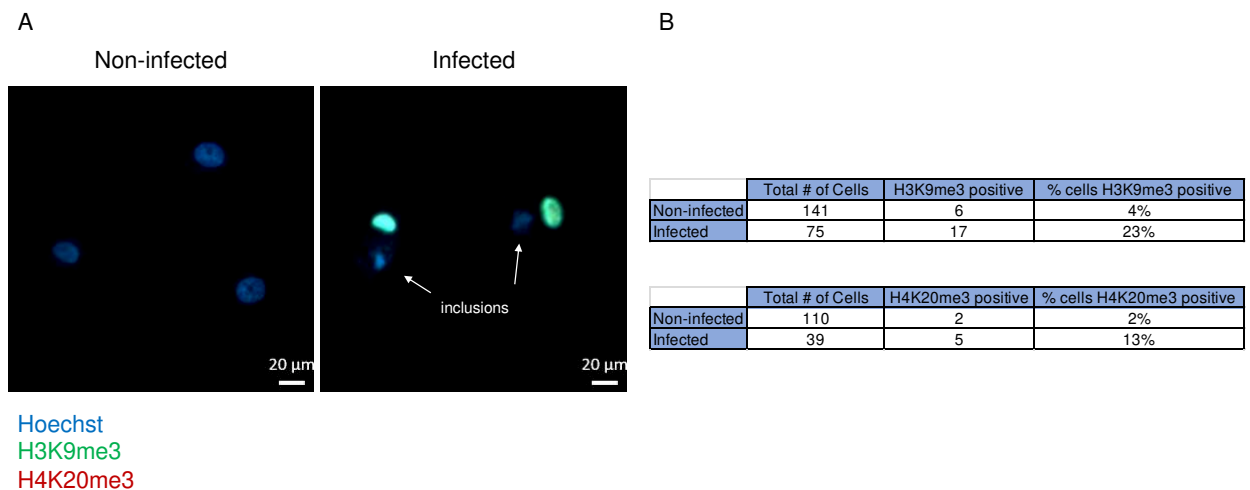


Figure 23 Histone methylation of patient-derived ectocervical epithelial cells. (A) Patient-derived cells were infected with *C. trachomatis* LGVL2 for 48 hours and then immunostained for H3K9me3 and H4K20me3. (B) Quantification of imaging data

Finally, to know whether these observations were specific to the human adapted strain *C. trachomatis*, we infected HeLa cells for 48 h with *C. muridarum*. We observed that this mouse adapted strain also elicited hypermethylation of H3K4 and H4K20, showing that histone hypermethylation is not specific of *C. trachomatis* infection (Fig 24).

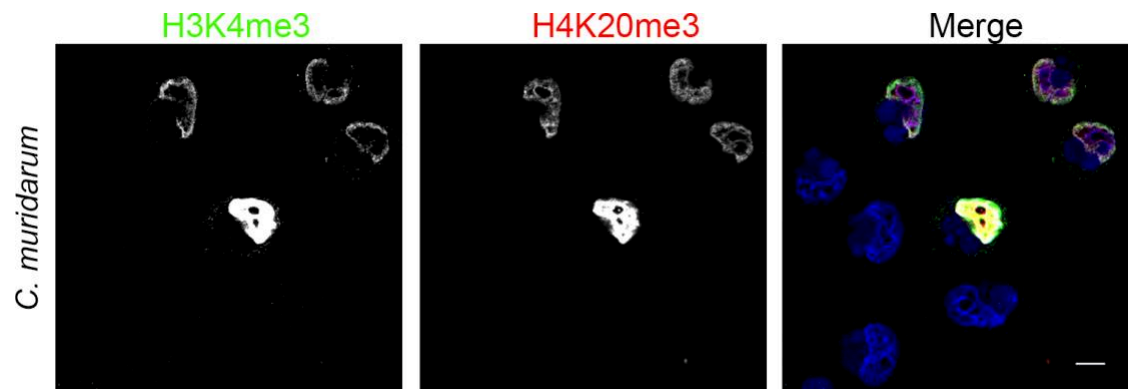


Figure 24 Histone methylation in cells with *C. muridarum*. HeLa cells were infected for 48 hours with *C. muridarum*, MOI 2, and then immunostained for H3K4me3 and H4K20me3.

In 2010, Pennini *et al.* characterized NUE, a chlamydial effector secreted by the bacteria that localizes to the host cell nucleus. NUE was shown to have histone methyltransferase activity *in vitro*, but its targets *in vivo* remain unknown (Pennini *et al.*, 2010). Through chemical mutagenesis, the Validvia lab has obtained and isolated several mutant strains of *C. trachomatis* (Kokes *et al.*, 2015). One of these strains contained a nonsense mutation in NUE, which we designate here as L2 NUE*. To determine whether NUE could be responsible for the histone hypermethylation observed during late infection, cells were infected with L2 NUE*, or with the parental strain used in the mutagenesis strategy, as a control (Fig 25). The level of H3K9me3 and H4K20 me3 methylation in cells infected with L2 NUE* was similar to that observed in cells infected with the parental strain, indicating that hypermethylation of histones during late infection is independent of NUE activity.

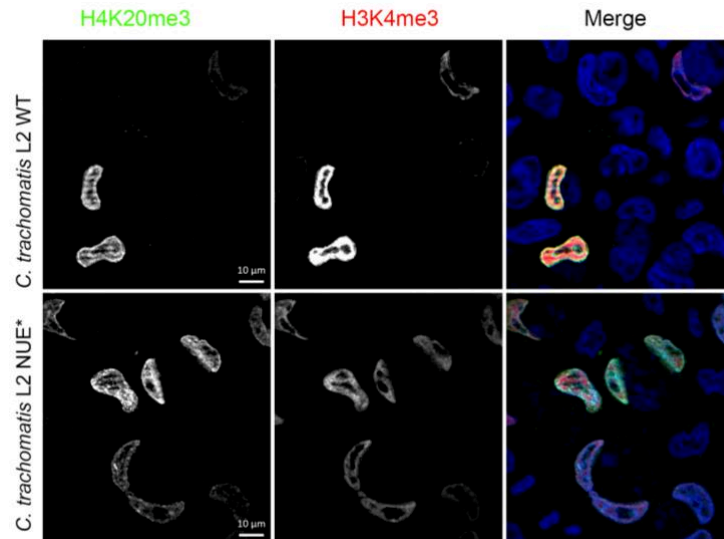


Figure 25 Immunostaining of HeLa cells infected for 48 hours with *C. trachomatis* L2 WT or *C. trachomatis* L2 NUE*

II. Infected cells are sensitized to histone demethylase activity

The observation that several histone marks were simultaneously up-regulated late in infection suggested a common mechanism. Histone methylation and demethylation are achieved by dedicated enzymes, histone methyltransferases and histone demethylases, respectively. We sought to determine if the increase in histone methylation during infection was due to increased histone methyltransferase activity, or rather a consequence of decreased histone demethylase activity. It

did not seem likely that the hypermethylation we had observed was due to increased histone methyltransferase activity, as the common co-factor for these enzymes, S-adenosyl methionine (SAM), is imported by the bacteria and, if anything, would be depleted from the host cytoplasm during infection (Binet *et al*, 2011).

We thus hypothesized that global histone hypermethylation could be a consequence of a decrease in activity of histone demethylases. In support for this hypothesis, we observed that infected cells displayed high sensitivity to JIB-04, a small molecule inhibitor that has been shown to inhibit the jumonji domain found in JmjC demethylases both *in vitro* and *in vivo* (Fig 26) (Wang *et al*, 2013, Parrish *et al*, 2018). In uninfected cells, JIB-04 did not induce any detectable histone methylation, at least by immunofluorescence. However, in infected cells treatment with JIB-04 induced increased trimethylation of H3K9 and H4K20 compared to the wild-type infection.

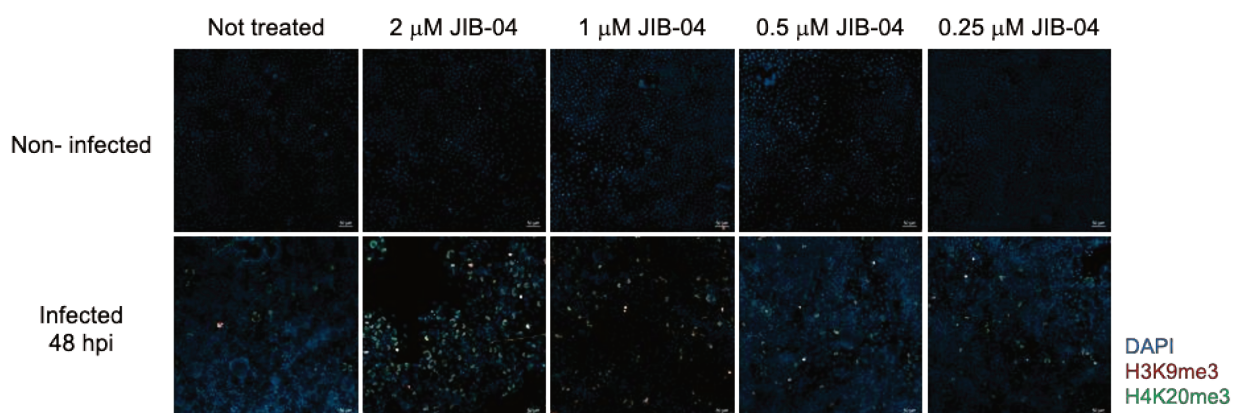


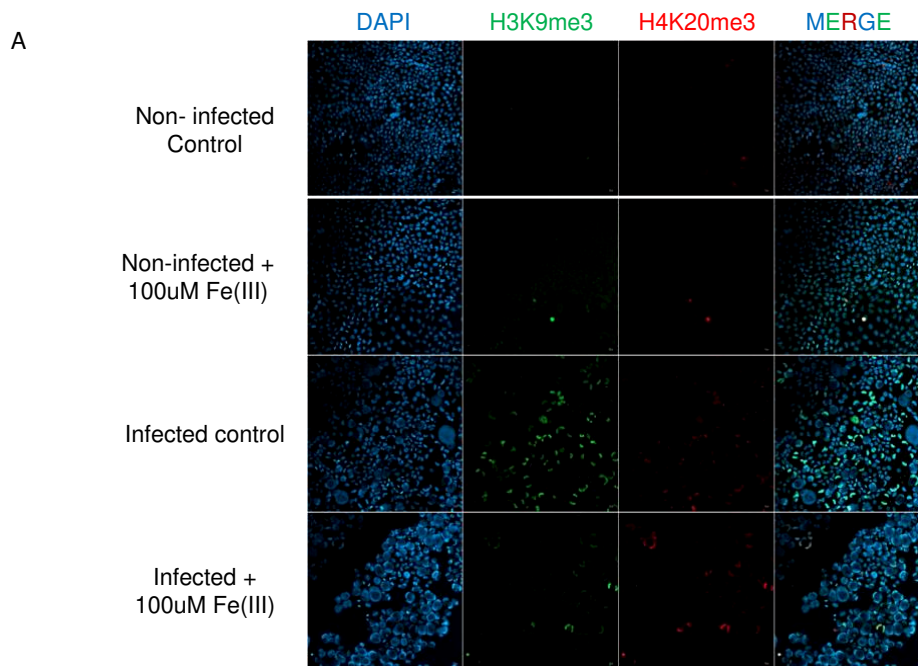
Figure 26. JIB-04 treatment induces increased methylation of H3K9 and H4K20. Non-infected and *C. trachomatis*-infected cells were treated with JIB-04 at doses ranging from 0.25 to 2 μ M.

These data suggests that infected cells are sensitized to histone demethylase activity, particularly the JmjC histone demethylases, and that the equilibrium between methylation and demethylation is disturbed late in infection. The JmjC demethylases are a large family of demethylases capable of modifying histones at multiple residues such as H3K9, H3K27, H3K4, and H4K20, which is consistent with the modifications observed to be upregulated during late infection. These demethylases require iron and α -ketoglutarate as co-factors to carry out their function, both of

which are consumed by *C. trachomatis* during infection. We then sought to determine which of these co-factors are rate-limiting for JmjC demethylases during infection.

III. Iron is not the limiting co-factor for JmjC demethylase activity during infection

To test whether iron was the limiting co-factor for JmjC demethylases, we supplemented culture medium of infected and non-infected cells with iron (III) citrate and observed the levels of histone methylation by immunofluorescence. There was a decrease in the level of histone trimethylation of H3K9 and H4K20 upon iron citrate supplementation, indicating that intracellular iron levels in infected cells might become limiting for JmjC demethylase activity (Fig 27A). However, this conclusion needs to be taken with caution as intracellular iron levels have been shown to regulate the activity of several enzymes of the TCA cycle and might therefore indirectly influence the level of TCA cycle intermediate, α -ketoglutarate, the second co-factor of JmjC demethylases (Oexle *et al*, 1999; Tesler *et al*, 2019).



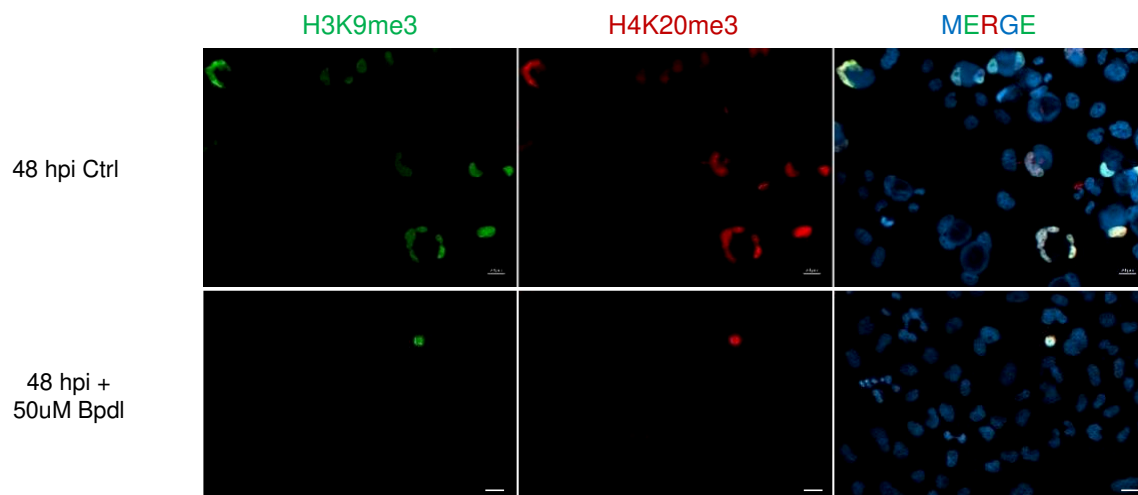


Figure 27 Effect of iron on histone methylation during infection (A) Non-infected and infected cells treated with 100 μ M Iron (Fe III) citrate and immunostained at 48 hpi. (B) Infected cells treated with 50 μ M Bpdl and immunostained at 48 hpi for H3K9me3

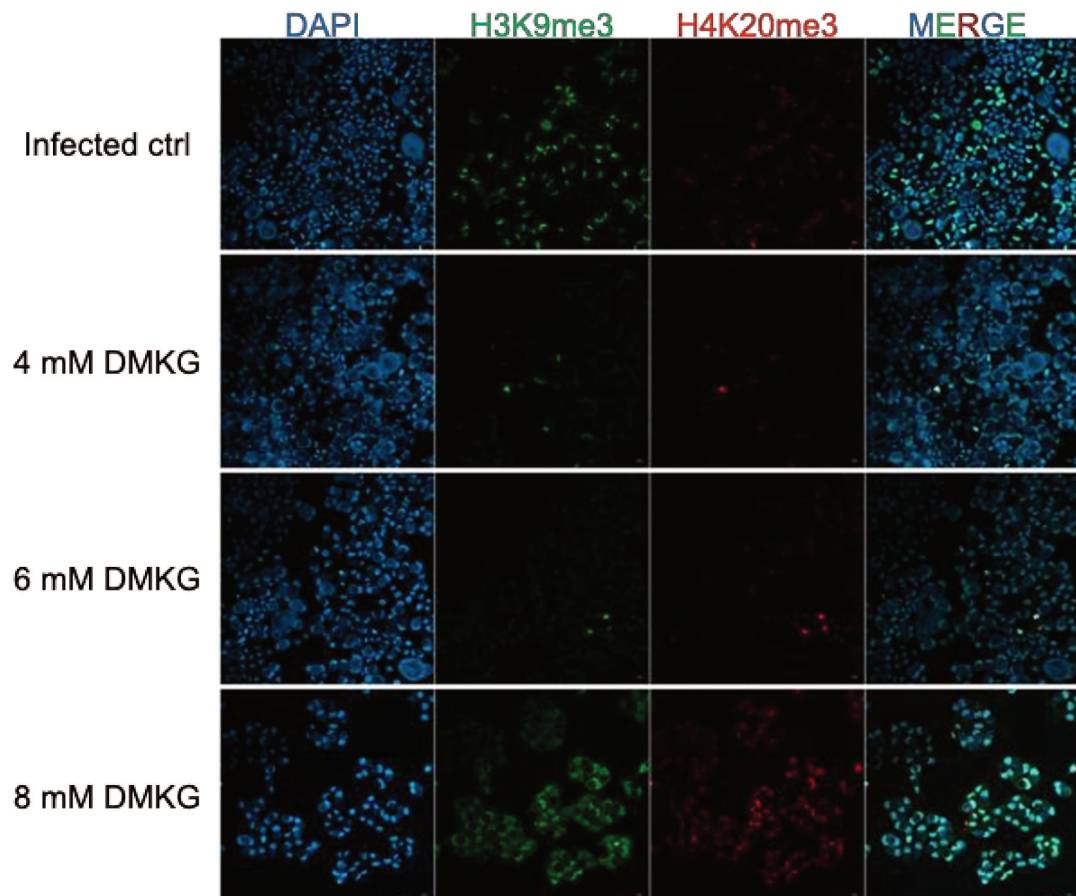
As an alternative approach to determine whether iron was the limiting factor for demethylase activity, we used the iron chelator, 2,2 – bipyridyl (Bpdl). Bpdl has been shown to sequester iron in infected cells, and to delay chlamydial development (Thompson and Carabeo, 2011). Bacterial development was strongly inhibited at this concentration of iron chelator, indicating that the iron concentration was below what it would have been normally in infected cells (Fig 27B). However, histone methylation was not detected in cells infected for 48 h in the presence of 50 μ M Bpdl, indicating that even the level of iron limitation reached in these conditions was not sufficient to induce histone hypermethylation. We concluded from these experiments that a decrease in iron availability in the cytoplasm and nucleus cannot account for the histone hypermethylation observed late in infection.

IV. DMKG supplementation during infection prevents histone hypermethylation

Next, we wanted to determine if increasing the intracellular level of α -ketoglutarate would affect histone methylation. α -ketoglutarate is not cell permeable on its own, so to increase its intracellular levels, it may be added in the form of an ester such as Dimethyl-ketoglutarate (DMKG). After entering the host cell cytosol, DMKG is hydrolyzed by host cell esterases into α -ketoglutarate and has been shown to increase intracellular levels of α -ketoglutarate (Baracco *et al*, 2019).

Cells were infected and 2 hours later were treated with 4, 6, or 8 mM DMKG (Fig 28A). At 48 hpi, cells were fixed and analyzed by immunofluorescence. Less methylation of H3K9 and H4K20me3 was observed in infected cells treated with 4 and 6 mM DMKG. Infected cells treated with 8 mM DMKG had an increase in the overall level of histone methylation at these marks, suggesting that infected cells may be sensitive to levels of α -ketoglutarate that are too high. It was determined that 5 mM DMKG was the ideal dose to prevent hypermethylation during late infection (Fig 28B).

A



B

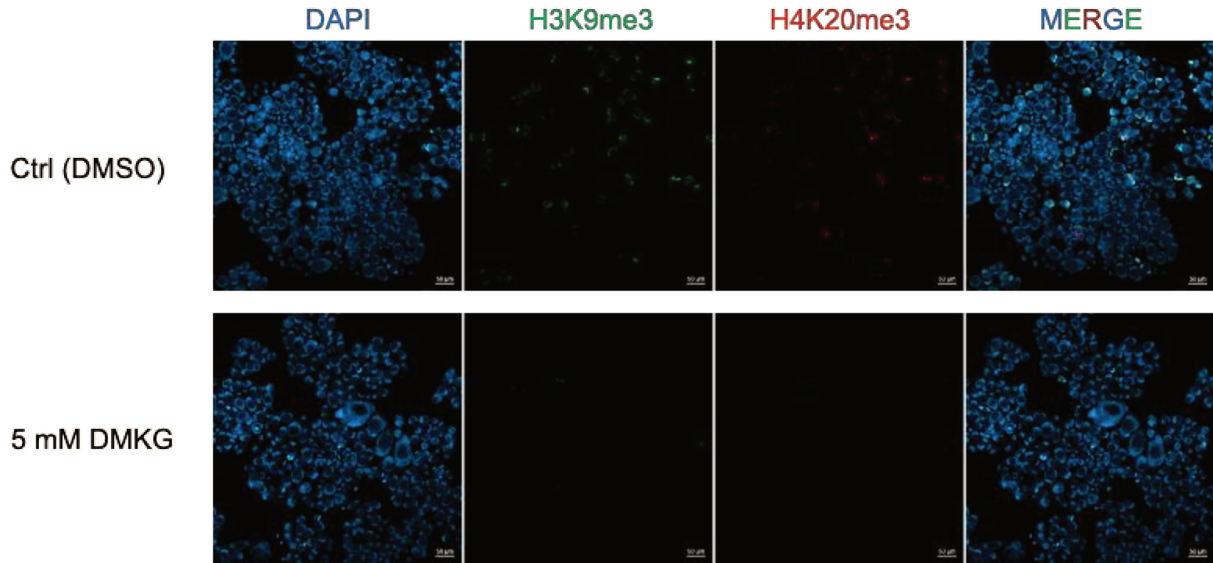
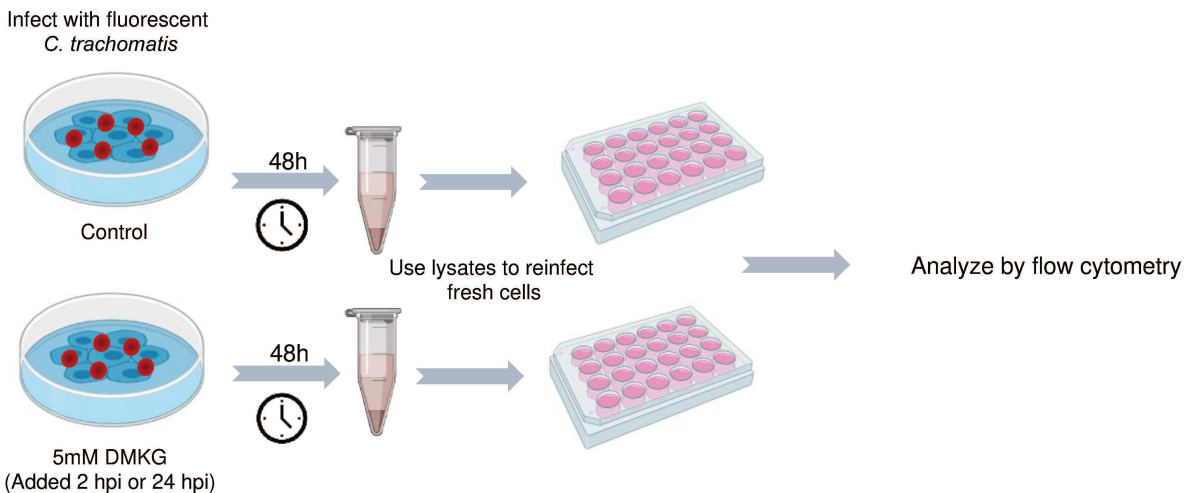


Figure 28 Effect of DMKG supplementation on histone methylation during infection. (A) Infected cells were treated 2 hpi with a range of doses of DMKG 2-8 mM and analyzed at 48hpi via immunofluorescence. (B) Infected cells treated 2 hpi with 5 mM DMKG were immunostained at 48 hpi

However, when further characterizing the effect of 5 mM DMKG on infection, we observed a significant decrease in infectious progeny (Fig 29). This was concerning as the decrease in hypermethylation could then be due to delayed infection. To decrease the negative impact of DMKG on bacterial growth, the treatment time was postponed until 24 hpi. Progeny assays performed on infected cells with DMKG supplementation added 24 hpi showed that in this case, the decrease in infectious particles collected 48 hpi was marginal (Fig 29 A&B).

A



B

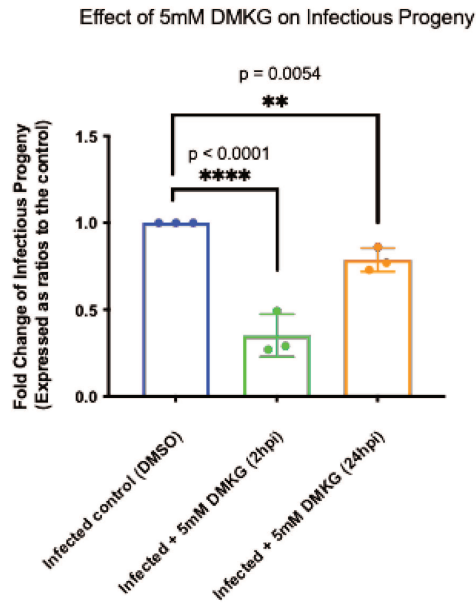
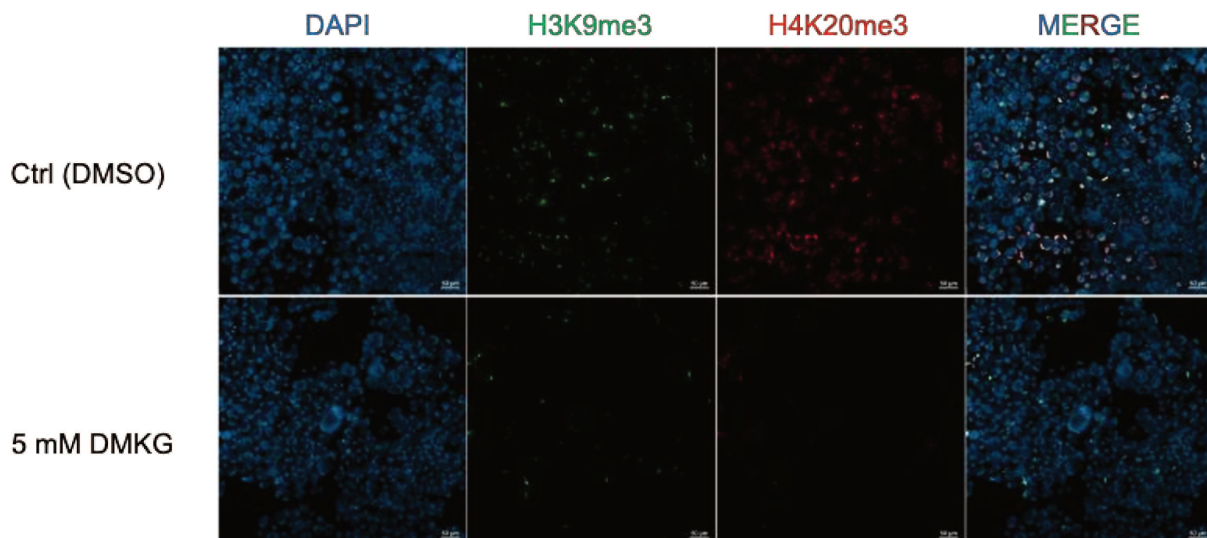


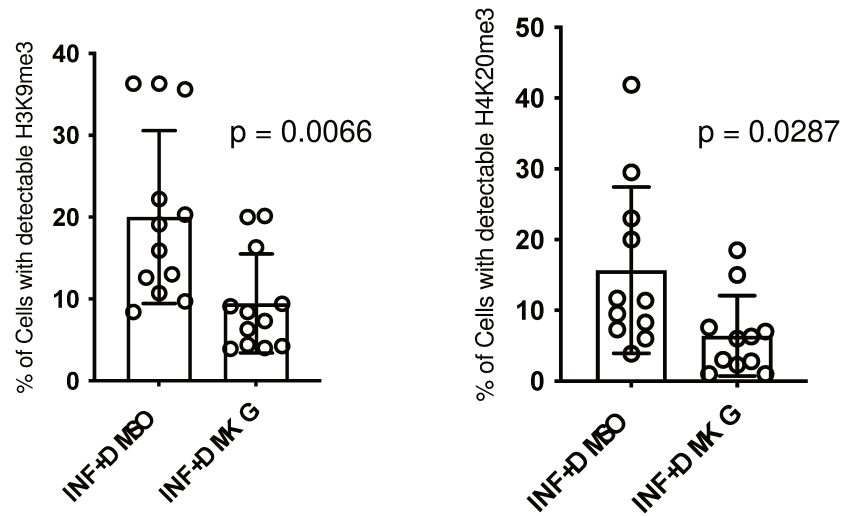
Figure 29 Effect of DMKG supplementation on infectious progeny. (A) Schematic of progeny assay. Fluorescent bacteria are used to infect cells for 48 hours. Lysates from the primary infection are used to infect a fresh plate in serial dilution. The second infection is analyzed by flow cytometry and used to calculate IFU (B) Quantification of progeny assays comparing addition time point for DMKG supplementation. The histogram displays the mean \pm standard deviation of three independent experiments and the p-value of the unpaired Student's t-test.

Infected cells with DMKG supplementation at 24 hpi displayed a significant reduction of histone methylation of H3K9me3 and H4K20me3 at 48 hpi compared to the control (Fig 30A&B). Methylation was also prevented at H3K4me3 and H3K79me2 (Fig 30 C). Together, these results indicate that DMKG supplementation prevents histone hypermethylation during late infection.

A



B



C

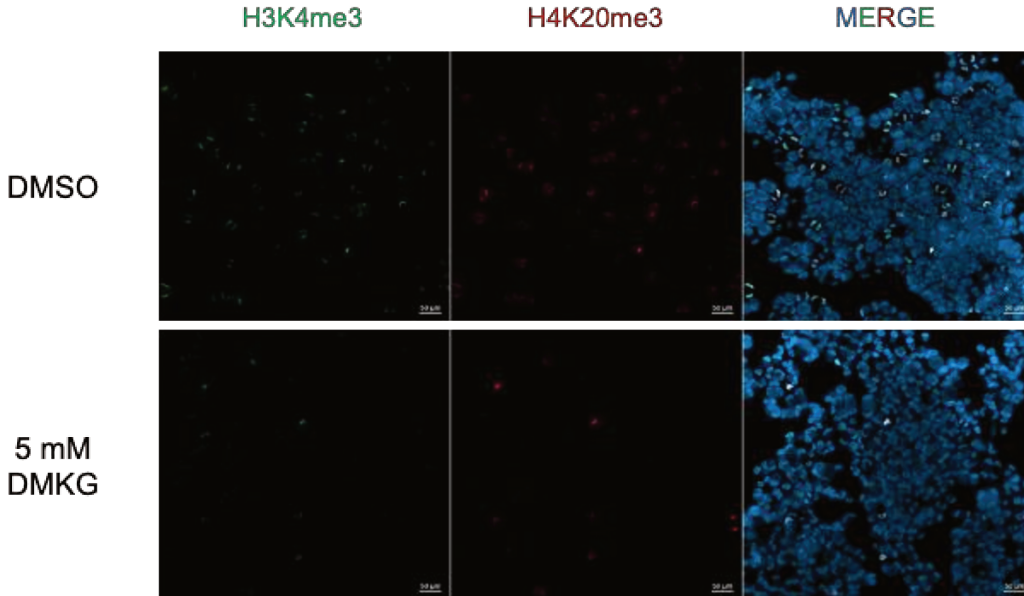


Figure 30 Effect of DMKG supplementation on histone methylation (A) Infected cells treated with DMKG at 24 hpi and analyzed by immunofluorescence at 48 hpi (B) Quantification of H3K9me3 and H4K20me3 methylation during infection with DMKG supplementation. Analysis of three independent experiments was performed in GraphPad Prism 9.0 and subjected to an unpaired t-test. (C) Infected cells treated with DMKG at 24 hpi and immunostained for H3K4me3 and H4K20me3

V. Reducing mitochondrial α KG consumption does not prevent histone hypermethylation

The oxoglutarate dehydrogenase (OGDH) complex is the TCA enzyme complex responsible for converting α -ketoglutarate into succinate. Conversion of α -ketoglutarate to succinyl-CoA by OGDH has potentially two synergetic effects on JmjC demethylases: it depletes α -ketoglutarate levels, and it increases succinate level, which competitively inhibit the JmjC demethylases via their α -ketoglutarate domains, (Xiao *et al*, 2012). In an attempt to decrease the succinate to α -ketoglutarate ratio, and thus restore JmjC demethylase activity, OGDH expression was silenced using siRNA. siRNA transfection was optimized to decrease OGDH levels prior to infection and to maintain decreased OGDH levels throughout infection. Transfection conditions were tested and verified by qRT-PCR (Fig 31B). It was determined that cells should be transfected with siRNA against OGDH for 30 hours prior to infection, and retransfected just prior to infection to maintain low levels of OGDH through the duration of infection.

Using the optimized transfection conditions, infected cells were analyzed by immunofluorescence (Fig 31A). Silencing OGDH did not prevent histone hypermethylation at H3K9me3 and H4K20me3 during late infection. Thus, against our expectations, this observation suggests that the host TCA cycle does not contribute significantly to the succinate/ α -ketoglutarate ratio during infection.

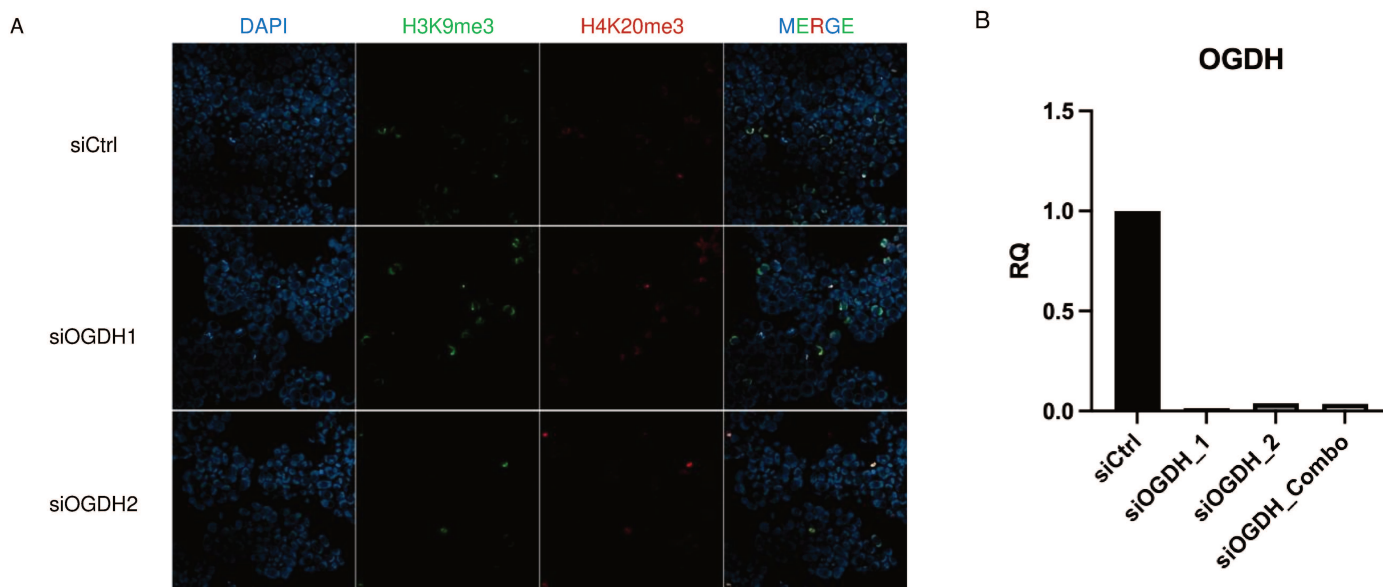


Figure 31 Silencing oxoglutarate dehydrogenase (OGDH) does not affect histone methylation during late infection (A) Immunostaining of cells transfected with siRNA against OGDH, 30 hours later cells were retransfected and infected with *C. trachomatis* LGVL2, and then cells were analyzed by immunofluorescence at 48 hpi. (B) Transcript levels of OGDH were measured in parallel via qRT-PCR to quantify siRNA efficacy. There were two different siRNA targets for OGDH that were tested independently and in combination. Quantification was performed using the delta CT method, and OGDH transcripts were normalized to 36B4. qRT-PCR verification that siRNAs decrease expression of OGDH. The expression of each target gene is shown relative to that in cells treated with the control siRNA.

VI. DMKG supplementation during infection influences host transcriptome

A microarray study was carried out to assess how DMKG supplementation affects the host transcriptome during late *C. trachomatis* infection (Fig 32). Cells were infected with *C. trachomatis* LGVL2 at MOI 3 24 hours after infection 5 mM DMKG or an equal volume of DMSO, the culture medium was renewed with medium containing 5 mM DMKG or an equivalent volume of DMSO and at 48 hpi samples were collected for RNA extraction.

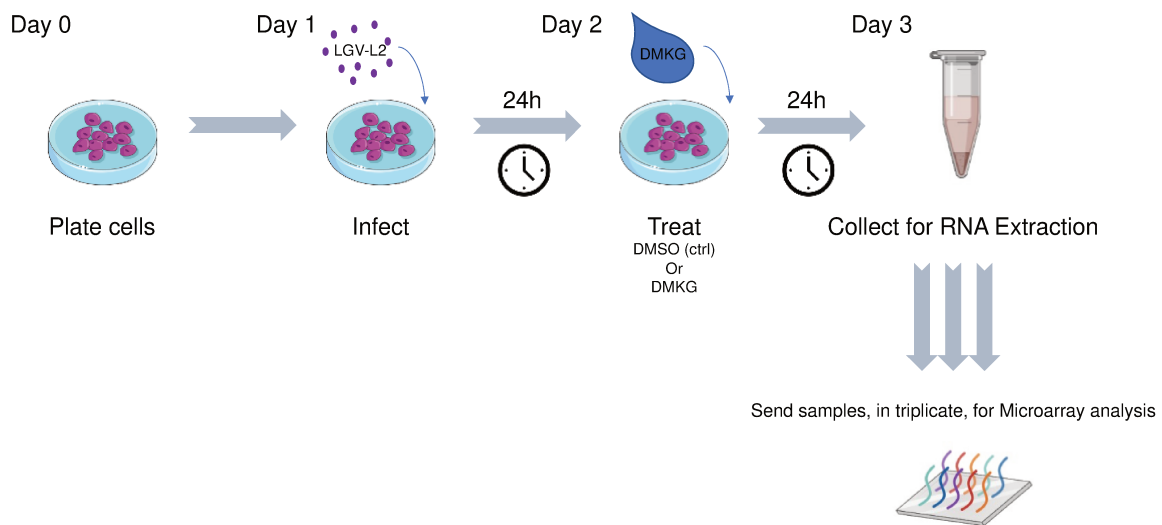


Figure 32 Scheme for microarray sample preparation.

The data was analyzed with the Transcriptome Analysis Console software from Thermo Fisher Scientific, and the differentially regulated elements (DRE) were defined with a cut-off of $|\text{Fold-increase}| > 2$ and a $p\text{-value} < 0.05$. We refer here to elements rather than genes because we will see

later than only a portion of these sequences correspond to protein- coding genes. The number of elements differentially up or down regulated between any conditions are split fairly evenly (Fig 33A). When comparing the total number of DREs in infected samples compared to non-infected samples, it was interesting to observe that ~1/3 of the DREs were no longer differentially expressed with DMKG supplementation, suggesting that increasing intracellular levels of α -ketoglutarate during infection affects the transcriptional host response to infection (Fig 33B).

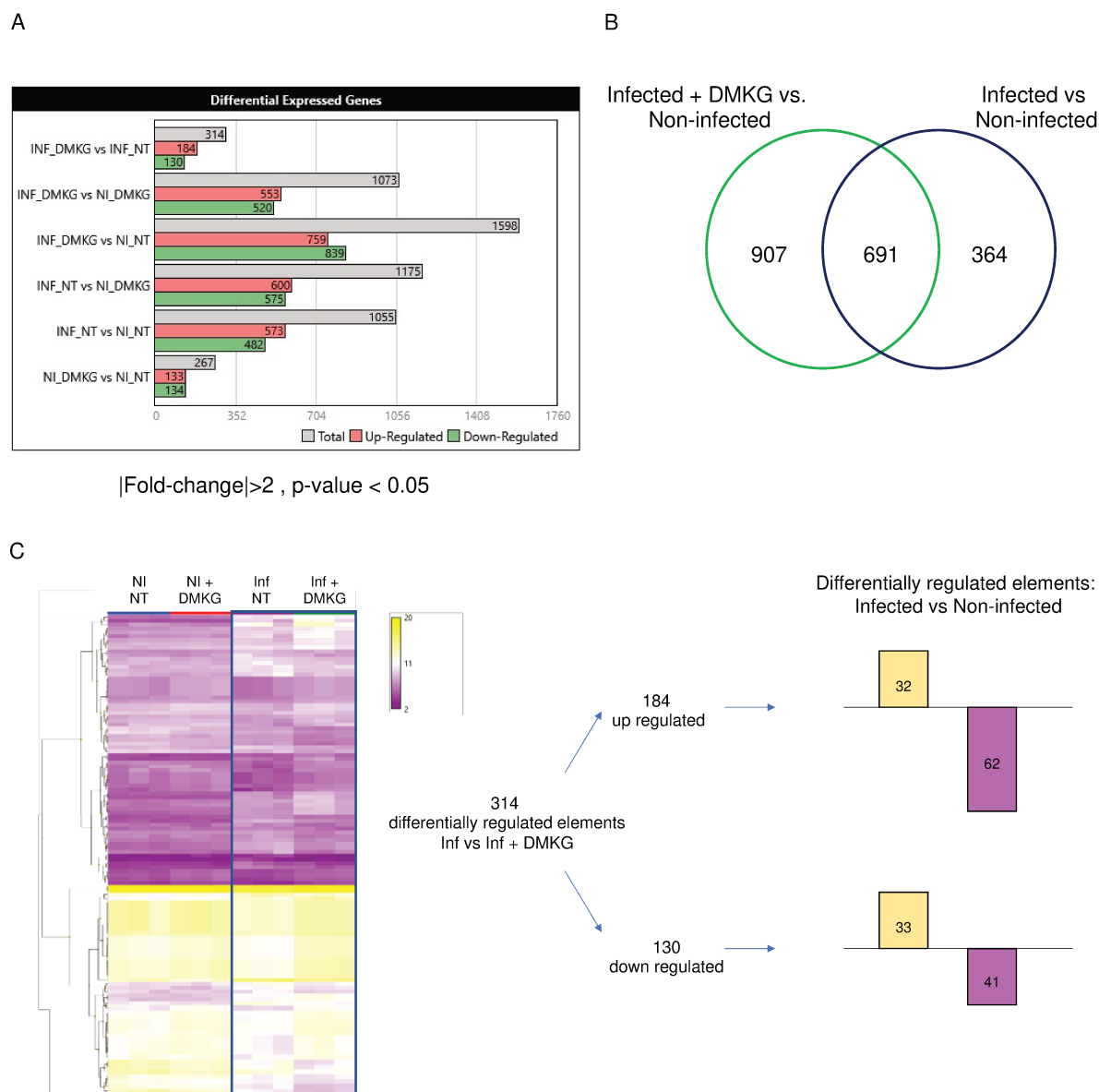


Figure 33 Microarray analysis of DMKG supplementation during infection. (A) Quantification of genes differentially expressed between each condition (B) Venn diagram comparing differentially expressed genes between non-infected cells and infected cells with or without DMKG supplementation. (C) Hierarchical clustering across all conditions of genes differentially expressed between normal infection conditions and infection with DMKG supplementation.

We next investigated the differences in transcription between normal infection and infection with DMKG supplementation. There are 314 DREs under these conditions with 184 gene upregulated and 130 down regulated. This gene set under normal infection conditions are expressed differently (Fig 33C). For instance, among the 184 elements that are up-regulated with DMKG supplementation, 32 correspond to transcripts that are up-regulated by infection, and 62 to transcripts that are down-regulated by infection (the remaining 90 elements showing less than a 2-fold difference in transcription upon infection). Therefore, the shift in transcription from infection to infection with DMKG supplementation is not one direction. In other words, DMKG supplementation enhances the transcription of some of the elements that were already up-regulated by infection, while it dampens the transcription of others. The same observations were made on elements down-regulated by infection. Overall, the effect of DMKG really depends on the transcript, just as methylation can repress or activate gene transcription depending on the mark considered and its position relative to the genetic element.

Interestingly, only 115 of the 314 DRE corresponded to transcripts of a protein-coding gene. The rest of the elements fell into non-coding RNA of various sorts (pseudogenes, lncRNA, piRNA). One interesting category among those transcripts that did not code for genes are several tRNAs, which seem to be downregulated in infection, and recovered by DMKG treatment.

Analysis of the 115 protein-coding genes was carried out using several protein pathway enrichment algorithms such as: WikiPathways, STRING, Kegg, Reactome and GeneOntology. All of them indicated that the p53 transcriptional network was differentially regulated by the addition of DMKG. We thus chose 2 of the 4 genes highlighted in this network to validate the microarray data by RT-qPCR: GADD45A (Growth arrest and DNA damage-inducible protein GADD45 alpha) and PMAIP1 (PMA-induced protein 1, also called NOXA). Both genes were up-regulated by infection, with an additional boost upon DMKG supplementation, confirming the micro-array data (Fig 34).

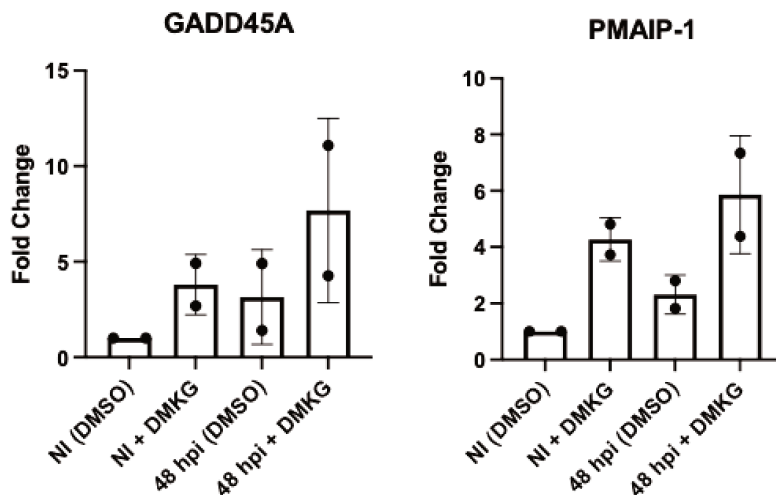


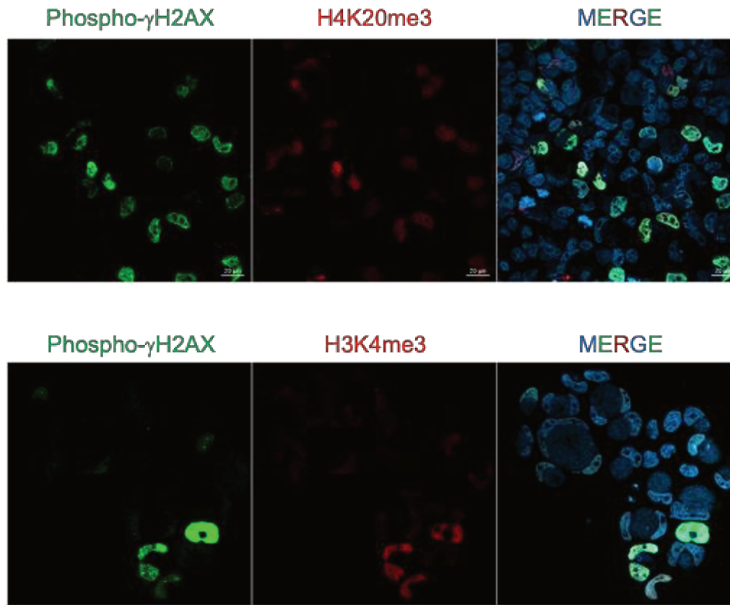
Figure 34 qRT-PCR verification of microarray data. mRNA transcripts of GADD45A and PMAIP-1 transcripts were measured by real-time RT-PCR and normalized to *actin* transcripts following the $\Delta\Delta C_t$ method. The data are presented as relative mRNA levels compared to uninfected cells treated with DMSO. Data from 2 independent experiments are shown.

More stringent analysis of DREs only differentially regulated under infection with DMKG supplementation narrowed the number of significant DREs down to 250, of which 45 are protein-coding. Protein pathway enrichment algorithms were applied to this list, and the MAPK signaling cascade was found to be most prominently regulated. There were 6 proteins from this pathway which were differentially regulated: FGF2 (Fibroblast growth factor-2), EFNA1 (Ephrin-A1), EPHA2 (Ephrin type-A receptor 2), HSPA1A (Heat shock 70 kDa protein 1A) CACNB2(Calcium Voltage-Gated Channel Auxiliary Subunit Beta 2), AND DUSP6 (Dual specificity protein phosphatase 6).

VII. DMKG supplementation during infection circumvents DNA damage

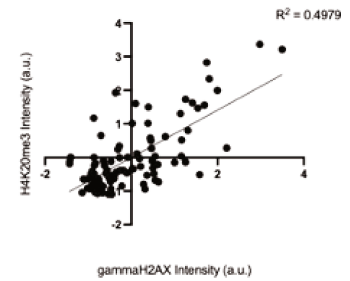
GADD45 is a DNA damage-inducible protein, a chromatin alteration previously observed in *C. trachomatis* infected cells (Chumduri *et al*, 2013). Phosphorylation of the Ser-139 residue of the histone variant H2AX, forming γ H2AX, is an early cellular response to the induction of DNA double-strand breaks and is a commonly used marker of DNA damage (Mah *et al*, 2010). To determine if DNA damage was linked to the histone hypermethylation phenotype we have uncovered, we co-stained infected cells with γ H2AX and H3K4me3 or H4K20me3. We observed a very good correlation of phosphorylation and methylation levels between these marks, meaning that DNA damage is most abundant in nuclei that display histone hypermethylation (Fig. 35 A&B).

A



B

Correlative levels of γ H2AX and H4K20me3 during late infection



Correlative levels of γ H2AX and H3K4me3 during late infection

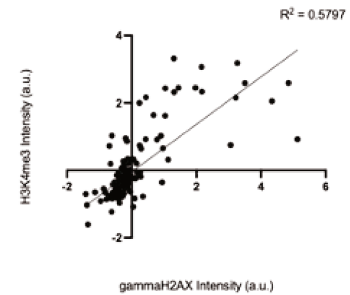


Figure 35 DNA damage occurs in infected cells with increased histone methylation. (A) Immunofluorescence of infected cells at 48 hpi (B) Correlative levels of γ H2AX with H4K20me3 and H3K4me3. Fluorescence intensity measurements were standardized and normalized using Z-normalization. GraphPad Prism 9.0 was used to plot the Z-scores and determine the R-squared

Chumduri *et al* reported an inhibition of the DNA repair machinery in cells infected by *C. trachomatis*, with a failure to recruit the DNA damage response proteins phosphoATM and 53BP1 to the damaged sites (Chumduri *et al*, 2013). We did not reproduce this observation, as in our hands phosphoATM and 53BP1 co-stained γ H2AX positive nuclei in infected cells (Fig. 36).

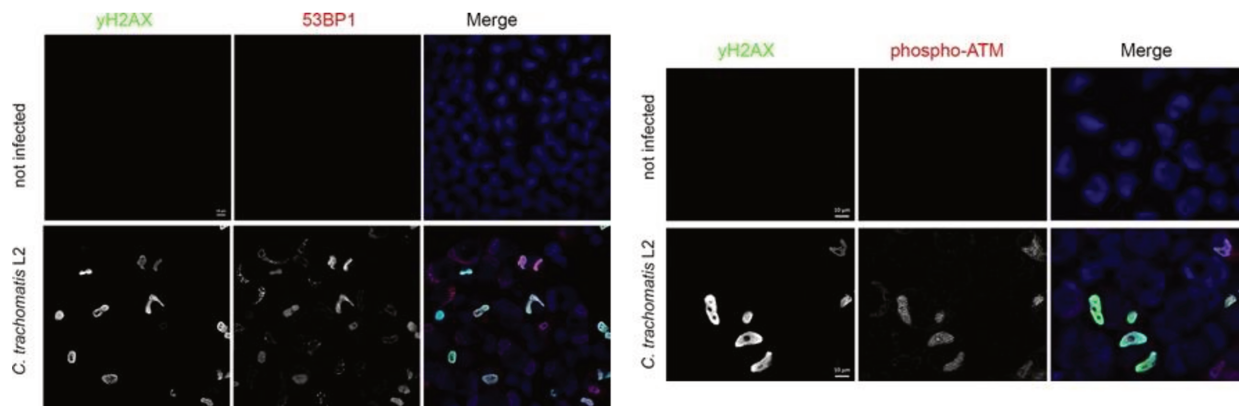


Figure 36 *C. trachomatis* infection does not inhibit DNA repair machinery. HeLa cells were infected MOI 2 with *C. trachomatis* LGVL2. At 48 hpi, cells were fixed and immunostained as indicated.

Finally, we hypothesized that if DNA damage and histone hypermethylation were linked, preventing histone hypermethylation should also affect DNA damage. Indeed, we found that the γ H2AX signal was greatly reduced in cells supplemented with 5 mM DMKG (Fig 37 A&B). This observation indicates that DMKG supply as an important consequences not only on the epigenome of the host, but also on chromatin integrity

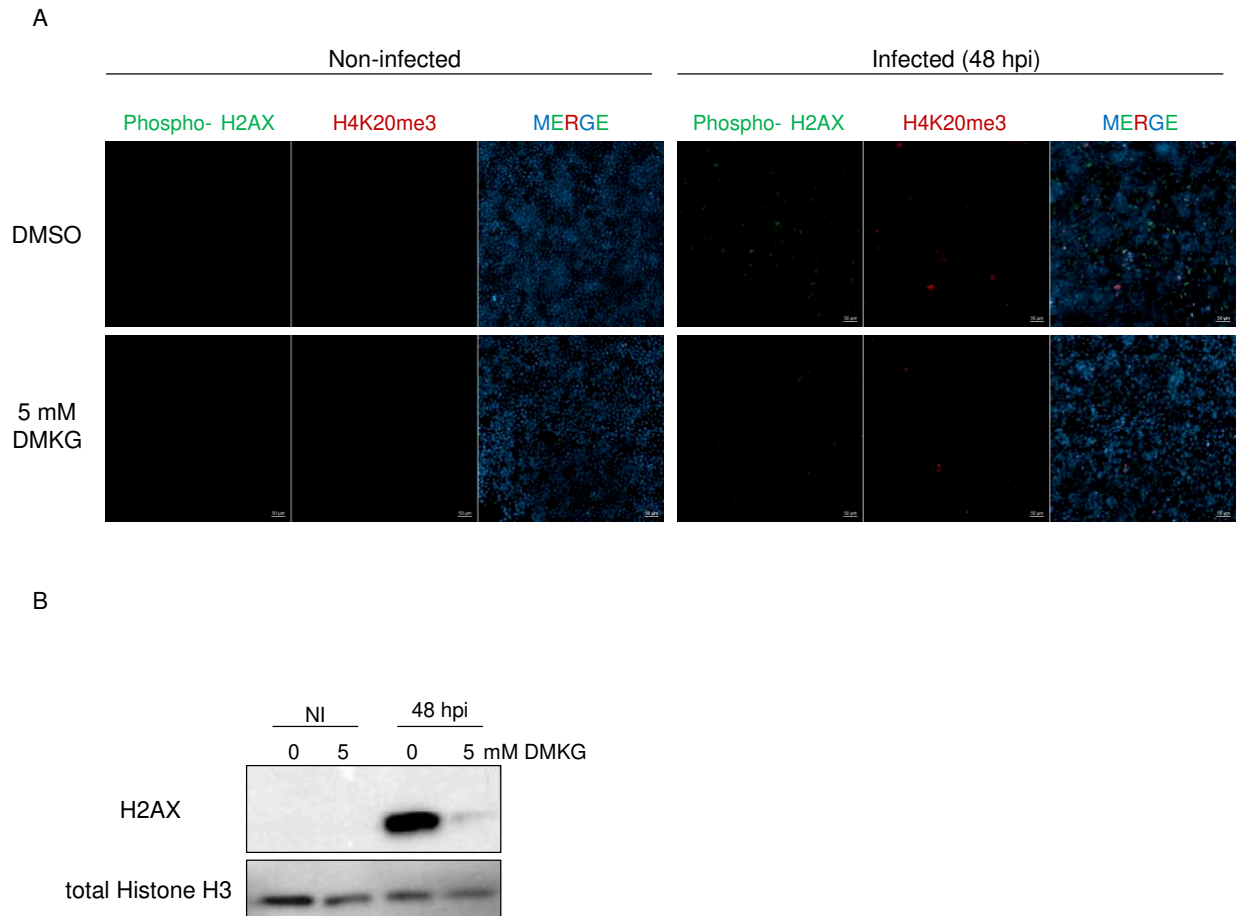


Figure 37 Effect of DMKG supplementation on DNA damage during infection (A) Immunostaining of infected cells treated with DMKG or DMSO at 24 hpi and then fixed at 48 hpi (B) Western blot analysis of isolated nuclei

DISCUSSION

Modifications made to chromatin regulate DNA accessibility and influence gene transcription. Only in more recent years are we learning about how metabolism plays a role in chromatin modifications, and that perturbations in metabolism affect chromatin regulation and subsequent transcription.

C. trachomatis is known to influence host cell activities to survive and relies on its host for nutrients. The bacteria grossly shift the metabolism of their host in an effort to obtain nutrients, though little is known about the effect of *C. trachomatis* infection on chromatin. In this work we set out to explore the mechanism by which hypermethylation occurs during late infection, and how it affects host cell transcription.

I. Hypermethylation of histone occurs during late to *C. trachomatis* infection

An increase in histone methylation at multiple residues (H3K9, H3K4, H3K27, H3K79, and H4K20) was observed during late infection (Fig 21A). These results are consistent with another study in which Chumduri *et al*, displayed increased histone methylation during late infection, largely through analysis of whole cell lysates of Western blots (Chumduri *et al*, 2013). What was striking about our findings was the co-regulation of the different histone modifications within the same cell (Fig 21A&B). Cells with up-regulation of one histone modification usually expressed another, despite the seemingly antagonistic roles of certain marks. For example, we found that H4K20me₃, a histone PTM associated with heterochromatin formation and gene repression, co-expressed in cells with up-regulated methylation at H3K4me₃ or H3K79me₂, which are marks associated with gene activation (Hyun *et al*, 2017).

The hypermethylation phenotype was also observed in primary epithelial cells obtained from the ectocervix, suggesting that this phenotype is physiologically relevant (Fig 23). Hypermethylation of histones was also observed in HeLa cells infected with mouse-specific *C. muridarum*, indicating that this phenotype is relevant for other *Chlamydiaceae* (Fig 24).

II. Histone hypermethylation is caused by reduced demethylase activity

The methylation state of a given histone residue can be altered by histone methyltransferases and histone demethylases. We first investigated whether the increased methylation could be due to the action of a bacterial protein. One such chlamydial effector, NUE, has been shown to possess histone methyltransferase activity *in vitro* (Pennini *et al*, 2010). However, NUE did not appear to be the likely culprit behind this hypermethylation phenotype as cells infected with a strain of *C. trachomatis* containing a nonsense mutation in NUE (L2 NUE*) displayed similar levels of histone methylation to that of infection with the wild-type strain (Fig 25).

There are a number of histone methyltransferases and demethylases and each enzyme is usually only capable of changing the methylation state of one histone residue. Therefore, it is not likely that the hypermethylation observed during late infection is the result of a singular enzyme. Because the upregulation of methylation occurs at multiple marks simultaneously, it was therefore logical to investigate the role of co-factors that are shared between histone methyltransferases or histone demethylases. It has been shown that S-adenosyl-L-methionine, the common co-factor for histone methyltransferases, is consumed by the bacteria during infection, therefore it is unlikely that the hypermethylation phenotype observed is a result of increased histone methyltransferase activity (Binet *et al*, 2011).

We turned our attention to histone demethylases and sought to determine whether infected cells are sensitive to demethylase activity. JIB-04, a JmjC-domain inhibitor that targets the large JmjC domain-containing family of demethylases, was used to treat infected and non-infected cells (Wang *et al*, 2013, Parrish *et al*, 2018). In uninfected cells, there was no detectable increase in histone methylation. However, it was observed that at the same concentrations JIB-04 induced increased histone methylation in infected cells compared to their untreated controls (Fig 26). These results indicate the methylation state of *C. trachomatis*-infected cells is held in balance, and that infected cells are sensitized to demethylase activity.

III. Shifting Metabolites during infection influences histone modification

The largest family of histone lysine demethylases are the jumonji domain-containing demethylases. They require oxygen, iron, and α -ketoglutarate, a TCA cycle intermediate, as co-factors to carry out their function.

We first investigated the role of iron on histone demethylase activity, as iron is known to be necessary for normal *C. trachomatis* development (Raulston 1997). Supplementing infected cells with iron (Fe^{3+}) citrate resulted in a decrease in observable histone methylation (Fig 27A). However, it is important to note that iron is also a necessary co-factor for many of the TCA cycle enzymes responsible for generating intermediates like α -ketoglutarate (Oexle *et al*, 1999; Tesler *et al*, 2019). To determine whether iron was limiting, infected cells were treated with iron chelator, Bpdl, which has been shown to effectively sequester iron during *C. trachomatis* infection to the point that it halts bacterial development (Carabeo and Thompson, 2011). It was observed that Bpdl treatment of non-infected cells was not enough to induce histone methylation, but the same concentration was enough to inhibit bacterial development (Fig 27B). These results indicated that iron is not the limiting factor for demethylase activity during *C. trachomatis* infection.

α -ketoglutarate is an energy-generating TCA cycle intermediate that has been implicated in a number of other roles, such as: amino acid formation, signaling, and tissue repair (Zdzisińska *et al*, 2017). *C. trachomatis* have an incomplete TCA cycle and must rely on their host to acquire TCA cycle intermediates to generate energy (Fig 9) (Stephens *et al*, 1998). While glucose is their preferential carbon source, Ilfee-Lee and McClarty demonstrated that the bacteria are capable of utilizing α -ketoglutarate as a carbon source by glucose starving the host cells and supplying only α -ketoglutarate, among other gluconeogenic carbon sources (Iliffe-Lee and McClarty, 2000). While their study indicated that α -ketoglutarate was a poor substitute for glucose as a carbon source, it is worth noting that they did not use a cell permeable source of α -ketoglutarate, which could explain why bacterial development was low. To determine if increasing intracellular levels of α -ketoglutarate could enhance demethylase activity, dimethyl-ketoglutarate (DMKG) was added to the culture media. DMKG is a cell permeable ester form of α -ketoglutarate that, once it enters the cytosol, is rapidly hydrolyzed by host cell esterases and has been used to increase

intracellular levels of α -ketoglutarate (Baracco *et al*, 2019). After optimizing the doses of DMKG (Fig 28A), it was determined that 5 mM DMKG was sufficient to reduce histone methylation during late infection by (Fig 28B). Note that reversion of histone hypermethylation by supplying DMKG was also reported in other studies. In epidermal stem cells, DMKG addition resulted in reduced trimethylation of H3K27me3 (Baksh *et al*, 2020). In human embryonic stem cells engaged into neuroectodermal differentiation, DMKG supplementation caused a decrease in H3K4, H3K9, H3K27, H3K36 trimethylation marks (TeSlaa *et al*, 2016).

DMKG supplementation affected bacteria development. During the initial experiments, DMKG was added 2 hpi and cells were processed 48 hpi (Fig 28). It was noted that the inclusions in the DMKG-treated cells were smaller than those of the infected control, so progeny assays were performed to determine if DMKG treatment was affecting infectious progeny (Fig 29A). It was found that addition of DMKG at 2 hpi induced a significant decrease in infectious progeny, that could be partially rescued by moving the addition time to 24 hpi (Fig 29B). Addition of DMKG 24 hpi still prevented hypermethylation during late infection at multiple histone lysine residues (Fig 30). With this experimental set-up, the reduction on bacterial progeny is of about 20%. One preliminary experiment indicates that treatment of infected cells with 50 nM doxycycline reduced the progeny by 50% without affecting histone hypermethylation. Quantification of the results is pending and should allow us to rule out the possibility that the reduction in histone hypermethylation with DMKG supplementation during infection is accounted for by a reduction in bacterial growth. At this point it is unclear why DMKG supplementation reduces bacterial proliferation. It could be because of the indirect effect this treatment has on host gene expression, and thus on the environment faced by the bacteria. Alternatively, it could result from an imbalance in the host metabolism that affects the nutrients required for optimal bacterial growth. In an attempt to address this question, we treated DMKG-supplemented cells with low concentration (0.5 μ M) of the demethylase inhibitor JIB-04. We observed that the negative effect of DMKG on bacterial growth persisted in this experimental set-up, which preserved histone hypermethylation (data not shown). This result suggests that the detrimental effect of DMKG is exerted at the metabolic level, rather than via its consequence on chromatin structure.

IV. α -ketoglutarate depletion in infection is likely driven by bacterial metabolism

JmjC histone demethylases are influenced by metabolites other than their α -ketoglutarate co-factor. They may be regulated by fellow TCA cycle intermediates, succinate and fumarate, which act as competitive inhibitors of α -ketoglutarate, thus rendering these demethylases sensitive to intracellular levels of all three metabolites (Xiao *et al*, 2012). Interestingly, it has been shown that fumarate and succinate levels are increased during *C. trachomatis* infection (Fig 38) (Rother *et al*, 2018). One caveat of this study is that the location (cytoplasm, inclusion lumen, bacteria?) of these compounds is not known. Still, it supports the hypothesis that the ratio of succinate + fumarate over α -ketoglutarate increases in the host cytoplasm and could account for the demethylase inhibition.

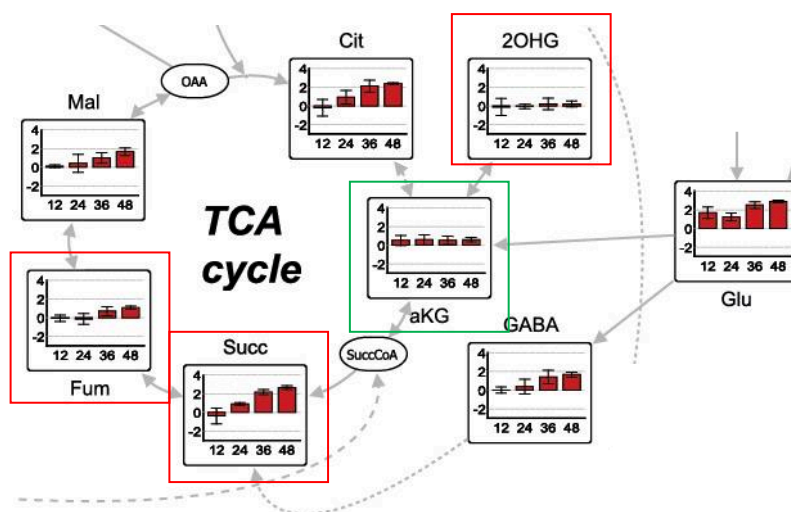


Figure 38 Levels of TCA metabolites during *C. trachomatis* infection. HeLa cells were infected with *C. trachomatis* LGV L2 at MOI 1 or left uninfected for 12, 24, 36 and 48 h. Metabolite levels were analyzed using gas chromatography-mass spectrometry (GC-MS). Histograms represent the log2 values of the ratios of the fold changes between infected and non-infected samples (mean \pm SD) Boxed in red are the TCA intermediates capable of inhibiting JmjC histone lysine demethylases via competitive inhibition with α -ketoglutarate (boxed in green). Abbreviations of the metabolites: 2OHG: 2-Hydroxyglutarate; aKG: α -ketoglutarate; Cit: Citrate; Fum: Fumarate; GABA: γ -Aminobutyric acid; Glu: Glutamate; Mal: Malate; Succ: Succinate. (Adapted from Rother *et al* 2018).

To investigate whether decreasing intracellular succinate would restore JmjC demethylase activity, OGDH of the OGDH complex was silenced with siRNA (Fig 31). The OGDH complex is responsible for conversion of α -ketoglutarate to succinyl-CoA, which is further converted into succinate. Since silencing OGDH was sufficient to increase α -ketoglutarate levels in stem cells and reduced H3K27me3 levels (Baksh *et al*, 2020), we opted for a similar strategy. Though the siRNA reduced expression of OGDH (Fig 31B), it did not prevent hypermethylation of histones during late infection (Fig 31A). These data suggests that α -ketoglutarate that transits through the mitochondrial TCA cycle only marginally contributes to the α -ketoglutarate pool in infected cells and is not sufficient to replenish what is consumed by the bacteria. It has been well established that the bacteria readily take up glutamine, a precursor of α -ketoglutarate through glutaminolysis (Rajeeve *et al*, 2020). Our current hypothesis is that glutamine rerouting towards the inclusion acts as a sink that deprives host enzymes of α -ketoglutarate, and thereby limits the enzymes that depend on this co-factor. Of note, α -ketoglutarate itself, might be diverted, as metabolic labelling of purified bacteria suggested that several bacterial amino acids can be made *de novo* from host TCA-derived dicarboxylates such as malate, oxaloacetate or α -ketoglutarate (Mehlitz *et al*, 2017). In support of this hypothesis, it is important to note that the truncated TCA cycle of the bacteria starts with α -ketoglutarate dehydrogenase, as it lacks the upstream enzyme, isocitrate dehydrogenase (Fig 9). It would thus ideally be fed with direct α -ketoglutarate import from the host. Interestingly, the enzymes of this truncated TCA cycle are much more abundant in EBs than in RBs (Saka *et al*, 2011), indicating that this need for α -ketoglutarate increases after RB to EB conversion. This timing fits well with our observation that histone hypermethylation occurs late in infection.

V. *C. trachomatis* infection and DNA damage

Consistent with the study by Chumduri *et al*, we observed an increase in phosphorylation of DNA damage mark, γ H2AX, during late infection (Fig 35A) (Chumduri *et al*, 2013). Remarkably, we made the observation that cells with increased DNA damage also displayed histone hypermethylation (Fig 35A&B). Furthermore, DMKG supplementation prevented phosphorylation of γ H2AX during late infection in a similar fashion to methylation at histone lysine residues (Fig 37A&B). Taken together, these data establish a link between histone

hypermethylation and DNA damage. There are two non-exclusive hypotheses that could account for this link: histone hypermethylation might favor DNA damage, and/or it might impair DNA damage repair (DDR). In support for this second scenario, a recent article showed that the increase in succinate to α -ketoglutarate ratio resulted in aberrant hypermethylation of H3K9 at loci surrounding DNA breaks. This hypermethylation masks a local H3K9 trimethylation signal that is essential for the proper execution of DDR (Sulkowski *et al*, 2020). Chumduri *et al* reported that the DDR machinery was blocked in infected cells, with an absence of p-ATM or 53BP1 staining in γ H2AX positive nuclei (Chumduri *et al*). We did not reproduce these observations, as we observed increased phosphorylation of γ H2AX in nuclei of infected cells with increased p-ATM or 53BP1 (Fig 36). However, the pattern of distribution of these different markers shows some heterogeneity, and we are currently developing image analysis tools to quantify these images and determine whether DNA damage repair is defective in cells displaying histone hypermethylation.

VI. DMKG supplementation changes infection transcriptome

A microarray study was carried out to investigate how preventing histone hypermethylation by DMKG supplementation influenced host transcription (Fig 32). Between each of the conditions, the number of elements differentially up- or down- regulated was split fairly evenly (Fig 33A). Interestingly, $\sim 1/3$ of DREs normally differentially regulated during infection were no longer so with DMKG supplementation (Fig 33B). Hierarchical clustering was performed on the DREs which were differentially regulated during infection with DMKG supplementation (Fig 33C). There was no overall shift in transcription in which a global increase or decrease in gene regulation was detected. These results may have been anticipated based on the methylation marks we observed (Fig 21A). Some of these marks are associated with gene expression (H3K4me3 and H3K79me3) and others with gene repression (H3K9me3 and H4K20me3), and the resulting transcriptome seems to be a balance of these antagonistic marks (Hyun *et al*, 2017). Thus, restoring demethylase activity through DMKG supplementation, would have mixed effects transcriptionally that will depend on the histone residue in question and its location on the chromosome.

There were 115 protein-coding genes found to be differentially transcribed during infection with DMKG supplementation. They were analyzed with protein pathway enrichment algorithms: STRING, Kegg, Reactome, and GeneOntology.

One of the pathways indicated from the protein pathway enrichment algorithms was the p53 transcriptional network in which GADD45A, CCNG2, PMAIP1, and SESN2 were differentially regulated. The p53 transcriptional network is comprised of pathways involved in DNA damage response, apoptosis, and cell cycle regulation; all pathways known to be dysregulated during *C. trachomatis* infection. qRT-PCR analysis of GADD45A and PMAIP1 confirmed the microarray results and displayed that while both genes undergo increased transcription during infection, that DMKG supplementation during infection increases their transcription even further (Fig 34). Interestingly, it has been shown that intracellular levels of α -ketoglutarate are linked to p53 activity (Morris *et al*, 2019). Morris *et al*, demonstrated that restoring p53 expression in p53 deficient cells or supplementing them with α -ketoglutarate induced similar transcriptional profiles, with a prominence for the p53 signaling network. Human cells infected with *C. trachomatis* undergo a very pronounced reduction in p53 expression (Siegl *et al*, 2014; González *et al*, 2014).

Our system, in which we supplement p53-low cells (as a consequence of infection) with DMKG, thus shows similarity with the study by Morris *et al*, which also supplemented p53-deficient cells with DMKG. In both cases, the p53 transcriptional network stands out among the differentially regulated pathways. The molecular mechanism behind this observation remains unclear at this point.

It is also interesting to note at this point that GADD45A, which we confirmed to be upregulated in infection by qRT-PCR, promotes DNA demethylation (Li *et al*, 2015). DNA hypomethylation was recently described in testicular cells infected by *C. trachomatis*, and GADD45 upregulation could be implicated (Bryan *et al*, 2020).

MAPK signaling was also implicated by the protein pathway enrichment analysis, in which the genes FGF2, EPHA2, EFNA1, HSPA1A, CACNB2, and DUSP6 are differentially regulated. Several reports have shown that the MAPK pathway is upregulated during the infectious cycle and is important for optimal bacterial development (Buchholz and Stephens, 2007; Chen *et al*, 2010; Olive *et al*, 2014; Zadora *et al*, 2019). Differential regulation of the MAPK signaling cascade influences cell survival and could be advantageous for the bacteria. One remaining question is whether histone hypermethylation is implicated in the regulation of this pathway, or whether DMKG plays a role through another independent mechanism. To address this question, we plan to measure the level of these transcripts in infected cells treated with a low dose of the JmjC demethylase inhibitor, JIB-04. Indeed, we have observed that JIB-04 exacerbates histone hypermethylation in infected cells (Fig 26). Thus, any gene regulation that is driven by histone hypermethylation should be amplified by JIB-04 treatment.

Interestingly, over half of the DREs found to be differentially regulated during infection with DMKG supplementation were non-protein-coding RNAs, the majority of which were tRNAs. Importantly, infection alone already seems to decrease tRNA levels, a finding that has never been reported. These results need to be verified by Northern blot. If true, this loss in tRNA could explain the block in protein translation recently reported in *C. trachomatis* infected cells (Ohmer *et al*, 2019), and it will be important to understand its molecular basis.

Concluding remarks

This work demonstrated that *C. trachomatis* induces hypermethylation of histones during late infection at multiple residues simultaneously. This phenotype is a result of decreased histone lysine demethylase activity due to decreased intracellular levels of their shared co-factor, α -ketoglutarate. Demethylase activity of α -ketoglutarate-dependent JmjC demethylases can be restored by increasing intracellular levels of α -ketoglutarate via the supplementation of infected cells with DMKG, a cell permeable form of α -ketoglutarate. DMKG supplementation results in decreased histone methylation during late infection and circumvents DNA damage. Increased intracellular α -ketoglutarate also changes the transcriptome of infected cells with differential regulation of the

p53 transcriptional network, and MAPK signaling. Overall, this work demonstrates that shifts in metabolism and nutrient availability caused by an intracellular microbe impacts the epigenome of its host, with broad consequences on chromatin integrity and gene expression.

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