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“EFECTO DE LA RELOCALIZACIÓN DE HP1 A LA REGIÓN DE HETEROCROMATINA PERICENTROMÉRICA EN LA GENERACIÓN DE INESTABILIDAD CROMOSÓMICA DESPUÉS DEL TRATAMIENTO CON AGENTES DESESTABILIZADORES DE HETEROCROMATINA.”

TESIS

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ABREVIATURAS

ACA: Anti-centromere antibody

CCAN: Constitutive centromere-associated network

CENP-A: Centromeric protein A

ChIP: Chromatin immunoprecipitation

CIN: Chromosome instability

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

HDAC: Histone deacetylase

HP1: Heterochromatin protein 1

KL1: Katanin-like 1

KMN network: KL1, Mis12, and Ndc80 complex

Mis12: Minichromosome instability 12; MIND kinetochore complex component

Mis14: Minichromosome instability 14; kinetochore-associated protein NSL1

ncRNAs: Non-coding RNAs

Ndc80: Kinetochore protein NDC80 homolog

TSA: Trichostatin A

WIF1: WNT inhibitor factor 1

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RESUMEN

Antecedentes: Las proteínas de heterocromatina 1 (HP1) son importantes en el establecimiento, propagación y mantenimiento de la heterocromatina constitutiva. Se ha sugerido que la organización y estabilidad de la región pericentromérica es crucial para la correcta segregación centromérica durante mitosis. Participan en el reclutamiento y direccionamiento de la proteína Mis12 hacia el centrómero durante interfase. Alteraciones en HP1 pueden llevar a la perdida de la incorporación de Mis12 al cinetocoro. Por lo tanto, la estructura del centrómero y la relajación de cinetocoro promovidas por la ausencia de Mis12 podrían influenciar la inducción de inestabilidad cromosómica (IC), al reducir la capacidad del cinetocoro para anclarse con los microtúbulos. **Objetivo:** El objetivo de nuestro estudio fue determinar si alteraciones en la localización de las proteínas HP1 inducidas por tricostatina A (TSA) modifican el reclutamiento de Mis12 al centrómero, y si cambios en la presencia de las HP1 y la metilación de H3K9 en la cromatina centromérica afectan la expresión de transcritos RNAnc promoviendo un incremento de la IC en células HCT116 y WI-38. **Material y métodos. Para confirmar la generación de IC,** se realizaron cultivos de las líneas celulares humanas WI-38 y HCT116, y se evaluó el efecto de tratamiento con TSA con el protocolo de bandeo G. Se realizaron inmunofluorescencias de cultivos en células HCT116 y WI-38 para observar la localización a las proteína HP1, Mis12, CENP-A y modificaciones de histonas expuestas a TSA. Para observar el efecto del tratamiento en los niveles de proteína total, se aislaron proteínas de los cultivos celulares tratados con TSA por 24 y 48 h, subsecuentemente se revelaron por quimioluminiscencia mediante Western Blot. Se analizó la abundancia de las proteínas en las regiones de satélite alfa y 2 mediante inmunoprecipitaciónes de la cromatina (ChIP) partiendo de cromatinas aisladas de células mitóticas e interfásicas, así como de los tratamientos con TSA , y éstos se evaluaron por PCR en tiempo real. Se aisló el RNA de cultivos tratados con TSA, y se analizó la expresión del RNAnc del satélite alfa y satélite 2 por tiempo real y con el método de doble delta CT, donde se utilizó a GAPDH como gen de expresión constitutiva. **Resultados y Discusión:** Nuestros resultados muestran que la reducción de marcas de histonas asociadas con heterocromatina en la cromatina centromérica inducidos por TSA, reducen la presencia de HP1 en el centrómero de células normales WI-38 y que dicha reducción está asociada con un arresto en ciclo celular e IC. Sin embargo, en células HCT116, la proteína HP1 se relocaliza hacia la cromatina centromérica en respuesta al tratamiento con TSA, incluso después de un decremento de H3K9me3 en los nucleosomas centroméricos. El enriquecimiento de HP1 se asoció con un incremento en la IC, sugiriendo un mecanismo de respuesta en la cromatina centromérica y pericentromérica que aumenta la presencia de las proteínas HP1 en dicha regiones, posiblemente asegurando la segregación cromosómica a pesar de que se promueva la IC. Nuestros resultados proveen un nuevo acercamiento al panorama epigenético de la cromatina centromérica y el papel de las proteínas HP1 en la estabilidad cromosómica.

ABSTRACT

Background: Heterochromatin protein 1 (HP1) is important in the establishment, propagation, and maintenance of constitutive heterochromatin, especially at the pericentromeric region. HP1 might participate in recruiting and directing Mis12 to the centromere during interphase, and HP1 disruption or abrogation might lead to the loss of Mis12 incorporation into the kinetochore. Therefore, the centromere structure and kinetochore relaxation that are promoted in the absence of Mis12 could further induce chromosome instability (CIN) by reducing the capacity of the kinetochore to anchor microtubules. The aim of this study was to determine whether alterations in the localization of HP1 proteins induced by trichostatin A (TSA) modify Mis12 and Centromere Protein A (CENP-A) recruitment to the centromere and whether changes in the expression of HP1 proteins and H3K9 methylation at centromeric chromatin increase CIN in HCT116 and WI-38 cells. **Methods:** HCT116 and WI-38 cells were cultured and treated with TSA to evaluate CIN after 24 and 48 h of exposure. Immunofluorescence, Western blot, ChIP, and RT-PCR assays were performed in both cell lines to evaluate the localization and abundance of HP1α/β, Mis12, and CENP-A and to evaluate chromatin modifications during interphase and mitosis, as well as after 24 and 48 h of TSA treatment. **Results:** Our results show that the TSA-induced reduction in heterochromatic histone marks on centromeric chromatin reduced HP1 at the centromere in the non-tumoral WI-38 cells and that this reduction was associated with cell cycle arrest and CIN. However, in HCT116 cells, HP1 proteins, together with MIS12 and CENP-A, relocated to centromeric chromatin in response to TSA treatment, even after H3K9me3 depletion in the centromeric nucleosomes. The enrichment of HP1 and the loss of H3K9me3 were associated with an increase in CIN, suggesting a response mechanism at centromeric and pericentromeric chromatin that augments the presence of HP1 proteins in those regions, possibly ensuring chromosome segregation despite serious CIN. Our results provide new insight into the epigenetic landscape of centromeric chromatin and the role of HP1 proteins in CIN.

Keywords: HP1, centromeric chromatin, TSA, chromosome instability, CENP-A.

1. Introducción

El centrómero es un locus esencial que se requerire para que se realice una correcta segregación del material genético durante la mitosis y la meiosis. Dicha región funge como plataforma sobre la cual se establece el cinetocoro, por lo que es una estructura vital para la unión de los microtúbulos del huso mitótico, que son unen para guiar el movimiento cromosómico durante la división celular. Los centrómeros son fundamentales para dicha tarea la cual se conserva en muchos organismos, sin embargo, existe una sorprendente variabilidad en la secuencia estructural y organización del centrómero entre los eucariontes. Los centromeros de eucariontes se caracterizan por presentar una variante de la Histona 3 (H3) conocida en mamíferos como la proteína centromérica A (CENP-A, por sus siglas en ingles) [1]. Los centromeros se encuentran localizados cercanos o embebidos dentro de secuencias repetidas de DNA, aunque solo se ha encontrado especificidad en dichas secuencias repetidas en levaduras. El centrómero de levaduras se determina por una secuencia de DNA de 125pb que se ensambla en un elemento sencillo de nucleosomas Cse4, el cual posee la capacidad de capturar un microtúbulos [2]. Otros organismos carecen de esta especificidad de secuencia, de tal forma que incluso el DNA centromérico dentro de una especie difiere entre cromosomas.

En humanos, los centrómeros se definen con repetidos ricos en adenina y timina denominados satélites α, los cuales están constituidos por monómeros de 171 pb repetidos en tándem, generando arreglos de alto orden que se extienden desde 0.2-5 Mb [3]. En cromosomas humanos, la proteína CENP-A esta localizada sobre secuencias de DNA del satélite α, sin embargo, la unión de esta proteína no parece ser secuencia especifica, ya que

CENP-A esta confinada a solo una porción de los arreglos de mega bases del centrómero. Dicha proteína no se une a secuencias de satélites α dispersas en el genoma, ni tampoco a centrómeros inactivos que ocurren naturalmente en cromosómas dicéntricos humanos los cuales contienen dos regiones de repetidos de satélite α [4, 5]. Además, en el caso de neocentromeros humanos, estos se forman *de novo* en regiones típicamente de DNA no repetido, y también pueden formarse localmente en regiones cercanas al centrómero o a cientos de kilobases de distancia del centrómero borrado dentro de regiones pobres en genes con pocas secuencias repetidas [6].

La deposición de CENP-A hacia el centrómero está mediada por la chaperona de histona "HJURP" (de sus siglas en ingles: Hollyday junction recognition region protein). En particular, el dominio N-terminal de HJURP es responsable de la unión especifica y estequiométrica hacia el complejo CENP-A/H4 [7, 8]. La expresión de la chaperona HJURP es altamente regulada, ya que una perturbación en su expresión conlleva a defectos en la mitosis [9].

Los monómeros de satélite α contienen un motivo de 17 pb conocido como la caja B o caja CENP-B, el cual es reconocido por la proteína centormérica B (CENP-B) [10]. La proteína CENP-B es importante durante el ensamblaje de centrómeros *de novo* y para el correcto posicionamiento de los nucleosomas en el centrómero, con la excepción del cromosoma humano Y, el cual carece de cajas B en el satélite α, y por lo mismo no se une a la proteína CENP-B. A pesar de esto todas las demás proteínas centroméricas se encuentran reclutadas en el centrómero del cromosoma Y [11, 12]. Cabe resaltar, que dentro de las secuencias de satélite α que carecen de cajas B o aquellas que presenten mutada la caja B, no se forma

eucromatina ni en el DNA Y alfoide, ni en forma en cromosomas artificiales [4, 13]. Lo anterior sugiere que CENP-B es esencial para la formación del centrómero y que el satélite α es la secuencia preferencial para la incorporación *de novo* de CENP-A. Sin embargo, no todas las secuencias de satélites α tienen la capacidad de dar lugar a centrómeros *de novo* [13].

El ambiente de la cromatina ha ido adquiriendo mayor importancia en la determinación de centrómero y su establecimiento, a medida que aumentan los estudios en el área, sin embargo, los elementos de cromatina y genómicos necesarios para el establecimiento y mantenimiento del centrómero, aun se desconocen. Se ha sugerido que la secuencia de DNA por si misma no es suficiente para el establecimiento y función del centrómero , lo cual apoya las teorías postuladas que involucran mecanismos epigenéticos o cromatínicos en la formación del centrómero [14]. Dichos mecanismos epigenéticos y de cromatina han tomado importancia en los últimos años en el entendimiento del centrómero, por lo que los explicaremos a detalle a continuación.

1.1 Cromatina centromérica y epigenética.

El estudio del ambiente de la cromatina en centrómeros normales de humanos ha tenido como obstáculo la naturaleza de las secuencias repetidas y las regiones de secuencias compartidas entre regiones no homologas de los centrómeros, lo cual dificulta la evaluación de estos por medios moleculares para el análisis de grandes bloques de cromatina. Se sabe que la cromatina centromérica en humanos y otros organismos se caracteriza por arreglos de nucleosomas que contienen a CENP-A donde se encuentran intercalados nucleosomas

con H3K4me2 [15]. La proteína CENP-A es una variante de histona H3, la cual se encuentra localizada solo en los centrómeros funcionales, y es sobre esta histona donde el cinetocoro eventualmente se ensamblará [16, 17]; esta proteína representa una marca epigenética necesaria para la activación del centrómero. Recientemente, por medio de estudios estructurales de alta resolución se ha observado el heterotetrámero CENP-A/H4, el cual muestra diferencias estructurales importantes con los nucleosomas H3/H4 canónicos. A su vez los análisis estructurales por cristalografía de la proteína CENP-A humana, mostraron las diferencias con la histona 3 canónica, en particular el bucle 1 que contiene dos residuos de aminoácidos extra (Arg 80 y Gly 81), los cuales pudieran ser los responsables en estabilizar la cromatina centromérica que contiene a CENP-A [18].

Las propiedades físicas globales del nucleosoma en la cromatina centromérica son alteradas por las diferencias mediadas por CENP-A entre CATD (CENP-A centromere targeting domain, por sus en ingles) y H4 en el principio de la interfase, esta interacción convierte el nucleosoma en una estructura mas rígida en comparación al nucleosoma canónico [19, 20]. Dichas diferencias son esenciales para la incorporación de nucleosomas CENP-A al centrómero, y revelan la contribución de análogos de histonas a estructuras especializadas de cromatina en el centrómero que difieren de la típica heterocromatina y eucromatina.

Los centrómeros en aves (gallo) también contienen nucleosomas CENP-A, los cuales están entremezclados con nucleosomas H3K9me3, a su vez, se presenta en menor proporción H3K4me2 [21]. En especies de plantas, como el maíz, el centrómero esta enriquecido con H3K9me2 y H3K9me3, y presentan poco enriquecimiento de H3K4me2. Los dominios de H3 centroméricos en maíz están entremezclados con H3K27me1 [22, 23], y por medio de

estudios alta resolución en dichos centrómeros, se ha mostrado la presencia de genes activos dentro de la región, que están asociados con enriquecimiento de H3K4me2 y acH4, lo cual sugiere que los centrómeros estas organizados como regiones discretas de eucromatina rodeadas por regiones de heterocromatina enriquecidas con H3K9me2 [24]. A su vez, la marca de histona H3K4me2 se ha descrito como un componente esencial del ambiente del cinetocoro en vertebrados, el cual es requerido para el mantenimiento y función a largo plazo de dicha estructura [25].

Los resultados mencionados anteriormente sugieren que la cromatina centromérica varia entre especies. A pesar de las diferencias reportadas en las modificaciones de histonas, algunos modificaciones de histonas y proteínas asociadas podrían no haberse identificado, debido a las limitaciones en la resolución causada por la naturaleza repetida de estas regiones.

La inactivación centromérica (se refiere a centrómeros sin la incorporación de CENP-A) se considera un fenómeno epigenético; donde los centrómeros inactivos pueden adoptar configuraciones de cromatina incompatible con el mantenimiento del centrómero [26]. El ambiente de la cromatina en los centrómeros difiere de la configuración usual activa e inactiva. Lo anterior se ha sugerido en estudios con cromosomas artificiales a los cuales se les integró satélite α y con construcciones inducibles TetO las cuales están ligadas a proteínas modificadores de la cromatina, que son tanto represores o activadores transcripcionales, donde ambos eliminan la función del centrómero en los cromosomas artificiales. Entonces, ambientes eucromáticos o heterocromáticos son incompatibles con el ensamblaje y mantenimiento de CENP-A [27]. Otros estudios han propuesto que para la

formación de neocentromeros funcionales no son necesarios grandes dominios de heterocromatina ni de eucromatina [28]. Sin embargo, a la fecha no es claro si cambios en la cromatina tienen la capacidad de promover la remoción de las proteínas centroméricas (CENP), o si la pérdida de CENP permite que la heterocromatina remplace a la cromatina centromérica. Aunque, se ha observado que los nucleosomas que contienen H3 remplazan activamente sitios ocupados por CENP-A cuando esta proteína es reducida, sin embargo cuando se sobre expresa CENPA, remplaza los sitios de unión de nucleosomas H3 [15]. Considerando que la H3K9me3 es una marca que posee una función de factor limitante o antagonista a la cromatina CENP-A, la abundancia de cromatina que contiene CENP-A comparado con la heterocromatina podría ser mas importante que solo una simple asociación de presencia y ausencia de esta modificación particular [29, 30].

La metilación de la H3K27 es otra marca epigenética que define a la heterocromatina. En algunas especies de plantas, dicha modificación se ha asociado con el centrómero, en donde el enriquecimiento de H3K27me2/me3 promueve la inactivación de dos centrómeros del cromosoma tricéntrico encontrado en trigo [31] (**Figura 1**). Siendo así, las modificaciones de marcas epigenéticas tanto asociadas a estados de heterocromatina como de eucromatina reducen la capacidad del centrómero para generar un cinetocoro funcional, entonces, la modificaciones a la cromatina pueden causar perdida de función de la región centromérica durante mitosis, lo cual apoyala importancia de un modelo epigenético en el establecimiento y regulación de una cromatina centromérica única.

1.2 El Cinetocoro

En eucariontes, la correcta segregación cromosómica requiere que cada cromosoma

interaccione apropiadamente con los microtúbulos de uso mitótico, los cuales proveen el andamiaje estructural mediante el cual se realiza la segregación cromosómica. Dicha interacción está mediada por un complejo macromolecular conocido como el cinetocoro, el cual es una estructura compuesta por mas de 90 proteínas [32] (**Figura 1**). El cinetocoro facilita la interacción entre la cromatina centromérica y los microtúbulos con el fin de asegurar la bi-orientación de los cromosomas en la placa metafásica y la en la segregación cromosómica durante anafase [33].

Durante la fase S, la proteína CENP-A esta distribuida de manera equitativa en ambas cromatidas hermanas, donde la CENP-A recién sintetizada no es incorporada a la cromatina centromérica hasta la telofase y G1 [16]. El significado de este ritmo anormal de generación de nucleótidos con CENP-A aún no esta bien elucidado, sin embargo, se ha sugerido que la incorporación de esta proteína en esas fases del ciclo celular puede representar un mecanismo defensivo en contra de errores en la anexión y en el ensamblaje subsecuente de estructuras de cinetocoro a sitios no centroméricos causado por la acumulación temporal de ncleosomas CENP-A en la replicación e incorporación de otras histonas [34]. En algunos organismos, como en *Drosophila*, la proteína CENP-A por si misma es suficiente para asegurar la formación del cinetocoro, sin embargo en células de humanos para el correcto ensamblaje del cinetocoro se requieren proteínas adicionales a la histona fundadora [35]. A la fecha, se han realizado muchos acercamientos con el fin de identificar los componentes del centrómero de mamíferos, de las proteínas ya bien caracterzadas como formadoras de cinetocoro se encuentran: CENP-A, CENP-B, CENP-C, CENP-I, CENP-H, CENP-T y CENP-W entre otras proteínas, las cuales se han definido como la red constitutiva asociada a centrómero (CCAN, de sus siglas en ingles

"Constitutive Centromere Associated Network") [1, 32, 36, 37]. Basado en varios análisis funcionales se ha sugerido que estas proteínas del CCAN juegan un papel estructural en la formación de un cimiento estable para el ensamblaje dinámico del cinetocoro y para proveer un ambiente adecuado para la incorporación de CENP-A recién sintetizada [1, 32]. También ha sido sugerido que el CCAN puede funcionar como un control para la dinámica de los microtúbulos [36].

Recientemente se ha descrito un complejo nuevo capaz de interactuar de manera estable con los nucleosomas H3, denominado CENP-T/W [36]. A pesar de que la organización molecular precisa de la población de nucleosomas H3-CENP-T/W se desconoce, se ha sugerido que se encuentra distribuida cercanos a los nucleosomas CENP-A (Figura 1). Las consecuencias funcionales de estos eventos de incorporación de nucleosomas CENP-A serian la expansión del compartimento H3-CENP-T/W adentro de la cromatina centromérica post-replicativa. Esto sugiere que el complejo CENP-T/W tiene un papel funcional en la formación del cinetocoro después de la replicación del DNA [38].

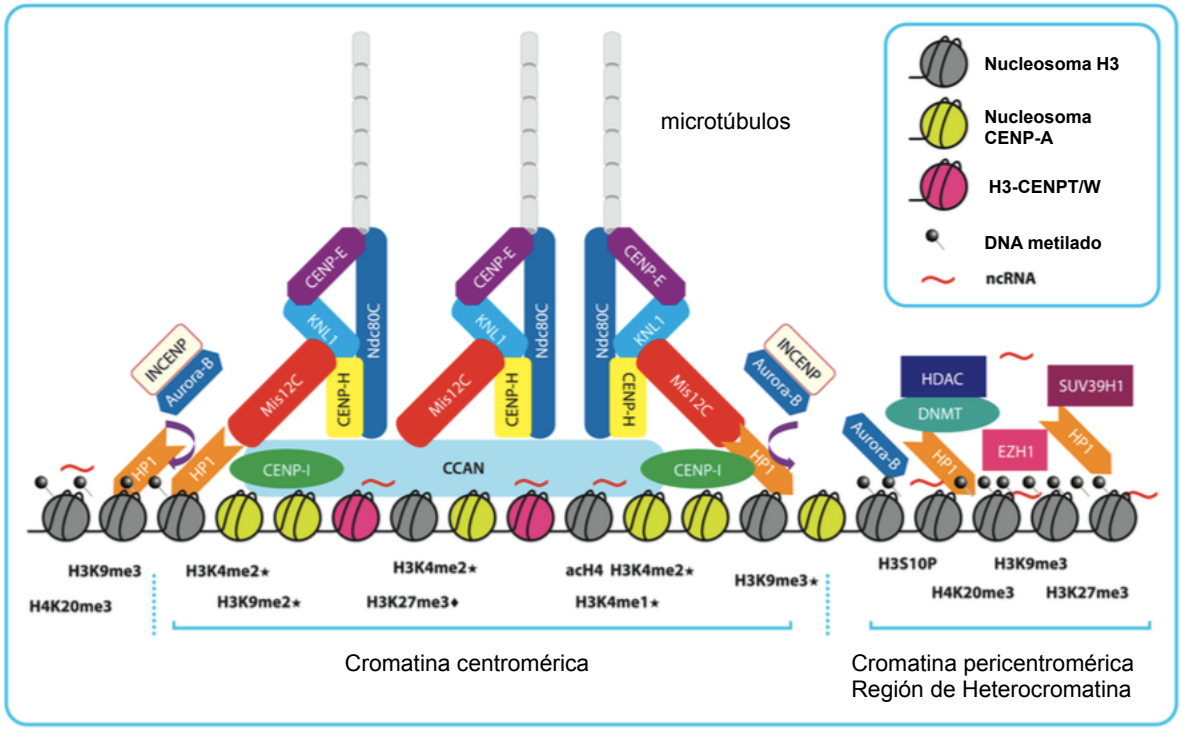

Figura 1. *Representación esquemática de la cromatina centromérica y pericentromérica y la formación del complejo epigenético fundador del cinetocoro. La cromatina centromérica es moldeada principalmente por la presencia de los nucleosomas rígidos CENP-A, que están intercalado con complejos H3-CENP-T/W y nucleosomas H3, los cuales presentan marcas represivas, H3K9me2/3 y H3K27me1/3 (* representa marcas encontradas en especies de plantas y ∗ representa marcas encontradas en mas de una especie), y marcas activas, H3K4me2 y H4ac, así como RNAnc que dan lugar a la fundación del centrómero. Figura obtenida de [39].*

Se ha demostrado que las proteínas que pertenecen al CCAN se encuentran asociadas a la cromatina centromérica a lo largo de todo el ciclo celular. En conjunto con CENP-A, estas pueden formar un ambiente estable para el ensamblaje de la estructura del cinetocoro mitótico. Se ha observado que la actividad de unión al DNA o la directa interacción con la proteínas CENP-A para varias de las proteínas pertenecientes al CCAN a lo largo del ciclo celular, como CENP-C y CNP-I/H [10, 32, 40].

La placa externa del cinetocoro y la corona fibrosa se ensamblan al iniciar la mitosis, estas contienen proteínas requeridas para interacciones con los microtúbulos. Dicha proteínas incluyen aquellas que tienen actividad de unión directa con los microtúbulos, como es el caso del complejo KL1, Mis12, Ndc80 (en conjunto forman la red KMN) [32], CENP-E y el complejo Ska1 [41], los cuales son factores transitorios que modulan esta interacción o monitorean el anclaje [34] (**Figura 1**).

Se han realizado experimentos sobre la tensión de los microtúbulos en células humanas tratadas con taxol, lo cual ha ayudado a comprender la arquitectura interna del cinetocoro en presencia o ausencia de tensión en los cinetocoros. Estos estudios identificaron cambios en la organización de la capas estructurales, donde la ausencia de tensión conlleva a la reducción en la distancia entre las proteínas internas del cinetocoro, como CENP-C, y el complejo Ndc80. Sin embargo, la localización de las proteínas internas del cinetocoro permanece sin modificaciones [42]. La reducción de la tensión del cinetocoro también provoca rearreglos sorprendentes en los componentes de la red KMN, y se sugire que los complejos de proteínas del cinetocoro son mas dependientes de la tensión por la interacción con microtúbulos.

A pesar que el cinetocoro parece ser una estructura estable durante mitosis, se ha sugerido que las proteínas del cinetocoro son altamente dinámicas a lo largo del ciclo celular. Aunque las proteínas que conforman el CCAN están presentes en el centrómero de forma constitutiva , algunas proteínas de cinetocoro externo, como el complejo Mis12 (Mis12C) y KLN1, se reclutan durante la fase G2 [43]. Debido a que el reclutamiento de estos

complejos es la base de ensamblaje en el centrómero, la incorporación de los componentes restantes para generar la estructura capaz de unirse con lo microtúbulos ocurren durante profase y prometafase

De las mas de 90 proteínas que contribuyen en el ensamblaje de cinetocoro, cuatro grupos de proteínas se han sugerido como bases del ensamblaje en el centrómero, debido a sus funciones conocidas, denominadas de: unión, andamiaje, chaperonas y estabilizadores estructurales [44]. Sin embargo, una característica de los cinetocoros de vertebrados es la reorganización masiva que toma lugar durante la mitosis. En un periodo de tiempo de menos de una hora, el cinetocoro es capaz de reclutar mas de 40 componentes mitóticos de una forma jerárquica y después estas proteínas se desensamblan para regresar a un estado de interfase. Se ha propuesto que este proceso podría ser controlado por la presencia de la envoltura nuclear, la cual restringe proteínas al núcleo de tal forma que estas no son capaces de asociarse al cinetocoro hasta que ocurra la eliminación de la envoltura nuclear [45]. Aunque, trabajos recientes han demostrado que al menos algunas proteínas están presentes dentro del núcleo sin estar localizados en el cinetocoro, sugiriendo que la formación del cinetocoro durante mitosis no necesariamente se bloquea para ensamblarse por la envoltura nuclear [43]. A demás, las modificaciones post-traduccionales podrían regular la formación del cinetocoro. Se ha observado que la proteína similar a ubiquitina (SUMO) modifica a CENP-I, y cuando CENP-I es SUMOilada extensivamente , se vuelve blanco de degradación. Por lo tanto, para que esta proteína se incorpore al cinetocoro durante mitosis, es necesaria la degradación de los grupos SUMO por la proteasa de SUMO SENP6 [46].

1.3 La Proteína de heterocromatina 1: función y relación con el cinetocoro.

La proteína de heterocromatina 1 (HP1) fue descubierta en Drosophila m. como un supresor dominante en el fenómeno de variegación por efecto de posición (PEV- de sus siglas en ingles "Position effect variegation") y después se observó su participación en la formación de la heterocromatina pericentromérica [47]. A pesar de que en estudios iniciales las proteínas HP1 se asociaron a la formación de la heterocromatina, especialmente en regiones centroméricas y pericentromericas, se ha vuelto cada vez mas claro que HP1 posee múltiples funciones y esta también presente en regiones eucromáticas activas transcripcionalmente [48]. HP1 también juega un papel importante en la cohesión de las cromátidas hermanas, mantenimiento del telómero y reparación del DNA [49–51]. En humanos dichas funciones se realizan de manera especifica por los tres subtipos de HP1: HP1α, HP1β y HP1γ [52, 53]. Dichas proteínas son codificadas por el gen CBX5, localizado en el cromosoma 12q13.13. Esta constituido por 6 exones que codifican a la proteína HP1α, de 22KDa; El gen CBX1, localizado en el cromosoma 17q21.32, constituido por 6 exones que codifican a HP1β, de 21.4 KDa; y el gen CBX3, localizado en el cromosoma 7p15.2, constituido por 8 exones que codifican para HP1γ de 20.1 KDa (NCBI genes ID 23468, 10951 y 11335 respectivamente). La estructura es altamente conservada como se ejemplifica para HP1β (**Figura 2**).

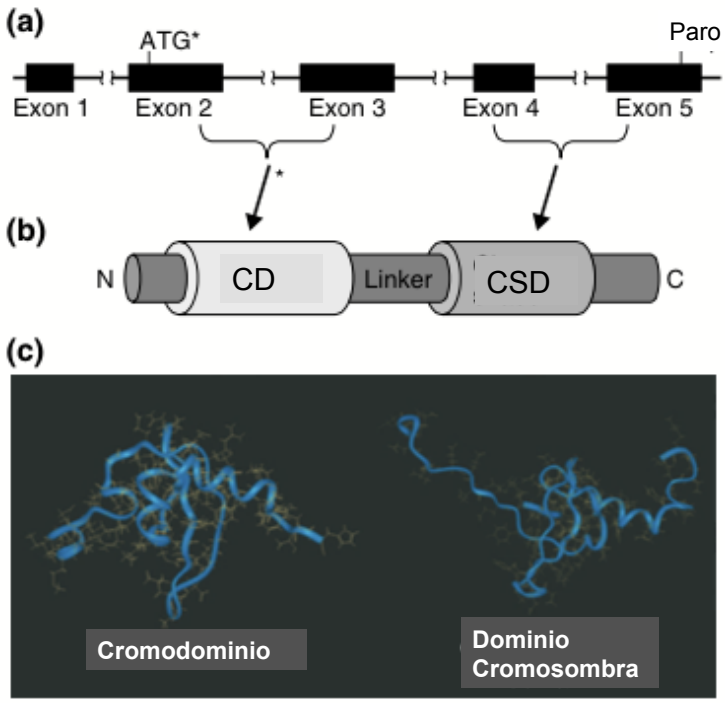

Figura 2. *Estructura de la proteína HP1 y del gen CBX5 del cual codifica. a) Estructura del gen conservada de Drosophila a humanos. El gen consiste de cinco exones separados por cuatro intrones (en humanos son 6 exones). El sitio de inicio (ATG) y el de termino están indicados (PARO). Los paréntesis indican los exones involucrados en la formación de los dominios cromodominio (CD) y cromosombra (CSD). b) Estructura linear de la proteínas HP1. N, termino amino; C, termino carboxilo. c) Estructura tridimensional de los dominios CD y CS de HP1β de murinos. obtenidas del Protein Data Bank; figura tomada de [54].*

Las proteínas HP1 se unen a la histona H3 que ha sido metilada en la lisina 9 por enzimas metil-tranferasas especificas, como G9a y SUV39H1; una vez unida HP1 recluta a SUV39H1 hacia el DNA, lo cual propaga la metilación a lo largo de la cromatina [55]. Dicha relación entre HP1 y SUV39H1 esta conservada en los organismos, como se ve en los homólogos de SUV39H1 presentes en *Saccharomyces pombe*, Swi6 y Clr4, lo cual sugiere la conservación evolutiva del mecanismo de formación de heterocromatina [56]. Se ha demostrado que tanto Swi6 como otros factores, se requieren para el establecimiento de

los centrómeros *de novo*, pero no para su mantenimiento [57].

La función de HP1 es muy importante en el establecimiento, propagación y mantenimiento de la heterocromatina constitutiva [58], especialmente en la región pericentromérica donde ha sido bien caracterizado el enriquecimiento de las marcas H3K9me3 y H4K20me3, hipo acetilación de H3 y H4, así como una alta tasa de metilación en la secuencias de repetidos satelitales [59, 60].

La metilación de DNA consiste en la adición covalente de grupos metilo al carbono 5 de la citosina, dando lugar a la 5-metil citosina [61, 62]. Debido a la yuxtaposición a la cromatina centromérica, se ha sugerido que la organización y la estabilidad de la región pericentromérica es crucial para asegurar una correcta segregación cromosómica durante mitosis; por lo tanto, esta región es importante para inestabilidad genómica [59, 63]. En estudios recientes en humanos y en moscas, se demostró que la red KMN interactúa con HP1, HP1 participa en el reclutamiento y direccionamiento de Mis12C hacia el centrómero durante interfase [64–66]. También se ha sugerido que el reclutamiento de la proteína Mis12 sebrealiza por HP1 [65]. Cada subunidad de HP1 consta de un cromodominio, el cual reconoce a la metilación de H3K9, una bisagra implicada en la regulación de interacciones proteína/DNA/RNA y un dominio cromosombra, el cual es responsable de la dimerización y de las interacciones con diferentes proteínas que contengan el motivo consenso denominado PXVXL [54]. Estas propiedades de las proteínas HP1 dan origen a su función como un adaptador que permite que otras proteínas interactúen con la cromatina. Mediante análisis in vitro, se ha sugerido que Mis12C dimeriza con HP1, debido a la interacción con el motivo PVIHL localizado en los residuos 209-213 de la proteína NSL1

(miembro de complejo Mis12) la cual es responsable de la unión con el motivo PXVXL de HP1[64, 67], lo cual sugiere la manera en la que HP1 podría reclutar a Mis12 a la cromatina centromérica.

Los resultados mencionados anteriormente, dieron origen a las siguientes preguntas. Si la H3K9me3 sirve como un marcador de frontera o un antagonista de CENP-A, ¿Por qué una proteína que se requiere en la cromatina centromérica de una manera dependiente de SUV39H1 juega un papel en el direccionamiento de Mis12C hacia el cinetocoro? ¿Será la participación de HP1 regulada de una manera dependiente del ciclo celular? Aunque el enriquecimiento de H3K9me2 y H3K9me3 se ha encontrado en el centrómero, otras marcas como la H3K4me2 y H3K27me3 se han encontrado intercaladas en la cromatina del centrómero. Análisis in vivo demostraron que la localización de HP1α y HP1β en humanos presenta un rol especifico a diferentes tiempos durante el ciclo celular. La localización de ambas proteínas en la heterocromatina centromérica es intercambiada durante interfase y metafase. Específicamente durante metafase, HP1β (la cual se encuentra preferentemente en la cromatina centromérica) se remplaza por HP1α (la cual se encuentra típicamente localizada en la cromatina pericentromérica y telomérica). Dichos intercambios son mediados por diferencias en la secuencia del dominio cromosombra [68]. Recientemente, por análisis de interacción in vitro se demostró que el dímero DSN1-NSL1 es crucial para la unión HP1, Ndc80C y KNL1C, y en estudios tanto de HP1 como NDC80 sugirieron que estos pueden poseer una región de unión idéntica o que se sobrelapa. Por lo tanto, HP1α y Ndc80C son competidores para unirse con Mis12, [67]. Para que Ndc80 se pueda localizar en el cinetocoro, es necesario desplazar a HP1α de su unión con Mis12. El intercambio ocurre rápidamente de una manera coordinada durante mitosis junto con el proceso de

segregación cromosómica [69]. La eliminación o una reducción en las proteínas HP1 se ha visto asociada a la formación de algunos tumores y también es posible que la ausencia de HP1 promueva una perdida de la incorporación de Mis12C hacia el cinetocoro. Esto tendría efecto en la estructura del centrómero y generaría una relajación del cinetocoro promoviendo inestabilidad cromosómica (IC) [70] (**Figura 3**, **4A-B**).

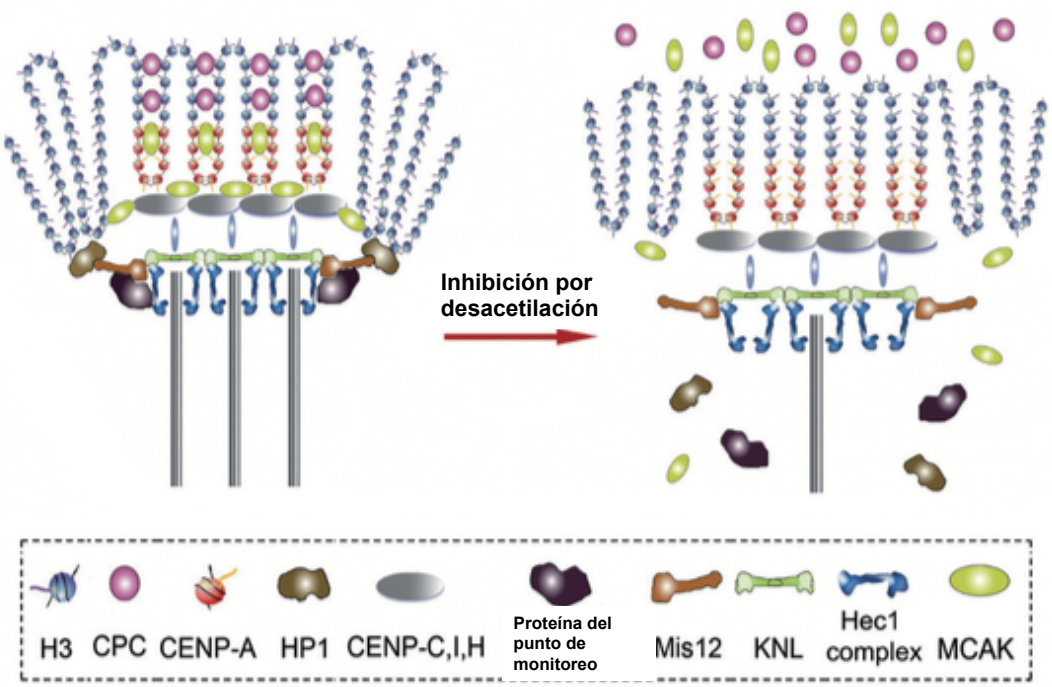

Figura 3. *Modelo del efecto de la relajación de la cromatina en el cinetocoro interno. La inhibición de la desacetilación de histonas incrementa la acetilación de las mismas y altera la localización normal de HP1, resultando en una organización de la cromatina pericentromérica ectópica. La cromatina pericentromérica aberrante elimina la dinámica de reclutamiento de la base de la placa externa. Esto elimina al complejo pasajero cromosómico (CPC) de la región centromérica y reduce la capacidad del complejo Mis12 y Hec1 para unirse a los microtúbulos. El efecto en múltiples vías de esta relajación resulta en un arresto en prometafase y en inestabilidad cromosómica [71].*

Existe evidencia que sugiere que la localización de HP1α en la heterocromatina centromérica durante mitosis, contribuye a la estabilidad de la cohesión de la cromatidas

hermanas y a la activación de punto de monitoreo de cinetocoro, de manera que la reducción de HP1α en el centrómero metafásico pude ser una causa de IC en células cancerosas [72, 73]. La formación de centrómeros y cinetocoros en eucariontes aparenta ser directa o indirectamente regulada por mecanismos epigenéticos, en particular, esta hipótesis se apoya por el hecho de que varios componentes de cinetocoro se relacionan con factores epigenéticos. Varios estudios al respecto sugieren que la desregulación de componentes epigenéticos en el complejo del cinetocoro conlleva a defectos cromosómicos y al desarrollo de inestabilidad cromosómica [74–76].

1.4 Relación entre la epigenética y el centrómero con la inestabilidad cromosómica.

Se han descrito dos modelos principales de la inestabilidad genética. El primer modelo se asocia con la inestabilidad microsatelital (IMS), mientras el segundo describe la IC. Los microsatélites son secuencias de DNA que varían en longitud entre individuos pero no durante el curso de la vida de un solo individuo, donde la longitud permanece constante. Los cambios anormales en el tamaño de los microsatélites que suceden en un individúo se refieren como IMS. Este fenómeno esta asociado con enfermedades como el cáncer; aproximadamente 15% de las neoplasias colorectales presentan un fenotipo IMS [77].

La IC se puede desarrollar por dos vías principales, la primera está relacionada con anormalidades en el número de cromosomas, lo cual ocurre principalmente debido a la ganancia o perdida de cromosomas completos; la otra está asociada con rearreglos o cambios estructurales anormales de los cromosomas. Donde los cambios estructurales de los cromosomas, como translocaciones de fragmentos de cromosomas, sucede

principalmente por rupturas. Este fenómeno se asocia a errores mitóticos que posibilitan fallas en la segregación cromosómica, que podrían conllevar a la oncogénesis [78]. En particular, están involucradas regiones lábiles de DNA, conocidas como sitios frágiles. Los cuales son *loci* específicos que son propensos a rompimientos y re arreglos [79]. Dichos sitios provocan rearreglos de regiones genómicas grandes por inserciones, deleciones y translocaciones derivan en IC. Por lo tanto, el fenómeno de IC promueve alteraciones en la expresión de oncogenes, perdida de genes supresores de tumores y deleciones de algunos genes, como aquellos que codifican a micro RNAs [80].

La IC en el cáncer esta asociada con pobre diagnostico en tumores sólidos y resulta en variaciones fenotípicas que promueven resistencia a fármacos [81]. La IC es una causa probable de la heterogeneidad de células tumorales [81]. Una de la hipótesis mas aceptadas indica que estos tumores adquieren resistencia a la terapia y por lo tanto a bajas tasas de supervivencia.

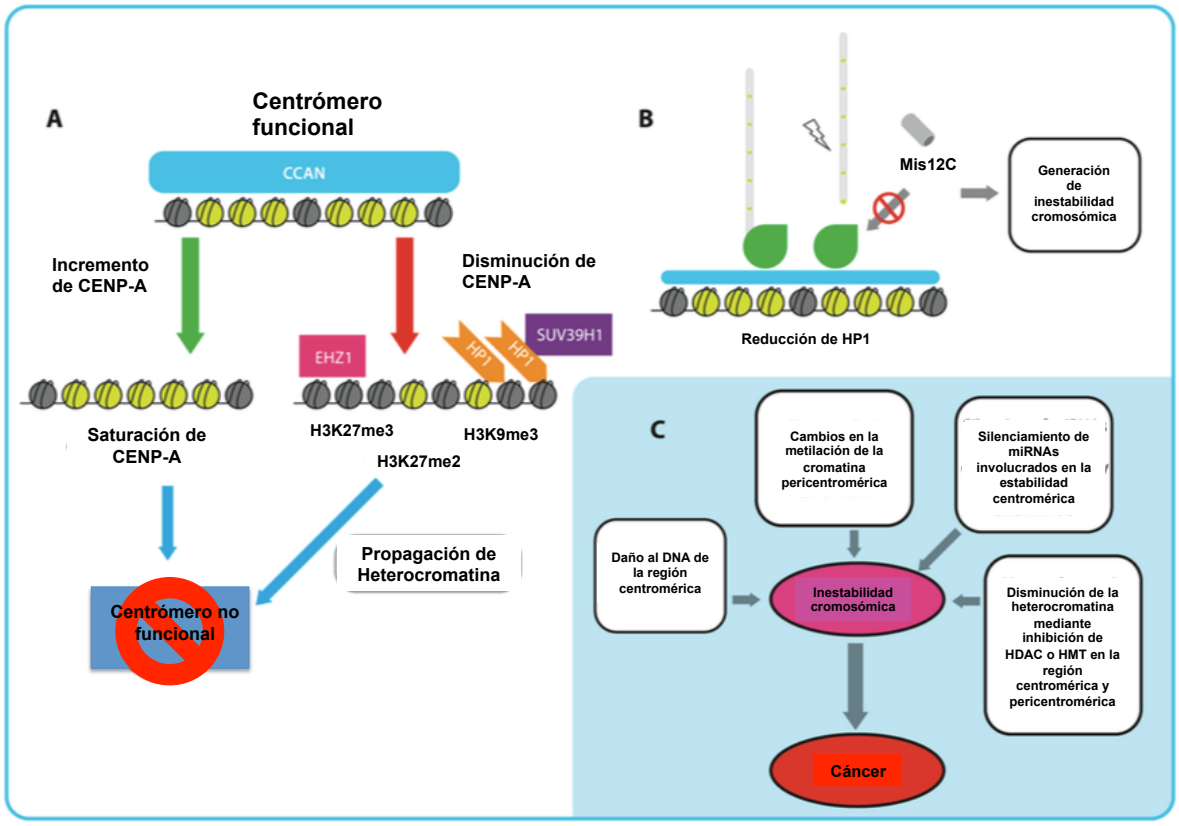

Figura 4. *Disociación epigenética del centrómero y sus implicaciones en la inestabilidad cromosómica (IC). A) Representación esquemática de la disociación del centrómero por medio de la sobre-expresión de CENP-A y por heterocromatinización. B) La depleción de HP1 promueve una reducción en la localización del complejo Mis12, lo cual puede afectar la incorporación de microtúbulos y da lugar a IC. C) Generación de IC por diversos mecanismos en la regiones centroméricas y pericentromericas como una posible causa de cáncer, figura obtenida de [39].*

Existe evidencia reciente que involucra procesos epigenéticos en la generación de IC [64]. La transformación celular y desarrollo de cáncer se han asociado con modificaciones en el balance epigenético y alteraciones en la expresión genética e IC [82] (**Figura 4B-C**). Uno de los procesos epigenéticos primarios involucrados en el desarrollo de diversas enfermedades es la metilación del DNA. En particular, hipermetilación local asociada principalmente a islas CpG e hipometilación global son los principales cambios que se

observan en cáncer [62, 83, 84]. Este fenómeno esta presente en la mayoría de los tumores y sugiere que la metilación de DNA es importante en la transformación oncogénica [85, 86]. Se ha sugerido que la inestabilidad epigenética representa una alternativa a la inestabilidad cromosómica en el cáncer [85]. Muchos tipos de cáncer exhiben diferencias en el grado de metilación que da origen a una sub-clasificación de tumores llamada el "Fenotipo metilador de islas CpG o CIMP (por sus siglas en ingles)". Esta clasificación representa grupos de tumores diferentes clínica y etiológicamente, que se caracteriza por inestabilidad epigenética [87]. En estudios de cáncer de colon aunque existe inestabilidad genómica, entendida como mutaciones que afectan la expresión o función de los genes; no presentan IMS ni IC pero si presentan el fenotipo CIMP, sugiriendo la importancia de la regulación epigenética en el cáncer [88]. Los tumores positivos a CIMP son clínicamente distintos de aquellos pacientes que no la presentan, dichas diferencias podrían contribuir a mejorar el entendimiento del origen de los tumores.

Un componente implicado en la IC es la proteína CENP-A. Esta proteína se encuentra sobre-expresada en cáncer colorectal primario [89]. En particular, los niveles de expresión de la proteína Rb se han asociado con la sobre-expresión de CENP-A y la inducción de aneuploidias hipodiploides, debido a la presencia de un motivo E2F en el gen CENPA humano [90, 91]. Una hipótesis novedosa es que la sobre-expresión de CENP-A podría causar expansión de CENP-A en la heterocromatina centromérica y hacia los brazos de los cromosomas, interfiriendo con el ensamblaje del complejo del cinetocoro, y de tal forma se convierte en una causa de inestabilidad cromosómica [90] (**Figura 4A**). La sobre-expresión y deslocalización de la chaperona depositadora de CENPA, "HJURP", se ha observado en líneas celulares de cáncer de pulmón y se asocio con inducción de IC e inmortalidad de

células tumorales [9]. En estudios clínicos, la sobre-expresión de HJURP se asoció con un incremento en la sensibilidad a radioterapia y con un decremento en la tasa de supervivencia en pacientes con cáncer de mama [92].

Por otra parte, la ganancia o pérdida de modificaciones postraduccionales de histonas se ha asociado con el silenciamiento génico a nivel local, así como a la presencia de rearreglos de cromatina a nivel global; ambos fenómenos tienen un efecto profundo en la función local de la célula y pueden promover el surgimiento de ciertas enfermedades. Un ejemplo de desregulación de proteínas modificadoras de histonas, incluye a las histona metiltranferasas como EZH2, y SUV39H1, que provocan una desregulación del complejo policomb y HP1, al afectar las marcas H3K27me3 y H3K9me3 [93]. Adicionalmente, la inhibición o reducción de niveles de HDAC en la región centromérica promueve la acumulación de H3K9ac y H4K14ac y resultan en defectos en la segregación cromosómica debido a la inhibición dependiente de acetilación de H3K9me3 [70, 94, 95] (**Figura 4C**).

Por lo tanto, es claro que las alteraciones que modifiquen la estructura de la cromatina son importantes en la estabilidad cromosómica. Particularmente la coexistencia de componentes epigenéticos, como H3K9me3 y HP1 (que se encuentran altamente enriquecidos en la cromatina pericentromérica y secuencias satélites), podrían fortalecer la hipótesis de que HP1 no solo es un componente que estabiliza la heterocromatina, también es una proteína que funciona como andamiaje en el ensamblaje de cinetocoro junto con CENP-A. La alteración en la presencia de HP1 en la región pericentromérica podría promover la IC; de tal manera que una disminución de HP1 también podría participar en el desarrollo del cáncer y otras enfermedades [49, 64].

1.5 Transcritos RNA no codificantes centroméricos y su relación con el cinetocoro

Aunque la función de los centrómeros esta muy conservada en los organismos, la secuencia de DNA que conforma al centrómero no muestra conservación evolutiva. La cromatina que conforma al centrómero se localiza en secuencias de DNA repetidas especie-especificas que se suponía carecían de transcripción. La naturaleza epigenética del centrómero es en cambio altamente conservada, donde la histona CENP-A es esencial para la formación del cinetocoro, como se mencionó anteriormente.

Sin embargo, la presencia de transcritos de RNA no codificante (RNAnc) a partir de los repetidos centroméricos y pericentroméricos, se asocian como otro factor epigenético determinante en la organización del centrómero. En levaduras, repetidos de DNA pericentroméricos son transcritos y procesados por la maquinaria del RNAi en siRNA, los cuales guían la deposición de marcadores de heterocromatina como H3K9me3 y HP1 a la heterocromatina pericentromérica [95]. La vía del RNAi también se ha observado en el establecimiento de la heterocromatina pericentromérica en plantas y células animales [96, 97], sin embargo, la reducción de Dicer en líneas celulares humanas causan disminución en la heterocromatina pericentromérica, esta no afecta la unión de la proteína CENP-A, ni de CENP-C al centrómero, lo que sugiere que los RNAi no son requeridos para la función del centrómero, mas bien para la formación de la estructura de la heterocromatina [98]. El dominio del centrómero que contiene nucleosomas CENP-A en células humanas y en *Drosophila* esta enriquecido por la marca de eucromatina H3K4me2, y se ha visto que esta cromatina es permisible para la transcripción y no afecta la función de cinetocoro, incluso se ha observado la presencia de RNAnc y de transcripción por la RNAPII en el cinetocoro

mitótico [99, 100]. A la fecha, es desconocido como estos RNAs participan en la formación o estabilización a larga escala de la estructura de la cromatina centromérica.

Estos transcritos de RNA centroméricos son esenciales para la localización proteínas centroméricas con es el caso de CENP-C. La inhibición de la RNAPII durante mitosis promueve una reducción de CENP-C en el cinetocoro al disminuir la expresión del RNAnc centromérico y promueve la generación de cromosomas rezagados durante la mitosis [100]. En el repetidos de DNA del satélite menos de cromosomas de ratones el cual se encuentra en la constricción primaria, se ha descrito la acumulación de RNA transcritos del satélite menor en los cromocentros, que son agrupaciones de varios centrómeros, y se ha propuesto que estos RNAnc participan en la formación de complejos centroméricos específicos [101]. Recientemente se observó que los transcritos del satélite menor son componentes integrales de la fracción de cromatina con CENP-A y estos se asocian con las proteínas endógenas del complejo pasajero cromosómico como Aurora B, Survivina e INCENP, al inicio de la mitosis. La presencia de este RNA potencia la actividad de cinasa de INCENP en la histona 3 encontrada en el centrómero. También, favorece la interacción entre Aurora B y Survivina, así como con CENP-A, apoyando el papel estructural de los RNA centroméricos en la estabilización de complejos asociados al centrómero [102].

Los transcritos centroméricos son cruciales en la función y ensamblaje de centrómero-cinetocoro, cambios en la expresión de estos RNAs o mutaciones en la vía metabólica del RNA inducen inestabilidad cromosómica, fallas en la segregación y aneuploidia, facilitando el proceso de tumorigénesis [103].

Recientemente se observó que la proteína CTCF en los repetidos de satélite 2 humano promueve la reducción de la proteína Vigilina y de HP1α en la región, promoviendo la transcripción de transcrito de satélite 2 [104]. Lo que sugiere que la transcripción del satélite 2 necesita disminución en la heterocromatina constitutiva y después estos RNAnc funcionan en *cis* formando heterocromatina y estabilizándola, lo cual indica que hay un dinamismo en estructura de la heterocromatina. Aunque se desconoce el efecto en la composición de la cromatina centromérica en la transcripción de RNAnc centromérica, así como la capacidad de que estos transcritos de interactuar con proteínas de heterocromatina como HP1 y su importancia en el desarrollo de enfermedades como el cáncer.

2. PLANTEAMIENTO DEL PROBLEMA

Se ha sugerido que alteraciones en lo niveles de expresión, función y localización de la proteína HP1 se relacionan con la generación de tumores. La ausencia de HP1 podría dirigir una pérdida de la incorporación de Mis12 hacia el cinetocoro. Por lo tanto, la estructura del centrómero y la relajación del cinetocoro promovidas por la ausencia de HP1 y de Mis12, podrían inducir inestabilidad cromosómicas al reducir la capacidad de unirse a los microtúbulos del cinetocoro . Diversos estudios sugieren que la desregulación de componentes epigenéticos en el complejo del cinetocoro pueden dar lugar en defectos cromosómicos y al desarrollo de inestabilidad cromosómica. Los defectos en la composición de la cromatina centromérica afectan la determinación del centrómero y su establecimiento. Sin embargo, las modificaciones de la cromatina que son necesarias para el establecimiento y mantenimiento del centrómero siguen siendo desconocidas. Se ha sugerido que la secuencia del DNA por si misma no siempre es suficiente para el establecimiento y la función del centrómero [14], lo cual apoya a las teorías que postulan un papel relevante de mecanismos epigenéticos [39]. Por lo tanto, nuestro estudio se enfocara en determinar si la alteraciones en la localización de las proteínas HP1 modifican el reclutamiento de Mis12 hacia el centrómero y si los cambios en la expresión de las proteínas HP1 y la metilación de H3K9 en la cromatina centromérica dan lugar a la generación de inestabilidad cromosómica observada en incremento en la generación de aneuploidias.

Los niveles de transcripción de los transcritos centroméricos son esenciales para la formación y función centrómero-cinetocoro, cambios en la expresión de estos RNAncs o

mutaciones en la vía metabólica de RNA inducen inestabilidad cromosómica, fallas en la segregación y aneuploidias, facilitando la tumorigénesis [103].

2.1. HIPOTESIS

La movilización de proteínas HP1α y HP1β a la región de heterocromatina pericentromérica y centromérica provocada por el tratamiento con Tricostatina A, reducirá la heterocromatina y el reclutamiento de Mis12 y CENP-A en el centrómero generando así inestabilidad cromosómica.

2.2. OBJETIVO GENERAL

Determinar el efecto de la alteracion en la localización de HP1α y HP1β sobre el reclutamiento de Mis12 hacia el centrómero y analizar si los cambios en la presencia HP1α, HP1β y la metilación de H3K9 en la cromatina centromérica afectan la expresión de transcritos RNAnc que estarán relacionados a un incremento en la inestabilidad cromosómica.

2.3. OBJETIVOS PARTICULARES

1. Observar la generación de inestabilidad cromosómica en respuesta a los tratamientos con TSA en células WI-38 y HCT116.
2. Evaluar la localización de las proteinas HP1α, HP1β, MIS12 y de las modificaciones postraduccionales de histonas H3K4me3, H3K9ac, H3K9me2/3 y la variante CENP-A, durante interfase y mitosis, asi como post tratamiento con TSA en células

HCT116 y WI-38.

3. Determinar el efecto del tratamiento con TSA en la abundancia de la proteinas HP1α, HP1β, H3K4me3, H3K9ac, H3K9me3 y CENP-A en células HCT116 y WI-38.
4. Evaluar la presencia de HP1α, HP1β, MIS12 y de las modificaciones postraduccionales de histonas H3K4me3, H3K9ac, H3K9me2/3 y la variante CENP-A en la cromatina de los repetidos pericentromericos (satélite 2) y centromericos (satélite α) durante interfase y mitosis, asi como post tratamiento con TSA en células HCT116 y WI-38.

2.4 DISEÑO EXPERIMENTAL

1. **Inducción de inestabilidad cromosómica.**

   Se evaluara la capacdad de inducción de aneuploidias por el tratamiento con TSA durante 24 y 48 h en células WI-38 y HCT116, contando el numero total de cromosomas teñidos en 50 células por duplicado.

2. **Efecto en la localización nuclear**

   Se evaluará la localización celular de las proteinas HP1 α / β, Mis12, H3K9me3, H3K9ac, H3K4me2, y su relación con el centromero observado por CENP-A en celulas WI-38 y HCT116 en interfase y mitosis y despues del tratameinto con TSA por 24 h y 48 h, mediante la técnica de inmunofluoresencia epifluorescente y confocal.

3. **Abundancia de proteinas**

   Observaremos la abundancia de proteina total en la proteinas HP1 α / β, Mis12, CENP-A, H3K9me3, H3K9ac, H3 y actina en células WI-38 y HCT116 sin

tramiento y despues del tratameinto con TSA por la técnica de electroforesis e inmuno blot (western blot).

4. **Inmunoprecipitación de la cromatina,**

   Observaremos el enriquecimiento de las proteinas HP1 α / β, Mis12, CENP-A, H3K9me3, H3K9ac, H3K4me2, durante interfase y mitosis, asi como despues del tratameinto con TSA por 24 y 48h, en el promotor de WIF1, GAPDH, secuencias de satélite α y satélite 2 de la células WI-38 y HCT116.

5. **Expresión del RNAnc de satélite α y satélite 2,**

   Observaremos el efecto del tratamiento con TSA en la expresión de los transcritos centroméricos (satélite α) y pericentromericos (satélite 2), mediante qRT-PCR en la lineas celulares WI-38 y HCT116.

6. **Interacción del RNAnc satelite α con las proteinas HP1 α y CENP-A,**

   Evaluaremos si el RNAnc satélite α interactua con las proteinas HP1 α y CENP-A, mediante Inmunoprecipitación de RNA (RIP) en células HCT116.

3. MATERIA Y MÉTODOS.

Anticuerpos.

Se utilizaron los siguientes anticuerpos:

ANTICUERPOS PRIMARIOS	ESPECIE	MARCA	IF	CHIP	WB
ACA	anti-ratón	Antibodies Inc, Davi, CA, USA 15-235-F	1;200	X	X
Mis12	anti-cabra	Santa Cruz sc-107750	1;200	4µg	
H3K4me2	anti-conejo	Millipore 07-030	1;200	3µg	X
H3K9ac	anti-conejo	Abcam ab10812	1;200	2.4µg	1;250
H3K9me2	anti-ratón	Abcam ab1220	1;200	3µg	X
H3K9me3	anti-conejo	Abcam ab 8898	1;200	3µg	1;250
H3K9me3	anti-conejo	Diagenode CS-056-050	1;250	1.5-3µg	X
CENP-A	anti-ratón	Abcam ab13939	1;200	5µg	1;200
HP1α	anti-cabra	Abcam ab77256	1;100	4µg	1;300
HP1β	anti-rata	Abcam ab10811	1;100	4µg	1;200
Anti-GFP	anti-conejo	Abcam ab290	1;100	4µg	X
H3 N-Terminal	anti-conejo	Sigma H9289-200µl	X	1.5µg	1;300

ANTICUERPOS SECUNDARIOS	ESPECIE	MARCA	IF	WB
Alexa Fluor 488	anti-ratón	Invitrogen A11001	1;200	X
Alexa Fluor 488	anti-conejo	Invitrogen A11008	1;200	X
Alexa Fluor 488	anti-cabra	Invitrogen A11078	1;200	X
Cy3	anti-ratón	Millipore AP124C	1;200	X
Cy3	anti-conejo	Millipore AP1132C	1;200	X
Goat anti mouse HRP IgG	anti-ratón	Zynmed 31440	X	1:10000
Goat anti-rabbit IgG	anti-conejo	Santa Cruz Biotechnology, Inc sc-2301	X	1:15000
Chicken anti-goat	anti-cabra	Millipore AP163P	X	1:15000

Concentración inhibitoria 50 (IC_{50}) después del tratamiento con TSA

Las células humanas WI-38 (Fibroblastos de pulmón fetal) y HCT116 (Carcinoma colorectal) se obtuvieron de ATCC (ATCC CCL-75 y CCL-247), y se mantuvieron con medio Eagles's Minimum Essential (EMEM) (ATCC) y el medio McCoy (Gibco), respectivamente, se

suplementaron los medio con suero bovino fetal (Gibco) al 10% y se agregaron antibióticos, se incubaron las células a 37ºC en una atmosfera de 5% CO_2. Para obtener la IC50 las células se incubaron a las concentraciones de TSA (Sigma T8552-5MG) de 1, 3, 5, 8, 12 y 15 μM a 37ºC por 24 y 48 h en una atmosfera húmeda. Las líneas celulares fueron autentificadas para evitar asegurar que no hubiera contaminación con otras líneas celulares. Las concentraciones se determinaron cultivando 80 000 células incubadas toda la noche a 37ºC en cajas de 24 pozos que contenían 0.5 ml de medio. Después de la exposición a TSA las células se lavaron con PBS y se incubaron en medio fresco por 24h. Las células se fijaron con etanol al 70% a -20ºC, se lavaron con PBS y se tiñeron con 1% de cristal violeta. Después se lavaron y la tinción fue solubilizada con 33% de acido acético, donde se midió la absorbancia por medio de un lector de ELISA a 570nm. El análisis se hizo por triplicado en tres experimentos biológicos independientes. Los valores de IC_{50} se calcularon por medio de un análisis de regresión lineal de los datos dosis dependiente, utilizando los puntos en la región exponencial de la curva. La concentración usada para los experimentos con TSA fue inferior a la IC_{30} obtenida a partir de los valores de IC_{50} que fueron 4.9 μM para las células HCT116 y 9.4 μM para las células WI-38, con el fin de que observáramos el efecto en la remodelación de la cromatina y no la toxicidad del fármaco.

Inmunofluorescencias

Las células WI-38 y HCT116 se cultivaron en cubre objetos de 18mm (PEARL 7201) en medio EMEM y McCoy, respectivamente y fueron suplementadas con 10% de suero bovino fetal y antibióticos, estas fueron incubadas a 37ºC en una atmosfera de CO_2 al 5%. Se fijaron la células con paraformaldehido al 2% (PFA) en PBS 1X (pH 7.4) por 10 minutos seguidos por una permeabilización de 0.4% de IGEPAL (Sigma CA-630) en PBS por 10 minutos a temperatura

ambiente, después se incubaron con 0.5% de buffer de bloqueo BSA. Para cada par de anticuerpos primarios, se determino la concentración optima de uso en experimentos preliminares. Los fluoroforos de los anticuerpos secundarios fueron Alexa Fluor 488 (Invitrogen A11001 anti mouse, A11008 anti rabbit y A11078 anti goat) para la fluorescencia verde y Cy3 conjugado (Millipore AP124C anti mouse y AP1132C anti rabbit) par ala fluorescencia roja. Con la excepción de ACA (Antigene anti-centromero), el cual fue visualizado por que tenia un anticuerpo secundario conjugado de fluoreseina. El DNA fue teñido con DAPI. Las células se observaron por microscopia de fluorescencia utilizando un Zeiss Axio Imager.A2; las imágenes fueron analizadas utilizando el software AxioVision 4.8. Las células fueron también observadas por microscopia confocal laser Zeiss LSM 710 DUO; y las imágenes se analizaron con el software Zen 2008.

Tratamiento con TSA

A las células HCT116 y WI-38 que se encontraban en crecimiento exponencial sobre cubre objetos se les agrego medio que contenían 1μM/ml de TSA (Sigma T8552-5MG) durante 24 h y 48 h, realizando cambios de medio diarios. Se realizaron lavados de PBS, se fijaron a las células con PFA y estas se utilizaron para análisis de inmunofluorescencia como se describió anteriormente. Para el aislamiento de cromatina, las células fueron cultivadas en cajas de cultivo de 100mm y tratadas de la misma manera que las cultivadas en cubre objetos.

Electroforesis e Inmunoblot

Despues de tratamiento con TSA, las células WI-38 y HCT116 se cosecharon en buffer de lisis conteniendo 50mM Tris-HCL pH 7.5, 150mM NaCl, 1% Nonident P40, 0.5% deoxycolato y el coctel de inhibidores de proteasas completo (Roche) seguida por una breve sonicación. Después, se

cargaron 30 μg de proteína en un gradiente desnaturalizante 10-20% o un gel de SDS-poliacrilamida al 16% el cual fue subsecuentemente transferido a un papel de nitrocelulosa. Transcurridas 2 h de incubación en PBS que contenía albumina al 5%, los blots fueron expuestos a los siguientes anticuerpos primarios: anti-HP1α (1:300), anti-HP1β (1:200), H3 N-terminal (1:300), anti-H3K9me3 (1:250), anti-H3K9ac (1:250), and anti-CENP-A (1:200). Los blots fueron incubados 1 h a temperatura ambiente con anticuerpos conjugados con peroxidasa: goat anti-mouse IgG (1:10 000 Zymed 31440), goat anti-rabbit IgG (1:15 000 Santa Cruz Biotechnology, Inc. Sc-2301) y chicken anti-goat IgG (1:15 000 Millipore AP163P) subsecuentemente fueron revelados por quimioluminiscencia (ECL kit Millipore, USA) en placas Kodak X-Omat. Como control negativo el anticuerpo primario no estuvo presente durante el procedimiento.

Construcción y localización de proteínas HP1

Las proteínas HP1 verde fluorescentes fueron obtenidas de Addgene plasmido pBCHGN 17652 (HP1α-GFP), 17651 (HP1β-GFP) y 17650 (HP1γ-GFP). Se transfectaron células HeLa con cada tipo de plásmido HP1-GFP usando el protocolo Lipofectamina LTX y reactivo Plus (Invitrogen 15338-100) y se cultivaron en medio Opti-MEM (Gibco) a 37ºC con CO_2 por 24 h. La HeLa transfectadas fueron enriquecidas por medio de un sorteador de células BD FACSAria II y se cultivaron en cubre objetos para análisis de microscopia utilizando el Axio-Imager A2 o se cultivaron para realizar un análisis de inmunoprecipitación de la cromatina.

Inmunoprecipitación de la cromatina (ChIP)

El ChIP se realizó utilizando el OneDay ChIP kit (Diagenode Kch-onedIP-180) siguiendo las indicaciones del fabricante. Para todos los experimentos se realizaron al menos dos preparaciones de cromatina de los controles independientes y de las células tratadas con TSA. Para obtener la

cromatina de células mitóticas e interfásicas, las células control y tratadas con TSA se expusieron a 2 µg/ml de nocodazol por 12 h; las células mitóticas se aislaron por el método de shake-off; y se uso un sorteador de células fluorescentes activas (FACS) para seleccionar a la población de células que tenían un enriquecimiento de células mitóticas de 90%. Utilizando este método, las células interfasicas permanecían en la cajas de cultivo y fueron cosechadas separadamente de las células mitóticas. Cada cromatina fue fijada con 1% de formaldehido y se contaron las células para asegurar que se usaran $1x10^6$ células para cada IP. La cromatina fue extraída y el ChIP se realizó siguiendo las instrucciones del fabricante. Como control negativo se uso un anticuerpo normal de conejo IgG (sc-2027, Santa Cruz Biotechnology).

Los resultados obtenidos son representativos de los experimentos con triplicados independientes que fueron usados para calcular la desviación estándar. Para balancear las diferencias en las cantidades de ChIP y de Input en el qPCR, la eficiencia de amplificación (EA) se calculó dentro del 10% del input. El nivel de enriquecimiento fue calculado de la EA de los amplicones experimentales específicos contra la EA de la amplificación con el IgG , los cuales fueron amplificados por triplicado en un qPCR "fast optical 96 reaction plate" (Applied Biosystems). El qPCR se realizó usando un Thermo Maxima SYBR Green/ROX 1 PCR Master mix (Thermo Scientific K0222) en un equipo de tiempo real "Step One Plus" (Applied Biosystems 4376600).

Oligos para ChIP

Los oligos para ChIP que fueron utilizados para el análisis son lo siguientes: 5'-TCGTTCCCAAAGTCCTCCTGTTTC-3' (Fwd) y 5'-TCCGCAGCCGCCTGGTTC-3' (Rev) para el promotor de GAPDH; 5'-AGCCCTTCCCGCTCTTCTGTT-3' (Fwd) y 5'-CGGCAGAGACGTAAGACTGGCAAA-3' (Rev) para el promotor de WIF1; 5'-

ATCGAATGGAAATGAAAGGAGTCA-3’ (Fwd) y 5’-GACCATTGGATGATTGCAGTCA-3’ (Rev) para el satélite 2 de cromosoma 4 humano (ab85781); 5’-AAGGTCAATGGCAGAAAAGAA-3’ y 5’-CAACGAAGGCCACAAGATGTC-3’ (ab85782) para el satélite alfa de cromosoma 1 humano; and 5’-GAAGTTTCTGAGAATGCTTCTG-3’ (Fwd) and 5’-CTCACAGAGTTGAACCTTCC-3’ (Rev) para los monómeros de 175 pb del satélite alfa humano.

Preparación de cromosomas y conteo

La células WI-38 y HCT116 fueron cultivadas en cubre objetos de 22x22 mm, cuando tenían una confluencia de alrededor del 70%, las células fueron tratadas con TSA por 24 y 48 h. Las medios de cultivo fueron removidos y remplazados con medio fresco después de 24 h. Después, las células fueron tratadas por 3 h (para las HCT116) y por 24 h (para las WI-38) con 80ng/ml de colcemid (KaryoMAX GIBCO 15210-040), debido a que las células WI-38 les tomaba mas tiempo dividirse en comparación a las HCT116, sequido se incubaron por 30 minutos a 37ºC con solución hipotónica (75 mM KCl) que fue calentada a 37ºC previamente. Las células se fijaron por tres lavados de 2 minutos con una solución metanol/acido acético 3:1 y se secaron con aire a presión. Se realizo un protocolo estándar de bandeo G con una solución de tripsina-Giemsa para teñir los cromosomas mitóticos. Se contaron 50 metafases por duplicado para cada experimento por medio de un citogenetista certificado.

Extracción de RNA y RT-PCR

La extracción del RNA se hizo con Trizol y se cuantificaron bajo condiciones optimas, después se realizo un reverso-trascripción para obtener el cDNA utilizando el kit “GeneAmp RNA PCR core kit” (N808-0143 Applied Biosystems) de acuerdo a las instrucciones del fabricante. El cDNA

resultante fue utilizado como templado para las subsecuentes amplificaciones de qPCR usando los oligos específicos para el monomer de 175 pb del satélite-α (5'-GAAGTTTCTGAGAATGCTTCTG-3' (Fwd) y 5'-CTCACAGAGTTGAACCTTCC-3' (Rev)) y para el repetido del satélite 2 del cromosoma 1 humano (ab85781) (ATCGAATGGAAATGAAAGGAGTCA-3' (Fwd) y 5'- GACCATTGGATGATTGCAGTCA-3' (Rev).

Interacción de los trascritos centroméricos con la proteína HP1α por RIP

Se realizó el ensayo de inmunoprecipitación por RNA (RIP de sus siglas en ingles "RNA inmunoprecipitation") utilizando 10 millones de células HCT116 por inmunoprecipitación a las cuales se les extrajo la cromatina por el método de RIP publicado por Sun BK y Lee JT [105]. Se agregó formaldehído a una concentración final de 1% para llevar a cabo el entrecruzamiento de las interacciones de las proteínas y los ácidos nucleicos y posteriormente se detuvo la reacción con glicina 125 mM. El botón de células se lavó con PBS y se agregó cocktail inhibidor de proteasas (Cell Signaling) para evitar la degradación de las proteínas. Se lisaron las células resuspendiendo en Buffer A (5mM PIPES (pH 8.0), 85 mM KCl, 0.5% NP40, 1X Cocktail inhibidor de proteasas, inhibidor de RNAsa Superase.In (50U/mL) (Ambion), se centrifugó la fracción nuclear y se lavó con Buffer A sin NP-40, posteriormente se resuspendió en Buffer B (1% SDS, 10mM EDTA, 50mM Tris-HCl pH (8.1), inhibidor de proteasas e inhibidor de RNAsas). En este Buffer se sonicó la cromatina y se diluyó 10 veces en Buffer IP (0.01% SDS, 1.1% Tritón X-100, 1.2mM EDTA, 16.7mM Tris (pH 8.1), 167mM NaCl, inhibidor de proteasas e inhibidor de RNAsas) y se separó 1 mL por cada IP en tubos de 1.5 mL. A cada tubo se agregaron 4 µg de anticuerpo anti-HP1α (Abcam, Ab77256) u 8µg de anticuerpo anti-CENPA (Abcam,

Ab13939) y 1 µg de anti-IgG y se dejaron rotando durante 16 h para permitir la formación de los complejos inmunes. Transcurrido el tiempo, se recuperaron los complejos con 50 µl de perlas A/G y se lavaron cinco veces con el siguiente orden de buffers:

1: Lavado Low-salt: (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH 8.1), 150mM NaCl). 2: Lavado High-salt: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl (pH 8.1), 500mM NaCl). 3: Lavado LiCl: 0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl (pH 8.1)). 4: TE pH 8 (10 mM Tris-Cl pH 8, 1 mM EDTA) 5: TE pH 8.

Se eluyeron los complejos en Buffer de Elución (1% SDS, 0.1M $NaHCO_3$, Inhibidor de RNAsa (50 U/ml)) y se revirtió la reacción de entrecruzamiento con NaCl y se hizo tratamiento con proteinasa K. Se extrajo el RNA y se detectó mediante RT-qPCR utilizando primers específicos del satélite α.

Análisis Estadístico

La significancia estadística se determinó con la prueba "T de Student" o por un análisis de varianza de una vía (ANOVA). Todos los resultados se expresaron como la media ±SEM, y se uso un valor de significancia de $p<0.05$. Se realizó una " prueba de Levine" para comparar a significancia estadística del control contra el tratado de 24 y 48 h de TSA, o del tratamiento de 24 contra el de 48 h de TSA, con un valor de significancia de $p<0.05$ en los conteos de metafases. El análisis estadístico se realizó usando el software GraphPad Prism 5.

4. Resultados

Se utilizo la concentración de 1 μM de TSA por 24 y 48 h, debido a que a esta concentración encontramos una inducción significativa de IC, así como una remodelación de la cromatina centromérica, y cambios en la regulación de la transcripción de RNAnc provenientes del satélite-α y de satélite 2. Adicionalmente, dicha concentración esta por debajo del IC30 en la células utilizadas (IC50= 49 y 9.4 μM para HCT116 y WI-38 respectivamente).

El tratamiento con TSA indujo aneuploidia en ambas líneas celulares (**Figura 5A**). El 26% de las células WI-38 presentaron aneuploidia después de 24 h de tratamiento, y esta frecuencia permaneció después de 48 h de tratamiento. En el caso de las células HCT116, el 47% de la células presentaron aneuploidia después de 24 h de tratamiento, pero esta frecuencia disminuyó a 22% a las 48 h de exposición a TSA. La células WI-38 presentaron perdidas de mas de 6 cromosomas y ganancias de mas de 20 cromosomas (**Figura 5B**) , mientras que las células HCT116 mostraron niveles mas altos de aneuploidia después d e24 h de tratamiento, pero 24 h después, pocas pérdidas y ganancias de cromosomas fueron observadas (**Figura 5C, Tabla 1**). El 32% de las células WI-38 fueron tetraploides; después de 48 h de exposición, el 19.6% de la células permanecían tetraploides, indicando que las células no pudieron segregar adecuadamente (**Tabla 1**). Contrastando con las células HCT116, donde solo los 4% de las células se encontraban en 4n después de 24 h de tratamiento, y no se encontraron células tetraploides después 48 h de tratamiento, indicando que estas células no se arrestaban en mitosis (**Tabla 1**).

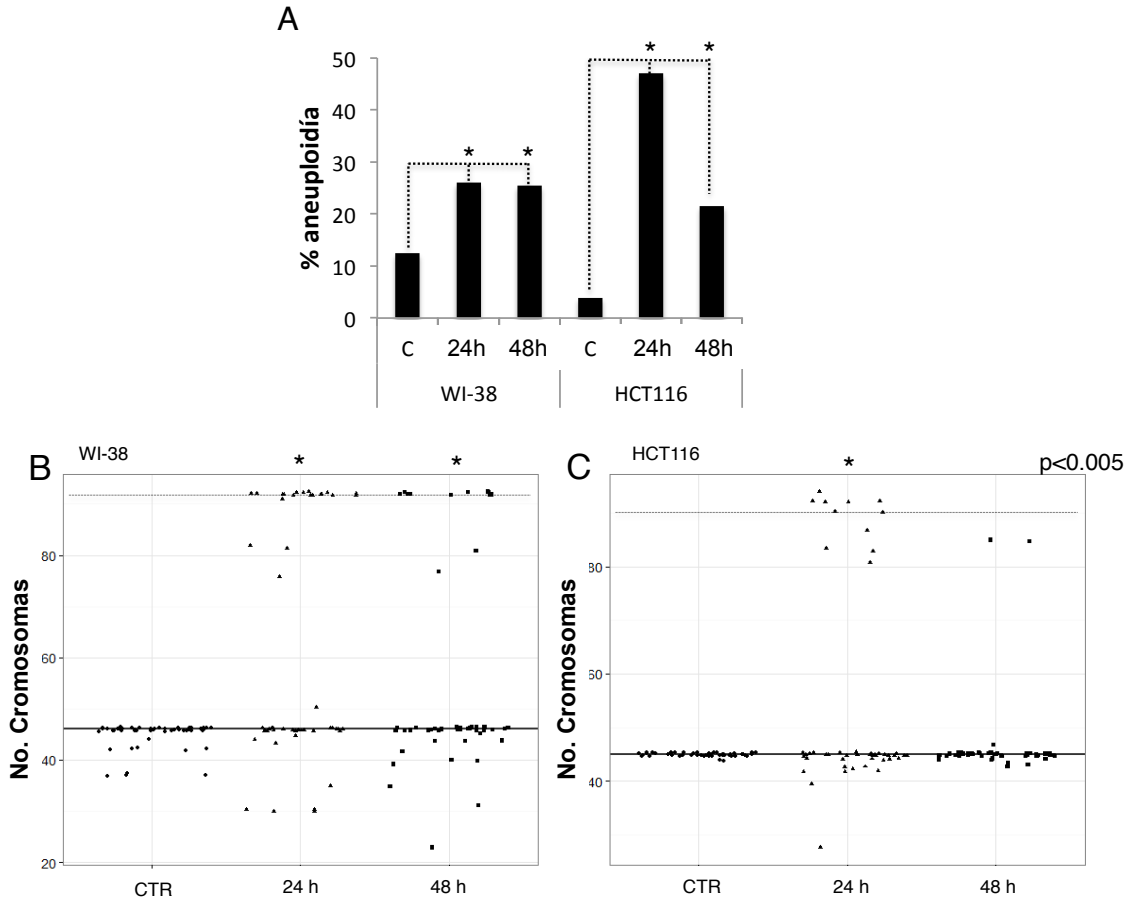

Figura 5. *El tratamiento con tricostatina (TSA) genera inestabilidad cromosómica principalmente en la línea celular HCT116. Las células fueron tratadas con 1μM de TSA por 24 y 48h, arrestadas en mitosis con colcemida (80ng/ml), y goteadas y teñidas mediante el protocolo de bandas G, después se contó el numero total de cromosomas en 50 células. (**A**) El porcentaje de aneuploidia fue superior a 26% después de 24 y 48 h de tratamiento con TSA en células W-38, y el efecto fue mas pronunciado en células HCT116 después de 24 h (47%), y disminuido a 21% después de 48 h de exposición. (**B-C**) Representación del conteo de numero de cromosomas de los controles y los tratamientos de 24 y 48 h de TSA en células WI-38 (**B**) y HCT116 (**C**), mostrando las ganancias y perdidas después del conteo; la línea blanca designa a las células 2n, y la línea punteada designa a las células 4n. La prueba Krustal-Wallis con una $p<0.05$ comparo los valores con el control (CTR).*

Tabla 1. Análisis del numero total de cromosomas presentados en cada célula, después del tratamiento con TSA por 24 y 48 h.

	WI-38			HCT116		
	Control	**24h**	**48h *,****	**Control**	**24h**	**48h ****
Moda	42 (87.5%)	21(42%)*,**	28 (54.9%)	49 (96.08%)	25 (54.9%)*,**	40 (78.43%)
Hipodiploidía	6 (12.5%)	8 (16%)*,**	11 (21.56%)	2 (3.92%)	14 (27.5%)*,**	7 (13.73%)
Hiperdiploidía	0	5 (10%)*,**	2 (3.92%)	0	10 (19.6%)*,**	4 (7.84%)
Tetraploidía	0	16 (32%)*,**	10 (19.6%)	0	2 (3.9%)*,**	0
TOTAL	**48**	**50**	**51**	**51**	**51**	**51**

La pérdida fue considerada debajo de 2n y la ganancia por arriba de 2n.
* Levene test $p < 0.001$ tratamiento *vs.* control.
** Levene test $p < 0.05$ 24h tratamiento *vs.* 48h tratamiento

4.1 Las proteínas HP1 se localizan en la cromatina centromérica en respuesta al tratamiento con TSA.

Con el fin de observar la localización de HP1α y HP1β a lo largo del ciclo celular y su asociación con H3K9me3 y CENP-A, se realizaron ensayos de inmunofluorescencia en células WI-38 (**Figura 6**) y HCT116 (**Figura 7**). En las células WI-38 se exploró la localización nuclear de H3K9me3 con CENP-A, las cuales estaban enriquecidas en el *locus* centromérico y las regiones adyacentes. Dicho enriquecimiento persistió en las células mitóticas (**Figura 6A**). Ya que H3K9me3 es una modificación epigenética que es reconocida por el dominio cromodominio de la proteína HP1, y debido a la importancia de las proteínas HP1 en el adecuado alineamiento de los cromosomas y la progresión mitótica [106, 107], evaluamos la localización nuclear de las isoformas HP1α y HP1β comparando con la de CENP-A. Observamos diferencias en la localización de las isoformas de HP1 en la región centromérica: donde HP1α co-localizó con CENP-A, y HP1β ocupo otras regiones de la cromatina (**Figura 6A**). Por lo tanto, a pesar de que

ambas isoformas juegan un papel critico en el establecimiento y mantenimiento de la heterocromatina, ambas proteínas parecen tener un papel diferente en términos de la cromatina centromérica.

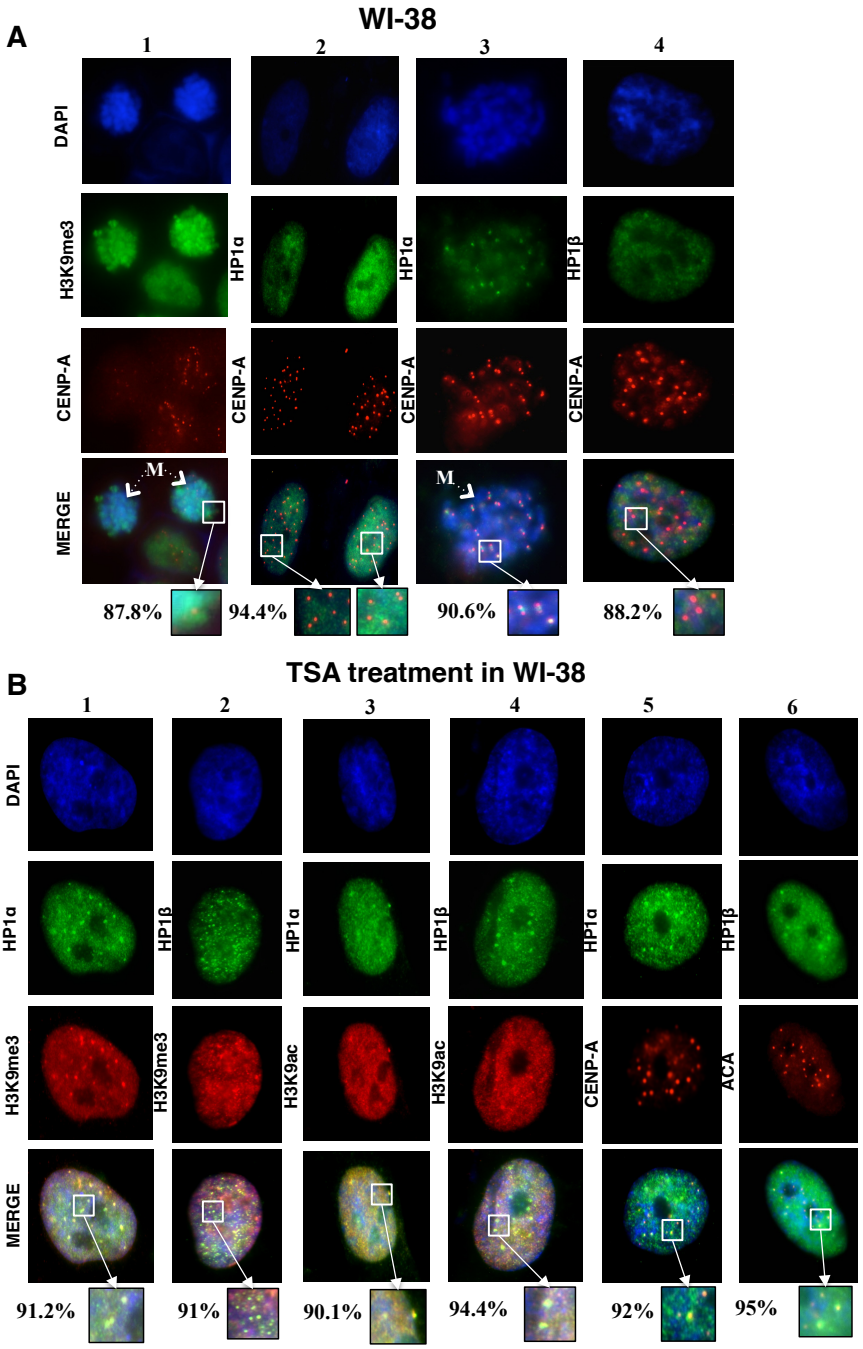

Figura 6. *Localización centromérica de HP1α y HP1β en células WI-38 bajo condiciones basales y después de*

tratamiento con TSA. A) Localización por microscopia de fluorescencia en células WI-38 de CENP-A con H3K9me3 (línea 1), HP1α (línea 2-3) y HP1β (línea 4). B) Localización de cromatina por microscopia de fluorescencia en células WI-38 tratadas con TSA comparando H3K9me3 con HP1α (línea 1) o HP1β (línea 2) H3K9ac con HP1α (línea 3) o HP1β (línea 4), localización centromérica de HP1α comparada con CENP-A (línea 5), y HP1β comparada con ACA (línea 6). El DNA esta marcado con DAPI; las imágenes mostradas representan la distribución de proteína más común después del análisis de 100 células (%); las cajas representan una magnificación de los resultados de inmunofluorescencia; M, células mitóticas.

Para observar el efecto de antagonizar la heterocromatina pericentromérica y centromérica, tratamos a las células WI-38 con 1 μM de TSA, lo que promueve una relajación y modulación de la expresión génica [107, 108]. Con el fin de evaluar el efecto en el ciclo celular, las células fueron tratadas por 24 y 48 h, reincorporando la droga con medio fresco cada 24 h. El efecto del tratamiento fue observado en la localización nuclear de las proteínas por medio de ensayos de microscopia de fluorescencia. La TSA redujo los niveles de H3K9me3 como era esperado, y también redijo los niveles de HP1α y HP1β. Observamos *foci* claros de H3K9me3 que permanecían después del tratamiento, y dichos *foci* co-localizaron con las isoformas HP1α y HP1β (**Figura 6B**). También observamos que los *foci* de las proteínas HP1ocupaban las mismas regiones que CENP-A, sugiriendo que ambas, H3K9me3 y HP1α/β, están presentes en la región centromérica definida por los anticuerpos de CENP-A y ACA. Estos resultados podrían sugerir que H3K9me3 esta siendo preservada en el centrómero y que ambas proteínas HP1 se acumulan en la cromatina centromérica en respuesta al tratamiento con TSA (**Figura 6B**).

Después determinamos si la dinámica y localización de H3K9me3, HP1α y HP1β, eran conservadas en una línea celular proveniente de cáncer la cual no presentara una previa IC, como es el caso de HCT116. Para este propósito, realizamos ensayos de microscopia de fluorescencia

para observar a la localización centromérica de las proteínas HP1. Durante interfase co-localizaron H3K9me3 con CENP-A. Mientras que en miosis, ya se que co-localizaban o que se encontraba un enriquecimiento en la región colindante con el centrómero (**Figura 7A**). Como era esperado, las isoformas de HP1 se localizaban similarmente en ambas líneas celulares, sugiriendo un comportamiento conservado en la cromatina para H3K9me3 y las proteínas HP1. Cuando se trataron con TSA, las células HCT116 también presentaron H3K9me3 en las regiones de CENP-A, y dichas regiones también estaban ocupadas por las isoformas de HP1 (**Figura 7B**).

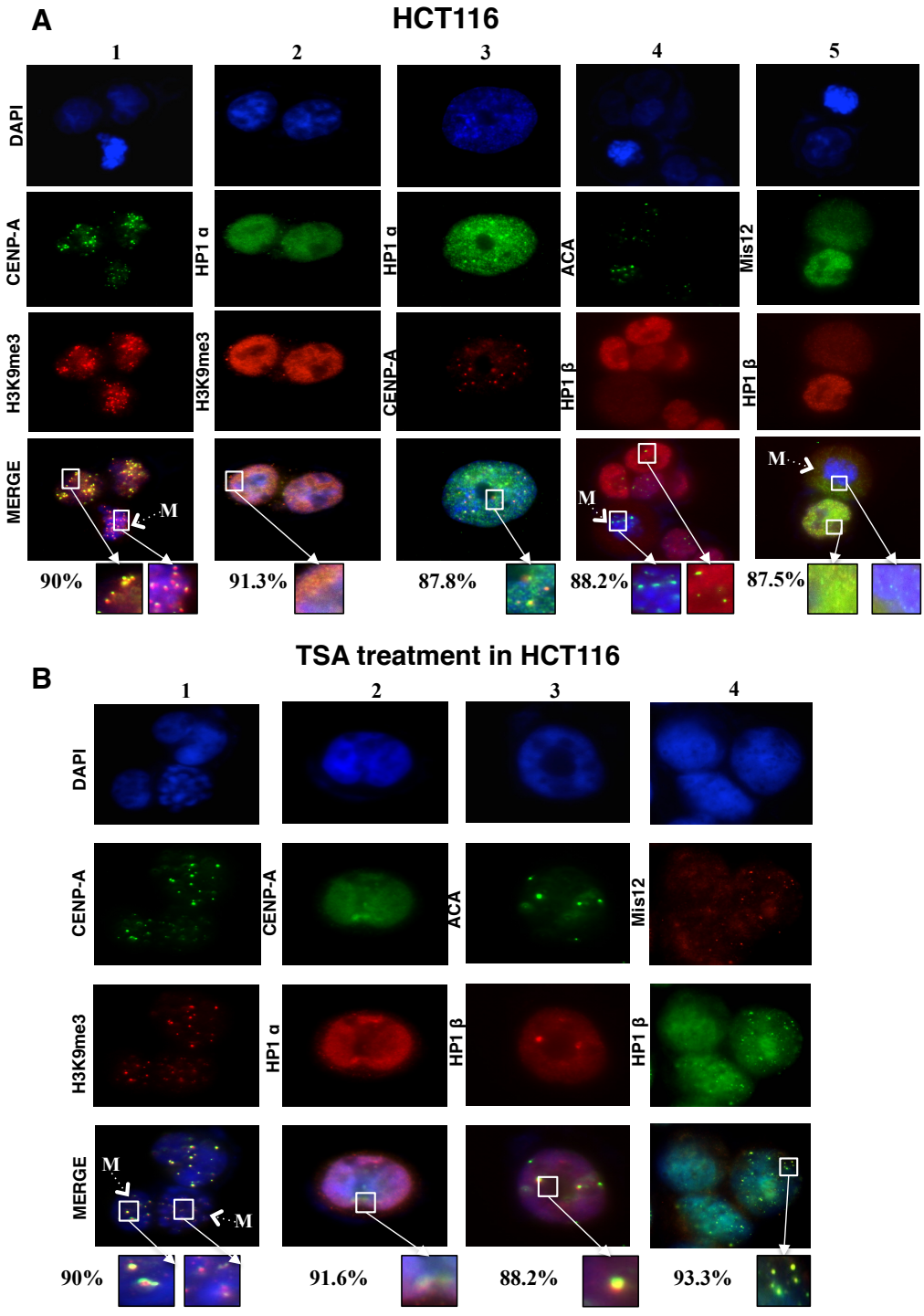

Figura 7. *Localización centromérica de HP1α y HP1β en células HCT116 bajo condiciones basales y después de tratamiento con TSA. A) Localización por microscopia fluorescente de cromatina en células HCT116 de CENP-A con H3K9me (línea 1) o HP1α (línea 2), H3K9me3 con HP1α (línea 3), ACA y HP1β (línea 4), y HP1β con Mis12 (línea 5).*

B) Localización centromérica de proteínas después de un tratamiento con TSA de H3K9me3 y CENP-A (línea 1), CENP-A y HP1α (línea 2), ACA y HP1β (línea 3) y co-localización de Mis12 con HP1β (línea 4). El DNA esta marcado con DAPI; las imágenes mostradas representan la distribución de proteína más común después del análisis de 100 células (%); las cajas representan una magnificación de los resultados de inmunofluorescencia; M, células mitóticas.

Nosotros no observamos una co-localización aparente en las marcas de eucromatina H3K9ac y H3K4me2 con CENP-A, lo cual indica que las marcas de cromatina abierta estas presentes en la regiones centroméricas en una baja frecuencia en ambas líneas celulares (**Figura 8A-B**). Considerando que HP1 ha sido asociado con el complejo Mis12, determinamos si la localización de Mis12 es afectada por TSA. De manera interesante, Mis12 mostró una fuerte correlación con la localización de HP1β y también se enriqueció en los foci de HP1β que eran promovidos por TSA, sugiriendo que esta proteína del cinetocoro esta asociada a HP1 no solo durante mitosis, si no también durante interfase (**Figura 7 A-B**).

Para determinar si los cambios en la localización de las proteínas HP1 eran relacionados con alteraciones en los niveles totales de proteína, realizamos ensayos de inmunoblot en ambos tipos celulares antes y después del tratamiento con 24 y 48 h de tratamiento con TSA. Observamos una reducción significativa de HP1α solo en células HCT116 después de 24 h; no observamos cambios significativos en células WI-38. HP1β disminuyo en las células WI-38 después de 24 h de exposición a TSA, pero los niveles original fueron restaurados después de 48 h. No observamos cambios en la abundancia de HP1β en las células HCT116 después de tratamientos con TSA (**Figura 9A**). No encontramos cambios significativos en la H3K9ac después del tratamiento con TSA, como era esperado. Sin embargo, si observamos una tendencia a la acumulación de

acetilación después de las 48 h de tratamiento en células WI-38. A pesar de lo anterior los niveles de H3K9me3 disminuyeron significativamente, y esto fue mas notoria después de las 48 h de tratamiento, sugiriendo que no solo la TSA disminuía esta marca de heterocromatina, si no también promovía acetilación que no afecto significativamente la acetilación del residuo H3K9 (**Figura 9A**). Una posible explicación es que la acetilación podría ocurrir con mayor frecuencia en otros residuos de lisina de H3 y H4. En suma, nuestros resultados sugieren que la reducción de H3K9me3 después del tratamiento con TSA es de mayor manera la razón por la que se dan cambios en la localización de HP1 y no por alteraciones en la traducción de las proteínas HP1.

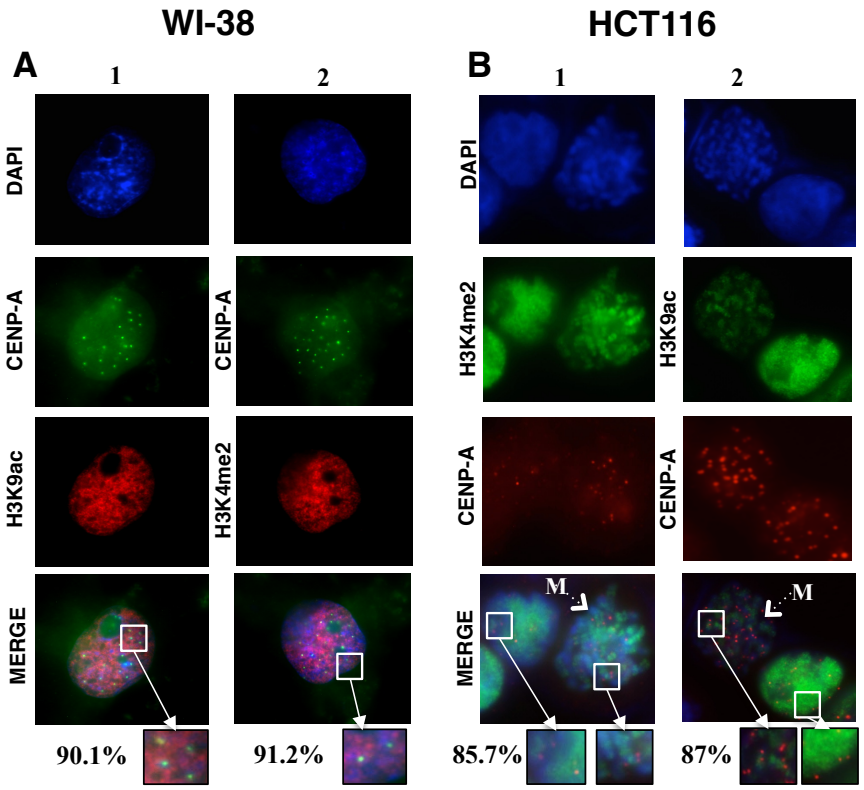

***Figura 8**. Localización de H3K4me3, H3K9ac y Mis12 en células WI-38 y HCT116 en condiciones basales. A) Localización de la cromatina por microscopia de fluorescencia en células WI-38 de CENP-A con H3K4me2 (línea 1) y H3K9ac (línea 2). B) Localización de la cromatina por microscopia de fluorescencia en células HCT116 de CENP-A con H3K4me2 (línea 1) y H3K9ac (línea 2). El DNA esta marcado con DAPI; las imágenes mostradas representan la*

distribución de proteína más común después del análisis de 100 células (%); las cajas representan una magnificación de los resultados de inmunofluorescencia; M, células mitóticas.

Con el fin de confirmar la localización de las proteínas HP1 realizamos un transfección con construcciones HP1α, β y γ-GFP. Observamos que la localización nuclear de cada isoforma, y como era esperado, HP1α y β se asociaron con la periferia nuclear y otras regiones de DNA teñidas con DAPI. Estas regiones están asociadas con territorios de heterocromatina y co-localizan DNA teñido con DAPI. En contraste, HP1γ se vio en una diferente localización y no estaba enriquecida en la periferia nuclear, mas bien, mostró una distribución de *foci* mas claros (**Figura 9B**). Nosotros observamos en células arrestadas en mitosis que HP1β-GFP esta asociada de mayor manera con la cromatina en los cromosomas en comparación a nuestras construcciones para otras isoformas (**Figura 9C**).

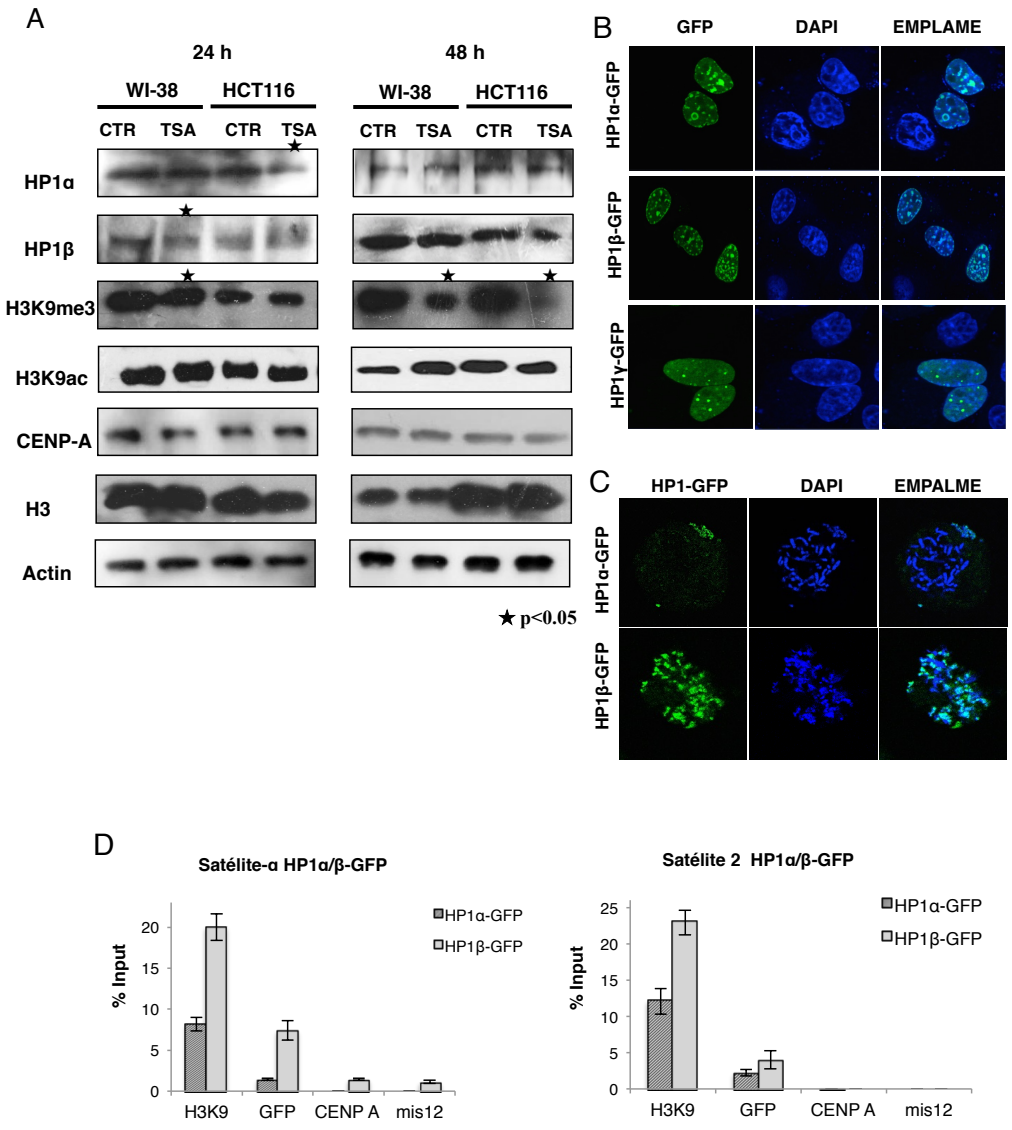

Figura 9. *A) Contenido de proteína en células HCT116 y WI-38 que fueron tratadas con TSA. Western blot representativo de los niveles de HP1α, HP1β, H3, H3K9me3, H3K9ac y CENP-A, después de tratamientos con 1 μM de TSA por 24 y 48 h. Los experimentos fueron realizados en tres determinaciones independientes que fueron realizadas por duplicado para cada condición experimental; el asterisco rojo representa una $p<0.05$ comparado con el valor del control (CTR) obtenido con la prueba de T de student. B) Localización de las proteínas HP1-GFP durante interfase y mitosis. Las células HeLa transfectadas con HP1α-GFP, HP1β-GFP y HP1γ-GFP co-localizan con el DNA teñido con DAPI en células interfasicas. C) localización mitótica de las proteínas HP1-GFP; a pesar de que las proteínas HP1 están presentes durante mitosis, HP1β-GFP esta enriquecida en los cromosomas mitóticos. D) inmunoprecipitación de la cromatina en celulas HeLa transfectadas con las proteínas HP1-GFP con anticuerpos*

específicos anti-GFP, H3K9me3, CENP-A y Mis12, realizados para determinar su unión con los repetidos de satélite-α (izquierda) y satélite 2 (derecha).

Para confirmar que la localización de las proteínas HP1 observadas mediante inmunofluorescencia esta presente en la cromatina centromérica, evaluamos el enriquecimiento de estas proteínas por el ensayo de ChIP después de la transfección con HP1α-GFP y HP1β-GFP, en los repetidos de satélite-α de las regiones centroméricas y los repetidos del satélite 2 de la región pericentromérica en células HeLa transfectadas. Para esto inmunoprecipitamos a H3K9me3, CENP-A, Mis12 y a GFP de las construcciones quiméricas de HP1. Donde observamos que HP1α-GFP y HP1β-GFP se asociaron con las secuencias de las regiones de satélite-α y satélite 2; de manera sorprendente, la transfección con HP1β-GFP incremento la abundancia de H3K9me3 en ambas secuencias satelitales (**Figura 9D**). Sin embargo, no pudimos evaluar por análisis de ChIP a las células mitóticas transfectadas, debido a que la sobre expresión de HP1α/β-GFP arresto a las células en G2/M, sugiriendo que el incremento de H3K9me3 podría inhibir la presencia de CENP-A en los repetidos de satélite-α.

4.2 Dinámica de la cromatina centromérica durante ciclo celular

Para determinar la presencia de HP1 en la cromatina centromérica y su dinámica durante ciclo celular, seleccionamos células HCT116 con un estable segregación cromosómica. Tratamos a esta células con nocodazol por 12 h y aislamos las células mitóticas por shake-off. Después realizamos un ensayo de ChIP para marcas de activación (H3K4me2 y H3K9ac), marcas represivas (H3K9me2/me3), CENP-A, Mis12, HP1α y HP1β en células mitóticas e interfásicas. Como controles para cromatina abierta y represiva, evaluamos las regiones promotoras de lo genes

GAPDH y WIF1. Como era esperado, el promotor de GAPDH se encontró enriquecido con marcas asociadas a la activación génica H3K4me2 y H3K9ac; encontramos que la abundancia de estas marcas se incrementó durante mitosis, pero este incremento era proporcional a la ganancia de H3 total en la región (**Figura 10A**). Usamos el promotor génico del Gen WIF1 como control positivo para silenciamiento génico debido a su rolo como inhibidor de WNT, el cual encontramos enriquecido de H3K9me2 y H3K9me3 después de la diferenciación. Encontramos que dicha región está enriquecida con marcas represivas, y que hay un incremento de las mismas durante mitosis (**Figura 10B**).

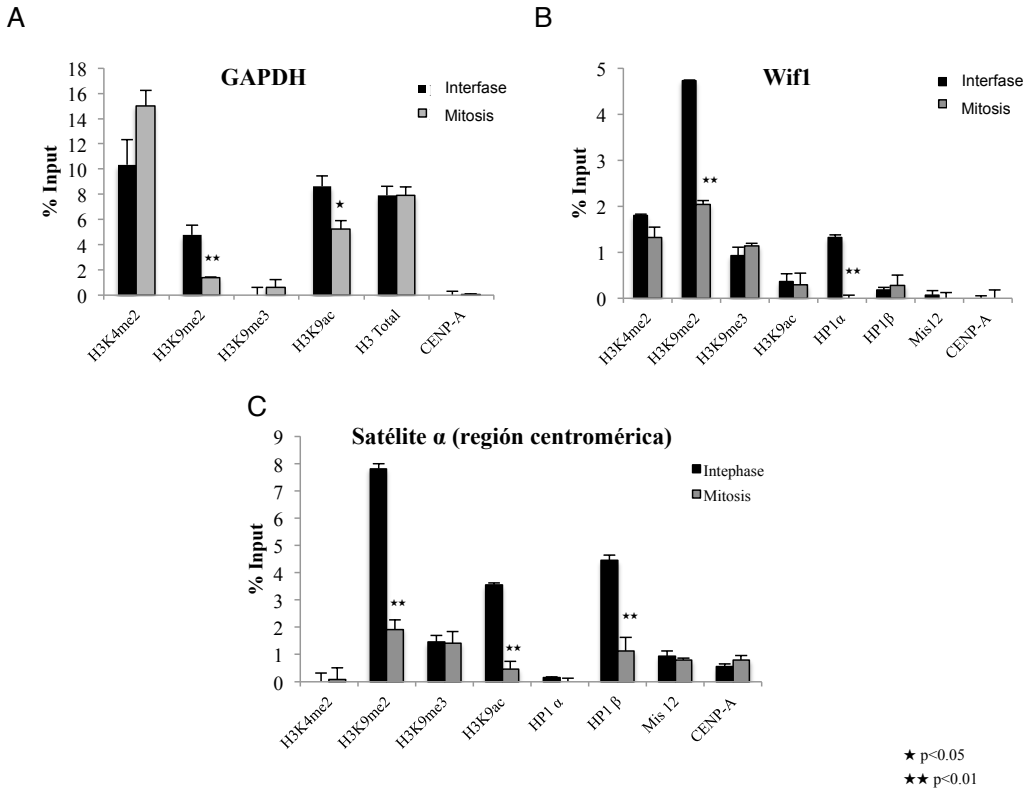

Figura 10*. Controles del ensayo de inmunoprecipitación de la cromatina para marcas epigenéticas activa y represivas durante interfase (negro) y mitosis (gris). A) Se analizó al promotor del gen GAPDH para marcas de estado abierto de cromatina. B) Se analizó al promotor del gen WIF1 para marcas represivas. C) Se observó el panorama epigenético sobre la secuencia consenso de repetido del satélite α durante ciclo celular. Se observan los cambios en el enriquecimiento de H3K4me2, H3K9me2/3, H3K9ac, HP1α, HP1β, Mis12 y CENP-A. ∗ p<0.05, ∗∗*

p<0.01 indican las diferencias significativas entre interfase y mitosis, evaluadas por la prueba t-Student.

Debido a que la cromatina centromérica ha sido pobremente estudiada por análisis de ChIP y que hay resultados contrastantes en diferentes modelos, diseñamos oligos para el repetido global de satélite-α y analizamos la secuencias del monómero de 171 pb. Nuestros resultados muestran que H3K9me2 y HP1β están presentes durante interfase y mitosis, mientras que las marcas de histonas H3K4me2, H3K9ac y H3K9me3 fluctúan a lo largo del ciclo celular (**Figura 10C**).

Sin embargo, los arreglos de repetidos de satélite-α varían en los centrómeros de los cromosomas y los nucleosomas CENP-A/H3 están dispersos en la secuencia centromérica, por lo tanto diseñamos oligos que son mas específicos para la región de satélite-α y satélite 2 de un cromosoma y así acotar el análisis de cambios de la cromatina en dichas regiones. Evaluamos el satélite 2 de la región pericentromérica del cromosoma 1, la cual esta enriquecida con H3K9me2 y H3K9me3 durante interfase; la proteína HP1β también esta presente durante esta fase (**Figura 11A-B**) debido a que el satélite 2 es una región de heterocromatina constitutiva ya bien conocida. De manera interesante, observamos un incremento de 2 magnitudes de H3K4me2 y una reducción del 50% de H3K9ac en células mitóticas. Nosotros no observamos la presencia de Mis12 y CENP-A en el satélite 2. Nos preguntamos si esta misma modulación ocurre en células no tumorales como las células WI-38. Por lo tanto, exploramos las mismas regiones del satélite 2 durante interfase y mitosis y encontramos que en células en interfase, H3K9me3 es abundante junto con HP1α, lo cual es típico de dominios de heterocromatina constitutiva pericentromérica (**Figura 11C**). Durante mitosis se redujo H3K9me3, pero HP1α se enriqueció fuertemente, sugiriendo la importancia de HP1α en la heterocromatina pericentromérica durante la segregación cromosómica (**Figura 11C**). Observamos un incremente inesperado de CENP-A en la cromatina del satélite 2 durante mitosis

(**Figura 11C**).

Sin embargo, en la región del satélite α del cromosoma 1 encontramos un panorama epigenético mas heterogéneo, en el cual las marcas de histonas represivas y activas coexisten a lo largo del ciclo celular (**Figura 11D-E**). Observamos un incremento significativo de CENP-A durante mitosis, mientras el enriquecimiento de otras modificaciones H3 fue reducido (**Figura 11D**). La región del satélite α en células WI-38 también estaba enriquecida con CENP-A y HP1α. A pesar de que H3K9me3 fue reducida durante mitosis , esta continuo presente en el centrómero (**Figura 11F**), lo cual sugiere que HP1α tiene un rol diferente en el centrómero en esta línea celular comparado con la las células HCT116. En contraste con los resultados de inmunofluorescencia, detectamos HP1α y HP1β en la región especifica del satélite α, y su presencia fluctúa ligeramente durante mitosis (**Figura 11E-F**).

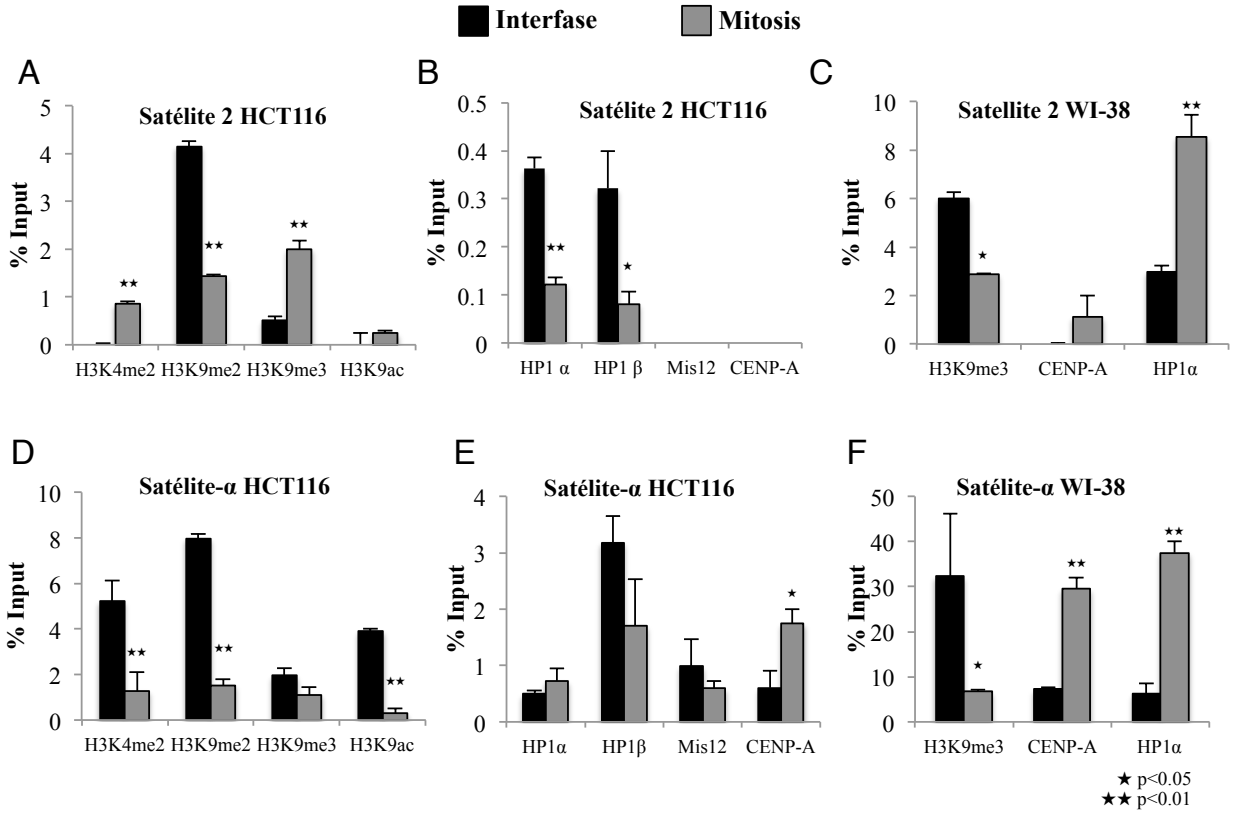

Figura 11. *Cambios epigenéticos y dinámica de proteínas en las regiones de los satélite 2 y satélite α durante interfase y mitosis en células HCT116 y WI-38. Se realizó un análisis de qPCR de los repetidos de satélite 2 (A-C) y satélite α (D-F) a partir del DNA obtenido del ensayo de ChIP en interfase (negro) y mitosis (gris) de las proteínas H3K9me2, H3K9me3, H3K9ac, HP1α, HP1β, Mis12 y CENP-A. Se empleo el anticuerpo IgG de conejo como control negativo. ⋆p<0.05, ⋆⋆p<0.01 indican las diferencias significativas entre interfase y mitosis, evaluadas por la prueba t-Student.*

4.3 El tratamiento con TSA promueve que la proteínas HP1 se re-localicen a la cromatina centromérica en HCT116 pero no en WI-38.

El tratamiento con TSA provoca cambia la localización nuclear de las proteínas HP1 [107, 108]; por lo tanto nosotros realizamos un ensayo de ChIP después de la exposición con HCT116. Encontramos que la TSA disminuye la abundancia de H3K9me3 y H3K4me2 en las regiones de satélite 2 después de las primeras 24 h de tratamiento. Dicha reducción la encontramos asociada con la perdida de HP1α y HP1β después de 24 h de exposición (**Figura 12A-B**). De manera sorprendente, HP1α y HP1β fueron restablecidas en la cromatina de satélite 2 después de 48 h de exposición de TSA, incluso después de que la H3K9me3 fuera dramáticamente disminuida por el tratamiento (**Figura 12A-B**). En las regiones de satélite-α al igual que en las de satélite, se presento una perdida de H3K4me2 y H3k9ac y H3K9me3 después de las primeras 24 h de tratamiento con TSA, pero después de 48 , H3K4me2 y H3K9ac fueron restablecidas en el centrómero aunque con menor abundancia (**Figura 12D**). Después de 24 h de tratamiento, CENP-A fue incrementada en 10 magnitudes en la cromatina de satélite-α y permanecía enriquecida 10 veces después de 48 h de TSA (**Figura 12E**). Como observamos en los experimentos de inmunofluorescencia, HP1α y HP1β fueron enriquecidas 6 y 4 veces respectivamente, y dicho aumento den abundancia fue proporcional al incremento de Mis12 en la cromatina del satélite-α, sugiriendo que la presencia de

Mis12 esta proporcionalmente asociada con la abundancia de HP1 en el centrómero (**Figura 12E**). Utilizamos el promotor del de GAPDH como control para evaluar los efectos de tratamiento con TSA; aunque no vimos un incremento en la H3K9ac, si observamos una perdida de H3K9me3 como resultado del tratamiento, sugiriendo que H3K9ac no es esencial para regulación de GAPDH (resultados no mostrados).

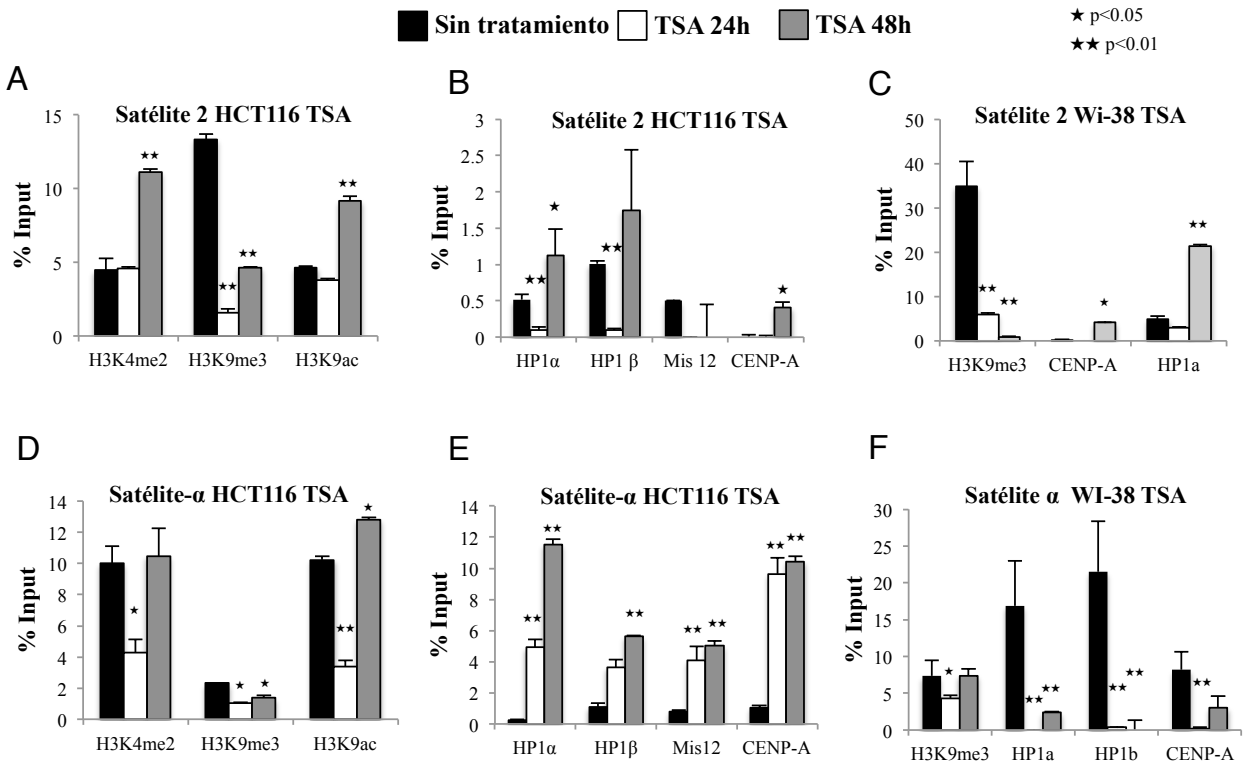

Figura 12. *Cambios epigenéticos y dinámica de proteínas en las regiones del satélite 2 y satélite α después del tratamiento con TSA por 24 y 48 h en células HCT116 y WI-38. Se analizo mediante qPCR los repetidos del satélite 2 (A-C) y satélite (D-F) a partir del DNA obtenido del ChIP con los anticuerpos H3K4me2, H3K9me3, H3K9ac, HP1α, HP1β, Mis12 y CENP-A, en células sin tratamiento (negro) y tratadas con TSA por 24 (blanco) y 48 h (gris). Se empleo IgG de conejo como control negativo. ∗p<0.05, ∗∗p<0.01 indican las diferencias significativas entre las células sin tratamiento, y aquellas tratadas con TSA por 24 y 48 h, evaluadas por la prueba t-Student.*

Ya que en las células HCT116 se encontraron cambios en la marcas de histonas y en los niveles de

las proteínas HP1 después de tratamientos con TSA, nos preguntamos si la esta modulación ocurriría también en células normales. Por lo tanto, tratamos a las células WI-38 con TSA por 24 y 48 h y realizamos ensayos de ChIP precipitando con anticuerpos para H3K9me3, CENP-A y HP1. Exploramos las mismas regiones de satélite 2 durante ciclo celular y encontramos que células interfásicas, H3K9me3 es abundante junto con HP1α, lo cual es típico de un dominio de heterocromatina constitutiva (**Figura 9C**). Durante mitosis H3K9me3 es reducida, pero HP1α es incrementada, sugiriendo un papel de HP1α en la heterocromatina pericentromérica durante la segregación cromosómica (**Figura 9C**). El satélite-α de la células WI-38 también esta enriquecido con CENP-A y HP1α; a pesar de que H3K9me3 se encuentra reducida durante mitosis, esta permanece en el centrómero (**Figura 9F**), sugiriendo que en esta línea celular, HP1α juega un papel importante en el centrómero que no es observado en HCT116.

El tratamiento de 24 h con TSA redujo H3K9me3 en las regiones de satélite 2 y casi abolió esta marca de histona a las 48 . Por lo tanto, HP1α es reducida ya que su marca lectora es disminuida (**Figura 12C**). En contraste con las observaciones hechas en HCT116, después de 24 h de exposición a TSA en las células WI-38 HP1α y HP1β son reducidas en el satélite-α y no fueron restablecidas después de 48 h, a pesar de que los niveles de H3K9me3 fueron restablecidos (**Figura 12E**). Estos resultados sugieren que la co-localización observadas por inmunofluorescencia son localizados en regiones especificas de la cromatina centromérica y pericentromérica que no fueron observados en la región centromérica del cromosoma 1.

4.4 Interacción de los transcritos centroméricos satélite α con HP1α, e inducción de sobre expresión de los RNAnc satélite-α y satélite 2 por TSA.

Se realizó un ensayo de inmunoprecipitación de RNA (RIP), con el fin de determinar si hay existe asociación entre HP1α y los transcritos centroméricos. Se inmunoprecipitaron las proteínas HP1α y a CENPA, este ultimo sirvió de control positivo ya que la interacción el RNAnc satélite α ha sido reportada recientemente [109]. Por otra parte, se utilizó el anticuerpo inespecífico IgG como control interno. Analizamos mediante RT-qPCR y encontramos un enriquecimiento de tres veces del control positivo CENP-A y de 0.4 veces de la proteína HP1α. Este resultado sugiere una asociación entre HP1α y los transcritos de satélite α (**Figura 13**).

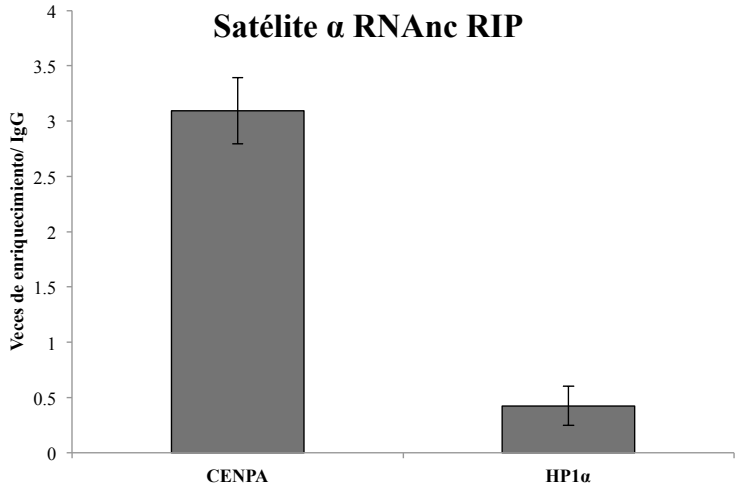

Figura 13. *Inmunoprecipitación de las proteínas CENPA y HP1α asociadas al RNAnc satélite α tras inmunoprecipitación (RNA-IP) en la fracción nuclear. Las barras muestran el error estándar del triplicado técnico. Se normalizó con el valor del IP con el anticuerpo inespecífico IgG.*

Extrajimos RNA de las células WI-38 y HCT116 sin tratamiento y con tratamiento con TSA por 24 y 48 h y realizamos una PCR de reverso transcripción para detectar los niveles de expresión del los RNAnc provenientes del satélite-α y satélite 2. Ambos repetidos transcribieron a moléculas de

RNAnc. La exposición a TSA indujo una sobre expresión de satélite-α después de 24 y 48 h en ambas células, HCT116 y WI-38. La sobre-expresión tendió a ser incrementada significativamente después de 48 h de exposición comparada con la observada a las 24 h de TSA (**Figura 14**). Encontramos que el satélite 2 también se sobre expresó significativamente después de los tratamientos con TSA y dicha sobre expresión incremento aun mas después de 48 h de exposición en ambas líneas celulares (**Figura 14**).

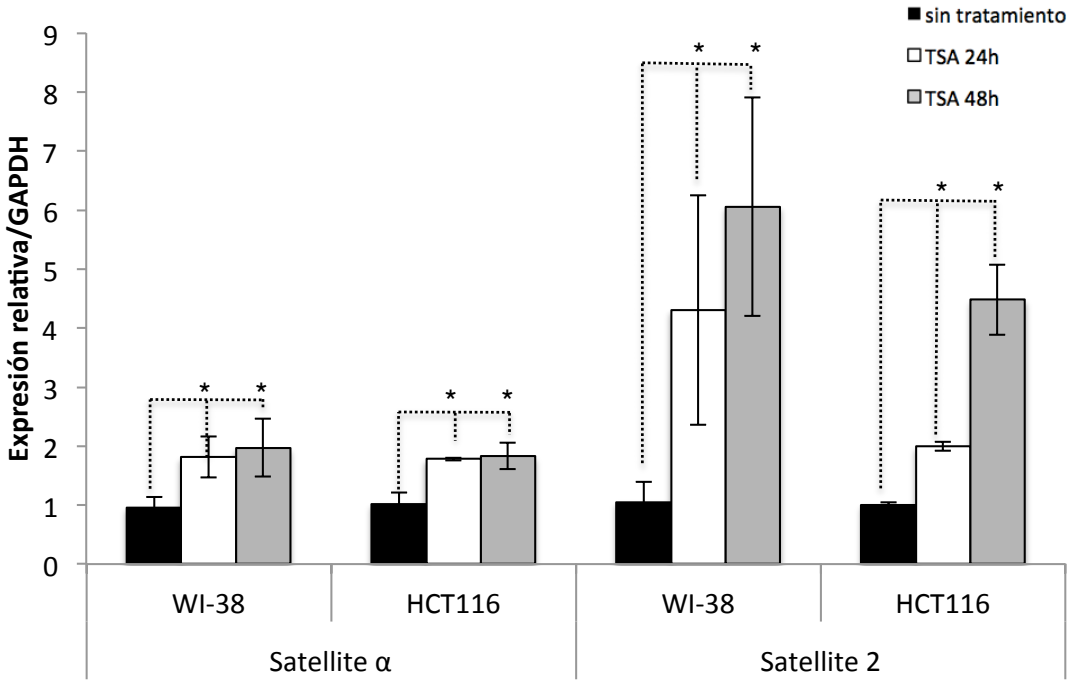

Figura 14. *RNAnc proveniente de los repetidos de satélite-α y satélite 2 se sobre-expresan con tratamientos con TSA. El cDNA fue transcrito de células control (negro) y células tratadas con 1 μM de TSA por 24 y 48 h, donde el RNAnc trascrito a partir del satélite-α y satélite 2 se sobre expreso en células WI-38 y HCT116 después del tratamiento.* ∗$p<0.05$*, indica las diferencias significativas entre las células sin tratamiento, y aquellas tratadas con TSA por 24 y 48 h, evaluadas por la prueba t-Student.*

5. Discusión

5.1 Las proteínas HP1α y HP1β se localizan en las regiones centroméricas después de exposición a TSA en las células HCT116 mientras son reducidas en WI-38.

Nuestros resultados proveen un acercamiento adicional al panorama epigenético de la cromatina centromérica y del papel de l HP1α y HP1β en la segregación cromosómica. También presentamos evidencias de las diferencias de organización de la cromatina centromérica y la localización de HP1 en respuesta a tratamientos con TSA en células normales y cancerosas. Estas diferencias se asociaron a IC, posiblemente resultante de modificaciones en la cromatina causadas por hiperacetilación de histonas, o perdidas de H3K9me, y H4K20me. En diferentes modelos de células y modelos animales se han atribuido fenotipos mitóticos aberrantes por perdidas pericentromérica de H3K9me3, cambios de H4K20me y una regulación anormal de HDAC; donde la IC ha sido observada en todos los estadios de la mitosis como un incremento de cromosomas desalineados en metafase, no disyunción en anafase, cromosomas rezagados en telofase, altas tasas de aneuploidia y aparición de micro núcleos en la citocinesis o en G1 temprana [75, 76, 110].

La proteína HP1 se une a la H3 que ha sido metilada en el residuo de lisina 9 por la enzima SUV39H1 [55], donde la función de HP1 es importante en el establecimiento, propagación y mantenimiento de la heterocromatina constitutiva [58], especialmente den la región pericentromérica, la cual esta enriquecida en H3K9me3 y H4K20me3, hipoacetilación de H3 y H4, y una alta tasa de metilación en las regiones de repetidos de satélites [56, 60].

Observamos que HP1α y HP1β junto con H3K9me3 esta localizados en la regiones aledañas al centrómero y que dicha localización de HP1 esta preservada durante mitosis, a pesar de que HP1α

y HP1β fueron disociadas de otras regiones cromosómicas. Estos resultados son consistentes con reportes previos en donde las proteínas HP1 se disocian a gran escala en células en G2 [111]. De manera interesante, concomitante con la disociación en heterocromatina, HP1α co-localiza con proteínas pasajeras durante G2, y la inhibición o eliminación de Aurora-B/AIM provoco una retención de HP1 en la cromatina centromérica durante mitosis [70, 112]. La reducción en la inmunolocalización de HP1 durante mitosis también podría ser atribuida con la presencia de histonas acetiladas en los cromosomas mitóticos, lo cual disminuye la accesibilidad del anticuerpo a las regiones N-terminal de las histonas, como fue observado para la fosforilación de la serina 10 de H3 [74]. Aunque estos resultados están a favor de otra hipótesis, donde las proteínas HP1 poseen una función en la cromatina centromérica durante el ciclo celular.

El tratamiento con TSA promueve una híperacetilación de histonas que es visible en la periferia nuclear y una reducción de muchas regiones de heterocromatina en el núcleo [107, 113]. De manera notable, las regiones de heterocromatina constitutiva, definidas por un retardo en el tiempo de replicación y un enriquecimiento de HP1, son excluidas de los dominios de híper acetilación [107]. Debido a dicha exclusión por acetilación, evaluamos si el impacto de un tratamiento de corto plazo con TSA modifica las regiones centromérica y pericentromérica provocando un incremento de las proteínas HP1. Encontramos que tanto HP1α y HP1β son enriquecidas en *foci* que co-localizan con H3K9me3 y CENP-A, sugiriendo que ambas proteínas HP1 no solo permanecen en la heterocromatina pericentromérica, si no también son enriquecidas en la heterocromatina constitutiva y se expanden a la cromatina centromérica en respuesta a un tratamiento con TSA. De manera interesante, también observamos que en las células HCT116 se da una reducción de H3K9me3 y HP1 de manera general en el núcleo después de un tratamiento con TSA, pero durante interfase aparecen claros *foci* enriquecidos con HP1 los cuales co-localizan

con CENP-A y H3K9me3, y dicha co-localización continua durante mitosis. Estos resultados son contradictorios con los que han sido reportados recientemente en células murinas, donde las proteínas HP1 son reducidas de la cromatina centromérica y son dispersadas en el núcleo después de un tratamiento de baja dosis de TSA [114]. Con respecto a ésto, se ha observado que a partir de un inhibición de la acetilación de la heterocromatina, HP1 se dispersa reversiblemente en el núcleo, y se ha reportado que tratamiento con 5-aza-2-deoxicitidina e inhibidores de HDACs en células primarias humanas provocan un reclutamiento dinámico de las proteínas HP1 la cromatina centromérica, también sugiriendo que la movilización de HP1 después del tratamiento podría promover una protección de la estructura y función del cinetocoro y que las proteínas HP1 se comportan de manera diferente en células humanas y de ratones a pesar de su conservación [106, 114, 115].

Después determinamos si la transfección con construcciones de proteínas HP1-GFP pudiera causar también una localización en la cromatina centromérica. De manera notable, HP1β-GFP se enriqueció de mayor manera en los cromosomas mitóticos en comparación a la HP1α-GFP. La transfección de proteínas HP1-GFP genero un arresto de ciclo celular, aunque las células se liberaron del dicho arresto, sugiriendo que HP1 afecta la transición G2/M por un mecanismo desconocido. A la fecha, poco se sabe de los efectos de HP1 en mitosis; aunque en reportes previos se ha observado una reducción de HP1 causada por defectos promovidos por TSA durante mitosis [74, 107]. Para corroborar estos resultados, realizamos un ensayo de ChIP y analizamos las secuencias repetidas de satélite-α y satélite 2 con el fin de observar la cromatina centromérica y pericentromérica. Nuestros resultados muestran que la transfección de HP1β-GFP incrementan la H3K9me3 en ambas secuencias satélite, resultando en una reducción significativa de CENP-A y Mis12 hacia el centrómero. Con respecto a esto ultimo, se ha reportado que los nucleosomas H3

remplazan a los sitios de ocupación de CENP-A cuando esta proteína se disminuye, pero cuando es sobre expresada, CENP-A puede remplazar los sitios de H3 [15, 30]. Sin embargo, aun permanece desconocido si los cambios en la cromatina promueven un a disociación de CENP-A o si la remoción de CENP-A permite que la heterocromatina remplace la cromatina centormérica. En suma, nuestros resultados sugieren que un incremento en la presencia de HP1 y las modificaciones de marcas de histonas en la heterocromatina reducen la unión de CENP-A, posiblemente afectando la capacidad de centrómero a generar el cinetocoro.

5.2 La TSA induce cambios diferenciales en la cromatina centromérica y pericentromérica así como en la inducción de IC en células HCT116 y WI-38.

Los centrómeros contienen nucleosomas CENP-A entremezclados con nucleosomas que contienen H3K9me2 y H3K9me3 y que exhiben un enriquecimiento bajo en los niveles H3K4me2 [21, 23]. Nosotros encontramos que en células HCT116, la H3K4me2 esta presente de manera constante en la cromatina centromérica durante interfase y mitosis. Ha sido reportado que H3K4me2 es una marca esencial para el ambiento de la cromatina en el cinetocoro de vertebrados y que es requerida para el mantenimiento y función del mismo a largo plazo [25]. Las marcas de H3Kme2/3 y H3K9ac fluctúan a lo largo del ciclo celular. Este resultado es interesante, ya que la H3K9me3 se ha reportado incrementado importantemente durante G2/M en mamíferos, mientras la abundancia de H3K9me2 permanece constante durante el ciclo celular [75]; Nuestros resultados concuerdan con un incremento en H3K9me3 en la cromatina del satélite 2, en de regiones de satélite-α no cromosoma específico, sin embargo, no vimos un incremento significativo en la región del satélite-α proveniente del cromosoma 1 durante mitosis. De manera interesante, encontramos una relación inversa en las células WI-38. Donde la H3K9me3 se redujo en el satélite 2 durante mitosis.

Sorprendentemente, dichos resultados sugieren que la abundancia de H3K9me3 en las regiones de satélite 2 durante mitosis no es igual en todas las células, por lo menos no es así entre HCT116 y WI-38.

A su vez, observamos que la abundancia de HP1α y HP1β cambia a lo largo del ciclo celular; una posible explicación, es que las fluctuaciones puedan deberse a un intercambio entre HP1α y HP1β durante mitosis. Esto específicamente durante metafase, donde HP1β es remplazada con HP1α. Dichos intercambios podrían ser mediados por diferencias en la secuencia del dominio cromosombra de HP1 [68]. Aunque no observamos un incremento de HP1α en las células mitóticas de HCT116, si observamos un incremento de HP1α en la cromatina centromérica de WI-38. De manera interesante, cuando usamos aproximaciones globales las diferencias entre las células no son aparentes, pero analizando localmente las diferencias son expuestas. Entonces, los intercambios dependen del tipo de célula y del contexto celular.

Existe evidencia creciente de que la red KMN (KL1, Mis12 y el complejo NDC80) en humanos es un compañero de unión con HP1, donde HP1 puede participar en el reclutamiento y direccionamiento del complejo Mis12 hacia el centrómero durante interfase, por una directa interacción con Mis14 [64–66]. En las células HCT116, observamos que durante interfase, HP1α y HP1β están presentes junto con Mis12 en la cromatina centromérica, concordando con reportes previos. En contraste, durante mitosis, Mis12 no se encuentra enriquecida en los mismos sitios, aunque HP1α y HP1β son también reducidas. Una posible explicación podría ser los dominios de interacción de HP1 y Mis12; ya que HP1α y NDC80 son competidores en la unión con Mis12, sugiriendo que estas proteínas tienen sitios de unión idénticos o sobrelapados [67]. Por lo tanto, para que el complejo Ndc80 se pueda localizar en el cinetocoro, es necesario que HP1α sea

desplazada en mayor parte de su unión con el complejo Mis12. Dando como resultado, que los complejos Mis12 y Ndc80 tengan un papel importante en el cinetocoro externo durante metafase pero no en la cromatina centromérica.

LA TSA puede influenciar la acetilación de histonas en las regiones pericentromericas, como ha sido reportado en modelos celulares a bajas dosis, peor requiere de varios ciclos celulares para su efecto [107]. En contraste, con un tratamiento a corto plazo con 1 μM de TSA, observamos que tanto HP1α como HP1β son relocalizadas a la cromatina centromérica que fue enriquecida con H3K9me durante interfase y mitosis en ambas líneas celulares. Nuestros resultados difieren con otros reportes de la región de satélite III, donde la H3K9ac se incrementa, pero no hay cambios en la abundancia de H3K9me3,me3 después de 15 h de tratamiento con TSA y otros inhibidores de HDAC [106, 116]. Estos resultados sugieren que la cromatina en la regiones pericentromericas responde diferente al tratamiento con TSA.

Adicionalmente, observamos una reducción significativa en la H3K9me3 junto con una deslocalización de HP1α y HP1β después de 24 h de tratamiento en la heterocromatina pericentromérica constitutiva de las regiones del satélite 2. Este resultado es similar a los resultados de un estudio en células HeLa después de un tratamiento de corto plazo con TSA [107]. Sin embargo, observamos que después de 48 h de tratamiento con TSA en células HCT116, ambas HP1 son recuperadas en las regiones de satélite 2 aunque no se observo una recuperación de H3K9me3, sugiriendo que este mecanismo podría estar implicado en la localización pericentromérica de HP1.

De manera notable, en la cromatina centromérica, la exposición a TSA redujo H3K9me3 y

paradójicamente afecto la marcas asociadas a cromatina activa, como H3K4me2 y H3K9ac; Sin embargo, después de 24 y 48 h de tratamiento las proteínas HP1 fueron enriquecidas a niveles proporcionales a Mis12 y CENP-A en la cromatina centromérica de células HCT116. Estos resultados soportan nuestros resultados por inmunofluorescencia, en donde Mis12 esta asociada con HP1β durante interfase, y después del tratamiento con TSA Mis12 co-localizo con HP1β en los foci asociados al centrómero. Por lo tanto, la localización de Mis12 parece estar intrínsecamente asociada con HP1β durante el ciclo celular. Sorprendentemente, en células normales, este fenómeno no se presentó, y tanto H3K9me3 y las proteínas HP1 se reducen, sugiriendo que el mecanismo que promueve a HP1 a moverse hacia el centrómero en HCT116 no esta funcionando en las células WI-38.

Nuestros resultados muestran que en respuesta TSA ocurren diferentes defectos en la cromatina centromérica en células HCT116 y WI-38, junto con la reducción de la marcas de heterocromatina en regiones de cromatina pericentromérica. Se ha sugerido que la inhibición de HDAC antes de mitosis esta asociada con defectos en la condensación de cromosomas, rezagos cromosómicos, formación de micro núcleos e impedimentos en el apareo de microtúbulos con el cinetocoro debido a una perdida de la función del centrómero y a alteraciones en la composición de cinetocoro así como de la dinámica de los microtúbulos, sugiriendo que una condensación no adecuada induce arresto celular e IC [71, 74, 107]. Dichos defectos mitóticos han sido asociados también con una reducción de la heterocromatina pericentromérica y con desplazamientos de HP1α o disminuciones de HP1β y la Aurora-B en esta región durante fase-S y G2. Siendo así, dichos efectos en la región pericentromérica podrían dirigir a un ensamblaje del cinetocoro deficiente durante mitosis [71, 107, 108].

Analizamos la generación de IC en las células HCT116 y Wi-38 después de un tratamiento con TSA. Dicho tratamiento genero anuploidias en ambas líneas celulares. Encontramos un porcentaje importante de células tetraploides después de la exposición a TSA en las células WI-38. Lo anterior podría ser explicado por la capacidad de la TSA para detener la transición G2/M [117]. Mientras en células HCT116, observamos una baja frecuencia de células tetraploides (4%), sugiriendo que las células están siendo liberadas del arresto G2/M, promoviendo defectos en la segregación cromosómica. Aunque la IC generada disminuyo a la mitad después de 48 h en HCT116. Con respecto a esto, ha sido reportado en células HeLa que 24 h de tratamiento con 1 μM de TSA induce aproximadamente un 20% de células apoptóticas [113], por esta razón nosotros razonamos que la reducción en la IC podría ser relacionada con la muerte celular de las células aneuploides por que la TSA disminuye la proliferación y promueve la muerte celular y apoptosis al inducir la actividad de la caspasa 3/7 [118].

En resumen, observamos que los efectos citotóxicos de la TSA son mas pronunciados en las células HCT116 comparados con WI-38. Una razón de la citotoxicidad de la TSA a altas dosis, puede deberse a por su efecto de interrupción en dos puntos de monitoreo del ciclo celular: el punto de monitores de G2 y en el punto de monitoreo del uso mitótico [117, 119]. La interrupción de ambos puntos de monitoreo resulta en una salida prematura de la células a una mitosis abortiva, posiblemente seguida de apoptosis. Sin embargo, un reporte reciente en células fibroblásticas de ratón NIH 3T3 no observó efectos significativos en los índices mitóticos y en la inducción de micro núcleos, excepto por tratamientos de 48 h bajo condiciones de alta concentración con inhibidores de HDAC [114], esto es similar a observado en las células de fibroblastos de pulmón humanos WI-38, donde estas células podrían ser mas resistentes a la TSA que las células HCT116.

5.3 La exposición a TSA afecta la transcripción de RNAnc del satélite-α y satélite 2 y esta asociado a la generación de IC.

Existe evidencia *in vivo* de que hay RNAlnc transcritos de los repetidos de satélite-α, y se ha demostrados la unión de estos transcritos no codificantes con la proteína asociada el cinetocoro CENP-C [99]. Esta unión esta asociada con la actividad de la RNAP II y de factores de trascripción que están localizados en el centrómero, lo cual promueve la transcripción de RNAnc de satélite-α durante mitosis [100]. El RNAnc del satélite-α asociado al cinetocoro es de un tamaño correspondiente a las unidades de 171nt repetidas de satélite-α [99]. Es interesante que el dominio de CENP-C que interacciona con el RNAnc satélite-α es homologo con el dominio de bisagra de HP1 [99, 120]. La región de bisagra de HP1 conecta los dominios cromosombra y cromodominio y es requerida para la localización de estas proteínas a la heterocromatina centromérica [120, 121]. Nosotros observamos que la proteína HP1α interacciona con el ncRNA del satélite-α, y dicha interacción podría jugar un papel en la regulación y localización de HP1 cuando la expresión de este RNAnc es modificada. Por lo tanto, el ncRNA del satélite-α podría estar implicado en la regulación y localización de las proteínas HP1 en la cromatina centromérica, a pesar de que H3K9me3 este reducida.

Nosotros observamos que el RNAnc proveniente del satélite 2 se encuentra sobre expresado por la TSA en células normales y cancerosas, en donde la marca de heterocromatina H3K9me3 se encuentra seriamente reducida, y que HP1 es reducida después de 24 h de tratamiento. Este resultado es similar a evidencia reciente que sugiere que HP1α y la proteína vigilina al estar presentes en la región de satélite 2 pericentromérica regulan a la baja la expresión de RNAnc del satélite 2 [104]. Por lo tanto, la reducción de HP1α y vigilina en los repetidos de satélite 2 promueven una sobre expresión de este transcrito no codificante [104]. Estos resultados sugieren

que la reducción de H3K9me3 por la TSA promueven la perdida de HP1α y HP1β en la región pericentromérica. Esta perdida de heterocromatina promueve una sobre expresión del satélite 2, y es posible que este mismo mecanismo ocurra en otros repetidos centroméricos. Sin embargo se necesitan mas estudios para entender la función de los RNAnc del satélite-α y el satélite 2.

Nuestros resultados indican que los RNAnc de los satélites podrían estar implicados en la regulación de HP1 en la cromatina centromérica y pericentromérica. Encontramos una asociación entre la sobre expresión de satélite-α y satélite 2 y la generación de IC en células HCT116. Esta asociación podría ser explicada por otros reportes, en donde transcritos provenientes de repetidos del satélite menor en células de murinos son importantes en el ensamblaje de complejos de proteínas haca el centrómero [102]. A demás, en modelos de ratones los defectos en la segregación cromosómica son consistentes con un impedimento de la función centromérica que es asociada con la acumulación de pequeños transcritos centroméricos [101].

Los RNAnc centroméricos y pericentroméricos son importantes en la regulación epigenética de la arquitectura y función del centrómero humano. Los transcritos no codificantes del satélite-α y del satélite 2 podrían representar ejemplos individuales de las vías que regulan el ensamblaje de la heterocromatina en mamíferos, posiblemente al contribuir en la asociación de proteínas del cinetocoro y de la heterocromatina.

En resumen, encontramos diferencias en la respuesta al tratamiento con TSA en la cromatina centromérica de células HCT116 y WI-38. Basándonos en estos resultados, junto con la reducción de las marcas de heterocromatina en las regiones de cromatina pericentromérica, proponemos el modelo mostrado en la **Figura 15**, en donde la células HCT116 y WI-38 presentan una distribución

similar de las proteínas HP1 y las modificaciones de la cromatina en las regiones centromérica y pericentromérica. Sin embargo, después del tratamiento con TSA H3K9me3 se redujo, mientras H3K9ac y CENP-A se incrementó en ambas líneas celulares. A demás, en células HCT116 las proteínas HP1 se reincorporaron en las regiones pericentromérica y centromérica a pesar del estado de metilación de la marca lectora. Sugiriendo que un mecanismo desconocido, que podría el RNAnc centromérica u otras proteínas podrían reclutar a HP1 a la zona. Esto contrasta con lo ocurrido en células WI-38, donde la proteínas HP1 se perdieron de la cromatina centromérica después del tratamiento. La exposición a TSA promovió IC, especialmente en las células HCT116, donde se vio una baja tasa de arresto al ciclo celular comparándolo con las células WI-38. Estos resultados sugieren que el panorama epigenética de la cromatina centromérica y pericentromérica conlleva a diferencias en la promoción de IC en respuesta a TSA en células tumorales y células no tumorales.

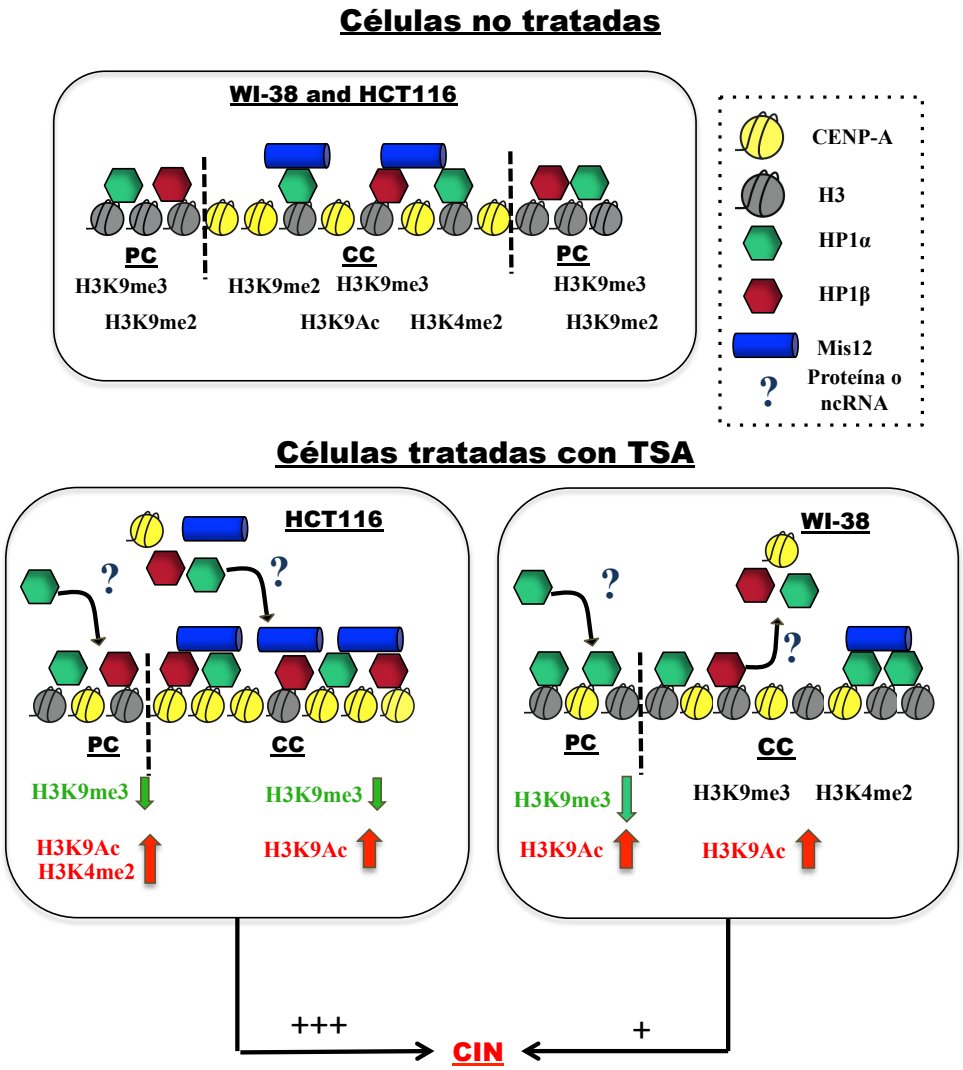

Figura 15. *Efecto de la exposición de TSA en la localización HP1α/β y las modificaciones en la cromatina centromérica y pericentromérica, así como en la relación con la inducción de IC en células HCT116 y WI-38.* ***ARRIBA****: Las células WI-38 y HCT116 presentan un estado basal similar en la localización de HP1α/β en la cromatina centromérica (CC) y pericentromérica (PC). En PC, se encuentra enriquecida H3K9me2/3 junto con las proteínas HP1; en CC, CENP-A esta enriquecida en mitosis, mientras H3K9me2/3, H3K4me2 y H3K9ac, así como HP1α y HP1β y MIS12 fluctúan en interfase y mitosis. ABAJO: Después del tratamiento con TSA en células HCT116, H3K9me3 se redujo significativamente en PC y CC, resultando en un incremento de H3K4me2 y H3K9ac en la región del satélite 2; las proteínas HP1α/β se redujeron significativamente. Sin embargo, Estas se recuperaron en la zona por un mecanismo desconocido que podría incluir otras proteínas o a el trascrito RNnc centromérico. H3K9ac y CENP-A se enriquecieron en CC, junto con HP1α/β y Mis12. Las células HCT116 proliferaron presentando bajos niveles de*

arresto celular y exhibieron IC en el 50% de las células. En ambas líneas celulares, PC presentó reducción de H3K9me3, mientras H3K9ac y HP1 se incrementaron; aunque Hp1 y CENP-A fueron reducidas en CC, no fue significativa la reducción de H3K9me3, a pesar de que H3K9ac se incrementó. Se genero IC, pero fue menor a la presentada en células HCT116.

6. Conclusión

Los resultados presentados en este trabajo proveen de un nuevo acercamiento al panorama epigenético de la cromatina centromérica y la función de las proteínas HP1α y HP1β en la segregación cromosómica y por lo tanto en la estabilidad de la división celular. También presentamos evidencia de diferencias en la organización de la cromatina centromérica y de la localización de HP1 en respuesta a tratamientos con TSA en células HCT116 y WI-38. Dichas diferencias están asociadas con la IC que resulta de una perturbación en la cromatina causada pro la reducción de H3K9me3 y la inducción de hiperacetilación por TSA. Los efectos de la TSA son mas pronunciados en células malignas y transformadas HCT116 comparado con la células WI-38, promoviendo una mayor perdida en la segregación cromosómica y generación de IC. Adicionalmente, creemos que una de las razones por las los efectos en la generación de IC y en el arresto al ciclo celular por la TSA, podría se la desregulación del ambiente de la cromatina centromérica y pericentromérica, el donde componentes epigenéticos como los novedosos RNAnc podrían estar implicados en la estabilidad de dichas regiones, dando pie a la posibilidad de que la regulación epigenética del centrómero podría alterar la respuesta de las líneas tumorales a tratamientos con TSA. Sin embargo aun quedan muchas preguntas por contestar en cuanto a la epigenética del centrómero y como esta regula el ensamblaje del cinetocoro, así como su importancia en la segregación cromosómica, y la comunicación establecida entre la cromatina centromérica y pericentromérica.

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Differential distribution of HP1 proteins after trichostatin a treatment influences chromosomal stability in HCT116 and WI-38 cells

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Abstract

Background

Heterochromatin protein 1 (HP1) is important in the establishment, propagation, and maintenance of constitutive heterochromatin, especially at the pericentromeric region. HP1 might participate in recruiting and directing Mis12 to the centromere during interphase, and HP1 disruption or abrogation might lead to the loss of Mis12 incorporation into the kinetochore. Therefore, the centromere structure and kinetochore relaxation that are promoted in the absence of Mis12 could further induce chromosome instability (CIN) by reducing the capacity of the kinetochore to anchor microtubules. The aim of this study was to determine whether alterations in the localization of HP1 proteins induced by trichostatin A (TSA) modify Mis12 and Centromere Protein A (CENP-A) recruitment to the centromere and whether changes in the expression of HP1 proteins and H3K9 methylation at centromeric chromatin increase CIN in HCT116 and WI-38 cells.

Methods

HCT116 and WI-38 cells were cultured and treated with TSA to evaluate CIN after 24 and 48 h of exposure. Immunofluorescence, Western blot, ChIP, and RT-PCR assays were performed in both cell lines to evaluate the localization and abundance of HP1α/β, Mis12, and CENP-A and to evaluate chromatin modifications during interphase and mitosis, as well as after 24 and 48 h of TSA treatment.

Results

Our results show that the TSA-induced reduction in heterochromatic histone marks on centromeric chromatin reduced HP1 at the centromere in the non-tumoral WI-38 cells and that this reduction was associated with cell cycle arrest and CIN. However, in HCT116 cells, HP1 proteins, together with MIS12 and CENP-A, relocated to centromeric chromatin in response to TSA treatment, even after H3K9me3 depletion in the centromeric nucleosomes. The enrichment of HP1 and the loss of H3K9me3 were associated with an increase in CIN, suggesting a response mechanism at centromeric and pericentromeric chromatin that augments the presence of HP1 proteins in those regions, possibly ensuring chromosome segregation despite serious CIN. Our results provide new insight into the epigenetic landscape of centromeric chromatin and the role of HP1 proteins in CIN.

Keywords

HP1, Centromeric chromatin, TSA, Chromosome instability, CENP-A

Background

Heterochromatin protein 1 (HP1) binds to histone H3 proteins that have been methylated at lysine 9 by SUV39H1, thereby propagating the methylation along chromatin [1]. HP1 function is highly important for the establishment, propagation, and maintenance of constitutive heterochromatin [2], especially at the pericentromeric region, which is enriched with H3K9me3 and H4K20me3 marks, hypoacetylated H3 and H4, and highly methylated

regions along most of its satellite repeats [3,4]. Due to its juxtaposition with centromeric chromatin, it has been suggested that the organization and stability of the pericentromeric region are crucial for correct chromosomal segregation during mitosis; therefore, this region is important for genome stability [3,5].

HP1 also plays a role in centromeric sister chromatid cohesion [6], telomere maintenance, and DNA repair [7]. In humans, these functions are performed in a specific manner by each of the three identified HP1 subtypes: HP1α, HP1β, and HP1γ [8,9]. HP1 protein localization differs in the interphase nucleus, with HP1α typically found in pericentric and telomeric chromatin and HP1β normally found in heterochromatin regions [10].

Live cell microscopy analyses have demonstrated that the localizations of human HP1α and HP1β have specific functions at different points of the cell cycle. An exchange between human HP1α and HP1β has been observed at centromeric heterochromatin during mitosis [10]. This exchange is mediated by differences in the chromoshadow domain sequences of these proteins [10]. Increasing evidence has shown that the Knl1-Mis12-Ndc80 (KMN) protein complex is a binding partner of HP1 in humans, in which HP1 might participate in recruiting and directing Mis12, a kinetochore complex component that is a subunit of the KMN network that resides at the centromere during interphase and stably associates with the kinetochore during mitosis [11-13]. The disruption or abrogation of HP1 is believed to lead to tumor formation, and the absence of HP1 might also lead to the loss of Mis12 incorporation into the kinetochore. Therefore, the centromere structure and kinetochore relaxation that are promoted by the absence of Mis12 could further induce chromosome instability (CIN) by reducing the capacity of the kinetochore to anchor microtubules [14].

These findings suggest that the deregulation of epigenetic components in the kinetochore complex could result in chromosomal defects and CIN development. Furthermore, it has become increasingly clear that chromatin composition affects centromere determination and establishment. Nevertheless, the genomic and chromatin modifications that are necessary to establish and maintain the centromere remain unknown. It has been suggested that the DNA sequence alone is not always sufficient for centromere establishment or function [15], supporting theories that postulate the involvement of epigenetic mechanisms [16]. Thus, the aim of our study was to determine whether alterations in the localization of HP1 proteins modify Mis12 recruitment to the centromere and whether changes in the expression of HP1 proteins and H3K9 methylation at centromeric chromatin lead to an increase in CIN.

To address these questions, we evaluated HP1 proteins during the cell cycle. In addition, we treated cells with trichostatin A (TSA), an inhibitor of histone deacetylase (HDAC) enzymes, to indirectly antagonize centromeric heterochromatin and HP1 binding by reducing H3K9me3 abundance. Our results show that the TSA-induced reduction in heterochromatic histone modification of centromeric chromatin reduced HP1 levels at the centromere in WI-38 cells and that this reduction was associated with cell cycle arrest and CIN. However, in HCT116 cells, HP1 proteins relocated to centromeric chromatin in response to TSA treatment, and this re-localization was induced even after H3K9me3 was reduced in the centromeric nucleosomes. The enrichment of HP1 proteins in HCT116 cells was associated with increased CIN, suggesting a response mechanism in the centromeric and pericentromeric chromatin that augments the presence of HP1 proteins in those regions, possibly ensuring chromosome segregation despite serious CIN.

Results

Chromosome instability is induced by TSA

HP1 proteins and H3K9me3 have been shown to play an important role in chromosome stability. There are several reports on the different types of CIN promoted by TSA treatment in a wide range of concentrations and periods of exposure [17-19]. Therefore, we evaluated if treatments with TSA promoted a similar effect in the induction of CIN in WI-38 and HCT116 cells.

TSA induced aneuploidy in both cell lines (Figure 1A). After TSA treatment for 24 h, 26% of WI-38 cells were aneuploid, and this frequency was maintained for at least 48 h post-treatment. In contrast, 47% of HCT116 cells were aneuploid after TSA treatment for 24 h; however, this frequency was lower (22%) after treatment for 48 h. WI-38 cells lost more than 6 chromosomes or gained more than 20 chromosomes (Figure 1B). A high number of HCT116 cells were aneuploid after 24 h of treatment; however, after 48 h, the rate of chromosomal gains and losses was reduced (Figure 1C, Table 1). After TSA treatment for 24 h, 32% of WI-38 cells were 4n; after treatment for 48 h, 19.6% of the cells remained 4n, indicating that WI-38 cells could not properly segregate following TSA treatment (Table 1). Only 4% of HCT116 cells were 4n after treatment for 24 h, and no 4n cells were found after 48 h (Table 1).

Figure 1 Trichostatin A (TSA) treatment generates chromosome instability primarily in HCT116 cells. Chromosome counting was performed after cells were treated with 1 μM TSA for 24 and 48 h. **(A)** The percentage of aneuploidy was greater than 26% after the 24 and 48 h TSA treatments in WI-38 cells, and the effect of TSA was more pronounced in HCT116 cells after 24 h (at 47%) but decreased to 21% after 48 h of exposure. **(B-C)** The representation of the number of chromosomes from the controls and the 24- and 48-h TSA-treated WI-38 **(B)** and HCT116 cells **(C)**, showing gains and losses after counting; the black line designates the 2n cells, and the dotted line designates the 4n cells. The total number of chromosomes in 50 cells was counted. The Kruskal-Wallis test yielded $p < 0.05$ compared with the values of the control (CTR).

Table 1 Analysis of total chromosome number in each cell after 24 and 48 h of trichostatin A (TSA) treatment

	WI-38			HCT116		
	Control	**24 h ***	**48 h *,****	**Control**	**24 h**	**48 h ****
Mode	42 (87.5%)	21(42%)	28 (54.9%)	49 (96.08%)	25 (54.9%)*	40 (78.43%)
Loss	6 (12.5%)	8 (16%)	11 (21.56%)	2 (3.92%)	14 (27.5%)*	7 (13.73%)
Gain	0	5 (10%)	2 (3.92%)	0	10 (19.6%)*	4 (7.84%)
4n	0	16 (32%)	10 (19.6%)	0	2 (3.9%)	0
Total	**48**	**50**	**51**	**51**	**51**	**51**

Loss was considered below 2n, and gain was considered above 2n.
* Levene?s test $p < 0.001$; treatment versus control.
** Levene?s test $p < 0.05$; 24-h treatment versus 48-h treatment.

Centromeric chromatin dynamics during the cell cycle

To observe the localization of HP1α and HP1β proteins throughout the cell cycle, as well as their association with H3K9me3 and CENP-A, we performed immunofluorescence assays in

WI-38 (Figure 2A) and HCT116 (Figure 3A) cells. In WI-38 cells, we explored the nuclear localization of H3K9me3 and CENP-A, both of which were enriched at centromeric loci and neighboring regions. This enrichment persisted in mitotic cells (Figure 2A). Because H3K9me3 is the epigenetic modification that is recognized by the HP1 protein chromodomain, and given the importance of HP1 proteins for proper chromosome alignment and mitotic progression [11,19], we evaluated the nuclear localization of the HP1α and HP1β isoforms together with CENP-A. We observed little difference in the localization of both HP1 isoforms at the centromere. HP1α was localized to regions neighboring CENP-A, which are likely pericentromeric heterochromatin, and also occupied other chromatin regions. HP1β showed a similar localization pattern (Figure 2A). Therefore, although both isoforms play a critical role in establishing and maintaining heterochromatin, they might play different roles in terms of the surrounding centromeric chromatin.

Figure 2 Centromeric localization of HP1α and HP1β in WI-38 cells under basal conditions and after TSA treatment. (A) WI-38 cell fluorescent microscopy localization of CENP-A with H3K9me3 (lane 1), HP1α (lane 2-3) and HP1β (lane 4). **(B)** Chromatin localization by fluorescent microscopy in WI-38 cells after TSA treatment comparing H3K9me3 with HP1α (lane 1) or HP1β (lane 2) H3K9ac with HP1α (lane 3) or HP1β (lane 4), the centromeric localization of HP1α compared with CENP-A (lane 5), and HP1β compared with ACA (lane 6). The DNA is marked with DAPI; the images show the most common distribution of proteins after the analysis of 100 cells (%); the boxes represent a magnification of the immunofluorescence results; M, mitotic cell.

Figure 3 Centromeric localization of histone marks, HP1α, and HP1β in HCT116 cells under basal conditions and after TSA treatment. (A) HCT116 chromatin localization by fluorescent microscopy of CENP-A with H3K9me3 (lane 1) or HP1α (lane 2), H3K9me3 and HP1α (lane 3), ACA and HP1β (lane 4), and HP1β and Mis12 (lane 5). **(B)** Centromeric localization of H3K9me3 and CENP-A (lane 1), CENP-A and HP1α (lane 2), ACA and HP1β (lane 3) and Mis12 co-localization with HP1β (lane 4). The DNA is marked with DAPI; the images show the most common distribution of the proteins after the analysis of 100 cells (%); the boxes represent a magnification of the immunofluorescent results; M, mitotic cell.

We then determined whether the dynamics and localization of H3K9me3, HP1α, and HP1β are conserved in a cancer cell line with no CIN and with stable chromosome segregation such as HCT116 cells. For this purpose, we performed immunofluorescence microscopy assays to observe the centromeric localization of HP1. H3K9me3 co-localized with CENP-A during interphase; during mitosis, it either co-localized or was enriched in the region neighboring the centromere (Figure 3A). As expected, the HP1 isoforms showed similar patterns of localization in both cell lines, suggesting conserved chromatin behavior for both H3K9me3 and HP1 (Figure 3A). We observed almost no co-localization of the euchromatic marks H3K9ac and H3K4me2 with CENP-A, indicating that open chromatin marks are present at centromeric regions at a low frequency in both cell lines (Additional file 1: Figure S1A-B). Considering that HP1 has been associated with the Mis12 complex, we observed that HP1 co-localizes with Mis12 during interphase, but this localization changes slightly during mitosis (Figure 3A).

To confirm the presence of HP1 at centromeric chromatin and to assess its dynamics during interphase and mitosis, we selected HCT116 cells with stable chromosomal segregation. We treated these cells with nocodazole for 12 h and isolated the mitotic cells using the shake-off method. We then performed a ChIP assay for activating (H3K4me2 and H3K9ac) and

repressive (H3K9me2/me3) histone marks, as well as for CENP-A, Mis12, HP1α, and HP1β, in mitotic and interphase cells. As controls for open and closed chromatin, we evaluated the GAPDH and WIF1 promoter regions, respectively. The GAPDH promoter, as expected, was enriched with the markers of gene activation H3K4me2 and H3K9ac; the abundance of these marks was increased during mitosis, but the increase was proportional to the gain in total H3 in the region (Additional file 2: Figure S2A). We used the WIF1 gene promoter region as a positive control for gene silencing due to its role as a WNT inhibitor; this promoter is known to be enriched with H3K9me2 and H3K9me3 modifications after cell differentiation during embryonic development. We found that this region was enriched with repressive marks and that these marks were increased during mitosis (Additional file 2: Figure S2B).

Because centromeric chromatin has been poorly studied by ChIP analysis and because there have been contrasting results in different models, we designed primers for global satellite-α repeats and analyzed the 171-bp monomer sequence. Our results showed that H3K9me2 and HP1β are present during interphase and mitosis, whereas the presence of H3K4me2, H3K9ac and H3K9me3 histone marks fluctuate throughout the cell cycle (Additional file 2: Figure S2C).

However, the satellite-α repeat arrangement varies at the centromere, and CENP-A/H3 nucleosomes are scattered thorough the centromeric sequence. We therefore designed primers that were specific for satellite-α and satellite-2 regions of chromosome 1 to confine the analysis of chromatin changes to these regions. We evaluated chromosome 1 satellite-2 pericentromeric regions, which were enriched with H3K9me2 and H3K9me3 during interphase; as expected, HP1α and HP1β were also present during this phase (Figure 4A-B) because satellite-2 is a well-known heterochromatic region. Interestingly, we observed a 2-fold enrichment of H3K4me2 and a 50% reduction of H3K9me3 in mitotic cells. We did not observe Mis12 and CENP-A at the satellite-2 repeat. We then questioned whether the same modulation occurred in normal cells such as WI-38 cells. We explored the same satellite-2 regions during interphase and mitosis and found that in interphase cells, H3K9me3 was abundant alongside HP1α, which is typical of the pericentromeric constitutive heterochromatin domain (Figure 4C). During mitosis, H3K9me3 was reduced, but HP1α was heavily enriched, suggesting a role for HP1α at pericentromeric heterochromatin during chromosome segregation (Figure 4C). We observed an unexpected enrichment of CENP-A at satellite-2 chromatin during mitosis (Figure 4C).

Figure 4 Epigenetic changes and protein dynamics at satellite-2 and satellite-α regions during interphase and mitosis in HCT116 and WI-38 cells. qRT-PCR analysis of the satellite-2 **(A-C)** and satellite-α **(D-F)** repeats was performed on DNA obtained from anti-H3K4me2, H3K9me2, H3K9me3, H3K9ac, HP1α, HP1β, Mis12 and CENP-A ChIP assays in interphase (black) and mitotic (gray) cells. Normal rabbit IgG was employed as a negative control. $p < 0.05$ and $p < 0.01$ represent significant differences between interphase and mitosis, as evaluated by Student?s T-test.

Moreover, in the chromosome 1 satellite-α repeat region, a mixed histone epigenetic landscape was found, in which active and repressive histone marks were present throughout the cell cycle (Figure 4D-E). We observed a significant enrichment of CENP-A during mitosis, whereas the enrichment of known H3 modifications was reduced (Figure 4D). The satellite-α region in WI-38 cells was also enriched with CENP-A and HP1α. Although H3K9me3 was reduced during mitosis, it remained present at the centromere (Figure 4F), suggesting that HP1α plays a different role at the centromere in this cell line than in HCT116

cells. In contrast to the immunofluorescence results, we detected HP1α and HP1β in the specific satellite-α region, and their enrichment fluctuated slightly during mitosis (Figure 4E-F).

TSA treatment causes HP1 proteins to re-localize to centromeric chromatin in HCT116 but not WI-38 cells

To observe the effect of antagonizing heterochromatic regions of pericentromeric and centromeric chromatin, we treated WI-38 cells with 1 μM TSA, which leads to chromatin relaxation and gene expression modulation [20,21]. To evaluate the effect of TSA on the cell cycle, cells were treated for 24 and 48 h; the drug was reintroduced via fresh medium every 24 h. The effect of this treatment on protein nuclear localization was observed by fluorescence microscopy. TSA reduced H3K9me3 levels, as expected, and also reduced the protein levels of HP1α and HP1β (Figures 2A and 3B). Clear foci of H3K9me3 remained after treatment, and these foci co-localized with the HP1α and HP1β isoforms (Figures 2B and 3B). We also observed that these HP1 protein foci localized to the same regions occupied by CENP-A, suggesting that both H3K9me3 and HP1α/β are more enriched in the centromeric region, as defined by the localization of CENP-A and ACA. These results suggest that H3K9me3 was preserved at the centromere and that both HP1 proteins accumulate at centromeric chromatin in response to TSA treatment (Figures 2B and 3B). When treated with TSA, both HCT116 and WI-38 cells presented H3K9me3 at CENP-A foci, which were also occupied by both HP1 isoforms (Figures 2B and 3B).

Considering that HP1 has been associated with the Mis12 complex, we determined whether Mis12 localization was affected by TSA. Interestingly, Mis12 localization showed a strong correlation with HP1β localization and was also enriched at TSA-promoted HP1β foci, suggesting that this kinetochore foundation protein is associated with HP1β not only during mitosis but also during interphase (Figure 3B).

To determine whether these changes in HP1 protein localization were related to alterations in total protein levels, we performed an immunoblot assay in both cell types before and after the TSA treatments for 24 and 48 h. After 24 h, we observed a significant reduction in HP1α in HCT116 cells only; no significant changes were found in WI-38 cells. After 24 h of TSA exposure, HP1β was decreased in WI-38 cells; however, the original levels were restored after 48 h. No changes were observed in the abundance of HP1β in HCT116 cells or the levels of CENP-A in either cell line after TSA treatment (Figure 5). Moreover, we found no significant changes in H3K9ac levels after TSA treatment, although we did observe a tendency to accumulate acetylation after 48 h of treatment in WI-38 cells. Nevertheless, H3K9me3 levels were significantly decreased, especially after 48 h of TSA exposure, suggesting not only that TSA decreased this heterochromatin mark but also that TSA-promoted acetylation did not significantly affect H3K9 residues (Figure 5). One possible explanation is that acetylation might occur at a higher frequency on other H3 or H4 lysine residues. Taken together, our results suggest that the changes in HP1 localization are most likely due to the reduction in H3K9me3 after TSA treatment rather than to alterations in HP1 protein translation.

Figure 5 The protein content in HCT116 and WI-38 cells treated with TSA. Representative Western blot of HP1α, HP1β, H3, H3K9me3, H3K9ac and CENP-A levels after treatment with 1 μM TSA for 24 or 48 h. The experiments were conducted in 3 independent determinations that were performed in duplicate for each experimental

condition; the asterisk indicates $p < 0.05$ compared with the value of the control (CTR), as obtained by Student?s t-test.

TSA treatment leads to changes in the nuclear localization of HP1 proteins. Therefore, we performed a ChIP assay after TSA exposure in HCT116 cells. We found that TSA abolished the abundance of H3K9me3 at satellite-2 regions after the first 24 h of treatment; this abolishment was associated with the loss of HP1α and HP1β after 24 h of TSA exposure (Figure 6A-B). Surprisingly, HP1α and HP1β were reestablished at satellite-2 chromatin after 48 h of TSA exposure, even though H3K9me3 was dramatically diminished by the treatment (Figure 6A-B). H3K4me2 and H3K9ac were not significantly changed after 24 h of treatment, but were significantly increased after 48 h (Figure 6A), suggesting that TSA treatment promotes a significantly open chromatin state at the satellite-2 region after 48 h of exposure.

Figure 6 Epigenetic changes and protein dynamics at satellite-2 and satellite-α regions after treatment with TSA for 24 and 48 h in HCT116 and WI-38 cells. qRT-PCR analysis of the satellite-2 **(A-C)** and satellite-α **(D-F)** repeats was performed on DNA obtained from anti-H3K4me2, H3K9me3, H3K9ac, HP1α, HP1β, Mis12 and CENP-A ChIP assays in untreated cells (Black bar) and TSA-treated cells for 24 (white bar) or 48 h (gray bar). Normal rabbit IgG was employed as a negative control. $p < 0.05$ and $p < 0.01$ indicate significant differences between treated and untreated cells, as evaluated by Student?s t-test.

Satellite-α, in addition to satellite-2, regions exhibited losses in H3K4me2, H3K9me3, and H3K9ac after 24 h of TSA treatment. However, after 48 h, H3K4me2 was restored to the centromere, whereas H3K9ac was significantly increased (Figure 6D). Although H3K9me3 was significantly reduced by the TSA treatment, some fraction of this mark remained at the centromere (Figure 6D). After 24 h of treatment, CENP-A was increased by 10-fold at satellite-α chromatin and remained enriched by 10-fold after 48 h of TSA treatment (Figure 6E). As observed in the immunofluorescence experiments, HP1α and HP1β were enriched 6- and 4-fold, respectively, and this fold enrichment was proportional to the fold increase in Mis12 at satellite-α chromatin, suggesting that the presence of Mis12 was associated with the abundance of HP1 at the centromere (Figure 6E). We used the GAPDH gene promoter as a control to evaluate the effect of TSA treatment. Although we observed no increase in H3K9ac, we did observe a loss of H3K9me3 as a result of the treatment, suggesting that H3K9ac is not essential for GAPDH upregulation (data not shown).

Because HCT116 cells showed changes in histone marks and HP1 protein levels after TSA treatment, we next questioned whether the same modulation occurred in normal cells. Therefore, we treated WI-38 cells with TSA for 24 and 48 h and performed a ChIP assay using antibodies against H3K9me3, CENP-A, and HP1. TSA treatment for 24 h reduced H3K9me3 levels in satellite-2 regions and nearly abolished this histone mark after 48 h. Therefore, HP1α was reduced as its reader mark was diminished (Figure 6C). In contrast to the observations made in HCT116 cells, after 24 h of exposure to TSA in WI-38 cells, HP1α and HP1β were reduced at the satellite-α region and were not reestablished after 48 h, even though H3K9me3 levels were reestablished (Figure 6F). This result suggests that the co-localization observed by immunofluorescence was located at specific centromeric and pericentromeric chromatin regions and not at the chromosome 1 centromeric region.

Discussion

HP1α and HP1β localize at centromeric regions after TSA exposure in HCT116 cells, but their levels are reduced in WI-38 cells

In different cell lines and animal models, aberrant mitotic phenotypes have been attributed to a lack of pericentromeric H3K9me3, changes in H4K20me, and abnormal regulation of HDAC. CIN has been observed as increased chromosome misalignment in metaphase, nondisjunction in anaphase, and lagging chromosomes in telophase, and as high rates of aneuploidy and the appearance of micronuclei during cytokinesis or early G_1 phase [17,22,23].

HP1 is essential, especially in the pericentromeric region, which is enriched with H3K9me3 and H4K20me3 modifications, hypoacetylated H3 and H4, and highly methylated regions along satellite repeats [4,24,25]. Little is known regarding the effects of HP1 during mitosis; however, the reduction of HP1 by TSA-promoted mitotic defects has been previously reported [18,20]. We observed that HP1α and HP1β, together with H3K9me3, are located in pericentromeric regions and that the centromeric localization of HP1 is preserved during mitosis, although HP1α and HP1β are dissociated in other regions of the chromosome. Likewise, we observed changes in HP1α and HP1β abundance throughout the cell cycle at the centromeric and pericentromeric chromatin of chromosome 1. This result was consistent with a previous report in which HP1 proteins underwent large-scale dissociation in G_2-phase cells [26]. The change in HP1 localization during mitosis could also be attributed to the presence of acetylated histones on mitotic chromosomes, which decreases the accessibility of histone N-tails to the antibody, as was observed for H3 serine 10 phosphorylation [18,27].

Centromeres contain CENP-A nucleosomes interspersed with H3K9me2/3 nucleosomes but exhibit low levels of H3K4me2 enrichment [28,29]. In this regard, we observed clear HP1-enriched foci co-localizing with CENP-A and H3K9me3 during interphase; this colocalization continued throughout mitosis. In HCT116 cells, we found that H3K4me2 is reduced at centromeric chromatin during mitosis. It has been reported that H3K4me2 is an essential modification of centromeric chromatin that is required for its long-term maintenance and function, whereas the enrichment of H3K9me3 and H3K9ac fluctuate significantly throughout the cell cycle [22]. H3K9me3 has been reported to increase in abundance during G_2/M in mammals, whereas H3K9me2 abundance remains constant during the cell cycle [22,30,31]. Our results are consistent with an increase in H3K9me3 at satellite-2 chromatin and at non-specific satellite-α regions; moreover, no significant increase was detected at satellite-α regions upon analyzing chromosome 1 during mitosis. Remarkably, these results suggest that H3K9me3 abundance at satellite-2 regions during mitosis is not equal in all cells, as was previously suggested.

Increasing evidence has shown that the KMN network in humans is a binding partner of HP1 and that HP1 may participate in recruiting and directing the Mis12 complex to the centromere during interphase by direct interaction with Mis14 [11,12,32]. In HCT116 cells, we observed that during interphase, HP1α, HP1β and Mis12 are present at centromeric chromatin, in agreement with previous reports. In contrast, during mitosis, Mis12 was not enriched at the same site, although HP1α and HP1β were also reduced. This could be explained by the nature of the Mis12 interaction with HP1: HP1α and Ndc80 are competitive binders of Mis12, suggesting that these proteins have identical or overlapping binding sites [13]. For the Ndc80

complex to localize to the kinetochore, it is necessary to displace most of the HP1α from Mis12. As a result, Mis12 and the Ndc80 complex play a role at the outer kinetochore but not at mitotic centromeric chromatin during metaphase.

TSA induces differential changes in centromeric and pericentromeric chromatin and in CIN induction in HT116 and WI-38 cells

TSA treatment promotes histone hyperacetylation, which becomes visible at the nuclear periphery, as well as the reduction of many heterochromatin regions in the nucleus [20,33,34]. Due to this reduction of heterochromatin, we evaluated whether short-term TSA treatment modifies the centromeric and pericentromeric regions due to HP1 protein enrichment. We found that both HP1α and HP1β were enriched in foci that co-localized with H3K9me3 and CENP-A, suggesting that both HP1 proteins not only remained at pericentromeric heterochromatin but were also enriched at constitutive heterochromatin and expanded to centromeric chromatin. This result is similar to the findings of a study conducted in HeLa cells after short-term TSA treatments [20]. We also observed that H3K9me3 modifications and HP1 proteins are generally reduced in the nuclei after TSA treatment in HCT116 cells. Interestingly, this result is contradictory to a recent report in which HP1 protein localization to centromeric chromatin was reduced and scattered in the nucleus after the treatment of murine cell lines with low concentrations of TSA [35]. In this regard, it has been observed that upon inhibition of heterochromatin acetylation, HP1 disperses within the nucleus. Another report observed that HDAC inhibition caused the dynamic recruitment of HP1 proteins to pericentromeric chromatin in a primary human cell line, suggesting that HP1 mobilization after treatment could protect the kinetochore structure and function and that HP1 proteins behave differently in human and mouse cells [19,35-37].

TSA could influence histone acetylation at pericentromeric heterochromatin regions, as reported for low doses in other cell models, but requires several cell cycles to take effect [20]. In contrast, following short-term treatment with 1 μM TSA, we observed that HP1α and HP1β relocalized to centromeric chromatin, where H3K9me3, although reduced, was still present during interphase and mitosis in both normal and transformed cells. Our result is in contrast with the results of other reports indicating that, whereas H3K9ac was increased at the satellite-III region, the abundance of H3K9me2/me3 after treatment with TSA for 15 h TSA or with other HDACi treatments was not changed [19,38]. These results suggest that the chromatin at pericentromeric and centromeric regions responded differently to TSA treatment.

We observed that Mis12 localization appears to be intrinsically associated with HP1β during the cell cycle. Remarkably, this phenomenon did not occur in normal cells, and H3K9me3 levels were nearly unaffected by treatment, whereas both HP1 proteins, together with CENP-A, were reduced, suggesting that the mechanism that promotes HP1 protein localization to the centromere in HCT116 cells fails in WI-38 cells.

It has been suggested that the inhibition of histone deacetylation before mitosis is associated with improper chromosome condensation, which might induce mitotic checkpoint activation and CIN [18,20,39]. Such inhibition at the pericentromeric region might lead to deficient kinetochore assembly during mitosis [20,21,39]. However, such effects upon the kinetochore composition and microtubule dynamics were observed without an effect on Mis12 [39]. Although we agree that Mis12 was not globally affected, Mis12 was enriched at the

centromeric chromatin of chromosome 1 after TSA exposure, indicating that the effect on Mis12 is more fine-tuned.

TSA treatment induced aneuploidy in both cell types. We observed that the cytotoxic effects of TSA were more pronounced in HCT116 cells than in WI-38 cells. Additionally, we found a significant percentage of tetraploid cells after TSA exposure in WI-38 cells. One reason for the cytotoxicity of TSA used at high doses is thought to be the disruption of two cell-cycle checkpoints: the G2 phase checkpoint and the mitotic spindle checkpoint [40,41]. This dual checkpoint disruption results in the premature exit of cells from mitosis, possibly followed by apoptosis; it has been reported in HeLa cells that treatment for 24 h with 1 μM TSA induced apoptosis in approximately 20% of cells [33]. Therefore, we suggest that this cell death is caused by aneuploidy because TSA decreases proliferation and promotes apoptotic cell death by inducing caspase 3/7 activity [42]. However, a recent report in murine fibroblastic NIH 3 T3 cells observed significant effects on the mitotic index and micronuclei induction only upon treatment of high concentrations of HDACi for 48 h [35]. A similar phenomenon might be occurring in WI-38 cells, wherein these cells might be more resistant to TSA than HCT116 cells.

We show that differential changes in centromeric chromatin occur in HCT116 and WI-38 cells in response to TSA. Based on this result, together with the reduction of heterochromatin markers in pericentromeric chromatin regions, we propose the model shown in Figure 7, in which untreated HCT116 and WI-38 cells present a similar distribution of HP1 proteins and chromatin modifications at the centromeric and pericentromeric regions. However, after TSA treatment, H3K9me3 was reduced, whereas H3K9ac and CENP-A were increased at pericentromeric and centromeric chromatin in both cell lines. Furthermore, in HCT116 cells, HP1 proteins were recovered to the pericentromeric and centromeric regions regardless of the status of the reader mark. Suggesting that an unknown mechanism that could include other proteins or could involve an non-coding RNA transcript might be recruiting HP1 proteins [43-45]. In contrast, in WI-38 cells, the HP1 proteins were lost from centromeric chromatin after treatment. CIN occurred after TSA treatment, especially in HCT116 cells, in which very low levels of cell cycle arrest were promoted compared with those in WI-38 cells. These results suggest that the epigenetic landscape of centromeric and pericentromeric chromatin leads to the differential promotion of CIN upon TSA treatment in tumoral and non-tumoral cell lines.

Figure 7 The effect of TSA exposure on HP1α/β localization and centromeric and pericentromeric chromatin modifications, as well as on the relationship with CIN in WI-38 and HCT116 cells. Upper panel: Untreated WI-38 and HCT116 cells presented a similar localization of HP1α and HP1β along centromeric (CC) and pericentromeric chromatin (PC). At PC, H3K9me2/3 were enriched together with HP1 proteins; at CC, CENP-A was enriched during mitosis, whereas H3K4me2, H3K9me2/3 and H3K9ac modifications, as well as HP1α and HP1β and Mis12 proteins, fluctuate through interphase and mitosis. **Lower panel:** After treatment of HCT116 cells with TSA, H3K9me3 was significantly reduced at PC and CC, resulting in increased H3K4me2 and H3K9ac at PC satellite-2 regions; HP1α/β were initially significantly reduced. However, they later recovered by an unknown mechanism that could include other proteins or could involve an ncRNA PC transcript [43-45]. CC was enriched with H3K9ac and CENP-A, together with HP1α/β and Mis12 proteins. HCT116 cells proliferated with low levels of cell arrest and exhibited CIN in 50% of the cells. In both WI-38 and HCT116 cells, PC presented reduced H3K9me3, whereas H3K9ac and HP1 were enriched; moreover, CC was depleted of CENP-A and HP1,

and no significant reduction in H3K9me3 was observed, even though H3K9ac was increased. While CIN was still generated, it was reduced compared with HCT116 cells.

Conclusions

The data presented here provide new insight into the epigenetic landscape of centromeric chromatin, as well as into the role of HP1α and HP1β proteins in chromosome segregation and, by extension, cell division stability. We also present evidence of differences in the organization of centromeric chromatin and HP1 localization in response to TSA in the WI-38 and HCT116 cell lines. These differences are associated with CIN resulting from a chromatin disturbance caused by reduced H3K9me3 levels and TSA-induced hyperacetylation. The effects of TSA were substantially more pronounced in the malignant, transformed HCT116 cells than in WI-38 cells, leading to more significant chromosome mis-segregation and CIN. In addition, we believe that one cause underlying the effects of TSA-induced CIN and cell cycle arrest might be the deregulation of centromeric and pericentromeric chromatin regions, leading to the possibility that epigenetic regulation of the centromere might alter the response of tumor cell lines to TSA treatment. Nonetheless, many questions remain regarding the nature of centromere epigenetics, how these epigenetic modifications regulate kinetochore assembly and their role in chromosome segregation, as well as how communication is established between centromeric and pericentromeric chromatin.

Materials and methods

Antibodies

The following antibodies were used: anti-ACA (immunofluorescence (IF) dilution 1:200; Antibodies Incorporated, Davis, CA, USA 15-235-F); anti-Mis12 (C-13; IF dilution 1:80, ChIP 4 µg; Santa Cruz, Santa Cruz, CA, USA sc-107750); anti-H3K4me2 (IF dilution 1:200, ChIP 3 µg; Millipore Temecula, CA, USA 07-030); anti-H3K9ac (IF dilution 1:200, ChIP 2.4 µg; Abcam, Cambridge, MA, USA ab10812); anti-H3K9me2 (IF dilution 1:200, ChIP 3 µg; Abcam, ab1220), anti-H3K9me3 (WB dilution 1:250; Abcam, ab8898; IF dilution 1:200, ChIP 3 µg; Diagenode, Denville, NJ, USA CS-056-050); anti-CENP-A (IF dilution 1:200, ChIP 5 µg; Abcam, ab13939); anti-HP1α (IF dilution 1:100, ChIP 4 µg; Abcam, ab77256); anti-HP1β (IF dilution 1:100, ChIP 4 µg; Abcam, ab10811); anti-GFP (ChIP 4 µg; Abcam, ab290); and anti-H3 N-terminal (ChIP 1.5 µg; Sigma, St. Louis, MO, USA, H9289-200 µl).

Cell viability (IC_{50}) after TSA treatment

Human WI-38 and HCT116 cells were obtained from ATCC (CCL-75 and CCL-247). All cell lines were tested and authenticated and were maintained in Eagle?s Minimum Essential Medium (EMEM; ATCC) and McCoy (Gibco) medium, respectively, supplemented with 10% fetal bovine serum (Gibco) and antibiotics; the cells were incubated at 37?C in a 5% CO_2 atmosphere. The cells were treated with TSA (Sigma, T8552-5MG) at 37?C for 24 and 48 h. IC_{50} concentrations were determined by plating 80,000 cells in 24-well dishes containing 0.5 ml of medium and incubating overnight at 37?C; TSA was added when cultures reached 80% confluence. Cells were washed with PBS and fixed with 70% ethanol at -20?C, then washed in PBS and stained with 1% crystal violet. After washing, the stain was solubilized in 33% acetic acid, and the absorbance was determined in an ELISA reader at 570 nm. The analyses were performed in triplicate in three independent experiments. The IC_{50}

values were calculated by linear regression analysis of the dose-response data using the points in the exponential region of the curve. The concentrations used for the TSA experiments were below the IC_{50}: 4.9 μM for HCT116 cells and 9.4 μM for WI-38 cells.

Immunofluorescence

WI-38 and HCT116 cells were grown on 18-mm glass coverslips (PEARL 7201) with EMEM (ATCC) and McCoy medium (Gibco), respectively, supplemented with 10% fetal bovine serum (Gibco) and antibiotics, and the cells were incubated at 37?C in a 5% CO_2 atmosphere. The cell lines were fixed with 2% paraformaldehyde (PFA) in 1X PBS (pH 7.4) for 10 min, followed by permeabilization in 0.4% IGEPAL (Sigma CA-630) in PBS for 10 min at room temperature and incubated with 0.5% BSA blocking buffer. For each pair of primary antibodies, the optimal order of addition was determined in preliminary experiments. With the exception of ACA, which was visualized with a fluorescein-conjugated secondary antibody, the fluorophores on the secondary antibodies were Alexa Fluor 488-conjugated (Invitrogen, Life Technologies, M?xico; A11001 anti-mouse, A11008 anti-rabbit, and A11078 anti-goat) for green fluorescence and Cy3-conjugated (Millipore, Temecula, CA, USA; AP124C anti-mouse and AP1132C anti-rabbit) for red fluorescence. Following incubation with the primary and secondary antibodies, DNA was stained with DAPI. The cells were observed by fluorescence microscopy using a Zeiss Axio Imager A2 (Carl Zeiss?, Germany); the images were analyzed using the software AxioVision 4.8 (Carl Zeiss?, Germany). The cells were also observed by laser confocal microscopy using a Zeiss LSM 710 Duo (Carl Zeiss?, Germany); the images were ana lyzed using the Zen 2008 software (Carl Zeiss?, Germany).

TSA treatment

Exponentially growing HCT116 and WI-38 cells were cultured on glass coverslips for 24 and 48 h in medium containing 1 μM/ml TSA (Sigma, T8552-5MG), with daily media changes. The cells were washed with PBS, fixed with PFA, and used for immunofluorescence analysis, as described above. For treated chromatin isolation, WI-38 and HCT116 cells were cultured on 100-mm culture plates and treated in the same manner as the cell cultures grown on glass coverslips. We used 1 μM TSA for 24 h and 48 h because at this concentration, we found a significant induction of CIN or centromeric chromatin remodeling. In addition, this concentration was below the IC_{50} for both cell lines.

Electrophoresis and immunoblotting

After treatment with TSA, WI-38 and HCT116 cells were harvested in lysis buffer containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% deoxycholate, and the cOmplete Protease Inhibitors Cocktail (Roche) and were then sonicated. Then, 30 μg of protein was loaded onto a denaturing 10-20% gradient or 16% sodium dodecyl sulfate (SDS)-polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. After incubation for 2 h in a PBS solution containing 5% albumin, the blots were exposed to the following primary antibodies: anti-HP1α (1:300); anti-HP1β (1:200); anti-H3 N-terminal (1:300); anti-H3K9me3 (1:250); anti-H3K9ac (1:250); and anti-CENP-A (1:200). The blots were incubated for 1 h at room temperature with the following horseradish peroxidase-conjugated secondary antibodies: goat anti-mouse IgG (1:10,000 Zymed); goat anti-rabbit IgG (1:15,000 Santa Cruz Biotechnology, Inc.); and chick anti-goat IgG (1:15,000 Chemicon International). The signal was subsequently detected by chemiluminescence (ECL kit from

Millipore, USA) on Kodak X-Omat film. For the negative control, the primary antibody was omitted.

Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed using the OneDay ChIP kit (Diagenode, NJ, USA, KchonedIP-180), following the manufacturer?s instructions. For all experiments, at least two chromatin preparations from independent controls and TSA-treated cells were analyzed. To obtain mitotic and interphase cell chromatin, control and TSA-treated cells were exposed to 2 μg/ml nocodazole for 12 h; the mitotic cells were isolated by the shake-off method, and fluorescence-activated cell sorting (FACS) was used to select the population with 90% enrichment of mitotic cells. Using this method, the interphase cells remained on the culture plates and were harvested separately from the mitotic cells. Chromatin from each cell population was fixed with 1% formaldehyde, and the cells were counted to ensure that $1x10^6$ cells were used for each IP. The chromatin was then extracted, and ChIP was performed following the manufacturer?s instructions. As a negative control, we used a normal rabbit IgG antibody (sc-2027, Santa Cruz Biotechnology, USA).

The obtained results represent experiments from three separate amplifications that were used to calculate the standard deviation. To balance any difference in the amounts of ChIP products and input for qPCR, the amplification efficiency (AE) was calculated to within 10% of the input. The fold of the enrichment was calculated from the AE of specific experimental amplicons against the AE of the background IgG amplicon, which was amplified in triplicate by a fast optical 96-well qPCR reaction plate (Applied Biosystems). The qPCR reaction was performed using Thermo Maxima SYBR Green/ROX 1 PCR Master Mix (Thermo Scientific, K0222) with a StepOnePlus Real?Time PCR System (Applied Biosystems, 4376600). Total H3 immunoprecipitation was used to calibrate the increase in enrichment generated by chromosome duplication during mitosis.

ChIP primers

The primers used for the ChIP qPCR analysis were as follows: 5′-TCGTTCCCAAAGTCCTCCTGTTTC-3′ (Fwd) and 5′-TCCGCAGCCGCCTGGTTC-3′ (Rev) for the GAPDH promoter; 5′-AGCCCTTCCCGCTCTTCTGTT-3′ (Fwd) and 5′-CGGCAGAGACGTAAGACTGGCAAA-3′ (Rev) for the WIF1 promoter; 5′-ATCGAATGGAAATGAAAGGAGTCA-3′ (Fwd) and 5′-GACCATTGGATGATTGCAGTCA-3′ (Rev) for human chromosome 1 juxtacentromeric satellite-2 (Abcam, ab85781); 5′-AAGGTCAATGGCAGAAAAGAA-3′ and 5′-CAACGAAGGCCACAAGATGTC-3′ (Abcam, ab85782) for human chromosome 1 centromeric satellite-α; and 5′-GAAGTTTCTGAGAATGCTTCTG-3′ (Fwd) and 5′-CTCACAGAGTTGAACCTTCC-3′ (Rev) for the satellite-α 175-bp monomers.

Chromosome spread and counting

WI-38 and HCT116 cells were cultured on 22x22-mm glass coverslips until 70% confluence was reached; the cells were then treated with TSA for 24 or 48 h. The culture medium was removed and replaced with fresh medium after 24 h. The cells were treated for 3 h with 80 ng/ml colcemid (KaryoMAX GIBCO, USA 15210-040) to induce mitotic arrest and then incubated for 30 min at 37?C in hypotonic buffer (75 mM KCl) that had been pre-warmed to 37?C. The cells were fixed with three 2-minute washes in a 3:1 methanol:acetic acid solution

and air-dried. The G banding standard protocol was performed with trypsin-Giemsa solution to stain mitotic chromosomes. For each condition, a certified cytogeneticist evaluated 50 metaphases in duplicate.

Statistical analyses

Statistical significance was determined using Student?s t-test or one-way analysis of variance (ANOVA). All of the results are expressed as the mean ? SEM, and we used a significance value of $p < 0.05$. We performed Levene?s test to compare the significance of the control versus the 24- and 48-h TSA treatments or the 24- versus the 48-h TSA treatment, with a significance value of $p < 0.05$ in the metaphase counting analysis. Statistical analysis was performed using the GraphPad Prism 5 software?.

Abbreviations

ACA, Anti-centromere antibody; CCAN, Constitutive centromere-associated network; CENP-A, Centromeric protein A; ChIP, Chromatin immunoprecipitation; CIN, Chromosome instability; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HDAC, Histone deacetylase; HDACi, Histone deacetylase inhibitor; HP1, Heterochromatin protein 1; KL1, Katanin-like 1; KMN network, KL1, Mis12, and Ndc80 complex; Mis12, Minichromosome instability 12; MIND kinetochore complex component; Mis14, Minichromosome instability 14; kinetochore-associated protein NSL1; ncRNAs, Non-coding RNAs; Ndc80, Kinetochore protein NDC80 homolog; TSA, Trichostatin A; WIF1, WNT inhibitor factor 1

Competing interests

The authors declare that they have no competing interests.

Authors? contributions

LAH designed the methods and experiments, analyzed the results, and drafted the manuscript. RG-B designed the methods and experiments, analyzed the results, and drafted the manuscript. ES-R designed the methods and experiments, performed ChIP assays, and analyzed the results. RQ-B performed Western blot assays and analysis. EF-M performed the cytogenetic work and analysis. JD-C performed expression analysis and data interpretation, contributed to critically revising the manuscript. VdC performed ChIP assays and analyzed the results. JM performed cell viability and Western blot assays and contributed to the analysis. AL-S performed immunofluorescence imaging. CC contributed to designing the experiments and critically revising the article for intellectual content. All authors read and approved the final manuscript.

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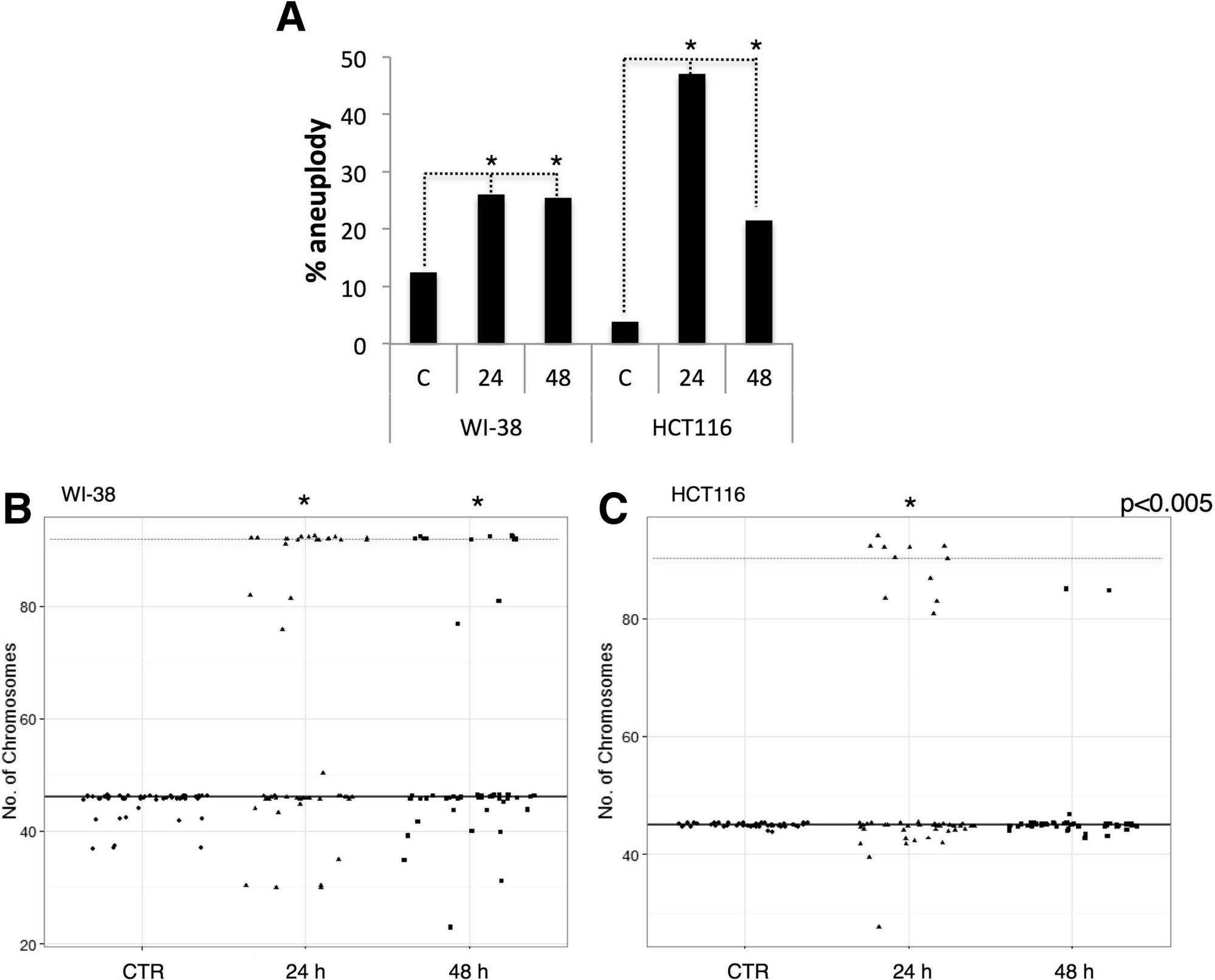
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No. of Chromosomes
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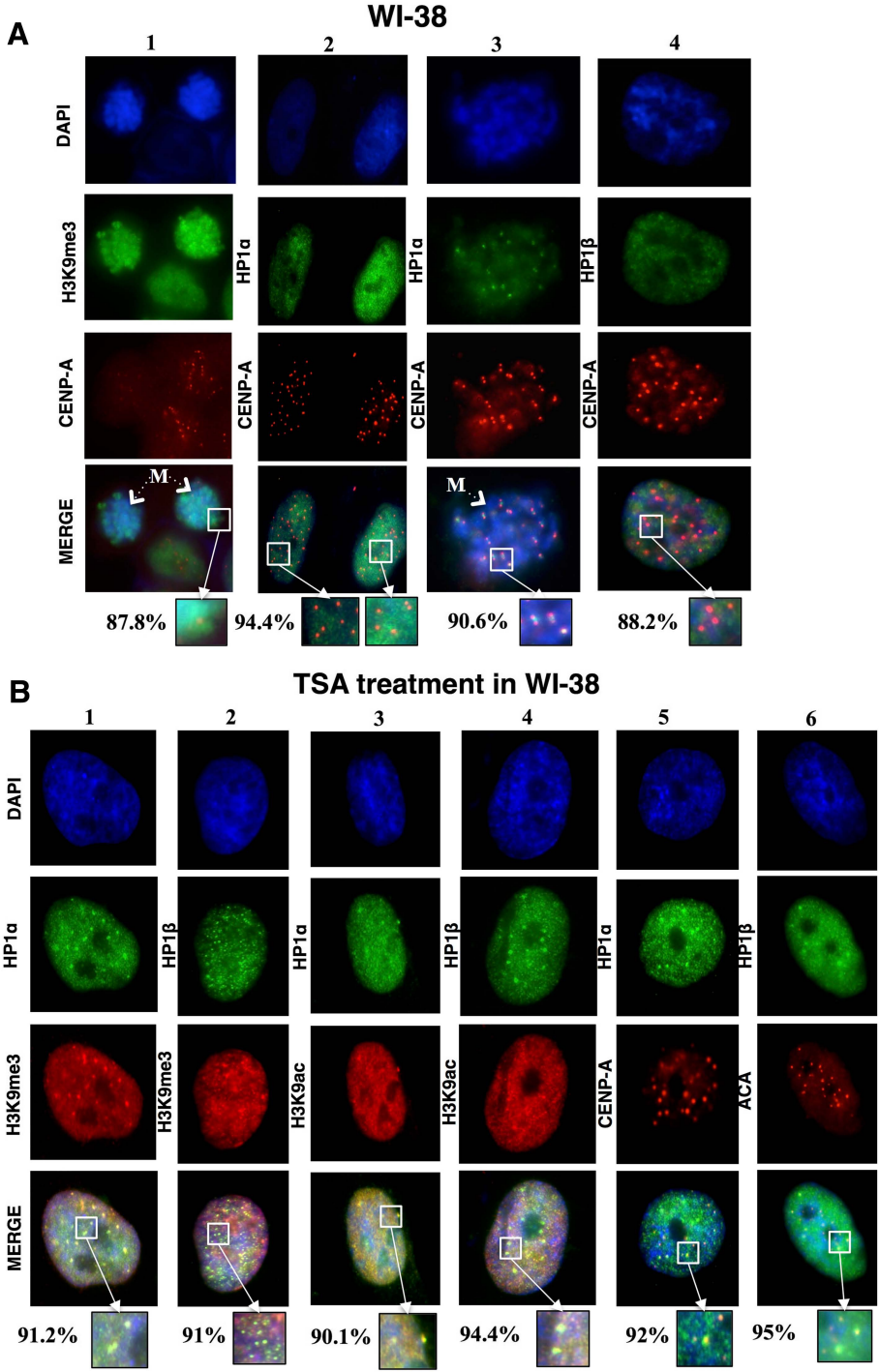
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3
4
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HP1α
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CENP-A
CENP-A
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CENP-A
MERGE
M
M
87.8%
94.4%
90.6%
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B
TSA treatment in WI-38
1
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DAPI
HP1α
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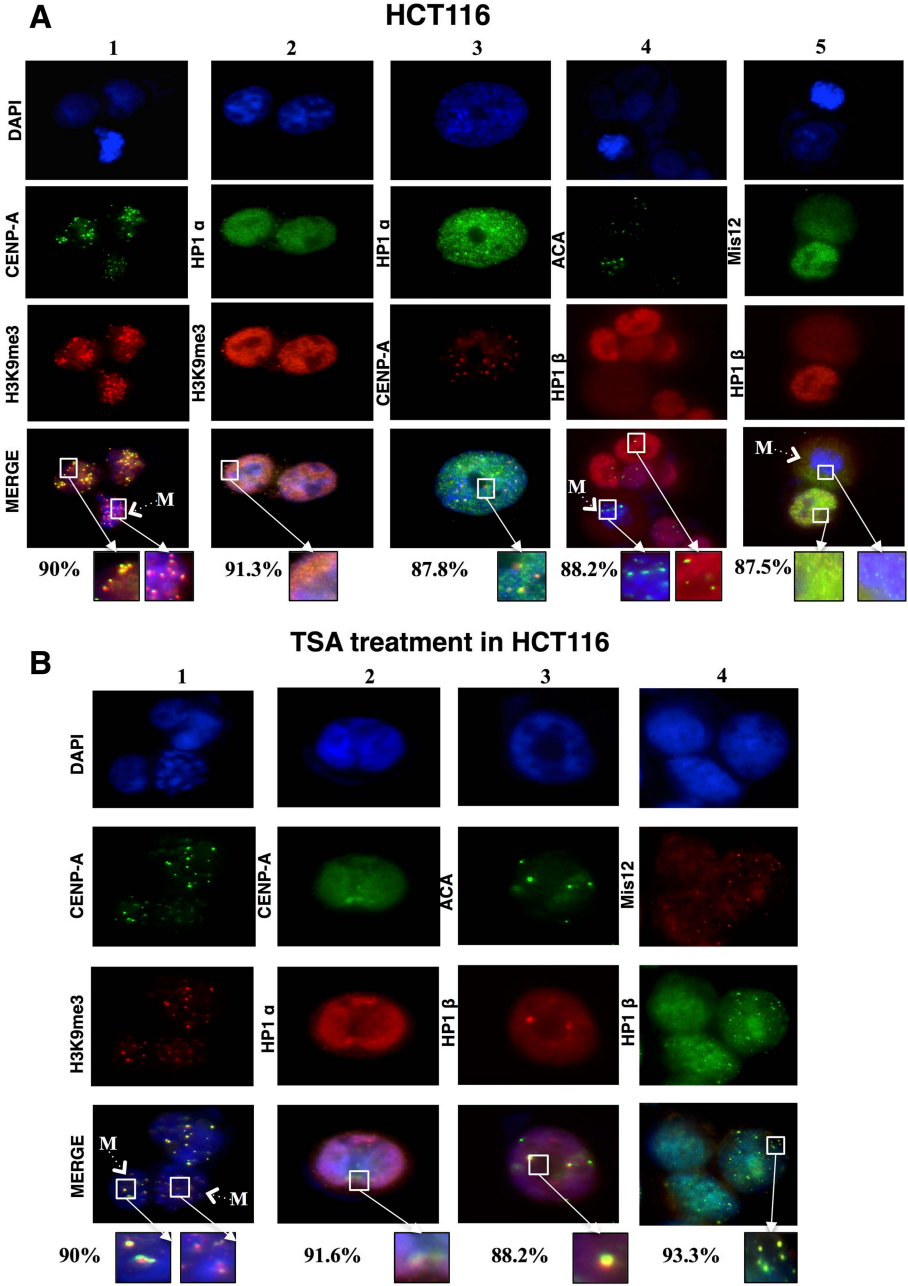
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ACA
Mis12
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CENP-A
HP1 β
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MERGE
M
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M
90%
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B
TSA treatment in HCT116
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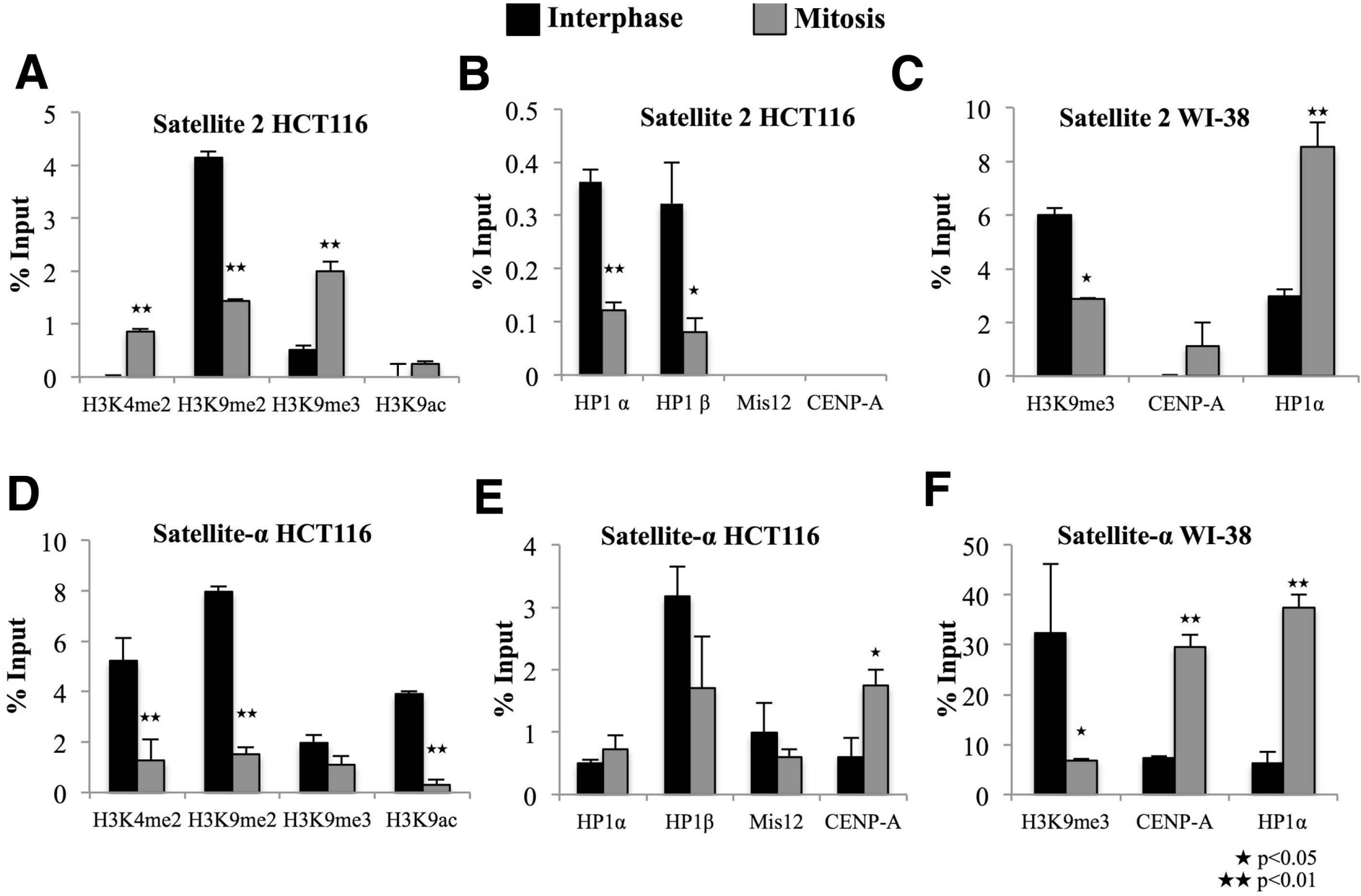
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HP1 β
Mis12
CENP-A
C
Satellite 2 WI-38
% Input
H3K9me3
CENP-A
HP1α
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H3K9me2
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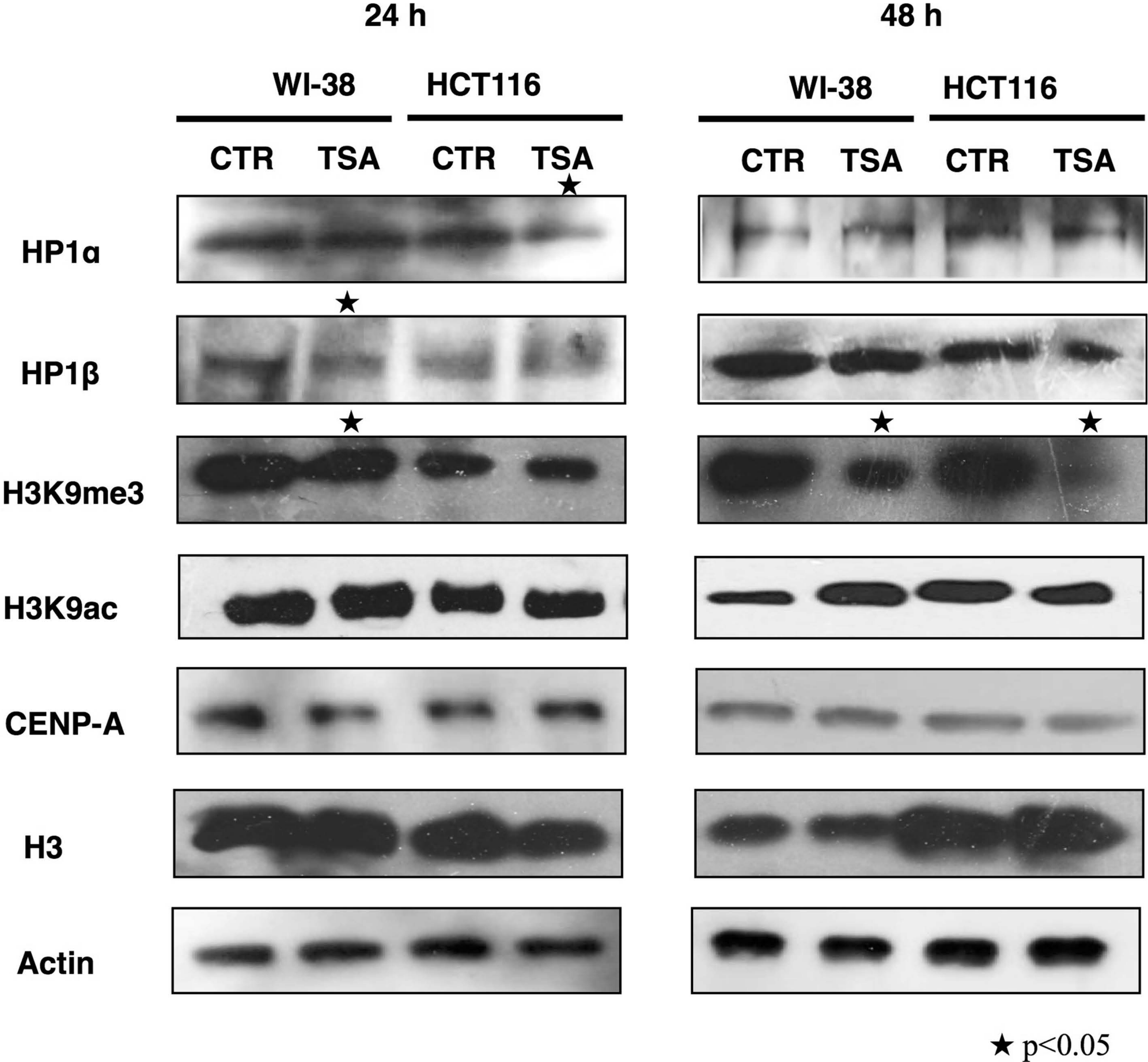
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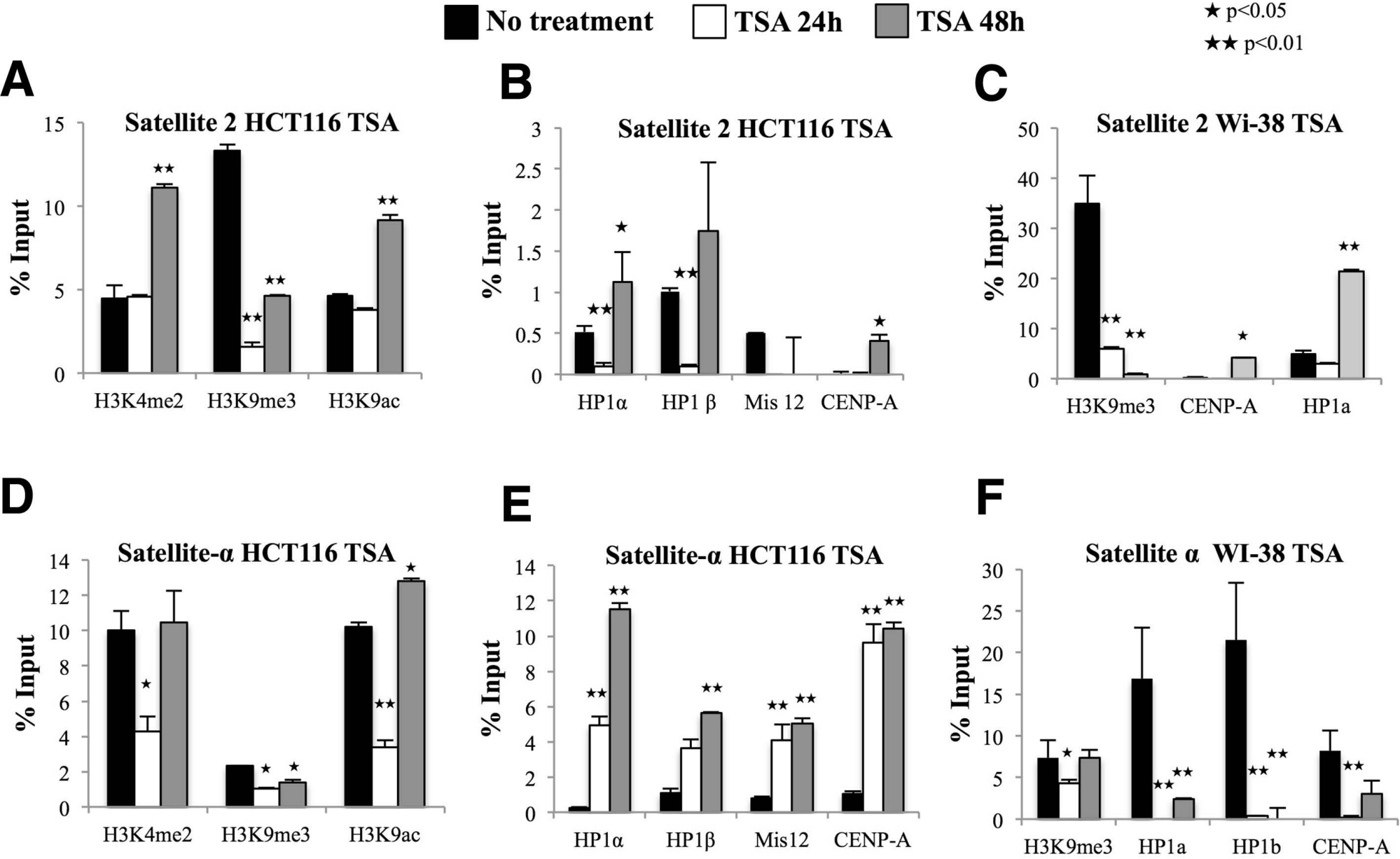
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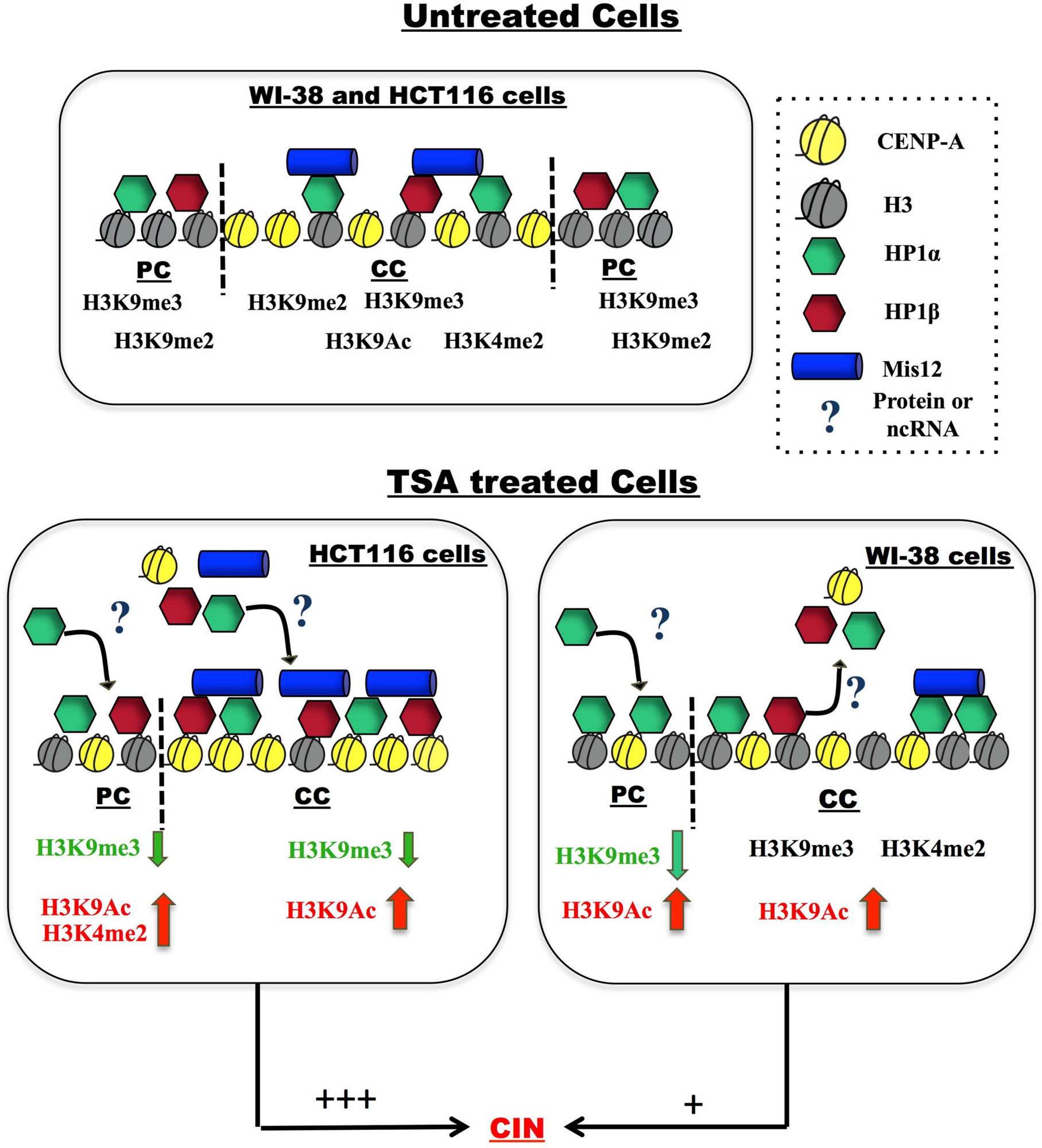
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H3K9me2
CENP-A
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HP1α
HP1β
Mis12
Protein or ncRNA
TSA treated Cells
HCT116 cells
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CC
H3K9me3
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Addtional files provided with this submission:

Additional file 1: Figure S1. Localization of H3K4me3, H3K9ac and Mis12 in WI-38 and HCT116 cells under basal conditions. (A) WI-38 cell fluorescent microscopy images of the localization of CENP-A with H3K4me2 (lane 1) and H3K9ac (lane 2). (B) Chromatin localization by fluorescent microscopy of CENP-A with H3K4me2 (lane 1) and H3K9ac (lane 2). DNA is marked with DAPI; the images show the most common distribution of the proteins after the analysis of 100 cells (%); the boxes represent a magnification of the immunofluorescence results; M, mitotic cell (615k)
http://www.celldiv.com/content/supplementary/s13008-014-0006-2-s1.jpeg

Additional file 2: Figure S2. Chromosome immunoprecipitation controls for active and repressive epigenetic marks during interphase (black) and mitosis (gray). (A) The GAPDH gene promoter was analyzed for open chromatin state marks. (B) The WIF1 gene promoter was analyzed for repressive marks. (C) The epigenetic landscape was observed using changes to the satellite-α repeat consensus sequence during the cell cycle. Changes in the enrichment of H3K4me2, H3K9me2/3, H3K9ac, HP1α, HP1β, Mis12 and CENP-A are shown. ˜ $p < 0.05$, ˜˜ $p < 0.01$ indicate significant differences between interphase and mitosis, as evaluated by Student's t-test (579k)
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Assembling pieces of the centromere epigenetics puzzle

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Key words: chromosome instability, kinetochore, DNA methylation, histone modifications

The centromere is a key region for cell division where the kinetochore assembles, recognizes and attaches to microtubules so that each sister chromatid can segregate to each daughter cell. The centromeric chromatin is a unique rigid chromatin state promoted by the presence of the histone H3 variant CENP-A, in which epigenetic histone modifications of both heterochromatin or euchromatin states and associated protein elements are present. Although DNA sequence is not regarded as important for the establishment of centromere chromatin, it has become clear that this structure is formed as a result of a highly regulated epigenetic event that leads to the recruitment and stability of kinetochore proteins. We describe an integrative model for epigenetic processes that conform regional chromatin interactions indispensable for the recruitment and stability of kinetochore proteins. If alterations of these chromatin regions occur, chromosomal instability is promoted, though segregation may still take place.

Introduction

The centromere is an essential locus that is required for the accurate segregation of genetic material during mitosis and meiosis. It serves as a platform upon which the kinetochore assembles; thus, it is a vital structure for mitotic spindle attachment that is required to guide chromosomal movements during cell division. Centromeres are vital in this task and serve a conserved role in many organisms; however, there is a surprising variability in the structure's sequence and organization among eukaryotes. Eukaryotic centromeres are characterized by the presence of a histone H3 variant known as centromeric protein A (CENP-A) in mammals.[1] Centromeres are located near or within repetitive DNA sequences, but sequence specificity has only been found in budding yeast. The budding yeast centromere is determined by a 125 bp DNA element that is assembled into a single Cse4 nucleosome, which captures a single microtubule.[2] Other organisms lack this sequence specificity in such a way that even centromeric DNA within the same organism varies among chromosomes.

In humans, centromeres are defined by AT-rich repeats called α satellites, which are based on a 171 bp monomer that is tandemly arranged into higher order arrays that extend from 0.2–5 Mb.[3] In human chromosomes, CENP-A is located on the α satellite DNA. However, its binding does not appear to be sequence-specific, as CENP-A is confined to just a portion of a given multi-mega base array. It also does not bind to scattered monomeric α satellite DNA or at inactive centromeres of naturally occurring dicentric human centromeres that contain two regions of α satellite DNA.[4,5] Neocentromeres are ectopic centromeres formed de novo typically at regions of non-repetitive DNA and may be formed locally at sequences near the centromere or hundreds of kilobases away from a deleted centromere in gene-poor regions with some repetitive sequence.[6]

The deposition of CENP-A to the centromere is mediated by the histone chaperone HJURP (Holliday junction recognition protein). In particular, HJURP's short N-terminal domain, is responsible for specific and stoichiometric binding to the CENP-A/H4 complex.[7,8] The expression of HJURP chaperone is tightly regulated since perturbation of its expression leads to mitotic defects.[9]

α satellite monomers contain a 17 bp motif, known as the CENP-B box that is recognized by centromere protein B (CENP-B).[10] CENP-B is important during de novo centromere assembly and for the proper phasing of centromeric nucleosomes, with the exception of human chromosome Y, which lacks CENP-B boxes at the α satellite and does not associate with CENP-B, although all other centromere proteins are recruited to this site.[11,12] Interestingly, in α satellites devoid of CENP-B boxes or those that contain mutated CENP-B boxes, euchromatic DNA and Y alphoid DNA do not form artificial chromosomes.[4,13] These findings suggest that CENP-B is essential for centromere formation and that α satellites are the preferred sequence for de novo CENP-A assembly. However, not all α satellite sequences can form de novo centromeres.[14]

To date, it has become increasingly clear that the chromatin environment has a relevant impact on centromere determination and establishment. Nevertheless, the necessary genomic and chromatin elements that establish and maintain the centromere are still unknown. Moreover, it has been suggested that DNA sequence alone is not always sufficient for centromere establishment or function, which supports theories postulating the involvement of epigenetic or chromatin based mechanisms.

In this review, we focus on centromere chromatin structure and its relationship with epigenetic regulation. We will also discuss centromere epigenetics as a cause of chromosomal instability

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(CIN) (for further review on centromere epigenetics, see refs. 15 and 16).

Centromere Chromatin and Epigenetics

One obstacle in the study of the chromatin environment at normal human centromeres is the nature of the repetitive sequence and shared sequence regions at non-homologous centromeres, making centromeres difficult to evaluate using molecular approaches for long-range chromatin organization analysis. It is known that centromeric chromatin in humans and flies are arranged as CENP-A nucleosomes that are interspersed with H3K4me2 nucleosomes.[17] CENP-A is a histone H3 variant found only at functional centromeres over which the kinetochore will eventually assemble;[18,19] it represents an epigenetic mark necessary for centromere activation. Recently, high-resolution structural data for a CENP-A/H4 heterotetramer have been reported, showing significant structural differences between CENP-A/H4 and the canonical H3-containing nucleosomes. Also, the crystal structure of the human CENP-A has been reported, showing specific differences with the H3 canonical histone, in particular the loop 1 contains two extra aminoacid residues (Arg 80 and Gly 81), which may stabilize centromere chromatin containing CENP-A.[20]

CENP-A-mediated differences at centromeric chromatin between CATD (CENP-A centromere targeting domain) and H4 beginning at interphase alter the global physical properties of the nucleosome, thus converting the nucleosome into a more rigid structure. This finding supports the existence of a CENP-A-driven self-assembly mechanism that mediates the maintenance of centromere identity.[21,22] These differences are essential for centromeric incorporation of CENP-A nucleosomes and reveal the contribution of the histone analog to a specialized chromatin structure at the centromere that differs from typical heterochromatin and euchromatin.

Chicken centromeres also contain CENP-A nucleosomes that are interspersed with H3K9me3 nucleosomes, although H3K4me2 is present in lower amounts.[23] In plants such as maize, centromeres are also enriched with H3K9me2 and H3K9me3, while exhibiting low enrichment levels of H3K4me2. Maize centromeric H3 domains are interspersed with H3K27me1 [24,25] and high resolution studies of these centromeres also revealed the presence of active genes within the region that are associated with H3K4me2 and acH4 enrichment, suggesting that centromeres are organized as euchromatic pockets surrounded by regions of heterochromatin enriched with H3K9me2 (**Fig. 1**).[26] Also, the H3K4me2 histone mark is an essential part of the chromatin environment of vertebrate kinetochore required for long-term maintenance and function.[27]

All together, these results suggest that centromeric chromatin varies among different species. In spite of these dissimilarities in histone modifications, some findings could be obscured due to resolution limitations caused by the nature of these regions.

Centromere inactivation (centromeres without CENP-A incorporation) is considered an epigenetic phenomenon. Therefore, inactive centromeres may adopt a chromatin configuration that is not compatible with centromere maintenance.[28] The chromatin environment in centromeres is different from the usual active and inactive chromatin configuration. It has been suggested by studying artificial chromosomes with integrated α satellite and TetO sequences constructed to tether chromatin-modifying proteins, both transcriptional activators and repressors, which disrupt centromere function on the artificial chromosomes. Thus, purely euchromatic and heterochromatic environments are incompatible with CENP-A assembly and maintenance.[29] It has also been suggested that neither large domains of euchromatic nor heterochromatic chromatin are required for the formation of functional neocentromeres.[30] A closer analysis using a transcriptional repressor, KAP1, targeted to the synthetic centromere of the artificial chromosome resulted in the depletion of CENP-C and CENP-H followed by depletion of CENP-A, thus providing more evidence for a hierarchy of centromere disassembly in which CENP-A is one of the last proteins to be removed from a centromere that is being inactivated.[31] However, it remains unclear whether the changes in chromatin promote CENP disassembly or if CENP removal allows heterochromatin to replace centromere chromatin. In this respect, it has been shown that histone H3-containing nucleosomes readily replace sites of CENP-A occupancy when this protein is depleted, but when overexpressed, CENP-A can replace sites of histone H3 assembly.[17] Considering that H3K9me3 is a marker that is thought to function as a limiting factor or antagonist of CENP-A chromatin, the overall ratio of chromatin containing CENP-A to heterochromatin may be more important than just the simple presence or absence of a particular modification.[32,33]

H3K27 methylation is another epigenetic mark that defines heterochromatin, and in some species of plants it has been associated with centromeres in which H3K27me2/me3 enrichment promotes centromere inactivation of two centromeres of a tricentric chromosome in wheat (**Fig. 1**).[34] Thus, the modification of histone marks in either a heterochromatic or euchromatic state abrogates the capacity of the centromere from generating a kinetochore. Therefore, modifications cause the loss of function to the region during mitosis, which supports the significance of an epigenetic model in the establishment and regulation of the unique centromeric chromatin (**Fig. 2A**).

The Kinetochore: A Macromolecular Protein Complex

In eukaryotes, accurate chromosome segregation requires each chromosome to interact appropriately with microtubules from the mitotic spindle, which provides the structural framework upon which chromosome segregation occurs. This interaction is mediated by a macromolecular complex known as the kinetochore, which is a structure composed of more than 90 proteins (**Fig. 1**).[35] The kinetochore must facilitate the interaction between centromeric chromatin and dynamic microtubules to ensure the bi-orientation of chromosomes on the metaphase plate and the segregation of sister chromatids at anaphase.[36]

During S phase, CENP-A is equally segregated between sister chromatids, but new CENP-A is not incorporated into

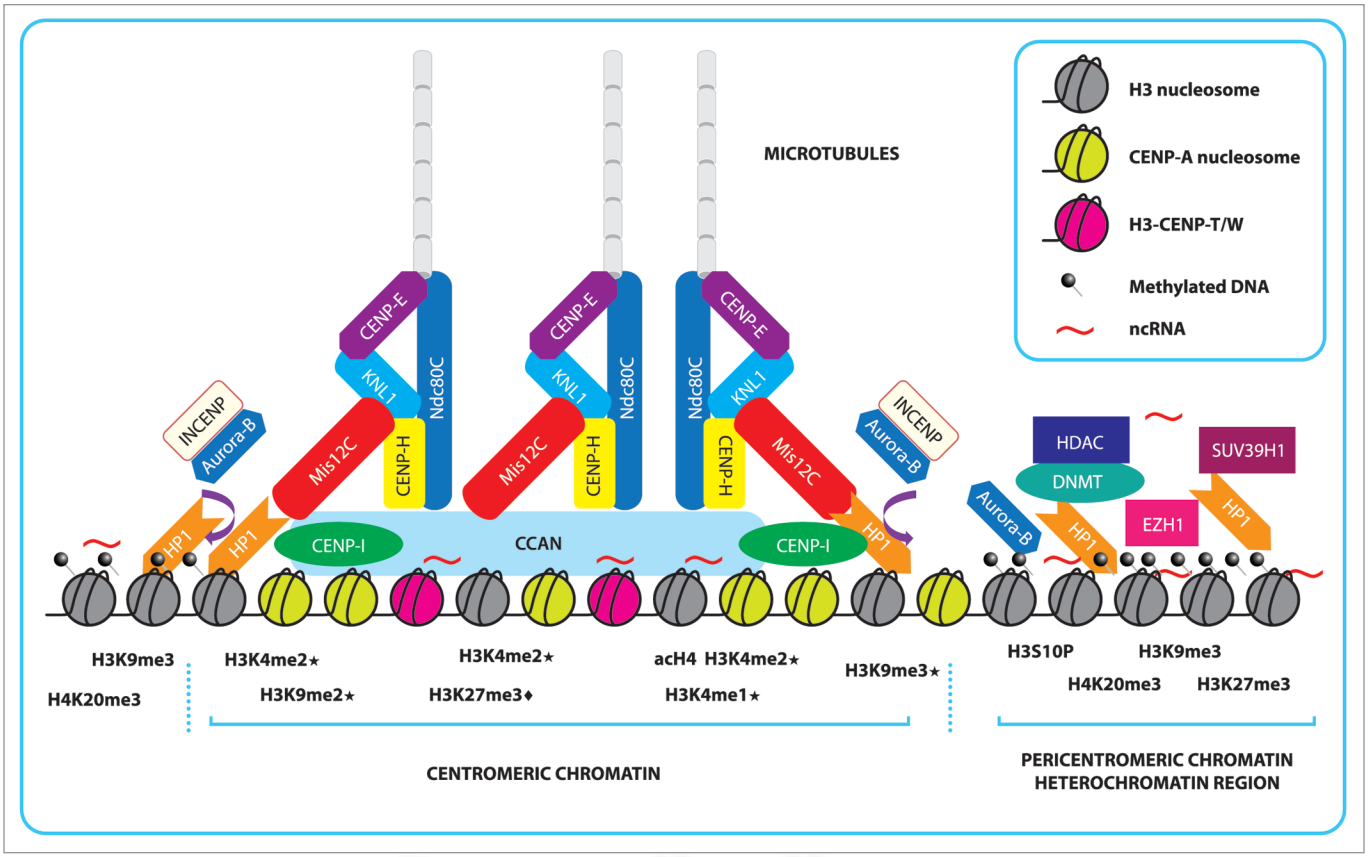

Figure 1. Schematic representation of centromeric and pericentromeric chromatin and the formation of an epigenetic complex that further shapes the kinetochore. Centromeric chromatin is shaped mainly by the presence of rigid CENP-A nucleosomes, interspersed H3-CENP-T/W complex and interspersed histone H3 nucleosomes, that present the repressive marks, H3K9me2/me3 and H3K27me1/3 (◆ indicates marks found only in plants species and ⋆ indicates marks reported in more than one species), active marks scattered throughout the region, H3k4me2 and acH4, and ncRNAs that form the foundation. This foundation then recruits the CCAN that will further link the other complexes that recruit the microtubules needed for chromosome segregation. In mammals, many of the CENP proteins that form the inner centromere are recruited by DNA interactions and histone mark-dependent proteins because there is evidence that Mis12C depends on HP1 for its incorporation into the kinetochore. Aurora-B/INCENP is recruited from methylated pericentromeric chromatin in order for Mis12C to interact with NDC80C (represented by the brown arrow). Aurora-B/INCENP dimer is removed from the kinetochore by phosphorylation of H3S10. Pericentromeric chromatin is mainly constitutive heterochromatin composed of repetitive satellite DNA that is heavily methylated and enriched with repressive marks, principally H3K9me3 and scattered H3K27me3, dependent on SUV39H1 and EZH1, which serve to recruit HP1, HDAC and DNMT. Pericentromeric chromatin is further stabilized and regulated by ncRNAs generated from these satellites regions.

centromeric chromatin until telophase and G1.[18] The significance of this abnormal timing remains elusive, but it has been suggested that this timing could represent a defense mechanism against misincorporation and the subsequent assembly of the kinetochore structures at non-centromeric sites caused by temporarily separating the incorporation of CENP-A from replication and the incorporation of other histones.[37] In some species, such as Drosophila, CENP-A alone is sufficient to ensure kinetochore formation; however, this is not true in human cells, and CENP-A alone is not sufficient for complete kinetochore assembly in mitosis.[38] Additional proteins are also required for the correct assembly. To date, many approaches have been attempted to identify core centromere components in mammals, and 15 proteins were identified and defined as the constitutive centromere associated network (CCAN).[1,35,39,40] It has been suggested based on several functional analyses that these proteins play a key structural role in forming a stable foundation for dynamic kinetochore assembly and for providing a proper environment for new CENP-A incorporation.[1,35] It was also suggested that CCAN might also function to directly control microtubule dynamics.[39]

Recently, the CENP-T/W complex has been shown to interact stably with histone H3-containing nucleosomes.[39] Although the precise molecular organization of the histone H3-CENP-T/W nucleosome population is not known, it has been suggested that they are interspersed closely with CENP-A nucleosomes (**Fig. 1**). The functional consequence of these assembly events would be an expansion of the histone H3-CENP-T/W compartment within post-replicative centromeric chromatin. The dynamic behavior of proteins within this compartment kinetically parallels the active establishment of the kinetochore complex. This suggests that the CENP-T/W complex plays a functional role in kinetochore formation following DNA replication.[41]

It has been shown that CCAN proteins remain associated with centromeric chromatin through the entire cell cycle. In

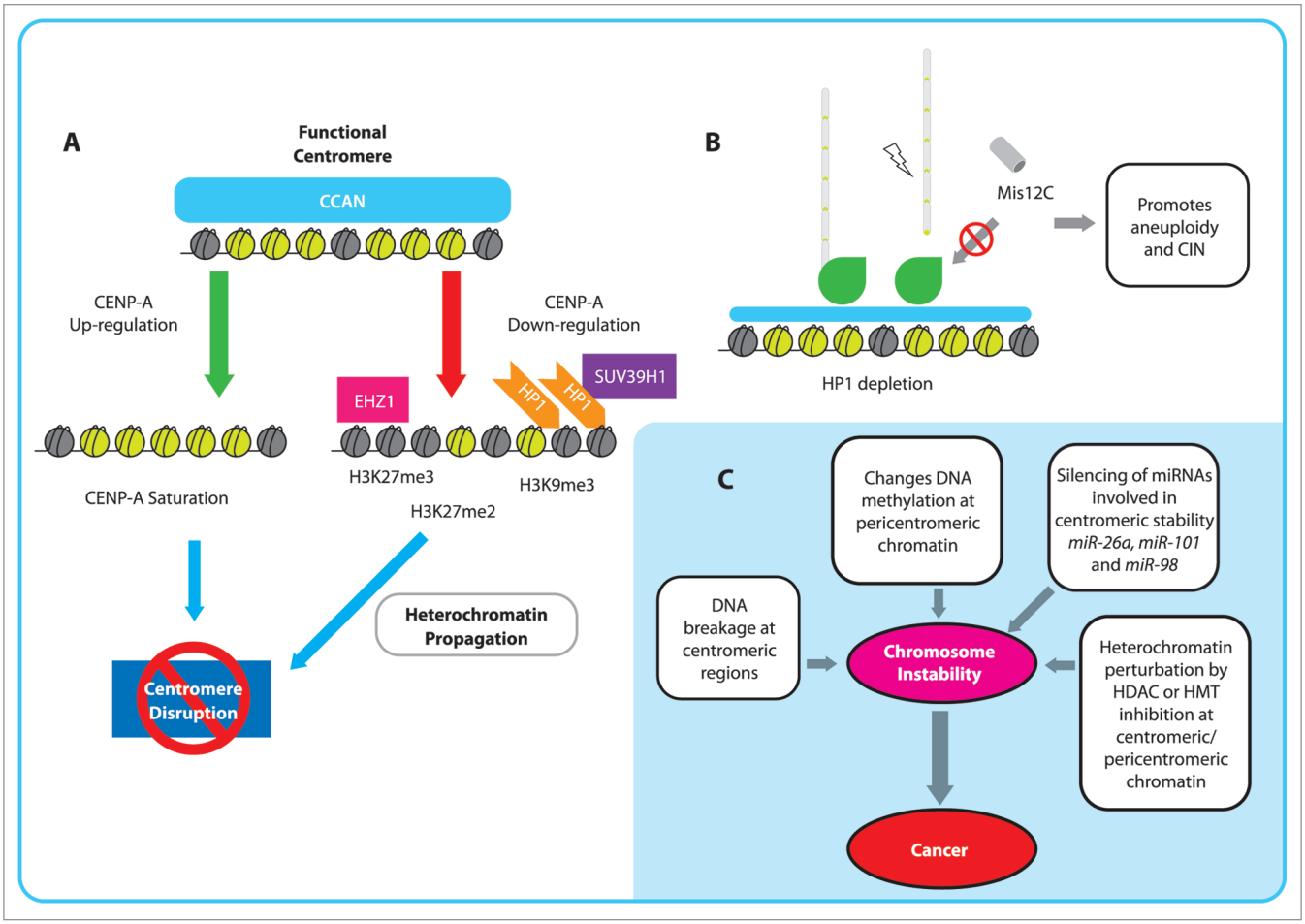

Figure 2. Epigenetic disruption of the centromere and implications for chromosomal instability (CIN). (A) Schematic representation of centromere disruption pathways by CENP-A upregulation and heterochromatization. (B) HP1 depletion causes that localization of Misc12C to the kinetochore is reduced, which may promote microtubule misincorporation and kinetochore unsteadiness generating aneuploidy and CIN. (C) Chromosomal instability generated by different mechanisms at centromeric and pericentromeric regions as a plausible early cause of cancer.

conjunction with CENP-A, they may form a stable environment for the assembly of the mitotic kinetochore structure. DNA binding activity or direct interaction with CENP-A has been observed for several CCAN proteins.[10,35,42]

The outer kinetochore plate and fibrous corona assemble upon entry into mitosis and contain proteins required for interactions with microtubules. These proteins include those with direct microtubule binding activity, such as the KL1, Mis12, Ndc80 complex (Ndc80C) (together forming the KMN network),[35] CENP-E and the Ska1 complex,[43] all of which are transient factors that modulate this interaction or monitor attachment status.[15]

Recent experiments on microtubule tension in human cells treated with taxol helped map the internal architecture of the kinetochore in the presence and absence of tension across kinetochore pairs. These studies identified surprising changes in the organization of the layer structure, where the absence of tension leads to a reduced distance between inner kinetochore proteins, such as CENP-C, and the microtubule interacting complex Ndc80. However, the localization of inner kinetochore proteins with respect to each other remained unchanged.[44] The reduction of tension across kinetochores also caused striking rearrangements of components of the KMN network, suggesting that some kinetochore-proteins complexes are more dependent than others on forces exerted by microtubule interactions.

Although the kinetochore appears as a stable structure during mitosis, it has recently been suggested that kinetochore proteins are highly dynamic through the cell cycle. Whereas the inner kinetochore proteins of the CCAN are present at the centromere in a constitutive manner throughout the cycle, some outer kinetochore proteins, such as the Mis12 complex (Mis12C) and KLN1, are recruited in G2.[45] Because this recruitment is suggested to prime centromere assembly, the assembly of remaining components occurs at prophase and prometaphase to generate the structure capable of binding to microtubules.

From the more than 90 proteins that contribute to kinetochore assembly, four groups have recently been suggested to prime centromere assembly given their known functions: linkers, scaffolds, chaperones and structural stabilizers.[15] However, a striking feature of the vertebrate kinetochore is the massive reorganization that takes place during mitosis. In a time period of less than one hour, the kinetochore recruits more than 40 mitotic components in a hierarchical manner and then subsequently

disassembles these proteins to return to an interphase state. It has been proposed that this process may be controlled by the presence of the nuclear envelope, which restricts proteins from the nucleus such that they are unable to associate with the kinetochore until nuclear envelope break down occurs.[46] However, recent work has demonstrated that at least some proteins are present within the nucleus at times when they are not localized at the kinetochores, suggesting that the formation of the kinetochore during mitosis is not necessarily blocked from assembly by the nuclear envelope.[45] Furthermore, post-translational modifications may regulate kinetochore formation. Recent work has demonstrated that the ubiquitin-like protein SUMO modifies CENP-I. When CENP-I is extensively SUMOylated, it is targeted for degradation. Thus, for this protein to become incorporated into the kinetochore during mitosis, the removal of the SUMO group by the SUMO protease SENP6 is required.[47]

Heterochromatin Protein 1: A Kinetochore Partner

Heterochromatin protein 1 (HP1) was first discovered in Drosophila as a dominant suppressor of position-effect variegation (PEV) and was later found to participate in the formation of compact heterochromatin in an array of pericentric heterochromatin.[48] Although initial studies demonstrated the role of HP1 in the formation of heterochromatin, especially in centromeric and pericentromeric regions, it has become increasingly evident that HP1 has multiple functions and is also present in actively transcribed euchromatic regions.[49] HP1 also plays a role in centromeric sister chromatid cohesion,[50] telomere maintenance[51] and DNA repair.[52] In humans, these functions are performed in a specific manner by each of the three subtypes of HP1 that have been identified: HP1α, Hp1β and HP1γ.[53,54]

HP1 binds to histone H3 that has been methylated at lysine 9 by SUV39H1 and, in turn, it recruits SUV39H1 to the DNA, which further propagates methylation along the chromatin.[55] This relationship between HP1 and SUV39H1 is conserved in their *Saccharomyces pombe* homologs, Swi6 and Clr4,[56] suggesting evolutionary conservation of this mechanism of heterochromatin formation. It has been demonstrated that Swi6 and other factors are required for the establishment of de novo centromeres, but not for their maintenance.[57]

The function of HP1 is highly important in the establishment, propagation and maintenance of constitutive heterochromatin,[58] especially at the pericentromeric region that has been demonstrated to be enriched in the H3K9me3 and H4K20me3 marks, hypoacetylated histones H3 and H4 and highly methylated regions along the satellites repeats.[59,60] Due to its juxtaposition next to centromeric chromatin, it has been suggested that the organization and stability of the pericentromeric region is crucial to ensuring correct chromosomal segregation during mitosis; therefore, this region is important for genome stability.[59,61] Increasing evidence has shown that the KMN network in humans and flies is a binding partner of HP1, where HP1 may participate in recruiting and directing Mis12C to the centromere during interphase (**Fig. 1**).[62-64] It is also suggested that the recruitment of Mis12 protein is performed by HP1.[63] Each HP1 subunit consists of a chromodomain, which binds to methylated H3K9, a hinge implicated in the regulation of the protein/DNA/RNA interactions, and a chromoshadow domain, which is responsible for dimerization and for the interactions with binding partners containing the defined motifs comprise of the PXVXL consensus.[65] These properties of the HP1 proteins give rise to its function as an adaptor that enables other proteins to interact with chromatin. Recently, in vitro analyses have suggested that Mis12C dimerizes with HP1, but that its interaction with the PVIHL motif located at residues 209–213 of protein NSL1 is largely responsible for binding to the PXVXL consensus.[62,66]

Moreover, these results give rise to the following question. If H3K9me3 functions as a boundary marker or an antagonizing marker for CENP-A,[33] why does a protein that is recruited to the centromeric chromatin in a Suv39 h-dependent manner play a major role in directing the Mis12C complex to the kinetochore? Is HP1 involvement regulated in a cycle-dependent manner? Even though enrichment of H3K9me2 and H3K9me3 has been found in the centromere, other marks, such as H3K4me2 and H3K27me3, have been found to be interspersed among centromeres, as previously discussed. In vivo microscopic analyses have demonstrated that human HP1α and HP1β localization has a specific role at different times during the cell cycle. Thus, the localization of human HP1α and HP1β to centromeric heterochromatin at interphase and metaphase is exchanged. Specifically, while in metaphase, HP1β (which is preferentially found at centromeric chromatin) is replaced by HP1α (which is typically located at pericentric and telomeric chromatin). These exchanges are mediated by differences in HP1 chromoshadow domain sequence.[67] Recently, in vitro protein interaction analyses demonstrated that the DSN1-NSL1 dimer is a crucial binding partner for HP1, Ndc80C and KNL1C. Therefore, HP1α and Ndc80C are competitive binders of Mis12C, suggesting that they have either identical or overlapping binding sites.[66] Hence, for Ndc80C to localize to the kinetochore, it is necessary to displace most of the HP1α from Mis12C. It is clear that this exchange must occur rapidly and in a coordinated fashion during mitosis if chromosome segregation is to occur.[68] The disruption or abrogation of HP1 is believed to lead to the formation of some tumors, and it may also be possible that the absence of HP1 may lead to the loss of incorporation of Mis12C into the kinetochore. Therefore, centromere structure and kinetochore relaxation further promote CIN[69] (**Fig. 2B**).

It has been proposed that INCENP localizes to the Aurora-B/AIM-1 complex in heterochromatin, where its kinase activity is required for the dissociation of HP1 from chromosome arms in mitotic cells. This process is mediated by the phosphorylation of H3S10. It has also been shown that Aurora-B/AIM1 regulates the localization of SUV39H.[70] These results indicate that Aurora-B/AIM1 is necessary for the regulated histone modifications involved in the binding of HP1 to centromere chromatin during mitosis. However, it is not sufficient by itself to completely regulate the localization of HP1 subtypes during mitosis, implying that other mechanisms are necessary for the event. Moreover, strong evidence suggests that the HP1α localized at the mitotic centromeric heterochromatin contributes to the stability of sister

chromatid cohesion or activation of the kinetochore checkpoint. Reduced HP1α at the metaphase centromere may be a cause of chromosomal instability in cancer cells.[71,72] Centromeres and kinetochore formation seem to be directly or indirectly regulated by epigenetic mechanisms in most eukaryotes. In particular, this hypothesis is supported by the fact that several kinetochore components are related to epigenetic factors. This finding suggests the deregulation epigenetic components at the kinetochore complex could lead to chromosome defects and the development of chromosomal instability.

Chromosomal Instability: Epigenetics and Centromere Involvement

Two major models of genetic instability have been described. The first model is associated with microsatellite instability (MIN), and the second describes CIN. Microsatellites are repeated sequences of DNA that vary in length among individuals but over the course of an individual's lifetime, the lengths remain constant. Abnormally long or short microsatellites of DNA are referred as MIN. This phenomenon may be associated with diseases such as cancer. Approximately 15% of colorectal cancers present a MIN phenotype.[73] Meanwhile, CIN can develop in two principal ways. One is related to abnormalities in chromosome number, which mainly occur due to the gain or loss of the whole chromosome (W-CIN); the other is associated with an abnormal organization of the chromosome (S-CIN). This faulty organization is characterized by structural changes of the chromosomes by gain, loss or translocation of chromosome fragments, which are mainly caused by breakage. This phenomenon is associated with mitotic errors that allow chromosome missegregation, which can lead to oncogenesis.[74] In particular, labile regions of DNA, known as chromosomal fragile sites, are heritable and contain specific loci that are especially prone to breakage and rearrangement.[75] These sites lead to rearrangements of large genomic regions by the insertions, deletions or translocations deriving in S-CIN. Thus, the CIN phenomenon promotes the expression of altered oncogenes, the loss of tumor suppressor genes and the deletion of several other genes, such as those encoding microRNAs.[76]

In cancer, CIN is associated with poor prognosis in solid tumors and results in phenotypic variations that promote drug resistance.[77] CIN is a likely cause of tumor cell heterogeneity.[77] One of the main hypotheses is that these tumors rapidly acquire multidrug resistance, leading to lower rates of disease-free survival.

It was previously believed that genomic instability develops from strictly genetic mechanisms. However, there is now some evidence that epigenetic processes are also involved. The perturbation of the epigenetic balance may lead to alterations in gene expression and CIN, resulting in cellular transformation and cancer development (**Fig. 2B and C**).[78] DNA methylation, one of the primary epigenetic processes, is performed by the addition of a methyl group to the cytosine base of DNA to form 5-methyl-cytosine (for more information, see refs. 79 and 80). DNA methylation has been linked to the silencing of imprinting genes, X-chromosome inactivation and repetitive elements, leading to chromosome stability.[81] A growing number of human diseases linked to epigenetic defects are currently being studied. In particular, DNA methylation in cancer gained attention with studies reporting that there is local hypermethylation, mainly at CpG islands and global hypomethylation in cancer.[82,83] This phenomenon is present in the majority of cancers, suggesting that it plays an important role in oncogenic transformation.[83-85] Recently, it has been suggested that epigenetic instability represents a theoretical alternative to genetic instability in cancer.[86] Several cancers exhibit high degrees of DNA methylation. The difference in the methylation degree gave rise to a tumor subclassification called a CpG island methylator phenotype or CIMP. This classification represents a clinically and etiologically distinct group of tumors that is characterized by epigenetic instability.[87]

It has been reported that some colon cancers that demonstrate genetic instability do not exhibit MIN nor CIN, but do present the CIMP phenotype, suggesting the importance of epigenetic deregulation in cancer.[88] The CIMP-positive tumors are clinically distinct from those in the rest of the patient population. These differences could help improve the understanding of the tumor's origins. Several human genetic disorders have been linked to epigenetic deregulation, such as Prader-Willi, Angelman and Fragile X syndrome,[89] but only one human genetic disease is currently known to arise from a germline mutation, namely the immunodeficiency, centromeric region instability and facial anomalies syndrome (ICF).[90] ICF is an autosomal recessive disease that involves spontaneous CIN and immunodeficiency. The molecular basis for this disease is related to the mutation of DNA methyltransferase 3B (*DNMT3B*). This disease is extremely rare and is characterized by profound immunodeficiency due to the absence of or significant reduction in the expression of at least two immunoglobulin isotypes.[91]

Diseases can have many causes, from a single nucleotide modification to structural changes at the chromosome level, genetic damage, chromosomal rearrangements, mutations or germinal and somatic deficiencies in genes associated with DNA repair.[92] However, the study of epigenetic components has become more relevant in the last two decades because of its implications in multiple cellular processes, such as transcriptional regulation, differentiation and genomic protection against viral infections. Deregulation of any of these processes is associated with the development of syndromes and diseases such as cancer.[93]

An epigenetic component implied in CIN is CENP-A. This protein has been reported to be overexpressed in primary colorectal cancer.[94] In particular, diminished levels of pRb have been associated with the CENP-A overexpression and the induction of hypodiploid aneuploidy. Bioinformatics analysis at the 5' upstream sequence of the human *CENPA* gene revealed a potential E2F motif. This observation could explain the increase of CENP-A transcript in pRb-depleted cells.[95,96] A novel hypothesis is that CENP-A overexpression might cause spreading along the centromere heterochromatin through chromosome arms and interfere with the correct kinetochore complex assembly, this being a cause of genomic instability.[96]

It has also been reported that the overexpression and mislocalization of the CENP-A chaperone HJURP has been observed in lung cancer cell lines. These observations were associated with CIN and immortality of cancer cells.[9] In clinical trials, the overexpression of HJURP was associated with an increased sensitivity to radiotherapy but with a decreased survival in patients with breast cancers.[97]

DNA methylation is another epigenetic process associated with neoplastic disorders in many reports.[98] Global hypomethylation and local hypermethylation are broadly represented in cancer, and it is suggested that they might promote CIN as a result of gene expression deregulation.[83,86,98,99] The gain or loss of histone marks is associated with gene silencing at a local level[100] and chromatin rearrangements at a global level; both have a profound effect on the local function of the cell and can promote certain diseases. Examples of histone modifying proteins include the deregulation of histone methyltransferases, such as EZH2, and the downregulation of HP1, either by the loss of H3K9me3 or gene mutations. Additionally, the inhibition or reduction of HDAC levels at the centromeric region promotes the accumulation of H3K9ac and H3K14ac, which is expected to cause a loss of chromosomal segregation due to the acetylation-dependent inhibition of H3K9me3 (**Fig. 2C**).[69,101,102]

Therefore, it is clear that aberrant changes that modify chromatin structure are important for chromosome stability. Particularly, the co-existence of epigenetic components, such as H3K9me3 and HP1, which are highly enriched at pericentromeric chromatin regions and satellites, may strengthen the hypothesis that HP1 is not only a component that helps establish heterochromatin (thereby making it a protein that is associated with genetic silencing) but is also an important scaffold protein that is involved in kinetochore assembly. If HP1 is disrupted, studies suggest CIN is promoted; therefore, HP1 disruption may lead to cancer.[51,62]

Non-Coding RNA: Covering the Centromere

Non-coding RNA (ncRNA) has become an increasingly studied field of research in epigenetic studies, is believed to be involved in chromatin regulation at the level of the centromeres and the kinetochore.[103] There are many types of ncRNAs including the following: small interfering RNAs (siRNAs), microRNAs (miRNAs) and long ncRNAs. The expression of these RNAs has a direct effect on chromosomal architecture. Moreover, the transcription of siRNA from the satellite regions that form and stabilize pericentromeric and centromeric DNA has been reported, and this transcription is conserved in species such as Drosophila, mice and humans. Although their function is not yet clear, it is possible that they are involved in the establishment and regulation of chromatin structure in pericentromeric and centromeric regions.

Fission yeast centromeres resemble those of human in their organization and epigenetic nature, but provide a simplified model for the study of complex regional centromeres. The discovery that ncRNA, specifically iRNA, directs chromatin modifying activities to outer repeats of fission yeast centromeres, become a key precedent concerning heterochromatin formation.[104] The modulation of such heterochromatin formation is engaged by RNA-induced silencing complexes (RISCs) which downregulate homologous gene expression.[105] Its effect in transcriptional gene silencing has been intensively studied in *Schizosaccharomyces pombe*, where members of the RNAi pathway such as: Dicer, Argonaute (ago1), Chp1 and the RNA-dependent RNA polymerase (Rdp1) plays an important role. Evidence suggest that at sites of active RNAi, chromatin-based activities drives the formation of self-enforcing loop coupling siRNA biogenesis to promote H3K9me2 expansion, where RNAPII transcription of centromeric repeats together with chromodomain proteins bound to H3K9me2 mediate recruitment of silencing factors.[106] Interestingly, in fission yeast, RNAPII transcribe centromeric pre-siRNAs at heterochromatin, which act in transcriptional gene silencing. These transcripts require a particular subunit of RNAPII, Rbp7, for initiation of centromeric siRNA precursor transcription that will drive centromeric chromatin silencing.[107]

For efficient production of centromere repeat homologous to be attained, siRNA is followed by the loading of RNA-induced transcriptional silencing effector complexes (RITS). By means of a component of RITS complex, Chp1, that contain a chromodomain that binds to H3K9me2 modification, RITS associates with heterochromatin repeats.[108] RITS, along with its encapsulated ss-siRNA, might be targeted to homologous chromatin via siRNA-nacent transcript complementarity. The siRNA response is amplified by the RNA-dependent RNA polymerase complex (RDRC), which promotes further dsRNA, and thus, more siRNA synthesis.[108]

Recently, a surveillance mechanism was proposed, where small RNA degradation products are generated independently of Dicer or RDRC activities that becomes loaded to Ago1. Such Ago1-priRNAs complexes engage homologous centromeric transcripts and recruit Clr4 to promote basal H3K9me2 levels that are sufficient to induce RNAi-mediated heterochromatin establishment.[109] However, similar Dicer-independent Ago mediated small regulatory RNA have been characterized in zebrafish and mice.[110,111]

Moreover, centromeric ncRNA transcript might have different functions rather than heterochromatin propagation and silencing. There is evidence that suggest that transcripts homologous to centromere-associated DNAs are detected in various organisms, since then centromeric transcripts have been found to associate with kinetochore proteins.[112-114] In fission yeast, it has been reported that Hrp1, an ATP-dependent remodeling factor (orthologous to *S. cerevisiae* chd1), affects CENP-A deposition.[115] In *S. pombe*, Hrp1 facilitates the assembly of CENP-A analogous to siRNA derived of outer repeats transcripts drive heterochromatin formation. Thus, Hrp1 facilitates the assembly of CENP-A chromatin, and becomes essential when MIs6 or CENP-A function is impaired. Also, Hrp1 acts at a subset of gene promoters to dissemble histone H3-containing nucleosomes close to the transcription start sites, allowing the deposition of CENP-A-nucleosome at the promoter of some genes in the centromere.[116] Such remodeling resembles the transcription-coupled replacement of H3.1, H2A with H3.3 and H2A.Z in metazoans.[117] The

fact that Hrp1 promotes H3-nucleosome eviction suggests that similar remodeling processes may occur at RNAPII promoters within centromeres. Therefore, it is possible that transcription within centromeres occurs merely as a consequence of having RNAPII promoters that might contribute to promote CENP-A deposition.[116] It is also possible that the discrete transcripts of 0.5 kb detected at centromere repeats could be processed into a specific class of small RNAs that have a roll in CENP-A chromatin formation and kinetochore assembly analogous to the siRNA derived from outer repeats transcripts drive heterochromatin formation. Interestingly, in *S. cerevisiae* it has been shown that CENP-A also tends to associate with some RNAPII promoters where RNAPII binding is enriched; however, not clear association with CENP-A depositions has been observed.[118]

In humans, recent analyses have found that the human chromatin remodeling factor FACT, whose function is implicated in transcription, interacts with affinity purified CENP-A chromatin.[1] Moreover, depletion of FACT was found to impair incorporation of newly synthesized CENP-A in chicken cells.[40] Taken together, these results might give evidence of possible transcripts regulated by FACT acting at centromere chromatin.

Hence, if centromeric chromatin in fission yeast and other organisms contains RNPII transcripts that are implicated in many processes at these regions, it is natural to think that such transcripts can be modulated through cell cycle and development. Such an example in development has been recently explored in mammals, where pericentromeric chromatin flanks the centromere; these regions are important in the stability of the centromere and in kinetochore formation. It has been observed that pericentromeric regions in mice, specifically the major satellite region, present high peaks of transcription from the zygote genome followed by rapid downregulation, which coincides with the organization of the chromocenters. Particularly, the paternal genome forward strand DNA was predominantly expressed, suggesting that this paternal bias might reflect the asymmetry in histone marks between maternal and paternal pericentric domains.[119] This evidence strongly supports the idea that pericentric satellites have an important functional role during embryo development.

In cancer, it has been reported that the ncRNAs transcripts are expressed in repetitive satellite regions in some solid tumors, suggesting that their deregulation could be involved in the carcinogenesis process.[120]

Alternatively, extensive studies of miRNAs have revealed their importance in the regulation of multiple gene targets, including key epigenetic components. Interestingly, the overexpression of EZH2 deregulates multiple miRNAs, especially miR-26a, miR-101 and miR-98, in many types of cancers, such as lung, gastric and nasopharyngeal cancer and glioblastoma. Given that ncRNAs are shown to play a key role in establishing the characteristic heterochromatin epigenetic patterns necessary for centromere regions, they may function as structural components.[120]

Conclusion and Final Remarks

Centromeric chromatin regions are highly structured and regulated during the cell cycle, leading to the formation of one of the most complex macromolecular machineries that function to maintain genetic information for progeny. It is clear that this huge assembly of proteins must be regulated at many levels and is not dependent on DNA sequence. Centromeres must be an epigenetically regulated region that is needed to generate a unique rigid chromatin state, as defined by the CENP-A nucleosome surrounded by a constitutive heterochromatin region. The conjunction of the centromeric chromatin and euchromatin and heterochromatin is indispensable for the recruitment and stability of kinetochore proteins. If alterations of these chromatin regions occur, chromosomal instability is promoted, although segregation may still take place. This disruption could occur in the early stages of cancer development, supporting the fact that epigenetic mechanisms might be one of the first steps of carcinogenesis. However, there are still many open questions regarding centromere epigenetics and how it regulates kinetochore assembly as well as how the communication between centromeric and pericentromeric chromatin is established.

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REVIEW ARTICLE

The role of the histone demethylase KDM4A in cancer

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Histone posttranslational modifications are important components of epigenetic regulation. One extensively studied modification is the methylation of lysine residues. These modifications were thought to be irreversible. However, several proteins with histone lysine demethylase functions have been discovered and characterized. Among these proteins, KDM4A is the first histone lysine demethylase shown to demethylate trimethylated residues. This enzyme plays an important role in gene expression, cellular differentiation, and animal development. Recently, it has also been shown to be involved in cancer. In this review, we focus on describing the structure, mechanisms, and function of KDM4A. We primarily discuss the role of KDM4A in cancer development and the importance of KDM4A as a potential therapeutic target.

Keywords KDM4A, histone demethylase, cancer, Jmjd2A, therapeutic target

Eukaryotic DNA is packed in a complex composed of RNA and proteins known as chromatin. The fundamental unit of this complex is the nucleosome, which is composed of 147 bp of DNA wrapped in 1.67 superhelical turns around the octameric histone core (composed of one pair each of histones H2A, H2B, H3, and H4). The DNA structure in chromatin leads to a five- to 10-fold DNA compaction (1). These compact structures negatively affect gene expression (2) and are modulated by several mechanisms, including histone posttranslational modifications such as the methylation of lysine and arginine residues, acetylation, the phosphorylation of serine and threonine, ADP-ribosylation, and the ubiquitination and SUMOylation of lysines. These modifications occur mainly at the histone N-terminal tail and promote either chromatin relaxation or compaction into a heterochromatin structure, affecting chromatin architecture and therefore gene transcription (3). One of the most-studied histone modifications is acetylation, which is controlled by acetyl transferases and deacetylases, suggesting that acetylation is a dynamic histone mark (4).

Lysine methylation is another prominently studied covalent histone modification. This histone mark can be recognized by at least four protein motifs: the chromodomain, the plant homeodomain zinc finger PHD, the Tudor domain, and the WD40-repeat domain (5–7). Proteins that contain these motifs are recruited by specific methylated lysines. However, the mechanism becomes more complex because lysine residues can be mono-, di-, or trimethylated, and the binding affinity of a protein for a particular modification might be affected by an adjacent modification (8,9). Histone lysine and arginine methylation were believed to be stable and irreversible modifications (10). However, approximately 30 enzymes capable of removing this covalent modification have been discovered to date. The search for histone demethylases began in the 1960s, when an enzyme that could remove a methyl group from mono- and dimethylated lysine residues was reported (11). Years later, the same research group partially purified a protein that had histone demethylase function (12,13); nevertheless, the molecular identity of this enzyme was not fully known for several decades. Not until 2004 was the first histone demethylase—lysine-specific demethylase 1 (LSD1), later renamed lysine (K) demethylase 1 (KDM1)—identified and characterized (14). This enzyme can remove the methyl groups from lysines 4 and 9 of the histone (H3K4me2/1 and H3K9me2/1, respectively), suggesting that this protein plays a role in the dynamic structure of chromatin and transcription (15,16). KDM1 belongs to the oxidase family that includes enzymes that can demethylate

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mono- and dimethylated residues using flavin adenine dinucleotide (FAD) as an electron acceptor (15,17). The oxygenase family, also known as the Fe(II) oxygenases, can demethylate mono-, di-, and trimethylated residues; this type of enzyme uses diatomic oxygen and α-oxoglutarate as cosubstrates (18–21). Lysine (K)-specific demethylase 4A (KDM4A, also known as JMJD2A, JHDM3A, and KIA0677) is categorized as a member of the Fe(II) oxygenase family.

In this review, we focus on the structure and function of the KDM4A protein, its role in cancer development, and the importance of this enzyme as a therapeutic target. For further review of the KDM4 family, see Shi et al., Whetstine et al., and Berry et al. (20–22).

KDM4A protein structure and enzymology

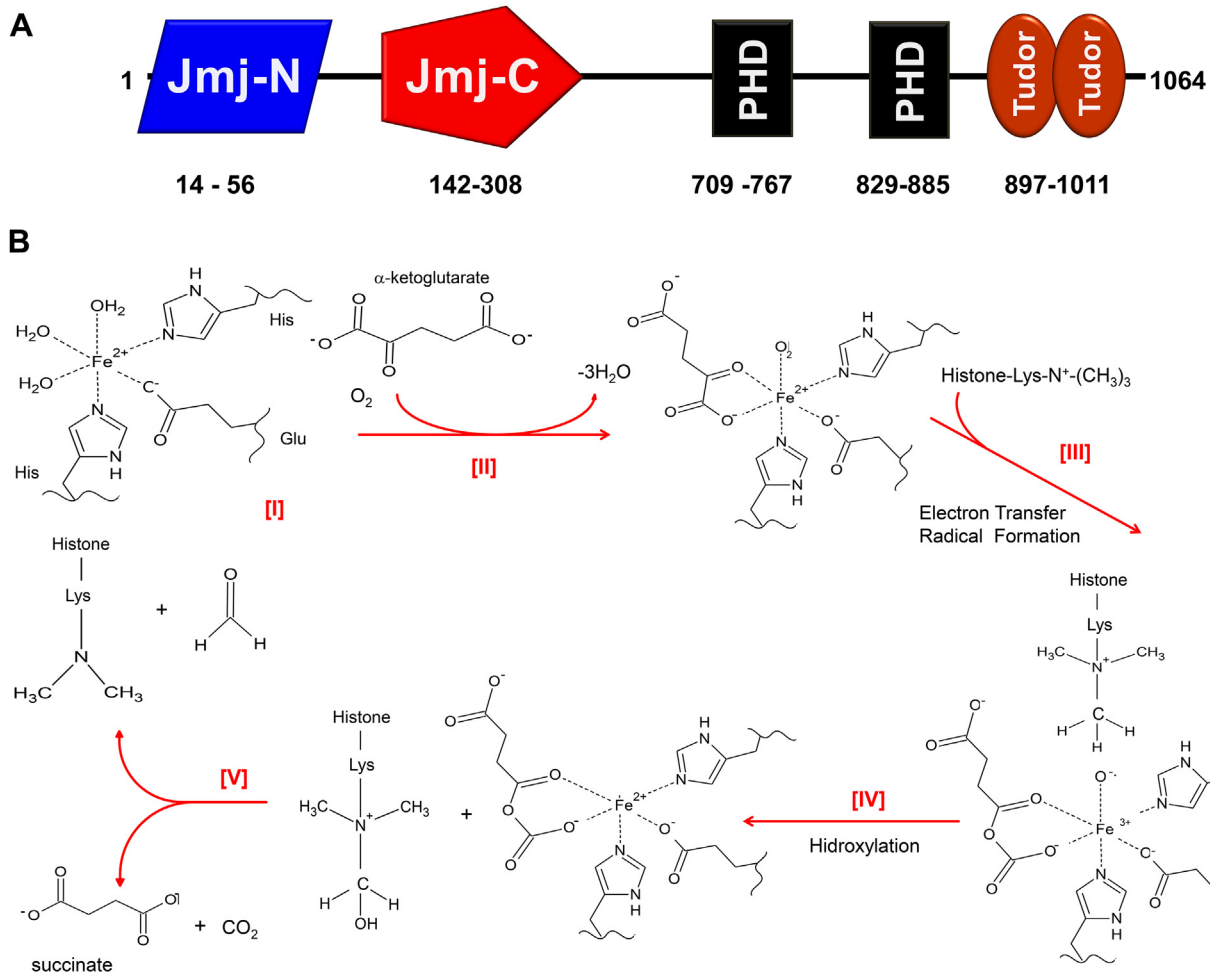
The *KDM4A* gene is a member of the Jumonji domain 2 (*JMJD2*) family and encodes a protein that contains JmjC and JmjN domains that form a composite active site, two PHD-type zinc finger domains, and two hybrid Tudor domains that form a bilobal structure, with each lobe resembling a normal Tudor domain (Figure 1A) (24,25). The function of the PHD fingers of KDM4A is not yet clear (22), in contrast to the functions of the PHD fingers present in other proteins, such as those in the NURF complex, which are known to bind to the H3K4me3 histone mark (26). The hybrid Tudor domains are formed by the exchange of the β3 and β4 chains; therefore, the electrostatic potential of the second Tudor domain is more negative than that of the first domain (21,27,28). Because of the folding of the hybrid Tudor domains of KDM4A, the side chain of H3K4me3 is inserted into the aromatic cage pocket of one Tudor domain, whereas the side chains of the other Tudor domain form intermolecular contacts; these domains also bind H4K20me3 peptides but in the opposite direction (28–32). Additionally, in vitro assays have demonstrated that this enzyme can demethylate di- and trimethylated residues at lysines 9 and 36 of histone 3 (H3K9me3/2 and H3K36me3/2, respectively), but this enzyme cannot demethylate monomethylated residues; in vivo however, KDM4A demethylates only trimethylated residues (18). KDM4A also has a higher affinity for H3K9me3 than for H3K36me3 (21,27,28). In particular, the H3K9me3 mark is associated with heterochromatic regions and transcriptional repression (8). Although H3K36me3 is associated with transcriptional repression in some models, it is primarily involved in transcription elongation by the RNA polymerase II, transcription initiation, alternative splicing, and DNA repair and recombination. For further review, see Pradeepa et al. (33).

Interestingly, the unusual KDM4A specificity for two regions with different sequences can be explained because the interplay between the enzyme and the histone peptides is governed by weak interactions such as hydrogen bonds and van der Waals interactions and by interactions with substrate backbone peptides (18,34). In addition, the N-terminal residues of H3K9me3 and H3K36me3 share a similar β-chain conformation, and the peptides bind in the same direction within the substrate-binding site (18,34). Thus, the trimethyl lysine is deposited in the catalytic site, which has an Fe(II) ion that is essential for the catalytic activity of the enzyme (18,34).

The proposed reaction mechanism of KDM4A is very similar to that of other Fe(II)-containing and α-ketoglutarate–dependent hydroxylases (Figure 1B). This process involves five general steps (35). (1) First, the active unbound Fe(II) ion is in a +2 oxidation state and is coordinated by two histidine residues, one glutamate residue, and three molecules of water. (2) Second, the α-ketoglutarate and diatomic oxygen are coordinated to the iron center, displacing the water molecules. (3) Third, a single electron transfer occurs from the Fe(II) ion to the oxygen molecule, leading to the formation of a peroxide radical that attacks the α-ketoglutarate and yields a mixed anhydride that is attached to the Fe^{3+}–hydroxyl radical. (4) Fourth, this highly reactive radical activates the carbon–hydrogen bond of the methyl group located on the methyl lysine by removing a proton and transferring the hydroxyl group to the carbon atom of the methyl group, leading to hydroxymethyl lysine formation. (5) Finally, the demethylation reaction proceeds with the spontaneous loss of formaldehyde from the hydroxymethyl lysine because the carbonyl is a good leaving group. Due to the hydroxyl group transfer, which leaves a gap in the coordination sphere of the Fe^{2+}, the mixed anhydride dissociates, producing succinate and carbon dioxide as byproducts. The union of three water molecules with the Fe^{2+} regenerates the original catalytic site (35).

In vitro studies have described the kinetic parameters of the KDM4A catalytic site (cKDM4A) (Figure 1C) (23); the k_{cat}/K_M (k_{cat} as the catalytic constant and K_M as the Michaelis constant) values represent how fast the enzyme reacts with the substrate once it encounters the substrate, where the values are proportional to the catalytic efficiency. The k_{cat}/K_M values of the dimethylated and trimethylated peptide (2.4×10^{-3} $(\mu mol/L)^{-1}$ min^{-1} and 3.0×10^{-2} $(\mu mol/L)^{-1}$ min^{-1}, respectively) show that cKDM4A has a stronger preference for the trimethylated substrate than the dimethylated substrate. Furthermore, a comparison of the k_{cat}/K_M values for a modified nucleosome and an analogue trimethylated peptide that has an aminoethylcysteine but behaves in a way similar to that of natural lysine residues suggests that the catalytic site of KDM4A predominantly recognizes the residues immediately surrounding the H3K9 and not additional structures on the nucleosome (Figure 1C). These data suggest that the catalytic site of KDM4A acts in a distributive manner and that the recognition of other chromatin features or modifications by the double Tudor or PHD domains of the entire demethylase may result in a tighter association, additional interactions, and an increase in demethylase activity (23).

Such interactions and protein–protein cross talk may play an important role in the regulation of KDM4A activity and processivity. In vivo, in the presence of chromatin histone marks or protein partners, the entire KDM4A may demethylate in a processive manner, and this regulation of KDM4A has significant implications on the specific output of KDM4 proteins in a context-dependent manner (31,36–39). Additionally, the demethylation activity toward H3K9me3 is influenced by other posttranslational modifications on the same peptide. Further studies of these cross-talk interactions at the peptide level are needed to obtain a more accurate understanding of the dynamics of epigenetic marks (40). Due to its catalytic activity, interactions, particular structure, and recognition ability, several functions have been attributed to KDM4A. Below, we describe some functions of KDM4A.



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Substrate	k_{cat}/K_M ((μmol/L)$^{-1}$min^{-1})	Catalytic significance
ARKme2STGGK	2.4*10^{-3}	Less efficient
ARKme3STGGK	3.0*10^{-2}	Most efficient
ARK$_c$me3STGGK	1.5*10^{-3}	Less efficient
	k_{max}/K_M ((μmol/L)$^{-1}$min^{-1})	
H3K$_c$me3 Nucleosome	6.7*10^{-4}	None efficient

Figure 1 The KDM4A demethylase protein structure and its biochemical mechanisms. (A) The KDM4A protein architecture consists of one JmjN domain and one JmjC domain, which contain an Fe(II) ion, two PHD domains, and two hybrid Tudor domains in the catalytic site. (B) The proposed reaction mechanism of KDM4A is very similar to that of other Fe(II)-containing and α-ketoglutarate–dependent hydroxylases. More details can be found in the text. The structures were created using ACD/ChemSketch from ACD/Labs Freeware, Toronto, Canada. (C) Kinetic parameters (k_{cat}/K_M) of KDM4A catalytic site and the catalytic significance of the values; ARKme2STGGK and ARKme3STGGK correspond to the H3K9me2 and H3K9me3 peptides, respectively; trimethyllysine analogue peptide and recombinant homogeneous H3K9me3 nucleosomes correspond to ARK$_C$me3STGGK and H3K$_C$9me3 (23).

KDM4A functions

Through KDM4A activity, H3K9me3 demethylation promotes an open chromatin state, contributing to the transcription activation of promoter regions (Figure 2A) (48). Regarding the functional impact of KDM4A-mediated demethylation of H3K36, the outcome is less clear.

Notably, H3K36 and H3K27 methylation are antagonistic histone marks, because nucleosomes that are methylated at H3K27 inhibit the enzymatic methylation of H3K36 and vice versa (49). Whereas H3K27 methylation is a characteristic repressive histone mark that is associated with the Polycomb group, H3K36me3 histone modification has been implicated in other processes that affect euchromatin functions. For

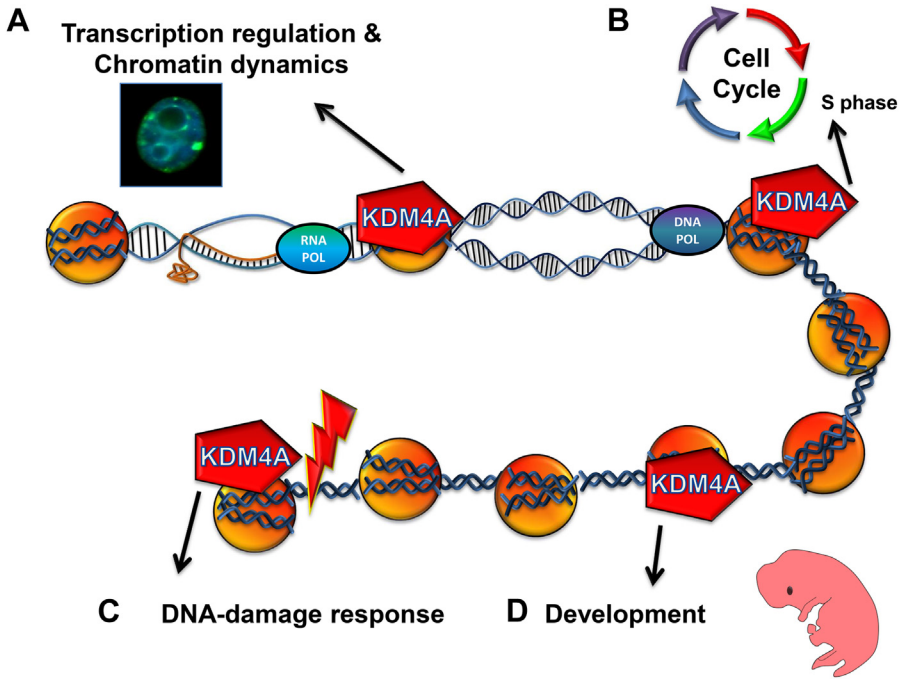


Figure 2 Biological implications of KDM4A. (A) KDM4A can act as a transcription repressor or activator and its chromatin dynamics, through its interactions with N-CoR, pRB, HDACs, and histones 3 and 4 (8,18,36,41–44). (B) In the cell cycle, KDM4A accelerates replication during S phase (45). (C) KDM4A associated with the DNA damage response avoids the recruitment of 53BP1 (46), the lightning bolt symbolizes DNA damage. (D) During development, KDM4A leads to the reduction of H3K36me3 in the X chromosome and is also involved in the activation of the *Myog* gene (47,43).

example, H3K36me3 is recognized by proteins that antagonize exon definition, affecting alternative splicing (50,51).

The role of H3K36me3 in transcription is controversial. Whereas some investigators have demonstrated that H3K36me3 couples with RNA polymerase II (RNA Pol II) in transcription elongation (52), others have shown that H3K36me3 is recognized by DNMT3A, promoting DNA methylation at nearby DNA regions and suggesting a DNMT3A-mediated gene repression link to H3K36me3 (53).

KDM4A is implicated in replication timing and genomic stability (45). *KDM4A* overexpression in human cells increases chromatin accessibility, coinciding with accelerated replication during S phase (Figure 2B). In contrast, a mutation in the *Caenorhabditis elegans* orthologue leads to increased replication timing and DNA damage, which induces p53-dependent apoptosis. KDM4A abundance is cell cycle-dependent, and this protein antagonizes the function of heterochromatin protein 1 gamma (HP1γ) (45).

Additionally, KDM4A is involved in the DNA damage response through the tandem Tudor domains of KDM4A and KDM4B that bind to the preexisting methylated residues of H4. After DNA damage, KDM4A/B is ubiquitinated by RNF8 and RNF168 and degraded by the proteasome, allowing the binding of 53BP1 to H4K20me2. Furthermore, *KDM4A* overexpression abrogates 53BP1 recruitment to DNA damage sites, suggesting a possible role of KDM4A in the DNA damage response (Figure 2C) (46).

In *C. elegans*, KDM4A appears to be involved in H3K36me3 reduction on the X chromosome, suggesting that this protein has a relevant role in germ cell development (Figure 2A) (47). In addition, in HeLa cells, KDM4A is associated with the repression of the achaete-scute complex homologue 2 (*ASCL2*) gene by acting as a cofactor of the nuclear receptor corepressor (N-CoR); this function requires its demethylase activity (18,41). KDM4A also interacts with histone deacetylases (HDACs) and retinoblastoma protein (pRb); this partnership is involved in the repression of E2F-dependent promoters (Figure 2A). However, the role of this protein as a demethylase has not been studied in this context (42). Remarkably, H3K9me3 demethylation of the myogenin (myogenic factor 4, or *Myog*) gene promoter during skeletal muscle differentiation of myoblasts into myotubes is performed by a ΔN-KDM4A isoform (Figure 2D) (43). These data suggest that functional KDM4A isoforms might also play a major role in the regulation of gene expression.

Genes repressed or activated by *Drosophila melanogaster* KDM4A (dKDM4A) were reported to not require KDM4A catalytic activity for expression; nevertheless, activated genes require its demethylase activity for proper expression (44). These findings suggest that some of these genes are indirect dKDM4A targets and that epigenetic regulation can be either dependent on or independent of the demethylase catalytic activity (44).

One study demonstrated that some dKDM4A-regulated genes were near one another, suggesting that genes controlled by this enzyme may require a common chromatin environment (44).

Interestingly, chromatin immunoprecipitation (ChIP) assays showed that the H3K36me3 levels were very low in both wild types and dKDM4A mutants (P/DdKDM4A) and that no detectable amounts of H3K9me3 were present in either wild types or mutants. These results indicate that many of the dKDM4A-controlled genes are modulated not by these histone marks but by other dKDM4A-dependent functions. A

Table 1 Summary of the role of KDM4A in several cancer types

Cancer type	KDM4A expression[a]	Associated with	References
Prostate	↑	AR, PSA	(61)
Colorectal	↑	p53, p21	(62)
Lung	↑	CHD5, Ras, ADAM12, CXL5	(63,64)
Breast	↑	ER, C-Jun, Cyclin D1	(65)
Squamous cell carcinoma, lymph node metastases	↑	FOS1, JUN	(66)
Bladder	↓	Patient survival, new biomarker for patient risk stratification	(67)
Bladder	↑	Involved in an early stage of human bladder carcinogenesis	(63)

[a] Arrows represent overexpression (↑), and underexpression (↓).

likely explanation for the changes found in mutants may be that they are due to the interaction of KDM4A with other proteins, such as pRb and N-CoR (Figure 2A) (41–44).

Histone modifications are important in chromatin structure. Global and local chromatin architecture alterations are common findings in tumors (54,55). The expression pattern of *KDM4A* has been suggested to be altered in several cancer types that involve such chromatin modifications. Here, we summarize some aspects of the role of KDM4A in cancer development.

The role of KDM4A in cancer development

KDM4A in genomic stability

Chromosomal instability and copy number alterations are common features in cancer (56); nevertheless, there is little information regarding the molecular mechanisms that demonstrate how copy number variations (CNVs) are involved in the timing of tumor progression. However, recent studies have demonstrated that the overexpression of *KDM4A* leads to focalized copy gains at the 1q12, 1q21, and Xq13.1 loci (57).

The 1q12 and 1q21 regions harbor several putative oncogenes (58,59) and are often amplified in multiple myeloma and lung cancer. In addition, primary tumors of different cancer types that exhibit *KDM4A* overexpression also have increased copy gains at 1q12, 1q21, and Xq13.1; however, surprisingly, it has been suggested that the KDM4A-mediated copy gain does not cause chromosome instability (57). The 1q12 loci copy gain also appears to occur in one cell cycle and is not stably inherited by daughter cells. Moreover, the sites with amplified copy numbers are re-replicated and have increased occupancy by DNA polymerase, KDM4A and MCM (57). The 1q12/1q21 copy gains may be associated with drug resistance in multiple myeloma and ovarian cancer cell lines (60).

These results suggest that 1q12 copy gains are not incorporated into the genome but exist as extrachromosomal DNA. Additionally, these results establish how copy number changes originate during tumorigenesis and provide evidence showing that the overexpression of specific chromatin modulators promotes these events (57).

KDM4A has been reported to be deregulated in several cancer types, such as prostate, bladder, colorectal, squamous cell carcinoma, and lung and breast cancers.

Prostate, bladder, and colorectal cancer

The overexpression of *KDM4A* has been observed in prostate cancer (Table 1), with KDM4A functioning as a coactivator of the androgen receptor (AR) under simulated conditions of low AR ligand levels. KDM4A appears to activate the basal transcription of prostate-specific antigen (PSA) (61).

These results could impact patients undergoing androgen ablation. *KDM4A* overexpression has been suggested to be involved in prostate tumors that become refractory to androgen ablation therapy (61).

In contrast, KDM4A levels were shown to be reduced in bladder cancer (Table 1). KDM4A and AR are absent in primary and advanced bladder tumors, suggesting that these proteins are involved in neither initiation nor tumor progression; however, these proteins might be involved in delaying the onset of carcinogenesis. The physiological significance of the AR and KDM4A losses in androgen signaling remains to be determined (67). Furthermore, there is an association between decreased levels of KDM4A and patient smoking, with KDM4A greater presence being associated with patient survival. Protein loss is correlated with a particularly aggressive bladder disease and poor prognosis in bladder cancer patients. These data suggest evidence of a possible new biomarker for patient risk stratification (67).

Although a recent study demonstrated that the expression levels of *KDM4A* are upregulated in bladder cancer tissue compared with normal bladder tissue, no significant differences among different tumor grades have been found. This finding suggests that the elevated expression of *KDM4A* could be involved in an early stage of human bladder carcinogenesis (63).

As in prostate cancer, *KDM4A* overexpression has been observed in colorectal tumors (Table 1). ChIP assays have shown that KDM4A and p53 are recruited to the *p21* gene promoter after Adriamycin-induced DNA damage. KDM4A reduction leads to increases in p53, p21, and the proapoptotic protein PUMA, thus inducing apoptosis in the HCT116 cell line model (62). Interestingly, *KDM4A* knockdown results in reduced cell proliferation, whereas *KDM4A* overexpression correlates with cell proliferation under low serum concentration conditions (61). Therefore, *KDM4A* overexpression could be advantageous in tumors, as they are often surrounded by stroma and extracellular matrix that limit the diffusion of growth factors, which resembles a low serum

environment. Thus, researchers have proposed that KDM4A promotes cell proliferation and survival in colon cancer (59) and that KDM4A inhibition may sensitize cells to chemotherapeutics such as Adriamycin (62).

Head and neck squamous cell carcinoma

The transcription factor activating protein 1 (AP-1) plays a critical role in metastasis and tumor growth. AP-1 is composed of two proteins, c-Jun and c-Fos. AP-1 activation can be partially mediated by the transcriptional activation of *JUN* and *FOS*. In addition, *JUN* and *FOS* undergo positive feedback with the recruitment of AP-1 to their own gene promoters, thereby enhancing AP-1 activation (68–71). When H3K9me3 is enriched in this region, AP-1 cannot be recruited. The demethylation of this histone mark, mediated by KDM4A, can promote the gene activation of *JUN* and *FOSL1* (66).

Furthermore, the abundance of this enzyme correlates with the abundance of JUN and FOSL1, increasing the activity of AP-1 in human squamous cell carcinoma tissues (66). Remarkably, *KDM4A* is overexpressed in lymph node metastases (66) and squamous cell carcinoma tissue compared with expression levels in normal tissues (Table 1) (63). These data suggest that KDM4A could be involved in squamous cell carcinoma invasion and metastasis of the head and neck (66).

Lung cancer

KDM4A is overexpressed in mouse and human lung cancer cell lines (Table 1). The depletion of KDM4A in the human lung cancer cell line A549, which bears an activated K-Ras allele, triggers senescence. Therefore, *KDM4A* could function as an oncogene that represents a target for Ras-expressing tumors (64). Additionally, KDM4A appears to be involved in the regulation of the tumor suppressor gene chromodomain helicase DNA binding protein 5 (*CHD5*). CHD5 targets p19ARF, which is involved in the p53 ubiquitination pathway (72). Thus, *KDM4A* overexpression may cooperate with Ras in the transformation of primary cells by blocking cellular p53-dependent senescence via CHD5 in lung adenocarcinomas (64).

Furthermore, the nuclear presence of KDM4A in neoplastic tissues such as lung carcinomas and non–small cell lung carcinomas (NSCLC) was detected, unlike in normal lung tissue (63,64). Surprisingly, no association between *KDM4A* expression and prognosis was observed. The above data suggest that *KDM4A* overexpression may be an early event in NSCLC carcinogenesis (63).

New candidate genes that appear to be upregulated by KDM4A through the demethylation of H3K9me3 have recently been reported in the A549 cell line, including three cancer-related genes, *ADAM12, CXCL5,* and *JAG1* (63). *ADAM12* is overexpressed in several types of human carcinomas (73–75) and enhances tumor cell growth by the proteolytic shedding of EGFR ligands (76). The *CXCL5* gene may be implicated in the promotion of tumor growth, progression, and angiogenesis (77). *JAG1* encodes a ligand involved in the Notch intracellular pathway and angiogenesis (78) and has also been implicated in enhancing cell proliferation by activating the canonical Notch signaling pathway (79).

Taken together, these results suggest that KDM4A may have a dual role in lung carcinogenesis by downregulating the tumor suppressor gene *CHD5* (64) and by activating tumor growth- and cell proliferation-related genes (63).

Breast cancer

In triple-negative breast tumors, the overexpression of *KDM4B* and *KDM4A* has been observed to correlate with the loss of H3K9me3, which is normally enriched in the pericentromeric region. This phenomenon may contribute to the development of aneuploidy and chromosomal instability in solid tumors and thus to tumor progression (80). However, other factors may cause increased KDM4A expression and promote chromosomal instability due to the loss of H3K9me3 in pericentromeric regions, such as the downregulation of the expression of the methyltransferase SUV39H1/2 (81) or of complexes that help correct chromosome segregation and tumor suppression, such as pRb, SWI/SNF, and mSds3 (82–85).

A study comparing breast cancer tissue and normal breast tissue found significant differences in several proteins that modify histones, including KDM4A (Table 1). These differences were associated with pathological and clinical parameters. However, further studies are required to determine the biological and clinical significance of this altered expression for each histone-modifier gene and for the different expression profile combinations (86). Moreover, the depletion of KDM4A by siRNA in breast cancer cell lines suppresses tumor proliferation, invasion, and migration (87,88).

Similarly, KDM4A has been proposed as an estrogen receptor coactivator (ERα) that forms a KDM4A-ERα complex, by which the overexpression of *KDM4A* increases estrogen-dependent transcription. Meanwhile, KDM4A depletion causes a transcriptional decrease of ERα target genes such as *CCND1*, which is overexpressed in breast cancer (89). Another protein that is also decreased after *KDM4A* downregulation is c-Jun. The inactivation of c-Jun causes cell cycle arrest. This protein, which is regulated by ERα, has important functions in cancer tissues, and its overexpression stimulates the invasion, migration, and formation of tumors (90).

Taken together, these associations suggest that KDM4A can coactivate both hormone signaling-dependent and signaling-independent genes and that it may regulate cell growth by influencing the expression of at least two oncogenes, *CCND1* and *c-Jun* (65).

Recently, *KDM4A* has been revealed to have a higher expression in infiltrating duct carcinoma than in benign lesions in situ at the mRNA and protein levels (91). The same study showed a negative correlation between the expression levels of *KDM4A* and ADP-ribosylarginine hydrolase 1 (ARH1). In contrast, the expressions of *KDM4A*, *p53,* and *ER* were positively correlated. Although the exact mechanism of KDM4A's involvement in human breast cancer is not yet clear, these results suggest that KDM4A has a role in the diagnosis of cancer and as a possible therapeutic target (91).

The ability of KDM4A to activate or repress transcription may be dictated by chromatin structure, the presence or

absence of other transcriptional regulators, stressful stimuli such as hormonal stimulation, and transcription factor recruitment (64,65). These data suggest that the reduction or inhibition of KDM4A may be beneficial for the treatment of different cancer types.

KDM4A as a potential therapeutic target

Given our understanding of the structures of demethylases, their catalytic reaction mechanisms, the selectivity of their methylation marks, and the implications of these marks in cancer, a great interest in developing inhibitors of these demethylases has emerged.

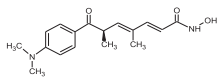
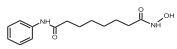
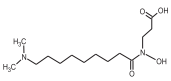
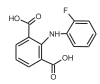
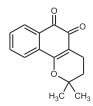
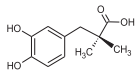
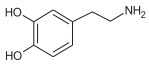
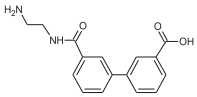
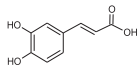
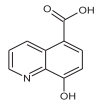
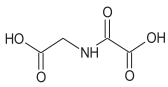
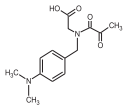
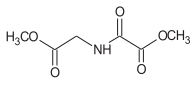
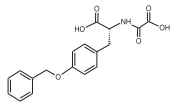
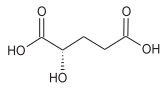
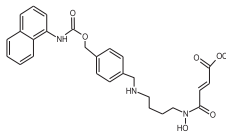
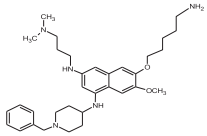
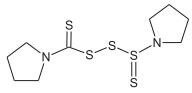
Many histone demethylase inhibitors have been described; these inhibitors can be classified into five groups: iron chelators, α-ketoglutarate analogues, catalytic site inhibitors, prodrugs, and zinc chelators (Table 2) (92–104). However, the lack of research on the selectivity and specificity of histone demethylases and thus the deficiency of our knowledge regarding nondesirable targets have prevented these inhibitors from progressing toward clinical research. Therefore, the use of such inhibitors remains in the preclinical phase (105).

These reported inhibitors include N-oxalylglycine (NOG) and its derivatives, which are analogues of the cosubstrate α-ketoglutarate. They inhibit KDM4A and other members of the KDM4 family via competition. In addition, another analogue of α-ketoglutarate is the oncometabolite 2-hydroxyglutarate, which also inhibits KDM4 enzymes by competition but is a weak antagonist of α-ketoglutarate (92–94). However, these chemicals are not very specific, because they target different α-ketoglutarate-dependent enzymes (92–94).

Another molecule that has inhibitory action is pyrimidine 2,4-dicarboxylic acid, whose mechanism of action is based on electrostatic interactions with a lysine residue within the active site; nevertheless, little is known about its uses in therapy (105). Additionally, hydroxamic acid and its derivatives, such as trichostatin A, most likely function as iron chelators to inhibit the catalytic activity of JmjC domain-containing demethylases such as KDM4A (65). Moreover, the main use of these compounds is against HDACs (106); therefore, the probable side effects make the future development of this class of compounds unpromising (105).

The structures of KDM4A revealed a Cys-His Zn(II) binding site that is close to the substrate binding spot, which bioinformatic analyses indicated was not present in any other histone demethylase subfamily. Therefore, an alternative method to inhibit the KDM4 family (95) would be to use compounds that chelate Zn(II) ions. One derivative of disulfiram is a potent KDM4A inhibitor; it changes the methyl lysine–binding site by the chelation of Zn(II) ions (95). This strategy may have potential for the development of selective inhibitors for those Jumonji protein subtypes containing a

Table 2 Classification of JumonjiC domain-containing demethylase inhibitors, according to the inhibition mechanism

Iron chelators					
 Trichostatin [94].	 Suberoylanilde hydroxamic [94].	 Internal hydroxamic acid [98].	 Pyidine 2,4-dicarboxylic acid [97].	 PDCA derivative [97].	 β-lapachone [99].
 [S]-[-]-carbidopa [99].	 Dopamine [99].	 8-hydroxyquinoline-5-carboxylic [96].	 3,4-dihydroxy cinnamic acid [101].	 2,2'-bypirydine derivative [100].	
α-ketoglutarate analogs					
 N-oxalylglycine [93].	 NOG-derivative [93].	 Dimethyl N-oxalylglycine [DMOG] [93].	 *N*-oxalyl D-tyrosine derivative [102].	 *[S]*-2-hydroxyglutaric acid [2-HG] [92].	
Prodrug		**Binds to the active site**		**Zinc chelators**	
 Methylstat [103].		 E67 [104].		 Disulfiram derivative [95].	

More details can be found in the text. The structures were created using ACD/ChemSketch from ACD/Labs Freeware, Toronto, Canada.

structural Zn(II) ion (KDM4) (96). Different chelants that may be involved in KDM4 inhibition are 8-hydroxyquinoline and its derivatives; 8-hydroxyquinoline chelates the Fe(II) ion with a bidentate structure and executes its inhibitory action via a carboxylic acid motif positioned toward the active site (97). Interestingly, there is a new compound that performs its inhibitory action by similarly chelating the Fe(II) ion and binding to the cosubstrate cleft (107). This compound consists of a peptide and an α-ketoglutarate analogue that are connected by a disulfide bridge. Although studies have revealed potent and partially high selectivity of this compound, its peptide nature may be an obstacle to the further development of these compounds into potential drugs due to cell permeability, intracellular stability, and other pharmacokinetic parameters (105).

The search for novel histone demethylase inhibitors provides a starting point for the development of new therapies with selective agents against aberrant epigenetic phenomena. However, further research is still needed for such therapies to become a reality.

Conclusions and final remarks

KDM4A has a dual role as an epigenetic transcriptional regulator. Knowing which conditions are required for it to activate or suppress a gene would be extremely interesting. Due to its structure, interactions, and pleiotropic activity, the role of KDM4A in cancer development may be more complex than originally believed. Further research is required to clarify how KDM4A affects cancer development and to create a comprehensive overview of the functions performed by this protein in various cancer types.

Another challenge is designing a drug that is selective for a subset of demethylases. This selectivity could potentially be achieved by linking the drug to at least three protein domains; however, the designed drug would be too large to penetrate the cellular membrane. In addition, an allosteric inhibitor that changes the conformation of the catalytic site of the enzyme without binding to this site could be designed. A recent computational screen identified putative allosteric sites that could be used for this purpose (108). However, more research is required to further experimentally identify and characterize these sites.

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