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► **To cite this version:**

Audrey Dujardin. Identification of Chromodomain on Y Like 2 (CDYL2) as an indispensable protein for a faithful mitosis. Molecular biology. Université de Lyon, 2021. English. NNT : 2021LYSE1134 . tel-03855841

HAL Id: tel-03855841

<https://theses.hal.science/tel-03855841>

Submitted on 16 Nov 2022

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N°d'ordre NNT :
2021LYSE1134

THESE de DOCTORAT DE L'UNIVERSITE DE LYON
opérée au sein de
l'Université Claude Bernard Lyon 1

Ecole Doctorale N° ED340
BMIC – Biologie Moléculaire, Intégrative et Cellulaire

Spécialité de doctorat : Biologie
Discipline) : Biologie Moléculaire

Soutenue publiquement le 05/07/2021, par :
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**Identification of Chromodomain on Y Like 2
(CDYL2) as an Indispensable Protein for a
Faithful Mitosis**

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Cette thèse a été préparée au sein du Centre de Recherche en Cancérologie de Lyon (UMR INSERM1052 / CNRS 5286), dirigé par le Docteur Patrick MEHLEN, dans l'équipe "Épigénétique et Cancer" créée en Juin 2014 et dirigée par le Dr. Peter MULLIGAN, sur le site du Centre Léon Bérard situé 28 rue Laennec, 69373 Lyon Cedex 08.

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Identification de CDYL2 (Chromodomain on Y Like 2) en tant que protéine indispensable pour une mitose fidèle

La sous-unité de la chromatine, le nucléosome, est constitué d'ADN et d'histones, des protéines pouvant être modifiées de manière covalente pour recruter d'autres protéines sur la chromatine. Souvent étudiés dans le contexte du contrôle de l'expression, les modifications d'histones sont également importantes pour la stabilité génomique. Une perte de la triméthylation de la lysine 9 de l'histone 3 (H3K9me3) aux péricentromères induit par exemple un manque de cohésion des chromosomes et une instabilité génomique. La protéine CDYL2 (Chromodomain on Y-Like 2) est un lecteur de H3K9me3. Alors que son paralogue CDYL1 a pu être lié à la répression génique, la réparation de l'ADN, la tumorigénèse et l'épilepsie, CDYL2 reste peu caractérisée. Pendant ma thèse, j'ai montré que CDYL2 est essentiel pour la stabilité génomique de la cellule. Avec un immunomarquage, j'ai observé que CDYL2 est une protéine principalement nucléaire, localisée aux péricentromères dépendamment de H3K9me3, et aux centrosomes. En utilisant notamment la spectrométrie de masse, j'ai montré que CDYL2 interagit avec de nombreuses protéines impliquées dans la mitose, tel que des facteurs épigénétiques répresseurs, CHAMP1 (Chromosome Alignment-Maintaining Phosphoprotein 1), et des protéines du complexe cohésine. En parallèle, j'ai collaboré à montrer que CDYL2 interagit avec la méthyltransférase d'histone EHMT2 et la recrute sur la région promoteur de miRNA-124. Grâce à un inhibiteur de EHMT2, j'ai aussi pu montrer que la méthylation de H3K9 induite par EHMT2 est nécessaire pour la répression de la transcription de miRNA-124. Dans l'ensemble, ma thèse participe à définir CDYL2 en tant que protéine importante pour la régulation de la transcription et la stabilité génomique.

Mots clés: Épigénétique, CDYL2, Mitose, Péricentromères, Instabilité génomique

Identification of Chromodomain on Y Like 2 (CDYL2) as an Indispensable Protein for a Faithful Mitosis

The chromatin subunit, the nucleosome, contains DNA and histone proteins that can be covalently modified to, among other functions, recruit binding proteins to the chromatin. Often studied in the context of gene expression regulation, histone modifications are also relevant for genomic stability. As an example, loss of trimethylated lysine 9 on histone 3 (H3K9me3) at pericentromeres induces defects in chromosome cohesion and genomic instability. Chromodomain on Y-like 2 (CDYL2) protein is an H3K9me3 reader. While its paralog CDYL1 has been linked with gene repression, DNA repair, tumorigenesis, and epilepsy, CDYL2 remains poorly characterized. During my thesis, I revealed that CDYL2 is essential for faithful mitosis as its knock-down induces mitotic defects, including chromosome misalignment and cohesion defects. Using immunofluorescence staining, I observed that CDYL2 is mostly a nuclear protein, localizing at pericentromeres, in an H3K9me3 dependent manner, and at centrosomes. Using notably mass spectrometry, I have shown that CDYL2 is interacting with many proteins involved in mitosis, such as epigenetic silencing factors, Chromosome Alignment-Maintaining Phosphoprotein 1 (CHAMP1), and proteins from the cohesin complex. In parallel, I collaborated to show that CDYL2 interacts with the histone methyltransferase EHMT2 and recruits it to the promotor region of miRNA-124. Using an EHMT2 inhibitor, I also revealed that H3K9 methylation induced by EHMT2 are necessary for miRNA124 transcription repression. Taken together, my thesis participates to reveal CDYL2 as an important protein for both transcription regulation and genomic stability.

Key words: Epigenetics, CDYL2, Mitosis, Pericentromeres, Genomic Instability

Remerciements

J'aimerais tout d'abord remercier chaleureusement **Dr. Claire Vourc'h** ainsi que **Dr. Christian Jaulin** pour avoir accepté d'être les membres rapporteurs de mon jury de thèse. Merci également à **Dr. Miguel Godinho Ferreira** et **Dr. Delphine PONCET** pour en être les examinateurs. Je suis heureuse et honorée de pouvoir discuter avec vous de ce projet sur lequel j'ai travaillé pendant un peu plus de trois ans.

Un grand merci à **Reini Fernandez de Luco** et **Franck Tirode**, membres de mon comité de suivi de thèse pour avoir accepté de suivre ce projet. Merci **Reini** pour ta gentillesse et tes questions toujours pertinentes. Merci **Franck** pour ton écoute objective et ton assurance.

Thank you, **Peter** for accepting me in your team and allowing me to do my thesis in your lab. Thank you for sharing your knowledge and point of view, both in science and in everyday life. I wish you all the best in your future research projects.

I would also like to acknowledge **Dr Thomas Jenuwein** for the MEF cells he kindly shared with our lab and which allowed me to show the dependance of H3K9me3 for CDYL2 pericentromeric localization.

Je tiens évidemment aussi à remercier tous les ex-membres de l'équipe Mulligan. Un énorme merci à **Maha** pour m'avoir transmis tous ses précieux protocoles. Merci pour avoir toujours été prête à discuter sur les manips et le projet. Un énorme merci pour ton amitié, le partage de nos moments de peine et de nos tranches de vie plus joyeuse. Rendez-vous à la prochaine bière ! Merci également à **Laetitia**, l'autre pilier de notre petite équipe. Merci Laetitia pour ton accueil chez toi et dans ton cœur. Moi et mon rejet de l'administratif te remercient également pour toutes les commandes que tu as passé pour moi, c'était parfait. Merci à toutes nos (petites ?) stagiaires qui sont passées dans cette équipe et ont égayé mes journées. Merci **Blanche** d'avoir été ma première stagiaire, merci pour ton travail avec les MEF qui a permis de faire avancer mon projet et que tu retrouveras dans ces pages. Merci **Manon** pour être celle que tu es, étonnamment proche de celle que je suis. Merci pour tes petits mails de nouvelles qui n'étaient peut-être pas réguliers mais toujours très précieux et plein de vie. Je te souhaite tellement plein (oui tellement plein) de bonheur et surtout (parce que je sais à

quel point c'est le plus important) plein de courage pour la fin de ta thèse. Merci **Tina** pour tes discussions toujours surprenantes et pour la création de tes dinosaures-parasites qui ont décoré longtemps mon bureau. Enfin, merci **Aurélie**, pour ta douceur et la boîte à sucre qui est toujours là !

Merci aussi à toutes les personnes qui sont passées à un moment ou à un autre par le 5^{ème} étage du Cheney A. Cela inclue bien évidemment l'équipe Bartholin à côté de laquelle j'en ai vécu des aventures ;-)

Merci **Véronique** pour ta générosité, nos échanges de post-it et nos SMS. Je te souhaite vraiment plein de bonheur pour la fin de ta thèse. Et comme dit, si le karma existe, elle ne peut que bien se finir pour toi. À moins que tu m'aies caché quelque chose d'important sur ta vie avant ta venue dans le bureau. J'espère vraiment te revoir souvent et arriver à jouer à Par Minou avec toi à défaut de connecter nos Switch. Merci **Victoire** d'avoir été un exemple de courage, de ténacité et de travail. Je te souhaite le meilleur également dans ton postdoc. Merci **Cassandra**, pour ton humour, ta joie de vivre et ta liberté. Elle m'a inspirée et j'espère que tu la garderas bien précieusement. Merci **Adrien** et **Sophie** pour votre présence et les échanges d'informations précieuses. Plein de courage à vous deux aussi pour la suite. Merci **Sylvie** pour avoir eu de nombreuses réponses à mes questions. Merci à **Agathe**, d'être passée presque en coup de vent à cet étage et d'avoir apporté un peu de soleil dans mes journées.

Merci à l'équipe des Maries (je pluralise les noms propres, oui) qui a partagé l'étage seulement au début de la thèse mais est restée dans mon cœur jusqu'au bout. Many thanks to you **Mister Reader**. I do miss you a lot and I do really hope you'll find a way that makes you truly happy. Don't hesitate to call whenever you want to go out, I'll be happy to join you. Merci **Ossama** de toujours passer prendre des nouvelles quand tu es dans le coin. Tu as un cœur en or, prends confiance et finis ta thèse en beauté si c'est ce que tu veux. Merci **Saidi** d'être à l'écoute et de te soucier de tout le monde, tout le temps. Garde cette si belle énergie qui fait croire à tout le monde que tu es un tout jeune post-doc. Garde cet humour qui sait si bien animer le labo. Thank you, **Chloe** for sharing your life with so much enthusiasm. I really did appreciate a lot to talk with you whenever I had the opportunity. Merci **Alexandra** pour ta gentillesse je crois légendaire. Merci **Ramdane** pour ton assiduité et ta motivation exemplaires dans tout ce que tu entreprends.

Dans mon stock de merci, j'en ai encore à distribuer à mes deux équipes d'adoption. Merci aux Ichims pour rendre mes journées en culture, et plus récemment les rares que je passe au bureau, plus joyeuses. Un énorme merci à **Kévin** évidemment. Je ne sais pas qui de toi ou de moi a été le plus surpris de découvrir que je partirai avant toi, et que ce serait de mon plein gré en plus. Merci pour m'avoir fait rire si souvent, merci de m'avoir prêté tes oreilles pendant que tes mains soignaient tes cellules, et merci de m'avoir permis d'y déverser tout ce qui me passait par la tête. Je compte sur toi pour venir m'embêter autant que tu veux dans ma maison (tu sais quoi ? Il y aura une piscine ! :-p). Un grand merci aussi à **Déborah** qui m'aura, par son accent, ramené un peu de ma Belgique natale. Merci aussi pour ta participation à l'embêtage de Kévin, un sport qui était quand même bien moins drôle sans toi. Merci à **Jade** grâce à qui le stock de culture était toujours plein sans que je n'aie rien à y faire. Merci aux Bernards pour me servir de refuge loin du Cheney A et pour toujours m'accueillir avec des gâteaux, des sourires et des histoires. En particulier évidemment, merci **Clotilde**, ma bella chica. Merci pour tous ces repas qu'on a pris ensemble à parler sans s'arrêter, pour tous ces soucis, ces espoirs, ces bières et ces résolutions (presque toujours dans cet ordre-là il me semble) que j'ai pu partager avec toi. Merci pour avoir vécu plus que des moments au labo avec moi. Merci pour être devenue une véritable amie.

Merci enfin à toutes les personnes du CRCL que j'ai croisé plus ou moins fréquemment et qui ont rendu cette thèse telle qu'elle est. Merci à **Inès** de faire en sorte qu'il y ait toujours "tout ce qu'il faut". Merci à **Christophe** pour tant donner à nous, petits amateurs de microscopie. Merci aux organisateur.rice.s des after-labs et à tou.te.s ceux qui y ont participé. Merci à ceux avec qui j'ai eu des conversations autour d'un verre. Merci à tou.te.s ceux qui m'ont nourri lors de pot de thèse. Merci à la team du laboratoire P3 pour m'avoir formé puis surveillé. Un énorme merci aux membres des petits génies de l'IHOPE. Vous faites un travail formidable et j'ai passé quelques après-midis mémorables à faire des volcans, une voiture-hélicoptère ou encore une histoire sur Scratch avec les enfants.

Ma thèse ne s'est pas que déroulée au sein du CRCL et je remercie toutes les personnes avec qui j'ai pu enseigner. Un merci particulier à **Sébastien Violot** pour sa confiance et sa disponibilité. J'ai beaucoup apprécié travailler et discuter avec toi. Merci aussi à l'école doctorale BMIC, **Roxane**, **Mathilde**, **Françoise**, **Mathias** et tous les membres du conseil. Merci

d'être à notre écoute, de proposer des solutions pertinentes à nos problèmes et de nous soutenir.

Ceux qui me connaissent personnellement savent à quel point j'aime remplir mon agenda de tas d'activités plus ou moins extravagantes. Ces activités m'ont permis de me changer les idées et de recharger mes batteries les jours difficiles. Mais surtout elles m'ont permis de rencontrer des personnes formidables dans cette ville qui m'était inconnue lorsque je suis arrivée pour mon stage de master.

Merci à la **Troupe Théâtrale de l'INSA** pour accepter des personnes hors INSA (donc moi). Merci de m'avoir permis de hurler, de courir et de sauter dans des amphithéâtres une fois par semaine pendant trois belles années. Merci particulièrement au groupe de Voyage vers l'Enfer. Notre pièce aurait été tout simplement magnifique si le coronavirus avait bien voulu nous laisser jouer. Vous étiez beaucoup trop beaux, le monde n'était pas prêt <3 Merci au **Centre Lyonnais de la Dentelle** pour m'apporter du papotage et me permettre de frimer avec mes œuvres. Merci à **Ghislaine** et **Valérie** pour me faire rire avec leurs anecdotes quotidiennes. Merci aux **Secouristes Français de la Croix Blanche** et tous les adhérents pour toutes ses heures passées ensemble, pour cette confiance, cette générosité et ces fous rires. Enfin last but not least comme on dit si bien, merci aux membres et ex-membres d'**Un Peu de Bon Science** ! Je vous aime tous et tant pour votre ouverture d'esprit et votre bienveillance. Merci pour toutes ces soirées, ces tournées et ces moments de vie partagés. Merci de me suivre sur la pente des animaux totems (#Ornithorynque4ever). Et surtout bravo pour ce que vous faites, c'est toujours magnifique. Je suis extrêmement fière et heureuse de vous avoir rencontré et de faire partie de votre équipe.

Merci aussi à tous les amis que je collectionne depuis la maternelle, et qui m'ont tous apporté du soutien et du bonheur. Merci à **Marie**, seule rescapée de Goxwiller. Merci à **Julia, Nathalie, Aïcha, Diane, Alexander** et **Lara**, la team ESK. Merci à la team Regio qui a rendu mes années de licence tout simplement magiques : bravo à tous pour votre parcours dans les études et vos débuts de carrière. Vous êtes tous incroyables <3. **Gauthier**, c'est officiel, tu pourras m'appeler Dr Drey. Quant à **Zak**, il me semble que tu me dois une blague. Ach, und **Christina**, was soll ich dir sagen ? *Eigentlich* mag ich dich sehr ;-) und ich wünsche dir alles bestens. Ich wünsche mir, dass wir noch oft zusammen stundenlang schwätzen werden. Merci à **Dona**, ma

belle Dona pour tous ces bons moments et ces belles vacances passées ensemble. Merci pour ta franchise et ton amitié. Et comme je sais ce que tu en penses, je ne te souhaite pas de bonne continuation ;-)

Merci **Edgar** pour m'avoir rassuré lors de mes moments de doute. Merci pour ta gentillesse sans faille, de me faire découvrir ta culture et tous les restaurants mexicains de la ville. Merci **Fabien** pour ta motivation et ton courage exemplaire. Je reste admirative de ton enthousiasme et de ta tenacité qui t'ont mené (tu me l'as annoncé il y a quelques heures) si rapidement et si brillamment au poste de chercheur que tu convoitais tant.

Enfin, j'aimerais formuler une mention spéciale à la team Villeurjeux. Vous êtes devenus tellement plus ces deux dernières années que vous méritez votre propre paragraphe, même si du coup il a l'air tout petit. Merci pour ces innombrables soirées jeux, makis, crêpes et/ou discussions pour refaire le monde. Merci **Jérémy** pour ton entrain permanent. Merci **Elen** pour ton calme et ton écoute posée. Merci **Bernard** pour tes anecdotes et, soyons fous, pour tes jeux de mots. Merci **Barbara** pour ta bienveillance envers les autres, bienveillance dont je n'ai pas encore trouvé les limites. Et surtout, surtout, merci à vous tou.te.s d'être prêt.e.s à devenir des pies. Vous savez quoi ? On va acheter une maison. D'ici la soutenance on aura probablement déménagé et notre aventure ne fait que commencer. <3

Il paraît qu'on choisit ses amis mais pas sa famille. Si j'avais eu l'occasion de le faire, je n'aurais pas pu mieux choisir. Merci à **Papy Lulu**, pour nous faire rire avec ses chansons sorties de nulle part. Merci à **Pépé** de vouloir comprendre ce que je fais et de le regretter au bout d'un quart d'heure d'explications. Merci à **Granny** de toujours prendre des nouvelles et de jouer avec brio à la radio familiale. Merci à **Papa** d'avoir répondu à mes questions d'enfants par « A ton avis ? ». Je n'aurais jamais pensé te remercier pour ça, mais au final c'était mes premières applications de la méthode scientifique. Merci à **Maman** de m'avoir toujours dit de faire ce que je veux face à mes questionnements d'orientation. C'est le meilleur conseil qu'on puisse recevoir et je vous souhaite à tous et toutes d'avoir des mamans comme ça. Merci à **Cédric** de m'avoir initié très tôt à l'art du débat entre deux personnes têtues. Merci à lui d'être devenu un homme incroyable, honnête et drôle. Merci à **Élodie** d'être toujours d'accord avec moi. Merci d'avoir un cerveau au moins aussi bizarre que le mien, d'être toujours pleine de bonne humeur et de courage.

J'aimerais finir ces remerciements avec la personne qui m'aura le plus soutenu au quotidien lors de cette thèse. En particulier avec ces confinements qui ont fait que tout reposait sur toi : merci **ViPe**. Merci de m'avoir consolé après les journées les plus dures. Merci d'avoir réussi systématiquement à me changer les idées lors de mes pauses. Merci de me laisser courir, tourner et sauter partout dans l'appart les jours où ça va bien. Merci de m'avoir épargné une bonne part de mes tâches ménagères lors des derniers mois. Merci surtout d'être toujours là ~~devant ton ordi~~ prêt à m'écouter patiemment peu importe ce que j'ai à dire et combien de temps cela dure.

En résumé : merci à tou.te.s, je vous aime.

*À tous les curieux,
Qui ont ouvert ces pages.*

*À tous ceux qui connaissent le Jeu :
Vous avez perdu. <3*

Résumé de la thèse en français

Le nucléosome, la sous-unité de la chromatine, est constitué d'un octamère d'histones autour duquel s'enroule l'ADN. Les histones sont sujets à de nombreuses modifications post-transcriptionnelles, ce qui a pour effet de modifier l'expression des gènes voisins. Les modifications d'histones sont également importantes pour la structure de la chromatine et la stabilité génomique de la cellule. C'est par exemple le cas de la triméthylation de la lysine 9 de l'histone 3 (H3K9me3), une modification enrichie au niveau de l'hétérochromatine constitutive qui comprend notamment les régions péri-centromériques (Saksouk et al., 2015). Une suppression de cette modification est liée à l'instabilité génomique et à un manque de cohésion des chromosomes en métaphase (Peters et al., 2001).

La cohésion correcte des chromosomes est principalement réalisée par le complexe cohésine. Cohésine est recrutée sur le chromosome humain en phase G1 du cycle cellulaire et y est libérée en deux phases (Morales and Losada, 2018). Au début de la mitose, la cohésine située sur les bras des chromosomes est relâchée par la voie de la prophase, alors qu'au niveau des péri-centromères, cohésine reste jusqu'au début de l'anaphase afin d'éviter une séparation prématurée des chromatides sœurs. L'interaction des kinétochores avec les microtubules lors de la mitose est également importante pour le déroulement d'une mitose fidèle. CHAMP1 (Chromosome Alignment Maintaining Phosphoprotein 1) est une protéine nécessaire à l'assemblage correct des kinétochores et à la stabilité des microtubules (Itoh et al., 2011). Une diminution de CHAMP1 provoque un défaut d'alignement des chromosomes.

Lors de ma thèse, j'ai étudié la fonction de CDYL2 (Chromodomain on Y Like 2), une protéine lectrice de H3K9me3. CDYL2 fait partie d'une famille de six protéines dont deux sont issues de gènes autosomaux. Les protéines de la famille CDYL ont deux domaines conservés : un chromodomaine qui leur permet d'interagir avec H3K9me3 (Fischle et al., 2008) et un domaine d'hydratase enoyl-CoA dont l'activité enzymatique permet à CDYL1 de catalyser la réaction de crotonyl-CoA en β -hydroxybutyryl-CoA (S. Liu et al., 2017). CDYL1 a également été révélé comme un répresseur de la transcription, notamment en recrutant des méthyltransférases d'histones (Abu-Zhayia et al., 2018; Mulligan et al., 2008). Enfin, il a été montré que CDYL1 est impliqué dans de nombreuses maladies comme la dépression, l'épilepsie ou encore certains cancers (Liu et al., 2019; Y. Liu et al., 2017a; Mulligan et al., 2008; Qi et al., 2014). **En revanche,**

CDYL2 reste une protéine très peu caractérisée et ce travail cherche à étudier sa fonction cellulaire.

Avant mon arrivée dans l'équipe, certaines expériences ont révélé que CDYL2 était potentiellement impliqué dans la mitose puisqu'une diminution de son expression induit des défauts mitotiques, incluant un défaut d'alignement des chromosomes sur le plan équatorial pendant la métaphase et un manque de cohésion des chromosomes. Avec des marquages d'immunofluorescence, j'ai montré que CDYL2 est une protéine nucléaire située notamment au niveau des péricentromères grâce à H3K9me3. D'autres analyses ont permis de mettre en évidence la localisation des CDYL2 au niveau des centrosomes. Enfin, pour compléter la caractérisation de CDYL2, une coimmunoprécipitation suivie d'une analyse de spectrométrie de masse a permis de révéler une liste d'interacteurs de CDYL2. Parmi cette liste, j'ai retrouvé des protéines épigénétiques impliquées dans la répression de la transcription génique, des protéines membre du complexe cohésine, ainsi que CHAMP1.

Suite à ces premiers résultats, trois hypothèses ont été formulées quant aux mécanismes impliquant CDYL2 dans la mitose et la stabilité génomique :

CDYL2 est impliqué dans la répression de l'expression des répétitions satellites aux péricentromères. En interagissant avec EHMT2 ou SETDDB1, trouvées dans la spectrométrie de masse et nécessaires pour la répression de l'expression des séquences satellites, CDYL2 pourrait recruter ces protéines sur la chromatine, au niveau des péricentromères, et ainsi participer à la répression des séquences satellites. L'instabilité génomique provoquée par une diminution de CDYL2 serait alors expliquée par une augmentation aberrante de l'expression des répétitions satellites.

CDYL2 participe à la régulation de la cohésion des chromosomes. En interagissant avec les protéines impliquées dans le complexe cohésine, CDYL2 pourrait par exemple participer au recrutement ou à la protection du complexe, notamment au niveau des régions péricentromériques. Une diminution de CDYL2 provoquerait une fragilité sur la cohésion des chromosomes et induirait ainsi des mitoses infidèles.

CDYL2 interagit avec CHAMP1 et lui est nécessaire pour la régulation des connexions kinétochores-microtubules. Une diminution de CDYL2 inhiberait la fonction de CHAMP1 et phénotyperait une mutation de CHAMP1 : un mauvais alignement des chromosomes pendant la métaphase provoquée par un mauvais assemblage des kinétochores.

Pour la première hypothèse, j'ai pu confirmer l'interaction de CDYL2 avec EZH2 et SETDB1 par coimmunoprécipitation suivie de Western Blot. Cependant, un changement de l'expression de séquences répétitives péricentromériques n'a pas pu être observé avec une réaction en chaîne par polymérase quantitative (qPCR). Une coimmunoprécipitation de chromatine (ChIP) suivie de qPCR n'a pas pu non plus révéler un changement de degré de méthylation de H3K9 au niveau des péricentromères après diminution de CDYL2. CDYL2 semble donc être dispensable pour la répression des séquences répétitives et satellites au niveau des péricentromères.

En parallèle, j'ai pu confirmer l'interaction de CDYL2 avec le complexe cohésine par coimmunoprécipitation et test de lien entre protéines (PLA). La localisation de cohésine au niveau des péricentromères pendant la mitose a pu être observée à l'aide d'un microscope confocal après immunomarquage. Cependant, malgré un manque de cohésion des chromosomes observé précédemment, une diminution de CDYL2 n'a pas semblé influencer la localisation de cohésine au niveau des péricentromères, suggérant que CDYL2, bien qu'elle est dispensable au recrutement de cohésine au niveau de l'hétérochromatine péricentromérique.

Enfin, j'ai pu également confirmer l'interaction de CDYL2 avec CHAMP1 par coimmunoprécipitation. Une perte de fonction de CHAMP1 induit une instabilité des microtubules mitotiques ainsi qu'un défaut de leur connexion aux kinétochores, ce qui provoque ensuite un défaut d'alignement des chromosomes sur le plan équatorial pendant la mitose. Bien que ce défaut d'alignement ait pu être observé après diminution de CDYL2, la stabilité des microtubules mitotiques et leur connexion aux kinétochores semblent préservés, indiquant que CDYL2 n'est pas nécessaire à la fonction de CHAMP1.

Pendant ma thèse, j'ai collaboré à l'étude de la fonction de CDYL2 dans le cancer du sein. J'ai montré par coimmunoprécipitation que CDYL2 interagit avec EHMT1 et EHMT2. En utilisant des inhibiteurs des activités enzymatiques de EHMT2 et EZH2, j'ai pu également révéler que

l'activité enzymatique de EHMT2 est nécessaire à la répression de l'expression de miR124 par méthylation de H3K9. Au final, l'étude a permis de conclure que CDYL2 recrute EHMT2 sur la région promotrice de miR124 et réprime son expression.

Dans l'ensemble, ma thèse a participé à l'élucidation de la fonction cellulaire de CDYL2. J'ai montré que CDYL2 est principalement nucléaire et se localise sur la chromatine au niveau des régions péri-centromériques grâce à la modification H3K9me3. Cette observation est dans la lignée des études *in vitro* précédemment menée sur la protéine qui montrait son affinité avec H3K9me3 (Fischle et al., 2008). Plus surprenante mais non moins intéressante est la localisation de CDYL2 au niveau des centrosomes pendant tout le cycle cellulaire. Reste à déterminer si cette localisation est dépendante d'une modification post-transcriptionnelle structurellement similaire à H3K9me3.

D'un point de vue fonctionnel, j'ai pu montrer que CDYL2 est une protéine indispensable au bon déroulé de la mitose. En étudiant les interacteurs de CDYL2, j'ai trouvé plusieurs candidats pouvant expliquer les phénotypes de mitoses infidèles observés après diminution de CDYL2. Ces candidats, comprenant EHMT2, SETDB1, le complexe cohésine ainsi que CHAMP1 ont tous pu être confirmé par une deuxième méthode, renforçant les premières observations. Cependant, malgré la fiabilité des techniques utilisées, un mécanisme reliant ses interactions avec la fonction de CDYL2 dans un contexte mitotique n'a pas pu être dévoilé. Des futures pistes de développement sont proposées dans ce travail afin d'explorer plus en avant la fonction cellulaire de CDYL2.

Dans le contexte de la cancérologie, provoquer une instabilité génomique est une stratégie qui est utilisée dans les traitements. Étant donné qu'une surexpression de CDYL2 profite aux tumeurs du sein en augmentant leur plasticité et leur agressivité (Siouda et al., 2020), une inhibition de CDYL2 dans ces tumeurs pourrait avoir un double intérêt thérapeutique : réduire l'agressivité et augmenter l'instabilité génomique des cellules cancéreuses, provoquant potentiellement leur mort.

TABLE DES MATIERES

1	Epigenetics: Transcription Regulation and Chromatin Organization.....	27
1.1	“Beyond Genetics” – A Historical Approach.....	27
1.2	Mechanism of Epigenetic Regulations	29
1.3	Epigenetics and Cancer.....	45
2	Genomic Instability	51
2.1	An Emerging Cancer Hallmark	51
2.2	Progression of a faithful mitosis	53
2.3	Mitosis From an Epigenetic Point of view	66
3	CDYL: A Multi-Tasked Histone Reader Family	69
3.1	A family to share the reading monopole of H3K9me3.....	69
3.2	CDYL Family.....	70
3.3	Cellular Function.....	74
3.4	Implication in Diseases	81
3.5	Targeting CDYL Proteins?.....	83
4	Hypothesis and Objectives.....	85
5	Key materials	89
5.1	Antibodies.....	89
5.2	Primers.....	90
6	Methods	93
6.1	Cell Culture	93
6.2	Transfections	94
6.3	Protein work	94
6.4	RNA work.....	97
6.5	Microscopy work	97

6.6	Cell Cycle Analysis.....	99
6.7	Chromatin Immunoprecipitation.....	99
7	CDYL2 biological function in mitosis – Preliminary Results	103
7.1	CDYL2 knock-down induces aberrant mitosis.	103
7.2	CDYL2 proteome.....	105
8	CDYL2 Biological Function in Mitosis - Thesis Results	107
8.1	CDYL2 is a nuclear protein, tightly bound to chromatin	107
8.2	CDYL2 localizes at pericentromeres in a H3K9me3 dependent manner	113
8.3	CDYL2 localizes at centrosomes throughout interphase and mitosis.....	118
8.4	CDYL2 interactome reveals many interactors involved in genomic stability and mitosis.....	121
8.5	Hypothetic mechanisms descriptions.....	124
8.6	CDYL2 seems dispensable for satellite repression	125
8.7	CDYL2 interacts with Cohesin but does not seem to regulate its level at pericentromeres.....	130
8.8	CDYL2 interacts with CHAMP1 but is dispensable for microtubules stability.....	135
9	CDYL2 Epigenetically Regulates MIR124 to Control NF-kB/STAT3-Dependent Breast Cancer Cell Plasticity	143
10	Results Discussion	193
10.1	CDYL2 localization and regulation through the cell cycle.....	193
10.2	Mechanisms of CDYL2 Implication in Mitosis	196
10.3	CDYL2 as a transcription repressor?	201
10.4	Further CDYL2 characterization	202
10.5	CDYL2 as a potential therapeutic target?	204
11	Conclusion	211
12	Bibliography.....	215

Abbreviations

APC: Anaphase Promoting Complex	MCM4: Minichromosome Maintenance 4
ATP: Adenosine Triphosphate	MECP2: Methyl-CpG-Binding Protein 2
	MHC: Major Histocompatibility Complex
BUB: Budding Uninhibited by Benzimidazoles	MPS1: Monopolar Spindle 1
CAF1: Chromatin Assembly Factor 1	NAD: Nicotinamide Adenine Dinucleotide
CARM1: Coactivator-Associated Arginine Methyltransferase 1	NCAP-H: Non-SMC Chromosome Associated Protein H
CDC20: Cell Division Cycling Protein 20	NF- κ B: Nuclear Factor κ B
CDK1: Cyclin Dependent Kinase 1	NIPBL: Nipped B-Like
CDYL: Chromodomain on Y-Like	NMR: Nuclear Magnetic Resonance
CENPA: Centromeric Protein A	NUMA1: Nuclear Mitotic Apparatus Protein
CHAMP1: Chromosome Alignment Maintaining Phosphoprotein 1	NuRD: Nucleosome Remodeling and Deacetylase
ChIP: Chromatin Immunoprecipitation	
CTBP1: C-Terminal Binding Protein 1	P/CAF: P300/CBP-Associated Factor
CTNNB1: Catenin beta 1	PD1: Programmed Cell Death
	PD1L1: PD1 Ligand 1
DNA: Desoxyribonucleic Acid	PDS5B: Sister chromatid cohesion protein PDS5B
DNMT: DNA Methyltransferase	PHD: Plant Homeodomain
DOT1L: Disrupter Of Telomer Silencing Protein 1 Like	PLK1: Polo-like Kinase 1
	POGZ: Pogo Transposable Element with Zinc-Finger Domain
ECH: Enoyl CoA Hydratase	PP2A: Phosphatase 2A
EHMT2: Euchromatic Histone Lysine Methyltransferase 2	PRC1: Polycomb Repressive Complex 1
EZH2: Enhancer of Zeste Homolog 2	PRMT: Protein Arginine Methyltransferase
	PRMT1: Protein Arginine Methyltransferase 1
FAD: Flavin Adenine Dinucleotide	
FDA: Food and Drug Administration	
H3K9me3: Histone 3 Lysine 3 trimethylated	RAD21: Double Strand Break Repair Protein Rad21 Homolog
HAT: Histone Acetyl Transferase	RC1: Repair Complex 1
HDAC: Histone Deacetylase	RCOR1: REST Corepressor 1
HKMT: Histone Lysine Methyltransferase	REST: RE1-Silencing Transcription Factor
HP1: Heterochromatin Protein 1	RhoA: Ras Homolog Family Member A
	RNA: Ribonucleic Acid
JMJD: Jumonji Domain Containing Protein	
KDM: Lysine Specific Demethylase	SAC: Spindle Assembly Checkpoint
	SAM: S-Adenosyl-L-Methionine
MAD2L2: Mitotic Arrest Deficient 2-Like Protein 2	SAM: S-Adenosylmethionine
MBD: Methyl-CpG Binding Domain	SCN8: Sodium Channel Protein Type 8
MBT: Malignant Brain Tumor	SET: Suv(var)3-9, Enhancer of Zeste and Trithorax
MCC: Mitotic Checkpoint Protein	SGO1: Shugoshin 1
	SIRT1: Sirtuin 1

SMC: Structural Maintenance of
Chromosome
STAG: Stromal Antigen
STAT3: Signal Transducer and Activator of
Transcription 3
SUV39H1: Suppressor of Variegation 3-9
Homolog 1

TCGA: The Cancer Genome Atlas
TET: Ten-Eleven Translocation

WAPL: Wings-Apart Protein Like
WT: Wild-Type

INTRODUCTION

1 EPIGENETICS: TRANSCRIPTION REGULATION AND CHROMATIN ORGANIZATION

1.1 "BEYOND GENETICS" – A HISTORICAL APPROACH

In 1930, Hermann Joseph Muller, an American geneticist, referenced abnormal phenotypes in *Drosophila* that could not be explained by genetics only (Muller, 1930). In 1952 and 1970, work in both *Rana pipiens* and *Xenopus laevis* lead to the observation that all somatic cells, despite different functions and shapes, contain the same DNA, holding all the information needed to form a complete organism when introduced into an enucleated egg (Briggs and King, 1952; Laskey and Gurdon, 1970). These observations strongly suggest that the genome cannot control all the events leading to phenotype establishment and that something "beyond genetics" should be involved. To describe this phenomena, we nowadays use the term "epigenetics" introduced by Waddington, which he defined as changes in phenotype that do not involve changes in genotype (Waddington, 1942).

In the following decades, an impressive number of publications revealed the mechanisms underlying this newly observed phenomenon. DNA methylation, corresponding to the

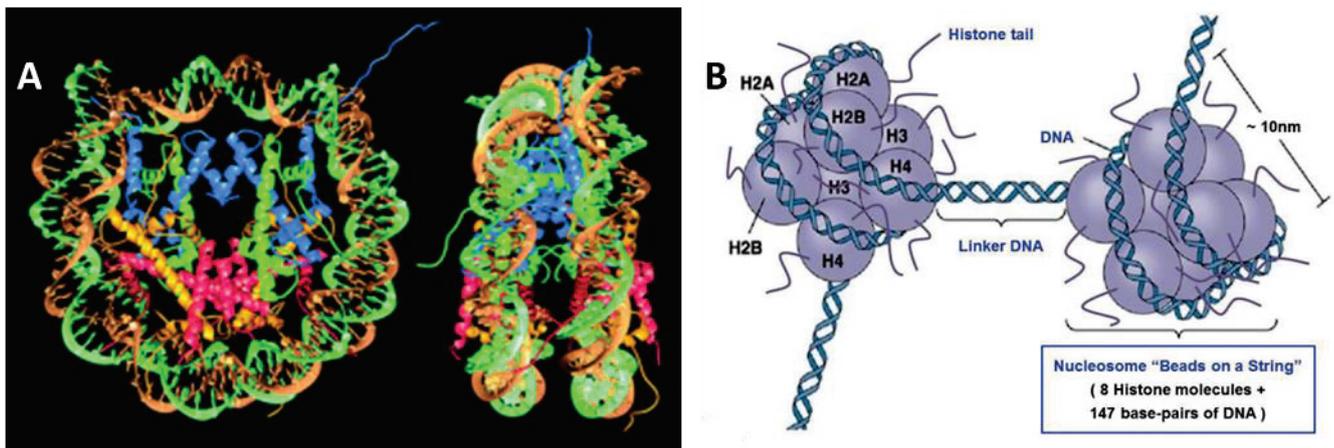


Figure 1: A nucleosome, corresponding to the chromatin subunit, is made out of a histone octamer, surrounded by a DNA strand. (A) The nucleosome structure revealed by X-ray crystallography (Luger et al. 1997). Brown and turquoise: DNA double helices; blue: H3; green: H4; yellow: H2A; red: H2B. (B) The nucleosome structure schematized by Young Zoon Kim, highlighting histone tails and nucleosome size (Kim, 2014).

covalent addition of a methyl group on cytosine base, was reported before the 50s by several groups (Hotchkiss, 1948; Johnson and Coghill, 1925) and its function in gene regulation observed approximately 30 years later (reviewed by Razin and Riggs, 1980). Also very important for the epigenetic field, was the discovery of histones. These small proteins were isolated from the nucleus and their diversity studied using notably chromatography (Crampton *et al.*, 1955). The organization of DNA around histones and the resulting subunit, the nucleosome, were proposed with more details in 1974 (Kornberg, 1974), and the X-ray structure, that can be seen on figure 1A, revealed in 1997 by Luger *et al.* (Luger *et al.*, 1997). Structurally, the nucleosome consists of a histone octamer packaging 147 bp of DNA wrapped 1.7x around the complex. Histones are small and conserved proteins, consisting of a globular and a tail domain. In the 60s, Allfrey *et al.* reported that histones could carry post-transcriptional modifications and suggested that these modifications could be linked with gene expression (Allfrey *et al.*, 1964). Since the 90s, various histone modifications, mostly localized on histone tails, have been observed and their function on gene regulation characterized.

Our understanding of epigenetic control of gene expression increased greatly since 2000 thanks to the use of more and more genome and proteome-wide analysis. The combination of genome-wide sequencing after chromatin immunoprecipitation (ChIP) in particular allowed us to characterize with precision the position of specific histone modifications, transcription factors, and DNA modifications (reviewed in Park, 2009). Similarly, the progress made on mass spectrometry techniques facilitated the discovery of interactions between epigenetic proteins and partners of specific histone modifications (Stunnenberg and Vermeulen, 2011). However, the more we detail epigenetic mechanisms, the more the mechanism seems multifaceted and tightly regulated by multiple actors. During the first discoveries of histone modifications, epigenetic modifications were believed to act as a code and would give us the ability to predict whether, when, and how the neighbour gene would be expressed (Jenuwein and Allis, 2001; Strahl and Allis, 2000). The code denomination now seems outdated and epigenetic pathways are more and more presented like a complex language (Allis and Jenuwein, 2016).

In the following chapters, I will review the current knowledge of the mechanism used by epigenetic factors to regulate both DNA transcription and chromatin organization. This review will include DNA methylation, histone modifications with a highlight on methylation, crosstalk mechanisms between the different modifications, histone variants, a brief introduction into the different chromatin structure, and transcription regulation by non-coding RNA. I will also discuss the importance of epigenetics in diseases, particularly in cancer and the advances made and to be done on therapies targeting epigenetics.

1.2 MECHANISM OF EPIGENETIC REGULATIONS

1.2.1 DNA methylation

The first description of DNA methylation – and thus the first modification on nuclear bases – was described as early as 1925 in *Tubercle bacillus* (Johnson and Coghill, 1925). Several hypotheses were given for DNA methylation function in the cell and a link with gene repression was confirmed by several groups in the mid-1970s (Holliday and Pugh, 1975; Razin and Riggs, 1980). Nowadays, we know that at least four different DNA modifications exist: methylation, hydroxylation, formylation, and carboxylation. Hydroxylation, formylation, and carboxylation are obtained through sequential oxidation of DNA methylation and were therefore considered to be intermediate for DNA demethylation (Tahiliani *et al.*, 2009). More recent work has highlighted, that they are also implicated in gene regulation (Branco *et al.*, 2012; Sun *et al.*, 2013), but methylation remains the best-characterized modification. Three DNA methyltransferases (DNMTs) have been identified in mammals and all three catalyze the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to cytosine C5. The three enzymes differentiate notably in their function: while DNMT1 is implicated for DNA methylation maintenance after replication, DNMT3a and DNMT3b are responsible for *de novo* establishment of DNA methylation, for example during embryogenesis (Hata *et al.*, 2002; Okano *et al.*, 1999).

Before the discovery of hydroxylation, DNA methylation was for a long time considered to be a rather stable modification, disappearing passively through a lack of maintenance of the modification on the new strand when DNA gets replicated (Rougier *et al.*, 1998). However, some observations have challenged this idea of stability and suggested the

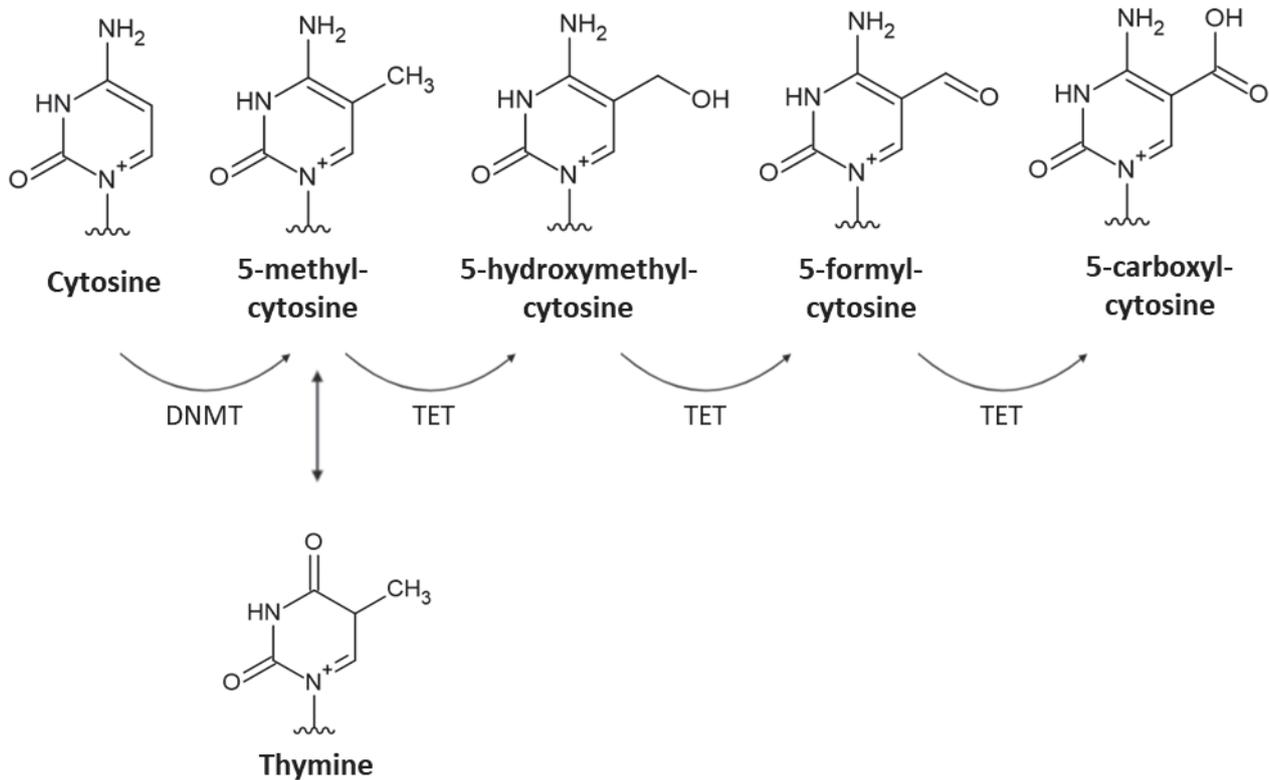


Figure 2: Cytosine modifications structure. Cytosine is C5 methylated by DNMT enzymes. Methylcytosine demethylation happens through subsequent oxidation of the methyl group to hydroxymethyl, formyl and carboxyl. Methyl-cytosine also has a similar structure to thymine and can be source of mutations.

existence of a more active demethylation mechanism. As an example, it has been observed that DNA undergoes a major global methylation loss in early zygotes before DNA replication occurs (Oswald *et al.*, 2000). A mechanism for DNA demethylation was proposed in 2009 with the description of DNA hydroxymethylation (Kriaucionis and Heintz, 2009; Tahiliani *et al.*, 2009). The Ten-Eleven Translocation (TET) family, consisting of three proteins – TET1, TET2, and TET3 – was shown to correspond to the enzymes responsible for this reaction (Ito *et al.*, 2010; Tahiliani *et al.*, 2009). The oxidation of methylated DNA by TET enzymes is illustrated on figure 2.

The first protein discovered which was able to recognize and bind to DNA methylation was methyl-CpG-binding protein 2 (MECP2) (Nan *et al.*, 1996). Soon after, four proteins, called methyl-CpG binding domain (MBD1-4) with a similar methylcytosine binding domain were discovered (Filion *et al.*, 2006). MBD2 and MBD3 can both be part of the Nucleosome Remodeling and Deacetylase (NuRD) complex, in a however mutually

exclusive manner (Guezennec *et al.*, 2006). Both proteins are playing a role in gene silencing by recruiting histone deacetylases to the chromatin. It is interesting to note that MBD3 has a poor affinity to DNA methylation due to a critical tyrosine being replaced by phenylalanine (Fraga *et al.*, 2003). MBD1 recruits chromatin-modifying enzymes to the chromatin and is mostly linked with transcription silencing (Fujita *et al.*, 2003; Ng *et al.*, 2000). Finally, MBD4 function is less characterized but seems implicated in DNA mismatch repair (Wong *et al.*, 2002). Except for MBD3, MBD proteins seem to have their strongest affinity for DNA methylation compared to other DNA modifications (Buchmuller *et al.*, 2020).

DNA methylation is mostly linked with transcription repression. It is found notably in permanently silenced regions like centromeres, telomeres, at repeat sequences, and on the inactive X-chromosome (Gonzalo *et al.*, 2006; Mohandas *et al.*, 1981; Weisenberger *et al.*, 2005; Yan *et al.*, 2010). DNA methylation is also found on so-called CpG islands, sites enriched in cytosine-guanine sequences and present in approximately 70 % of all mammalian promoters (Deaton and Bird, 2011). DNA methylation on these sites was shown to be linked with transcription silencing of downstream genes. However, DNA methylation was also found within actively transcribed gene bodies (Jones, 1999). It is now considered that DNA methylation functions are context-dependent, which brings new challenges to elucidate its effects. Other DNA modifications functions have been poorly characterized. Still, genome-wide mapping of DNA hydroxylation revealed a distribution at both active and repressed genes (Wu and Zhang, 2011), suggesting a context-dependent signification as well.

1.2.2 Histone Modifications

In the 60s, Vincent Allfrey gave some evidence for histone acetylation and suggested that histone post-transcriptional modifications are involved in gene transcription regulation (Allfrey *et al.*, 1964). The first histone acetyltransferase – the enzyme responsible for histone acetylation – was discovered three decades later by Brownell *et al.* (Brownell *et al.*, 1996). Very soon after, the first histone deacetylase was discovered too, indicating that histone acetylation is a reversible process (Taunton *et al.*, 1996). Lysine acetylation, lysine and arginine methylation, as well as serine phosphorylation are the most studied

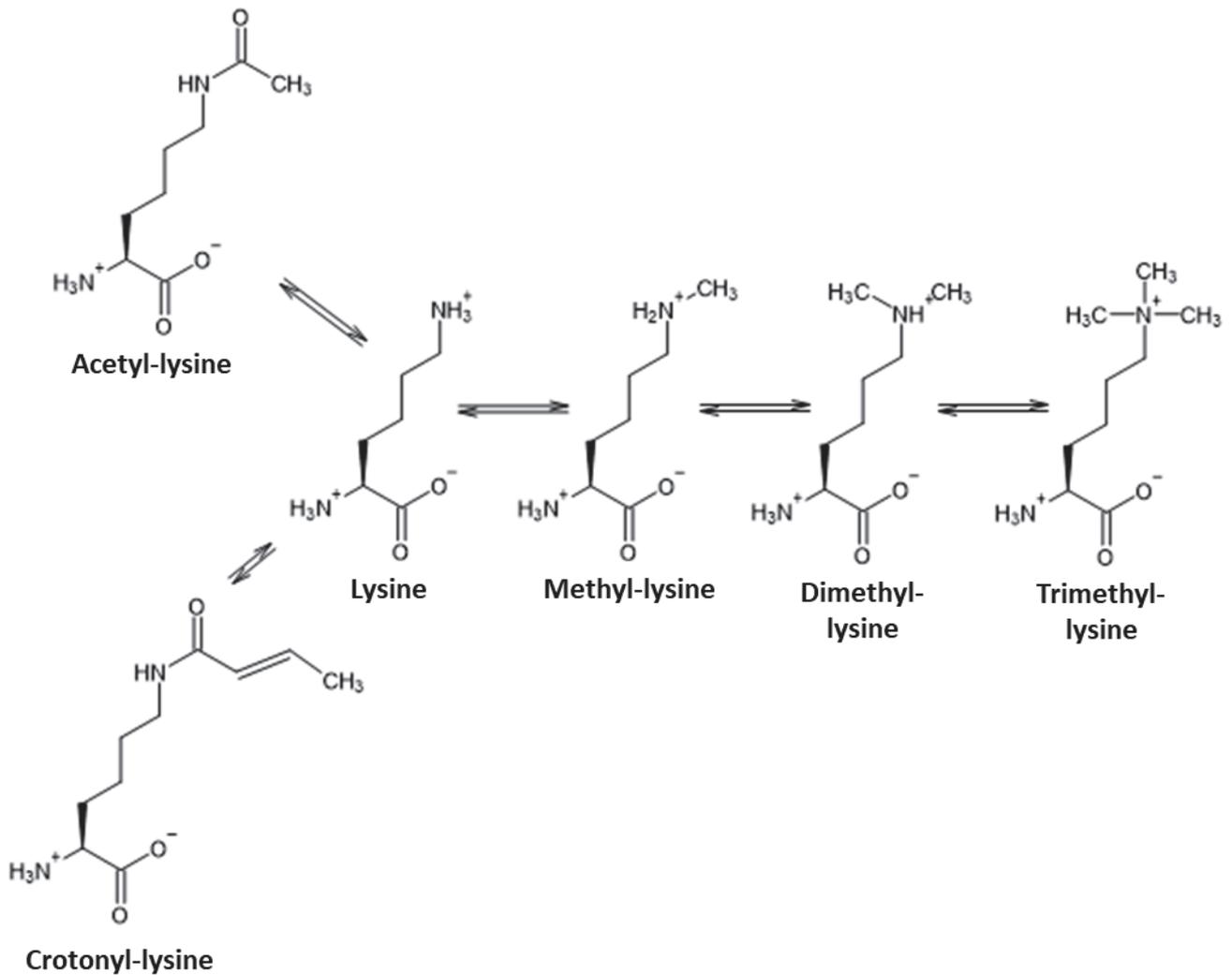


Figure 3: Lysine modifications structure. Lysine amino acids can be either acetylated, crotonylated or mono-, di- or trimethylated. The positive global positive charge of lysine in a physiological context is suppressed after acylation.

modifications, but other modifications as ubiquitylation, crotonylation, or sumoylation have also been described, and all are reversible. It is here interesting to note that lysines can be both acylated or methylated as described on figure 3. The enzymes responsible for their addition on histones were named “writers”, the ones for the reverse reaction the “editors” or “erasers” and the enzymes recognizing those modifications were designed as “readers”. In 2000, Strahl and Allis proposed a theory, called “the histone code”, saying that the combination of histone modifications leads to a specific biological outcome (Strahl and Allis, 2000). This would happen by the recruitment of reader proteins or transcription complexes at the wished loci. In addition to the recruitment of transcription proteins, some modifications, like acetylation and phosphorylation, neutralize histone tail

positive charge, which may induce weaker interactions with the DNA strand, changes chromatin structure and thus its accessibility (Zhang et al., 2017).

Histone acetylation, methylation, phosphorylation, and crotonylation are detailed in the next paragraphs, as well as their writers, editors, readers, and their broad functions.

1.2.2.1 Histone Acetylation

Histone acetyl-transferases (HAT) were the first histone modification proteins purified from ciliated protozoans in 1996 (Brownell *et al.*, 1996). These enzymes catalyze the transfer of the acetyl group from the cofactor acetyl-CoA to the ϵ -amino group of lysine (Racey and Byvoet, 1971). The HAT enzymes are classified into two types: A and B. The A-type enzymes are diverse and classified into three different sub-groups based on their amino acid sequence and their conformation. Each HAT can acetylate multiple sites and their specificity can be conferred by the complex they interact with (Grant *et al.*, 1997). Unlike the A-type, the B-type enzymes acetylate histones before they get deposited on chromatin. This is notably the case for acetylated lysines 5 and 12 from histone 4 (H4K5Ac and H4K12Ac), which are indeed acetylated by HAT-B enzymes (Haigney *et al.*, 2015). Those two modifications are necessary for Centromeric Protein A (CENPA) containing nucleosome deposition at the centromere (Shang *et al.*, 2016). It is interesting to note that, even though it is most common, histones are not only acetylated on the tail. As an example, H3K56 was found to be acetylated by hGCN5 following DNA damage (Tjeertes *et al.*, 2009).

Histone deacetylases – HDAC – are the enzymes catalyzing the removal of the acetylation marks deposited by HAT. The first HDAC purification and cloning was published one month after the report of the first HAT enzymes by Taunton and colleagues (Taunton *et al.*, 1996). Nowadays, eighteen enzymes have been identified in *Homo sapiens* and classified into four different classes (reviewed by Seto and Yoshida, 2014). Enzymes in the first and second classes are most closely related to the histone deacetylases scRpd3 and scHda1 respectively. Class IV only contains HDAC11. All three classes use a similar deacetylation mechanism, which necessitates a zinc metal ion. On the opposite, the third class, which contains enzymes related to scSir2, uses a mechanism involving Nicotinamide Adenine

Dinucleotide (NAD⁺). All HDACs can deacetylate various modifications, including acetylation on non-histone proteins. As an example, HDAC1 is responsible for p53 deacetylation (Ito *et al.*, 2002). Since HDACs are often found in big complexes that may contain several other HDAC proteins, it is difficult to investigate the specific function of each particular enzyme in the cell.

Histone acetylation was very rapidly linked to gene activation as the first HAT discovered was an ortholog to scGcn5, a known transcriptional activator in yeast (Brownell *et al.*, 1996), while the first HDAC was described as an ortholog of the budding yeast transcriptional co-repressor scRpd3 (Taunton *et al.*, 1996). Additionally, the silenced Xi chromosome in mammals was found to be hypoacetylated (Csankovszki *et al.*, 2001; Gilbert and Sharp, 1999) and ChIP-seq analysis revealed acetylated histones enriched at enhancers regions and throughout the transcribed region of active genes (Millar *et al.*, 2006; Roh *et al.*, 2005). The HAT/HDAC system, therefore, proposes a very interesting ON/OFF switch for gene activation. How acetylation is mechanistically linked to gene transcription was not clear from the beginning. It was first expected to induce changes in the chromatin structure through electrochemical charges. Indeed, naked lysine has a positive charge possibly inducing some interaction with the negative DNA strand and thus “closing” chromatin. Acetylation of the lysine residue will destroy this positive charge and this interaction with DNA, making the chromatin more open (Bode *et al.*, 1980; Walia *et al.*, 1998). In 1999 however, P300/CBP-Associated Factor (P/CAF), the first histone acetylation reader protein, containing a bromodomain, was described (Dhalluin *et al.*, 1999), putting light on the *trans* mechanism where the reader recruits various proteins at acetylated sites. Now, over forty proteins with bromodomain have been reported in humans. They do not only recruit transcription coactivators but also chromatin modelers, histone acetyltransferases, and methyltransferases. Other acetyl-lysine-reader proteins, containing a tandem plant homeodomain (PHD) fingers were also described (Zeng *et al.*, 2010). Their implication in transcription, DNA repair, replication, and condensation highlighted the importance of histone acetylation for numerous cellular functions (Feitoza *et al.*, 2017; Li *et al.*, 2018; Murr *et al.*, 2006; Sterner and Berger, 2000; Unnikrishnan *et al.*, 2010).

1.2.2.2 Histone Methylation

Histone methylation, unlike acetylation, includes several different modifications. Indeed two amino acids can get methylated: lysines and arginines. While the former can be mono-, di- or trimethylated, the latter can be monomethylated, symmetrically, or asymmetrically demethylated as illustrated on figures 3 and 4 respectively. The first Histone Lysine Methyltransferase (HKMT) enzyme, Suppressor of Variegation 3-9 Homolog 1 (SUV39H1), was reported in 1994 and shown to selectively trimethylate histone 3 lysine 9 (H3K9) six years later (Rea *et al.*, 2000; Tschiersch *et al.*, 1994). The enzymatic activity is made possible by the SET domain, whose name originates from Su(var)3-9, Enhancer of zeste and Trithorax, three proteins able to methylate histone lysines (Dillon *et al.*, 2005). In the mammalian genome, approximately 50 genes have been found to have a SET domain. HKMTs have specificity for both the modification localization

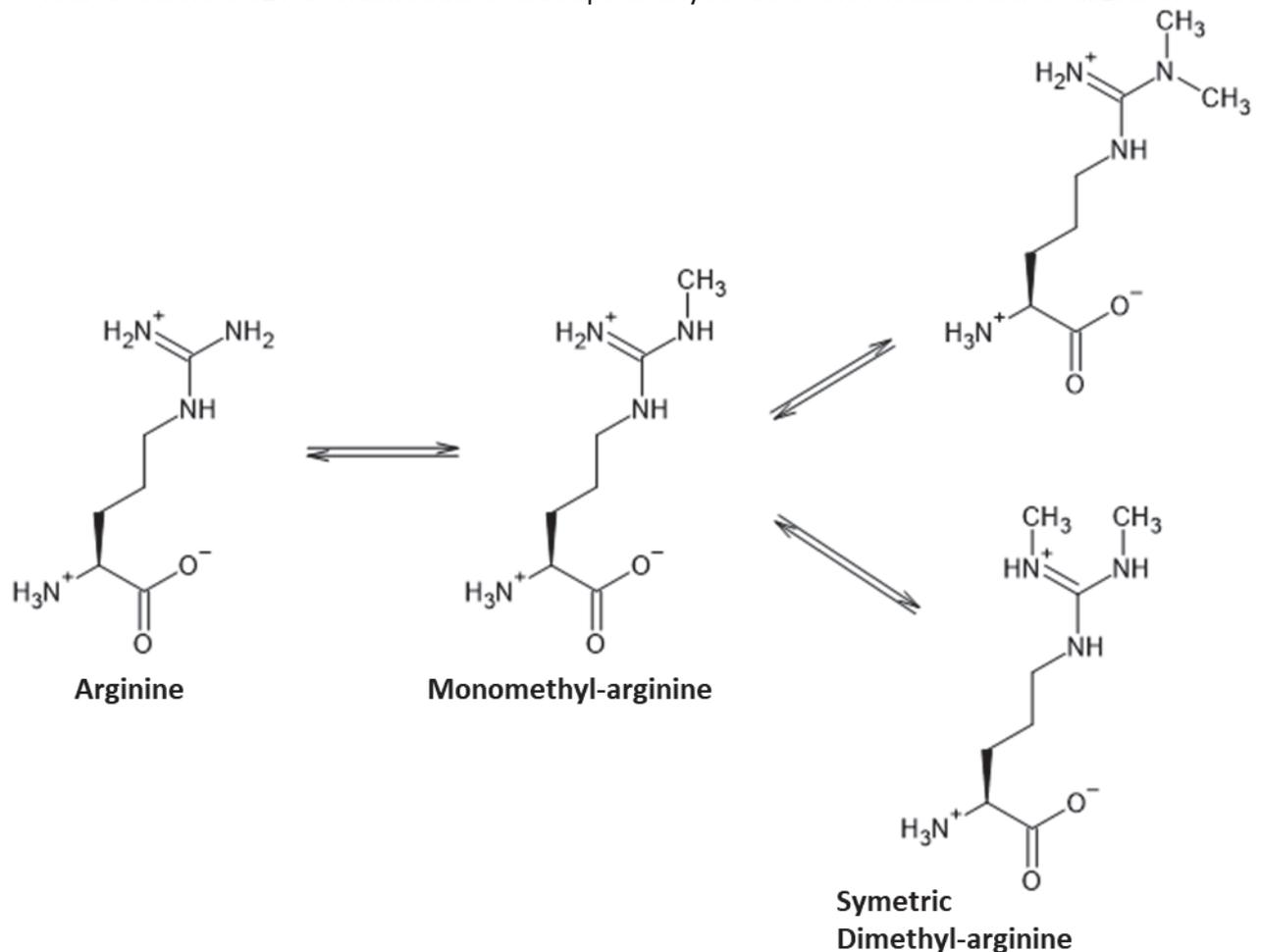


Figure 4: Arginine methylation structure. Arginine amino acids can be either mono- or dimethylated. Dimethylation happens either symmetrically or asymmetrically.

on the substrate and methylation. As an example, Euchromatic Histone lysine Methyltransferase 2 (EHMT2) mono- and dimethylates H3K9, Suv39h1 trimethylates the same lysine starting from H3K9me1, while Enhancer of Zeste Homolog 2 (EZH2) is responsible for H3K27 methylation (Cao and Zhang, 2004; Rea *et al.*, 2000; Tachibana *et al.*, 2002). Main histone methylation writer and eraser enzymes are presented on figure 5. The specificity is also observed when working *in vitro* with only one enzyme, suggesting it is independent of interactions with any complex (Collins *et al.*, 2005; Rea *et al.*, 2000). HKMTs without SET domain were also described and are responsible for methylation of lysine in the histone globular domain. This is for example the case for Disrupter Of Telomer Silencing Protein 1 Like (DOT1L) which methylates H3K79 (Feng *et al.*, 2002). All HKMT catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to a lysine ϵ -amino group. Every enzyme contains two distinct pockets: one is working as a binding site for SAM while the other is for the enzyme substrate. X-ray analysis has revealed that an aromatic residue, usually tyrosine or phenylalanine, is determinant to control the methylation degree of the SET enzyme (Collins *et al.*, 2005; Zhang *et al.*, 2003). The family of Protein Arginine Methyltransferases (PRMT) contains 11 members which are divided into two groups. The first contains proteins that catalyze mono- and asymmetric methylations, while the other contains enzymes responsible for mono- and symmetric methylations. Like for HKMT, PRMT enzymes transfer a methyl group from SAM and show a distinct pocket for their cofactor and the substrate (Copeland *et al.*, 2009).

Histone methylation was first believed to be a stable modification that could only get erased by diffusion through DNA replication, as the first HDMT description only got published in 2004. The described enzyme, Lysine-Specific Demethylase 1 (LSD1) uses Flavine Adenine Dinucleotide (FAD) as a co-factor (Shi *et al.*, 2004). As the demethylation mechanism requires protonation of the lysine, LSD1 is only able to demethylate mono- and dimethylation. Depending on the complex it is binding with, LSD1 can have different substrate, for example, complexed with REST Corepressor 1 (RCOR1) or Sirtuin 1 (SIRT1) it catalyzes H3K4 demethylation while with the androgen complex it demethylates H3K9 (Metzger *et al.*, 2005; Mulligan *et al.*, 2011; Shi *et al.*, 2005). Two years after the identification of LSD1, another class of histone demethylases was found, containing a Jumonji domain and demethylating lysine through another mechanism, which uses

iron (II) and α -ketoglutarate as co-factors, allowing them to work as well on trimethylation (Tsukada *et al.*, 2006). Like for HKMTs, histone demethylases have high specificity for both substrate and degree of methylation. As an example, the Jumonji proteins from the Jumonji Domain-containing protein 2 (JMJD2) subfamily can demethylate H3K9me3 and H3K36me3 but have only poor effects on the mono- and dimethylated forms (Cloos *et al.*, 2006; Couture *et al.*, 2007; Klose *et al.*, 2006). Demethylation of arginine was first described to go through deamination of the histone but JMJD6 was recently shown to be able to demethylate H3R2 and H4R3 even though it is not thought to be its main activity (Chang *et al.*, 2007).

Many different enzymes have been found to act as histone methylation readers and they are usually classified into two groups: the Royal superfamily, which contains chromodomain, tudordomain, and malignant brain tumor (MBT) proteins, and plant

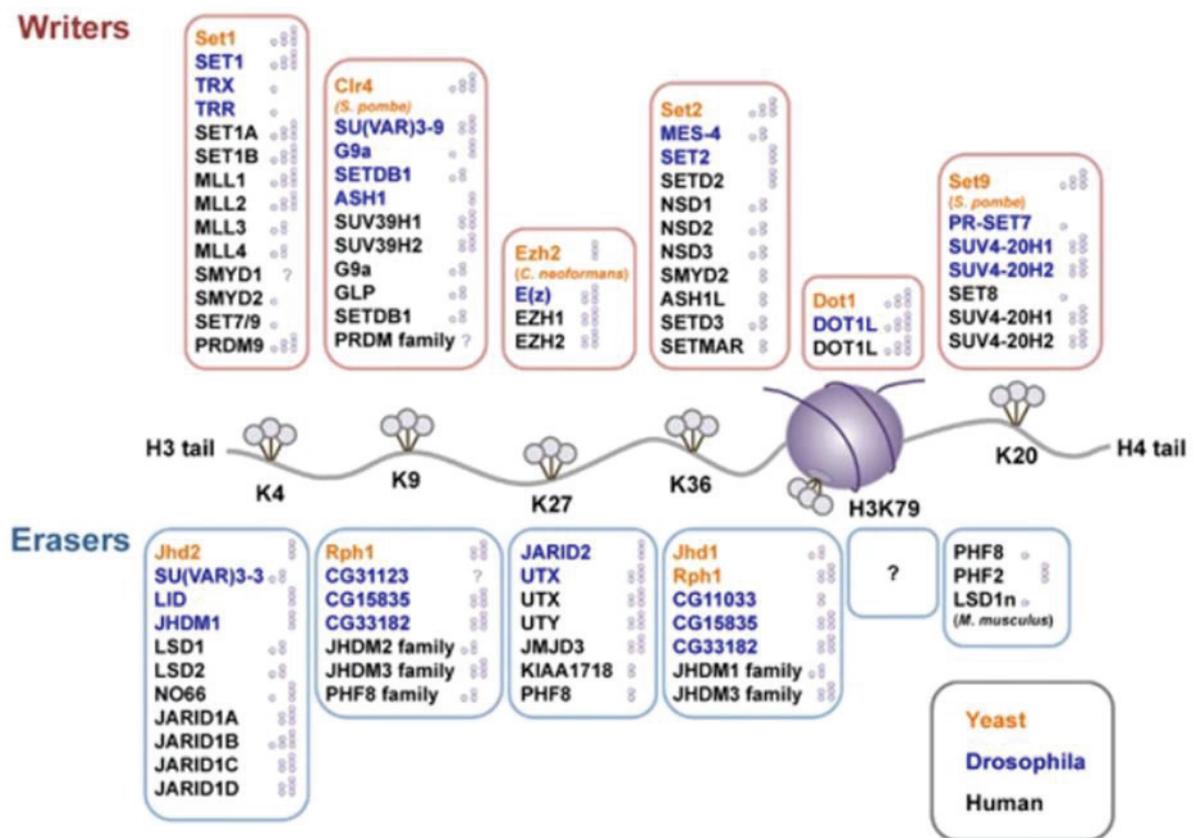


Figure 5: Principal histone methylation sites with their corresponding writers and editors proteins in *Saccharomyces cerevisiae*, *Drosophila Melanogaster* and *Homo sapiens*. The writers and erasers are depicted with their specificity for both the amino acid and the methylation state. Single circle: me1; two circles: me2; three circles: me3. Image taken from Hyun et al. (Hyun et al. 2017).

homeodomain (PHD) fingers. PHD domain proteins use a zinc-finger to interact with methylated histones and interact mainly with di- or trimethyl H3K4 (Org *et al.*, 2008; Wysocka *et al.*, 2006). The Royal superfamily interacts with methylated lysine through a pocket containing an aromatic cage. Since methylated lysine is mostly cationic in the physiological context, this cage containing aromatic residues allows cation- π -type interactions (Ma and Dougherty, 1997; Ruthenburg *et al.*, 2007). Polycomb proteins are probably the most famous complex interacting with H3K27me₃, however, the first methylation reader to be described in 2001 in humans was Heterochromatin Protein 1 (HP1) (Bannister *et al.*, 2001; Lachner *et al.*, 2001), a protein containing a chromodomain recognizing H3K9me₃ and a chromoshadow domain allowing HP1 to dimerize (Cowieson *et al.*, 2000). Another family of H3K9me₃ reader, the chromodomain on Y like (CDYL) family, is described with more precision in the third chapter of this introduction. Even though enzymes implicated in histone methylation are overall more specific than those involved in acetylation, some writers and erasers may also use non-histone proteins as substrate and several readers have been shown to interact with those modified proteins through their aromatic cages. For example, EHMT2 can self-methylated and is then recognized by CDYL chromodomain (Chin *et al.*, 2007; Fischle *et al.*, 2008).

Histone methylation is a modification highly conserved during evolution as it is present in unicellular organisms, in plants, in invertebrates as well as in mammals. In opposition to histone acetylation, histone methylation does not change the electrochemical charge of the histone tail and thus works through the recruitment or stabilization of downstream complexes. It is involved in many cellular processes and can either activate or repress gene transcription, however, each modification seems to have some functional specificity. H3K4 trimethylation for example is mostly associated with gene activation, as its di- and trimethylated forms are located on transcriptional start site (Guenther *et al.*, 2007; Santos-Rosa *et al.*, 2002). Similarly, H3K36 methylation is usually associated with active gene bodies (Guenther *et al.*, 2007; Wang *et al.*, 2007). While H3K9 monomethylation seems associated with gene activation as well (Barski *et al.*, 2007), higher methylated forms are linked with gene repression and are found in permanently silenced regions, as notably the Xi chromosome and satellite repeats (Bannister *et al.*, 2001; Keniry *et al.*, 2016; Nishibuchi and Déjardin, 2017). Similarly, H3K27me₃ was mostly described on

silenced regions and is highly enriched in the Xi silenced chromosome (Barski *et al.*, 2007; Li *et al.*, 2012). During development however some genes, referred to as bivalent, show a high amount of both activating (H3K4me3) and repressing (H3K27me3) modifications (Cui *et al.*, 2012; Stock *et al.*, 2007). It seems this conformation allows the gene to be poised and protects the cell from differentiation while keeping the gene ready to be activated. Histone arginine methylation is also associated with gene regulation as for example Coactivator-Associated Arginine Methyltransferase 1 (CARM1) and Protein Arginine Methyltransferase 1 (PRMT1), two arginine methyltransferases were found to have co-activator activities and can mediate hormone-dependent transcriptional stimulation via H3R17 or H4R3 methylation respectively (Li *et al.*, 2010, 2008; Selvi *et al.*, 2010).

1.2.2.3 Histone Phosphorylation

Histone phosphorylation can happen on histone threonine, tyrosine, and serine residues. As for the two previously described modifications, the modifications are found predominantly but not exclusively in the N-terminal histone tails (Tropberger and Schneider, 2010). The kinases proteins are catalyzing a transfer reaction from adenosine triphosphate (ATP) to the hydroxyl group of the target amino acids. Usually, the kinases are not specific to histones and have roles sometimes in the cytoplasm where they phosphorylate other proteins. Aurora B is for example responsible for H3S10 phosphorylation but also phosphorylates several proteins involved in the kinetochore formation (Bishop and Schumacher, 2002; Hirota *et al.*, 2005; Tanno *et al.*, 2010; Welburn *et al.*, 2010). It is however unclear how most of the kinases get recruited to the chromatin. Phosphatases, inducing the dephosphorylation reaction are poorly characterized in the context of histone modification.

Phosphorylation, like acetylation, is a modification affecting the electrochemical charge of the histone tail, thus possibly affecting chromatin folding and opening chromatin. Phosphorylation modifications show a highly dynamic pattern and are implicated in various dynamics cellular processes. Histone phosphorylation plays notably essential roles during mitosis, where its exact function is however not completely elucidated yet. Still, a decrease of H3T3 phosphorylation induces spindle assembly defects (Kelly *et al.*, 2010). Histone phosphorylation is also involved in DNA repair as H2AX phosphorylation is

necessary for the assembly of the damage response machinery to the chromatin (Podhorecka *et al.*, 2010). Functions in gene expression regulation, DNA replication, chromatin condensation, and apoptosis were also reported (Gurley *et al.*, 1974; Matsumoto *et al.*, 1980; Mukherjee *et al.*, 2006; Nakamura *et al.*, 2004; Yamamoto *et al.*, 2003).

1.2.2.4 Histone crotonylation

Histone acetylation, methylation, and phosphorylation are considered as the three main post-transcriptional modifications of histones. However, currently, over 10 different histone modifications have been reported. Some of these modifications like ubiquitylation, sumoylation, or ATP-ribosylation were described with corresponding reader proteins and function but many still need to be characterized. As the protein family CDYL, described in the third part of this introduction, was described to have potential crotonylase activity, I would like here to summarize the current knowledge available on histone crotonylation.

Histone crotonylation is a modification taking place on lysine residues and was first reported in 2011 from a mass spectrometry study aiming to find new histone modifications (Tan *et al.*, 2011). Several histone acetyltransferases were soon reported to have crotonylase activity *in vitro* and *in vivo* (Kollenstart *et al.*, 2019; Sabari *et al.*, 2015). It, therefore, seems that acetyl-CoA and crotonyl-CoA compete in the cell to regulate to some extent the balance between histone crotonylation and acetylation. This observation could additionally link epigenetic changes with metabolism (Sabari *et al.*, 2015). CDYL family, on the opposite, was shown to reduce histone crotonylation before the histones get modified through catalyzing Crotonyl-CoA into β -hydroxybutyryl-CoA (S. Liu *et al.*, 2017). Due to the structural similarities between crotonyl and acetyl groups (see Figure 3), it was rapidly hypothesized to have similar functions on gene activation. Crotonylation was indeed found enriched at some promotor and enhancer regions (Kollenstart *et al.*, 2019; Li *et al.*, 2016a; Sabari *et al.*, 2015). Still, crotonylation and acetylation have a different pattern *in vivo* suggesting crotonylation has, at least partially, non-redundant functions (Tan *et al.*, 2011). Notably, crotonylation is particularly enriched in post-meiotic cells at “escapees” genes, known to escape Xi chromosome silencing. YEATS domain

containing protein 2 (YEATS2) was described as a specific crotonylation reader, potentially explaining the different functions between acetylation and crotonylation (Andrews *et al.*, 2016; Li *et al.*, 2016b). Many aspects of this modification still remain unknown, however, crotonylation defects were already linked with diseases (Liao *et al.*, 2020; Liu *et al.*, 2019; Wan *et al.*, 2019) and it seems that it may gain in importance in the next years.

1.2.3 Crosstalks between Epigenetic Marks

Rapidly, it has been reported that several modifications influenced the deposition, interpretation, or erasure of other epigenetic modifications. Several modes of action have been described to link the different modifications together (reviewed by Lee *et al.*, 2010). The simplest mechanism involves substrate competition, as the same amino acids can carry several different modifications but only one at a time. For example, lysines can be acetylated, methylated, ubiquitylated, or crotonylated. H3K9Ac, therefore, does not only activate gene expression, but it also blocks H3K9me3 and thus gene repression. Some histone writers also have a reader domain corresponding to their product modification, which can induce some positive feedback loop on modification deposition. This is for example the case of the *S. pombe* homolog of SUV39, Clr4 which has a chromodomain, essential for heterochromatin spreading, in addition to its SET domain (Ivanova *et al.*, 1998). It is believed that binding of H3K9me3 by the chromodomain orientates Clr4 optimally to methylate the neighbor nucleosome, thus increasing its activity (Al-Sady *et al.*, 2013; K. Zhang *et al.*, 2008). A similar mechanism has also been observed in histone erasers, as for Lysine Specific Demethylase 5A (KDM5A), an H3K4 demethylase, which recognizes H3K4 unmodified and when bound to it, increases its enzymatic activity by 30-fold (Torres *et al.*, 2015).

Sometimes a modification is recognized by modifiers to induce another neighbor modification. This is for example the case of KDM4A and KDM4C, able to remove the repressive H3K9me3 mark and to recognize the activating H3K4me3 mark through a double-tudor domain (Pedersen *et al.*, 2016). This is also the case when the histone modifier enzyme belongs to a multicomplex. Complex components may be able to recognize specific modifications, allowing the enzyme to modify the neighbor nucleosome. In some cases, a modification can be event-dependent on another, as for

H3K4 and H3K79 methylation which can only exist after ubiquitylation of H2BK123 (Rhie *et al.*, 2013). Finally, the recognition of a modified or unmodified sequence by a reader or a substrate recognition site can be disrupted by an adjacent modification. A well-known example is the release of HP1 from H3K9me3 during mitosis as H3S10 gets phosphorylated (Fischle *et al.*, 2005; Hirota *et al.*, 2005).

Several crosstalks have also been reported between DNA and histone methylation. The modifications can work cooperatively, as for E3 ubiquitin-ligase UHRF1 whose interaction with H3K9me3 is significantly increased when the corresponding nucleosomal DNA is methylated (Bartke *et al.*, 2010). On the opposite, DNA can also inhibit the recognition by some readers. This is the case for KDM2A that can bind H3K9me3 only when DNA is unmethylated (Bartke *et al.*, 2010). Finally, there seems to be a reciprocal control between DNA methylation and histone modification. H3K9 methylation was for example proven to be necessary for DNA methylation (Tamaru and Selker, 2001), while MeCP2 can recruit HDAC to chromatin (Jones *et al.*, 1998; Nan *et al.*, 1998). This interplay adds a layer of complexities to gene transcription regulation through epigenetic mechanisms.

1.2.4 Histone variants

Histones do not only regulate chromatin through their modifications but also by themselves: indeed, histone 3 and 2A have several isoforms, which can be specific for chromatin regions or cellular events. Histone H2A has two universal variants: H2AZ and H2AX. The former is enriched at the transcription site, is anti-correlated with 5mC, and helps to recruit RNA polymerase II to chromatin (Adam *et al.*, 2001; Hardy *et al.*, 2009; Zilberman *et al.*, 2008), while the latter is an important recruiter of the DNA damage response when phosphorylated (Paull *et al.*, 2000; Rogakou *et al.*, 1998). An evolutionary more recent H2A variant is the macro H2A (mH2A) which can only be found in the animal reign and are notably enriched on the Xi chromosome in mammals, suggesting a role in transcription repression (Chadwick *et al.*, 2001; Costanzi and Pehrson, 2001). Histone 3 also has two universal variants. In contrast to canonical H3, which is loaded on chromatin during DNA replication with the Chromatin Assembly Factor 1 (CAF1) chaperone (Verreault *et al.*, 1996), H3.3 is deposited preferentially into actively transcribed

chromatin with the HIRA chaperone independently of DNA replication (Tagami *et al.*, 2004). The other H3 variant, called CENPA in humans is loaded on and defines centromeric regions (Black and Bassett, 2008) as will be further described in the paragraph on heterochromatin. It is interesting to note that in humans, the canonical H3 gene encodes for two different proteins H3.1 and H3.2 that differ at only a single residue. This small difference however is already able to induce changes in post-transcriptional modifications, revealing the importance of the histones sequences (Hake and Allis, 2006). The different histone variants and their deposition on chromatin is far from being fully characterized yet, but it reveals an additional complexity on the epigenetic mechanisms to regulate chromatin structure and gene expression.

1.2.5 Chromatin Structure and classification

After improving a cytological staining method, Emil Heitz described in 1928 two distinct structures in chromosomes which were designated as euchromatin and heterochromatin (Berger, 2019). The former was described as “open” regions, undergoing changes during the cell cycle, while the latter corresponds to condensed and compacted regions. Various attempts were made to further divide euchromatin and heterochromatin into more precise chromatin states depending notably on the histone modifications found in these regions. Analyzing the different histone modifications in human cells, Ernst and Kellis reported 51 distinct states (Ernst and Kellis, 2010). During the same period, work in *Drosophila melanogaster* classified the DNA in five different chromatin types depending on the chromatin interactors found; three types were considered as euchromatin and two as heterochromatin (Filion *et al.*, 2010).

1.2.5.1 Heterochromatin

Heterochromatin was first defined as the DNA that remains condensed throughout the cell cycle. It carries mostly repressive marks and is divided into two types: facultative and repressive heterochromatin (Déjardin, 2015; Filion *et al.*, 2010). Facultative heterochromatin is heavily marked by the H3K27me3 repressive mark and the presence of the polycomb repressor complex (Trojer and Reinberg, 2007). It corresponds mostly to genes silenced after development or differentiation. The repressed mammals' Xi chromosome for example is rich in facultative heterochromatin regions (Wutz, 2011).

Constitutive heterochromatin on the other hand consists of permanently silenced regions and is mostly marked by H3K9me3, the presence of HP1 and HP1 interacting proteins (Saksouk *et al.*, 2015; Wreggett *et al.*, 1994). Telomeres, centromeres, pericentromeres but also chromatin regions containing repetitive DNA sequences are examples of constitutive heterochromatin (Saksouk *et al.*, 2015).

Interestingly, centromeres are universally conserved heterochromatic structures but their DNA sequence is not. Additionally, centromeres are responsible for the kinetochore assembly but it was observed that centromeric DNA, consisting in humans of megabase sized arrays of satellite sequences, is neither necessary nor sufficient to mark the site of centromere formation (Black and Bassett, 2008). This suggests that centromeric features are controlled through something else, possibly epigenetics. Interestingly, CENPA, a variant of histone 3, is only found in centromeric regions and is an evolutionarily conserved protein among eukaryotes. CENPA depletion induces kinetochore assembly failure, while *de novo* CENPA localization on ectopic sites is enough to recruit several kinetochore proteins (Black and Bassett, 2008; Heun *et al.*, 2006), suggesting CENPA might be one of the keys for centromeric heterochromatin structure. However several studies reported that CENPA is not sufficient in vertebrates to define a centromere (Hooser *et al.*, 2001), meaning that other factors have to be involved.

Pericentromeres are regions flanking both sides of the centromeric regions. Similarly to centromeres, they show a non-conserved DNA sequence consisting of satellite repeat sequences. These satellite regions are epigenetically silenced by DNA and histone methylation, and their derepression was linked with dramatic genomic instability (Black and Bassett, 2008; Peng and Karpen, 2007; Saksouk *et al.*, 2015). Pericentromeres are highly enriched in H3K9me3 and H4K20me3 and these modifications are required for sister chromatid cohesion during mitosis (Inoue *et al.*, 2008; Peters *et al.*, 2001).

1.2.6 Small RNAs

In addition to histones and DNA modifications, gene expression can also be regulated post-transcriptionally through non-coding RNA. Non-coding RNA grew in importance since it was realized that only 2% of the genome is actually coding for genes (Amaral *et al.*,

2008). The rest serves notably as a template for non-coding RNAs. Non-coding RNAs can be classified into two categories depending on their size. Long non-coding RNA – over 200 nucleotides – are regulating gene expression through various mechanisms. They can for example act as molecular chaperones (Docter *et al.*, 2016) or recruit epigenetic regulators to chromatin (Kogo *et al.*, 2011; Tsai *et al.*, 2010).

Short RNAs are shorter than 200 nucleotides and can be classified into small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and microRNAs (miRNAs). Generally, short RNAs block translation by interacting with mRNA through specific base pairing (Petersen *et al.*, 2006). These RNAs are involved through interference binding in many processes, as differentiation, EMT, or mitosis, and were found deregulated in many cancers (Ji *et al.*, 2019; Winsel *et al.*, 2014; Zhao *et al.*, 2010).

1.3 EPIGENETICS AND CANCER

In 2000 and 2011, Hanahan and Weinberg reviewed in most famous work, the different characteristics a cell must obtain to promote tumor growth, invasion, and thus cancer (Hanahan and Weinberg, 2011, 2000). To acquire these characteristics, the cell must undergo changes influencing various aspects of its life, including transcription regulation, genomic stability, cellular plasticity, DNA replication, and repair. Genetic mutations were first reported as being responsible for most of the changes observed. However, epigenetics, as seen previously are implicated in those mechanisms and rapidly, scientists looked and found many epigenetic deregulations in cancer. Interestingly, epigenetic changes may explain the link observed between some external environment, like aging, and the increasing risk of cancer development (Dugué *et al.*, 2018; Issa *et al.*, 1994; Liu *et al.*, 2003). Nowadays, it is accepted that most if not all tumors show epigenetic alterations (Feinberg *et al.*, 2006). The importance of these changes, notably for tumor initiation and propagation remains however to be discussed. In opposition to DNA mutations, epigenetic modifications are known to be reversible. This is of particular interest for cancer therapy as it gives hope to the scientific community to find a drug, able to reverse the cancerous state of tumor cells by targeting proteins involved in epigenetics.

In the following subchapters, I will describe the epigenetic regulation which can be found in cancer and discuss their importance and relevance as tumor drivers. Finally, I will review the cancer therapies targeting epigenetic factors already in use or in clinical development and highlight the advantages and the remaining challenges of these therapies.

1.3.1 Epigenetic Deregulation in Cancer

Since epigenetics play a critical role in gene transcription, DNA replication and repair, and genome stability, aberrant patterns of epigenetic proteins can have significant effects on cancer initiation and progression. One of the first reported and most studied epigenetic deregulations in cancer is the global DNA hypomethylation (Feinberg and Vogelstein, 1983). Intriguingly, hypomethylation is observable even before the tumor is formed, in the pre-cancerous tissue, suggesting it could be an initiating event of the tumor and used for early diagnosis (Feinberg *et al.*, 2006; Feinberg and Vogelstein, 1983). Surprisingly, while the general DNA methylation decreases, CpG islands that are non-methylated in normal tissues show an increased methylation level, thus repressing gene expression, notably the expression of tumor-suppressor genes (Baylin, 2005; Issa *et al.*, 1994; Vrba *et al.*, 2013). Consistent with abnormal DNA methylation in many cancers, mutations in *TET* genes, mostly loss of function mutations, are also commonly found, notably in hematological malignancies (Delhommeau *et al.*, 2009; Langemeijer *et al.*, 2009). Repression of tumor suppressor genes can also be performed through deregulation of the Polycomb complex. EZH2 overexpression was for example found to correlate with poor prognosis in prostate and breast cancer (Bryant *et al.*, 2007; Kleer *et al.*, 2003; Varambally *et al.*, 2002).

Gene expression regulation is not the only mechanism that links epigenetics to carcinogenesis. Ectopic histone deposition can be linked with genome instability. This is for example the case for H3.3. Mutations in this histone or the complex responsible for its deposition were associated with increased telomere length and genomic instability in pediatric glioblastoma (Schwartzentruber *et al.*, 2012). Similarly, overexpression of CENPA – the H3 paralog specific for centromeric regions – was found in many cancers and increases genome instability (Amato *et al.*, 2009; Shrestha *et al.*, 2017; Tomonaga *et al.*, 2003). Finally, DNA methylation is prone to induce mutations. Indeed, methylated

cytosine tends to spontaneously deaminate and thus forms a thymidine. This thymidine forms a mismatch with guanine and if not corrected, can lead to a C-T mutation (Cooper *et al.*, 2010).

The epigenetic importance in cancer allows us to better understand the environmental events increasing cancer risk. This is particularly true with aging, as we know that when the organism is aging DNA methylation tends to decrease generally over the chromatin but increases at CpG islands, similar to what is observed in cancer (Dugué *et al.*, 2018; Issa *et al.*, 1994; Liu *et al.*, 2003). Epigenetics also allow us to link the cellular metabolism with cancer risk. For example, maintenance of unmethylated CpG islands requires vitamin C (Hore *et al.*, 2016), while methylation of DNA sequence needs folate (Kim, 2005). α -ketoglutarate and acetyl-CoA, described previously as important cofactors for histone modification, are also important metabolites, giving potential links with cancer development and cellular metabolism.

1.3.2 Epigenetic Events: Enough to Drive the Tumor?

Epigenetic changes are therefore extremely common in cancer and it is nowadays highly accepted that genetics and epigenetics have to work together to promote tumorigenesis. More interestingly, it is also observed that epigenetic mutations can sometimes be considered as "driver" mutations. Such modifications, positively selected by the tumor as it helps its progression, are notably recognized by their presence in many tumors. Impressively, global DNA hypomethylation was found in all tumors, even at very early stages, suggesting an essential role for cancer initiation and progression (Feinberg *et al.*, 2006). Mutations in histone writers or erasers are also very common as HKMT2, a histone methyltransferase was found mutated in close to 90% of follicular lymphoma (Morin *et al.*, 2011), while KDM6A, a histone demethylase is deregulated in up to 12 histologically distinct cancer (van Haaften *et al.*, 2009).

Epigenetic deregulation is therefore considered as a potential driver event in cancer initiation and progression and a potentially interesting target for chemotherapy. Remains the question: are those aberrant epigenetic modifications still necessary at later stages of cancer? Said in other words: would it be possible to reverse the cancerous hallmarks by

correcting those modifications? In very exciting experiments at the beginning of the century, Hochedlinger *et al.* showed it is possible to use mouse melanoma nuclei to clone a normal mouse (Hochedlinger *et al.*, 2004). This observation suggests, that epigenetic properties acquired by cancer, when reversed can lose their oncogenic characteristic and turn into normal tissue. Epigenetic modifications may thus be targeted in chemotherapies to decrease or stop tumor progression.

1.3.3 Between Success and Challenges: Targeting Epigenetic Factors in Cancer Therapy

The reversible aspect of epigenetic modifications, in opposition to DNA mutations, drove the attention of scientists and raised the hope to reprogram cancer cells into normal tissues. Azacitidine and decitabine, two drugs targeting DNMTs, were approved by the Food and Drug Administration (FDA) in 2004 and 2006 respectively, as the first drugs to target epigenetic proteins (Gore *et al.*, 2006; Issa *et al.*, 2005). Both drugs are cytidine-analog and thus can integrate DNA, where they trap DNMT leading to the proteasomal degradation of the writer and thus DNA methylation inhibition (Gore *et al.*, 2006; Issa *et al.*, 2005). DNMT inhibitors showed mixed results in solid tumors but are used with success in myelodysplastic syndrome. Histone modifications are targeted by therapies as well and in 2009, the first HDAC inhibitor was also approved by the FDA. HDAC inhibitors are used for cutaneous T-cell lymphoma but they show quite high toxicity (Grant *et al.*, 2007). This toxicity is probably due to the lack of specificity for HDAC inhibitors. Given the fact, that the HDAC themselves are involved in many reactions, it seems difficult to use them as a therapeutic target with low side-effects. Histone methylation, on the other hand, has very high specificity both for substrate and product, and may thus be more adapted for therapies. Several EZH2 inhibitors, blocking the repression of tumor suppressor genes are currently in clinical trials (Mohammad *et al.*, 2019).

Interestingly, many therapies combining a drug targeting epigenetic factors with another agent are currently being analyzed in clinical trials and may reveal high efficiency. This hope is based on two mechanisms. First, blocking epigenetic proteins might protect against resistance as it may inhibit cellular reprogramming. Second, targeting the epigenetic protein sometimes increases the effect of the first therapy. This is for example the case for immunotherapies as both HDACs and DNMT inhibitors upregulate Major

Histocompatibility Complex (MHC) class I genes and tumor antigens (Cruickshank *et al.*, 2018). Numerous epigenetic inhibitors are therefore tested in combination with Programmed Cell Death Protein 1 (PD-1) or Programmed Cell Death 1 Ligand 1 (PD1L1) antibodies to increase its effect. Several clinical trials with epigenetic inhibitors in combination with other drugs are currently being performed to evaluate their practical effectiveness (reviewed by Mohammad *et al.*, 2019).

To conclude, as promising as some therapies targeting epigenetic proteins might be, several challenges remain to be elucidated, notably the specificity of the different inhibitors, which is sometimes difficult to achieve either because of the lack of specificity of the target itself or because of the high structural similarities between the members of the same family. It is therefore mandatory to further study the epigenetics field to increase our capacity to predict relevant targets and find particularities that can be used to increase the specificity of future therapies.

2 GENOMIC INSTABILITY

2.1 AN EMERGING CANCER HALLMARK

2.1.1 Mechanism of Genomic Instability in Cancer

Genomic instability corresponds to an increase in genomic alterations in the cell. These alterations can be either small, as base-pair mutations, deletions, or insertions, or bigger, up to the loss, gain, or rearrangement of a complete chromosome. Genomic instability is not required for cancer as such, but strongly facilitates the acquisition of cancer hallmarks (Hanahan and Weinberg, 2011) and is a characteristic found in various tumors. Genomic instability can evolve from DNA damage, replication stress, or mitotic defects (Ovejero et al., 2020).

In normal tissue, DNA damage repair maintains genome integrity and stability after DNA damage. Depending on the type and timing of the damage, different repair mechanisms might be activated such as base excision repair, single-strand break repair, nucleotide excision repair, mismatch repair, non-homologous end-joining and homologous recombination. Interestingly, in cancer cells, however, pathways implicated in repair are often deregulated and can drive genomic instability, promoting tumor progression (Curtin, 2012).

Genomic instability can also arise from replication stress, corresponding to the blockage or overactivation of the replicative machinery (Sarni and Kerem, 2017). A high level of replication stress is strongly linked with double-strand breaks, increasing the risk that DNA repair machinery fails to repair every incident. Replication stress can also result from so-called R-loops, intermediate RNA-DNA hybrid structures. R-loops are normal transcriptional intermediates, but when they are happening at unscheduled timing, they result in double-strand breaks (Wimberly *et al.*, 2013). R-loops were additionally linked with genomic instability, notably in ER+ breast cancer (Stork *et al.*, 2016).

Finally, defects in mitosis can also commonly result in genomic instability, notably chromosome number instability. This is for example the case when sister chromatids are

not properly held together, when the checkpoints are not functional, when more than two centrosomes are present or when cytokinesis fails to happen (Barber et al., 2008; Chan, 2011; Itoh et al., 2011; Lylek et al., 2020). During my thesis I mostly focused on mitosis defects inducing genomic instability. Faithful mitosis is a complex process, involving many mechanisms, which are detailed with more details in the following chapter "Progress of a faithful mitosis".

2.1.2 Turning the enemy into an Ally: Inducing Genome Instability for Cancer Therapy

Genomic instability has proven to have pro-oncogenic activities, but interestingly, after a certain threshold, it seems that even tumor cells reach a point where the consequence of genomic instability becomes lethal. Recently, Andor *et al.* performed an analysis with more than 1000 tumors available from The Cancer Genome Atlas (TCGA) project (Andor *et al.*, 2017). They found that, indeed, tumors had a preferred genomic instability range, above which the instability is not viable. The authors propose two mechanisms to elucidate this idea. First, a large increase of chromosome number and size might slow down chromosome alignment during metaphase and require more energy to pull sister chromatids during anaphase, thus slowing the proliferation rate. Secondly, an increase in genomic instability, particularly in the case of cancer, would increase the copies of oncogenic genes and thus the production of oncogenic proteins. This, in turn, can amplify antigens expression, which may increase the immune recognition of the cancer cell.

Since too much genomic instability is also leading cancer cells to die, it might be of strategic therapeutic interest, depending on the number of genome instabilities already present in the tumor, to increase this instability even more and bring the tumor over the lethal threshold. Some chemotherapies used are already based on increasing the number of double-strand breaks. This is for example the case for alkylating agents and platinum-based drugs, such as bendamustine (Barman Balfour and Goa, 2001), melphalan (Thatcher et al., 1989), cisplatin (Dong *et al.*, 2017), or carboplatin (Boulikas and Vougiouka, 2004) (reviewed by Woods and Turchi, 2013). as for example cisplatin, which enhances double-strand breaks from irradiation. Irradiation, commonly used in cancer therapy is also working through the induction of DNA break (Prendergast et al., 2011). However, as described previously, promoting DNA breaks is not the only mechanism able to induce

genome instability. Additionally, drugs targeting mitosis and more specifically microtubules dynamics are successfully used in chemotherapy. This is for example the case of taxanes used against breast and ovarian cancer (Kelling et al., 2003; Long, 1994) and vinca alkaloids used against haematological malignancies (Zhou and Rahmani, 1992). Both drug families show great success but also a high toxicity as the drugs are non-specific to cancer cells and inhibit microtubules function in normal mitotic cells and in non-dividing cells. As a side effect of the chemotherapy, the inhibition of microtubules polymerization in peripheral neurons may thus induce neuropathie (Chaudhry et al., 1994). It is therefore of high interest to study in more depth the mechanism inducing genomic instability to find potential therapeutic targets with a similar or higher effectiveness but a lower toxicity than the currently used drugs.

To conclude, genomic instability is important for both tumor progression and cancer therapy and thus an important field of interest in oncology. Genomic instability can come through various mechanisms but during my thesis, I concentrated on genomic instability induced by mitotic defects. In the following subchapters, I will therefore review the different mechanisms relevant for a faithful mitosis.

2.2 PROGRESSION OF A FAITHFUL MITOSIS

2.2.1 Chromosome Formation and Segregation

During mitosis, chromatin structure undergoes major changes. Chromatids are dramatically condensed from prophase to telophase and sister chromatids are held together through a carefully regulated cohesion process. Those changes are mostly orchestrated by three complexes: cohesin, condensin I, and condensin II (Hirano, 2015). As their names indicate, cohesin is involved in sister chromatids cohesion while the two other complexes regulate chromosome condensation. The three complexes show a very similar ring-shaped structure (Gruber *et al.*, 2003; Haering *et al.*, 2002; Hirano, 2015). Briefly, cohesion between two sister chromatids is established during DNA replication (Lafont *et al.*, 2010; Samora *et al.*, 2016). The condensation happens in two waves during mitosis (Lipp *et al.*, 2007). During prophase, condensin II associates with chromatin while most cohesin complexes present on chromosome arms get released to allow chromatid

resolution (Losada et al., 2002). After nuclear envelope breakdown, condensin I, which is only cytoplasmic, gets access to the chromatin (Lipp et al., 2007). The remaining cohesin at pericentromeres is released at the onset of anaphase, allowing the sister chromatids to move to opposite poles (Waizenegger et al., 2000). This process is very important for an equitable distribution of chromosomes between the two daughter cells. The importance of cohesin to avoid chromosomal instability was highlighted in numerous studies (Barber et al., 2008; Leylek et al., 2020). Mutations in cohesin genes were found in several tumors showing chromosomal instability (Barber et al., 2008; Cucco et al., 2014). Interestingly, increasing cohesion leads to a decrease in genomic instability (Manning et al., 2014), suggesting a potential for cohesin as a cancer therapy target. It is however to note that genomic instability could not be detected in all tumors with mutated cohesin proteins (Balbás-Martínez et al., 2013; Taylor et al., 2014), suggesting that other functions of the complex, notably gene expression regulation, might induce other phenotypes that could benefit the tumor. Indeed, it was reported that cohesin complex is also involved in gene expression control (Busslinger et al., 2017; Kagey et al., 2010).

Cohesin is of particular importance in the cells, both for faithful mitosis and gene expression. The structure of cohesin and condensin complexes is described in the next-subchapter. The discovery of the exact mechanism of cohesion establishment, cohesin loading, and unloading has proven to be very challenging and our current knowledge is summarized in the following subchapters. Finally, I will briefly review the functions of cohesin outside mitosis and sister chromatid cohesion.

2.2.1.1 SMC Complex structure

Cohesin and condensin I and II are structurally very similar multiprotein complexes, which are sometimes designated as structural maintenance of chromosome (SMC) complexes due to their characteristic SMC heterodimers. SMC proteins have a long coiled-coil region separated by a hinge domain, allowing the protein to fold itself so that its N and C terminus interact together to form a globular head domain (Haering et al., 2002). SMC proteins form a V-shaped heterodimer by interacting at their hinge domain. For the cohesin complex, SMC1 dimerizes with SMC3 while for both condensin complexes SMC2 dimerizes with SMC4. Noteworthy in humans is also the SMC5-SMC6 complex which is involved in

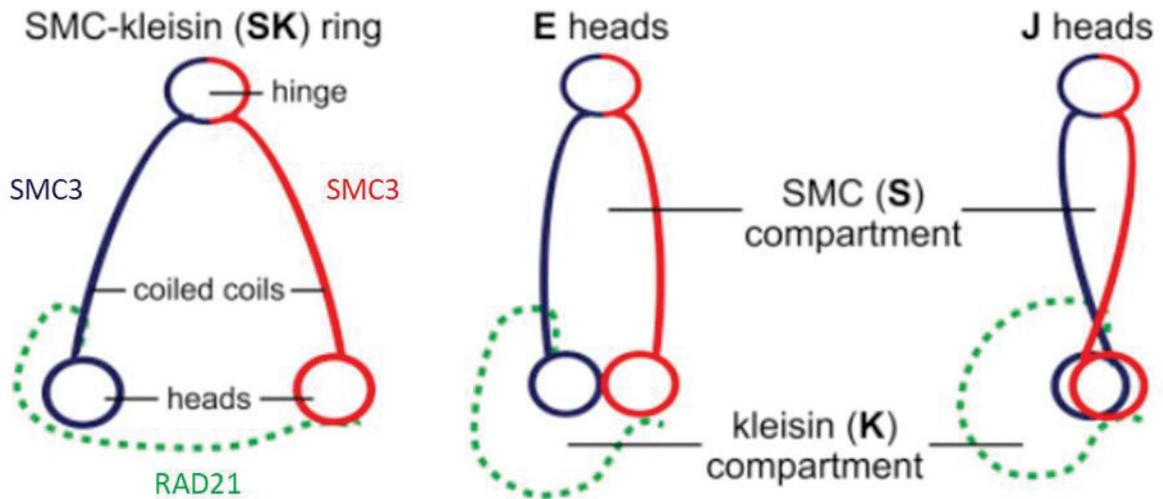


Figure 6: Cohesin complex consist of tripartite ring. Through interaction of SMC heads, the ring is divided into two compartement. It is believed that DNA is usually entrapped in the kleisin compartment. Image taken and adapted from Chapard et al. (Chapard et al. 2019).

DNA damage repair (Fujioka *et al.*, 2002). Two kleisin proteins, Double Strand Break Repair Protein Rad21 Homolog (RAD21) and Non-SMC Chromosome Associated Protein H (NCAPH) bind the head of both SMC proteins in cohesin and condensin respectively, and act as docking sites for other proteins (Haering *et al.*, 2002; Ono *et al.*, 2003). In cohesin, interactors of RAD21 include Stromal Antigen 1 or 2 (SA1 or SA2), two proteins containing a HEAT repeat domain, sister chromatid cohesion protein PDS5B, Wings-Apart Protein Like (WAPL), and Nipped-B-Like (NIPBL), proteins involved in cohesin loading and unloading as will be detailed later. In condensin, NCAPH is interacting with Non-SMC Chromosome Associated Protein G and D (NCAPG and NCAPD). Finally, the kleisin subunit closes the tripartite ring-shaped structure, which topologically entraps the DNA strand. Interestingly it was more recently proven that SMC1 and SMC3 heads also interact together, thus forming two compartments where DNA can be entrapped (Chapard *et al.*, 2019; Murayama and Uhlmann, 2015). In this conformation, three gates were described as being able to open to allow DNA entrapment as can be seen on figure 6: the entry gate between the SMC hinges, the inner gate between their heads, and the exit gate between RAD21 N-terminus and SMC3 head. Cohesin loading on the chromatin and thus the opening of the cohesin ring at the entry gate or inner gate can only be achieved with ATP hydrolysis at SMC heads (Arumugam *et al.*, 2003; Murayama and Uhlmann, 2015).

2.2.1.2 Cohesin loading...

While in budding yeast *Saccharomyces cerevisiae*, cohesin is loaded on the chromatin just before DNA replication (Uhlmann and Nasmyth, 1998), in vertebrate cells, the loading happens right after mitosis (Gerlich *et al.*, 2006; Morales and Losada, 2018). How exactly cohesin is loaded on chromatin is still unclear, and in the last decade, an impressive amount of publications tried to solve both structural and molecular mechanisms of DNA entrapment by the complex. As described in the paragraph on SMC complex structure and on figure 6, cohesin is now considered to have two compartments (Chapard *et al.*, 2019; Murayama and Uhlmann, 2015). In July 2019, Chapard *et al.* published their work which is strongly suggesting that DNA is entrapped in the compartment delimited by kleisin and SMC heads (Chapard *et al.*, 2019). They do however not exclude rare, possibly transient events, where DNA is entrapped in the compartment delimited by the SMC dimer.

Cohesin loading is enhanced by the NIPBL – MAU2 heterodimer, which consists of a globular domain, a body, and a hook (Chao *et al.*, 2015). The body and the hook were found sufficient to load cohesin on circular DNA, whereas the globular domain was only necessary *in vivo*, suggesting that it might act as a chromatin adaptor (Chao *et al.*, 2015). In *S. pombe* sister chromatid cohesion 2 (Scc2), NIPBL ortholog, promotes ATP hydrolysis at the heads of the SMC dimers (Petela *et al.*, 2018). NIPBL was found enriched around promoters of actively transcribed genes but shows little colocalization with cohesin (Busslinger *et al.*, 2017). This is explained by the fact that cohesin, after loading on chromatin, is relocated to CTCF-binding sites (Busslinger *et al.*, 2017). *In vitro* studies show that cohesin can slide on the DNA by itself. However, Kanke *et al.* revealed that ATP and thus possibly NIPBL-MAU2 loading complex is needed for cohesin sliding on chromatin (Kanke *et al.*, 2016). It was also proposed that cohesin is being pushed by RNA polymerase II (Busslinger *et al.*, 2017; Kanke *et al.*, 2016). How DNA entrapment in the cohesin ring is happening on a molecular level is still controversial. It has been proposed in 2006 that DNA enters the cohesin ring through opening of a gate at the SMC hinges, as holding the hinges together blocks its loading (Gruber *et al.*, 2006). ATP hydrolysis which is needed for DNA entrapment, on the other hand, promotes the opening of the “inner gate” of cohesin complex by dissolving the interaction between the SMC heads. Based on this, people have hypothesized that ATP hydrolysis would induce conformational changes

that would be transmitted to the hinge through the SMC coiled coils (Morales and Losada, 2018). The observation that post-translational acetylation on the structure of the coiled-coil of SMC induces ring opening at the hinge domains is suggesting a mechanism as to how conformational changes at the SMC heads can regulate their conformation at the hinge (Kulemzina *et al.*, 2016). In 2015 however, Murayama and Uhlmann published biochemical analysis, showing that WAPL, a known unloading factor, also has strong loading activities. They suggest that cohesin loading and unloading might happen through symmetrical processes and the NIPBL-MAU2 complex would promote loading through the inner gate (Murayama and Uhlmann, 2015). This model is however still controversial (Morales and Losada, 2018).

Until DNA replication, cohesin loading and unloading is extremely dynamic. To hold the complex on the chromatin and thus establish cohesion, SMC3 needs to get acetylated at lysines K105 and K106 by Establishment of Cohesion (ESCO) 1 and 2 (J. Zhang *et al.*, 2008). Both enzymes were found to be able to acetylate cohesin, with some redundancy as ESCO2 deficiency is viable in humans (Vega *et al.*, 2005). However, in this context, cohesion defects are visible, especially at centromeres, suggesting that ESCO2 has some unique functions in sister chromatid cohesion (Vega *et al.*, 2005; Whelan *et al.*, 2012). SMC3 acetylation by ESCO2 happens during DNA replication (Alomer *et al.*, 2017). Its recruitment depends on the prereplication complex assembly in *Xenopus laevis* egg extracts (Higashi *et al.*, 2012). Mechanistically, it is thought that SMC3 acetylation blocks ATP binding of the SMC heads, thus RAD21/SMC3 opening and DNA unloading (Chan *et al.*, 2012)(see next chapter for more details on unloading mechanism). In addition to SMC3 acetylation, cohesin is also protected from unloading through interaction with sororin (Nishiyama *et al.*, 2010; Schmitz *et al.*, 2007). Sororin binds PDS5 and inhibits its interaction with WAPL, two proteins essential for cohesin unloading (Nishiyama *et al.*, 2010).

2.2.1.3 ... and cohesin unloading mechanism

Before DNA replication, when cohesin shows a very dynamic interaction with chromatin, it is unloaded by PDS5 and WAPL (Kueng *et al.*, 2006; Shintomi and Hirano, 2009). WAPL interacts with cohesin through PDS5, which in turn interacts with RAD21. Together, they

induce an opening between SMC3 and RAD21 during interphase, allowing DNA exit out of the cohesin ring (Murayama and Uhlmann, 2015). How exactly the unloading complex helps the gate opening is unclear but ATP binding by the SMC heads seems necessary. After DNA replication, cohesion is established and sororin binds to PDS5, blocking its interaction with WAPL (Nishiyama *et al.*, 2010). From this point, cohesin can get released from chromatin through two different mechanisms, happening at precise cell cycle stages.

Most cohesin is released at prophase through the phosphorylation pathway – also called the prophase pathway. Cohesin unloading happens through phosphorylation of STAG proteins by Polo-Like Kinase 1 (PLK1) (Hauf *et al.*, 2005). In parallel, phosphorylation of sororin by Cyclin Dependent Kinase 1 (CDK1) and Aurora B, two kinases regulated by the cell cycle, induces its removal, leaving the possibility for WAPL to bind RAD21 and unload cohesin (Dreier *et al.*, 2011; Nishiyama *et al.*, 2013). WAPL depleted cells have problems separating their chromatids at anaphase (Haarhuis *et al.*, 2013; Nishiyama *et al.*, 2013), suggesting that cohesin removal at prophase helps topoisomerases to decatenate DNA and facilitates sister chromatid separation (Haarhuis *et al.*, 2013; Losada *et al.*, 2002). It is also possible that the prophase pathway allows quick reloading of cohesin after mitosis, as the complex is not destroyed by the unloading and only needs deacetylation of SMC3 by HDAC8 to be functional again (Deardorff *et al.*, 2012). Consistent with this last hypothesis, it is interesting to note that the prophase pathway is missing in yeast where cohesin loading happens in late G1, after new synthesis of RAD21 ortholog (Uhlmann *et al.*, 2000; Uhlmann and Nasmyth, 1998).

While most cohesin on chromosome arms is released through the prophase pathway, a small fraction of the complex remains at pericentromeres to hold sister chromatids together until anaphase. An important feature, as too early separation of sister chromatids would lead to unequal chromosome distribution between the two daughter cells and thus chromosomal instability. To protect cohesin, Shugoshin 1 (SGO1) and its partner phosphatase 2A (PP2A) locate at pericentromeres to antagonize phosphorylation from the prophase pathway (Kitajima *et al.*, 2006). Shugoshin localizes at pericentromeres through the recognition of H2A phosphorylation, which is mediated by Budding Uninhibited by Benzimidazoles 1 (BUB1) kinase (Kawashima *et al.*, 2010). Mechanistically,

once recruited to the pericentromeres, SGO1 binds cohesin and competes with WAPL affinity to STAG and RAD21 (Hara, 2014), thus inhibiting cohesin release. At the onset of anaphase, the remaining cohesin at pericentromeres is released through the cleavage of RAD21 by separase (Hauf *et al.*, 2001), allowing the sister chromatids to move to opposing poles.

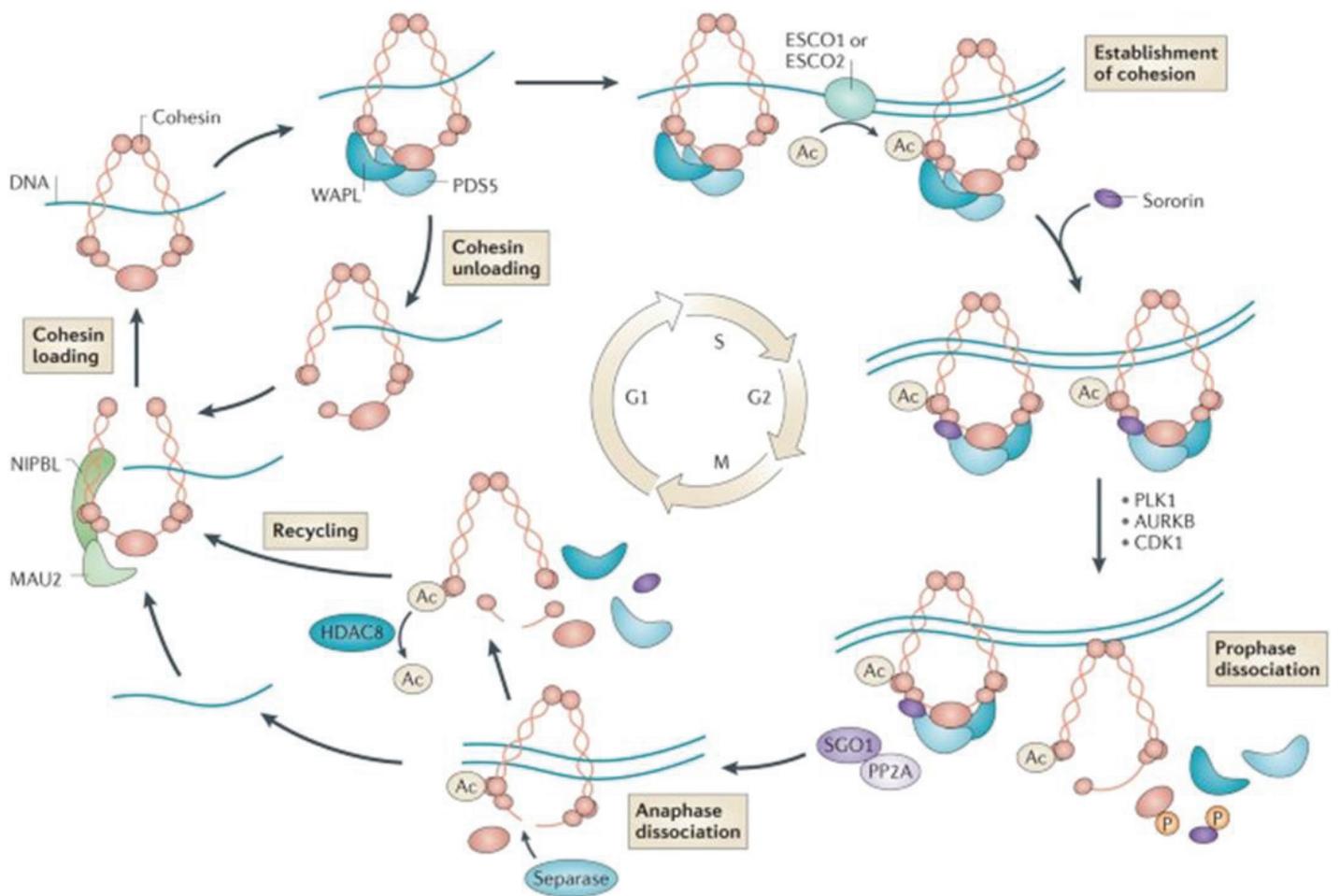


Figure 7: Cohesin loading and unloading mechanism during the cell cycle. Cohesin is loaded on the chromatin with the NIPBL-MAU2 complex and unloaded with PDS5B and WAPL. During G1 phase, cohesin is dynamically loaded and unloaded from the chromatin. After DNA replication, cohesion is established by ESCO1 or ESCO2 which acetylates SMC3 heads. Additionally sororin binds PDS5B, inhibiting WAPL interaction with PDS5B and thus cohesin unloading. At the onset of mitosis, cohesin at the chromosome arms is dissociated from the chromatin through phosphorylation of sororin and unloading by WAPL. At the pericentromeres cohesin release happens through RAD21 cleavage by separase at the onset of anaphase. Before a new loading cycle, cohesin is deacetylated by HDAC8. Image taken from Losada (Losada 2014).

2.2.1.4 Other cohesin functions

The canonical function of cohesin is as described earlier to hold sister chromatids together during cellular division. However, cohesin mutations were found to have effects in other various processes including development and DNA repair. In addition to holding DNA in an inter-chromatid way, cohesin is also responsible for enhancer clustering by bringing intra-chromatid DNA together, forming DNA loops, and bringing gene enhancer and promotor together. In this context, cohesin interacts additionally with mediator proteins, which are found to interact with both enhancer and promotor regions (Kagey et al., 2010). Consistent with this model, cohesin is found mostly at the promotor and enhancer regions of transcription active genes (Busslinger *et al.*, 2017). Additionally, it seems that cohesin is involved in gene repression as it was found to interact with the Polycomb Repressive Complex 1 (PRC1) complex and regulate its availability (Dorsett and Kassis, 2014).

Cohesin was also found to be relevant for DNA repair mechanisms. In *Schizosaccharomyces pombe*, cells with a mutation in RAD21, were deficient in double-strand break repair, while restoring RAD21 wild-type expression restored the pathway (Birkenbihl and Subramani, 1992). Mutations in SMC1, SMC3, or PDS5 leads to hypersensitivity to DNA damage induced by γ -irradiation (Sjögren and Nasmyth, 2001). Moreover, SMC1 and 3 were found in the Repair Complex 1 (RC1) (Jessberger *et al.*, 1996), and SMC1 gets phosphorylated by the serine threonine kinase ATM kinase after radiation, a modification that has proven to be essential for DNA repair (Yazdi *et al.*, 2002). How cohesin is mechanistically involved in the repair pathways remains unknown, but it potentially holds sister DNA sequences together to ensure a correct template for the repair (Hagstrom and Meyer, 2003a).

2.2.2 Connection between Chromosomes and Microtubules

2.2.2.1 The Kinetochore Complex: a brief overview

The kinetochore complex includes over eighty proteins (Cheeseman and Desai, 2008) and is responsible for the connection between centromeric DNA and microtubules during mitosis. Some proteins are localized permanently at the centromeres such as CENPA, while others such as CENPE and CENPF are recruited during specific cell cycle stages,

respectively prometaphase and prophase (Liao *et al.*, 1995; Thrower *et al.*, 1996). The kinetochore complex, in addition to its role of connection to microtubules, is also responsible for chromosome movement through controlling the polymerization and depolymerization of tubulin (reviewed by Cheeseman and Desai, 2008; Coue *et al.*, 1991). Attachment defects of microtubules with kinetochore induce aberrant chromosome segregation and chromosome number instability (Bakhoum *et al.*, 2009).

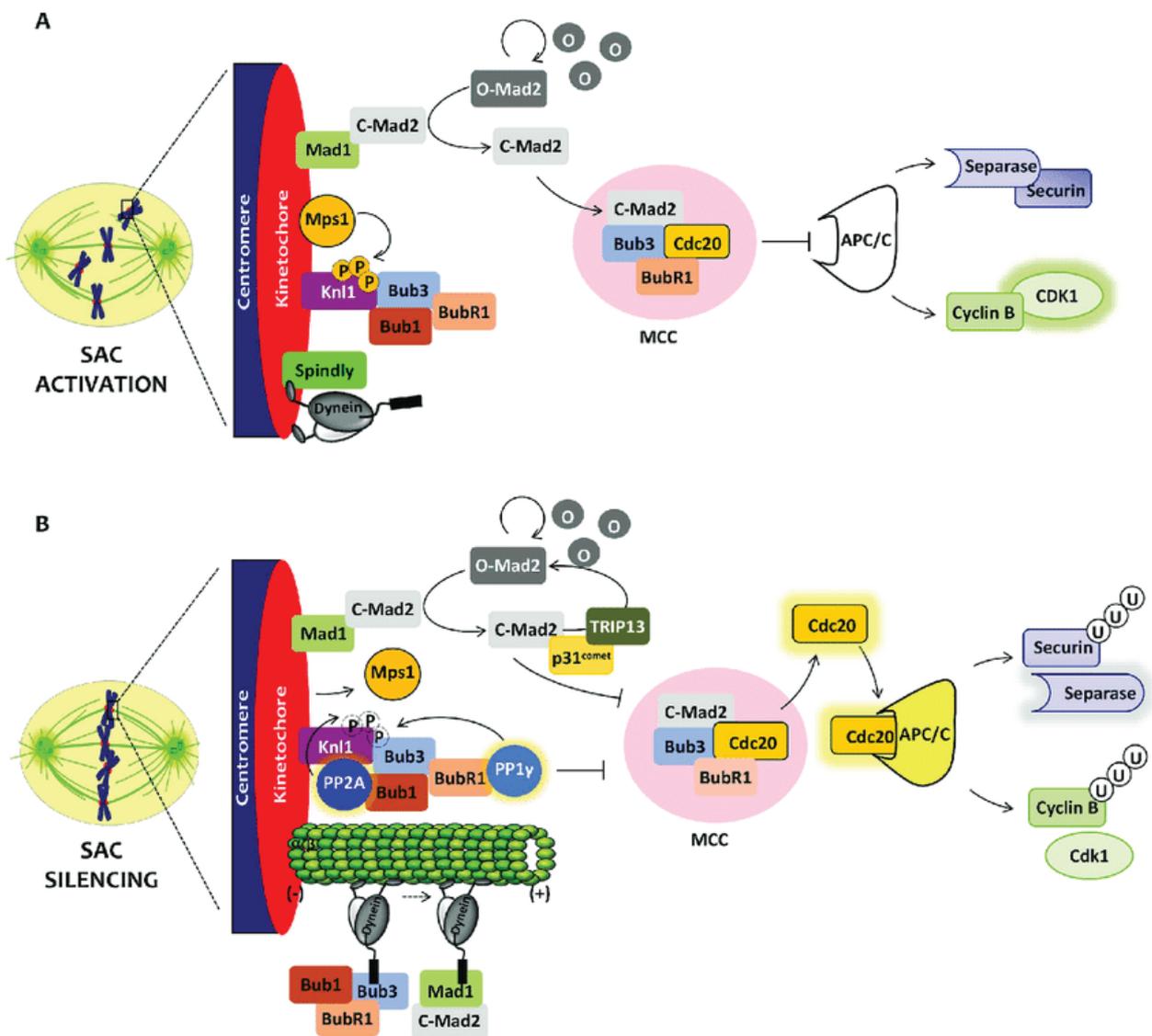


Figure 8: Mechanism of the Spindle Assembly Checkpoint (SAC). When the kinetochore is not connected to a microtubule, the SAC is activated: MPS1 is localized at the kinetochore and induces a phosphorylation cascade that leads to the formation of the MCC complex. The APC/C machinery is blocked and CDK1 and Separase inactivated. Once the kinetochore is connected, the SAC is satisfied and MPS1 released from the chromatin. CDC20 is therefore freed from the MCC complex and act as a co-activator for the APC/C machinery, which induces the degradation of securin and cyclin B, thus activating separase and CDK1. Image taken from Henriques *et al.* (Henriques *et al.* 2019).

In the following subchapter, I will focus on a protein discovered in the last decade to be necessary for kinetochore assembly, and which I found to be a CDYL2 interaction partner.

2.2.2.2 CHAMP1: A new player to take into account

In 2011, Itoh *et al.* described a novel protein, called chromosome alignment-maintaining phosphoprotein 1 (CHAMP1) and involved in kinetochore attachment (Itoh *et al.*, 2011). CHAMP1 – also designated as CAMP, C13orf8, or ZNF828 – is a zinc-finger protein conserved in vertebrates. It contains two Zinc-finger domain at its N-terminal and three at its C-terminal, while its middle region shows three characteristic repeat motifs: SPE, WK and FPE (Itoh *et al.*, 2011; Tanaka *et al.*, 2016). One zinc-finger at its C-terminal allows CHAMP1 to localize on the chromosome (Itoh *et al.*, 2011). The middle region possibly contains a nuclear localization sequence as a truncated protein missing the middle region is not able to localize in the nucleus (Isidor *et al.*, 2016). Finally, the FPE motif allows CHAMP1 to localize on spindle fibers and kinetochores (Itoh *et al.*, 2011). CHAMP1 was first identified as a Mitotic Arrest Deficient 2-Like Protein 2 (MAD2L2) interactor (Hara *et al.*, 2017; Tanaka *et al.*, 2016). The interaction could be detected in cells synchronized at both the onset of S-phase and mitosis and happens through the CHAMP1 WK motif (Hara *et al.*, 2017). CHAMP1 fragment is wrapped in the so-called “safety-bell” region of MAD2L2 at its C-terminus (Hara *et al.*, 2017).

On a cellular level, a knock-down of CHAMP1 induces misalignment of chromosomes at the metaphase plate (Itoh *et al.*, 2011; Tanaka *et al.*, 2016). No aberrant loss of cohesion could be observed but the interkinetochore distance was found shorter in CHAMP1 depleted cells and not all sister chromatids were attached to microtubules (Itoh *et al.*, 2011). Additionally, the kinetochore bound microtubules-fibers (K-fibers) were found to be irregular and unstable in cells treated with CHAMP1 interference RNA. How CHAMP1 regulates K-fibers stability and attachment to kinetochores could not be explained in detail yet, however, Itoh *et al.* observed an abolishment of MAD2L2, CENPE, and CENPF proteins at the kinetochores in cells lacking CHAMP1 (Itoh *et al.*, 2011; Tanaka *et al.*, 2016).

The phenotype observed after CHAMP1 knock-down can partially be explained by its interaction with MAD2L2 as CHAMP1 may recruit physically MAD2L2 to the kinetochore attachment region (Itoh *et al.*, 2011). However, this possibility was not further addressed in other studies and so remains somewhat hypothetical. CHAMP1 was also found to interact with Pogo transposable element with Zinc-Finger domain (POGZ) and HP1 proteins both interacting with the C-terminal region of CHAMP1 (Vermeulen *et al.*, 2010). Interestingly, POGZ also directly interacts with HP1 proteins but not through the PxVxL motif usually recognized by HP1 (Nozawa *et al.*, 2010). Instead, POGZ binds HP1 through its unique zinc-finger like motif. This raises the question of a potential competition between POGZ and other HP1 binding partners (Nozawa *et al.*, 2010).

On a clinical level, large scale sequencing revealed that CHAMP1 mutations, resulting mostly in truncated proteins, are potentially linked to intellectual disabilities and developmental delay. Decreased pain sensation, partial seizures, or difficulties in breathing were also described by some (Hempel *et al.*, 2015; Isidor *et al.*, 2016; Okamoto *et al.*, 2017; Tanaka *et al.*, 2016). When analyzed, the karyotype was always found normal (Hempel *et al.*, 2015; Isidor *et al.*, 2016), but lymphoblast cells from CHAMP1 mutated patients showed abnormal spindle and misaligned chromosomes as observed in *in vitro* analysis (Okamoto *et al.*, 2017). In mice, CHAMP1 was found in the developing brain at day E14,5, suggesting it plays a role in its development (Isidor *et al.*, 2016). Interestingly, while CHAMP1 depletion in mice was lethal soon after birth, HP1 β *-/-* mice also die in the neonatal period and show abnormal brain development and reduced proliferation of neuronal precursors (Isidor *et al.*, 2016). These observations thus raise the possibility that CHAMP1, POGZ, and HP1 can work together in brain development.

2.2.2.3 Spindle Assembly Checkpoint and Anaphase Promoting Complex

To obtain two identical daughter cells, with the same DNA content, it is essential to assure that, in each chromosome, each chromatid is attached to exactly one pole through microtubules. The Spindle Assembly Checkpoint (SAC) blocks mitosis until all kinetochores are correctly connected, avoiding a premature separation of sister chromatids, which would lead to unequal chromosome distribution. In yeast, SAC was found to be not essential for correct mitosis, but in mammals, mutations in SAC proteins were linked with

cancer and neurodegenerative disorders (Hayward *et al.*, 2019; Karra *et al.*, 2014; Ling *et al.*, 2014). Mechanistically, SAC relies on a cascade induced by the protein Monopolar Spindle 1 (MPS1) (Abrieu *et al.*, 2001; London and Biggins, 2014a). When there is no microtubule connection, MPS1 is localized at the kinetochore, where it induces a phosphorylation cascade to form the mitotic checkpoint complex (MCC) consisting of Cell Division Cycling protein 20 (CDC20), the mitotic checkpoint proteins BUB1B and BUB3, and the mitotic spindle assembly checkpoint protein MAD2 (Hayward *et al.*, 2019; London and Biggins, 2014b). The MCC complex interacts with the anaphase-promoting complex (APC) and acts as a pseudo-substrate to inhibit its action (Izawa and Pines, 2015). Once a microtubule gets attached to the kinetochore, the spindle assembly checkpoint is satisfied, MPS1 is released from the kinetochore and MCC stops to be released, as schematized in figure 8.

To be activated, APC needs to interact with its co-activator CDC20 released from the MCC (Izawa and Pines, 2015). APC mechanism then relies on proteasomal degradation, acting as an E3 ubiquitin ligase (Barford, 2011). Two targets of the APC are particularly essential for anaphase promotion: cyclin B and securin (Pines, 2011). Cyclin B degradation is essential for chromatids' motion as overexpressing a non-degradable mutant of Cyclin B blocks the chromosomes at the metaphase plate (Chang *et al.*, 2003). The mechanism behind this observation is however not elucidated. Securin, on the other hand, is a well-characterized protein acting as a chaperon for separase (Hornig *et al.*, 2002; Zhou *et al.*, 2020). When securin is degraded, separase is released and catalyzes the removal of the remaining cohesin at pericentromeres, as described previously. The cohesion between sister chromatids is thus lost and the chromatids are pulled by the microtubules in the direction of the centrosomes.

2.2.3 Centrosomes: The Microtubules Organisation Center

Centrosomes are organelles with various functions including cell motility, transport of vesicle or cell shape. They are particularly important for spindle organization and serve as an organizing center for the microtubules during mitosis. Centrosomes consist of two perpendicular centrioles surrounded by numerous proteins, notably γ -tubulin (Schatten, 2008). Centrosomes are tightly regulated by the cell cycle. During the early S-phase, the

centrosomes are duplicated (Lacey *et al.*, 1999). At the onset of mitosis, one centrosome will migrate to the opposite pole of the cell, preparing for bipolar chromosome attachment. Nuclear Mitotic Apparatus protein (NUMA1), a protein necessary for the anchorage of mitotic spindles, interacts with centrosomes structure transiently during mitosis (Tang *et al.*, 1994). Both centrosomes at the opposed pole of the cell will get attached via tubulin fibers to one chromatid of each chromosome, assuring an equal distribution of DNA content during anaphase.

Surprisingly, centrosomes were found to be not absolutely essential for bipolar mitosis, as in embryonic *Xenopus laevis* egg extracts, the functional bipolar spindle can be observed without the presence of centrosomes (Heald *et al.*, 1996). Additionally, *Drosophila melanogaster* genetically modified to avoid centrosomes duplication are still able to proceed into adult flies (Basto *et al.*, 2006). Heald *et al.* have suggested that bipolarity might be an intrinsic function of microtubules but recent studies have reported that NUMA1 is able to promote spindle polarization and bipolar orientation in centrosomes-lacking cells. In cells containing centrosomes, both pathways function in a redundant manner (Chinen *et al.*, 2020). Still, an aberrantly high number of centrosomes can induce defects in equal chromosome distribution and could be reported in most cancers studied, strongly suggesting that centrosomes are needed for correct mitosis (Chan, 2011; D'Assoro *et al.*, 2002). Additionally, centrosome amplification was linked to tumorigenesis in breast cancer patients and *Drosophila melanogaster* (Basto *et al.*, 2008; Salisbury *et al.*, 2004). It was proposed that centrosome amplification promotes chromosomal instability and thus cancer by inducing segregation errors and lagging chromosomes during anaphase (Ganem *et al.*, 2009).

2.2.4 Blocking the Cell Cycle

Mitosis is a critical event in cellular life and to ensure it happens in a controlled manner, so-called mitotic checkpoints can stop the mitotic progression at critical moments if the conditions are not fulfilled to continue mitosis faithfully, as the previously briefly described SAC. In the laboratory praxis, it is possible to use this checkpoint and other mechanisms to block the cell at a wished cycle stage. During my thesis, I have used several drugs, whose mechanisms are detailed below. Nocodazole and colchicine are two drugs,

derivatives of benzimidazole with a similar mechanism (Friedman and Platzer, 1978). They both interact with purified tubulin *in vitro* and inhibits microtubules polymerization (Friedman and Platzer, 1978; Hoebeke *et al.*, 1976). The cells treated with these drugs are thus blocked in prometaphase as microtubules cannot align the chromosomes on the equatorial cell plate (Ma and Poon, 2011). Another mechanism to block cells during mitosis is by using MG132, a well-known proteasome inhibitor (Lee and Goldberg, 1998). MG132, among other effects, inhibits the protein degradation usually induced by the anaphase-promoting complex, notably Cyclin B, and the treated cells, therefore, remain in metaphase. MG132 shows however a high toxicity due to its lack of specificity and the inhibition of most degradations. It is therefore a drug only adapted for imaging techniques, where a small fraction of synchronized cells is sufficient for analysis, and so a brief MG132 treatment can be used. Finally, cells can also get synchronized in the early S-phase using thymidine. The addition of thymidine to the cell media reduces the dCTP available in the cell, thus significantly slowing down DNA synthesis (Bjursell and Reichard, 1973; Bostock, 1971). Cells treated with double thymidine block are considered to be blocked at very early S-phase (Ma and Poon, 2011).

2.3 MITOSIS FROM AN EPIGENETIC POINT OF VIEW

During mitosis, epigenetic marks, in particular histone modifications, undergo major changes. Transcription during mitosis is mostly repressed to avoid DNA damage due to the highly condensed chromatin structure. A global acetylation loss is therefore observed on most residues, including for example on H3K9, H3K18, or H4K12 (Kruhlak *et al.*, 2001). The deacetylation may also be necessary for the dense packaging of nucleosomes during mitosis (Cimini *et al.*, 2003).

Specific histone residue phosphorylations are characteristic of mitosis. The first identified and most cited histone phosphorylation in mitosis is H3 Serine 10 phosphorylation (H3S10ph) (Hendzel *et al.*, 1997; Wei *et al.*, 1998). It is however not the only reported as H3 Serine 28 and H3 Threonine 3 phosphorylations (H3S28ph and H3T3ph) also show an impressive increase (Wang and Higgins, 2013). H3S10ph is mediated by Aurora B and starts at the onset of mitosis (Hirota *et al.*, 2005). By the end of prophase, chromosomes are extensively covered by the modification. It was rapidly shown that H3S10ph releases

HP1 proteins from the chromatin, even in the presence of H3K9 methylation (Fischle *et al.*, 2005; Hirota *et al.*, 2005). Similarly, H3S28 is phosphorylated as well by Aurora B and pieces of evidence suggest that H3S28ph releases polycomb proteins usually binding to methylated H3K27 (Gehani *et al.*, 2010; Hirota *et al.*, 2005). Why these methylated histone readers have to be removed and if this is the main function of H3S28 and H3S10 is not exactly known. Some hypothesize that HP1 removal may promote pericentromeres accessibility for mitotic factors such as Aurora B (Fischle *et al.*, 2005), and others suggest that removal of interacting factors may help chromosome condensation (Dormann *et al.*, 2006). Finally, H3T3 is phosphorylated by Haspin at early prophase as well and becomes highly enriched at inner centromeres by prometaphase (Dai *et al.*, 2005; Polioudaki *et al.*, 2004). As for the previously described modifications, H3T3ph potentially inhibits interactions of reader proteins with the neighboring methylated residues. It was indeed observed *in vitro* that proteins involved in gene transcription by binding methylated H3K4 were unable to do so upon H3T3ph (Couture *et al.*, 2007; Varier *et al.*, 2010), suggesting that H3T3ph participates in the general transcriptional repression happening during mitosis. H3T3ph was also proven to be involved in Chromosomal Passenger Complex (CPC) recruitment to inner centromeres through binding with survivin (Kelly *et al.*, 2010). Other phosphorylations as H2AT120ph or H3T11ph were also reported to be specific to mitosis but their function remains unknown (Wang and Higgins, 2013).

Histone methylation is also influenced by mitosis. This is for example the case of H4K20 which gets extensively monomethylated by KMT5A, starting in the S phase and decreasing in late mitosis with a peak during metaphase (Houston *et al.*, 2008). H4K20me was observed to colocalize with condensin on the chromatin, suggesting this modification is involved in the complex recruitment and chromosome condensation (Liu *et al.*, 2010). Further work to confirm and characterize their mechanistic link remains to be studied. Maintenance of H3K9me3 is also essential for mitosis, notably at pericentromeric regions, as its knock-down induces a lack of chromosome cohesion, condensation, and the cells are more prompt to genomic instability (Peters *et al.*, 2001). Indeed, in yeast Swi6, HP1 homolog, was shown to recruit cohesin to pericentromeres (Nonaka *et al.*, 2002). This exciting result first failed to be reproduced in mammals where HP1 was found to be dispensable for cohesin establishment (Koch *et al.*, 2008; Serrano *et al.*, 2009) even

though mice cells lacking H3K9me3 at pericentromeres revealed cohesion defects (Peters *et al.*, 2001). Recently, HP1 was shown to be involved in cohesin protection rather than loading in humans (Yi *et al.*, 2018). Indeed, HP1 was found to interact with and to help to recruit Haspin, a histone kinase, to the pericentromeres. Haspin was previously found to be necessary for the protection of pericentromeric cohesion of sister chromatids by phosphorylation and thus inhibiting WAPL activity (Dai *et al.*, 2006; Liang *et al.*, 2018). Finally, certain histone methylations, such as H3K4 methylation, are also particularly important during mitosis, even though they do not participate directly in the mitotic process, but allow the maintenance and transmission of gene transcription repression or activation through mitosis (Valls *et al.*, 2005).

In conclusion, many histone modifications are not only relevant for gene transcription regulation, but they are also necessary for the regulation of cellular events, such as mitosis. While the establishment and the effects of their knock-down are often well characterized, the exact mechanism linking histone modifications and their effects remains mainly obscure. During my thesis, I identified the chromodomain on Y-like 2 (CDYL2) protein, a poorly characterized H3K9me3 reader, as an important regulator of mitosis.

3 CDYL: A MULTI-TASKED HISTONE READER FAMILY

3.1 A FAMILY TO SHARE THE READING MONOPOLE OF H3K9ME3

Historically, HP1 was the first described reader for H3K9me3 (Bannister *et al.*, 2001; James and Elgin, 1986). However, it is not the only one that can be found in mammalian cells: in 1999, Lahn and Page described a family of proteins containing a conserved chromodomain, similar to the one observed in HP1 (Fischle *et al.*, 2008; Lahn and Page, 1999). Designated as chromodomain on Y like – CDYL – family, in reference to the Y chromosome localization of the first members discovered, these proteins were rapidly described in biochemical interactions studies to have a strong binding affinity for H3K9me3. Some observations even suggested, they may have a stronger interaction with the modification than HP1 proteins (Fischle *et al.*, 2008; Vermeulen *et al.*, 2010). Interestingly, the chromodomain is not the only common point between the two families. Indeed, while HP1 is known to dimerize through its chromoshadow domain (Brasher *et al.*, 2000), CDYL proteins were rapidly shown to trimerize through a conserved crotonyl-coA domain (Wu *et al.*, 2009).

In the last decade, many publications have emerged to characterize CDYL family members. Some cellular functions have started to emerge and several publications linked mutations or aberrant expression of CDYL family members with diseases, notably cancer and epilepsy. These results strongly suggest CDYL members are implicated in several important functions. Nevertheless, many remain unclear as to how these proteins work and what their exact role in the cell and organism is.

In the third and last part of my thesis introduction, I will present the current knowledge available on the CDYL family and its members. First, I will review the knowledge on the genes and proteins themselves, including a structural and functional analysis of the conserved domains. Second, I will highlight the cellular and molecular functions that have been already characterized and finally the importance of CDYL family members in human diseases and their potential as a new diagnostic tool or therapeutic target.

3.2 CDYL FAMILY

3.2.1 Evolution – Conservation

In *H. Sapiens*, six genes belong to the Chromodomain on Y Like Family. Four of them: *CDY1A*, *CDY1B*, *CDY2A*, and *CDY2B* are located on the Y chromosome, a localization that inspired their names. The two others: *CDYL1* – often written as *CDYL* in literature – and *CDYL2* are located on autosomal chromosomes, respectively chromosomes 6 and 16 in humans and chromosomes 13 and 8 in mice (Lahn and Page, 1999). The genes show high homology and the proteins they encode have two well-conserved domains: a chromodomain at the C-terminus and an Enoyl-CoA Hydratase domain (ECH) at the N-terminus (Fischle *et al.*, 2008; Wu *et al.*, 2009). Both domains are described with more details in the following subchapters.

CDYL family is also highly conserved during evolution. While *CDY* genes are only present in simians (Lahn and Page, 1999), *CDYL1* and *CDYL2* are found in most, if not all, mammals, in *Xenopus Laevis* and many fishes. Less complex organisms, such as *Sylophora pistillata* or *Drosophila Melanogaster* also have one autosomal copy of *CDYL* homolog – called HIPPI in *Drosophila* (Glenn and Geyer, 2019). It is believed that the progenitor of the *CDYL* family arose *de novo* via domain accretion and separated later into the two autosomal *CDYL*. As they are only present in simian mammals, it was first thought that *CDY* genes emerged recently in evolution (Lahn and Page, 1999). However, Dorus *et al.* argued in 2003, that the high sequence difference between *CDY* and their autosomal paralogs does not support this hypothesis and suggests that *CDY* might have appeared earlier, before mouse-human separation, and have disappeared in the mouse and non-simian mammals afterward. Indeed, between mouse and human *CDYL2*, the amino acid identity reaches 100% in both chromodomain and catalytic domain and drops to 80% in the less conserved linker regions (Dorus, 2003). On the other hand, human *CDYL2* and human *CDY* only reach 65% and 61% amino acid identity in the chromodomain and catalytic domain respectively. Similarly, between *CDYL1* and *CDY*, we observe 64 % amino acid identity, while on the opposite, *CDY* proteins show an impressive identity of 98 % between each other. *CDYL1* and *CDYL2* show moderate conservation as they have 51 % amino acid identity. However, the identity reaches 68 % for the ECH-domain and 72 % for the chromodomain. The

sequence homologies were calculated using Blast (blast.ncbi.nlm.nih.gov). Protein sequences are referenced as follows: CDYL1 Q9Y232; CDYL2 Q8N8U2; CDY1 Q9Y6F8 and CDY2 Q9Y6F7.

Due to their homology and based on our current knowledge, a redundancy between CDYL family proteins can yet not be excluded. However, as will be described in the next chapters, the phenotypes observed after CDYL proteins knock-down suggest it also, if not mostly, has necessary unique cellular functions.

3.2.2 Expression and Transcripts

Given their localization on the Y chromosome, it is likely that CDY proteins have a function in male reproduction and thus localized in masculine reproductive organs. Indeed, CDY was only reported in testis, notably in elongating spermatids (Lahn *et al.*, 2002; Lahn and Page, 1999). On the other hand, the autosomal *CDYL1* and *CDYL2* are expressed in most tissues (Franz *et al.*, 2009) and the proteins were found to be nuclear and localizing mostly at the heterochromatin (Abu-Zhayia *et al.*, 2018; Fischle *et al.*, 2008; Franz *et al.*, 2009). In mice and rats, where *CDY* does not exist, *CDYL1* was also found expressed in elongating spermatids and spermatids tail, suggesting the protein has additional functions compared to its human ortholog to compensate CDY loss (Franz *et al.*, 2009; Lahn *et al.*, 2002; Lahn and Page, 1999; Parab *et al.*, 2017).

Rapidly during the functional analysis of *CDYL1*, three transcripts were reported: *CDYL1a*, *CDYL1b*, and *CDYL1c*, whose differences only remain at their N-terminus. While *CDYL1b* has a very similar structure to *CDYL2*, *CDYL1a* has an additional 54 amino acids long sequence and *CDYL1c* has a complete deletion of its chromodomain and 178 amino acids of the linker region (Franz *et al.*, 2009). *CDYL1b* is the only transcript to have a functional chromodomain as explained in the chapter on chromodomain. *CDYL1b* is also the most abundant transcripts in many human tissues, explaining why most of the work was performed in this isoform. An exception lies in the testis where *CDYL1a* is expressed at the same level (Franz *et al.*, 2009). In addition to these different transcripts, CDYL proteins are possibly also fine-tuned by post-transcriptional modifications. Indeed *CDYL1* was found to be methylated by EHMT2 *in vitro* (Rathert *et al.*, 2008) and phosphorylated during DNA

damage response (Bennetzen *et al.*, 2010). A precise characterization of CDYL proteins post-transcriptional modifications, their biological relevance, and consequences were however never performed.

3.2.3 ECH Domain

In 2009, Wu *et al.* published the X-ray crystallography structural analysis of CDYL family proteins C-terminal conserved domains (Wu *et al.*, 2009). They described this domain as having a high structural similarity with already known Enoyl-CoA Hydratase (ECH) – also called crotonase – enzymes. ECH proteins are catalyzing the hydration of an enoyl-CoA into the corresponding hydroxyacyl-CoA (reviewed in Hamed *et al.*, 2008). Additionally, their catalytic domain allows those enzymes to trimerize and hexamerize – through trimers dimerization. As modeled in figure 9B, the trimerization capacity of CDYL proteins was confirmed and its structure analyzed in the same publication and Wu *et al.* do not exclude a possible hexamer formation. It remains however unknown if, *in vivo*, CDYL family proteins multimerize as homo- or heterotrimers.

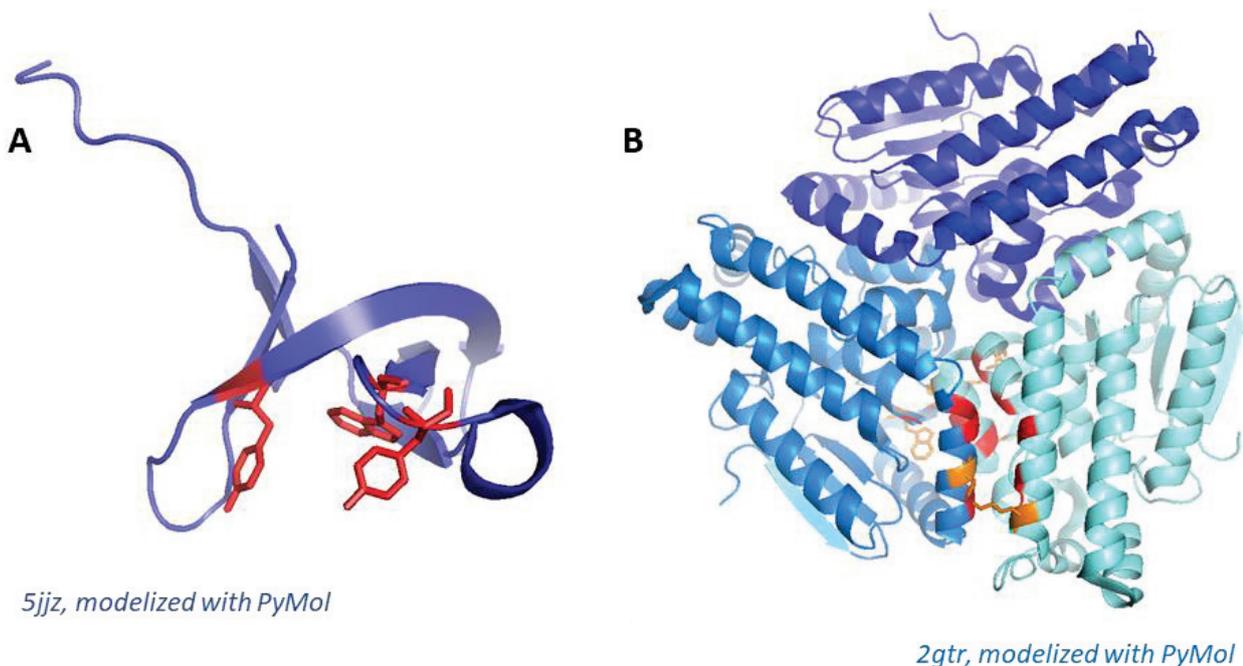


Figure 9: Modelisation of CDYL domains structure analysed with X-ray crystallography. (A) CDYL2 chromodomain (PDB 5jjz), in red are the amino acids forming the aromatic cages, allowing H3K9me3 specific binding. (B) Trimerisation of the enoyl CoA-Hydratase domain (PDB 2gtr). In red and orange are the amino acids predicted by Wu *et al.* to be responsible for respectively hydrophobic and H-bound interactions between the monomers.

When analyzing the structure of CDYL proteins ECH domain and comparing it to the other ECH-containing proteins, Wu *et al.* predicted the amino acids necessary for the enzymatic activity in CDYL1. They suggested the oxyanion hole is formed by L403 and L452 and the residue D483 might be responsible for proton transfer (Wu *et al.*, 2009). Recently, CDYL1 crotonylase activity was confirmed as it was shown to be able to catalyze the reaction from Crotonyl-CoA into β -hydroxybutyryl-CoA and thus prevent histone crotonylation (S. Liu *et al.*, 2017). As CDYL enzymatic activity was observed in elongated spermatids and was found to be important for male fertility, the author suggests it is implicated in the repression of sex chromosome-linked genes. The exact enzymatic activity of CDYL proteins however is still poorly studied, probably due, at least in part, to the lack of knowledge on crotonylation as a post-transcriptional protein modification. Indeed, its role in the histone code remains poorly characterized although it is now more and more linked with gene activation (S. Liu *et al.*, 2017; Wei *et al.*, 2017). Thus, if CDYL proteins are indeed able to protect histone from getting crotonylated, they may have a role in repression maintenance.

3.2.4 Chromodomain

The chromodomain – the domain which gave its name to the family – is conserved among all members and is localized at the N-terminal of CDYL proteins. Even though it is still not officially published, the structure of CDYL2 chromodomain was analyzed with X-ray crystallography and NMR and deposited on the protein databank PDB under the references 5jjz – which is modeled on Figure 9A – and 2dnt (deposited respectively by Dong *et al.* 2016 and Ruhul Momen *et al.* 2006). When compared with HP1, another human chromodomain protein, the CDYL2 chromodomain shows high structural similarities (Fischle *et al.*, 2008). It was therefore rapidly predicted to bind to histone trimethylation modifications. Fischle *et al.* showed in 2008, that CDYL chromodomain is indeed binding with specificity to the trimethylated lysine modification of ARK(S) motif. This motif can be found in histone proteins, where it is usually considered as a mark of repression and heterochromatin, like H3K9me₃, H3K27me₃, and H1.4K26me₃ but also in non-histone proteins as EHMT2 (EHMT2-K185) and EHMT1 (EHMT1-K174). CDYL2 and CDYL1a were found in this publication to bind these modifications, including to the non-histone proteins, *in vitro*. *In cellulo*, CDYL1b and CDYL2 localize at mouse

chromocenters, heterochromatic regions heavily enriched in H3K9me3 and this localization is dependent on both the histone modification and chromodomain integrity (Fischle *et al.*, 2008; Franz *et al.*, 2009). *In vitro*, CDYL proteins affinities to H3K9me3 and H3K27me3 were greatly reduced when the neighbor serine is phosphorylated (Fischle *et al.* 2008).

Chromodomains bind their target through an aromatic cage that interacts with the methylated modifications through cation- π interactions (reviewed in Taverna *et al.*, 2007). CDYL chromodomain makes no exception to this rule. In CDYL1b and CDYL2, the aromatic cage is formed by two tyrosines (Y7 and Y32) and one tryptophan (W29). In CDY proteins, the first tyrosine residue is replaced by phenylalanine (Fischle *et al.*, 2008). Exceptions to the H3K9me3 binding capacities in the CDYL Family are the CDYL1a transcript where the first tyrosine is missing and the CDYL1c transcript which misses the complete chromodomain. Surprisingly, the ECH domain and probably its multimerization capacity were shown to be necessary for the chromodomain to bind H3K9me3, as CDYL1b proteins depleted from their C-terminal was not able to bind chromatin (Franz *et al.*, 2009). Mutants lacking chromodomain and which have not lost their oligomerization capacities were proposed to have a regulator function on the H3K9me3 binding of other CDYL family proteins through trimerization. Indeed overexpression of CDYL1c in mouse cells was shown to be sufficient to displace CDYL1b from chromatin despite H3K9me3 marks (Franz *et al.*, 2009).

3.3 CELLULAR FUNCTION

3.3.1 Interactors

Proteomic-wide techniques, as coimmunoprecipitation followed by mass spectrometry, have gained in popularity in the last decades and have allowed us to find many interactors of CDYL proteins, in particular CDYL1 which is the most studied. Most interactions are recapitulated in Table 1. Those broad-ranged studies are of great help to find hypotheses for the proteins' cellular function. For CDYL1, the interactions with many epigenetic proteins involved in gene repression, including EHMT2 and HDAC, are noteworthy (Caron *et al.*, 2003; Mulligan *et al.*, 2008). Interestingly, transcription repressors were also found

Table1: Known CDYL proteins interactors.

	Interactor	interactor of	shown by	reciprocal shown by	Sources
	EHMT2	CDYL1; CDYL2	CoIP-MS; CoIP-WB	CoIP-WB; CoIP-MS	Mulligan et al. 2008 ; Siouda et al. 2020; Bantscheff et al. 2011
	EHMT1	CDYL1	CoIP-MS		Mulligan et al. 2008
	EZH2	CDYL1	CoIP-WB		Zhang et al. 2011
	HDAC1	CDYL1	CoIP-MS	Affinity Capture - MS; CoIP-WB	Mulligan et al. 2008; Hein et al. 2015; Caron et al. 2003; Bantscheff et al. 2011
	HDAC2	CDYL1	CoIP-MS	CoIP-WB	Mulligan et al. 2008; Caron et al. 2003; Bantscheff et al. 2011
	SETDB1	CDYL1	CoIP-MS		Mulligan et al. 2008
Epigenetic Factors	CBX1	CDYL1		Affinity Capture - MS	Hein et al. 2015
	YEATS4	CDYL2		Affinity Capture - MS	Hein et al. 2015
	SMARCB1	CDYL1	CoIP-MS		Mulligan et al. 2008
	RBBP4	CDYL1	IP - MS		Brantscheff et al. 2011
	SUZ12	CDYL1	CoIP-WB		Zhang et al. 2011
	CDYL1	CDYL1	two hybrid assay		Luck et al. 2020
	SNF5	CDYL1	CoIP-MS		Mulligan et al. 2008
Histones	H3	CDYL1; CDYL2	Affinity Capture - MS		Vermeulen et al. 2010
	H2AX	CDYL1		Affinity Capture - MS	Leung et al. 2017
	Rest	CDYL1	CoIP-MS		Mulligan et al. 2008
	CtBP1	CDYL1			Shi et al. 2003 ; Kuppaswamy et al. 2008
Transcription Factors	Wiz	CDYL1 ; CDYL2		Affinity Capture - MS; CoIP-WB	Hein et al. 2015 ; Kuppaswamy et al. 2008 ; Mulligan et al. 2008
	POU5F1 Oct4				Eiselleova et al. 2019
	MIER1	CDYL1	IP - MS		Brantscheff et al. 2011 ; Mulligan et al. 2008

	MIER2	CDYL1	IP - MS		Brantscheff et al. 2011
	ZSCAN5A	CDYL2		Affinity Capture - MS	Hein et al. 2015
	ZNF644	CDYL1	CoIP-MS	Affinity Capture - MS	Hein et al. 2015; Mulligan et al. 2008
	C10orf12	CDYL1		Affinity Capture - MS	Hein et al. 2015
Transcription Factors	ESR2	CDYL1		Affinity Capture - MS	Giurato et al. 2018
	NR2C2	CDYL1		BioID - MS	Yu et al. 2018
	SMARCA4	CDYL1	CoIP-MS		Mulligan et al. 2008
	ATF7IP	CDYL1	CoIP-MS		Mulligan et al. 2008
	PBRM1	CDYL1	CoIP-MS		Mulligan et al. 2008
Replication Fork	CAF1	CDYL1	CoIP-MS		Liu et al. 2017
	MCM	CDYL1	CoIP - MS; CoIP - WB	CoIP - WB	Liu et al. 2017
Others	MYH9	CDYL1		Affinity Capture - MS	Hein et al. 2015
	SRP72	CDYL1		Affinity Capture - MS	Hein et al. 2015
	HUWE1	CDYL2		Affinity Capture - MS	Hein et al. 2015
	CCT4	CDYL2		Affinity Capture - MS	Hein et al. 2015
	TPX2	CDYL1		Affinity Capture - MS	Hein et al. 2015
	NOP9	CDYL1		Affinity Capture - MS	Hein et al. 2015
	HTRA1	CDYL1	Two-hybrid assay		Murwantoko et al. 2004
	Srp72	CDYL1		Affinity Capture - MS	Hein et al. 2015
	PRMT5	CDYL2	Yeast-Two-hybrid		Weimann et al. 2013

to interact with CDYL1, notably C-Terminal Binding Protein 1 (CTBP1) or RE1-Silencing Transcription Factor (REST) (Mulligan *et al.*, 2008; Shi *et al.*, 2003). Other proteins, for example, Minichromosome Maintenance 4 (MCM4), a protein involved in replication fork, are interactors of CDYL proteins (Y. Liu *et al.*, 2017b). However, these broad-range analyses, as interesting as they are, could not always be characterized in the cellular environment and linked with a known function of CDYL proteins. Some interactions were further studied and are detailed in the following subchapters but many have not been explained until now and need further characterization.

Finally, I would like to highlight the controversial binding of CDYL1 with EZH2. Indeed this interaction was suggested in 2011 by Zhang *et al.* through pull-down assay (Zhang *et al.*, 2011), however, other convincing publications using broad range analysis like mass spectrometry did not notice an interaction between CDYL1 and EZH2 (Escamilla-Del-Arenal *et al.*, 2013; Maier *et al.*, 2015; Mulligan *et al.*, 2008). Similarly in our lab, I performed coimmunoprecipitation showing a strong interaction between CDYL2 and EHMT2 and EHMT1 and a very weak interaction with EZH2 (Siouda *et al.*, 2020).

In the following chapters, I will review the cellular functions of CDYL proteins, which include histone modifications, transcription repression, DNA repair, and function in development. Most of the studies are performed on CDYL1, even though, CDYL2 seems to share functional similarities. The phenotypes observed after a single knock-down of either CDYL1 or CDYL2 do however suggest the two proteins, despite similarities have unique functions.

3.3.2 Histone modifications

Many epigenetic readers show abilities to regulate histone modifications and CDYL proteins make no exception to it. Initially, before knowing the structure of their enzymatic domain, CDYL proteins were reported to act *in vitro* as histone acetyltransferases and suggested to be responsible for H4 hyperacetylation during spermatogenesis (Lahn *et al.*, 2002). This acetyltransferase activity is however controversial. While Franz *et al.* failed to reproduce it (Franz *et al.*, 2009), Caron *et al.* showed the acetyltransferase activity is inhibited in the presence of HDAC, which makes the author skeptical about a function *in vivo* (Caron *et al.*, 2003). The lack of HDAC in spermatids, however, may allow the

acetyltransferase function in this specific tissue. Indeed, recently, acetylation of tubulin from rat sperm flagella by CDYL1 was reported (Caron *et al.*, 2003; Parab *et al.*, 2017).

When the structure and the conservation of the CDYL ECH domain were revealed, it seemed possible that CDYL enzymatic activities would include decrotonylation (Wu *et al.*, 2009). The *in vivo* crotonylation relevance was long unknown, making progress on CDYL catalytic function difficult. In the last years, however, this post-transcriptional modification was more studied and histone crotonylation has arisen as a new activation marker. Recently, Liu *et al.* showed that the CDYL1 ECH domain can protect histones from this modification, by catalyzing the transformation of crotonyl-CoA into β -hydroxybutyryl-CoA, notably during mouse spermatogenesis (S. Liu *et al.*, 2017). Due to the high sequence homology between the Enoyl-CoA-hydratase domains of the different CDYL proteins, it seems likely that CDYL2 and CDY also have crotonase activities which however need to be further characterized.

Finally, CDYL1 seems to be implicated in the restoration of repressive histone modifications after DNA replication. CDYL1 was indeed shown to localize to replication sites and interact with both CAF1 and MCM4, two members of the replication fork complex (Y. Liu *et al.*, 2017b). In cells depleted for CDYL1, EHMT2, and EZH2, two writers necessary for histone methylation transmission fail to physically associate with the replication fork, suggesting that CDYL1 bridges the interaction and is necessary for histone methylation transmission during DNA replication (Y. Liu *et al.*, 2017b).

3.3.3 Transcription repression

The number of interactors of CDYL proteins having a function in gene repression suggests strongly that this family is implicated in processes of gene expression regulation. Indeed, rapidly, many genes were found to be repressed by CDYL proteins. This is notably the case of RhoA and BDNF, two proteins involved in brain development (Qi *et al.*, 2014; Qin *et al.*, 2017). Interestingly, CDYL1 was also found to interact with the neuronal gene repressor REST (Mulligan *et al.*, 2008) and to be necessary for the transcription regulation of REST target genes (Y. Liu *et al.*, 2017a; Mulligan *et al.*, 2008). Moreover, CDYL1 was shown to localize at the repressed X chromosomes in mouse embryonic stem cells (Escamilla-Del-

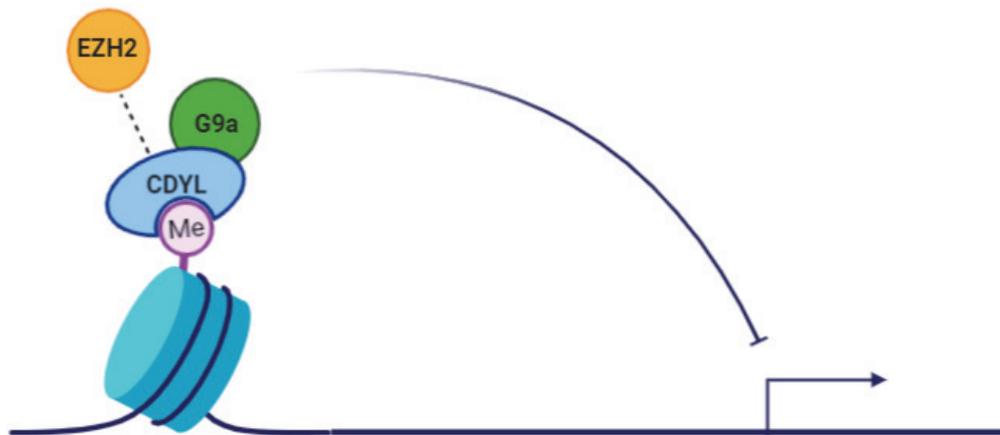


Figure 10: CDYL2 general mechanism for transcription silencing as reported by several studies. CDYL2 recruits the epigenetic writers G9a and EZH2 to chromatin, which methylate H3K9 and H3K27 respectively, thus maintaining transcription repression. Strong direct interactions were reported between CDYL proteins and G9a but interactions with EZH2 are less evident and possibly indirect.

Arenal *et al.*, 2013). Finally, CDYL proteins, also show repressive activities against miRNA genes, as was shown very recently by our lab. CDYL2 indeed controls the repression of miR-124, which targets genes involved notably in epithelial to mesenchymal transition (Siouda *et al.*, 2020). In most described cases, the repressive mechanism consists of CDYL proteins recruiting G9a and/or EZH2 to chromatin, thus indirectly regulating repressive methylation mark deposition (Y. Liu *et al.*, 2017a; Mulligan *et al.*, 2008; Siouda *et al.*, 2020). The mechanism of gene repression through CDYL proteins is schematized with our current knowledge on figure 10. In the next two paragraphs, I will focus on DNA repair and on development, two events where CDYL proteins seem to be implicated, mostly through their repressive function. If CDYL proteins have any functions that do not implicate transcription repression still needs to be investigated.

3.3.4 DNA Repair

In the cells, CDYL1b was found to localize at DNA breaks induced by endonuclease or microirradiation (Abu-Zhayia *et al.*, 2018; Y. Liu *et al.*, 2017b). Interestingly, the chromodomain was found to be dispensable for this interaction, as CDYL1b binds to poly(ADP-ribose) with its ECH domain, probably through the PAR-binding motif found between amino acids 535 and 542 (Abu-Zhayia *et al.*, 2018). Similar to the previously

described silencing mechanism, after CDYL1b is localized at DNA damage sites, EZH2 is recruited to the chromatin and participates in the transcriptional repression at double-strand breaks. As a result of this, cells lacking CDYL1b show a decreased capacity of homology DNA repair and an increased sensitivity to chemotherapy using DNA damage as their mechanism, as cisplatin (Abu-Zhayia *et al.*, 2018). Additionally, Liu *et al.* suggested that similar to its function after DNA replication, CDYL1b could recruit histone modifiers to induce the restoration of the repressive marks on the new DNA strand (Y. Liu *et al.*, 2017b).

3.3.5 In Development

There is, for now, no study clearly indicating a function of CDYL proteins in development. However one report claimed, without presenting supporting data, that *Cdyl1*^{-/-} mice were born in a Mendelian distribution but die within one or two hours after birth (Wan *et al.*, 2013). The author reported diaphragmatic breathing and cyanosis, suggesting a function of CDYL1 in the respiratory system development. CDYL1 was also reported to be highly expressed at embryonic day 12 in the brain and to be a necessary regulator of neuronal migration (Qin *et al.*, 2017). *In vitro* studies, suggested that CDYL1 is also involved in neuronal differentiation as induced pluripotent stem cells from *Cdyl1*^{-/-} mice were shown to be more prompt to spontaneous differentiation (Wan *et al.*, 2013). REST is a transcriptional repressor necessary for neuronal development and orchestrating many major epigenetic changes during neuronal differentiation (Ballas and Mandel, 2005; Nechiporuk *et al.*, 2016). Since CDYL1 was shown to interact with REST (Mulligan *et al.*, 2008), it might be that both proteins are working together in neurodevelopment and it would be of great interest to study CDYL1-REST interaction in this context. Finally, bioinformatics analysis linked CDYL1 with gastroschisis, suggesting CDYL1 protein may also be relevant for early developmental stages (Salinas-Torres *et al.*, 2019). All together, these observations strongly suggest that CDYL proteins are implicated and necessary for development.

3.4 IMPLICATION IN DISEASES

3.4.1 In Cancer

CDYL1 and CDYL2 were found overexpressed in wide-range analyses of cancer diseases, revealing them as new putative oncogenes (Kim *et al.*, 2014; Wu *et al.*, 2013) and tumor suppressors (Mulligan *et al.*, 2008) depending on the context. We recently found that in breast cancer, CDYL2 was not only found overexpressed in patients, a higher level of the protein is also correlating with a worse prognosis (Siouda *et al.*, 2020). In breast cancer cells, CDYL2 was found to repress miR124, a miRNA targeting Signal Transducer and Activator of Transcription (STAT3) and Nuclear Factor κ B (NF- κ B) and thus tumor migration and invasiveness. An increase in CDYL2 expression was shown to increase the metastatic potential of the tumor, while a decrease is reducing it (Siouda *et al.*, 2020). Additionally, some Single-Nucleotide Polymorphism (SNP) next to CDYL2 genes are correlating with prostate and breast cancer predisposition, suggesting CDYL2 may also be involved at earlier stages of tumor progression (Ghoussaini *et al.*, 2013; Michailidou *et al.*, 2013; Oh *et al.*, 2019). Noteworthy is the fact that CDYL1 circRNA may also be affecting tumor progression. Indeed, circCDYL1 expression was found altered in several types of cancer, though its effect and mechanism of action are still quite controversial (Cui *et al.*, 2019; Mei *et al.*, 2019; Okholm *et al.*, 2017; Sun *et al.*, 2019; Wei *et al.*, 2020).

Finally, CDYL expression in the tumor was found to correlate with resistance to farnesol (Efferth and Kuete, 2013). This information could be used as a prognostic for the treatment efficiency or to co-target CDYL in therapies using farnesol. A knock-out of CDYL1 was also shown to increase cisplatin efficiency *in vitro* by inducing DNA breaks sensitivity of the cancer cells (Abu-Zhayia *et al.*, 2018; Y. Liu *et al.*, 2017b) and the author suggest the possibility to co-target CDYL proteins in chemotherapy inducing DNA breaks. In both studies, a therapy targeting CDYL might increase the chance of therapy success. Studying the mechanisms by which CDYL is implicated in cancer and interacting with those drugs could be of great interest for future therapy design.

3.4.2 In Brain

As detailed earlier (see Transcription Repression and Function in development), CDYL1 is controlling transcription repression of several genes involved in the brain function and seems implicated in brain development. Still, CDYL1 might emerge as an important regulator of brain development, but its concrete function remains mostly unknown. Interestingly, a decrease in CDYL1 levels induces a higher susceptibility to epilepsy in mouse models (Y. Liu *et al.*, 2017a; Qin *et al.*, 2017). Two hypotheses possibly explain this: the derepression of Ras Homolog Family Member A (RhoA) during development and the derepression of Sodium Channel Protein Type 8 (SCN8). Increased levels of the former induce defects in neuronal migration and mobility, known to contribute to neurological and psychiatric disorders (Qin *et al.*, 2017). The latter reduces the neuronal threshold, making the mice more prompt to an epileptic episode (Y. Liu *et al.*, 2017a). On the other hand, overexpression of CDYL1 increases the resistance of mice to epileptogenesis (Y. Liu *et al.*, 2017a; Qin *et al.*, 2017). Moreover, in human patients, a single nucleotide modification 61 kb upstream of CDY1 was found to correlate with responsiveness to ketogenic dietary therapies (Schoeler *et al.*, 2018). Even though this study needs to be confirmed with a broader number of patients, this observation makes *CDYL1* an interesting gene to study more closely in an epileptic context.

In addition to effects on epilepsy, CDYL1 was also linked to stress-induced depression in mouse models (Liu *et al.*, 2019). It was observed that crotonylation levels are lower and CDYL1 levels higher in the medial prefrontal cortex in the mouse after inducing depressive disorders. An overexpression of CDYL1 in this region increased the sensitivity of the mice while downregulation protected the animals from stress-induced depression. These results showed that CDYL1 plays a critical role in stress-induced depression, possibly through its crotonase activity, and is a potential therapeutic target (Liu *et al.*, 2019).

3.4.3 In Fertility

Due to their expression profile in testis and their localization on the Y chromosome, it was rapidly suggested that *CDY* genes have a biological function for fertility. Several mutations of *CDY* genes were already linked with male infertility (Poli *et al.*, 2015). Localized knock out of *CDYL1* in mouse testis confirmed a function of CDYL proteins in spermatogenesis.

Male mice lacking CDYL1 in testis gave smaller and fewer litter compared to wild-type (WT) males. They also showed progressive infertility earlier during their life (Xia *et al.*, 2019). These observations suggested a role for CDY proteins in man fertility. The hypothesis of CDY proteins' involvement in spermatogenesis was strengthened by a study where CDY1 low expression correlated with failure of sperm recovery in testicular tissues (Heydarian *et al.*, 2016).

As CDY genes are not present in women and both CDYL1 and CDYL2 seem ubiquitously expressed, it is tempting to think that the CDYL family is only involved in men's fertility. But recently, a link between overexpression of CDYL1 and repetitive implantation failure (RIF) was found. Indeed, CDYL1 represses Catenin Beta 1 (CTNNB1) and was found downregulated in endometrial tissues of patients with RIF. The resulting overexpression of CTNNB1 then contributes to the poor endometrial receptivity in these women (Zhou *et al.*, 2020).

Interestingly, CDYL proteins have physical or functional interactions with sex hormones, even though these interactions were not characterized in detail yet. Deficits of testosterone and FSH were able to decrease CDYL1 levels (Gill-Sharma *et al.*, 2012). And broad range protein interaction analysis revealed that both CDYL1 and CDYL2 seem to interact with estrogen receptor ESR2 (Hein *et al.*, 2015).

3.5 TARGETING CDYL PROTEINS?

As discussed above, CDYL proteins were discovered twenty years ago and are getting more and more interesting as they show numerous indispensable cellular functions, and their aberrant expression is linked with various diseases, notably cancer, epilepsy, depression, and fertility. Brief studies also link CDYL proteins and circRNA to other diseases like gastroschisis, Hirschprung disease, and Myotonic Dystrophy Type 1 (Enguix-Riego *et al.*, 2016; Salinas-Torres *et al.*, 2019; Voellenkle *et al.*, 2019). Up to now, CDYL1b is the most studied candidate, but it is to be expected that the other proteins of the family, notably CDYL2, also show very interesting features. Due to the high implication of CDYL1 in disease, it was suggested to use it as a complementary diagnostic tool, notably for cancer (Mei *et al.*, 2019; Savci-Heijink *et al.*, 2019). Some studies suggest it may also be

used as a prediction tool, for the success of some therapies or surgeries (Efferth and Kuete, 2013; Heydarian *et al.*, 2016; Schoeler *et al.*, 2018). The efficiency of it needs however to be addressed in broader studies. *In vitro* studies have finally addressed the possibility to co-target it in chemotherapy to increase its efficiency (Abu-Zhayia *et al.*, 2018), while others have observed that decreasing CDYL1 level in the cortex region of mice, showing abnormally high levels of the protein, protects against depression thus raising the question to use it in depressive patients (Liu *et al.*, 2019).

In a not too far future, it can be thus envisaged to target CDYL proteins for therapy. Small molecules inhibiting CDYL chromodomain have already been described in the literature (Barnash *et al.*, 2016; Stuckey *et al.*, 2016). The high conservation between the different chromodomains in the CDYL family and with other chromodomain proteins as HP1 α , HP1 β , and HP1 γ , makes it difficult to obtain a perfect specificity for the drugs. However, by playing with organic radicals, potential satisfying specificity can be obtained (Yang *et al.*, 2019). To bypass the chromodomain specificity problems, drugs targeting other necessary CDYL domains or motif, notably its enzymatic pocket or its PAR motif, also have to be considered.

In conclusion, the CDYL family seems to be implicated in many different necessary processes and diseases. Up to now, our knowledge of this family is quite superficial and only future studies will help us understand all its implications, functions, and its relevance as a therapeutic target. My thesis, whose hypothesis and objectives are detailed on the following page, aims at characterizing CDYL2 biological function in the cell.

4 HYPOTHESIS AND OBJECTIVES

Taken together, CDYL2 belongs to the CDYL family, a family of H3K9me3 readers that were shown to be implicated in various diseases including several cancer types. CDYL1 in particular was shown to repress transcription through recruitment of EHMT2 and EZH2 and methylation of H3K9 and H3K27. CDYL1 recruitment of both methyltransferases is also involved in histone modification transmission. An overexpression of CDYL1 is correlating with chemoresistance and mental depression, while a downregulation was linked with increased susceptibility to epilepsy. However, very little is known about CDYL2. My thesis work therefore aimed at characterizing CDYL2 function in the cell.

Before my arrival in the team, preliminary results have revealed that a knock-down of CDYL2 induces several mitotic defects. During my thesis, I confirmed the importance of CDYL2 for faithful mitosis and characterized CDYL2 spatiotemporal localization in the cell. I performed an analysis of a CDYL2 immunoprecipitation followed by mass spectrometry dataset to identify potential interactors of CDYL2 relevant to the mitotic phenotype and verified the interactions with Proximity Ligation Assay or immunoprecipitations. These analyses were performed to answer the question: How is CDYL2 influencing mitosis? And what is the molecular mechanism behind CDYL2 implication in mitosis?

Three hypotheses were formulated following CDYL2 interactors characterization. First, could CDYL2 be involved in transcription regulation of repeats sequences, notably satellite repeats at pericentromeres? Second, is CDYL2 necessary for cohesin recruitment or establishment, notably at pericentromeres? And finally, is CDYL2 needed for CHAMP1 recruitment at the kinetochore?

During my thesis, I also collaborate to study the implication of CDYL2 in breast cancer. We observed that CDYL2 overexpression in breast cancer patients is correlating with a worse prognosis and found that it regulates breast cancer cell invasion and aggressiveness. I participated mostly in elucidating the molecular mechanism behind this regulation, notably by analyzing CDYL2 interactors and confirming mir124 repression through the recruitment of histone methyltransferases by CDYL2.

Overall during these three years, my work participated to provide the first studies characterizing CDYL2 molecular function and aimed at better understanding CDYL2 implications in mitosis.

Materials & Methods

5 KEY MATERIALS

5.1 ANTIBODIES

5.1.1 Primary Antibodies

Protein Target	Supplier	Reference
CDYL2	My BioSource	MBS821304
CDYL1	Diagenode	C15310243
Flag	Sigma	F3165
Actin	Merck	A3854
pericentrin	Abcam	Ab4448
SETDB1	Gift from Yi Zhang, Harvard Medical School	
DNMT1	Chromotek	2E8
EHMT2	Gift from Y. Nakatani lab, formerly Harvard Medical School	
CREST	Antibodies incorporated	15-235- 0001
α -Tubulin	Cell Signaling	#3873
H3K9me3	Abcam	Ab8898
H3K9me2	Abcam	Ab1220
IgG	Bethyl	P120-101
H3	Abcam	Ab1791
SMC1	Bethyl	A300-055A
RAD21	Bethyl	A300-080A
STAG1	Bethyl	A300-157A
PDS5B	Bethyl	A300-537A
CENPA	Genetex	GTX13939
CHAMP1	Bethyl	A304-216A
CENPI	MBL	PD032

5.1.2 Secondary Antibodies

Protein Target	Marker	Supplier	Reference
Rabbit (H+L)	HRP	Life Tech	#31460
Rabbit (L)	HRP	JacksonImmuno	211032171
Mouse (H+L)	HRP	Life Tech	G-21040
Rabbit	AlexaFluor 555	Life Tech	A-21428
Mouse	AlexaFluor 488	Life Tech	A-11029
Human	DyLight 650	abcam	Ab96914
Goat	Duolink in Situ PLA Probe PLUS	Sigma	DUO92006
Rabbit	Duolink in Situ PLA Probe MINUS	Sigma	DUO92002

5.2 PRIMERS

5.2.1 For mRNA Analysis

Primers	Sequence 5' - 3'
CDYL2	ACCAACGGGGGATTGAACCTGC GGTGTCCAGGGCATTGTTATCCGAGG
GAPDH	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTC

5.2.2 For repetitive Sequences

Primers	Sequence 5' - 3'
α -Sat	CTCACAGAGTTGAACCTCC GAAGTTTCTGAGAATGCTTCTG
SatII	ATCGAATGGAAATGAAAGGAGTCA GACCATTGGATGATTGCAGTCA
Negative Control	GGAACCTCTGCACCACTGT TGCAAGATGCTTCACACACA
LINE1ORF1	AGGAAATACAGAGAACGCCACAA GCTGGATATGAAATTCTGGGTTGA
LINE1ORF2	CAAACACCGCATATTCTCACTCA CTTCCTGTGTCCATGTGATCTCA

LINE1UTR	GAATGATTTTGACGAGCTGAGAGAA GTCCTCCCGTAGCTCAGAGTAATT
18S	GTAACCCGTTGAACCCATT CAAGCTTATGACCCGCACTT
K111	AAGAGCACCAGGATGCTTAATGCC AGTGACATCCCGCTTACCATGTGA FAM-TGCCGGTCCTAACAGTAGACTCAC-BHQ1
K222	CAGCGTTCTGGAATCCTATGT TGTATTGTGGTAACTGGGTATATGT FAM- ACCCACATGGCAGTGTCTGGATT-BHQ1

6 METHODS

6.1 CELL CULTURE

6.1.1 Cell lines

HeLa (ATCC, CCL-2), HeLa S3 (ATCC, CCL-2.2), HEK-293 (ATCC, CRL-1573), MDA-BD-231 (ATCC, HTB-26) and MEF (Jenuwein's Lab) cells were cultured in DMEM GlutaMAX medium (Gibco, 31966-047) supplemented with 10 % Fetal Bovine Serum (Gibco, 10270-106) and 1 % penicillin/streptomycin (Gibco, 15140-122). MCF7 (ATCC, HTB-22) cells were cultured in DMEM low-glucose medium (Gibco, 31885-049) supplemented with 10 % Fetal Bovine Serum (Gibco, 10270-106), 40 µg/mL gentamicin (Gibco, 15710-049) and 0,6 µg/mL insulin (NovoRapid). All cells were grown at 37°C and 5% CO₂ in a humidified incubator, passaged twice a week by trypsinization and regularly tested for mycoplasma contaminations using ATCC Universal Mycoplasma Detection Kit (ATCC, 30-1012K).

When indicated, for consequent biochemical experiments, HeLa S3 were cultured in suspension, in MEM medium, Joklik modified (Sigma, M8028), supplemented with 10 % Fetal Bovine Serum (Gibco, 10270-106) and 1 % penicillin/streptomycin (Gibco, 15140-122). Cells were grown under agitation using a 1L bioreactor at 37°C.

6.1.2 Synchronization

Cells were synchronised in S-phase using a double thymidine block as previously described (Ma and Poon, 2011). Cells were treated with 2 mM Thymidine (Sigma, T1895-5G) for 18 hours. Then released for 9 hours in fresh medium and treated again with 2 mM Thymidine for 15 hours. For mitosis synchronization, cells were treated with 2 mM thymidine for 24 hours, released for 3 hours and finally treated with 100 ng/mL Nocodazole (Sigma, M1404) for 12 hours. For synchronization before immunofluorescence assay, cells were treated for 16 hours with 100 ng/mL Nocodazole or for 2h with 10 µM MG132.

6.2 TRANSFECTIONS

6.2.1 Stable Transductions

For murine retroviruses, Phoenix cells were used, while human retroviruses were made in HEK293T. Cells were transfected with MSCV (Vector) or MSCV-CDYL2 plasmids described in Siouda *et al.* (2020), as well as with packaging plasmid when needed, using PolyJet (SignaGen, SL100688) and following manufacturer's instructions. Supernatant containing the viruses was taken 48 h after Transfection, filtered and added to the wished cells with 8 µg/mL polybrene. Cells were selected for 14 days using 2 µg/mL puromycin (Sigma, P8833). Expression was confirmed by western blotting, quantitative PCR and immunofluorescence.

6.2.2 RNAi Transfection

Interference RNA was transfected into cells using Interferin (Polyplus, 409-10) and manufacturer's protocol. Briefly, 500 ng esiRNA against CDYL2 and Luciferase (Control) were diluted in 100 µL OptiMEM medium (Gibco, 11058-021). 4 µL INTERFERin was added to the diluted RNA, mix thoroughly and incubated 10 minutes at room temperature. Meanwhile, medium was replaced by 500 µL fresh complete medium in each well. The INTERFERin-RNA complexes were added dropwise to each well. When a 6-well was necessary, 1 µg esiRNA was used and everything scaled accordingly to keep the same concentrations.

6.3 PROTEIN WORK

6.3.1 Whole Cell Protein Extraction

Cells were washed with PBS and lysed in 95°C hot NRSB- Buffer (2 % SDS; 10 % Glycerol; 62,5 mM Tris-HCl pH 6,8) freshly supplemented with 1 mM PMSF, 1 mM DTT, 1X PhoSTOP (Roche, 4906845001) and 1 X protease inhibitor cocktail (Roche, 04693132001). Lysates were boiled for 7 minutes at 95°C. Protein concentration was quantified using BCA Kit (ThermoScientific, 23225) and following manufacturer's instructions.

6.3.2 Coimmunoprecipitation

Cells were grown to sub-confluence in 15 cm tissue culture treated plates (Corning). Monolayers were washed three times with ice-cold PBS, scraped in cold PBS containing a protease inhibitor cocktail (Roche) and pelleted. The resulting cell pellets were lysed in lysis buffer (50 mM Tris pH8; 150 mM NaCl; 1 % NP40; 2 mM EDTA) containing protease inhibitor cocktail (Roche, 04693132001) phosphatase inhibitors cocktail (Roche, 4906845001). The lysates were mechanically homogenized using 25G syringes (BD, #300600), then incubated for 30 min at 4°C with rotation. Lysates were centrifuged at 12000 rpm 15 min 4°C to clear debris, treated with DNase I (Qiagen, #79254) and RNase A (Sigma, R4875), then precleared with protein A agaroses beads for 1 h at 4°C with rotation. Immunoprecipitation was performed by incubating indicated antibodies with the lysates overnight at 4°C with rotation, the prewashed protein A agaroses beads were incubated with the lysates for 2 hours 4°C with rotation.

The beads were washed 5 times with wash buffer (10mM Tris pH8; 1mM EDTA; 1mM EGTA; 150mM NaCl; 1% Triton) containing protease inhibitor cocktail (Roche, 04693132001) phosSTOP (Roche, 4906845001). The immune-precipitated beads were boiled in Laemmli buffer and then subjected to immunoblotting.

For Mass Spectrometry analysis, nuclear proteins were extracted prior to coimmunoprecipitation, cell pellet was swollen in 2 Volumes cold hypotonic Buffer (20 mM Tris pH 7,4; 10 mM KCl; 1,5 mM MgCl₂), incubated for 15 minutes at 4°C with occasional mixing, and cell membrane broken using tight Dounce (Wheaton USA). Nuclei were isolated through centrifugation for 5 minutes at 4 000 rpm and 4°C and broken with snap freezing. Nucleus were then resuspended in ½ Volume low salt concentration Buffer (20 mM Tris pH7,4; 25 % Glycerol; 1,5 mM MgCl₂; 0,2 mM EDTA; 20 mM EDTA; 20 mM KCl) and protein extracted by adding dropwise ½ Volume high salt concentration Buffer (low salt but 1200 mM KCl). The extract was incubated 45 minutes at 4°C with rotation and the matrix centrifuged for 20 min at 21 000 xg and 4°C. The supernatant was dialyzed 1h in 1 L Dialysis Buffer (20 mM Tris pH 7,4; 20 % Glycerol; 1,5 mM MgCl₂; 0,2 mM EDTA) with 300 mM KCl, 1h with 200 mM KCl and overnight to 100 mM KCl. Coimmunoprecipitation was performed on the dialyzed extract as previously described.

6.3.3 Mass Spectrometry

Proteins obtained after CDYL2 coimmunoprecipitation on nuclear extracts were resolved on SDS page gel and silver stained. The gel was cut in four parts (<55kDa; 55 kDa - 65kDa; 65 kDa - 100 kDa; 100 kDa – 150 kDa) to homogenize the protein content per mass spectrometry run. IgG control was sent as one part. Mass Spectrometry analysis were performed at Harvard Medical School, Taplin Mass Spectrometry Facility: after a trypsin digestion and liquid chromatography, peptides were run through a MS/MS system. Peptide lists were generated based on following criteria: have a DeltaCn of at least 0,08; minimum Xcorr of 1,8 for +1; 2,5 for +2 and 3,5 for + 3 and have a minimum peptide length of 7 amino acids.

6.3.4 Cell Fractionation

To separate nuclear proteins from the rest of the cells, a protocol similar to the Dignam protocol was used (Dignam *et al.*, 1983). Briefly, cells were swollen in hypotonic buffer, membrane broken using 25G syringe or tight Dounce when the Volume was superior to 1mL. Supernatant was kept as the S100 fraction. Nuclei were isolated through centrifugation and broken via freeze-thaw. Nuclear proteins were extracted using ½ Volume of low (20 mM Tris pH7,4; 25 % Glycerol; 1,5 mM MgCl₂; 0,2 mM EDTA; 20 mM EDTA; 20 mM KCl) and ½ Volume of high (same but 1200 mM KCl) salt concentration Buffer added dropwise at 4°C under constant stirring followed by 45 minutes incubation at 4°C under rotation. Chromatin was pellet for 20 minutes at 21 000 xg and 4°C. Supernatant was kept as the nuclear proteins fraction. The chromatin pellet was resuspended in 95°C hot NRSB- Buffer (2 % SDS; 10 % Glycerol; 62,5 mM Tris-HCl pH 6,8) freshly supplemented with 1 mM PMSF, 1 mM DTT, protease inhibitor cocktail (Roche, 04693132001), and 1X phosSTOP (Roche, 4906845001) and boiled for 7 minutes at 95°C to extract the tightly bound fraction.

6.3.5 Western Blot

Protein samples were quantified using BCA Kit (ThermoScientific, 23225) and usually 20 µg were boiled 3 min with Laemmli Buffer then resolved on Bis-Tris Gels (Invitrogen, NW04122BOX) using MOPS Buffer (Novex life technologies, NP0001). Samples were transferred on a Nitrocellulose membrane (GE Healthcare) in Towbin Buffer (25 mM Tris;

192 mM Glycine; 15 % Ethanol; 0,025 % SDS) for 1h at 350mA. Transfer efficiency and homogeneity were checked by staining the membrane with Red Ponceau. Membrane was then blocked in 5 % Milk in TBS + 0.1% Tween and incubated with primary antibody, described in section 5.1, overnight at 4°C. Membrane was incubated with secondary antibody for 1 h at Room Temperature. When the blotting samples were coming from a coimmunoprecipitation, light chain specific antibodies were used. Images were collected using ECL (Immobilon Western, WBKLS0500) and the ChemiDoc system (BioRad).

6.4 RNA WORK

6.4.1 RNA Purification and quantification

Cells were washed with PBS and lysed in RLT Buffer provided in Qiagen RNeasy kit (Qiagen, 74106). RNA was purified following kit's instruction. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814). The RT-qPCR was performed using the Fast SYBR Green 2X Master Mix (Applied Biosystems, #4385610) and an LC480 PCR machine (Roche). Primers are described in section 5.2. When analyzing Satellite repeats, qPCR was performed using previously described TaqMan probes (Zahn *et al.*, 2015). The experiments were done with SensiFAST reagent (Ozyme, BIO-86005) and following manufacturer's protocol.

miRNA was extracted using the miRNeasy Mini Kit (Qiagen, #217004) following the manufacturer's instructions, cDNA synthesized using miScript II RT Kit (Qiagen,#218161), and RT-PCR performed using miScript SYBR Green PCR Kit (Qiagen, #218073). Primers are described in section 5.2.

6.5 MICROSCOPY WORK

6.5.1 Immunofluorescence

Cells were seeded on sterilized coverslips. After the indicated treatment, cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Fixed cells were permeabilized by NP-40 0.5% at room temperature for 15 min and blocked with 5% FBS in PBS + 0.1% NP-40 at room temperature for 1 h. Cells were first probed with the indicated primary antibodies 1h at room temperature and then with Alexa dye tagged

secondary antibodies for 1h at room temperature. Antibodies references are recapitulated in section 5.1. When needed, Hoechst (abcam, ab145596) was added to the last washing step. Coverslips were mounted using mounting medium with DAPI (Vectashield; VECTOR Laboratories) and observed under the upright microscope (ZEISS AXIOIMAGER, SIP 60549). When stained for Tubulin, we used Dako Fluorescence Mounting Medium (S3023) and stained chromatin with 50 ng/mL Hoechst. Images were analyzed using Zen software.

When indicated, cells were preextracted 30 s to 3 min at room temperature prior to fixation using CSK Buffer (20 mM Hepes pH 8; 50 mM NaCl; 3 mM MgCl₂; 300 mM sucrose) + 0.01% Triton. Pre-extraction was followed by a classical Immunofluorescence but replacing PBS by CSK Buffer.

6.5.2 Proximity Ligation Assay

Cells were seeded, fixed, permeabilised and probed with indicated primary antibodies at 37°C as described for immunofluorescence assay. Coverslips were then incubated 1h at 37°C with a corresponding PLUS/MINUS pair secondary antibodies diluted 1 : 1000 (Sigma, DUO92002 and DUO92006). Ligation and Amplification were performed using PLA Duolink in Situ Detection Reagent Orange (Sigma, DUO92007-100RXN). Incubate slides 30 minutes at 37°C with Ligation Solution, then 100 minutes at 37°C with Amplification Solution. Coverslips were mounted using Dapi mounting medium (Vectashield; VECTOR Laboratories) and observed under the upright microscope (ZEISS AXIOIMAGER, SIP 60549). Images were analyzed using Zen software.

6.5.3 Metaphase Spread

Cells were treated with 0,1 µg/mL Colcemid for 2h. Mitotic cells were harvest with mitotic check off and swollen in suspension in 75 mM KCl for 15 minutes at 37°C. After centrifugation, supernatant was discard and cells fixed in Methanol : Acetic Acid (3 : 1). Microscope slides were washed with Ethanol and placed on a humid heating block at 85°C. Fixed cells were dropped from approximately 60 cm high on the slides. Chromosomes were stained using DAPI mounting medium (Vectashield, VECTOR Laboratories) and observed under the upright microscope (ZEISS AXIOIMAGER, SIP 60549). Images were analyzed using Zen software.

6.6 CELL CYCLE ANALYSIS

Cells were trypsinised and counted using standard protocol. 1 Million cells were rinsed with PBS, resuspend in 300 μ L PBS and fixed by adding dropwise 700 μ L cold Ethanol. After at least 1h incubation at 4°C, fixative was discarded, cells rinsed with 1 mL PBS and carefully resuspended in 250 μ L PBS. 250uL of 2x Staining Solution (50 μ g/mL Propidium Iodide (Sigma, P4864-10mL), 200 μ g/mL RNase A (Roche, 10109169001)) were added dropwise to the solution. The samples were incubated 1h at 4°C and analysed with FACS Calibur (Biosciences). Data was analysed with Flowing Software 2.

6.7 CHROMATIN IMMUNOPRECIPITATION

For CHIP-qPCR analysis, two 6-wells were transfected with esiRNA as previously described. Three days post transfection, cells were cross-linked by the addition of 1% methanol-free formaldehyde to the cell culture medium for 10 minutes, and the reaction quenched by the addition of glycine to a final concentration of 125 mM for 5 minutes. Monolayers were washed three times with ice-cold PBS, scraped in cold PBS containing a protease inhibitor cocktail (Roche) and pelleted. The resulting cell pellets were lysed in Lysis Buffer (0,5 % SDS; 0,1 % Na-deoxycholate; 1 mM EDTA; 0,5 mM EGTA; 10 mM Tris-HCl pH8,0; 100 mM NaCl; 1X protease inhibitor cocktail), incubated on ice for 10 minutes with rotation, then sonicated on ice to an average fragment size of 150 bp using a Branson sonicator. The sonicated lysate was centrifuged at top speed in a benchtop centrifuge at 4°C, the supernatant diluted five times in dilution buffer (1% Triton X-100; 1,5 mM EDTA; 30 mM Tris-HCl pH8,0; 100 mM NaCl; 1X protease inhibitor cocktail), and incubated over night at 4°C with 3 μ g antibodies. On following day, 30 μ L washed magnetic beads A or G were added to the lysates and incubated for 2h at 4°C. The beads were then pelleted and washed with two sequential additions each of wash buffer 1 (0.1% SDS, 1%Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8, 150mM NaCl), wash buffer 2 (0.1% SDS, 1%Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8, 500mM NaCl), wash buffer 3 (250 mM LiCl,1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8), and TE buffer (10mM Tris-HCl, 1mM EDTA,pH 8.0, 50 mM NaCl). Chromatin was eluted by incubating for 30 minutes at 65°C in elution buffer (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 1% SDS) with frequent vortexing. Crosslinks were reversed and eluates treated with RNase A (Sigma,

10109169001) overnight at 65°C, followed by 1h Proteinase K (0,2 µg/µL) treatment. DNA was extracted using Extraction Kit (Cell Signaling, 14209S) and qPCR performed with classic qPCR or Taqman protocol. Primers are described in section 5.2.

Results

7 CDYL2 biological function in mitosis – Preliminary Results

All preliminary results were performed by Dr. Maha Siouda prior to my arrival in the team.

7.1 CDYL2 KNOCK-DOWN INDUCES ABERRANT MITOSIS.

The main goal of this study was to characterize CDYL2 cellular function. To investigate it, CDYL2 protein was knocked down in HeLa and MCF7 cell lines using RNAi. Knock-down was confirmed in both cell lines on mRNA and protein level using qPCR and immunoblotting respectively (Fig. 11A). Our first observation was that after knock-down, cells had a lower proliferation rate. This was confirmed with a proliferation assay (Fig. 11B). After crystal violet staining, it was observed that cells transfected with CDYL2 RNAi gave fewer and smaller colonies. The colony number decreased to 63 % and 74 % of the control for HeLa and MCF7 respectively, while their average size was divided by approximately two (Fig. 11C). This suggests that CDYL2 is needed for vital functions of the cell or its proliferation.

To start exploring how CDYL2 RNAi slows down cell proliferation, live imaging of HeLa cells containing GFP-tagged histone H2B was performed, allowing us to observe the chromatin during the cell cycle. While in the cells transfected with control RNAi we could observe mitosis taking place in slightly less than ninety minutes, in cells transfected with CDYL2 RNAi, mitosis could last for over 5h (Fig. 11D). Interestingly, in CDYL2 knocked-down cells the chromosomes showed dramatic difficulties to align during metaphase, which can potentially explain the extremely long time needed for the cells to divide. In addition, instead of a well-defined bi-oriented metaphase plate, CDYL2 depleted cells showed tri- or quadripolar oriented plates in many mitotic cells or fail to align completely, suggesting that CDYL2 is a protein needed for faithful chromosome alignment during mitosis.

Correct chromosome condensation and cohesion are mandatory for efficient chromosome segregation (Dej *et al.*, 2004; Mazumdar *et al.*, 2004; Toyoda and Yanagida, 2006). To observe chromosome phenotypes, metaphase spreads were performed on

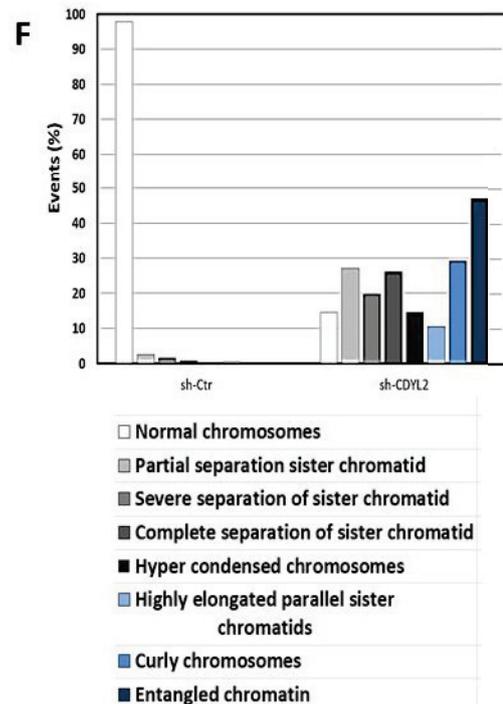
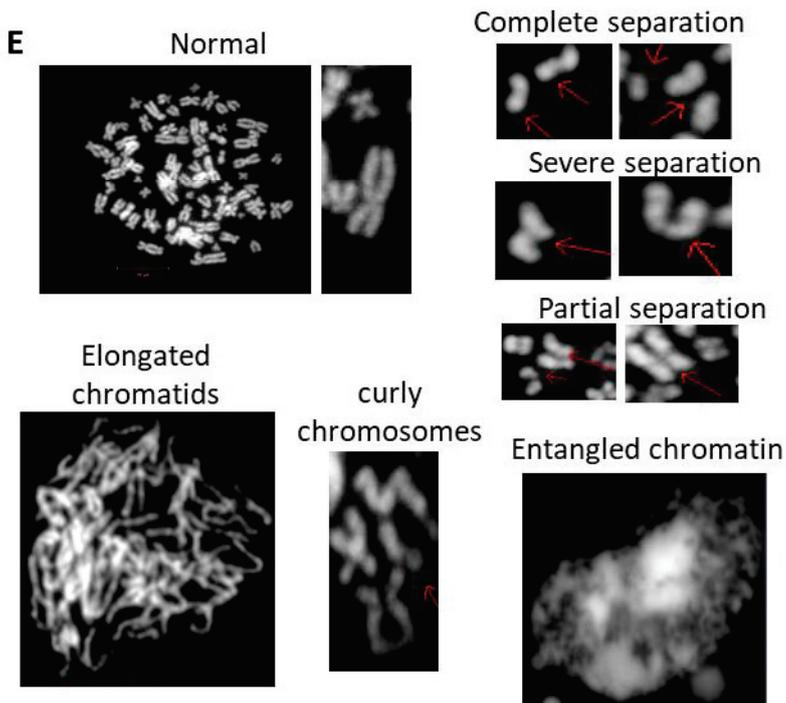
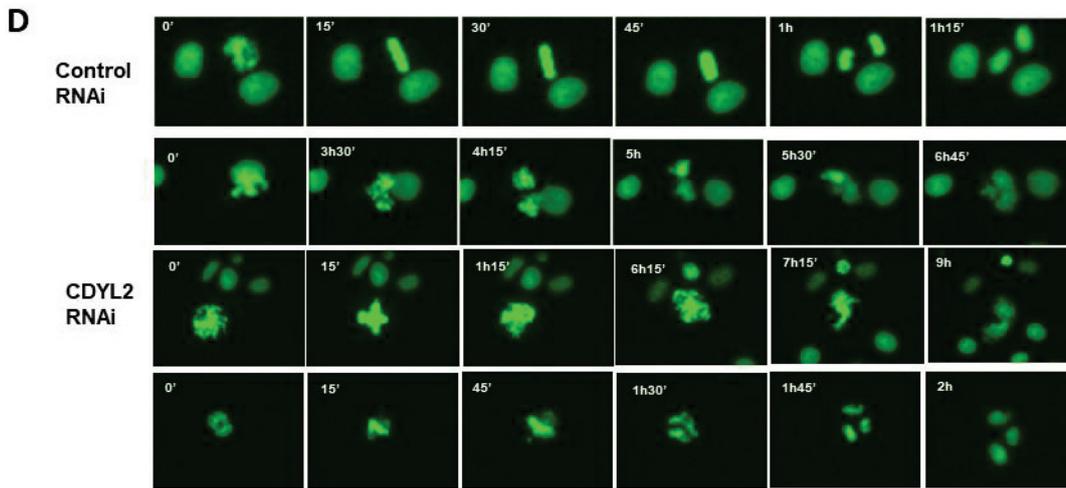
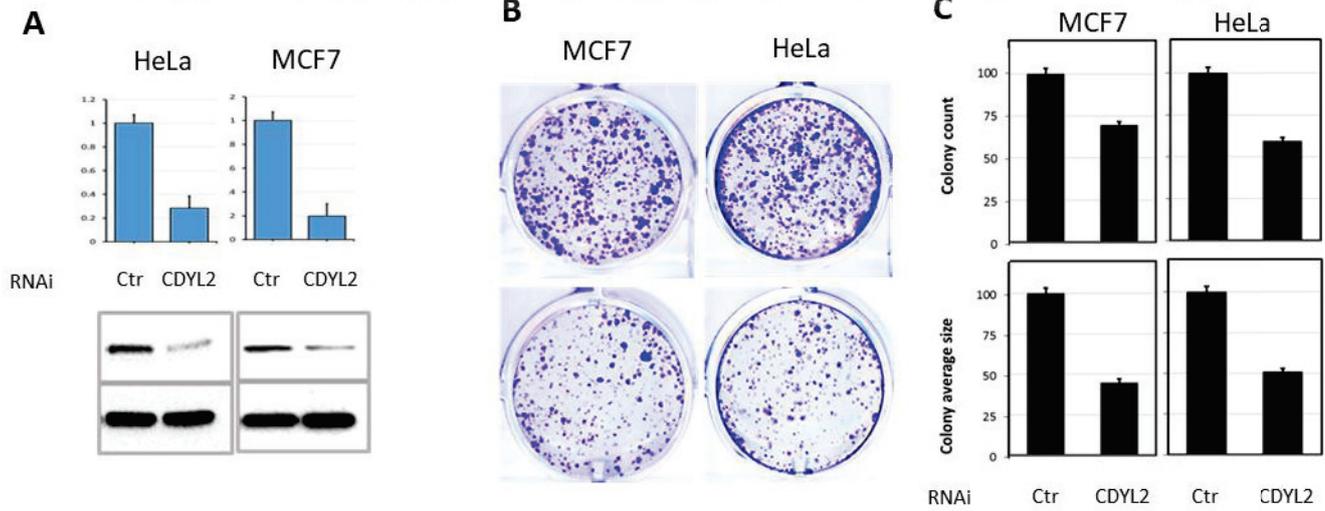


Fig. 11: CDYL2 knock-down induces mitotic defects and chromosome misalignment. (A) HeLa and MCF7 cells were transfected with CDYL2 esiRNA and the knock-down effect checked on mRNA and protein level. (B-C) Proliferation assay revealed less and fewer colonies in cells transfected with CDYL2 esiRNA (n=1). (D) Live imaging of HeLa cells with GFP-tagged H2B reveals difficulties in chromosome alignment during metaphase after CDYL2 knock-down. (E-F) Chromosome formation defects were observed and quantified in HeLa cells knocked down for CDYL2.

revealed well-defined chromosomes, with an X-shaped structure and clear restriction site at the centromeres, while again, a knock-down of CDYL2 induced several defects. These included lack of condensation, entangled chromatin and partial, severe, or complete loss of cohesion (Fig. 11E and F). In total, less than 15 % of the plated cells showed normal chromosomes against 97% in the control cells, which could explain the defects in mitosis observed in live imaging and the decreased proliferation rate. Taken together, the three assays strongly suggest that CDYL2 biological function is related to mitosis, potentially through chromosome formation, segregation, or their correct connection to microtubules.

7.2 CDYL2 PROTEOME

To investigate the mechanisms behind CDYL2 regulation of chromosome alignment, we looked for potential interaction partners using mass spectrometry analysis. Flag-tagged CDYL2 proteins were transgenically expressed in suspension HeLa by stable transduction of an expression vector. Coimmunoprecipitation using antibodies against the Flag-tag was performed and bound proteins eluted. The obtained eluate was run on an SDS-gel along with a negative control coimmunoprecipitate obtained by performing FLAG-IP on nuclear extracts from cells stably transduced with an empty vector (Mock). While the negative control was very clean, suggesting very few false positives in the CoIP, we observed many bands in the Flag CoIP (Fig. 12), indicating that CDYL2 is interacting with various proteins. The gel was sent to a mass spectrometry facility for the identification of the CDYL2-associated polypeptides. Three coimmunoprecipitations were performed as replicates and the returned interactomes were analyzed during my thesis.

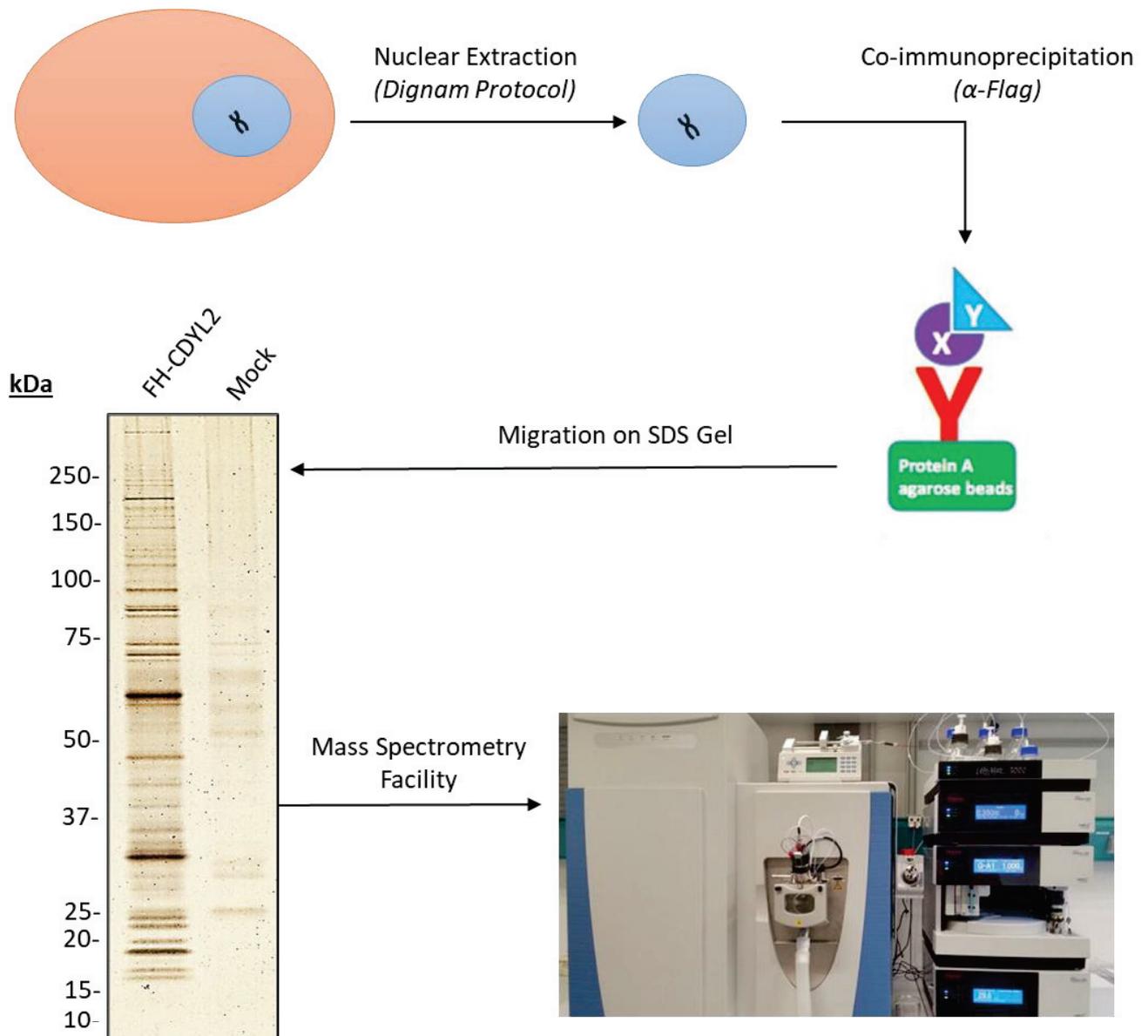


Fig. 12: Coimmunoprecipitation was performed in MCF7 expressing Flag-tagged CDYL2 and the precipitate analysed with mass spectrometry. Nuclear extracts were isolated from HeLa S3 cells expressing Flag-tagged CDYL2 using a Dignam protocol. Coimmunoprecipitation was performed against the Flag-tag. The immunoprecipitate was run on a SDS gel, silver-stained and compared with a negative control and sent for mass spectrometry analysis.

8 CDYL2 BIOLOGICAL FUNCTION IN MITOSIS - THESIS RESULTS

All the following experiments were performed by me.

8.1 CDYL2 IS A NUCLEAR PROTEIN, TIGHTLY BOUND TO CHROMATIN

Cyclins are proteins strongly regulating and regulated by the cell cycle. As an example, cyclin B expression is increased to up to 50-fold between mitosis and G1 phase and its expression is necessary for mitosis progression (Hwang *et al.*, 1995). Similarly, if CDYL2 is implicated in mitosis, its expression is potentially regulated through the cell cycle. To study CDYL2 regulation, suspension HeLa were synchronized with double thymidine block. A simple thymidine block does not guarantee a good synchronization of the cells as thymidine blocks all cells in S-phase, independently of the S-phase stage. A release is then performed to allow the cells to quit S-phase. The release is followed by a second thymidine block which will block the cells as they enter a new S-phase. Thus, after a double thymidine block, HeLa S3 cells are synchronized at the very beginning of S-phase. The cells were harvested every two hours until they reach the beginning of the next G1-phase, corresponding to a total of 14h. Synchronization efficiency was checked by staining with propidium iodide, a fluorescent DNA intercalating agent, and FACS cell cycle analysis. FACS Analysis allow us to measure the fluorescence per cell and thus can give a histogram of the number of cells per DNA content, corresponding to a cell cycle profile (Fig. 13A). The FACS analysis confirmed that the cells were nicely synchronized and that I had samples for each cell cycle stage, starting at early S-phase right after release, to G2 eight hours after release, mitosis mostly ten hours after release, and G1 from twelve to fourteen hours after release. The whole-cell extracts were immunoblotted and I observed a moderately lower level of CDYL2, at four and six hours after release, corresponding to mid and late S-phase respectively (Fig. 13B). Overall, CDYL2 is however expressed during the complete cell cycle and no dramatic changes were observed.

Because we previously described CDYL2 as a nuclear protein strongly bound to chromatin (Siouda *et al.*, 2020), I therefore investigated if a change of CDYL2 nuclear localization or

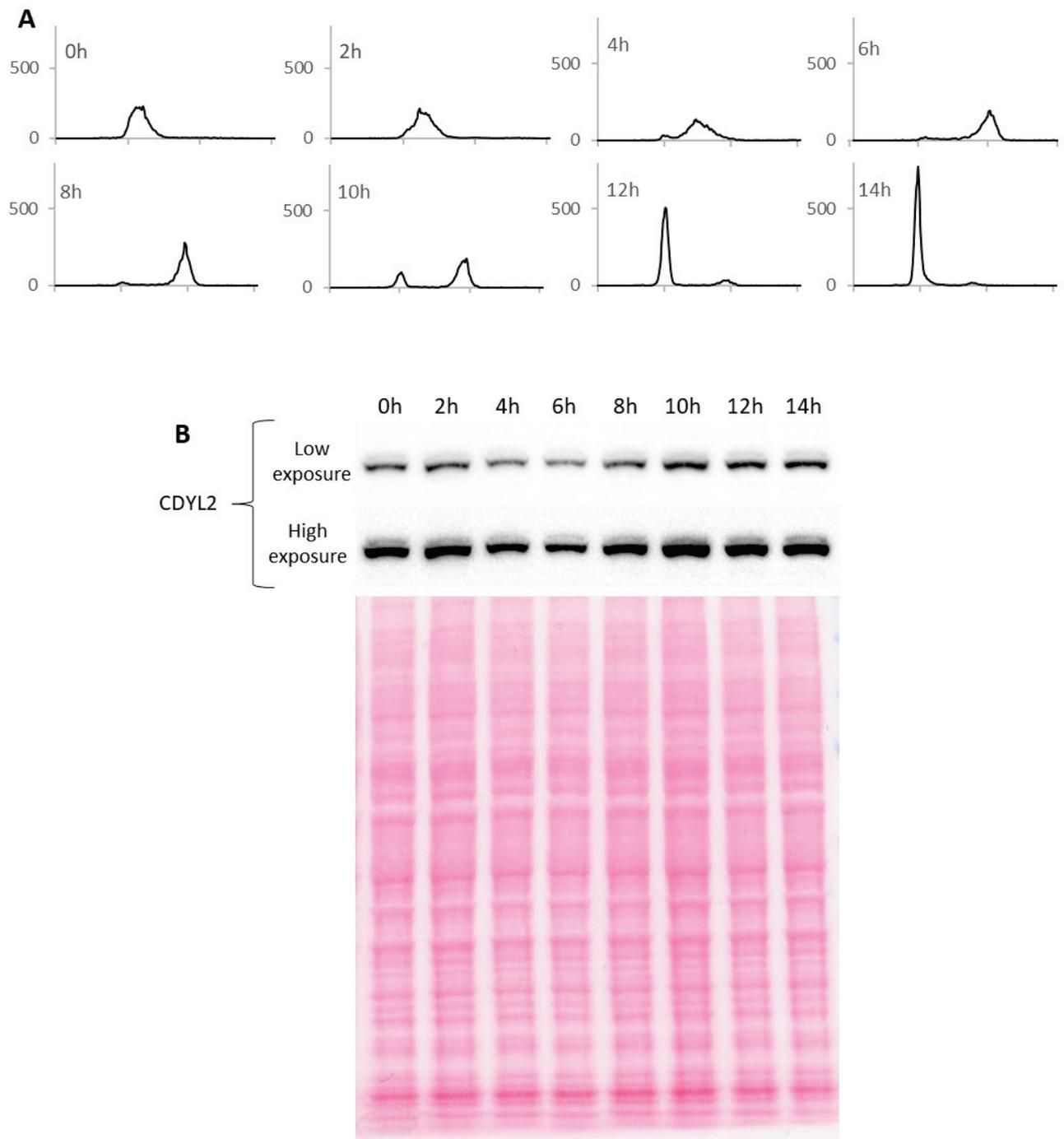


Fig. 13: CDYL2 level in the cell is lower during mid and late S-phase. (A) Synchronization efficiency was checked and cell cycle phase determined using FACS analysis. (B) CDYL2 level over the cell cycle does not seem to change. Experiment was performed twice, representative images are shown.

binding affinity to the chromatin would happen during the cell cycle. Taking the same synchronized samples as previously described, I separated the cytoplasmic, the nuclear, and the chromatin fractions. The efficiency of nuclei isolation was checked under the microscope using Trypan blue dye after douncing. Nuclei were distinguished by their small size and Trypan blue positivity. The efficiency of the nuclear and chromatin separation was also confirmed by Ponceau S staining of membranes after western blot protein transfer, revealing much lower levels of histones in the soluble nuclear protein fractions compared to the chromatin fractions. Consistent with our previous observations in MCF7 (Siouda *et al.*, 2020), I found that in HeLa cells as well, CDYL2 is mostly enriched in the tight chromatin fraction (Fig. 14A). Interestingly, I also observed that CDYL2 from the chromatin fraction runs slightly lower on the gel than in the other fractions, suggesting that two variants of CDYL2 with different affinities to chromatin co-exist in HeLa S3 cell line. It remains however to be elucidated if this variation comes from a post-transcriptional modification or a splicing variant. Over the cell cycle, the CDYL2 level on the chromatin matrix seems to be higher during the G1-phase (Fig. 14B). On the other hand, the nuclear fraction of CDYL2 increases just after mitosis (Fig. 14C). 10h after thymidine block release, corresponding to the sample containing the most mitotic cells, I also observed in the nuclear fraction, a signal usually corresponding to the size of CDYL2 observed on tight chromatin. This could suggest that during mitosis, CDYL2 is partially released from the chromatin in the nucleus. Mitosis being a fast event of approximately one hour, if most CDYL2 regulation happens during this stage, we cannot get a precise picture with an analysis every two hours, and the synchronization followed by fractionation technique may not be the most adapted one.

To have a more precise picture of CDYL2 regulation during mitosis, I performed immunofluorescence staining, which allowed me to observe CDYL2 localization during each stage of mitosis separately. During interphase, CDYL2 is only and always localized in the cell nuclei in both HeLa and MCF7 cell lines (Fig. 15A and B), coherent with the western blotting pattern obtained after cell fractionation (Fig. 14A). After nuclear envelope breakdown at prophase, CDYL2 seems at least partially released from chromatin and localizes to the cell cytoplasm (Fig. 15A and B), which is consistent with the previous hypothesis of chromatin CDYL2 release into the nuclear fraction during mitosis. However,

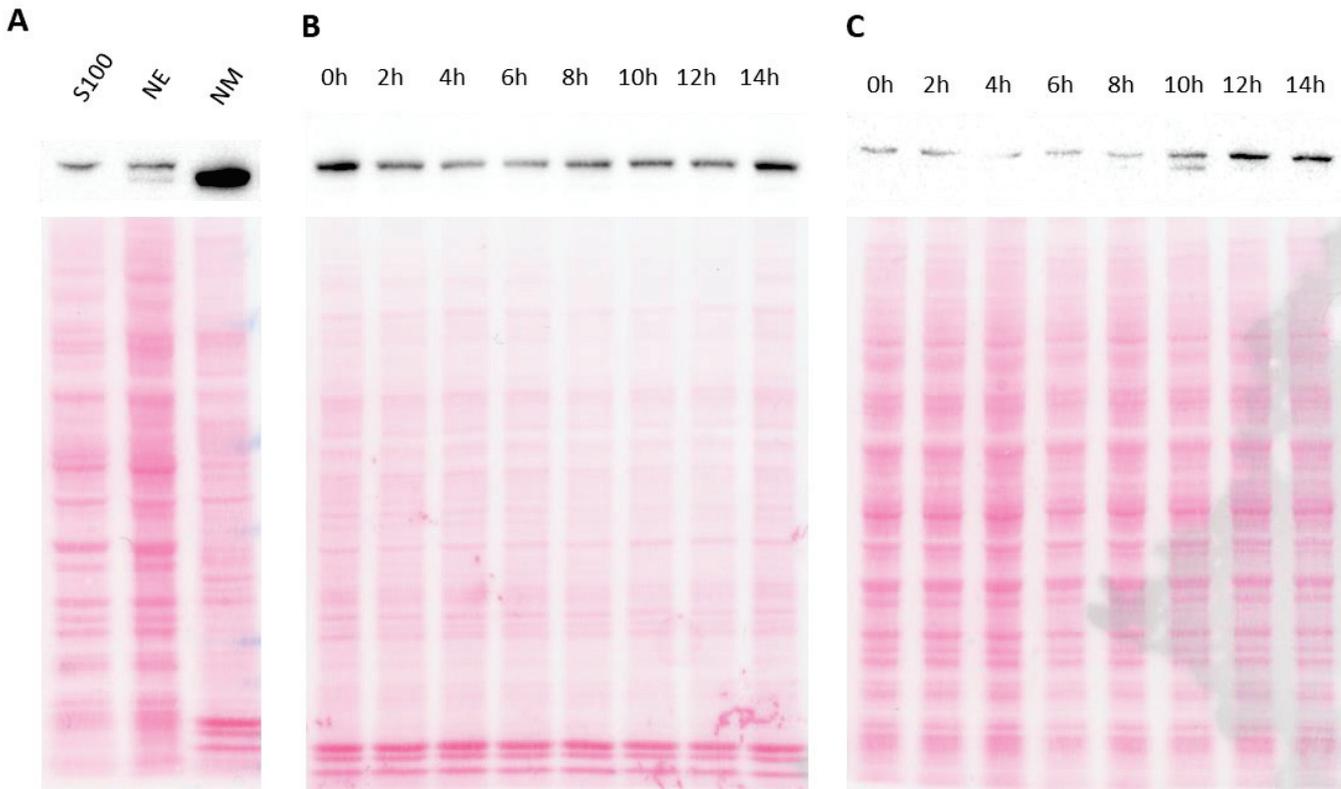
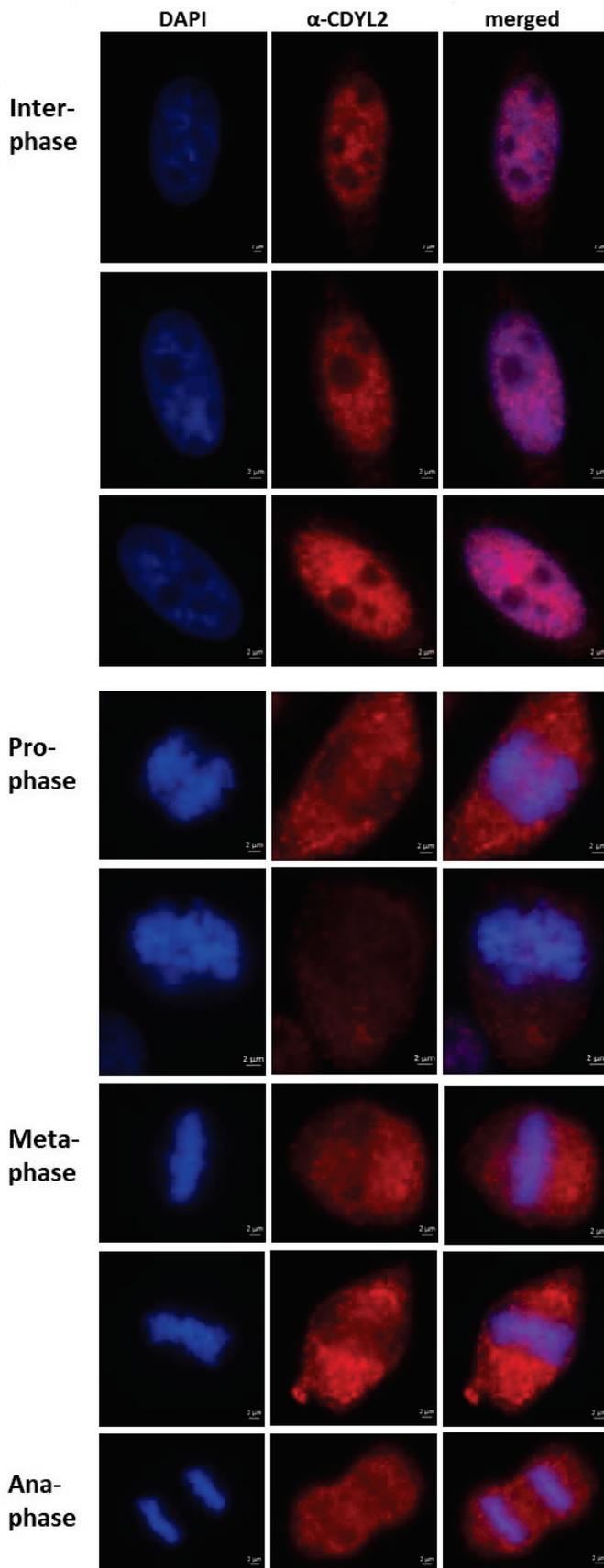


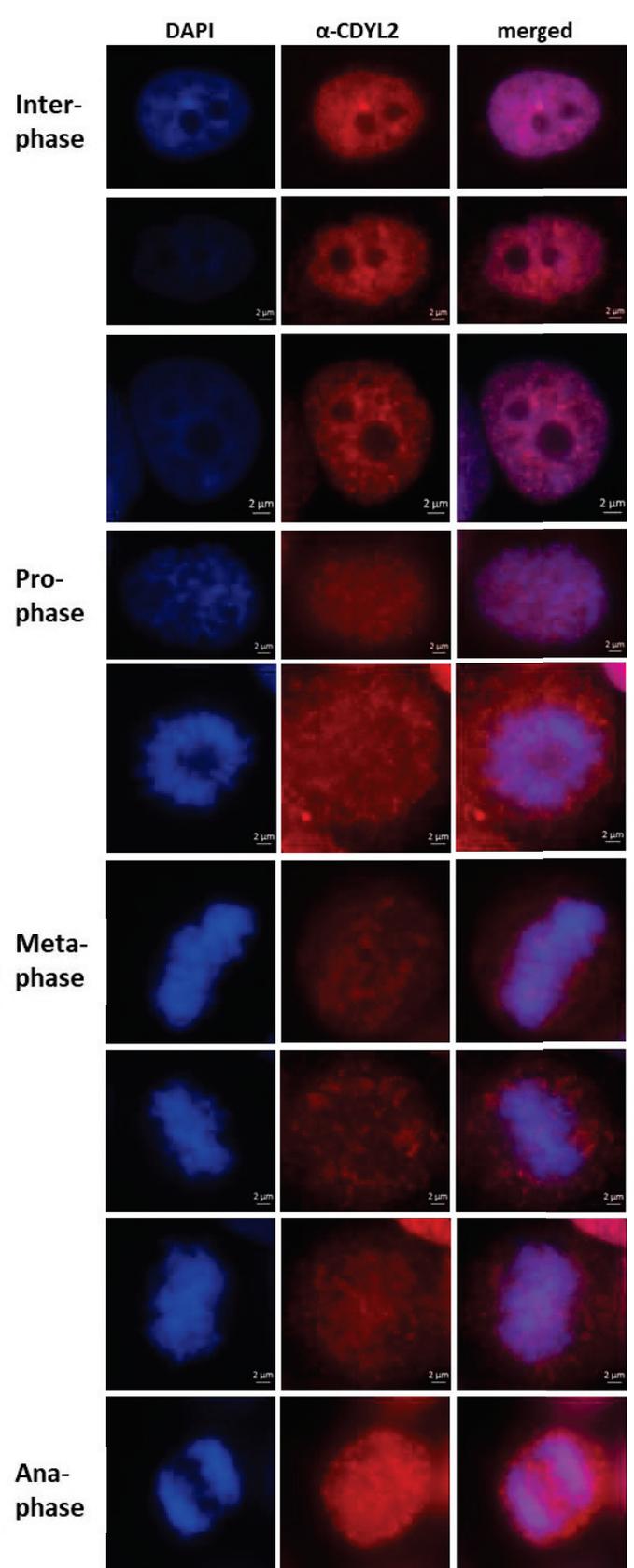
Fig. 14: CDYL2 level is higher on the chromatin during G1 and in the nuclear extract just after mitosis. (A) CDYL2 is mostly enriched in the nuclear matrix fraction. (B) CDYL2 level in the nuclear matrix is slightly higher in G1 phases compared to the rest of the cycle, while (C) in the nuclear extract its level increases right after mitosis. NE = Nuclear Extract; NM = Nuclear Matrix; experiment was performed twice and representative images are shown.

using pre-extraction techniques, releasing chromatin unbound proteins from the cell, I observed that a fraction of CDYL2 proteins remains on the chromatin throughout mitosis in the MCF7 cell line (Fig. 15D). This observation is consistent with observations made by Dr. Maha Siouda in non-published results using MCF10A cell line. In the HeLa cell line, on the other hand, CDYL2 could not be detected (Fig. 15C). The difference might come from HeLa sensitivity to the CSK pre-extraction technique. Indeed, in my hands, HeLa cells were much more rapidly affected by the pre-extraction. Limiting the extraction time as I did, might not be enough to protect CDYL2 localization on the chromatin during mitosis. Taken together, CDYL2 levels during the cell cycle are not dramatically changing as it is the case for Cyclin proteins, indicating that CDYL2 is probably not a simple collaborator, regulator or target of Cyclin proteins, but is rather part of a more complex mechanism.

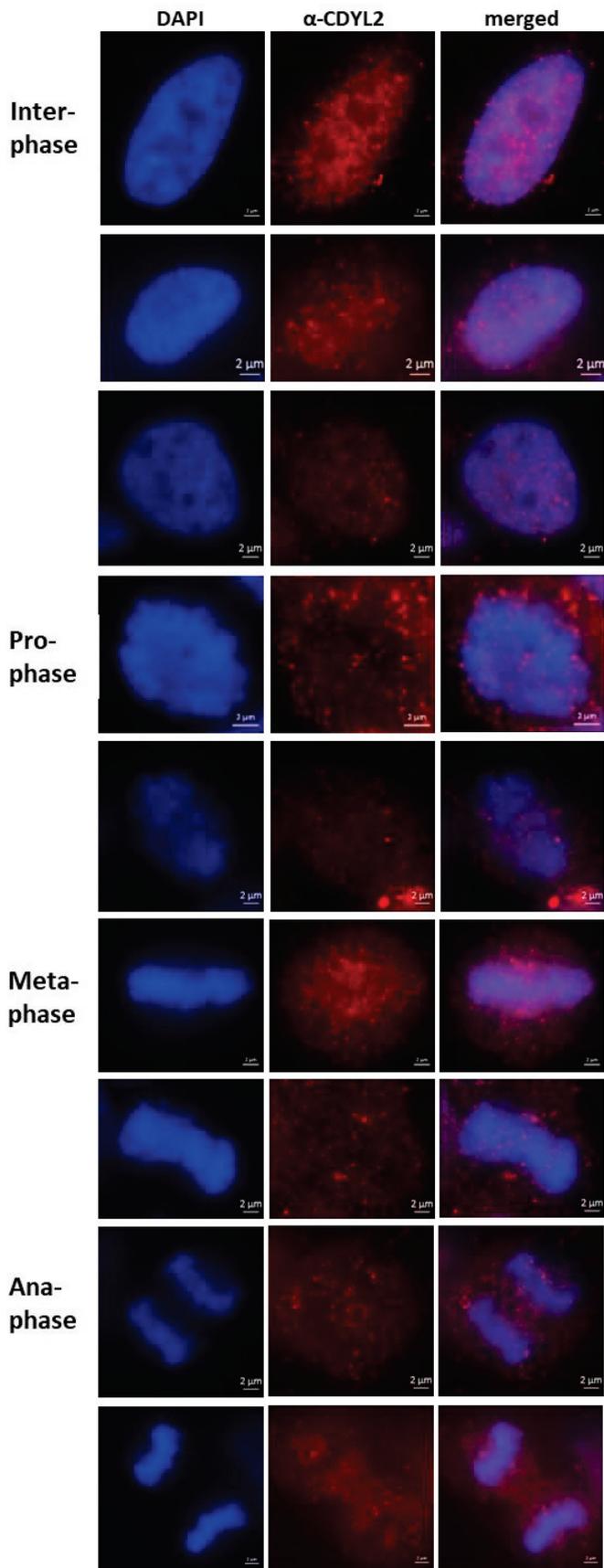
A HeLa without pre-extraction



B MCF7 without pre-extraction



C HeLa after pre-extraction



D MCF7 after pre-extraction

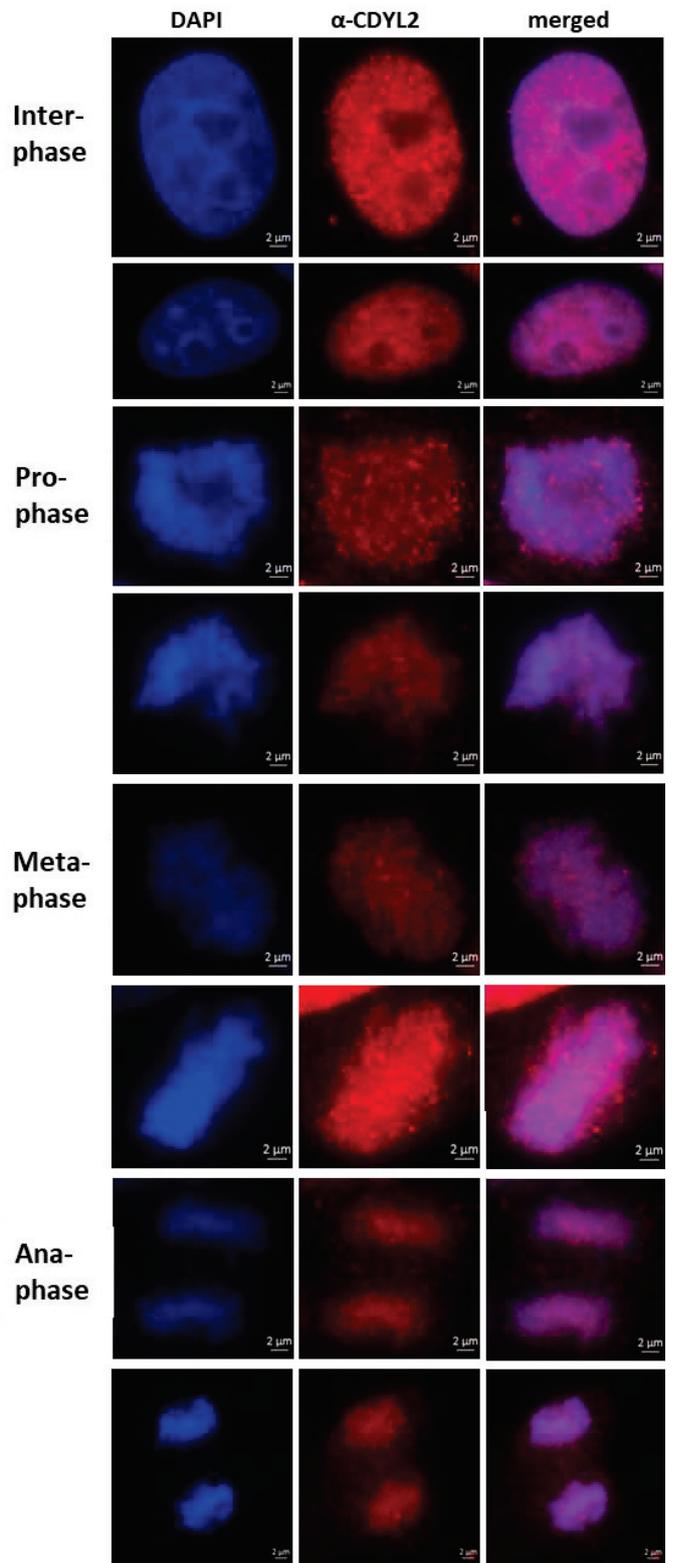
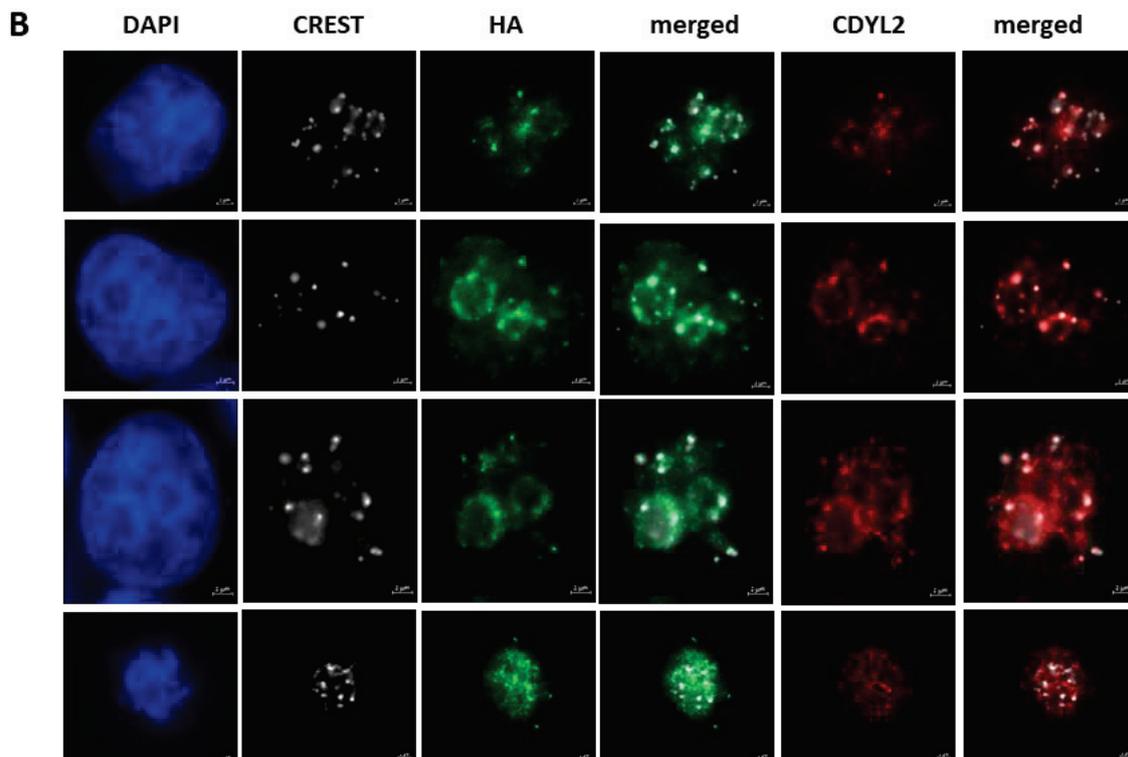
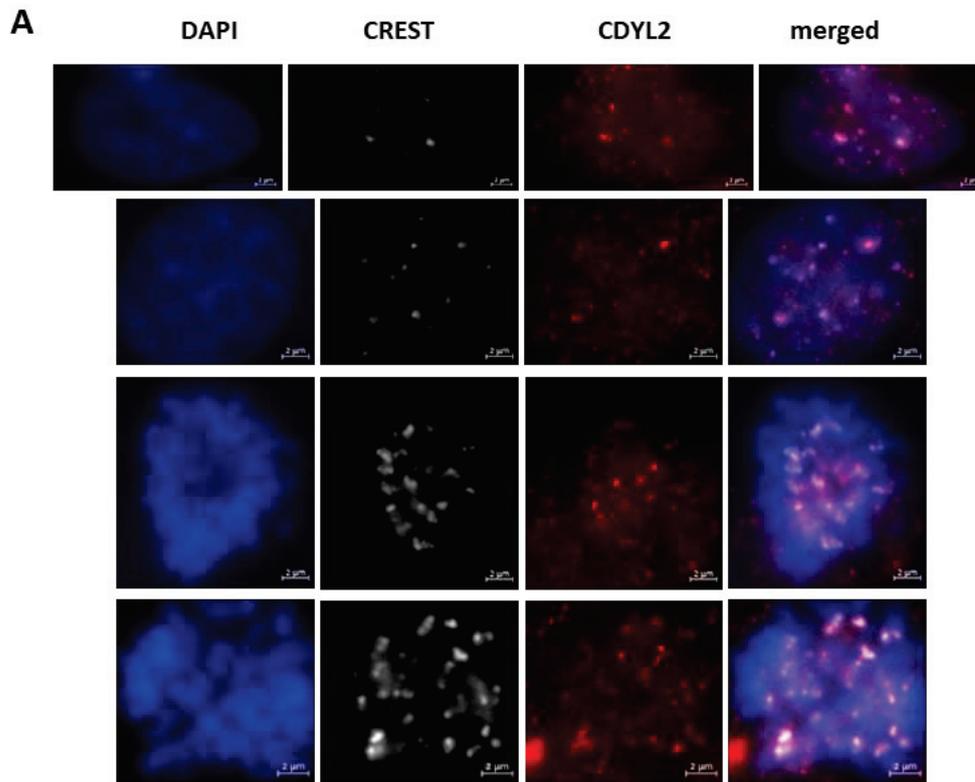


Fig. 15: During mitosis, only a small portion of CDYL2 remains on chromatin. (A) In HeLa and (B) MCF7 cells, CDYL2 is strongly enriched in the nucleus, most CDYL2 is released from chromatin during mitosis. (C,D) After CSK buffer pre-extraction, we observe that a small fraction of CDYL2 remains on the chromosomes during mitosis in MCF7 cells but not in HeLa. Experiments were performed three times, representative images are shown.

8.2 CDYL2 LOCALIZES AT PERICENTROMERES IN A H3K9ME3 DEPENDENT MANNER

Due to its chromodomain recognizing H3K9me3 as already described *in vitro* (Fischle *et al.*, 2008), CDYL2 is expected to bind to heterochromatin, which is notably enriched at centromeric, pericentromeric and telomeric chromatin. Interactions between chromodomain proteins and pericentromeres have already been described (Fischle *et al.*, 2008; Minc *et al.*, 1999), I wanted to look if this is also the case for CDYL2 in human cells. Using immunofluorescence co-staining against CDYL2 and CREST, a centromere antiserum, I found that CDYL2 is systematically localized near centromeres in interphase cells (Fig. 16A). This colocalization is even more striking using Flag-HA-tagged CDYL2 constructs, as can be seen in figure 16B, and remains after pre-extraction of cells with Triton X-100 before their fixation and staining (Fig. 16C), suggesting a strong interaction. As CDYL2 is implicated in mitosis, I wanted to analyze its colocalization with pericentromeres during this phase. In endogenous and overexpressing cells before pre-extraction, colocalization of CDYL2 with CREST signal was maintained, even though to a lower extent than in interphase cells. However, after CSK pre-extraction, I could not by using standard immunofluorescence techniques, observe a convincing enrichment of CDYL2 at pericentromeres during the different mitotic stages (Fig. 16C). On the opposite, it seems that the CDYL2 signal gets more diffuse around the chromatin, suggesting that CDYL2 binding to pericentromeric heterochromatin is at least weakened, if not lost during mitosis. This loss of interaction observed is consistent with the observation that *in vitro* H3S10ph, a marker of mitosis blocks CDYL2 chromodomain interaction with H3K9me3 peptides (Fischle *et al.*, 2008). I see here three possible hypotheses: either CDYL2 is regulating proteins involved in mitosis prior to the nuclear envelope breakdown, the fraction of CDYL2 remaining at the chromatin is enough for its function, or, like for chromosome cohesion and HP1, its function is independent of its chromatin binding

affinity (Kiyomitsu *et al.*, 2010). Still, as in my previous observations, CDYL2 remains diffusely localized on the chromatin.



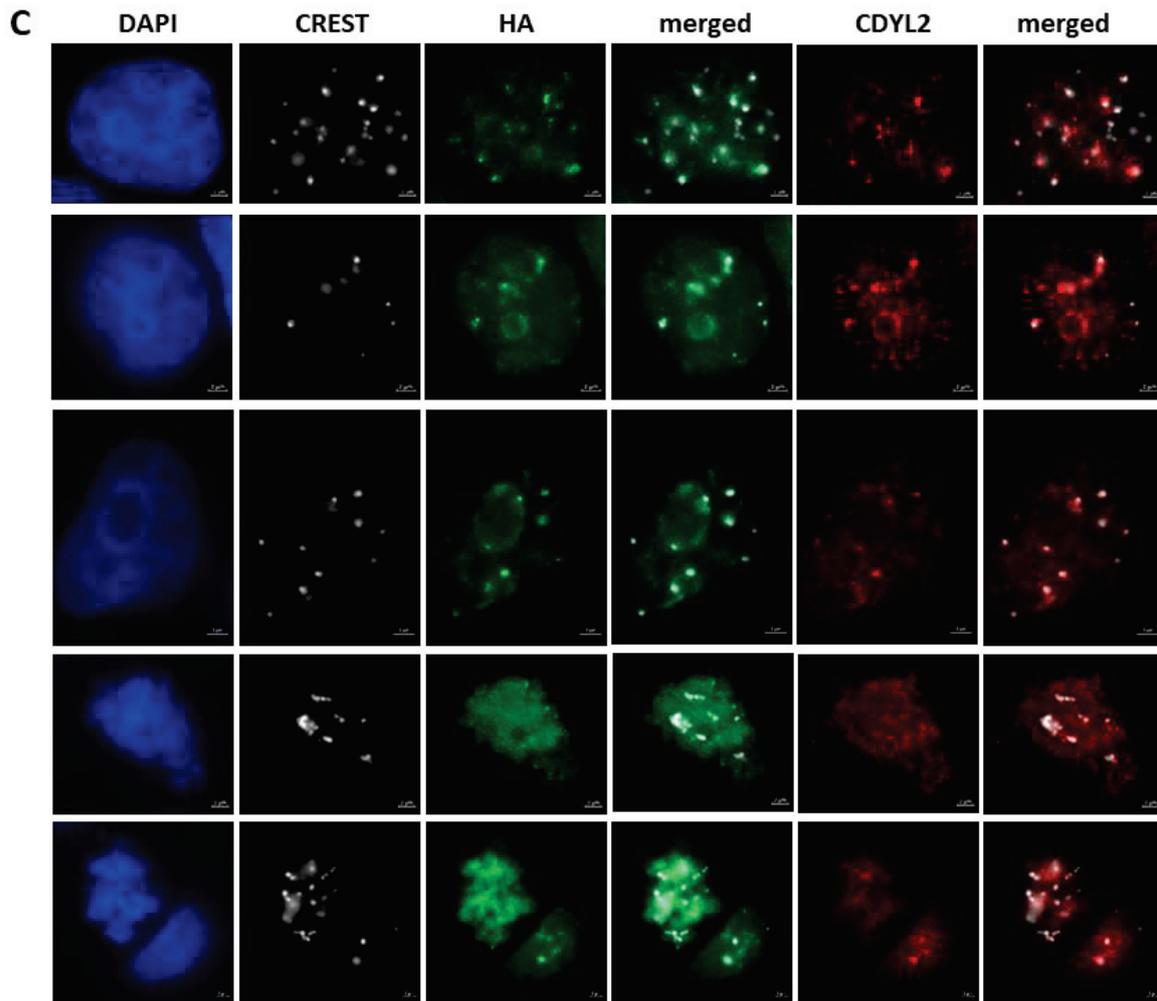


Fig. 16: CDYL2 colocalises with pericentromeres in MCF7 cells during interphase and partially during mitosis. (A) Endogenous CDYL2 in MCF7 cells localizes next to CREST proteins both in interphase and mitotic cells. (B) In cells overexpressing CDYL2 colocalisation of CDYL2 and CREST is always visible in interphase cells but is lost during mitosis. (C) When cells were treated with CSK prior to immunostaining, the same pattern was observed during interphase, but CDYL2 gave a diffuse signal in mitotic cells. Each panel is a representative experiment from a triplicate.

To further confirm CDYL2 localization at pericentromeres I performed chromatin immunoprecipitation followed by real-time PCR (ChIP-qPCR) on satellite sequences, found enriched at centromeres and pericentromeres. I used two primers pairs targeting α -satellite and satellite II repeats, specific for centromere and pericentromeres respectively. Both primers sets are commercially available. While primers against satellite II are specific to chromosome 1, the primers targeting α -Satellite are general to all chromosomes. I observed a strong increase of CDYL2 at those sequences compared to the negative control immunoprecipitation (Fig. 17). This increase was specific to the

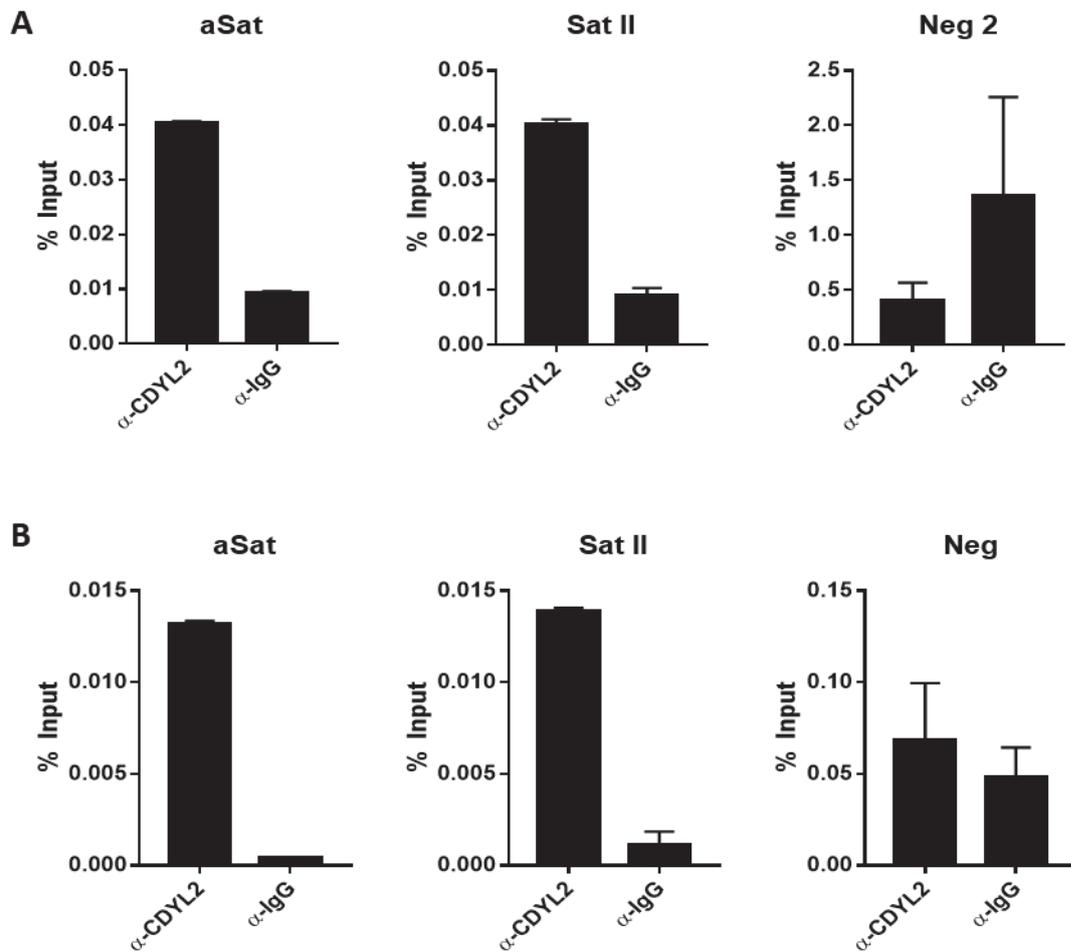


Fig. 17: CDYL2 localization on pericentromeric heterochromatin was confirmed using ChIP-qPCR. ChIP-qPCR analysis on satellite repeats confirmed CDYL2 enrichment on pericentromeres in (A) MCF7 and (B) HeLa cell lines. An unrelated genomic sequence was taken as a negative control. Samples were run as duplicates, experiment was performed twice. Shown are representative images. The standard deviations are represented as error bars.

satellite sequence, as it could not be observed in a random negative sequence. This observation thus confirmed that CDYL2 is enriched around pericentromeres both in HeLa and MCF7 cell lines.

To assess if CDYL2 is recruited to the pericentromeric regions through H3K9me3 modification, we obtained previously published mouse embryonic fibroblast (MEF) cells double knocked-out for the H3K9 trimethyltransferases *Suv39h1* and *Suv39h2*. These cells were already described to lack H3K9me3 modification at pericentromeres (Peters *et al.*, 2001). As these MEF cells expressed only a very low amount of CDYL2, we overexpressed CDYL2 protein using retrovirus and stained the cells using immunofluorescence. Similar to

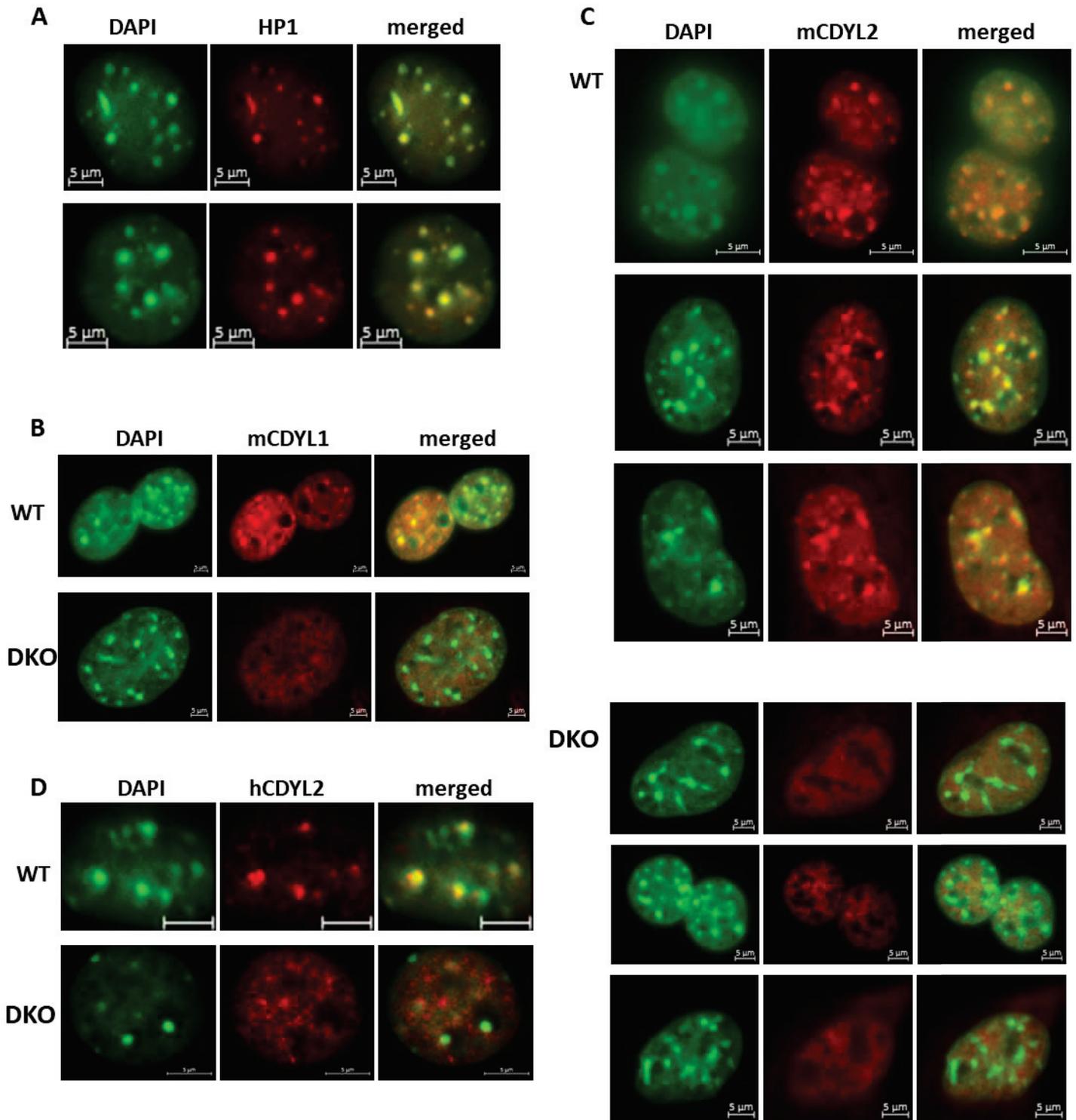


Fig. 18: CDYL2 localization on pericentromeres is H3K9me3 dependent. (A) HP1 and (B) mCDYL1 were used as positive controls and were found to colocalize with chromocenters, marked by a brighter DAPI staining. (C) mCDYL2 was found to be able to colocalize with chromocenters in wild-type (WT) cells. The colocalization could not be found in any cells double knock-out (DKO) against SUV39H1 and SUV39H2, where H3K9me3 at pericentromeres is lost. (D) Using hCDYL2, colocalization could also be observed in WT cells but never in DKO. Experiments were repeated three times with similar results. Shown are representative images

previous experiments performed (Fischle *et al.*, 2008), pericentromeres were marked with DAPI, as mice pericentromeric regions are AT-rich, thus having more affinity to DAPI and are stained brighter (Maison and Almouzni, 2004). To confirm that the assay is working in our hands, we used HP1 and CDYL1 as positive controls and observed a systematic and partial colocalization respectively (Fig. 18A and B) as previously described (Fischle *et al.*, 2008; Minc *et al.*, 1999). Interestingly, while in the wild-type cells mouse CDYL2 showed colocalization with pericentromeric satellite marked by DAPI, we could never observe a knock-out cell showing this colocalization. To the opposite, in knock-out cells, CDYL2 had a very diffuse pattern in the nucleus (Fig. 18C). The same pattern could be observed when expressing human flag-tagged CDYL2, confirming the localization conservation between humans and mice (Fig. 18D). Taken together, these data strongly suggests that CDYL2 localization at pericentromeres is dependent on H3K9me3 modification.

8.3 CDYL2 LOCALIZES AT CENTROSOMES THROUGHOUT INTERPHASE AND MITOSIS

When performing the fluorescence immunostaining against CDYL2 after cytoskeleton pre-extraction, I and Dr. Maha Siouda often observed one or two bright dots, just outside the nucleus or next to the chromatin, where centrosomes should be. As centrosomes are important organizational centers of the mitotic spindles and necessary for chromosome alignment, I wanted to confirm if CDYL2 is localizing at centrosomes. For this, both HeLa and MCF7 cells were immunostained for pericentrin, a marker of centrosomes, and CDYL2. Systematically, both at interphase and during mitosis, CDYL2 staining was enriched at the regions marked by pericentrin, indicating localization of CDYL2 at the centrosomes (Fig. 19). During mitosis, CDYL2 was enriched in the same way in the two opposed centrosomes (Fig. 20). As can be seen, this signal strongly decreased upon CDYL2 RNAi transfection (Fig. 19), confirming the specificity of our antibody and this colocalization.

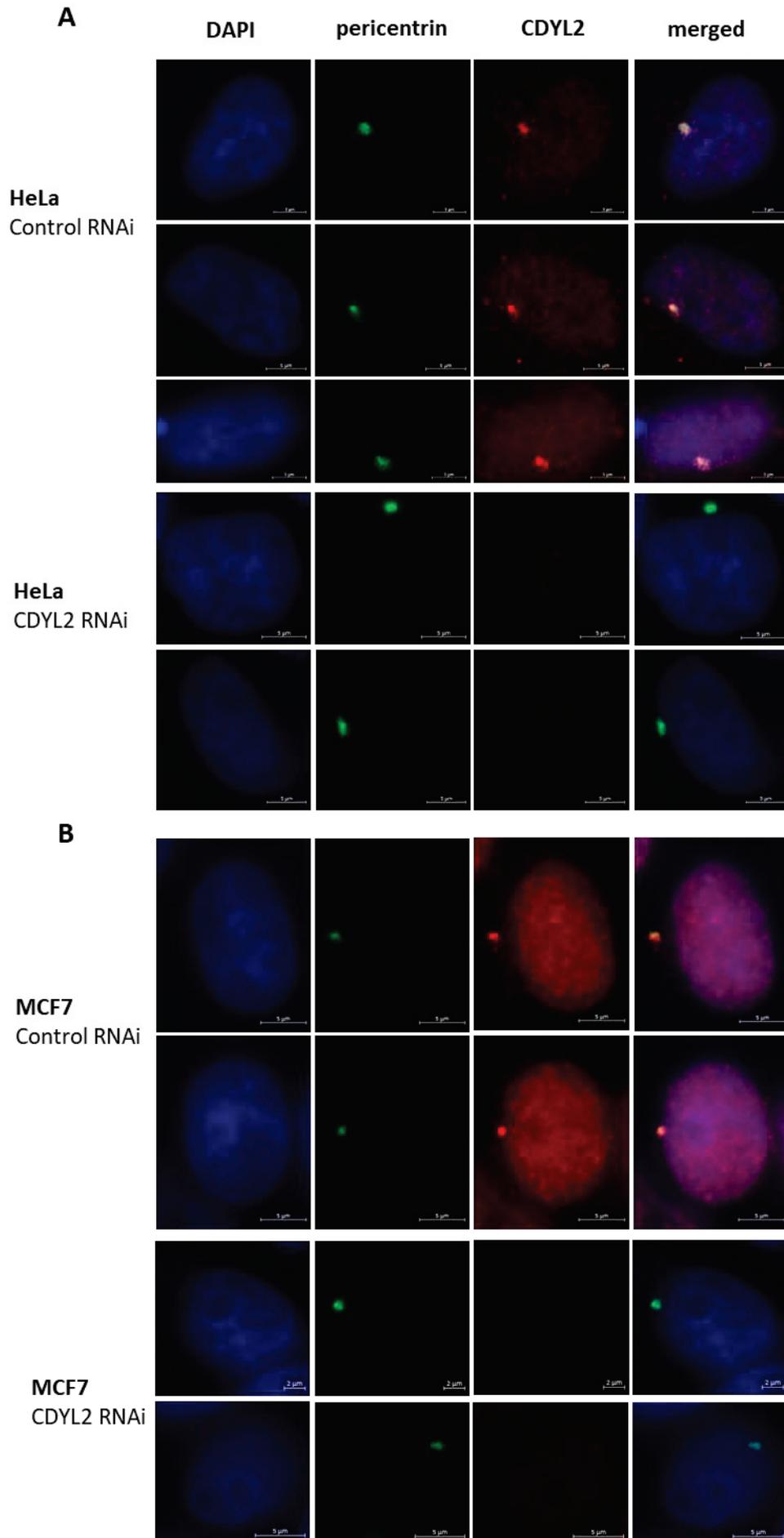


Fig. 19: CDYL2 is localised at centrosomes. CDYL2 colocalises with pericentrin in (A) HeLa and (B) MCF7 cells during interphase. CDYL2 signal is lost after CDYL2 RNAi transfection, confirming specificity of the signal. Experiment was performed once.

I took the opportunity of the cells previously transfected with CDYL2 RNAi and stained for CDYL2 and pericentrin to briefly investigate if CDYL2 knock-down would induce pericentrin defects, as chromosome misalignments observed in figure 11 could also be explained with centrosomes defects. In MCF7 cells knocked down for CDYL2, I could not observe any gross aberration in centrosomes number or position, even in cells with alignment defects. In HeLa, mitotic cells showing chromosome alignment problems revealed an aberrant number of centrosomes. However, in interphase cells, I did not observe cells with more than two centrosomes. Due to the absence of pericentrin phenotypes in interphase HeLa cells and MCF7 cells, I therefore speculated that the aberrant number of centrosomes is a consequence of the genomic instability induced by CDYL2 knock-down, rather than the mechanism by which the genomic instability is happening.

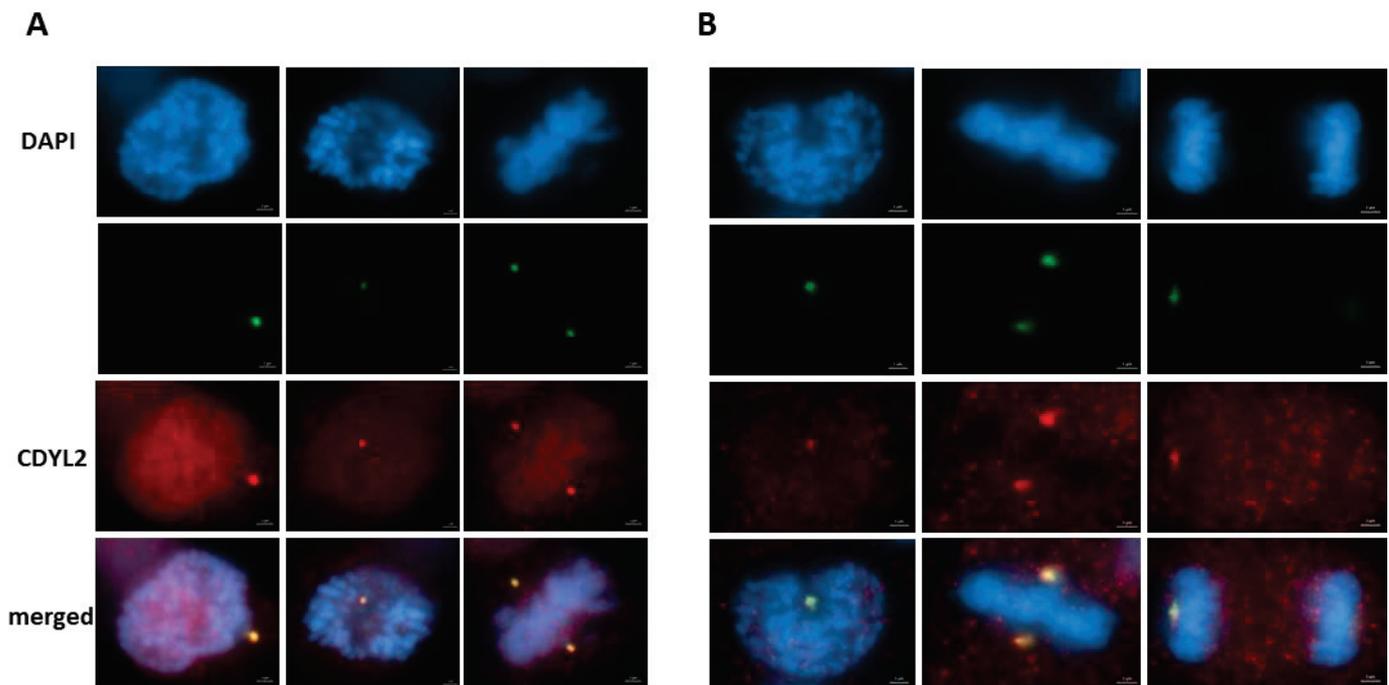


Fig. 20: CDYL2 is localised at centrosomes during mitosis. CDYL2 colocalises with pericentrin throughout mitosis in both HeLa and MCF7 cells. Experiments was performed twice and representative images are shown.

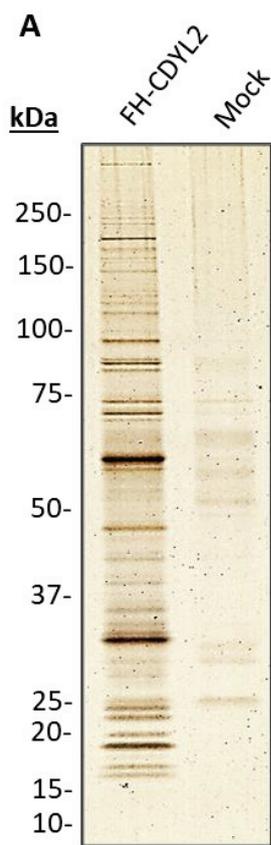
8.4 CDYL2 INTERACTOME REVEALS MANY INTERACTORS INVOLVED IN GENOMIC STABILITY AND MITOSIS

To start elucidating the mechanism behind CDYL2 regulation of mitosis, epitope CDYL2 was immunopurified and interaction partners identified by mass spectrometry. The protein lists obtained after mass spectrometry analysis were first classified using unique peptide numbers. However, this classification system is biased towards larger proteins, which can yield more unique peptides than smaller ones. For instance, despite being the bait, CDYL2 itself was never ranked in the top proteins, ranking behind higher molecular weight proteins. I, therefore, looked for a more quantitative classification. It appeared that it would not be possible to realize a perfectly quantitative ranking with the mass spectrometry protocol that was used. However, the intensity of the detected signal in mass spectrograms correlates to the total number of peptides detected, instead of the quantity of different peptides detected. The peak intensity, therefore, relates more closely to the number of proteins available in the sample. Using a ranking with the sum of the spectral peaks' intensities, brought CDYL2 as the most abundant protein in all the coimmunoprecipitation samples, confirming this ranking technique as being more adapted. The gel was silver-stained before sending it to the mass spectrometry facility to confirm the specificity of protein enrichments in the immunoprecipitation (Fig. 21A). Additionally, to avoid any false-positive results, only proteins that showed at least a 3-fold peptide enrichment in the CDYL2 IP compared to the negative control coimmunoprecipitate were considered. The triplicates were compared and I found 85 proteins common in the three IP-MS analyses. Interestingly, CDYL1 and several of its already described interactors as EHMT2, SETDB1, or ATF7IP (Mulligan *et al.*, 2008) were found in the list. CDYL1 and CDYL2 may therefore interact together.

This list of proteins found enriched in all three immunoprecipitations were further analyzed using various databases. From the Gene Ontology (GO) database, I found that four GO gene sets were significantly enriched: proteins involved in chromosome organization, chromosome modification, RNA binding, and cell cycle process (Fig. 21B-C). Interactions with proteins involved in chromosome organization, modification, or cell cycle process could at least partially explain defects in mitosis and were in my thesis

context of high interest. Interesting partners already described to be involved in mitosis are notably the SMC proteins, directly involved in chromosome cohesion and condensation (Hirano, 2015), MCM proteins involved in DNA replication and needed for cohesion establishment (Zheng *et al.*, 2018), or EHMT2, an epigenetic factor linked with satellite repression (Fritsch *et al.*, 2010).

I additionally performed a STRING (Szklarczyk *et al.*, 2017) analysis on the list of common proteins to see if some targets are already known to interact together. Based on its database, STRING highlighted four complexes (Fig. 21D-G; Fig. 32). The first complex included KARS, MARS, EPRS, IARS, QARS, and ADAR, which are aminoacyl t-RNA ligases. Since their action takes mostly place outside the nucleus, these interactors seem unlikely to be relevant for CDYL2 function on chromosome alignment during mitosis and the interactions were not further studied during my thesis. A second complex contained nucleopore proteins as NUP107 or NUP160 and was not further analyzed neither, due to time limitations. I also found a complex containing numerous epigenetic factors, like HDAC1, EHMT1, EHMT2, SETDB1, or CDYL1, known to be involved in gene repression (Fritsch *et al.*, 2010; Hassig *et al.*, 1998; Keniry *et al.*, 2016; Liu *et al.*, 2015; Mulligan *et al.*, 2008; Tachibana *et al.*, 2002). Interestingly, SETDB1 was described to be involved in major satellites and retrotransposons silencing through H3K9 trimethylation (Cruz-Tapias *et al.*, 2019). Additionally, SETDB1, EHMT1, and EHMT2 were described to coexist in a megacomplex that localizes at satellite promoters (Fritsch *et al.*, 2010). EHMT2 knock-down induced changes in the methylation profile at these promoters, suggesting it might be involved in satellite repeat repression (Fritsch *et al.*, 2010). The fourth complex contained SMC3 and Rad21 and SMC2, SMC4 and NCAPG, proteins from the cohesin and condensin complex, respectively (Hirano, 2015). Cohesin and condensin are the two main complexes responsible for mitotic chromosome structural transitions and are linked with both chromosome alignment and chromosome formation defects (Dej *et al.*, 2004; Hirano, 2015; Toyoda and Yanagida, 2006), raising as interesting potential interactors of CDYL2 to explain its function in mitosis control. Finally, I also noticed the presence of both POGZ and CHAMP1, two proteins interacting together and who have been described to be involved in genomic stability, notably by playing a role in kinetochore attachment to



B

GO Set Name [#Genes (K)]	Genes in Overlap (k)	k/K	p-value	q-value
Chromosome Organisation (1223)	40	0.033	2.83E-37	4.46E-33
RNA binding (1901)	33	0.017	1.34E-21	4.22E-18
Cell Cycle Process (1399)	26	0.019	1.45E-17	2.54E-14
Covalent Chromatin Modification (473)	17	0.036	3.11E-16	3.06E-13

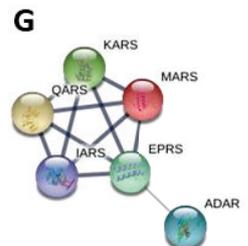
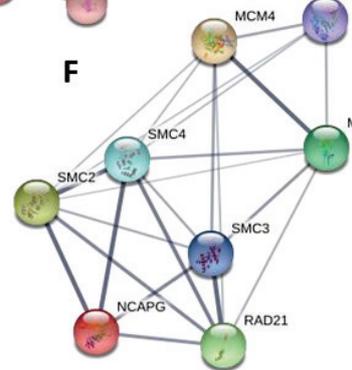
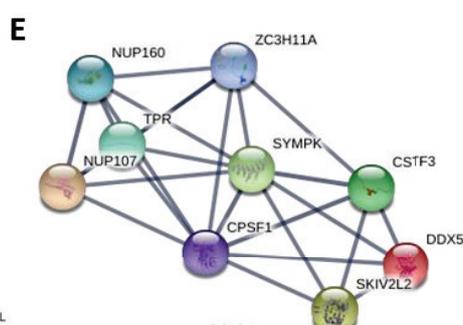
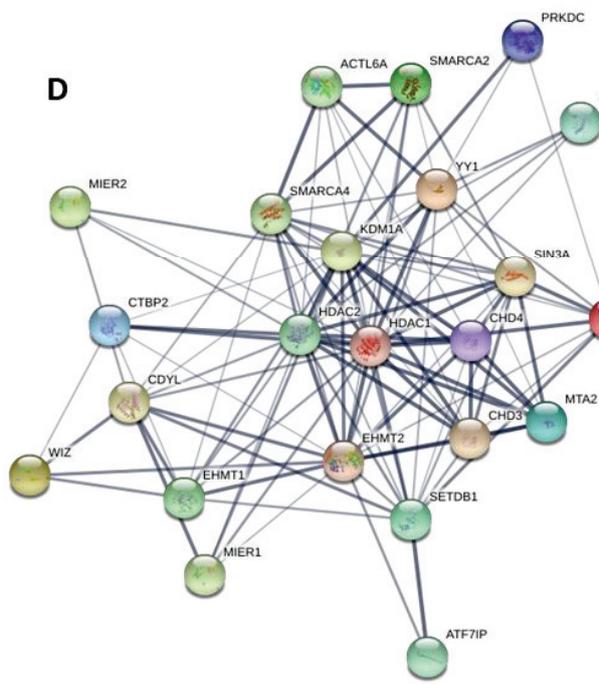
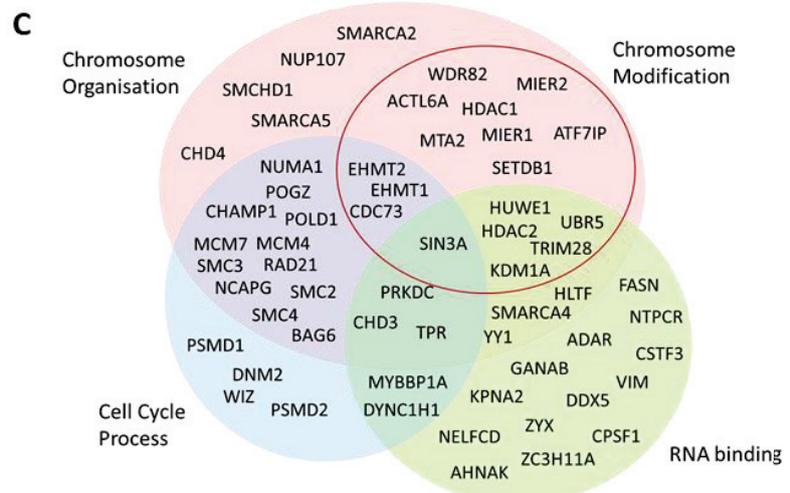


Fig. 21 : Characterisation of CDYL2 interactome by Mass Spectrometry reveals many proteins involved in mitosis and chromosome organisation. (A) Coimmunoprecipitation lysate of CDYL2 and negative control were separated on a SDS gel and the gel silver stained. (B,C) The proteins found in all three coimmunoprecipitations are involved in RNA binding, chromosome modification and organisation and cell cycle process. (D to G) Four big complexes were found in the proteins interacting with CDYL2, including SMC complexes and epigenetic repressive complexes.

microtubules (Itoh *et al.*, 2011; Okamoto *et al.*, 2017). In conclusion, various potential interactors of CDYL2 could explain its importance for mitosis.

8.5 HYPOTHETIC MECHANISMS DESCRIPTIONS

From my characterization of CDYL2 interactors and the analysis of its regulation and localization during the cell cycle, I studied three hypotheses during my thesis to explain CDYL2 necessity for mitosis.

1. CDYL2 could be responsible for satellite repeat sequence transcriptional repression:

CDYL2 localizes at pericentromeric regions and recruits epigenetic repressors as EHMT2 or SETDB1 by physically interacting with them. Interestingly, SETDB1 was already described to be involved in satellite sequence and other repeat elements repression, while EHMT2 was found necessary to repress retroviral repeat elements (Fritsch *et al.*, 2010). A knock-down of CDYL2 could, therefore, lead to aberrant expression of satellite repeats, which is known to induce genomic instability, centromere decondensation, and chromatid loss of cohesion (Zeller and Gasser, 2017).

2. CDYL2 might be involved in chromosome cohesion regulation: for this hypothesis, many mechanisms are possible. The most direct, given that proteins from the cohesin complex were found in the mass spectrometry analysis, is that CDYL2 could, like Swi6 in yeast (Nonaka *et al.*, 2002), promote cohesin recruitment to pericentromeric regions, by interacting with the complex. A knock-down of CDYL2 would then lead to a fragile sister chromatid cohesion and thus problems in chromosome alignment and defects in mitosis (Díaz-Martínez *et al.*, 2010).

3. CDYL2 might interact with CHAMP1 and be necessary for its function in regulating kinetochore-microtubules connection. A knock-down of CDYL2 would prevent CHAMP1

function in kinetochore formation and phenocopy CHAMP1 knock-down: failure of chromosome alignment, defects in kinetochore assembly, thus loss of connection between microtubules and centromeres and mimick the phenotype observed after CHAMP1 knock-down, notably chromosomes misalignment during mitosis (Itoh *et al.*, 2011).

8.6 CDYL2 SEEMS DISPENSABLE FOR SATELLITE REPRESSION

In previous studies (Benaissa, 2018; Siouda *et al.*, 2020), CDYL2 was found to have repressive activities. EHMT2 was found in my mass spectrometry studies to interact with CDYL2 (Fig. 21C and D) and its interaction was already confirmed in MCF7 in our previous publication (Siouda *et al.*, 2020). I wanted to additionally confirm CDYL2 interaction with SETDB1 and DNMT1 and for this performed a coimmunoprecipitation in MCF7 cell lines overexpressing CDYL2. In parallel, a coimmunoprecipitation with a non-specific antibody

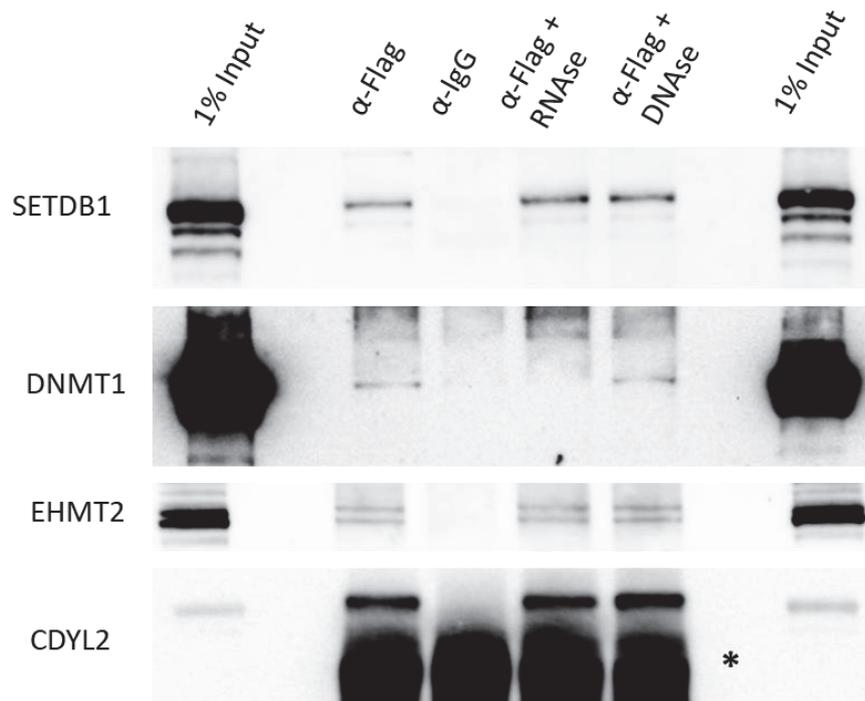


Fig. 22: CDYL2 interacts with EHMT2 and SETDB1 in a RNA and DNA independent manner. SETDB1, DNMT1 and EHMT2 were coprecipitated with Flag-tagged CDYL2 in MCF7 cells. All interactions remained after DNase treatment with the exception of interaction with DNMT1 which was lost after RNase treatment. *corresponds to the signal coming from the antibody denaturated heavy chain. Experiment was performed twice, representative images are shown.

was performed as a negative control. The obtained precipitates were immunoblotted and I could observe a specific interaction of CDYL2 with SETDB1 and DNMT1 (Fig. 22). To avoid artifact signaling that can arise through unwanted precipitation of DNA or RNA, coimmunoprecipitation on lysates treated with RNase or DNase was performed in parallel. EHMT2 and SETDB1 could still be detected confirming a physical interaction of CDYL2 with EHMT2 and SETDB1, independently of DNA or RNA presence (Fig. 22). Interaction between CDYL2 and DNMT1 was confirmed to be DNA independent, however, DNMT1 could not be detected in the precipitate treated with RNase, suggesting that RNA might be needed for CDYL2 interaction with DNMT1. Since SETDB1 and EHMT2 are necessary for satellite silencing and CDYL2 localizes at pericentromeres, I hypothesized that CDYL2 is implicated in recruiting the silencing factors to the pericentromeric heterochromatin and promotes satellite expression repression.

To verify whether satellite expression is controlled by CDYL2, I knocked down CDYL2 using RNAi and analyzed satellite messenger RNA levels using qPCR. I used two commercially available primers sets, targeting α -satellite and satellite II repeats, specific for centromere and pericentromeres respectively. While primers against satellite II are specific to chromosome 1, the primers targeting α -Satellite are general to all chromosomes. A transcription change in these sequences could not be observed after downregulation of CDYL2 (Fig. 23A and B). Using primers targeting the two open reading frames and the UTR regions of long interspersed nuclear element 1 (LINE1), the most abundant class of transposable elements in mammals (Yang and Wang, 2016), I also observed that a decrease of CDYL2 does not seem to influence retroviral element transcription. To strengthen my observations, I used Taqman probes designed by Zahn *et al.* in 2015, which target endogenous retroviral sequences K111 and K222 localized at the pericentromeric regions of respectively 9 and 15 chromosomes (Zahn *et al.*, 2015). K111 was also shown to be present on centromeric regions (Contreras-Galindo *et al.*, 2013). The linearity between the detected signal and the sample concentration was tested and confirmed for both probes using a dilution curve of the ChIP input samples (Fig. 24A-B). Centromeric and

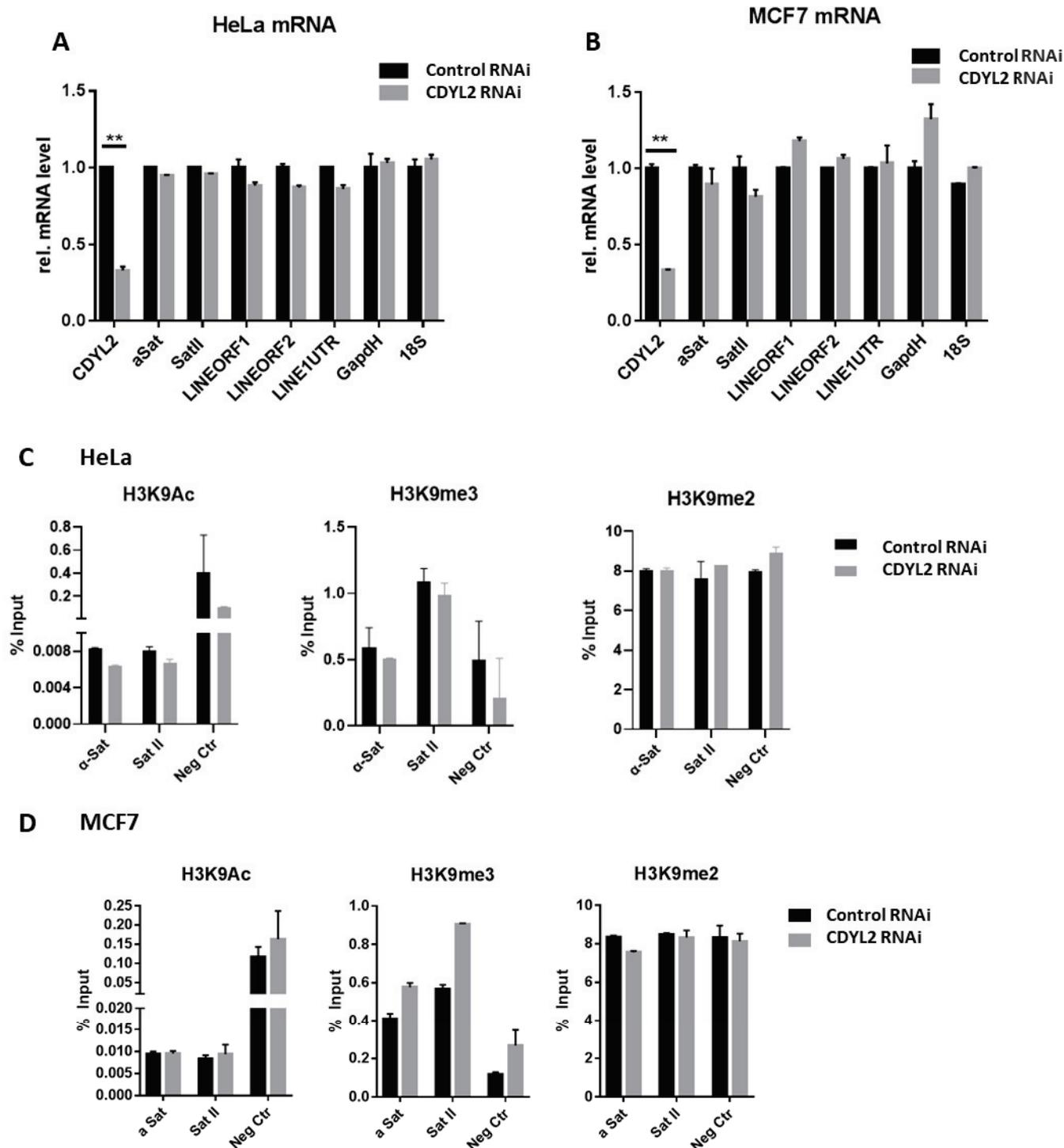


Fig. 23: CDYL2 seems dispensable for centromeric and pericentromeric Satellite repression. Satellite repeats mRNA are not found enriched in HeLa (A) and MCF7 (B) cells after knock-down of CDYL2. Experiments was performed thrice, representative results are shown. (C, D) ChIP on histone modifications and qPCR on satellite regions does not seem to reveal consistent and significant epigenetic changes at repetitive sequences in (C) HeLa and (D) MCF7. Experiments were performed twice, representative graphs are shown. Standard deviation between the sample replicates are represented as error bars.

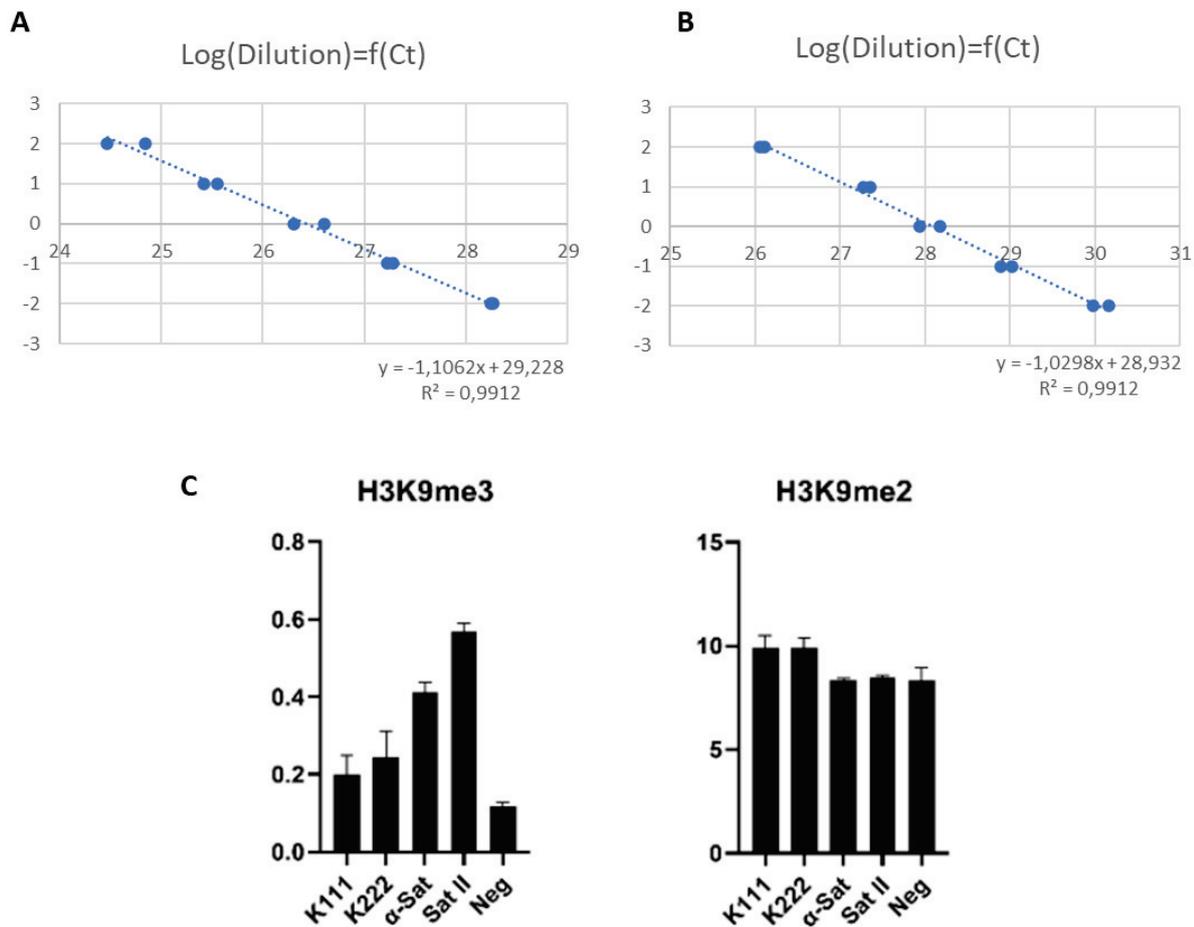


Fig. 24: TaqMan probes against K111 and K222 centromeric and pericentromeric repeat retroviral elements were validated in our hands. (A) K111 and (B) K222 linearity between the fluorescence signal of the probe and the initial concentration of the sequence was confirmed using a dilution sequence of input material from HeLa cells. Experiment was performed once. (C) K111 and K222 enrichment at pericentromeres and centromeres was confirmed with a ChIP against H3K9me3 and H3K9me2, respectively. In both cases, K111 and K222 were enriched compared to our negative control sequence. Experiments were performed twice. Representatives images are shown.

pericentromeric specificity was tested by us and Zahn and colleagues by performing ChIP on CENPA and H3K9me3. I observed that in my hands, K111 and K222 sequences are enriched after H3K9me3 ChIP in HeLa cells compared to a random negative sequence (Fig. 24C-D). However, a change in messenger RNA level after CDYL2 knock-down could again not be detected (Fig. 25A and B). In conclusion, it does not seem that CDYL2 is necessary for bulk repression of satellite sequences.

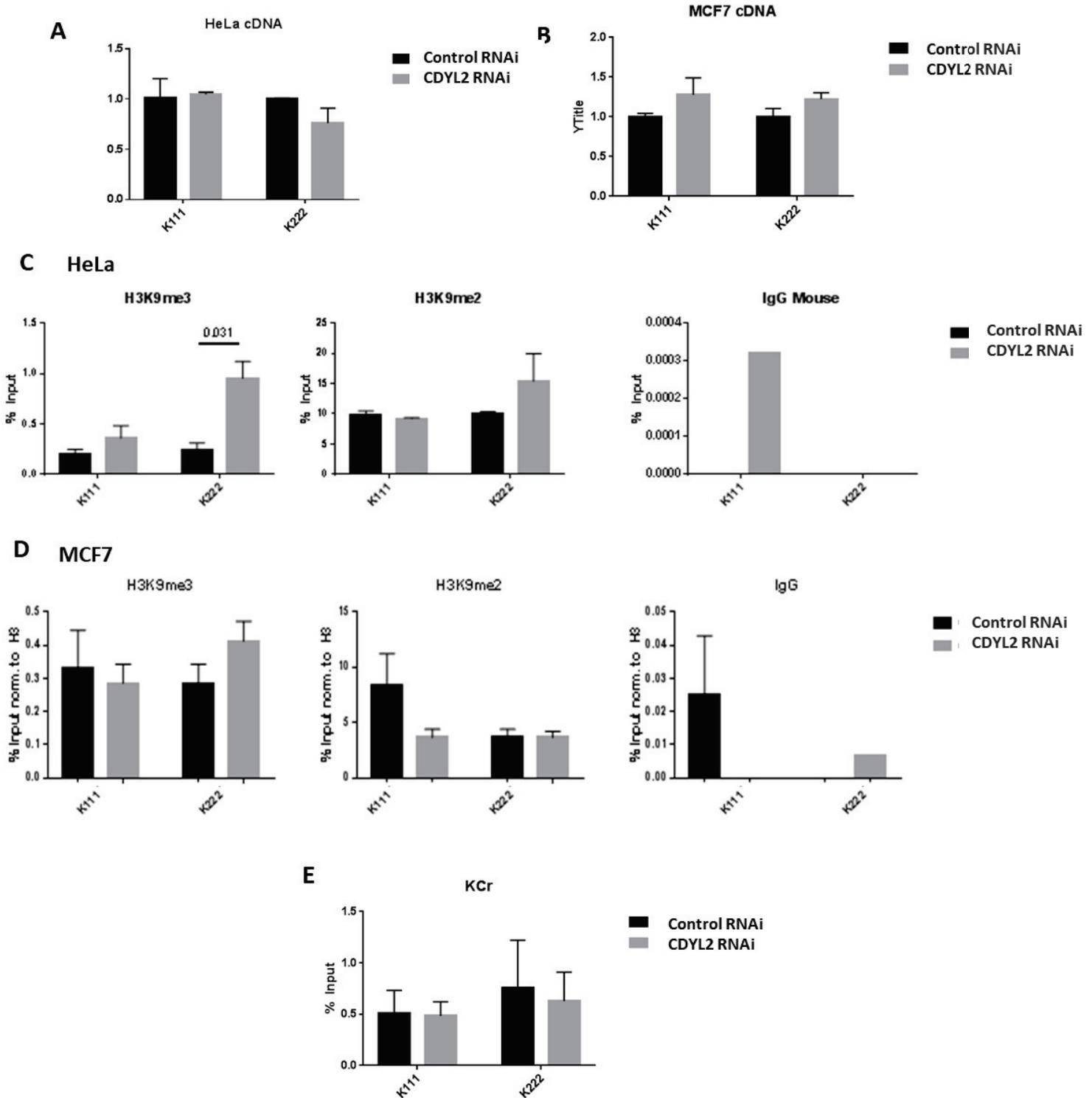


Fig. 25: CDYL2 seems dispensable for retroviral repeat elements K111 and K222. K111 and K222 repeats mRNA are not found enriched in HeLa (A) and MCF7 (B) cells after knock-down of CDYL2. (C, D) ChIP on histone modifications and qPCR on satellite regions does not seem to reveal consistent and significant epigenetic changes at repetitive sequences in (C) MCF7 and (D) HeLa. Experiments were performed twice, representative graphs are shown. Standard deviation between the sample replicates are represented as error bars.

Even though CDYL2 knock-down is not sufficient to promote repetitive sequences derepression, I asked if it can still induce histone modifications at these sequences and thus fragilize the silencing. Since CDYL2 was shown to induce epigenetic modifications at promoters by attracting EHMT2 to the promoters (Siouda *et al.*, 2020), I looked in particular for modifications on H3K9. I performed a ChIP assay against both H3K9 di- and trimethylation on HeLa and MCF7 cells treated either with Luciferase or CDYL2 RNAi. Disappointingly, I did not find any consistent or significant changes in H3K9 acetylation, di- and trimethylation in α -Satellite and Satellite II repeat regions (Fig. 23). Using TaqMan probes against K111 and K222, no reproducible increase of H3K9 methylation could be observed neither after CDYL2 RNAi infection (Fig. 25). Together, my data do not support a role for CDYL2 in pericentromeric satellite repeats repression, though due to the technical limitations of the PCR-based assays used this cannot be fully excluded.

8.7 CDYL2 INTERACTS WITH COHESIN BUT DOES NOT SEEM TO REGULATE ITS LEVEL AT PERICENTROMERES

A very interesting complex found in my mass spectrometry analysis is the SMC complex cohesin. One of the main functions of cohesin is to regulate chromosome cohesion, including at the pericentromeric regions during metaphase. As in *Schizosaccharomyces pombe*, Swi6, a chromodomain protein recognizing H3K9me3 was able to recruit cohesin to pericentromeric regions (Nonaka *et al.*, 2002), I hypothesized that CDYL2 might be involved in a similar mechanism in human. To confirm the mass spectrometry results, I first performed a CDYL2 immunoprecipitation followed by Western Blot. Non-specific antibodies were used in parallel as a negative control. I did observe an enrichment of RAD21 and PDS5B in the CDYL2 immunoprecipitate (Fig. 26A). Besides, using Proximity Ligation Assay (PLA), I wanted to observe if CDYL2 and cohesin colocalize in the cell. To test unspecific signaling from the primary antibodies, I performed a PLA with each antibody separately and observed no signal, suggesting that the antibodies are specific enough for this assay (Fig. 26B). As a positive control, I performed a PLA against SA1 and SMC1, two proteins interacting in cohesin, and obtained a strong signal in all cells. Finally, to ask whether cohesin and CDYL2 are colocalizing in the cell, I performed a PLA against CDYL2 and SA1. Interestingly, I could see a strong immunofluorescence signal in every cell

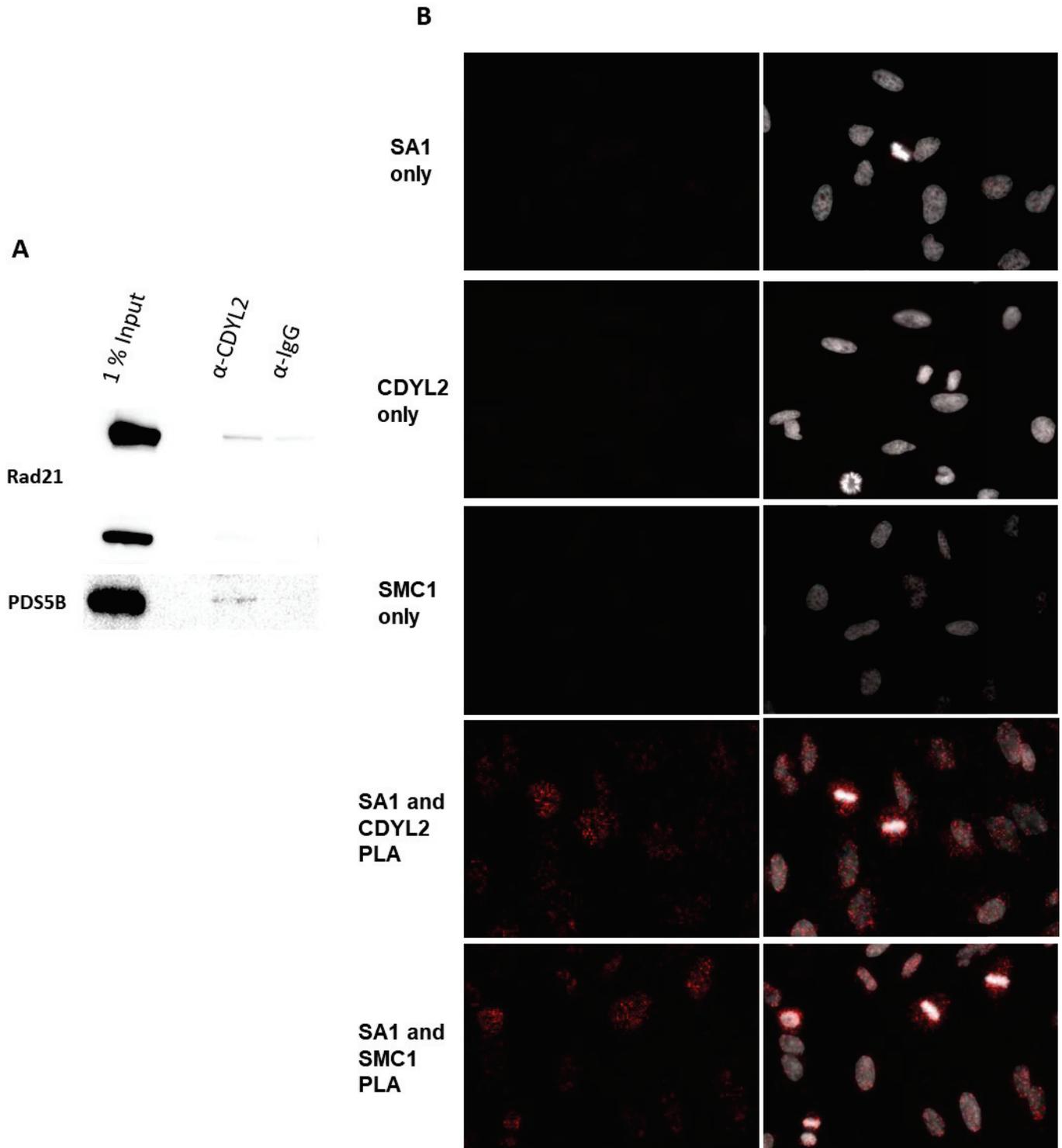
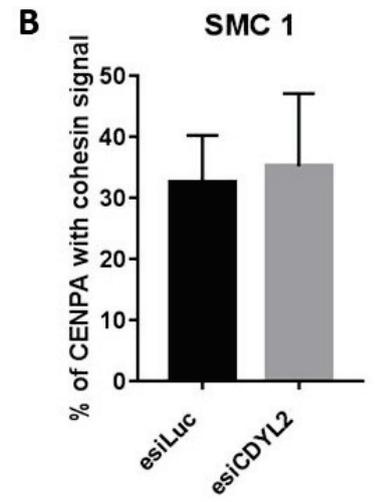
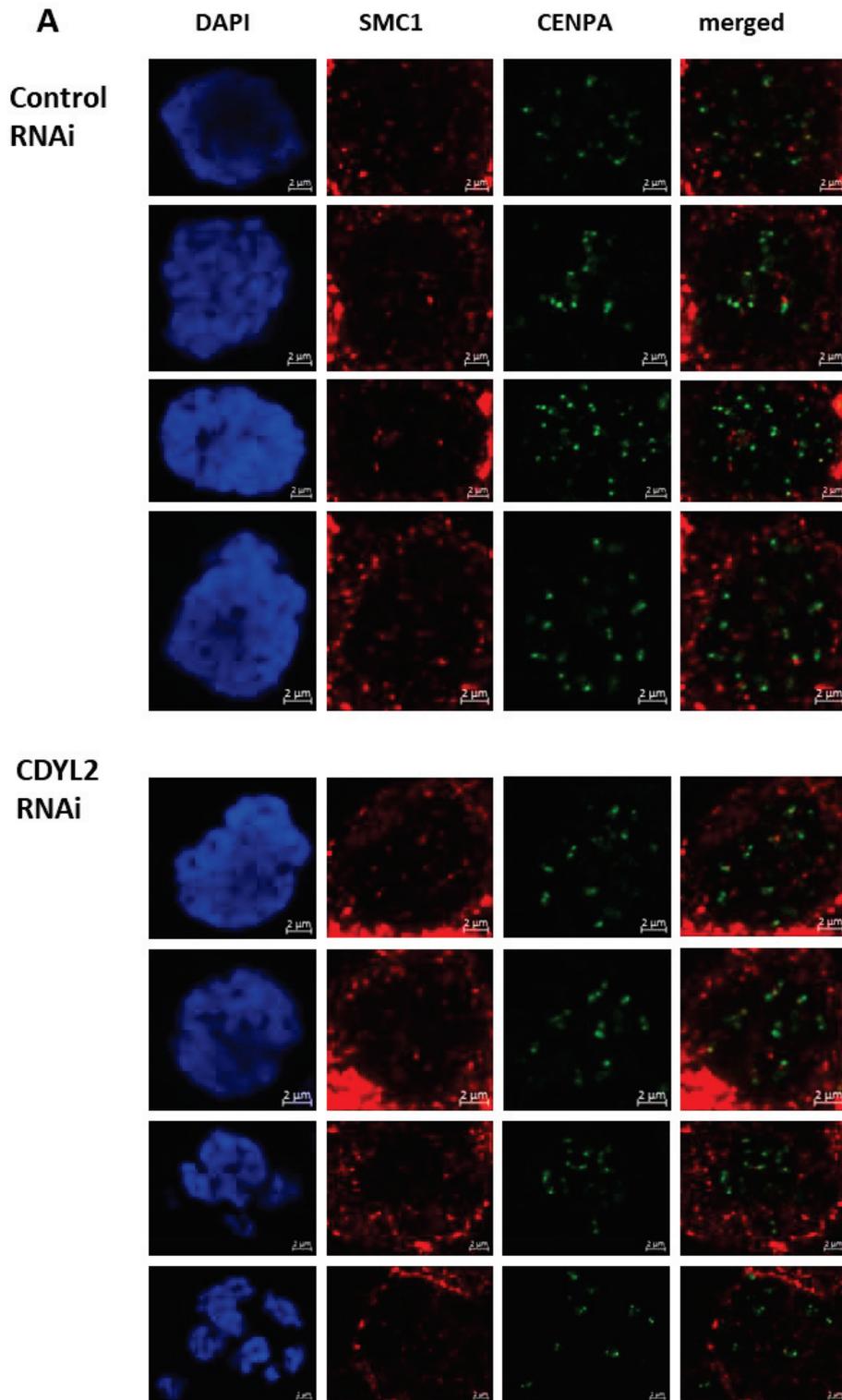


Fig. 26: CDYL2 seems to interact with Cohesin complex. (A) CDYL2 interaction with Rad21 and PDS5B was confirmed by coimmunoprecipitation followed by Western Blot analysis. Experiment was performed once. (B) Proximity Ligation Assay (PLA) confirmed a close proximity between SA1 and CDYL2. As a negative control, PLA was performed using each antibody individually and a PLA between SA1 and SMC1 was used as a positive control. Experiment was performed three times, representative images are shown.

observed, strongly suggesting that CDYL2 is often localized in the close neighborhood of cohesin (Fig. 26B). Interestingly, however, the proximity signal was not found only in the nucleus. Nor was the signal found where pericentromeres should be during mitosis. In fact, it seems that during metaphase the signal is rather enriched in the cytoplasm, consistent with the release of CDYL2 from the chromatin during mitosis observed in figures 15A-B and the pattern observed in SMC1 and SA1 PLA, but less consistent with my previous observation that CDYL2 remains, even though less tightly bound, on the chromatin during mitosis. Overall, my data strongly suggest a physical interaction between CDYL2 and the cohesin complex. However, whether CDYL2 is necessary for cohesin localization at pericentromeres remains to be elucidated.

To ask if lack of CDYL2 can induce changes in cohesin level at pericentromeres during metaphase, I performed SMC1 and SA1 immunofluorescence staining in cells knocked down for CDYL2, took images using confocal fluorescence, and quantified the CENPA signal colocalizing with cohesin, a protocol similar to what was done previously by Serrano *et al.* (Serrano *et al.*, 2009). The antibodies used to stain SMC1 and SA1 were commercial antibodies optimized for immunofluorescence and PLA. In my hands, they gave a strong nuclear signal. Numerous cells with aberrant chromosome alignment confirmed the efficiency of CDYL2 knocked-down. With immunofluorescence stainings, I could however not observe a significant decrease in cohesin localization to pericentromeres after CDYL2 knocked down with both antibodies, even in cells showing mitotic defects (Fig. 27). I did not observe a significant decrease of cohesin localization at pericentromeres during anaphase neither, suggesting a lack of adapted technique to observe cohesin loss of localization despite all my precautions, and making my data difficult to interpret. From all my data, it seems more than likely that CDYL2 and cohesin are interacting. However, I could not observe a specific decrease of cohesin at pericentromeres after CDYL2 knock-down.



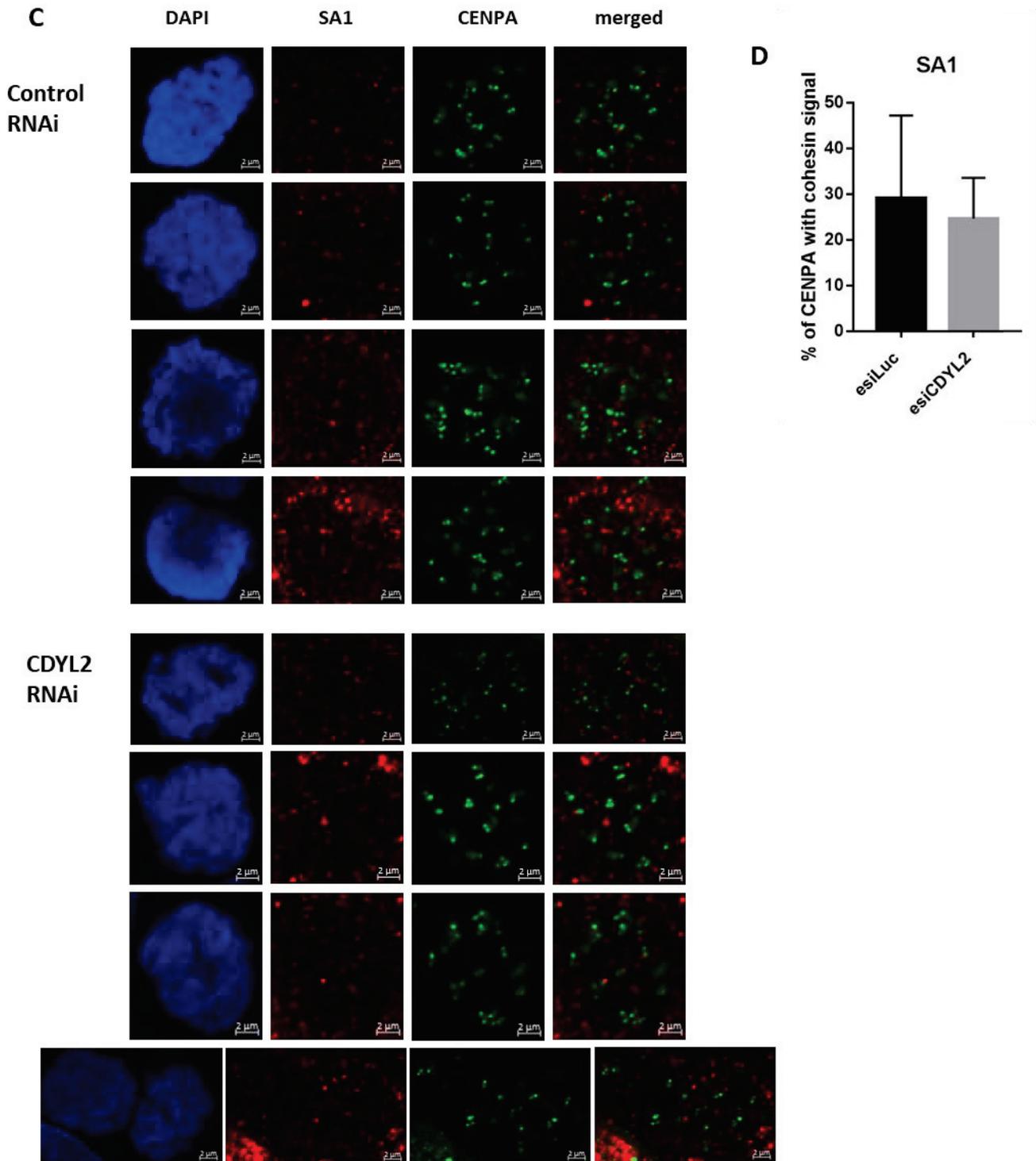


Fig. 27: CDYL2 does not seem to regulate cohesin level at pericentromeres during prometaphase. Costaining of CenpA and (A) SMC1 or (C) SA1 does not reveal a loss of Cohesin at pericentromeres after CDYL2 RNAi transfection, as quantified on (B) and (D). Error bars represent intercellular standard deviations. Experiments were performed twice. Representative images are shown.

8.8 CDYL2 INTERACTS WITH CHAMP1 BUT IS DISPENSABLE FOR MICROTUBULES STABILITY

It was previously described that CHAMP1 depleted cells induced a misalignment phenotype at metaphase, very similar to the phenotype observed after CDYL2 knock-down (Itoh *et al.*, 2011). Additionally, CHAMP1 was found necessary for kinetochore protein localization at the centromeres and microtubules stabilization. The CHAMP1-POGZ complex was present in the CDYL2 immunoprecipitation mass spectrometry analysis. I thus hypothesized that CDYL2 might work with CHAMP1-POGZ and be relevant for kinetochore assembly or microtubules stabilization. To confirm the interaction between CDYL2 and CHAMP1, I performed a co-immunoprecipitation on HeLa and MCF7 cells. A negative control using a non-specific antibody was done in parallel. I could detect a specific CDYL2 signal in the coimmunoprecipitation after immunoblotting, confirming the interaction revealed by the mass spectrometry (Fig. 28). The interaction was fainter in HeLa cell lines compared to MCF7, most likely because CDYL2 level was already observed by our lab to be lower in HeLa cell lines compared to MCF7.

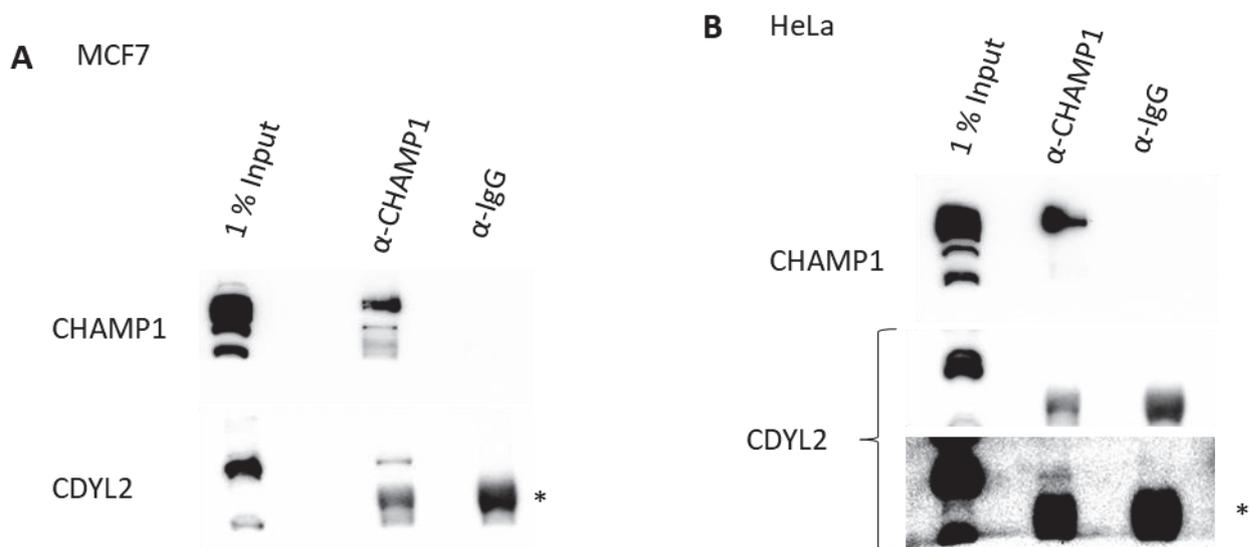


Fig. 28: CDYL2 interacts with CHAMP1 in both MCF7 and HeLa cell lines. Interaction between CDYL2 and CHAMP1 was confirmed with a reverse coimmunoprecipitation on CHAMP1 in both (A) MCF7 and (B) HeLa cells. Coimmunoprecipitation with a non specific IgG antibody was performed in parallel as a negative control. *corresponds to the denaturated heavy chain of the antibodies. Experiment was performed once.

I then wanted to study if CDYL2, like CHAMP1, is necessary for kinetochore assembly or mitotic microtubules stabilization. To answer this question, I stained RNAi transfected cells for microtubules and kinetochores using α -Tubulin and CENPA, similar to the protocol described by Itoh *et al.* (Itoh *et al.*, 2011). No difference in microtubules attachment to kinetochore could be seen compared to the control (Fig. 29), even in cells showing alignment defects, suggesting kinetochore assembly is not the mechanistic origin of CDYL2 knocked-down phenotype. I saw no sign of microtubules defects in interphase cells neither (Fig. 30). Finally, I wanted to test if CDYL2 is necessary for microtubules stabilization, and for this, I incubated the cells for ten minutes at 4°C to test their resistance to the cold. The efficiency of the cold incubation was confirmed by the stringent loss of α -Tubulin staining in interphase cells, where the microtubules are known to be cold-labile (Fig. 30). However, again no difference between Luciferase and CDYL2 RNAi treated cells could be observed, even in mitotic cells showing chromosome alignment defects (Fig. 31). This suggests that CDYL2 implication in mitosis is not related to microtubules' stability nor attachment to the kinetochore. It is therefore unlikely that CDYL2 is necessary for CHAMP1 activity at the centromeres.

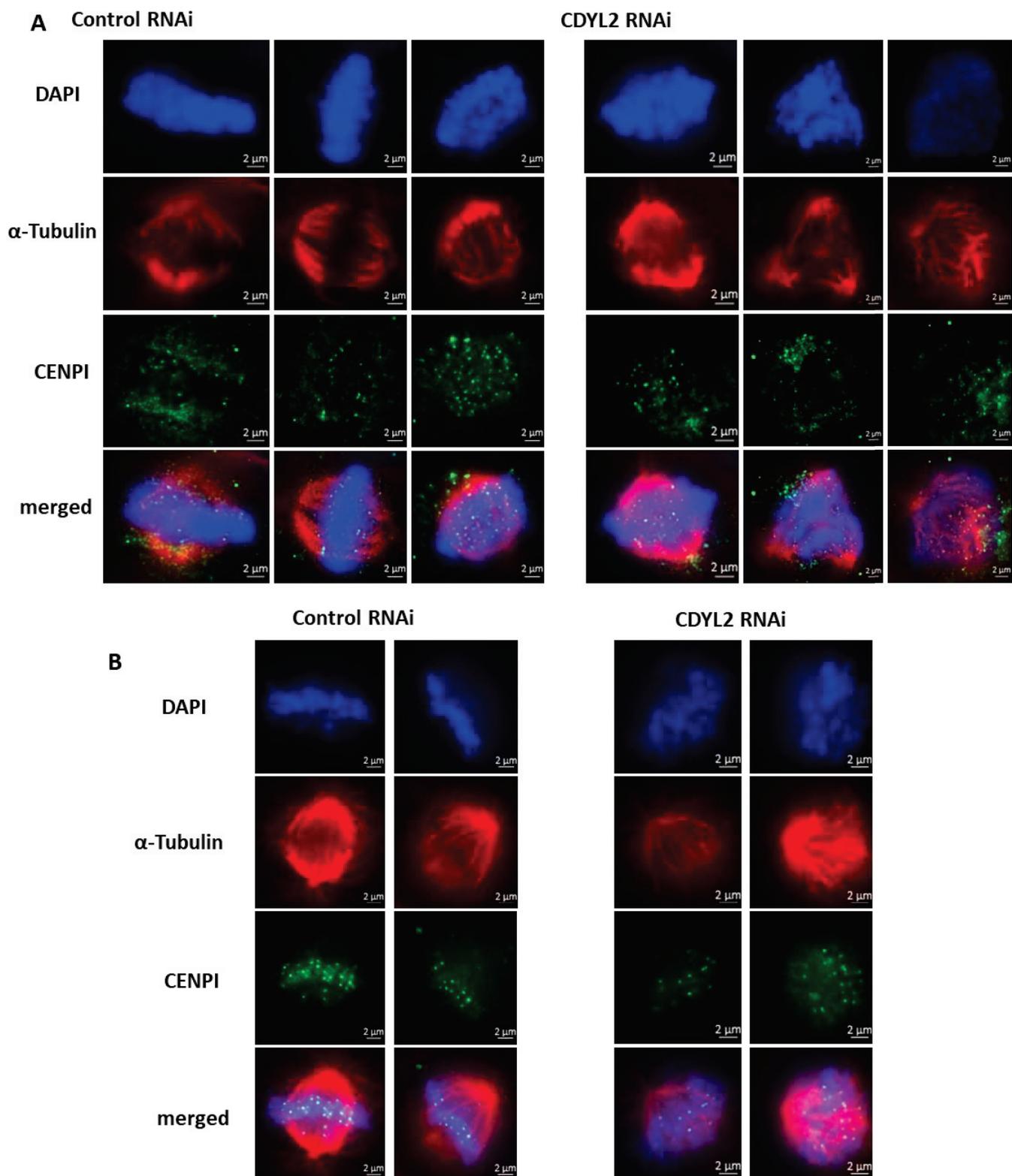


Fig. 29: CDYL2 seems to be dispensable for microtubules-kinetochores connection. Knock-down of CDYL2 was performed in both (A) HeLa and (B) MCF7 cells, followed by staining against Tubulin and CENPI. No significant difference was observed in the microtubules structure or their connection to kinetochores after CDYL2 esiRNA treatment. Experiment was performed twice. Representative images are shown.

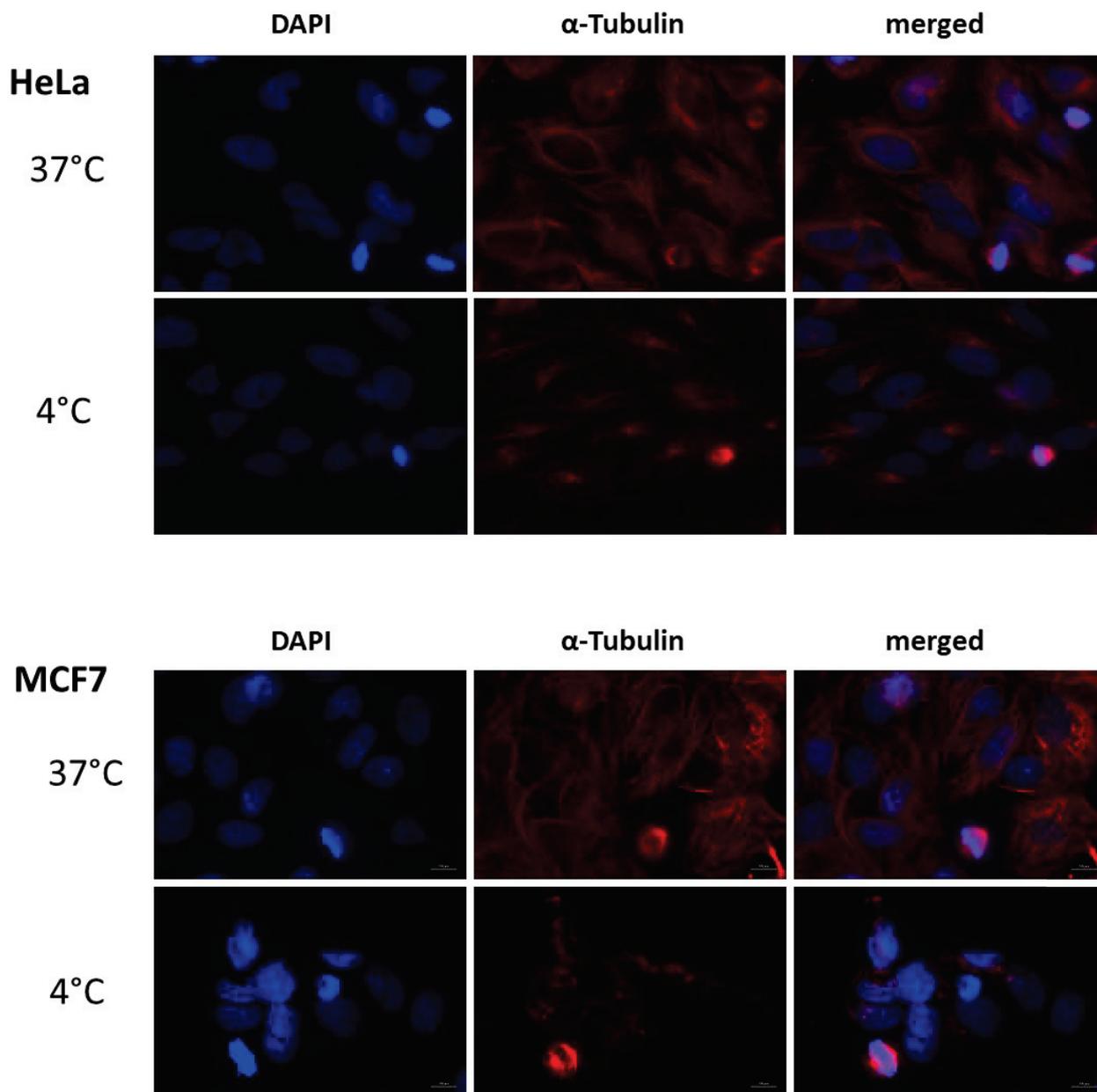


Fig. 30: Cold incubation of the cells prior to staining destroyed cold-labile microtubules. We confirmed that the cold incubation was sufficient to destroy cold-labile microtubules. Mitotic microtubules are cold stable and were preserved. Experiment was performed once.

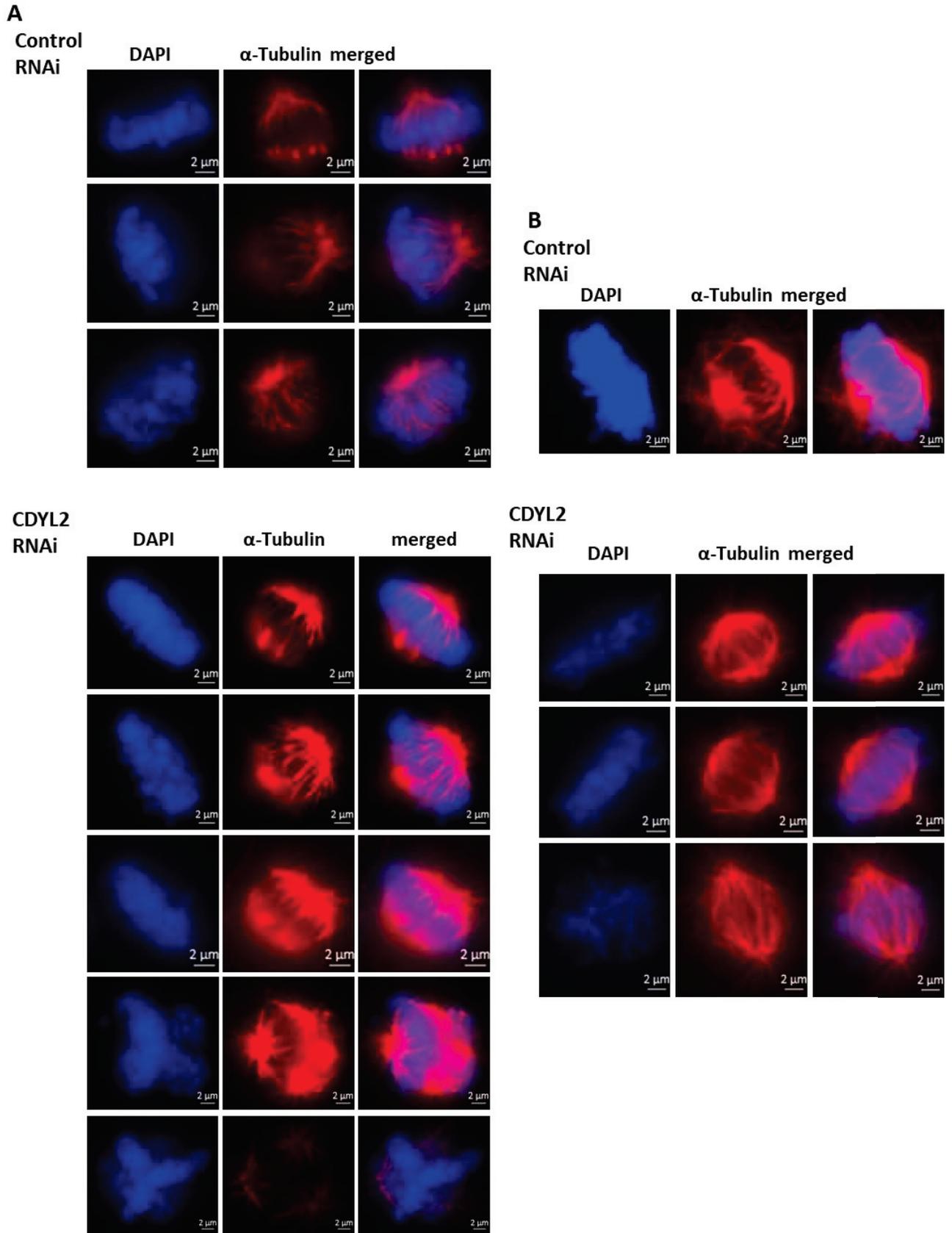


Fig. 31: CDYL2 seems to be dispensable for microtubules stability. Knock-down of CDYL2 was performed in both (A) HeLa and (B) MCF7 cells, followed by cold incubation and staining against Tubuli. No significant difference was observed in the microtubules structure or their stability to kinetochores after CDYL2 RNAi treatment. Experiment was performed once.

Table 2: Proteins common in all three Flag-CDYL2 coimmunoprecipitation.

Uniprot Acc	Uniprot Symbol	MWT(kDa)	Unique Mock	Total Mock	Unique IP	Total IP	Intensity Mock [a.u.]	Intensity IP [a.u.]
Q8N8U2	CDYL2	56,46	0	0	0	33	164	0
P78347	GTF2I	112,35	16	16	16	53	92	3900000
Q9Y232	CDYL1	66,44	0	0	0	25	83	0
P31327	CPSM	164,83	23	23	23	86	183	3800000
Q13547	HDAC1	55,07	4	4	4	17	34	1300000
Q14204	DYHC1	532,07	6	6	6	153	162	520000
P78527	PRKDC	468,79	24	24	25	126	141	2800000
P33993	MCM7	81,26	6	6	6	37	41	480000
Q8N108	MIER1	57,95	0	0	0	20	34	0
P33991	MCM4	96,5	0	0	0	34	65	0
Q7Z6Z7	HUWE1	481,59	0	0	0	71	92	0
Q92769	HDAC2	55,33	1	1	1	14	37	41000
P25705	ATPA	59,71	6	6	6	22	26	1400000
P42285	SK2L2	117,73	7	7	7	38	46	810000
Q6VMQ6	MCAF1	136,31	0	0	0	30	47	0
O95347	SMC2	135,57	0	0	0	41	47	0
P41252	SYIC	144,41	5	5	5	39	42	870000
Q99460	PSMD1	105,77	7	7	7	29	35	980000
O60264	SMCA5	121,83	2	2	2	29	36	260000
P50570	DYN2	98	5	5	5	33	61	280000
P08243	ASNS	64,33	6	6	6	22	29	950000
O94776	MTA2	74,98	3	3	3	15	17	470000
O95071	UBR5	309,16	0	0	0	66	83	0
P13674	P4HA1	61,01	4	4	4	19	25	210000
Q9UQE7	SMC3	141,45	8	8	11	44	50	1100000
P28340	DPOD1	123,55	4	4	4	30	33	580000
O95785	WIZ	178,56	5	5	6	18	25	590000
Q14839	CHD4	217,87	0	0	0	44	51	0
Q96KQ7	EHMT2	132,29	0	0	0	22	39	0
Q9H9B1	EHMT1	141,38	0	0	0	25	41	0
P55265	DSRAD	135,98	1	1	1	24	26	31000
Q13263	TIF1B	88,49	6	6	7	21	29	490000
P51531	SMCA2	181,17	0	0	0	36	40	0
Q15047	SETB1	143,07	0	0	0	30	41	0
Q12996	CSTF3	82,87	6	6	6	20	26	350000
Q9NTJ3	SMC4	147,09	0	0	0	39	43	0
Q14697	GANAB	106,81	0	0	0	24	35	0
Q6PJ19	CDC73	60,54	5	5	5	19	28	350000
Q9UNF1	MAGD2	64,91	0	0	0	15	16	0
P07814	SYEP	170,48	10	10	10	36	43	1400000
P56192	SYMC	101,05	0	0	0	13	16	0
P51532	SMCA4	184,53	0	0	0	18	19	0
P17844	DDX5	69,1	3	3	3	13	15	600000
P52292	IMA1	57,83	3	3	3	10	13	280000
Q14980	NUMA1	238,12	3	3	3	40	41	94000
Q15046	SYK	68	3	3	3	13	15	450000
A6NHR9	SMHD1	226,23	2	2	2	38	39	59000
Q9BQG0	MBB1A	148,76	4	4	4	20	22	160000
Q92797	SYMPK	141,06	2	2	2	21	23	100000
P42166	LAP2A	75,45	3	3	3	18	19	450000
O96019	ACL6A	47,43	1	1	1	9	10	330000
Q10570	CPSF1	160,78	4	4	4	26	33	360000
Q14527	HRLF	113,86	0	0	0	20	22	0
P57740	NU107	106,31	0	0	0	17	18	0
P46379	BAG6	119,33	0	0	0	15	15	0
P49327	FAS	273,25	2	2	2	30	36	110000
Q12760	NU160	162,02	1	1	1	17	19	29000
Q96JM3	CHAP1	89,04	0	0	0	16	16	0
Q8IXH7	NELFD	66,2	2	2	2	12	14	310000
Q13200	PSMD2	100,14	6	6	6	18	18	1400000
O00268	TAF4	110,05	2	2	2	7	8	430000
Q8N344	MIER2	59,91	0	0	0	7	8	0
Q86WB0	NIPA	55,23	0	0	0	7	8	0
O75152	ZC11A	89,08	0	0	0	12	14	0
Q8NBJ5	GT251	71,59	2	2	2	12	13	260000
O60341	KDM1A	92,84	0	0	0	12	12	0
Q12873	CHD3	226,45	0	0	0	17	18	0
Q96ST3	SIN3A	145,08	0	0	0	13	15	0
P47897	SYQ	87,74	2	2	2	11	11	110000
Q9BPK3	CND3	114,26	0	0	0	14	15	0
O60216	RAD21	71,64	0	0	0	12	13	0
P24666	PPAC	18,03	0	0	0	3	3	0
Q09666	AHMK	628,7	0	0	0	30	31	0
Q7Z3K3	POGZ	155,24	0	0	0	17	22	0
P08670	VIME	53,62	0	0	0	7	8	0
Q9BSD7	NTPCR	20,7	1	1	1	4	5	84000
P56545	CTBP2	48,91	0	0	0	5	5	0
P12270	TPR	267,13	0	0	0	16	16	0
Q9UBS4	DJB11	40,49	1	1	1	4	4	220000
Q12768	STRUM	134,2	1	1	1	9	10	28000
Q15942	ZYX	61,24	0	0	0	3	3	0
Q6UXN9	WDR82	35,06	1	1	1	3	3	180000
Q8N135	INADL	196,25	0	0	0	10	10	0
P25490	TTY1	44,68	0	0	0	4	4	0
Q641Q2	FA21A	147,09	0	0	0	3	3	0

9 CDYL2 EPIGENETICALLY REGULATES MIR124 TO CONTROL NF- κ B/STAT3-DEPENDENT BREAST CANCER CELL PLASTICITY

During my thesis, I also participated in the following project evaluating the function of CDYL2 in breast cancer and its effect on breast cancer invasiveness and aggressiveness. I participated particularly in the experiments and elaborations of figures 7A, S6A, and S7 of the published manuscript.

Briefly, Dr. Maha Siouda observed that high levels of CDYL2 in breast cancer tumors correlated with a worst prognosis of the patient (publication Fig. 1). RNA-sequencing allowed us to analyse the changes induced in the mRNA profile by an overexpression of flag-tagged CDYL2 in MCF7 breast cancer cell line. The transcriptional changes observed are linked with malignant progression of the tumor and several genes expression involved in EMT transition were found deregulated upon CDYL2 overexpression (publication Fig. 2). *In vitro* and *in vivo* invasion assays revealed that MCF7 cells become more invasive upon CDYL2 overexpression (publication Fig. 3F and 3G). Interestingly, MDA-MB-231, an aggressive breast cancer cell line, become less invasive upon CDYL2 knock-down induced by RNAi (publication Fig. 4).

In breast cancer, active NF- κ B/p65 and STAT3 pathways are inducing Epithelial-to-Mesenchymal Transition (EMT), linked with tumour invasion (Marotta et al., 2011; Yang et al., 2014; Zhou et al., 2008). A knock-down of p65 or STAT3 in MCF7 cells overexpressing CDYL2 rescued the invasive phenotype of MCF7 overexpressing CDYL2, indicating that CDYL2 increases the invasion potential of breast cancer tumors through both pathways (publication Fig. 5). Interestingly, ChIP-seq analysis revealed that CDYL2 binds upstream of the *MIR124* gene (publication Fig. 6A). Mir124 was previously shown to regulate EMT and both NF- κ B/p65 and STAT3s signaling (Cao et al., 2018; Hatziapostolou et al., 2011; Lv et al., 2011). Additionally, the overexpression of CDYL2 in MCF7 cell lines decreased the expression of MIR124, while the knock-down of CDYL2 in MDA-MB-231 increased the expression. We therefore investigated by which mechanism CDYL2 regulates MIR124 repression.

From the mass spectrometry analysis performed for the mitosis project, CDYL2 seems to interact with EHMT2, a known histone methyltransferase linked with transcription repression. We thus performed a coimmunoprecipitation against CDYL2 and observed an interaction with both EHMT2 and EHMT1 (publication Fig. 7A). A reverse coimmunoprecipitation against EHMT2 was performed in parallel and further confirmed the interaction with CDYL2. A coimmunoprecipitation against a non-specific antibody was done in parallel as a negative control. Finally, a coimmunoprecipitation was performed against EZH2. The presence of a strong SUZ12 portion binding to EZH2 confirmed the quality of the precipitate. However the presence of CDYL2 signal in EZH2 coimmunoprecipitate could not be detected. Under conditions that increased western blot sensitivity of detection, a weak signal of EZH2 and SUZ12 could be detected in CDYL2 coimmunoprecipitate, suggesting a weak interaction between the proteins is possible (publication Fig. S6A).

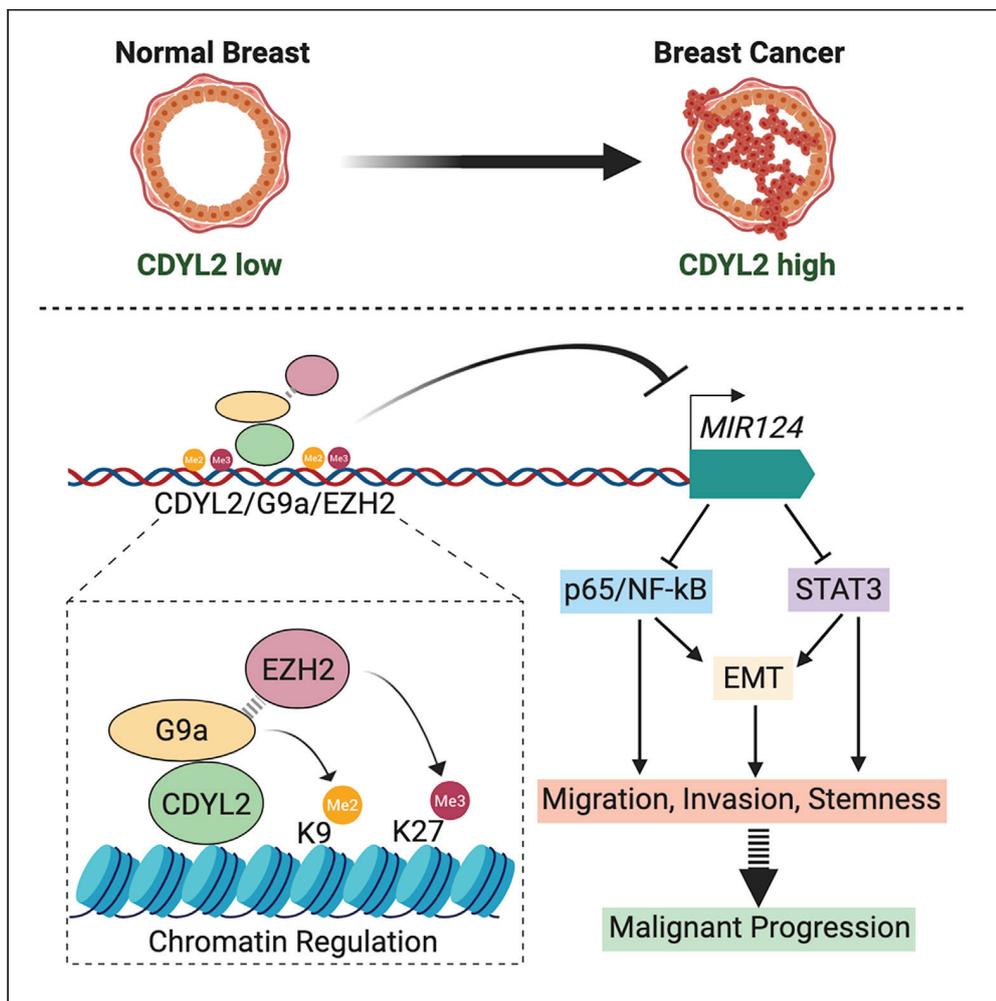
Performing, ChIP-qPCR analysis, we observed that an overexpression of CDYL2 increases H3K9 methylation and both enzymes level at the promotor regions of miR-124 (publication Fig. 7B and 7C), while on the opposite a knock-down of CDYL2 decreases their level (publication Fig. 7D and 7E). Using UNC0642, an EHMT2 inhibitor (Kim et al., 2016), I have shown that the enzymatic activity of EHMT2 is essential for miR124 repression through CDYL2, as UNC0642 treatment in MCF7 cells expressing exogeneous CDYL2 significantly increases miR-124 expression (publication Fig. S7). On the opposite, using CPI-169, an inhibitor of EZH2 (Bradley et al., 2014), it seems that EZH2 enzymatic activity seems dispensable for miR-124 silencing (publication Fig. 7B).

Taken together, our work allowed us to reveal that overexpression of CDYL2 increases the aggressivity and invasiveness of breast cancer cells, while its downregulation decreases it. This invasiveness is controlled through the NF- κ B signal, which is itself regulated by miR-124. Interestingly, CDYL2 is repressing miR-124. We studied the mechanism linking CDYL2 to miR-124 expression and found that CDYL2 locates at the upstream of miR-124 gene, where it recruits EHMT2. CDYL2 at the promotor regions is also necessary for EZH2 presence. EHMT2 finally methylates the upstream region of miR-124 gene, which leads to its repression, similar to previous description in the literature My participation to this

publication brought key elements to elucidate the molecular mechanism by which CDYL2 is silencing miRNA124 expression and thus controlling epithelial-to-mesenchymal transition.

Article

CDYL2 Epigenetically Regulates *MIR124* to Control NF- κ B/STAT3-Dependent Breast Cancer Cell Plasticity



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HIGHLIGHTS

Up-regulation of CDYL2 is common in breast cancer and correlates with poor prognosis

CDYL2 regulates enrichment of methyltransferases G9a and EZH2 at *MIR124* genes

microRNA-124 regulation by CDYL2 impacts STAT3 and NF- κ B signaling

CDYL2 regulation of EMT, migration, invasion, and stemness is STAT3/NF- κ B dependent

Siouda et al., iScience 23,
101141
June 26, 2020 © 2020 The
Authors.
[https://doi.org/10.1016/
j.isci.2020.101141](https://doi.org/10.1016/j.isci.2020.101141)



Article

CDYL2 Epigenetically Regulates
MIR124 to Control NF- κ B/STAT3-Dependent
Breast Cancer Cell Plasticity

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SUMMARY

Epigenetic deregulation of gene transcription is central to cancer cell plasticity and malignant progression but remains poorly understood. We found that the uncharacterized epigenetic factor chromodomain on Y-like 2 (CDYL2) is commonly over-expressed in breast cancer, and that high CDYL2 levels correlate with poor prognosis. Supporting a functional role for CDYL2 in malignancy, it positively regulated breast cancer cell migration, invasion, stem-like phenotypes, and epithelial-to-mesenchymal transition. CDYL2 regulation of these plasticity-associated processes depended on signaling via p65/NF- κ B and STAT3. This, in turn, was downstream of CDYL2 regulation of MIR124 gene transcription. CDYL2 co-immunoprecipitated with G9a/EHMT2 and GLP/EHMT1 and regulated the chromatin enrichment of G9a and EZH2 at MIR124 genes. We propose that CDYL2 contributes to poor prognosis in breast cancer by recruiting G9a and EZH2 to epigenetically repress MIR124 genes, thereby promoting NF- κ B and STAT3 signaling, as well as downstream cancer cell plasticity and malignant progression.

INTRODUCTION

A key feature of epigenetic processes is their ability to establish and maintain the expression level of genes in a manner that is durable, yet can be altered when necessary. To this end, the deposition of histone lysine methylation marks on chromatin is tightly regulated. However, deregulation of epigenetic factors can cause pathologic changes in cell identity and function and is a near-universal feature of cancer cells. Such perturbations offer therapeutic opportunities, and several drugs targeting epigenetic regulators are in use or under investigation as cancer treatments. These include inhibitors of the histone methyltransferases EZH2 and G9a, which respectively impart the H3K27me3 and H3K9me2 marks on chromatin (Dawson and Kouzarides, 2012; Jambhekar et al., 2019; Simó-Riudalbas and Esteller, 2015). However, despite recent progress, the epigenetic regulation of cellular plasticity in cancer remains poorly understood, with several putative epigenetic factors still uncharacterized. Addressing these issues could uncover new epigenetic drug targets for cancer treatment.

Most tumors are of epithelial origin, but epithelial cells are inherently resistant to several key steps in malignant progression. Molecular and cellular changes that render carcinoma cells more mesenchymal-like are associated with increased propensity to migrate and invade the surrounding tissues (Puisieux et al., 2014; Shibue and Weinberg, 2017). This so-called epithelial-to-mesenchymal transition (EMT) is also linked to the emergence of cancer stem cells (CSC), a subset of cells within a tumor mass that are highly efficient at seeding new tumor growth and in the case of breast cancer, more efficient at forming cellular aggregates called mammospheres *in vitro* (Shibue and Weinberg, 2017). In breast cancer, different tumor subtypes and prognosis correlate with distinct EMT states. Tumors expressing the estrogen receptor alpha (ER), but not the human epidermal growth factor (EGF) receptor 2 (HER2), are more epithelial-like, less invasive, and have better prognosis, whereas those triple-negative (TN) for expression of ER, HER2, and the progesterone receptor (PR) are more mesenchymal-like, invasive, and have worse prognosis (Sarric et al., 2008).

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<https://doi.org/10.1016/j.isci.2020.101141>



However, the acquisition of EMT-like features in a subset of cells within the ER+/HER2- tumor could drive the malignant progression of these cancers.

The gene expression changes underlying EMT and stemness result from interconnected regulatory systems involving transcription factors, epigenetic factors, and non-coding RNAs. In breast cancer, active forms of the transcription factors p65/NF- κ B and STAT3 promote EMT, migration, invasion, and stemness (Marotta et al., 2011; Yang et al., 2014; Zhou et al., 2008). Misregulation of EZH2 and G9a can also induce these cellular processes (Chang et al., 2011; Curry et al., 2015; Dong et al., 2012), as can aberrant silencing of the tumor suppressive microRNA-124 (miR-124) (Ji et al., 2019; Lv et al., 2011; Wang et al., 2016a), itself a regulator of p65/NF- κ B and STAT3 signaling (Cao et al., 2018; Hatzia Apostolou et al., 2011; Mehta et al., 2017; Olarerin-George et al., 2013). Recently, EZH2 was implicated in miR-124 repression in renal carcinoma cells (Zhou et al., 2019), supporting an interplay between these pathways. However, by and large, epigenetic regulation of EMT and stemness in cancer remains poorly understood.

In this study, we investigated the molecular and cellular functions of the putative epigenetic factor chromodomain on Y-like 2 (CDYL2) in breast cancer. This is a member of the CDYL family of genes, which includes two autosomal homologs in humans, CDYL1/CDYL and CDYL2 (Dorus et al., 2003). The family is defined by the presence of an N-terminal chromodomain that binds to methylated histone H3 lysine 9 (H3K9) and H3K27 residues (Fischle et al., 2008; Franz et al., 2009) and a C-terminal domain homologous to enoyl coenzyme A hydratase/isomerase enzymes (Dorus et al., 2003). CDYL1 is implicated in cancer as a candidate oncogene or tumor suppressor, depending on the context (Mulligan et al., 2008; Wu et al., 2013), and its epigenetic mechanism involves its interaction with and regulation of several other epigenetic factors, notably the H3K9 methyltransferases G9a/EHMT2, GLP/EHMT1 and SETDB1/ESET (Mulligan et al., 2008), and EZH2 (Zhang et al., 2011). By contrast, very little is known about the roles of CDYL2 in physiology or disease or its putative epigenetic mechanism.

A potential role for CDYL2 in cancer was suggested by a genome-wide association study that identified an intronic SNP in CDYL2 associated with cancer risk (Michailidou et al., 2013). Here we show that CDYL2 expression is also frequently up-regulated in breast cancer, and that high expression correlates with poor outcome in the estrogen receptor-positive/human EGF receptor 2-negative (ER+/HER2-) and TN subtypes. We propose that high levels of CDYL2 expression promote epigenetic repression of MIR124 genes by increasing G9a and EZH2 recruitment and H3K9 and H3K27 methylation at upstream regulatory regions. This, in turn, contributes to CDYL2 induction of NF- κ B and STAT3 signaling, consequent induction of EMT genes, and increased cell motility, invasiveness, and stemness. These findings implicate CDYL2 as candidate proto-oncogene and therapeutic target in breast cancer.

RESULTS

High CDYL2 Expression Level in Breast Cancer Is Associated with Poor Prognosis

Datamining revealed that CDYL2 mRNA is up-regulated in four breast cancer cohorts within The Cancer Genome Atlas (TCGA) (Cancer Genome Atlas Network, 2012) (Figures 1A and S1A). Similarly, the NCBI GEO datasets GSE10780 (Chen et al., 2010) and GSE21422 (Kretschmer et al., 2011) identified CDYL2 up-regulation in invasive ductal breast carcinomas as well as ductal carcinoma *in situ*, compared with normal breast tissues (Figure 1A). Analysis of the paired Clinical Proteomic Tumor Analysis Consortium (CPTAC) (Ellis et al., 2013) and TCGA datasets revealed that CDYL2 protein expression correlated with mRNA levels (Figure 1B). We found that both TCGA mRNA and CPTAC protein levels for CDYL2 across breast cancer subtypes also showed similar patterns, being higher in the ER+ forms than TN forms (Figures S1B and S1C). We next asked if the expression level of CDYL2 correlates with clinical outcome. Patients were subdivided into three categories based on their expression of the ER, PR, and HER2, namely, ER+/HER2-, ER+/HER2+, and receptor TN. This revealed that high expression of CDYL2 correlated with worse survival in both ER+/HER2- and TN subtypes (Figures 1C and 1D), but not ER+/HER2+ (Figure 1E). We also analyzed the expression of CDYL2 in normal breast tissues over the course of breast cancer progression, across all breast cancer types, ER+/HER2- and TN. This showed up-regulation of CDYL2 from the earliest pre-metastatic stage (pN0) in all three patient cohorts (Figures S1D-S1F). To further probe a possible association between CDYL2 expression and breast cancer progression, we examined its correlation with cancer gene expression signatures in the Molecular Signature Database (MSigDB) (Subramanian et al., 2005). This uncovered a positive correlation between CDYL2 expression in both ER+/HER2- and TN breast cancer and the Rizki_tumor_invasiveness-2D-UP signature (Figures S1G and S1H), corresponding to genes

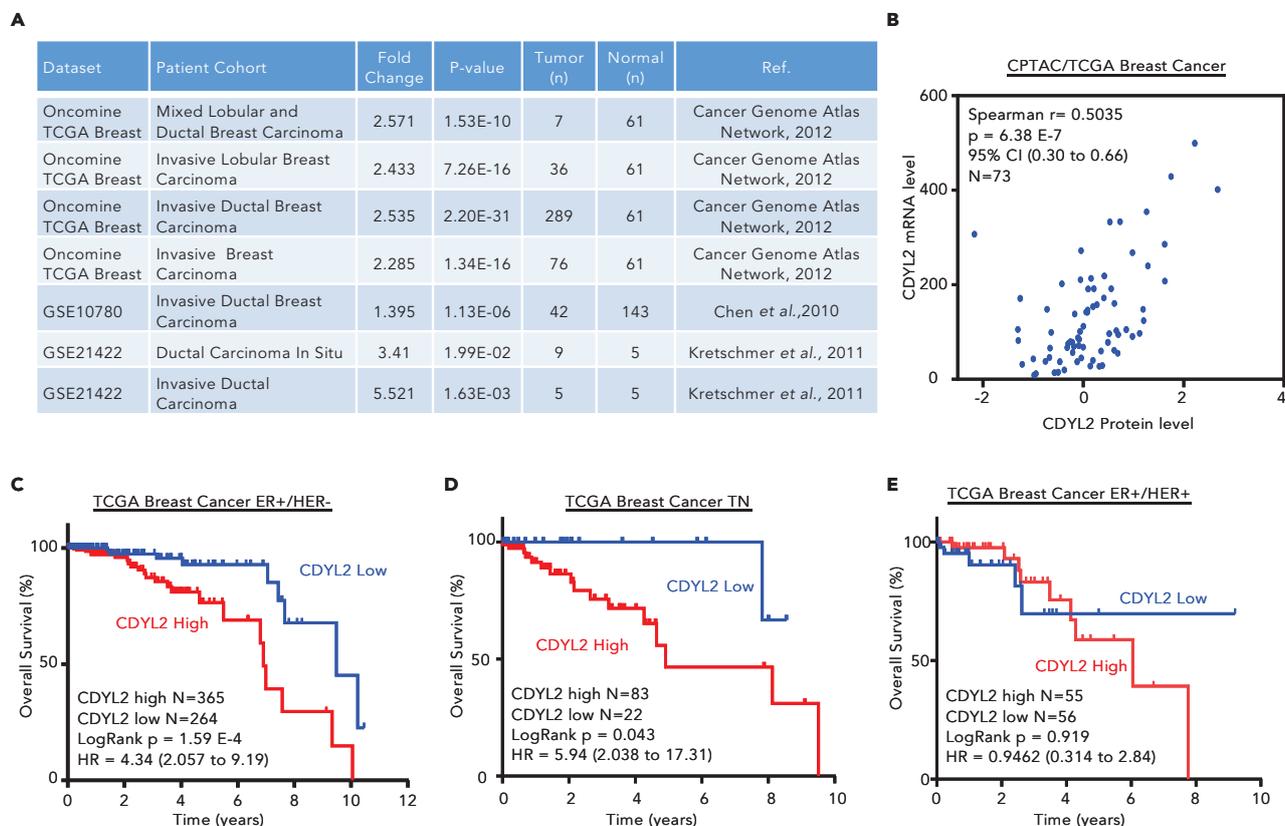


Figure 1. High CDYL2 Expression Level in Breast Cancer Is Associated with Poor Prognosis

(A) CDYL2 mRNA expression in breast tumors compared with normal tissues, as derived from the Oncomine database and GEO2R analysis of the indicated GEO datasets.

(B) Paired analysis of CDYL2 mRNA (TCGA, RNA-seq) and protein levels (CPTAC, mass spectrometry) in individual tumor specimen.

(C–E) Kaplan-Meier overall survival (OS) analysis performed from TCGA breast cancer subtypes: ER+/HER2– (C), triple negative (TN) (D), and ER+/HER2+ (E) using best cutoff of CDYL2 expression (high and low). Significance using log rank p value and hazard ratio (CI).

up-regulated in an invasive breast cancer cell line relative to the non-invasive precursor cell line from which it was derived (Rizki et al., 2008). Finally, we extended our analysis to other cancer contexts, revealing an association between CDYL2 expression level and survival in colorectal carcinoma, rectal adenocarcinoma, lung squamous cell carcinoma, and lung adenocarcinoma (Figures S11–S1L). These findings identify CDYL2 as a gene commonly up-regulated in breast cancer and a candidate modulator of cancer progression and patient survival in the ER+/HER2– and TN subtypes, and other cancer forms.

CDYL2 Over-Expression in the Non-invasive Breast Cancer Cell Line MCF7 Induces Transcriptional Changes Associated with Malignant Progression

To ask if CDYL2 up-regulation could induce oncogenic transcriptional and cellular changes, we stably expressed a CDYL2 cDNA in the non-invasive breast cancer cell line MCF7 (MCF7-CDYL2) or empty vector (MCF7-Vector) (Figure 2A). CDYL2 over-expression did not affect cell growth (Figure S2A), whereas RNA sequencing (RNA-seq) revealed striking differences between MCF7-CDYL2 and MCF7-Vector cells, with 693 genes up-regulated and 174 genes down-regulated at least 2.5-fold (Figure 2B; Table S1). Gene set enrichment analysis (GSEA) of genes up- or down-regulated in MCF7-CDYL2 cells revealed positive associations with EMT, metastasis, invasive versus non-invasive ductal carcinoma, breast cancer relapse in bone, and atypical ductal hyperplasia compared with non-cancerous breast tissue (Figures 2C and 2D). A number of genes from these GSEA signatures were validated by qRT-PCR, focusing on genes that are individually associated with breast cancer cell plasticity and malignant progression. These include the proto-oncogenes *SOX2*, *KLF4*, *MYC*, *MUC1*, *FOS*, *FOSL1/Fra-1*, and *JUN* (Alam et al., 2014; Bakiri et al., 2015; Jiao et al., 2010; Kufe, 2013; Nair et al., 2014; Piva et al., 2014; Yu et al., 2011), and the secretory

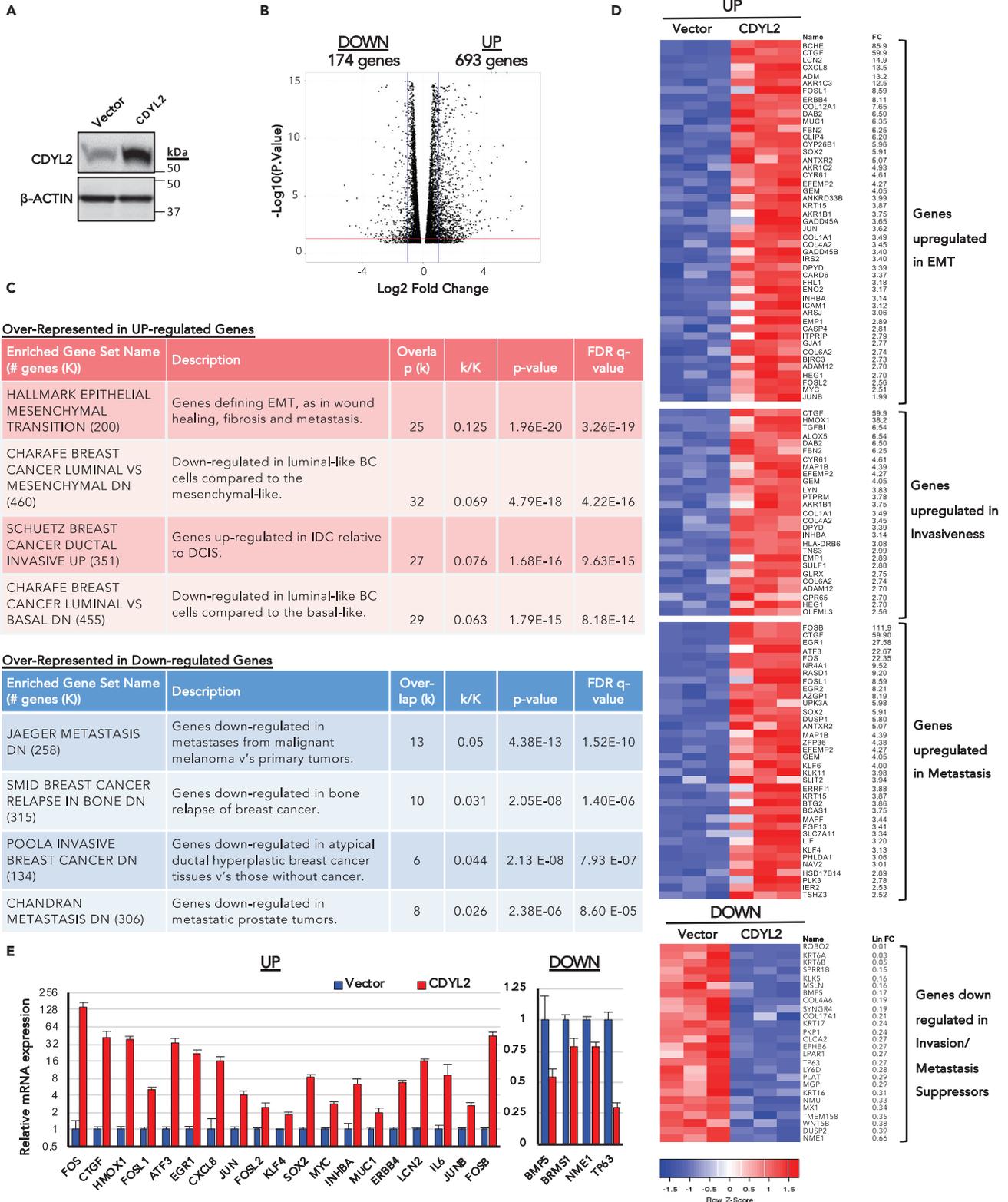


Figure 2. CDYL2 Over-Expression in the Non-invasive Breast Cancer Line MCF7 Induces Transcriptional Changes Associated with Malignant Progression

(A) Western blot analysis of CDYL2 and beta-actin expression in MCF7-CDYL2 and MCF7-Vector cells.
(B) Volcano plot showing genes up- or down-regulated at least 2.5-fold at an adjusted p value less than 0.05 (t test).
(C) Selected molecular signatures over-represented in either the up- or down-regulated gene sets from (B).
(D) Heatmap showing expression of genes from molecular signatures in (C) in the triplicate RNA-seqs.
(E) qRT-PCR validation of selected differentially expressed genes from (C). Mean of three independent experiments \pm S.D. All differential expressions were significant at $p < 0.05$ (t test).

molecules *LCN2*, *CTGF*, *CXCL8*, *INHBA*, and *IL6* (Rhodes et al., 2004, p. 8; Shimo et al., 2006; Singh et al., 2013, 2013; Sullivan et al., 2009, p. 8) (Figure 2E). Down-regulation of the tumor suppressor *TP63*, breast cancer metastasis suppressor *BRMS1*, and cytokine *BMP5*, which regulate EMT, metastasis, and stemness, among other processes (Gatti et al., 2019; Romagnoli et al., 2012), was also confirmed (Figure 2E). Together, these insights suggest that CDYL2 over-expression can induce transcriptional changes associated with malignant breast cancer, potentially by promoting EMT, invasiveness, and metastasis.

CDYL2 Over-expression in MCF7 Cells Induces EMT-like Changes, Migration, Invasiveness, and Mammosphere Formation

Further probing if CDYL2 might induce EMT-like changes in MCF7 cells, we assessed the expression of a panel of established EMT markers. qRT-PCR analysis revealed CDYL2 up-regulation of mesenchymal markers *TWIST1*, *SNAI1*, *FN1*, *VIM*, *CTNNB1*, and *SNAI2* (Figure 3A). Western blotting revealed down-regulation of epithelial marker E-Cadherin and up-regulation the mesenchymal markers Vimentin (*VIM*), *TWIST1*/*Twist*, and *SNAI1*/*Snail* (Figure 3B). However, CDYL2 over-expression did not alter the levels of ER-alpha (Figure 3B), down-regulation of which can induce EMT (Dhasarathy et al., 2007), suggesting an independent mechanism. Notably, 3 weeks after MCF7 cells were transduced with the CDYL2 over-expression construct a change in cell morphology occurred, with loss of the cobblestone-like morphology of monolayers, replaced by a more fibroblast-like morphology (Figure 3C), similar to previous descriptions of EMT in MCF7 (Lin et al., 2014; Yin et al., 2008).

To further investigate the possibility that CDYL2 over-expression induced EMT in MCF7 cells, we analyzed the expression of the cell surface adhesion molecules EpCAM and CD49f. It was previously demonstrated that low expression levels of EpCAM are associated with both EMT and breast cancer stem cells. The EpCAM⁻/CD49f⁻ subpopulation of transformed mammary epithelial cells was especially tumorigenic, confirming high tumor-initiating, stem-like capacity. On the other hand, the EpCAM⁺/CD49f⁻ subpopulation was shown not to form tumors (Kim et al., 2015). We found that over-expression of CDYL2 in MCF7 cells results in the appearance of a substantial population of EpCAM⁻/CD49f⁻ cells (Figure S2C, top panels). Notably, CDYL2 over-expression also induced an EpCAM⁻/CD49f⁻ cell population in another ER⁺ breast cancer cell line, Cama-1 (Figure S2C, center panels).

Among the primary contributions of EMT to malignant progression is increased cancer cell migration and invasion. *In vitro* assays revealed that the MCF7-CDYL2 cells migrated more proficiently across a microporous membrane compared with controls (Figures 3D and 3E). Using an adaptation of this assay to test for invasive capacity, wherein the porous membrane was first overlaid with a Matrigel barrier, we found that MCF7-CDYL2 cells also had increased invasive capacity relative to controls (Figure 3F).

We then probed the effect of CDYL2 on MCF7 cell invasion and metastasis *in vivo*. Both MCF7-CDYL2 and control cells were fluorescently labeled and injected into the perivitelline space of zebrafish embryos. The presence of tail metastases was monitored by fluorescence microscopy 24 h later. MCF7-Vector cells rarely produced metastases (3.57% of fish), whereas MCF7-CDYL2 cells did so in 21.57% of cases (Figure 3G).

To test if CDYL2 over-expression additionally induced stem-like characteristics in MCF7 cells, we first performed a mammosphere assay. This is a functional assay to assess the enrichment of stem-like cells in a population. MCF7-CDYL2 cells yielded both more and larger mammospheres compared with controls (Figures 3H–3J). Consistent with this, flow cytometry analysis revealed that CDYL2 over-expression in MCF7 cells increased the fraction of cells bearing the stem-like antigenic profile of CD44-high/CD24-low cells compared with controls (Figures 3K and 3L). Taken together, this series of studies indicates that CDYL2 over-expression in MCF7 cells promotes a number of cellular phenotypes associated with cellular plasticity and malignant progression.

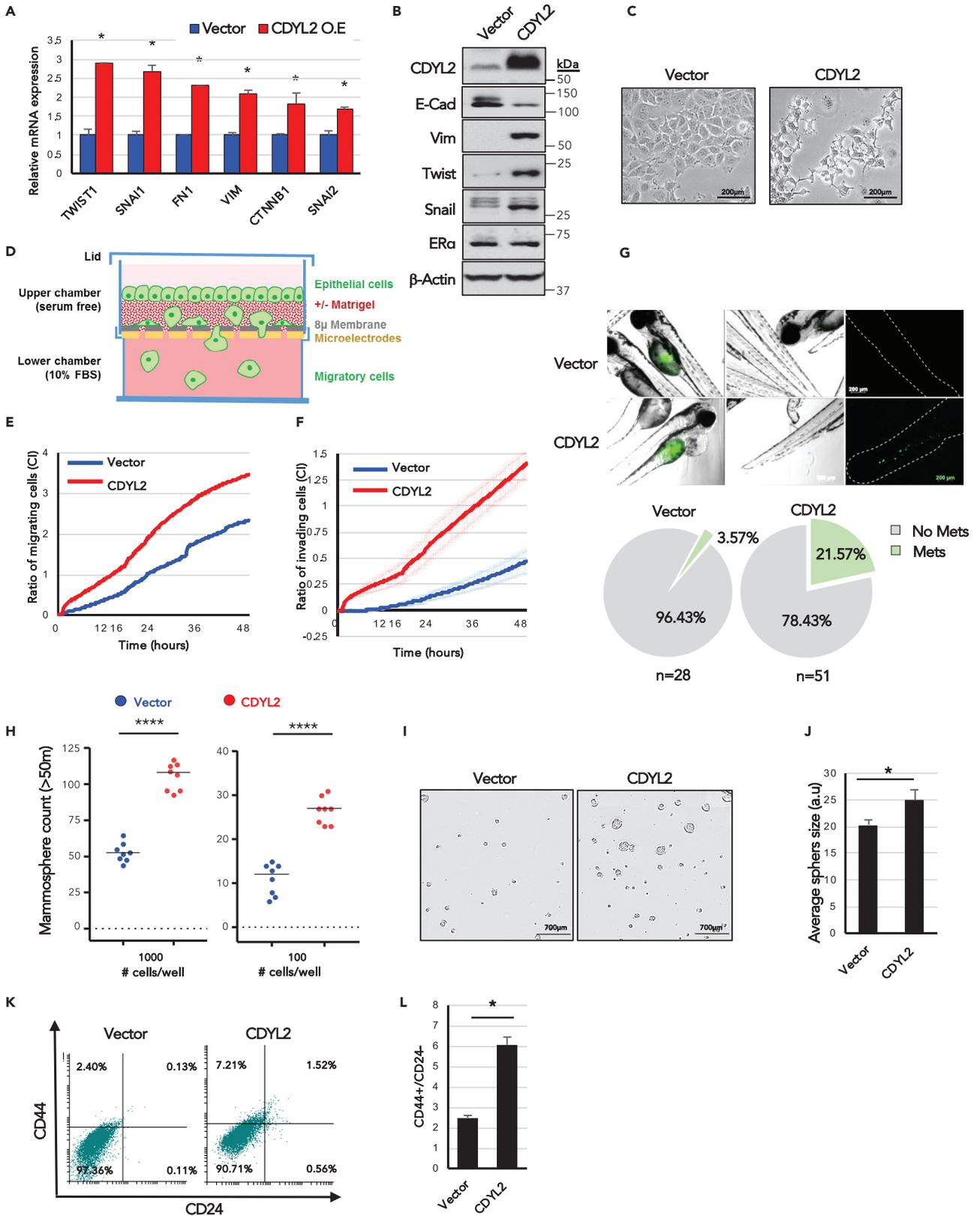


Figure 3. CDYL2 Over-Expression in MCF7 Cells Induces EMT-like Changes, Accompanied by Increased Migration, Invasiveness, and Mammosphere Formation

- (A) qRT-PCR analysis of a panel of EMT marker genes, normalization to GAPDH. Shown is mean \pm SD of three experiments. Differences significant at $p < 0.05$ (t test).
- (B) Western blot analysis of a panel of EMT markers, ER-alpha, CDYL2, and beta-actin.
- (C) 10 \times phase contrast micrograph of MCF7-CDYL2 and MCF7-Vector control. Scale bar, 200 μ M.
- (D) Schematic diagram of the xCELLigence quantitative, real-time migration and invasion assay system.
- (E) xCELLigence assay comparing the relative migration efficiency (Cell Index, CI) of MCF7-Vector and MCF7-CDYL2 cells. Both (E) and (F) show technical quadruplicates \pm SD. Experiments were repeated at least three times with similar results.
- (F) Invasion assays were performed as in (E), except that the porous membrane separating the upper and lower chambers of the transwell was first overlaid with Matrigel.
- (G) Zebrafish embryo cell invasion and migration assay. Shown are micrographs illustrating the metastasis of fluorescently labeled MCF7-CDYL2 or MCF7-Vector from the site of injection to the tail. Scale bar, 200 μ M. Quantification of the percentage of embryo exhibiting tail metastases is shown below. Experiments were repeated three times.
- (H) Mammosphere formation in MCF7-CDYL2 cells compared with MCF7-Vector controls. Cells were plated at the indicated seeding number per well in 96-well plates. Mammospheres with size $>50 \mu$ m were counted after 8 days. Shown is a scatterplot of the results of a representative of three independent experiments indicating the median (black bar) and t test significance (**** $p < 0.0001$).
- (I) Representative 4 \times phase microscopy images of mammospheres counted in (H). Scale bar, 700 μ M.
- (J) Mammosphere diameters were determined by image analysis. Shown is mean + SD of eight wells in which 1,000 cells were seeded. t test significance (* $p < 0.05$).
- (K and L) FACS analysis of antigenic profile associated with breast cancer stem cells (CD44+; CD24-low/negative). Shown are representative FACS scatterplots (K) and the mean of three independent experiments \pm S.D. (L). t test significance (* $p < 0.05$).

RNAi Knockdown of CDYL2 in the Invasive Breast Cancer Cell Line MDA-MB-231 Diminishes the Expression of EMT Markers and Inhibits Migration, Invasion, and Mammosphere Formation

We next analyzed the effect of CDYL2 loss of function in the highly invasive, cancer stem cell-enriched, mesenchymal-like breast cancer line MDA-MB-231. CDYL2 expression was inhibited by RNAi (Figures 4A and 4B) and high-throughput RNA-seq analysis performed. This revealed that compared with CDYL2 over-expression in MCF7 cells, the effects of its knock-down on MDA-MB-231 cell gene expression were more moderate, with no genes up- or down-regulated 2.5-fold or greater, except for CDYL2 itself (Table S2). However, using a fold-change cutoff of 1.25, we identified 204 genes up-regulated and 129 genes down-regulated (Figure 4C, Table S2). These gene lists were subjected to over-representation analysis using the GSEA molecular signatures database. This revealed that the down-regulated gene set was enriched in transcripts associated with EMT, metastasis, mammary stem cells, and invasive ductal carcinoma (Figures 4D and 4E). This suggests that CDYL2 knock-down might suppress EMT, metastasis, and stemness. To determine if this is the case, we performed essentially the same suite of assays used to probe the effect of CDYL2 over-expression on MCF7 cells (Figure 3).

We first confirmed by qRT-PCR that CDYL2 RNAi reduced the levels of a number of transcripts associated with EMT, namely, JUN, MYC, SNAI2, FOSL1, and TWIST1 (Figure 4F). Immunoblotting revealed induction of E-cadherin expression and diminished expression of Vimentin, Fibronectin, and Twist (Figure 4G). Fluorescence-activated cell sorting (FACS) analysis revealed that CDYL2 RNAi also induced the appearance of a subpopulation of EpCAM+/CD49- MDA-MB-231 cells (Figure S2C, lower panels), which was shown to be associated with loss of tumorigenicity (Kim et al., 2015). However, we did not observe morphological changes in these cells indicative of a mesenchymal-to-epithelial transition (MET), possibly because the duration of the RNAi treatment was not long enough for such phenotypes to emerge. Nonetheless, transduction of MDA-MB-231 cells with three independent small hairpin RNA (shRNA) sequences targeting CDYL2 (Figure S3A) did result in epithelial-like morphological changes after 2 weeks, with cells forming cobblestone-like monolayers (Figure S3B). These cells also exhibited down-regulation of a number of genes associated with EMT induction that were strongly up-regulated in MCF7-CDYL2 cells (*FOS*, *FOSB*, *JUNB*, *CXCL8*, *CTGF*, *LCN2*, *MUC1*, *ERBB4*), but not down-regulated in MDA-MB-231 cells treated with transient CDYL2 RNAi (Figure S3C).

The effect of CDYL2 RNAi on motility and invasiveness of MDA-MB-231 cells was evaluated using *in vitro* assays, revealing that transient or stable RNAi of CDYL2 dramatically reduced the migratory and invasive ability of MDA-MB-231 cells, relative to controls (Figures 4H, 4I, S3D, and S3E). *In vivo*, CDYL2 RNAi significantly impaired the ability of MDA-MB-231 cells to metastasize from the perivitelline site of injection to the tail of zebrafish embryos (Figure 4J), indicating suppression of the invasive and/or migratory capacity. Knockdown of CDYL2 by transient or stable RNAi treatment also resulted in fewer and smaller mammospheres relative to negative controls (Figures 4K–4M and S3F). While FACS analysis revealed that the

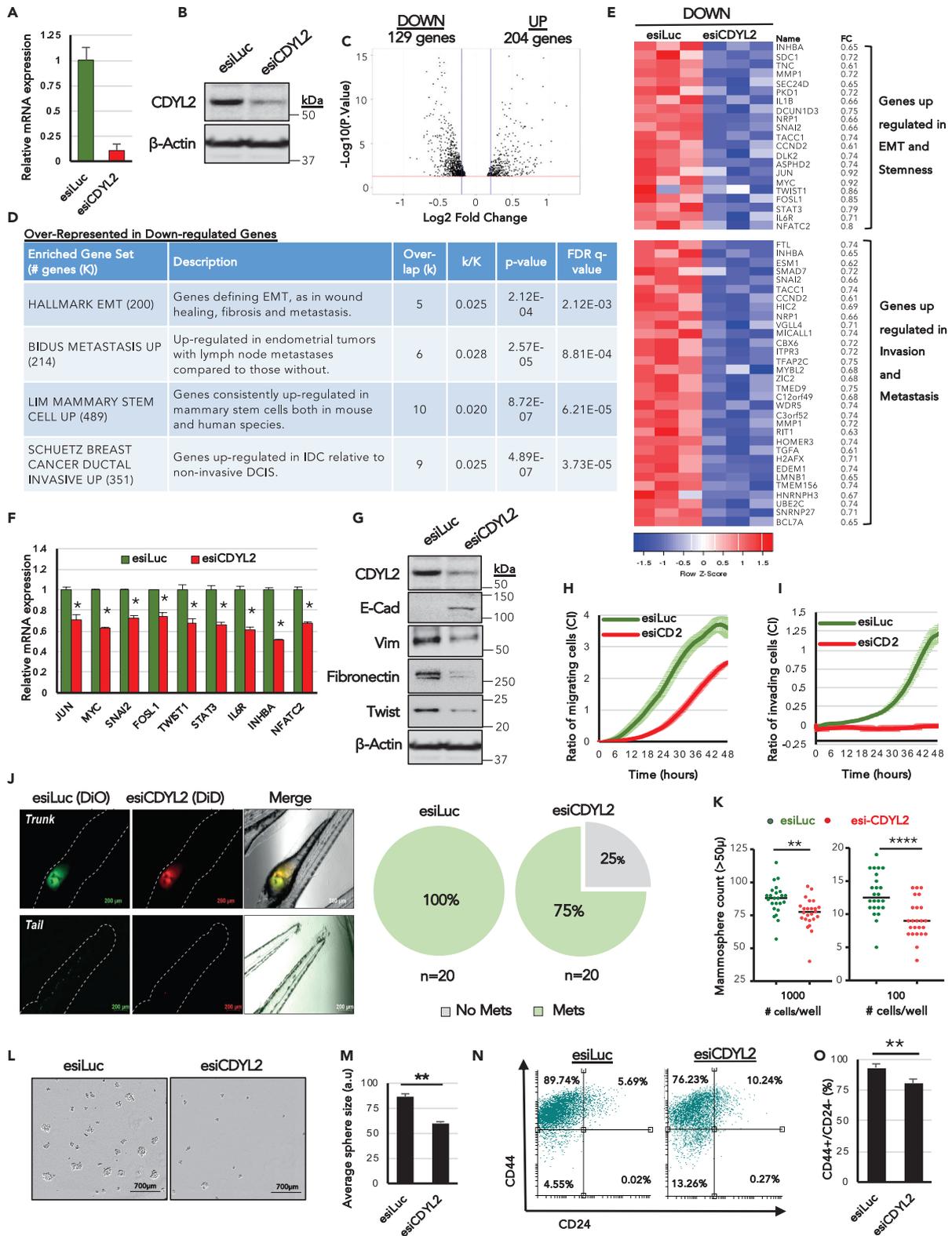


Figure 4. RNAi Knockdown of CDYL2 in the Invasive Breast Cancer Cell Line MDA-MB-231 Induces Transcriptional and Phenotypic Changes Associated with Inhibition of Malignancy

(A and B) CDYL2 knock-down validated by RT-qPCR (A) and western blotting (B).
(C and D) (C) Volcano plot showing genes up- or down-regulated at least 1.25-fold. (D) Selected molecular signatures from the MSigDB database that were over-represented in either the up- or down-regulated gene sets from (C).
(E) Heatmap showing expression of selected genes from (D) in the triplicate RNA-seq.
(F) qRT-PCR validation of differential expression of selected genes from (D). Expression normalized to GAPDH. Data are the mean \pm SD of three independent experiments. All differential expressions significant at $p < 0.05$ (t test).
(G) Western blot analysis of a panel of EMT markers, CDYL2, and beta-actin.
(H) xCELLigence assay comparing the relative migration efficiency of MDA-MB-231 cells treated with esiLuc or esiCDYL2 (esiCD2). Both (H) and (I) show technical quadruplicates \pm SD. Experiments were repeated at least three times with similar results.
(I) Invasion assays were performed as in (H), except that the porous membrane separating the upper and lower chambers of the transwell was first overlaid with Matrigel.
(J) Fluorescence microscopy analysis of with esiLuc or esiCDYL2 on the migration of MDA-MB-231 from the perivitelline space of zebrafish embryos to the tail. Representative images are shown. Scale bar, 700 μ M. Quantification of the percentage of embryos exhibiting tail metastases (Mets) is shown to the right. Experiments were repeated three times with similar results.
(K) Mammosphere formation in MDA-MB-231 cells treated with esiLuc or esiCDYL2. Cells were plated at the indicated seeding number per well in 96-well plates. Mammospheres with size >50 μ m were counted after 8 days. Shown is a scatterplot of the results of a representative of three independent experiments indicating the median (black bar) and t test significance (** $p < 0.01$; **** $p < 0.0001$).
(L) Representative 4 \times phase microscopy images of mammospheres counted in (K) are shown. Scale bar, 200 μ M.
(M) The diameters of mammospheres from (K) were determined using image analysis software. Shown is the mean \pm SD of eight wells in which 1,000 cells were seeded. t test significance (** $p < 0.01$).
(N and O) FACS analysis of antigenic profile associated with breast cancer stem cells (CD44 $^{+}$; CD24-low/negative). Shown are representative FACS scatterplots (N) and the mean of three independent experiments \pm SD. (O). t test significance (** $p < 0.01$).

majority of control RNAi-treated MDA-MB-231 cells were CD44-high/CD24-low, CDYL2 RNAi induced a population of CD44-low/CD24-low cells, indicative of loss of stemness (Figures 4N, 4O, and S3G). Collectively, these assays indicate that CDYL2 is required for MDA-MB-231 cell migration, invasion, and stemness, as well as the full expression of its mesenchymal-like state.

Regulation of p65/NF- κ B and STAT3 Signaling by CDYL2

Gene expression signature analysis of the effects of CDYL2 over-expression in MCF7 cells or knockdown in MDA-MB-231 revealed a potential role in regulating NF- κ B/TNF-alpha and STAT3/interleukin-6 signaling (Figure 5A). Given the importance of these signaling pathways in controlling cancer cell EMT, stemness, motility, and invasiveness, we asked if their regulation by CDYL2 might contribute to its regulation of these cellular processes. Consistent with the transcriptomic analysis, over-expression of CDYL2 in MCF7 cells increased the levels of tyrosine 705-phosphorylated STAT3 (Figure 5B), the active form of this protein. It also increased the levels of serine 536 phosphorylation on the NF- κ B TF p65 (Figure 5B), indicating an increase in canonical NF- κ B pathway signaling. By contrast, the levels of both phosphoproteins were diminished in CDYL2 RNAi-treated MDA-MB-231 cells (Figure 5C). We also probed the levels of total p65 and STAT3 proteins, as well as beta-actin, as a loading control. The total p65 levels were not affected by either CDYL2 over-expression or RNAi, whereas total STAT3 levels were higher in MCF7-CDYL2 cells compared with controls (Figure 5B) and down-regulated after CDYL2 RNAi in MDA-MB-231 cells (Figure 5C).

We then asked if CDYL2 induction of genes associated with EMT, invasion, and stemness in MCF7 cells might be dependent on signaling via p65/NF- κ B and STAT3. STAT3 and p65 were knocked down by transient transfection with small interfering RNA (siRNA) in both MCF7-Vector and MCF7-CDYL2 cells. A non-targeting siRNA was used as a control (Figures 5D and 5E). qRT-PCR analysis then revealed that p65 RNAi down-regulated several genes associated with NF- κ B signaling, namely, *CTGF*, *EGR1*, *FOS*, *IL6*, *CXCL8*, *INHBA*, *JUN*, *MYC*, *SNAI1*, *KLF4*, *SOX2*, and *TWIST1* (Figure 5F). Similarly, STAT3 RNAi down-regulated several genes associated with STAT3 signaling, including *FOS*, *TWIST1*, *SOX2*, *JUN*, *MUC1*, *INHBA*, *IL6R*, *IL6ST*, and *TNF* (Figure 5G). Strikingly, RNAi knockdown of either p65 or STAT3 potently suppressed both invasiveness (Figures 5H and 5I) and mammosphere induction by CDYL2 (Figures 5J and 5K). Taken together, these analyses indicate that p65/NF- κ B and STAT3 signaling is regulated by CDYL2, and that both pathways are required for CDYL2 induction of invasion and mammosphere formation in MCF7 cells.

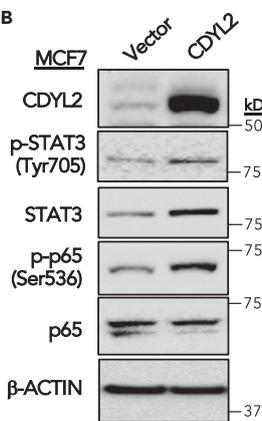
CDYL2 Binds Upstream of MIR124 Genes and Regulates miR-124 Expression

Consistent with the possibility that CDYL2 might be an epigenetic regulator of transcription, we found that it was enriched in the nucleus of both MCF7 and MDA-MB-231 cells, with a significant fraction present in the

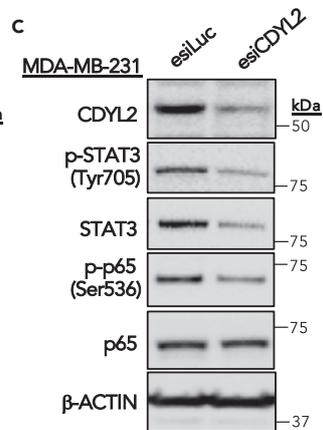
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Cell-Line	Input Gene List	Enriched Gene Set Name (# genes (K))	Description	# Genes in Overlap (k)	k/K	p-value	FDR q-value
MCF7	Up-regulated in MCF7-CDYL2	HALLMARK TNFA SIGNALING VIA NFKB (200)	Genes regulated by NF-κB in response to TNF.	40	0.2	1.97E-40	9.86E-39
MCF7	Up-regulated in MCF7-CDYL2	STAT3 02 (147)	Genes having at least one occurrence of the transcription factor binding site V\$STAT3 02 (v7.4 TRANSFAC) in the regions spanning up to 4 kb around their transcription starting sites.	14	0.095	2.03E-10	3.2E-09
MDA-MB-231	Down-regulated by esiCDYL2	HALLMARK TNFA SIGNALING VIA NFKB (200)	Genes regulated by NF-κB in response to TNF.	7	0.035	1.23E-06	2.06E-05
MDA-MB-231	Down-regulated by esiCDYL2	DASU IL6 SIGNALING UP (59)	Genes up-regulated in normal fibroblasts in response to IL6.	4	0.067	1.98E-05	7.25E-04

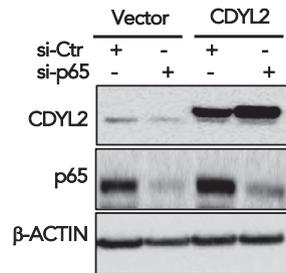
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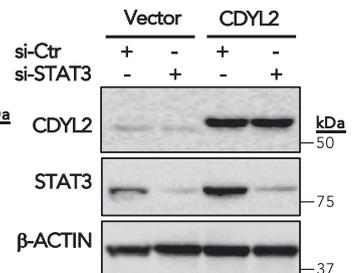
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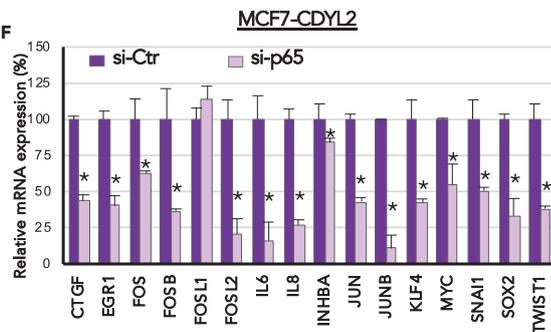
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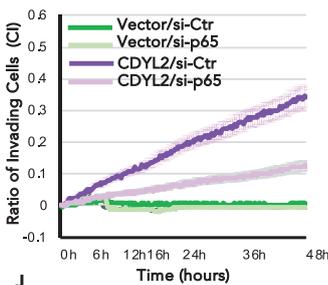
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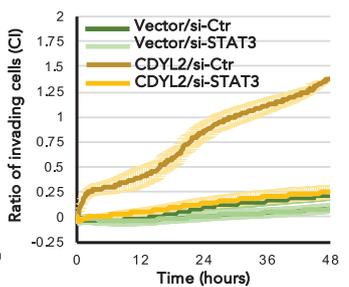
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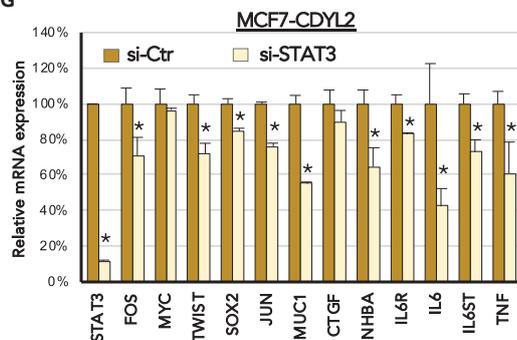
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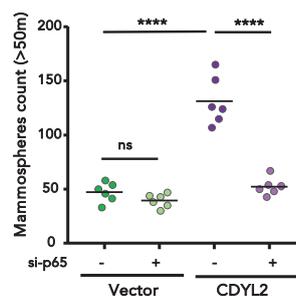
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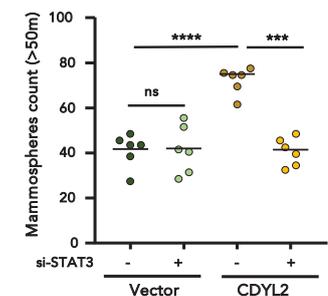


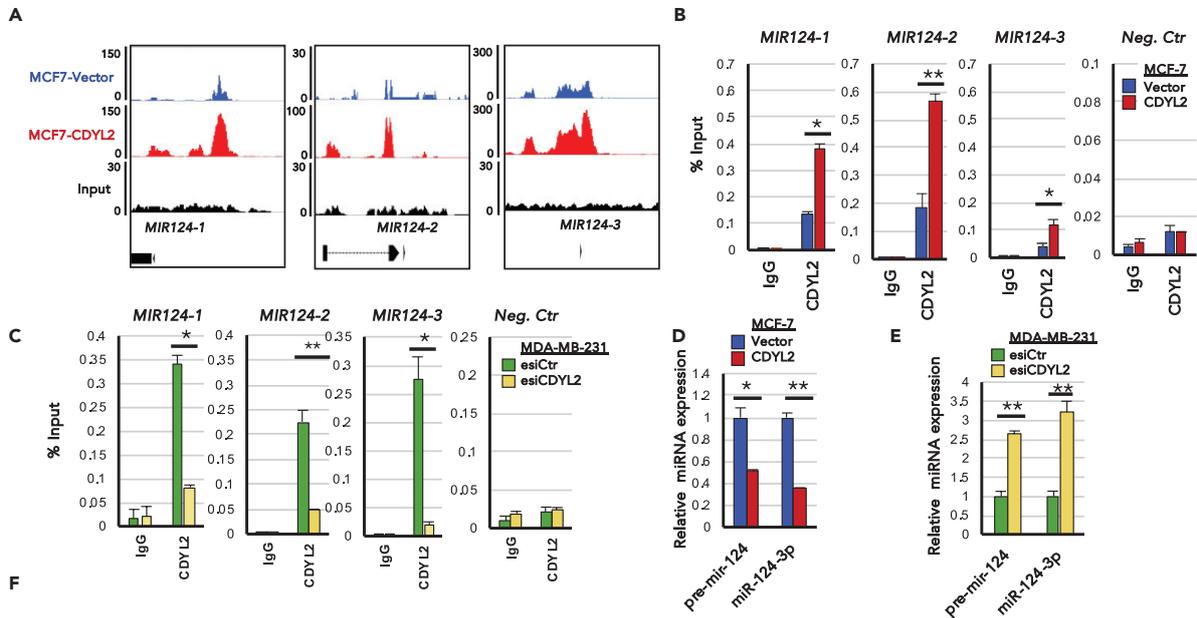
Figure 5. CDYL2 Regulation of NF- κ B and STAT3 Signaling Contributes to Its Induction of Invasion and Mammosphere Formation

(A) Selected gene expression signatures enriched in the indicated RNA-seq datasets.
(B) Western blot of Ser536-phosphorylated p65 and Tyr705-phosphorylated STAT3 in MCF7-Vector versus MCF7-CDYL2. The levels of total p65, STAT3, and β -actin were also probed.
(C) As for (B), except comparing MDA-MB-231 cells treated with esiLuc or esiCDYL2.
(D and E) Western blot validation of RNAi knockdown of p65 (D) or STAT3 (E) in MCF7-Vector and MCF7-CDYL2 cells. β -actin is shown as loading control.
(F) qRT-PCR analysis of the effect of RNAi knockdown of p65 on the expression of a panel of NF- κ B target genes that were up-regulated in MCF7-CDYL2 compared with MCF7-Vector cells. Data are represented as mean of three independent experiments \pm SD. All differences were significant at $p < 0.05$ (t test).
(G) As in (F), except the effect of RNAi knockdown of STAT3 on the expression of a panel of its target genes up-regulated in MCF7-CDYL2 compared with MCF7-Vector cells was evaluated.
(H and I) xCELLigence invasion assays of MCF7-CDYL2 in MCF7 cells treated with either control RNAi or siRNA targeting p65 (H) or STAT3 (I). Graphs are representative of three independent experiments in quadruple runs per condition. Error bars represent the SD of quadruplicate readings at each time point.
(J and K) Mammosphere assay of MCF7-CDYL2 in MCF7 cells treated with either control RNAi or siRNA targeting p65 (J) or STAT3 (K). Mammospheres from 1,000 seeded cells with size >50 μ m were counted after 8 days. Shown is a scatterplot of the results of a representative of three independent experiments indicating the median (black bar) and t test significance (*** $p < 0.001$; **** $p < 0.0001$; ns, not significant).

chromatin fraction (Figures S4A–S4C). To identify where on chromatin CDYL2 is bound, we performed CDYL2 chromatin immunoprecipitation (ChIP) in both MCF7-Vector and MCF7-CDYL2 cells followed by Illumina sequencing (ChIP-seq). This revealed several genomic loci that were more enriched in CDYL2 in the MCF7-CDYL2 cells compared with vector controls, including upstream of all three members of the *MIR124* gene family (Figure 6A; Data S1, S2, and S3). Owing to their implication in tumor suppression, and the ability to regulate both p65/NF- κ B and STAT3 signaling, EMT, invasion, and stemness (Cao et al., 2018; Hatzia Apostolou et al., 2011; Ji et al., 2019; Lv et al., 2011; Mehta et al., 2017; Olarerin-George et al., 2013; Wang et al., 2016a, 2016b), we decided to investigate CDYL2 regulation of the *MIR124* genes further. We confirmed CDYL2 enrichment upstream of *MIR124* genes using ChIP-qPCR (Figure 6B). A non-reactive IgG was used as negative ChIP control, whereas qPCR analysis did not detect enrichment of CDYL2 at an unrelated sequence (Figure 6B). We reasoned that CDYL2 repression of *MIR124* genes might contribute to its regulation of STAT3 and NF- κ B signaling in MCF7 and MDA-MB-231 cells. In agreement with this possibility, CDYL2 RNAi diminished its levels upstream of *MIR124* genes in MDA-MB-231 (Figure 6C), with a corresponding increase in the expression of both the precursor (pri-miR-124) and mature (miR-124-3p) forms of microRNA-124 (Figure 6E). Stable knock-down of CDYL2 using three independent shRNAs also increased levels of both pre-miR-124 and miR-124-3p (Figure S5). In complementary analysis, both *MIR124* transcripts were down-regulated by CDYL2 over-expression in MCF7 (Figure 6D). Supporting the notion that the alterations of miR-124 levels were sufficient to affect cell function, analysis of the MCF7-CDYL2 and MDA-MB-231 esiCDYL2 RNA-seq data showed that miR-124 target genes were commonly up-regulated in the former and down-regulated in the latter (Figure 6F). The miR-124 GSEA signature was also positively correlated with CDYL2 mRNA expression across all samples in the TCGA breast cohort, as well as in the ER+/HER2– and TN subtypes (Figures 6G–6I). Differential expression of several of the genes up-regulated in MCF7-CDYL2 cells or down-regulated in MDA-MB-231 cells treated with CDYL2 RNAi was validated by RT-qPCR (Figures 6J and 6K). In suppression assays, a miR-124-3p mimic strongly diminished the levels of the active, phosphorylated forms of both p65 and STAT3 (Figure 6L). miR-124-3p also suppressed the total levels of STAT3 protein (Figure 6L). In complementary experiments, a neutralizing anti-miR-124-3p oligonucleotide rescued esiCDYL2 suppression of phospho-p65 and phospho-STAT3 levels in MDA-MB-231 cells, compared with a control non-targeting anti-miR oligonucleotide (Figure 6M). The reduced total STAT3 levels observed upon esiCDYL2 treatment were also rescued by anti-miR-124-3p treatment (Figure 6M). These findings indicate that CDYL2 regulates miR-124 levels, possibly by its binding upstream of *MIR124* genes, and that control of miR-124-3p levels by CDYL2 contributes to its regulation of NF- κ B and STAT3 signaling.

CDYL2 Interacts with G9a, GLP, and PRC2 Complex Components EZH2 and SUZ12

Because CDYL2 is enriched at *MIR124* genes and negatively regulates miR-124 expression, we asked if it might promote an epigenetically repressive chromatin environment at these loci. However, the epigenetic mechanism of CDYL2 is not known. By analogy with CDYL1, we speculated that it may form a complex with the H3K9 di-methyltransferases G9a, GLP, or SETDB1 (Mulligan et al., 2008) and the Polycomb Repressive Complex 2 (PRC2) core components EZH2 and SUZ12 (Zhang et al., 2011). Using immunoprecipitation (IP) assays we found that anti-CDYL2, but not a control IgG, efficiently recovered endogenous CDYL2 from MCF7 lysates and co-immunoprecipitated (coIP) G9a and its heterodimeric partner (Tachibana et al., 2005), GLP (Figure 7A). After long exposure of the western blot membrane, we also detected the presence of small amounts of EZH2 and SUZ12 in the CDYL2 IP, but at a much lower percentage of input compared



Gene set	Gene Set Name (# genes (K))	Over-lap (k)	k/K	p-val.	FDR
Up-regulated in MCF7-CDYL2	TGCCTTA MIR124A ^a (552)	18	3.20E-02	8.96E-06	8.25E-05
Down-regulated in MDA-MB-231 CDYL2 RNAi	TGCCTTA MIR124A ^a (552)	10	1.80E-02	2.57E-06	6.31E-05

^a Genes having at least one occurrence of the motif TGCCTTA in their 3' untranslated region. The motif represents putative target (that is, seed match) of human mature miRNA hsa-miR-124a (v7.1 miRBase).

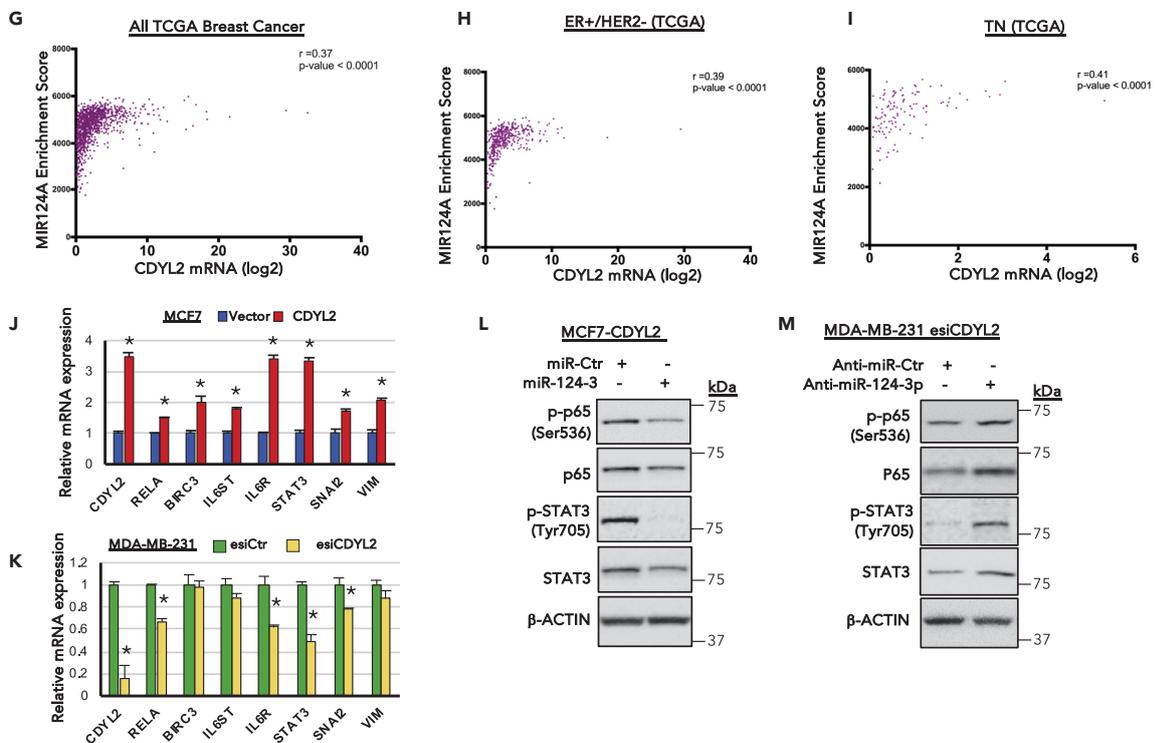


Figure 6. CDYL2 ChIP-Seq Analysis Identifies miR-124 as a Mediator of CDYL2 Regulation of STAT3 and NF- κ B Signaling

(A) Relative enrichment of CDYL2 upstream of *MIR124* genes in MCF7-Vector cells (blue) and MCF7-CDYL2 (red), as revealed by ChIP-seq analysis. Input control and gene positions relative to the peaks are shown below.

(B) ChIP-qPCR validation of data presented in (A). ChIP-qPCR signal at an unrelated negative control sequence is also shown. Shown is the mean enrichment as a percentage of input of three independent experiments, \pm SD. (* $p < 0.05$; ** $p < 0.01$, t test)

(C) As in (B), except CDYL2 or IgG ChIP was performed using chromatin prepared from MDA-MB-231 cells treated with esiLuc or esiCDYL2.

(D) qRT-PCR analysis of pre-miR-124 and miR-124-3p levels in MCF7-CDYL2 and MCF7-Vector cells. Expression was normalized to an unrelated miRNA. Data represent the mean of three independent experiments \pm SD. Significance determined by test (* $p < 0.05$; ** $p < 0.01$).

(E) As in (D), except qRT-PCR analysis was performed using microRNA (miRNA) prepared from MDA-MB-231 cells treated with esiLuc or esiCDYL2.

(F) Selected gene expression signatures enriched in the indicated RNA-seq datasets.

(G–I) Correlation between CDYL2 expression and the GSEA signature “TGCCTTA MIR124A” in the indicated TCGA breast cancer patient cohorts. The linear regression r and p value are indicated.

(J) qRT-PCR analysis of the expression of miR-124-3p target genes in MCF7-CDYL2 and MCF7-Vector cells. Data represented as mean of three independent experiments \pm SD. (* $p < 0.05$, by t test).

(K) As in (J), except qRT-PCR analysis was performed using RNA prepared from MDA-MB-231 cells treated with esiLuc or esiCDYL2.

(L and M) Western blot of phosphorylated p65 (Ser 536), total p65, phosphorylated STAT3 (Tyr 705), and total STAT3 in MCF7-CDYL2 cells treated with a miR124-3p mimic or miR control (L) or in MDA-MB-231 cells co-treated with esiCDYL2 and either an anti-miR-124-3p oligonucleotide or a control anti-miR (M). Data are representative of three independent experiments.

with G9a and GLP, suggesting a low abundance or labile interaction (Figure S6A). Reciprocal coIP assays confirmed G9a interaction with CDYL2, but did not identify CDYL2 association with EZH2 (Figures 7A and S6A). These data indicate that CDYL2 forms a complex with G9a and GLP and may interact marginally with EZH2 and SUZ12.

CDYL2 Regulates the Enrichment of G9a and EZH2 Upstream of *MIR124* Genes, as well as that of Their Cognate Methylation Marks H3K9me2 and H3K27me3

We next asked if CDYL2 might control the levels of G9a and EZH2 at a promoter-proximal region upstream of *MIR124* genes. ChIP-qPCR assays indicated that CDYL2, G9a, and EZH2 were enriched upstream of all three *MIR124* genes in both MCF7 and MDA-MB-231 cells (Figures 7B, 7C, S6B, and S6C). The enrichment of both methyltransferases was increased by CDYL2 over-expression in MCF7 cells (Figures 7B and S6B) and diminished by CDYL2 RNAi knockdown in MDA-MB-231 cells (Figures 7D and S6C). Increased levels of H3K9me2 and H3K27me3 were also observed upstream of *MIR124* genes in MCF7-CDYL2 (Figures 7C and S6B), whereas levels of H3K9me2 and H3K27me3 at these loci were decreased upon CDYL2 RNAi in MDA-MB-231 (Figures 7E and S6C). The levels of total histone H3 at these loci were not affected by CDYL2 over-expression in MCF7 or its knockdown in MDA-MB-231 (Figures 7C and 7E, left panels). The same pattern of alterations was not observed at two independent control sequences (Figures 7C and 7E, right panels; Figures S6B and S6C). These findings indicate that in addition to interacting with G9a, and weakly so with EZH2, CDYL2 positively regulates the enrichment of both methyltransferases upstream of *MIR124* genes, as well as those of the histone marks they regulate. To further probe the involvement of G9a and EZH2 in CDYL2 down-regulation of miR-124 levels, we treated MCF7-CDYL2 cells with the G9a/GLP inhibitor UNC0642 (Kim et al., 2017) or the EZH2 inhibitor CPI-169 (Bradley et al., 2014). We observed that UNC0642 treatment indeed resulted in increased levels of miR-124-3p (Figure S7A), relative to vehicle controls. However, treatment with CPI-169 at a range of doses did not affect miR-124-3p levels after 3, 4, or 5 days of treatment (Figure S7B and data not shown). This result further supports the importance of G9a/GLP methyltransferase activity in regulating miR-124 levels, but argues that inhibition of EZH2 enzymatic activity is not sufficient to antagonize CDYL2-mediated miR-124 down-regulation.

DISCUSSION

Despite the emergence of epigenetic factors as important regulators of cancer cell plasticity and malignant progression, the underlying molecular mechanisms remain poorly understood. This is due in part to insufficient characterization of several putative epigenetic factors, including CDYL2. Our study shows that CDYL2 is frequently misexpressed in breast cancer and provides a proof of principle that this could promote cellular phenotypes associated with malignant progression. We present the first insights into the genes and cellular pathways of CDYL2 controls, and the epigenetic mechanisms it engages. Based on our findings, we propose that CDYL2 up-regulation contributes to poor prognosis in breast cancer by inducing epigenetic deregulation of genes and pathways important in tumorigenesis (*MIR124*, NF- κ B, STAT3), resulting in cellular changes central to malignant progression (EMT, migration, invasion, stemness) (schematic diagram, Figure 7F).

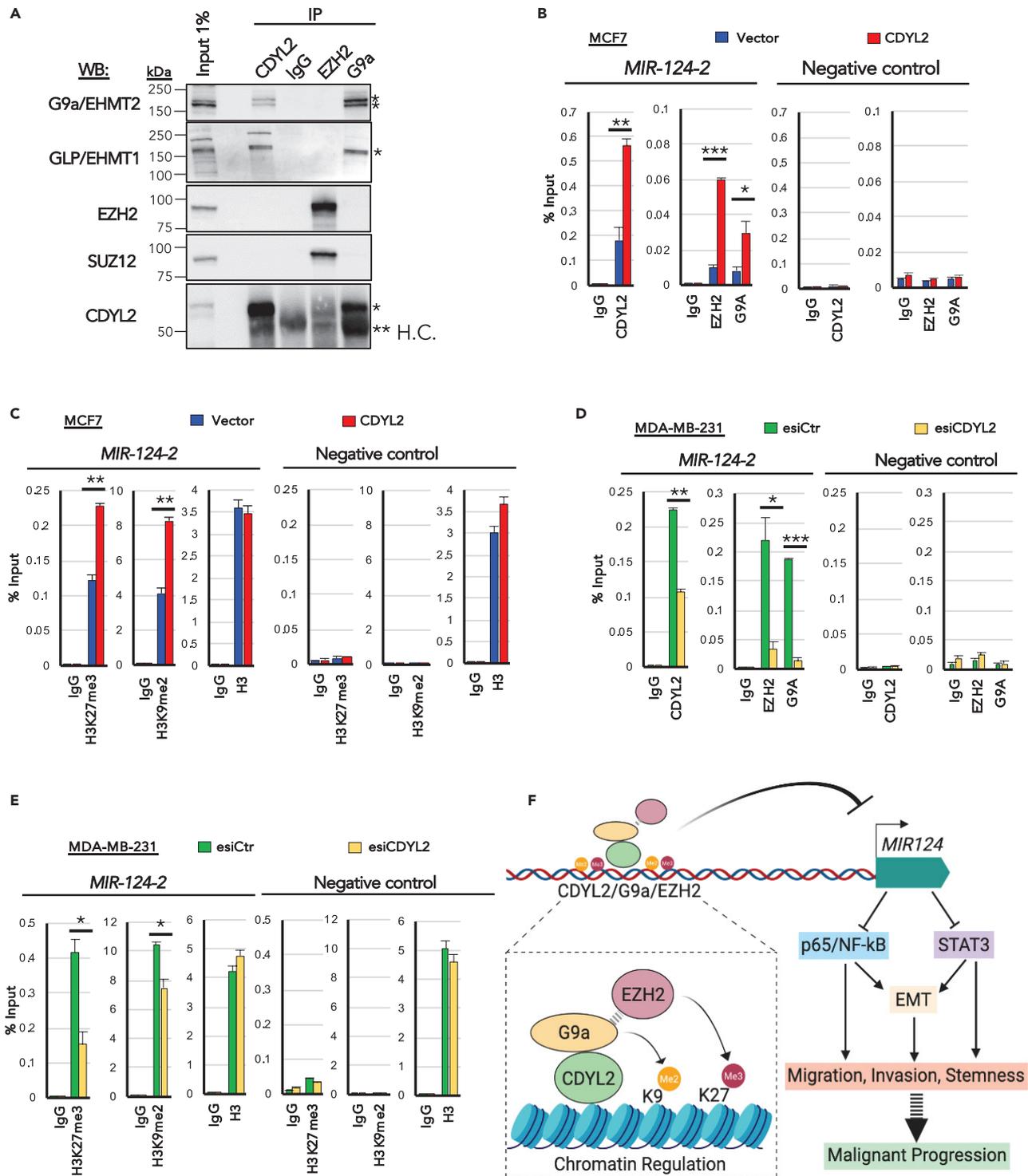


Figure 7. CDYL2 Interaction with G9a, GLP, EZH2, and SUZ12 and Its Regulation of G9a, EZH2, H3K9me2, and H3K27me3 Levels Upstream of *MIR124-2*

(A) Immunoprecipitation (IP) of CDYL2, EZH2, and G9a was performed on MCF7 cell lysates and the presence of the indicated proteins in the resulting IP eluates determined by western blotting (WB). A non-specific IgG was used as negative control. Input lysate was used to assess the relative strength of each coIP signal. The experiment was repeated three times with similar results. (*specific band; **H.C., IgG heavy chain).

Figure 7. Continued

(B) ChIP-qPCR analysis of the relative occupancy of CDYL2, EZH2, and G9a upstream of *MIR124-2* in MCF7-CDYL2 compared with MCF7-Vector cells. IgG, negative control ChIP. qPCR analysis was also performed at an unrelated negative control sequence. Shown is the mean enrichment as a percentage of input of three independent experiments, \pm SD. Significance was determined by t test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

(C) As in (B), except using antibodies specific to H3K9me2, H3K27me3, H3, and IgG. These ChIP analyses were conducted using the same lysates as in (B), so are paired analyses.

(D and E) Experiments were conducted as described in (B) and (C), except using chromatin lysates prepared from MDA-MB-231 cells treated with esiCDYL2 or esiLuc.

(F) Schematic model of the proposed contribution of CDYL2 to epigenetic regulation of *MIR124*, cell signaling, and malignancy-associated cellular processes.

Although we predicted CDYL2 to be an epigenetic repressor of transcription due to its homology to CDYL1 (Dorus et al., 2003; Fischle et al., 2008), this was not previously demonstrated. We have shown that CDYL2 is localized in the nuclear fraction of cells and binds to chromatin upstream of the *MIR124* genes. Further implicating CDYL2 as an epigenetic repressor of transcription, CDYL2 over-expression in MCF-7 cells both increased its enrichment upstream of *MIR124* genes and decreased the levels of *MIR124* transcripts. Treatment of MCF7-CDYL2 cells with the G9a/GLP inhibitor UNC0642 increased expression of miR-124-3p, indicating that the catalytic activity of G9a and/or its heterodimeric partner GLP (Tachibana et al., 2005) is important for the CDYL2-mediated repression of *MIR124* genes. Meanwhile, CDYL2 RNAi in MDA-MB-231 cells had the opposite effect. Our coIP data suggest that CDYL2 might regulate G9a levels at *MIR124* genes via a mechanism involving physical association of the two factors, whereas they only weakly support this possibility in the case of EZH2. We speculate that an indirect mechanism could account for the strong effects of CDYL2 over-expression and RNAi on EZH2 enrichment upstream of *MIR124* genes, such as the previously described regulation of EZH2 levels on chromatin by G9a methyltransferase activity (Mozzetta et al., 2014). However, the inhibition of the catalytic activity of EZH2 using the small molecule CPI-169 was not sufficient to de-repress miR-124 expression in MCF7-CDYL2 cells. This indicates that recruitment of the EZH2 methyltransferase activity to *MIR124* genes by CDYL2 is not sufficient for their repression. Taken together, our findings are consistent with the idea that CDYL2 regulation of G9a enrichment at *MIR124* genes promotes their transcriptional repression, likely via local increases in H3K9me2. Although CDYL2 also regulated the enrichment of EZH2 and H3K9me3 levels upstream of *MIR124* genes, the weak interaction between CDYL2 and EZH2/SUZ12 and failure of CPI-169 to reverse CDYL2 repression of *MIR124* genes in MCF7 cells suggests that these may be indirect and not sufficient for CDYL2 repression of miR-124.

MIR124 genes are emerging tumor suppressors commonly silenced in various cancers including breast (Conaco et al., 2006; Sullivan et al., 2009, p. 124; Wang et al., 2016a, p. 124; Wang et al., 2016b, p. 124). miR-124-3p directly targets STAT3 mRNA and antagonizes p65/NF- κ B by inhibiting multiple components of its signaling pathway. It also regulates EMT, migration, invasion, and stemness (Ji et al., 2019; Lv et al., 2011, p. 124; Wang et al., 2016a, p. 124). Importantly, we showed that CDYL2 over-expression up-regulated the levels of active STAT3 and p65 in MCF7 cells, whereas CDYL2 RNAi down-regulated their levels in MDA-MB-231 cells. These observations were consistent with the effects of CDYL2 gain or loss of function on miR-124 levels, suggesting a functional association. Further supporting this possibility, CDYL2 up-regulation of STAT3 and NF- κ B signaling in MCF7 cells was suppressed by a miR-124-3p mimic, whereas CDYL2 RNAi down-regulation of STAT3 and NF- κ B signaling was rescued by a miR-124-3p inhibitor. Our data suggest that CDYL2 might regulate STAT3 levels via direct targeting by miR-124-3p, as the levels of total STAT3 were increased or diminished in accordance with CDYL2 gain or loss of function. Owing to the diversity of miR-124-3p targets implicated in NF- κ B regulation across different cell types, it is challenging to determine which ones might connect CDYL2 with NF- κ B regulation. Based on our studies, candidates include BIRC3, RELA, and STAT3 in MCF7 cells, all of which were up-regulated upon CDYL2 over-expression and are known to link miR-124 with NF- κ B regulation (Cao et al., 2018, p. 3; Mehta et al., 2017; Wang et al., 2016a). In MDA-MB-231 cells, they include RELA and STAT3, which were down-regulated upon CDYL2 RNAi.

In accordance with the known effects of constitutive NF- κ B and STAT3 signaling in cancer cells (Banerjee and Resat, 2016; Huber et al., 2004, p.; Wu et al., 2009), stable over-expression of CDYL2 induced migration and invasiveness in MCF7 cells *in vitro*. Likewise, whereas control MCF7 cells injected into zebrafish embryos seldom metastasized, cells over-expressing CDYL2 frequently did so. In complementary experiments, we showed that CDYL2 RNAi suppressed migration and invasion of MDA-MB-231 cells, which are normally highly invasive. CDYL2 RNAi also diminished the metastatic potential of MDA-MB-231 cells

injected into zebrafish embryos. NF- κ B and STAT3 signaling also promote the emergence of breast cancer stem cells, which are believed to play crucial roles in malignant progression (Marotta et al., 2011; Shostak and Chariot, 2011; Wang et al., 2016a; Zhou et al., 2008). We showed that CDYL2 over-expression augmented mammosphere formation in MCF7 cells, suggesting an increase in the proportion of stem-like cells in the culture. Consistent with this, we also observed an increase in the proportion of cells expressing the breast cancer stem cell marker profile CD44-high/CD24-low. In complementary assays, CDYL2 RNAi in MDA-MB-231 cells decreased both mammosphere formation and the fraction of CD44-high/CD24-low cells. These findings support the notion that CDYL2 not only promotes breast cancer cell migration and invasion but also stemness. As was the case for invasion, CDYL2 induction of both invasiveness and mammosphere formation was suppressed by RNAi knockdown of either NF- κ B or STAT3, indicating key roles of these pathways in CDYL2 regulation of cancer cell biology.

It has been proposed that in certain malignancies, including breast, molecular and cellular changes that promote the emergence of mesenchymal-like cells constitute a key enabling step in the process of malignant progression (Huber et al., 2004; Puisieux et al., 2014; Sarrío et al., 2008; Shibue and Weinberg, 2017). We found that stable over-expression of CDYL2 induced morphological and molecular changes in the normally non-invasive, epithelioid, MCF7 cells, strongly indicative of an EMT. While transient transfection of MDA-MB-231 cells with siRNA targeting CDYL2 altered the expression of some genes consistent with a partial loss of mesenchymal cell identity, and impaired invasiveness and mammospheres formation in these cells, it did not induce any striking morphological changes. This is consistent with a partial reversal of the EMT state. However, when CDYL2 was knocked down for a longer duration using lentiviral RNAi vectors, we observed clusters of cells forming epithelial-like cobblestone monolayers after 2 to 3 weeks, suggesting a more advanced EMT reversal. These cells also exhibited down-regulation of several EMT-related genes (*LCN2*, *CTGF*, *FOSB*, *JUNB*, and *MYC*) that were over-expressed in MCF7-CDYL2 cells but not down-regulated by transient knock-down of CDYL2 in MDA-MB-231. This difference in gene expression may account for the observation of changes in cell morphology only after prolonged CDYL2 knock-down. In genetic suppression experiments, we found that the EMT-like gene expression program activated in MCF7-CDYL2 cells was antagonized by RNAi knock-down of either STAT3 or p65. These data argue that CDYL2 induction of migration, invasion, metastasis, and stemness in breast cancer cells might be due in part to its regulation of EMT states via up-regulation of NF- κ B and STAT3 signaling.

Overall, our studies are consistent with an oncogenic effect of CDYL2 over-expression in breast cancer. This might contribute to the poor prognosis of the patients with ER+/HER2- and TN breast cancer whose cancers express high levels of CDYL2. Although not studied in depth here, we also observed a correlation between high CDYL2 expression and poor prognosis in lung and colorectal carcinomas, hinting at a wider role in cancer. Given the emergence of epigenetic factors as viable therapeutic targets in cancer, our study supports the further evaluation of CDYL2 as a candidate drug target in breast cancer, and potentially other malignancies.

Limitations of the Study

Our study provides several lines of molecular and cellular evidence supporting roles for CDYL2 in regulating EMT, stemness, and cancer cell migration and invasion. Future studies should further extend these findings using murine models of breast cancer cell growth, invasion, and stemness. Although we showed that both G9a and EZH2 were involved in the mechanism of CDYL2 regulation of *MIR124* genes, several molecular details remain to be fully elucidated. For instance, given the weak colP between CDYL2 and EZH2, it remains to be determined how over-expression or knock-down of CDYL2 exerts comparably strong effects on EZH2 chromatin levels at *MIR124* genes. We speculate that this may be due to the previously described ability of G9a to regulate EZH2 enrichment at certain chromatin loci in a manner that depends on an intact G9a histone methyltransferase activity (Mozzetta et al., 2014), but this remains to be demonstrated in our models. An important aspect of this study is that it links the previously uncharacterized CDYL2 with pathways and genes of established importance in breast cancer, notably *MIR124* genes and signaling via the STAT3 and NF- κ B pathways. However, we do not exclude the possibility that CDYL2 regulation of other genes and pathways may also contribute to the cellular phenotypes we observed, and the role of CDYL2 in cancer.

Resource Availability

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Peter Mulligan (peter.mulligan@inserm.fr).

Materials Availability

All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement.

Data and Code Availability

The published article includes all datasets generated or analyzed during this study. They are also available via NCBI GEO: GSE150320.

METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.101141>.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance provided by Aurelie Jullien and Valery Attignon. We thank Christophe Ginestier, Toufic Renno, and Hichem Mertani for helpful technical discussions and sharing of protocols. This work was funded by grant number PLBIO2016-180 from l'Institut National du Cancer, grant number AJE20131128936 from La Fondation pour la recherche médicale, and financial support from le Centre Léon Bérard, and l'Institut national de la santé et de la recherche médicale, France (Inserm).

AUTHOR CONTRIBUTIONS

M.S.: execution and analysis of majority of experiments; writing of original and updated manuscript; supervision and mentorship of other project participants; co-ordination with other co-authors. P.M.: conceptualization, funding acquisition, project administration, supervision and mentorship of other project members, investigation, formal analysis, writing of original and updated manuscript. P.S. and H.G.: conceptualization, project administration, funding acquisition, formal analysis; M.A.M.-P.: formal analysis; A.D.D.: investigation; L.B.B.: investigation; M.O.: conceptualization, investigation; G.I.: conceptualization and investigation; L.T., A.V., M.R., J.-P.F., V.L., S.N.M., A.P., J.B., and B.G.: formal analysis.

DECLARATION OF INTERESTS

The authors have no conflicts of interest to declare.

Received: December 20, 2019

Revised: March 31, 2020

Accepted: May 4, 2020

Published: June 26, 2020

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iScience, Volume 23

Supplemental Information

CDYL2 Epigenetically Regulates

MIR124 to Control NF- κ B/STAT3-Dependent

Breast Cancer Cell Plasticity

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Supplemental Information:

Supplemental Figure Legends:

Figure S1: CDYL2 expression level is upregulated in a variety of human cancer and is associated with poor prognosis. Related to Figure 1.

(A) Oncomine analysis of cancer cohorts revealed upregulation of CDYL2 mRNA in breast, colorectal, esophagus cancers and leukaemia, but a downregulation in lymphoma.

(B,C) CDYL2 mRNA **(B)** and protein **(C)** expression in the indicated breast cancer sub-types, as derived from analysis of the TCGA and CPTAC breast cancer cohorts. P-values were calculated by t-test. NS: not significant.

(D-F) Expression of CDYL2 in normal breast tissues over or breast cancer at the indicated AJCC stages (pN0 – pN3) across all TCGA breast samples **(D)** or in the ER+/HER2- **(E)** and TN **(F)** sub-types. P-values were calculated by t-test. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001.

(G,H) Analysis of the correlation between CDYL2 expression in both ER+/HER2- **(G)** and TN breast cancer **(H)** and the 'Rizki_tumor_invasiveness-2D-UP' GSEA signature.

(I,J) Kaplan–Meier overall survival (OS) analysis performed from TCGA colorectal cancer **(I)**, or rectal adenocarcinoma **(J)**. High or low CDYL2 mRNA was based best cut-off. Significance using LogRank p-value and Hazard Ratio (CI) are indicated.

(K,L) Kaplan–Meier overall survival (OS) analysis performed from TCGA lung squamous cell carcinoma **(K)** or lung adenocarcinoma **(L)**. High or low CDYL2 mRNA was based respectively on best cut-off of and highest versus lowest quartiles. Significance using LogRank p-value and Hazard Ratio (CI) are indicated.

Figure S1

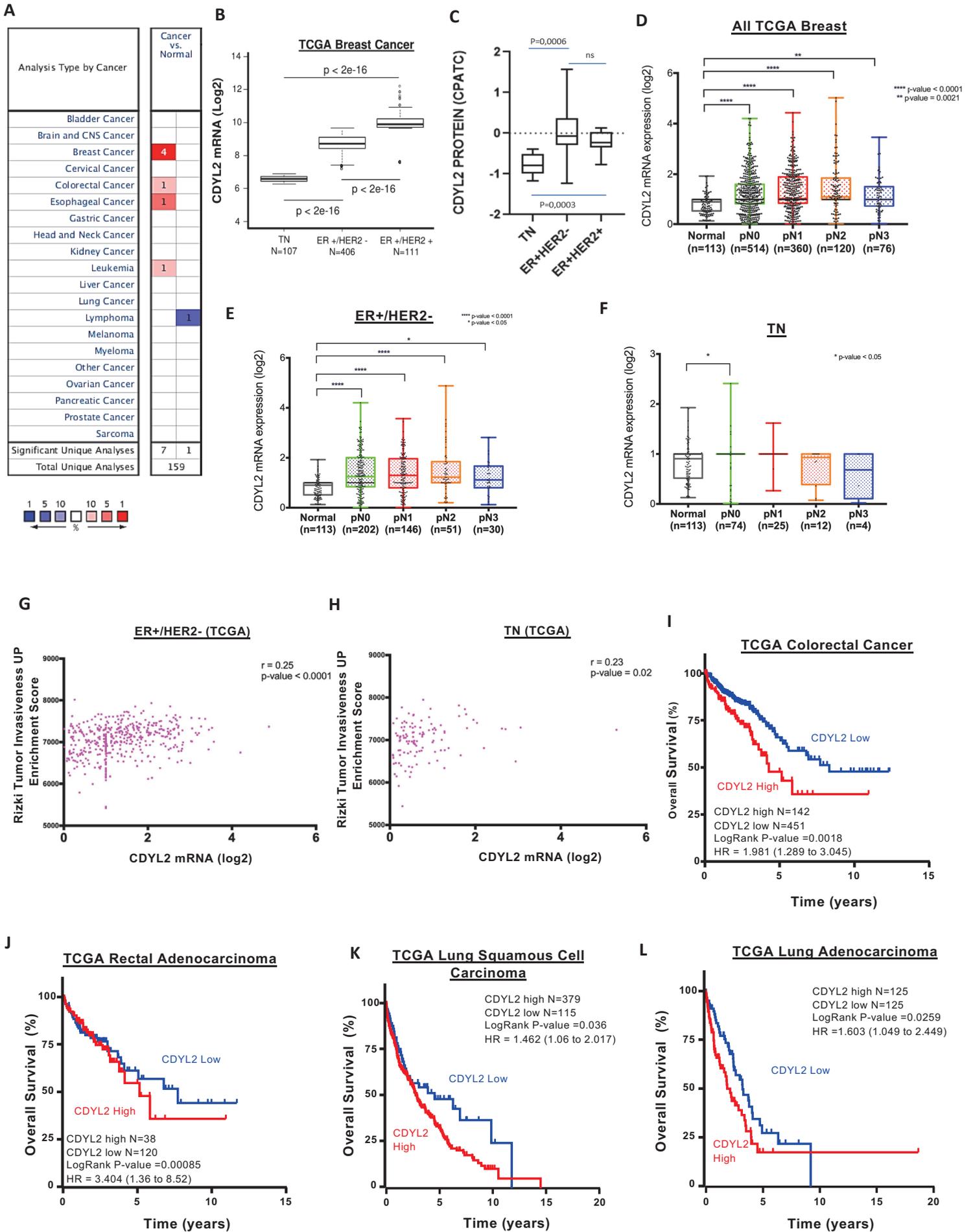


Figure S2: Analysis of the effect of CDYL2 over-expression on MCF7 cell growth and the EpCAM/CD49f profile of MCF7-CDYL2, Cama1-CDYL2 and MDA-MB-231 cells treated with CDYL2 siRNA. Related to Figures 3 and 4.

(A) Cell proliferation was monitored for 5 days by live imaging using the *Incucyte Zoom*. Images were taken every 2 hours. Values plotted represent mean \pm SD of confluency from three experiments.

(B) Colony formation assay of MCF7-CDYL2 or control (MCF7-Vector) that were seeded at low density, grown for 1 week then stained with Crystal Violet. Colonies count and size were quantified using *Fiji* image analysis software. Significance was determined by T-test ($p > 0.05$).

(C) FACS Scatter plots showing the expression of EpCAM and CD49f antigens on the indicated cell lines either expressing an empty vector or CDYL2 over-expression vector (MCF7, Cama-1) or treated with a control or CDYL2 siRNA (MDA-MB-231).

Figure S3: Stable knockdown of CDYL2 in the invasive breast cancer cell line MDA-MB-231 induces transcriptional and phenotypic changes associated with inhibition of malignancy. Related to Figure 4.

(A) CDYL2 knockdown was confirmed by western blotting as well as protein levels of EMT markers Vimentin (VIM) and fibronectin (FN). Beta-actin was probed as a loading control.

(B) 10X phase contrast micrograph demonstrating morphological differences consistent with mesenchymal to epithelial transition between MDA-MB-231 with stable knock of CDYL2 (sh-CDYL2) compared to control (sh-Ctr). Scale bar, 100 μ m.

(C) q-RT PCR validation of selected genes involved in loss of EMT. Values are normalized to GAPDH. Data are represented as mean of three independent experiments \pm S.D. Significance was determined by t-test (differences shown were significant at $p < 0.05$).

(D,E) stable CDYL2 sh-RNA in MDA-MB-231 cells decreased the rate of migration across a porous membrane **(D)** and suppressed invasiveness of cells across porous membranes overlaid with Matrigel **(E)**. Graphs shown are representative of three independent experiments in quadruple runs per condition.

(F) Mammospheres formation from cells plated at the indicated seeding number per well in 96-well plates, mammospheres with size $> 50\mu$ m counted after 8 days. Shown is a scatter plot of the results of a representative of three independent experiments indicating the median (black bar), and T-test significance (** $p < 0.01$; *** $p < 0.001$).

(G) FACS analysis of antigenic profile associated with breast cancer stem cells (CD44⁺/CD24^{low}/-).

Figure S3

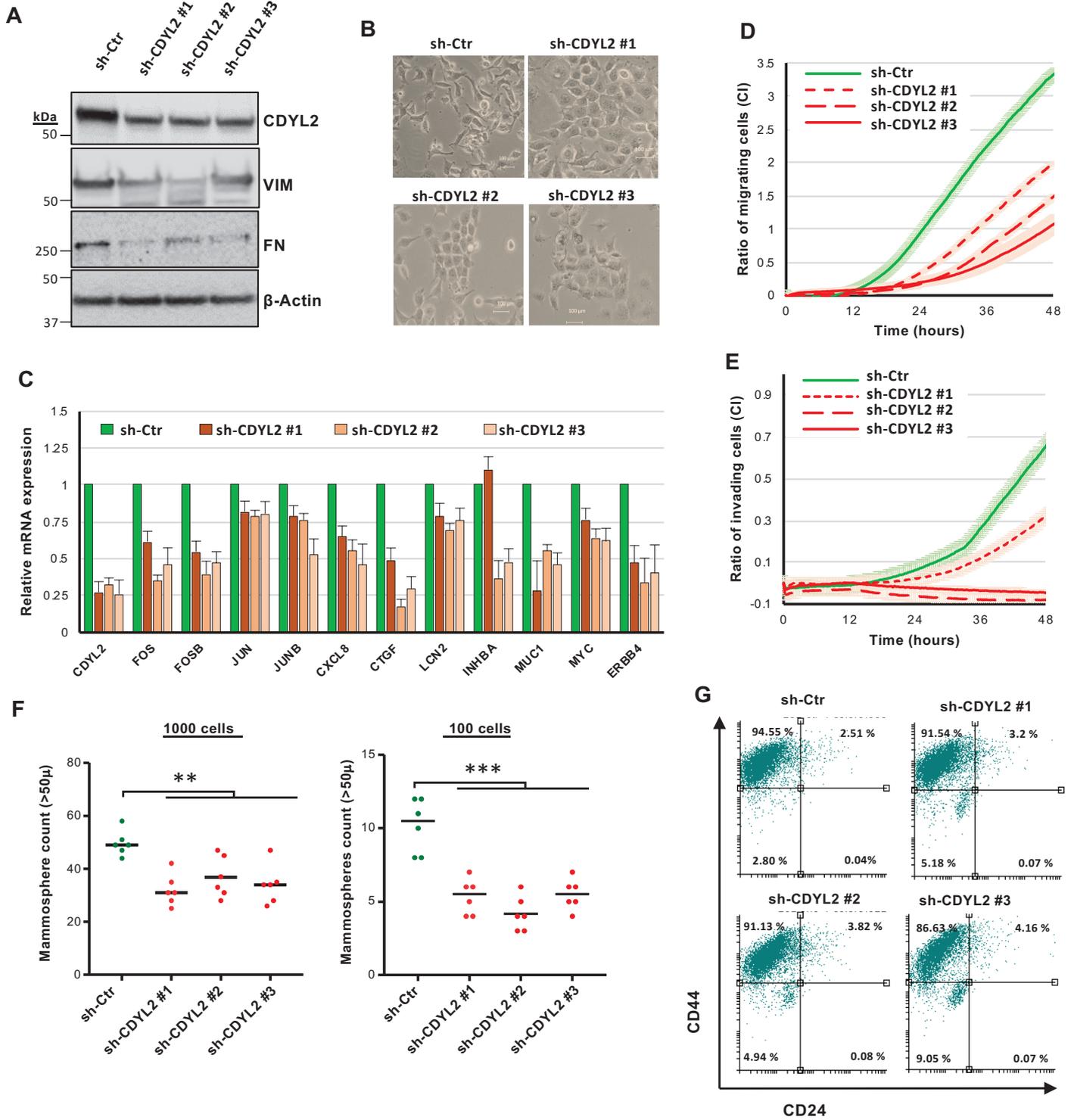


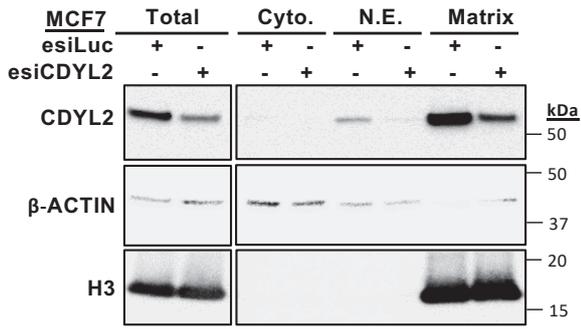
Figure S4: Nuclear localization of CDYL2. Related to Figure 6.

(A) Western blot detection of endogenous CDYL2 in several cellular fractions of MCF7 cells treated with RNAi against CDYL2 (esiCDYL2) or control (esiLuc). Total = whole cell lysate; Cyto.= Cytoplasmic fraction ; N.E. = Soluble nuclear extract ; MX = Matrix, or tight chromatin fraction.

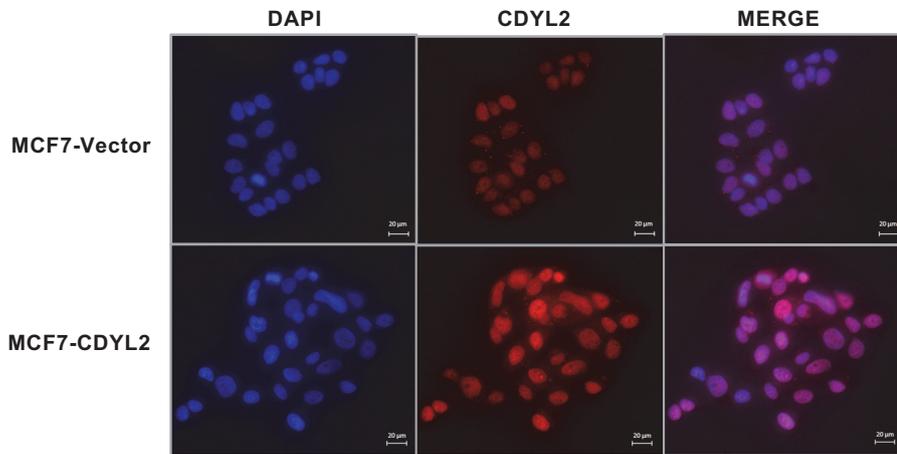
(B,C) Immunofluorescence detection of CDYL2 (red) in MCF7-Vector and MCF7-CDYL2 cells **(B)** and MDA-MB-231 cells **(C)**. Nuclear DNA is detected by DAPI staining.

Figure S4

A



B



C

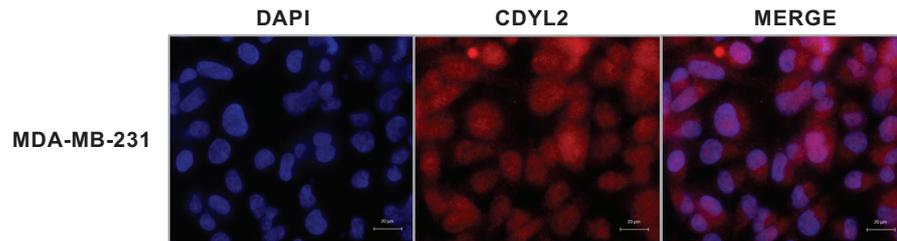


Figure S5: Stable knockdown of CDYL2 in the invasive breast cancer cell line MDA-MB-231 induces increase levels of MIR124. Related to Figure 6.

(A) The expression levels of both the precursor and mature 3' strand of mir-124 were measured by qRT-PCR. Values are the mean +/- S.D. of triplicate experiments. The differences with control are all significant with T-test (*p <0.05).

Figure S5

A

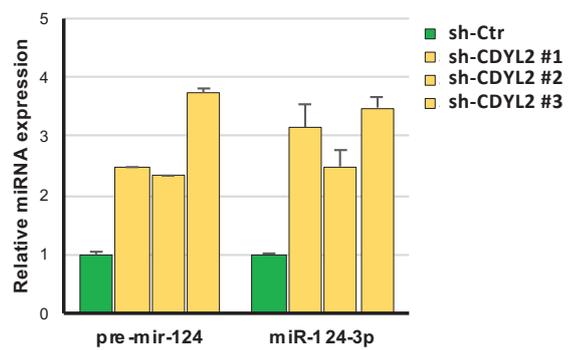


Figure S6: CDYL2 interacts with the histone methyltransferase G9a and regulates its enrichment and that of EZH2 at the *MIR124* promoter, as well as that of their cognate methylation marks. Related to Figure 7.

(A) Endogenous co-immunoprecipitation of G9a/EHMT2, EZH2 and CDYL2 using their respective endogenous antibodies. The immuno-precipitated proteins were visualized by immunoblotting. Non-specific IgG was used as a control for background binding. H.C.*= IgG Heavy Chain cross-reactivity from the IP antibody.

(B,C) ChIP-qPCR analysis of the enrichment CDYL2, G9a, EZH2, H3K9me2, H3K27me3 at *MIR124-1*, *MIR124-3* or an additional negative control region (Neg. Ctr. #2) in MCF7-CDYL2 compared to MCF7-Vector **(B)** or MDA-MB-231 treated with esiCDYL2 or esiLuc **(C)**. Enrichment is presented as percentage of Input. Values are the mean +/- S.D. of triplicate experiments. T-test: * p < 0.05, ** p<0.01; ns= not significant, p > 0.05).

Figure S6

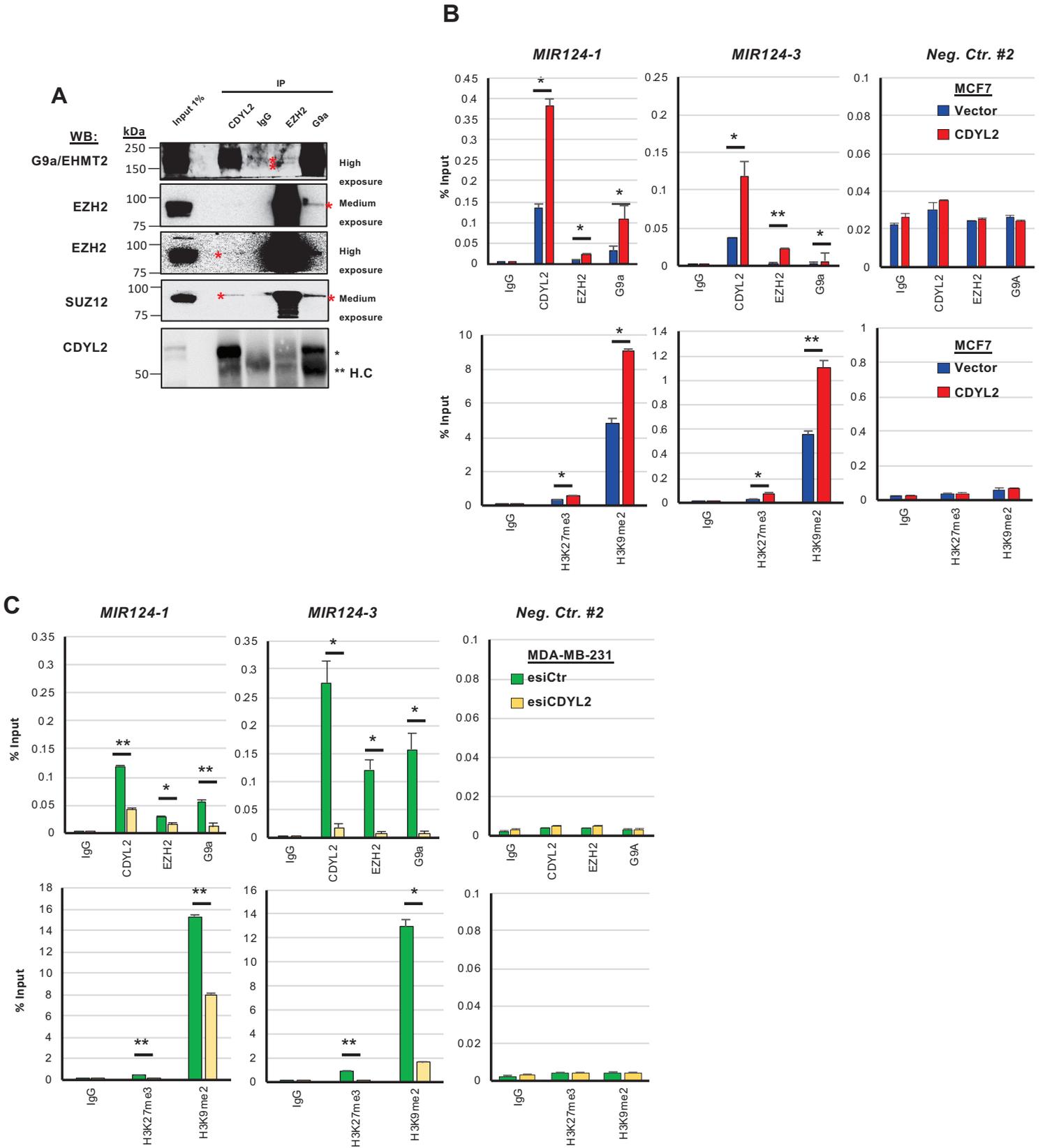


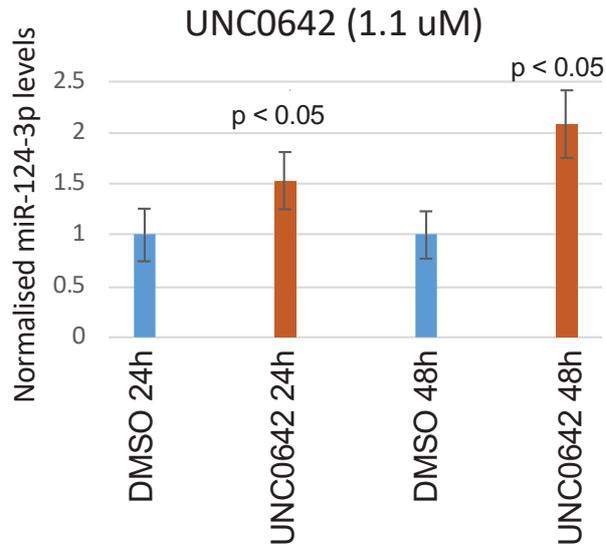
Figure S7: Analysis of the effect of the G9a/GLP inhibitor UNC0642 or the EZH2 inhibitor CPI-169 on miR-124 expression in MCF7-CDYL2 cells. Related to Figure 7.

(A) qRT-PCR analysis of miR-124-3p levels in MCF7-CDYL2 cells treated with 1.1 μ M UNC0642 for 24h or 48h. A DMSO vehicle control was included for each time point. Expression was normalized to an unrelated miRNA. Data represent the mean of three independent experiments \pm S.D. Significance determined by test, as indicated.

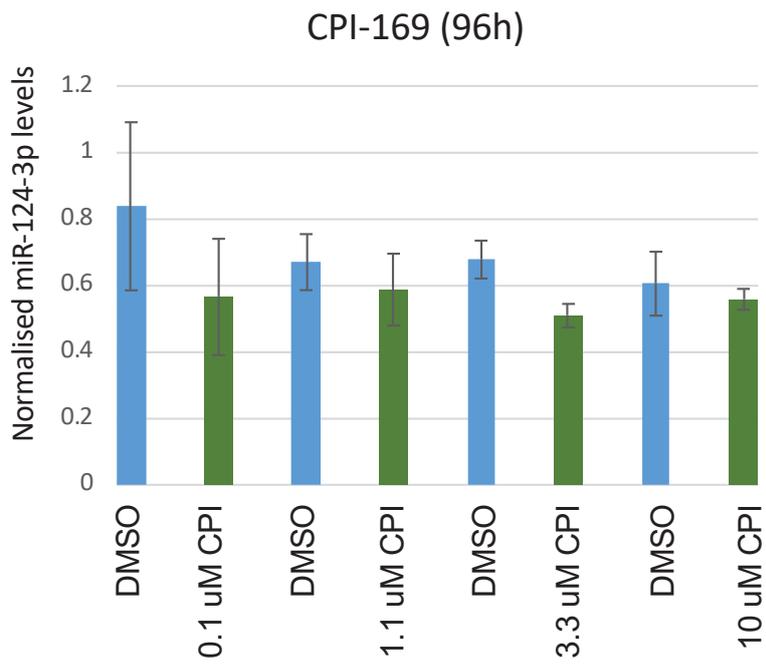
(B) qRT-PCR analysis of miR-124-3p levels in MCF7-CDYL2 cells treated with the indicated concentrations of CPI-169 for 96h. Matching DMSO vehicle controls were included for each concentration of drug used. Expression was normalized to an unrelated miRNA. Data represent the mean of three independent experiments \pm S.D. No significant differences were detected by t-test.

Figure S7

A



B



Transparent Methods:

Cell culture

MCF7 cells (ATCC, HTB-22) and their derivatives were grown in DMEM Low Glucose (Gibco, 31885-023) supplemented with 10 % of FBS (Gibco, 10270-106), 40 µg/mL of gentamicin (Gibco, 15710-049) and 0.6 µg/mL of insulin (NovoRapid, 3525909). MDA-MB-231 cells (ATCC, HTB-26) and Cama-1 cells (ATCC, HTB-21) were grown in DMEM GlutaMAX (Gibco, 10566016) supplemented with 10 % of FBS (Gibco, 10270-106), 1 % penicillin/streptomycin (Gibco, 15140122). Cells were grown at 37°C and 5% CO₂ in a humidified incubator and passaged every 2 – 4 days by trypsinization. Sustained expression of ER-alpha in MCF7 and Cama-1 was validated regularly by western blotting and immunofluorescence. Cells were regularly tested for mycoplasma using a commercial kit (ATCC, 30-1012K), and cultures renewed from low passage stocks every two months or less.

Stable expression of CDYL2 in MCF7 and Cama-1 cells

CDYL2 cDNA was cloned by PCR from an MCF7 cDNA library using the primers in Table S3 and Phusion polymerase (NEB, M0530), and inserted into the Gateway pENTR-D-TOPO vector (Invitrogen, K240020). Sequencing on both strands confirmed that the cDNA corresponded to a published CDYL2 sequence (Genbank, NM_152342.2). The cloned cDNA was then transferred into MSCV plasmid (Addgene # 41033) using LR Clonase (Invitrogen, 11791100), and the resulting expression construct validated by sequencing. MCF7 or Cama-1 cells were then stably transduced with MSCV (Vector) or MSCV-CDYL2 retroviruses and selected for 14 days using 2 µg/mL puromycin (Sigma, P8833). Expression was confirmed by western blotting and immunofluorescence using CDYL2 antibody.

RNA interference and microRNA treatments

For transient RNAi, MDA-MB-231 cells were transfected with CDYL2 esiRNA (Sigma, EHU042511), esiLuciferase (Sigma, EHUFLUC), on-target plus p65 siRNA (Dharmacon, L-003533-00), or on-target plus STAT3 siRNA (Dharmacon, L-003544-00-0005) or on-target plus control siRNA (Dharmacon, D-001810-01-05) using Interferin reagent (Polyplus, 409-10)

according to the manufacturer's instructions. Cellular assays and analysis were performed between 48 and 72h post-transfection, according to the experiment. For stable RNAi, MCF7 cells were transduced with non-targeting control shRNA pLKO.1 lentiviruses (Sigma, SHC016) or shRNA-pLKO targeting CDYL2 (Sigma, shCDYL2 #1, TRCN0000359078; shCDYL2 #2, TRCN0000130741; shCDYL2 #3, TRCN0000129278), selected for 14 days using 2 µg/mL puromycin (Sigma, P8833). Cellular assays and analysis were then performed between weeks 2 and 4 post-transduction. The Hsa-miR-124-3p MISSION microRNA Mimic (Sigma, HMI0086) and or a Negative Control miRNA mimic (Sigma, HMC0002) were transfected into MCF7-Vector or MCF7-CDYL2 cells using Interferin reagent (Polyplus). Samples were harvested for analysis 48 – 72h post-transfection. The hsa-miR-124-3p Inhibitor (Qiagen, YI04102198-ADA), and its corresponding negative control (Qiagen, YI00199006-ADA) were co-transfected into MDA-MB-231 cells along with either esiCDYL2 or esiLuciferase siRNA, using Interferin reagent. Samples were harvested 72h later for analysis.

Antibodies and reagents

The following antibodies were used: CDYL2 (MyBiosource, MBS821304), ER α (Santa Cruz, sc-8002), β -Actin-HRP (Sigma, A3854), Vimentin (Dako, M0725), E-cadherin (BD 610682), Snail/Slug (Abcam, ab85936), Twist (Abcam, ab50887), Phospho-NF- κ B p65 (Ser536) (Cell Signaling Technologies, #3031), total p65 (Cell Signaling Technologies, #3034), phospho-STAT3 (Tyr705) (Cell Signaling Technologies, #9145), total STAT3 (Cell Signaling Technologies, #9139), CD44-FITC (Miltenyl Biotec, 130-113-341), CD24-PE (Miltenyl Biotec, 130-095-953), APC anti-human CD326 (EpCAM) Antibody (Biolegend, 324208), PerCP/Cy5.5 anti-human/mouse CD49f Antibody (Biolegend, 313618), EZH2 (Cell Signaling Technologies #5246S), ChIP-grade EZH2 (Diagenode, C15410039), SUZ12 (Cell Signaling Technologies #3737S), H3K9me2 (Abcam, Ab1220), H3K27me3 (Diagenode, C15410069), rabbit IgG (Bethyl, P120-101), H3 (Abcam, Ab1791). The G9a and GLP antibodies were gifts from Y. Nakatani lab (Ogawa et al., 2002). UNC0642 and CPI-169 were obtained from Cayman Chemicals, re-suspended in cell-culture grade DMSO (Sigma), and used as described.

Immunoblot

Cells were washed with PBS, lysed in ice-cold RIPA buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM PMSF, all from Sigma) containing protease inhibitor cocktail (Roche, 04693132001) phosphatase inhibitors cocktail (Roche, 4906845001), and sonicated briefly. Cleared lysates were boiled in Laemmli buffer, resolved on Bis-Tris NuPage Gels (Invitrogen), transferred to Nitrocellulose (GE Healthcare), and probed according to the primary antibody manufacturer's protocols. Images were collected using the ChemiDoc system (BioRad).

Co-Immunoprecipitation

Cells were grown to sub-confluence in 15 cm tissue culture treated plates (Corning). Monolayers were washed three times with ice-cold phosphate buffered saline (PBS), scraped in cold PBS containing a protease inhibitor cocktail (Roche) and pelleted. The resulting cell pellets were lysed in lysis buffer (50mM Tris pH8; 150mM NaCl; 1% NP-40; 2mM EDTA; all from Sigma) containing protease inhibitor cocktail (Roche, 04693132001) and phosphatase inhibitors cocktail (Roche, 4906845001). The lysates mechanically homogenized on ice using 25G syringes (BD, #300600), then incubated for 30 min at 4°C with rotation. Lysates were centrifuged at 12000 rpm for 15 min at 4°C. The resulting supernatants were treated with DNase I (Qiagen, #79254) and RNase A (Sigma, R4875), then precleared with protein A agaroses beads for 1 hour 4°C with rotation. Immunoprecipitation was performed by incubating indicated antibodies with the lysates overnight at 4°C with rotation, followed by addition of prewashed protein A agaroses beads for 2 hours 4°C with rotation. The beads were then washed 5 times with wash buffer (10mM Tris pH8; 1mM EDTA; 1mM EGTA; 250mM NaCl; 1% Triton) containing protease inhibitor cocktail (Roche, 04693132001) phosphatase inhibitors cocktail (Roche, 4906845001). The immune-precipitated beads were boiled in Laemmli buffer and then subjected to immunoblotting.

Immunofluorescence

Cells were seeded on sterilized coverslips. Forty-eight hours after seeding, cells were fixed with 4% fresh electron microscopy-grade paraformaldehyde (Fisher, 50-980-487) diluted in PBS (Sigma) for 15 minutes at room temperature. Fixed cells were permeabilized by NP-40 0.5% (Sigma) in PBS at room temperature for 15 minutes and blocked with 5% FBS in a PBS/0.1% NP-40 buffer, at room temperature for 1 hour. Coverslips were then probed with

the indicated primary antibodies 1h at room temperature and then Alexa dye tagged secondary antibody (see above) for 1 h at room temperature. Coverslips were mounted using DAPI mounting medium (Vectashield; Vector Laboratories) and observed under the upright microscope (Zeiss axioimager, SIP 60549), images were analyzed using Zen software (Zeiss).

Flow cytometry

The cells were labeled with anti-CD44-PerCP-Cy 5.5, anti-CD24-PE, APC anti-human CD326 (EpCAM) Antibody, PerCP/Cy5.5 anti-human/mouse CD49f Antibody, according to the manufacturer's instructions. All analyses were performed using a BD FACSCalibur flow cytometer and BD CellQuest software (BD Biosciences).

Gene expression

RNA was extracted using TRI-reagent (Sigma, T9424), and cDNA synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368814). miRNA was extracted using the miRNeasy Mini Kit (Qiagen, #217004) following the manufacturer's instructions, and cDNA synthesized using miScript II RT Kit (Qiagen,#218161). The RT-qPCR was performed using the Fast SYBR Green 2X Master Mix (Applied Biosystems, #4385610) and an LC480 PCR machine (Roche). Primers are described in Table S3.

RNA-seq and enrichment analysis

RNA libraries were prepared with the TruSeq Stranded Total-RNA kit and sequenced on a Illumina NextSeq sequencing machine. After careful quality controls, raw data were aligned on the human genome (hg38) with STAR v2.7.0f (Dobin et al., 2013) and default parameters. Read counts on each genes of the Gencode annotation v29 were produced by STAR.

Unless otherwise specified, the analyses were performed using R (v 3.4.4) and illustrations produced with the ggplot2 (Wickham, H., 2016) and ggpubr packages. Starting from raw counts, we used the R package DESeq2 (Love et al, 2014) (v1.14) to perform the differential expression analyses. Each time the design was set as $\sim REP + TYPE$, where REP refers to the replicate number (paired analysis) and TYPE to the treatment group. Differential expression was tested using the Wald test, and p-values were corrected with the Benjamini-Hochberg method. To test the pathway enrichment of a list of genes, we used the R packages

clusterProfiler (Yu et al, 2012) (v 3.8.1), msgidbr (v 6.2.1), org.Hs.eg.db (v 3.5.0) and reactomePA (v 1.24) (Carlson, M, 2013). We tested the list of genes against pathways from msigdb hallmarks, GO molecular functions, KEGG and Reactome. Over-representation p-values were corrected with the Benjamini-Hochberg method.

Chromatin immunoprecipitation

For MDA-MB-231 ChIP-qPCR analysis, cells grown in 6-well plates were treated as described, then cross-linked by the addition of fresh 1% methanol-free formaldehyde to the cell culture medium for 10 minutes, followed by the addition of glycine to a final concentration of 125 mM for 5 minutes. Monolayers were washed three times with ice-cold PBS, scraped in cold PBS containing a protease inhibitor cocktail (Roche) and pelleted. Chromatin lysates were prepared from the cross-linked pellets, and ChIP assays performed using a commercial kit (Cell Signaling Technologies, #9003), with the indicated antibodies. Quantitative PCR was performed using the primers listed in Table S3. Input chromatin was purified in parallel in each ChIP assay and used to validate the chromatin digestion efficiency, and to determine the percentage of input recovered in each ChIP assay.

For MCF7 ChIP-seq and ChIP-qPCR analysis, cells were grown to sub-confluence in 15 cm tissue culture treated plates (Corning), cross-linked, washed and pelleted as described for MDA-MB-231 cells, above. The resulting cell pellets were pre-treated by incubating in lysis buffer A (Lysis Buffer 1 (50 mM HEPES pH 7.5; 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, 1× protease inhibitors) for 10 minutes at 4°C, then lysis buffer B (10 mM Tris-HCl pH 8.0; 200 mM NaCl; 1 mM EDTA; 0.5 mM EGTA; 1× protease Inhibitors) for 10 minutes at room temperature, as previously described (Lee et al., 2006). They were then lysed in buffer C (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, 1× protease inhibitors) supplemented with 0.5% SDS, incubated on ice for 30 minutes with occasional vortexing, then sonicated on ice to an average fragment size of 150 bp using a sonicator (Branson, Digital Sonifier 450). The sonicated lysate was centrifuged at 12,000 r.p.m. in a benchtop centrifuge at 4°C, the supernatant diluted five times in buffer C, and 1 mL aliquots of this added to 50 µL of magnetic protein A beads (Invitrogen) pre-coated with 5 µg of anti-CDYL2 IgG. These were incubated overnight at 4°C with rotation, then pelleted and

washed with two sequential additions each of wash buffer 1 (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8, 150mM NaCl), wash buffer 2 (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8, 500mM NaCl), wash buffer 3 (0.25M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10mM Tris-HCl, pH 8), and TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0, 50 mM NaCl). Chromatin was eluted by incubating for 30 minutes at 65°C in elution buffer (50 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 1% SDS) with frequent vortexing. Crosslinks were reversed by overnight incubation at 65°C, and eluates treated with RNase A (Sigma) for 2h, followed by Proteinase K for 2h, then extracted using a classical Phenol-Chloroform/Ethanol precipitation protocol.

ChIP-seq

Pair end DNA sample libraries were sequenced using Illumina. Raw sequences were aligned to human genome hg19, using Bowtie 2.0 (Langmead and Salzberg, 2012) with paired-end parameters. Normalized and subtraction bigwig files were obtained using deepTools (Ramírez et al., 2016). Analysis of ChIP-Seq data was in the galaxeast.fr instance. Significant peaks were called using MACS2 (Zhang et al., 2008, p. 2). Called peaks were annotated using Homer_AnnotatePeaks.

Colony formation assay

To form adherent colonies, 3000 cells were seeded in 12 well tissue-culture treated plates. After 14 days of growth, all colonies were stained with crystal violet solution (crystal violet 0.05%, formaldehyde 1%, methanol 1%) for 20min, washed extensively with water. Colonies were counted and their size was measured using *Fiji* software.

Migration and invasion assays

Real-time cell migration and invasion were measured using the *xCELLigence* RTCA DP apparatus (Acea Biosciences, Inc.) according to the manufacturer's protocol. Briefly, 40,000 cells were prepared in serum-deprived medium and then added to the upper chambers of *CIM-16 plates* (AceaBio, #5665817001). Complete medium containing 10% FBS was added to the lower chamber. For invasion assays, Matrigel diluted in serum-free medium at 500µg/mL (BD Biosciences, #354234) was added to the upper chamber and allowed to set before adding

cells. Migration across the membrane separating the two chambers was expressed as the Cell Index (CI).

Mammosphere formation assay

Cells were seeded at several densities (10, 50, 100, 1000 cells per well) in 96-well ultra-low attachment plates (Corning, #3474) with MEBM Basal Medium (Lonza, CC-3151) containing 2% B-27 (Invitrogen, 17504-044), 20 ng/mL EGF (Sigma, E9644), 4 µg/mL insulin (Novo Nordisk, # 3525909), and 2 µg/mL hydrocortisone (Sigma, H0135). Mammospheres were cultured for 1-2 weeks, with image collection approximately every three days starting at day 8. Whole-well images were taken with the *IncuCyte ZOOM* System (Essen Bioscience) using a 4X phase contrast objective. Mammosphere diameter and the number of mammospheres >50µm were determined by image analysis using Fiji software (Fiji).

Zebrafish embryo metastasis assay

Zebrafish embryos were raised under standard experimental conditions. Cells trypsinated, resuspended in serum-free media, and stained with lipophilic dyes DiO or DiD from the Vybrant Multicolor Cell-Labeling Kit (Invitrogen, V22889) for 20 minutes at 37°C, then washed resuspended in PBS 1x. 48 hours post-fecundation, the embryos were dechorionated and anesthetized with tricaine (Sigma-Aldrich, E10521). The anesthetized embryos were subjected to microinjection. 20nl of cell suspension, which represent approximately 300 labeled human cells, were injected into perivitelline space of each embryo. The injected zebrafish embryos were immediately placed at 30°C for 24 hours in presence of N-phenylthiourea (Sigma-Aldrich, P7629) to inhibit melanocyte formation. For metastasis assessment, The anesthetized embryos were evaluated using the fluorescent microscope Axio Observer Zeiss microscope (Zeiss).

Statistical analysis

CDYL2 expression levels from *TCGA* were stratified based on molecular markers such as ERα and HER2 expression by IHC. Correlation analysis between CDYL2 RNA and protein levels were performed using *GraphPad Prism7*. For overall survival analysis, patients were divided into

two groups (low and high CDYL2) using the expression level of CDYL2 and best cutoff. Kaplan-Meier survival plots, log-rank p-values, hazard ratios were calculated using GraphPad Prism7.

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Discussion

10 RESULTS DISCUSSION

10.1 CDYL2 LOCALIZATION AND REGULATION THROUGH THE CELL CYCLE

Despite showing more and more relevance in diseases (Kim *et al.*, 2014; Siouda *et al.*, 2020; Wu *et al.*, 2013), including several cancer types, CDYL family proteins remain poorly characterized, and their cellular functions mostly unknown. This is particularly the case for CDYL2, which was shown by us and others to be implicated in breast cancer growth and invasiveness (Siouda *et al.*, 2020; Yang *et al.*, 2020). In this study, my work aimed at characterizing CDYL2 cellular function and provided strong evidence that CDYL2 is necessary for faithful mitosis. Knocking down CDYL2 induced notably a strong defect in chromosome alignment during metaphase and defects in chromosome shape. Defects in chromosome shape include loss of cohesion between sister chromatids, entangled chromatids, and curly chromosomes (Fig. 11). These phenotypes are similar to some extent to the ones observed after the knock-down of proteins involved in mitosis, notably proteins involved in the cohesin complex, in H3K9 trimethylation, or CHAMP1. Indeed, knock-down of RAD21 in HeLa cells for example induced aberrant separation of sister chromatids and triaxial alignment of chromosomes during metaphase (Díaz-Martínez *et al.*, 2010). Lack of cohesion establishment, by knocking-down ESCO proteins also induced aberrantly separated sister chromatids and curly chromosomes (Ivanov *et al.*, 2018). Lack of sister chromatids cohesion in metaphase could also be observed in cells lacking H3K9me3 at pericentromeres after knock-out in SUV39h in MEF cells (Koch *et al.*, 2008) and HeLa cells knocked-own for HP1 α (Kiyomitsu *et al.*, 2010). Finally, the defects in chromosomes alignment I observed after CDYL2 knocked-down was similar to the one observed after CHAMP1 knock-down (Itoh *et al.*, 2011). Taken together, CDYL2 seems implicated in mitosis, but its mechanism in this context remains unknown. My thesis aimed at characterizing CDYL2 implication in mitosis and the mechanisms behind it, which would be the first analysis of a CDYL protein in the mitotic context.

Since CDYL2 is implicated in mitosis, I wondered if its level is regulated by the cell cycle. It seems, from my experiments that the total level of CDYL2 is lower during the mid and late

stages of the S-phase (Fig. 13). Similarly, its level on the chromatin seems to increase around mitosis and stays higher during the G1-phase, suggesting a function in these times (Fig. 14). The change of CDYL2 level during the cell cycle was however not as dramatic as the one observed with cyclins, proteins directly regulating cell cycle progress (Hayward *et al.*, 2019; Hwang *et al.*, 1995). This may suggest that CDYL2 is regulating mitosis through an indirect mechanism, potentially by interacting or regulating essential proteins or complexes, as cohesin, CHAMP1, or repressor complexes.

CDYL family was described to bind H3K9me3 modification *in vitro* (Fischle *et al.*, 2008). *In vivo*, using the MEF cell line, CDYL1 was found to colocalize with pericentromeres in an H3K9me3 dependent manner (Fischle *et al.*, 2008; Franz *et al.*, 2009). Fischle and colleagues also briefly studied the localization of CDYL2 in the same cell line model but remained inconclusive whether the protein localizes at pericentromeric heterochromatin (Fischle *et al.*, 2008). In MCF7 and HeLa cells, I found a systematic colocalization between CDYL2 and CREST during interphase as no interphase cells could be found lacking it (Fig. 16). To confirm that this localization is dependent on H3K9me3, I obtained MEF cells lacking this modification after knocking-out of the SUV39H1 and SUV39H2 proteins. This model was already used to describe HP1 α and CDYL1 localization at pericentromeres through H3K9me3 (Fischle *et al.*, 2008). We confirmed that the technique is working in our hands by using cells expressing both proteins as positive controls (Fig. 18A and B). In mouse embryonic fibroblasts, the colocalization between CDYL2 and pericentromeres was not systematic in opposition to HeLa and MCF7 cells (Fig. 18 D and C). This difference can be explained by a variation in infection efficiency as, due to the low levels of CDYL2 in MEF cells, the endogenous protein cannot be stained correctly for immunofluorescence. We thus used as was described by Fischle and colleagues, a retroviral infection to increase CDYL2 level and this infection probably induces intercellular variation for the protein expression. The lower colocalization frequency found in cells transfected with flag-tagged human CDYL2 despite high CDYL2 signal can potentially come from the Flag-tagged, disturbing the binding between CDYL2 chromodomain and H3K9me. It can also be explained by a lower transfection efficiency or due to the species change. Still, the fact that human CDYL2 can localize to pericentromeric regions of mouse cells, indicated that the protein is highly conserved. Taken together, we observed that CDYL2 localization at

pericentromeres is dependent on H3K9me3, similar to what was observed previously by others for CDYL1 (Franz *et al.*, 2009).

I could also observe CDYL2 localization at pericentromeres in mitotic MCF7 cells, however, this colocalization disappeared upon removal of soluble proteins using CSK buffer treatment before immunostaining (Fig. 16C), suggesting a weaker interaction of CDYL2 with the chromatin during mitosis compared to interphase cells. Interestingly, mitosis is notably epigenetically characterized by phosphorylation of H3S10 by Aurora B. My observation is therefore concordant with *in vitro* analysis showing that H3S10ph inhibits CDYL proteins chromodomain interaction with H3K9me3 (Fischle *et al.*, 2008). Interestingly, HP1, another chromodomain protein, despite its interaction with H3K9me3 also being inhibited by H3S10ph (Hirota *et al.*, 2005), is still localized at pericentromeres during mitosis through interaction with NSL1, allowing it to stay at pericentromeres during mitosis (Kiyomitsu *et al.*, 2010). As I could observe colocalization of CDYL2 and CREST in mitotic cells when they were not pre-extracted and a fraction of CDYL2 remained on the chromatin in MCF7 cells pre-extracted before immunofluorescence staining (Fig. 15), a similar mechanism may be working for CDYL2. However, since only a small fraction remains and is easily removed with pre-extraction, it is also possible that most CDYL2 function at pericentromeres is completed before mitosis onset. In HeLa cell lines the small fraction at pericentromeres during metaphase could not be observed (Fig. 15). In my hands, HeLa cells were more sensitive to the pre-extraction, one hypothesis is thus that the remaining fraction of CDYL2 during mitosis in HeLa cells is washed away during the pre-extraction.

Finally, I also observed that CDYL2 is localized at centrosomes throughout the cell cycle (Fig. 19 and 20). As CDYL2 is an epigenetic protein, recognizing histone methylation, it was first surprising to find it outside of the nucleus. However, CDYL family chromodomains were shown in *in vitro* analysis to recognize non-histone proteins as well, such as methylated EHMT2 (Fischle *et al.*, 2008). It would be interesting to study if CDYL2 chromodomain is necessary for its localization at the centrosomes and if it recognizes non-histone protein methylation.

10.2 MECHANISMS OF CDYL2 IMPLICATION IN MITOSIS

Phenotypes induced by knock-down of CDYL2 indicate that CDYL2 is necessary for a faithful mitosis. Its localization during the cell cycle suggest that its function takes place at pericentromeres or centrosomes. To better apprehend the mechanism of CDYL2 action and find potential interactors, we performed a broad-range mass spectrometry analysis. This unbiased interactome analysis is the first one performed on CDYL2. From the previous CDYL2 characterization and the proteins found in the mass spectrometry analysis, three hypotheses were formulated to explain CDYL2 implication in mitosis and are discussed in the following paragraphs: (i) Considering CDYL2 localization at pericentromeres and its interaction with epigenetic silencing factors, is CDYL2 implicated in pericentromeric repeat sequences silencing? (ii) Considering again CDYL2 enrichment at pericentromeres, loss of chromosome cohesion upon CDYL2 knock-down, and the presence of cohesin proteins in the mass spectrometry analysis, is CDYL2 interacting with cohesin to regulate its recruitment at pericentromeres? and (iii) Since CDYL2 is interacting with CHAMP1, a protein known to be indispensable for chromosome alignment and microtubules-kinetochore attachment, is CDYL2 working with CHAMP1 to connect microtubules and kinetochores?

10.2.1 CDYL2 as a pericentromeric repetitive sequence repressor

Several epigenetic proteins involved in transcription repression were found in the mass spectrometry analysis, as EHMT1, EHMT2, SETDB1, DNMT1, HDAC1 and HDAC2 (Fig.21). I confirmed CDYL2 interaction with EHMT1, EHMT2, SETDB1 and DNMT1 using coimmunoprecipitation followed by Western Blot (Fig. 22). Interestingly, WIZ, a protein known to interact with both EHMT1 and EHMT2 and recruiting both complex to chromatin (Bian *et al.*, 2015), was found in the mass spectrometry analysis, but also in previous broad-range interaction analyses (Hein *et al.*, 2015; Kuppaswamy *et al.*, 2008). CDYL1 was also found to interact with WIZ protein (Mulligan *et al.*, 2008). To further validate the interaction with epigenetic repressor, I performed as well a reverse immunoprecipitation of EHMT2 (Siouda *et al.*, 2020). False-positive interactions can sometimes be reported in coimmunoprecipitation assays when the proteins are all binding to the same polynucleotide strand that gets immunoprecipitated as well. To avoid such artifacts, the

immunolysates were treated with DNase and RNase and the interactions between EHMT2 and SETDB1 could still be reported, strongly validating CDYL2 interaction with the two proteins. Interestingly, EHMT2 and SETDB1 were already described to interact with CDYL1 and work together to repress gene transcription (Mulligan *et al.*, 2008).

We and others already described CDYL1 and CDYL2 to have repressive functions, by recruiting EHMT2 and potentially EZH2 to chromatin and induce histone dimethylation and trimethylation (Caron *et al.*, 2003; Y. Liu *et al.*, 2017a; Mulligan *et al.*, 2008; Siouda *et al.*, 2020). I further confirmed during my thesis, that CDYL2 is interacting with epigenetic repression factors and hypothesized that CDYL2 could be necessary for the repression of satellite repeats at pericentromeres. Indeed, a derepression of centromeric and pericentromeric satellite sequences and accumulation of their transcripts are linked with genomic instability and notably a loss of chromosome cohesion (Bouzinba-Segard *et al.*, 2006; Hall *et al.*, 2012). I used commercially available primer pairs for α -Satellite and chromosome 1 specific Satellite II and did not observe a change in satellite repeat expression, neither could we observe a change in their H3K9 methylation grade (Fig. 23). I then used Taqman probes targeting retroviral repeat sequences K111 and K222. K222 was shown to be specific to pericentromeres, while K111 is present on both centromeric and pericentromeric regions (Contreras-Galindo *et al.*, 2013; Zahn *et al.*, 2015). These probes are additionally present in multiple copies on multiple chromosomes. The specificity of the sequences was further confirmed as I obtained their enrichment after a ChIP on H3K9me3, a marker of pericentromeres compared to ChIP performed with a negative control antibody (Fig. 24C). The original publication describing the development of the probes additionally tested them in CENPA and CENPB ChIP assays (Contreras-Galindo *et al.*, 2013; Zahn *et al.*, 2015). This confirmed the specificity of both probes to the detection of pericentromeric and centromeric, rather than non-repeat sequences and validate the pertinence to use them to observe histone methylation changes at those regions. Finally, the linearity between the fluorescence of the probes and the sequence quantity was verified in my hands using a dilution curve of input samples (Fig. 24A and B). However, CDYL2 RNAi did not induce a change in K111 or K222 repeat elements expression as determined by qPCR or in the methylation grade of their genomic sequence (Fig. 25). It remains however possible, even though unlikely, that CDYL2 is regulating

repeat elements transcription that is not covered by the probes I used. However, the observation of CDYL2 being dispensable for repeats sequences repression is concordant with a previous publication, where several chromodomain proteins, including CDYL2, were found to be dispensable for retroviral repeats sequences silencing in mouse embryonic stem cells (Maksakova *et al.*, 2011). To conclude this hypothesis more confidently, it could be interesting to perform broad range RNA-seq after CDYL2 knock-down with parameters allowing us to analyze precisely repeats expression.

10.2.2 CDYL2 as a Regulator of Chromosome Cohesion

Pericentromeres are also a region where sister chromatid cohesion is established and has to be maintained during metaphase. Other chromodomain proteins were previously described to be implicated in chromosome cohesion. This is for example the case for Swi6 in yeast, which recruits cohesin complex to pericentromeres (Nonaka *et al.*, 2002). In humans, HP1 α and HP1 γ , two Swi6 homologs, also regulate cohesion at metaphase, but they do it by recruiting Haspin, a cohesin protector, and not through direct binding of cohesin (Yi *et al.*, 2018). The mass spectrometry analysis of CDYL2 immunoprecipitation revealed interactions of the protein with both RAD21 and SMC3, two proteins of the cohesin complex. Interaction between CDYL2 and RAD21 could be confirmed in MCF7 cell lines with endogenous levels of CDYL2 through coimmunoprecipitation and immunoblotting (Fig. 26A). Even though they are not always considered as strict members of the complex, PDS5 proteins, which include PDS5A and PDS5B are stoichiometric interactors and regulators of cohesin (Litwin and Wysocki, 2018). Interaction of CDYL2 and PDS5B was observed in MCF7 cell line through coimmunoprecipitation followed by immunoblotting, further confirming the interaction between CDYL2 and the cohesin complex. Finally, the interaction was further validated with PLA between CDYL2 and SA1 in HeLa cell lines. Close proximity between the two proteins could be detected in all cells, both at interphase and mitotic phases (Fig. 26B). Specificity of the detected signal was tested by performing a PLA with either CDYL2 or SA1 antibody. In both cases, when using only one primary antibody, no fluorescence signal could be detected, confirming the close proximity observed between SA1 and CDYL2 (Fig. 26B). Interestingly, the proximity between CDYL2 and SA1 did not seem enriched at pericentromeres but was rather found overall in the nucleus. In particular, in mitotic cells, proximity signals were found out of

chromatin as well. The cytoplasmic proximity signal between CDYL2 and SA1 during mitosis could indicate that both proteins remain close after dissociation of cohesin from the chromatin. Overall, my data support a physical interaction of CDYL2 with the cohesin complex.

Through physical interaction with the cohesin, CDYL2 could, similar to Swi6 function in *S. pombe*, recruit the complex to the pericentromeric heterochromatin. Immunofluorescence staining followed by confocal microscopy analysis was used to observe cohesin localization at pericentromeres after CDYL2 RNAi, similar to what was described previously (Serrano *et al.*, 2009). Using this technique, I could not see a significant difference in cohesin loading specifically at pericentromeres (Fig. 27), indicating that CDYL2 is dispensable for cohesin localization at pericentromeres during metaphase. CDYL2 seems therefore not necessary for cohesin complex loading on the chromatin and protection during early mitosis as are Swi6 or HP1 (Nonaka *et al.*, 2002; Yi *et al.*, 2018, p. 1).

10.2.3 CDYL2 as a CHAMP1 partner

Another interactor protein found in the mass spectrometry analysis that could have explained the mitotic defects observed after CDYL2 knock-down is CHAMP1. CHAMP1 is a protein necessary for the recruitment of kinetochore proteins to the centromeres and thus kinetochore-microtubule attachment (Itoh *et al.*, 2011; Okamoto *et al.*, 2017). CHAMP1 interaction with CDYL2 could be confirmed in both HeLa and MCF7 cell lines with a reverse coimmunoprecipitation (Fig. 28). CDYL2 signal in CHAMP1 coimmunoprecipitation seems stronger in MCF7 cells, a difference which can potentially be explained by the fact that MCF7 has a higher level of CDYL2 proteins (non-published results). Finally, POGZ, a known interactor of CHAMP1 was also found in the mass spectrometry analysis, which further validate the interaction between CDYL2 and CHAMP1.

Knock-down of CHAMP1 induces defects in chromosome alignment during metaphase (Itoh *et al.*, 2011; Okamoto *et al.*, 2017), similar to the phenotype observed after CDYL2 knock-down (Fig. 11D), suggesting that both proteins may work together. However, CDYL2

knock-down did not have any observable effects on kinetochore-microtubules attachment, even in cells showing strong alignment defects (Fig. 29). It is therefore unlikely that CDYL2 is necessary for the activity of CHAMP1 at kinetochores. CHAMP1 is however poorly characterized and it may have additional functions where CDYL2 interaction is needed and that does not involve microtubules' connection to kinetochores. Whether CHAMP1 and CDYL2 interaction is necessary for CDYL2 function on chromosome alignment remains unanswered and would need additional experiments to be elucidated. Still, CHAMP1 was also found to interact with HP1 in previous publications (Vermeulen *et al.*, 2010), it, therefore, seems that CHAMP1 and H3K9me3 readers are linked. The effects of these interactions remain however to be elucidated.

10.2.4 Additional hypotheses to investigate CDYL2 implication in mitosis.

During my thesis I observed that CDYL2 is necessary for a faithful mitotic progress. However, despite a convincing proteome-wide analysis of CDYL2 interactors, I could not explain the mechanism behind CDYL2 regulation of mitosis and several hypotheses are still open for investigation. As an example, instead of being necessary for cohesin loading on the chromatin, CDYL2 may be needed for cohesion establishment. Cohesion on the chromosome is established during DNA replication through SMC3 acetylation by ESCO1 or ESCO2 (Whelan *et al.*, 2012; J. Zhang *et al.*, 2008). The MCM complex, localized at the replication fork, is notably required for ESCO2 recruitment on the chromatin and thus cohesin acetylation (Higashi *et al.*, 2012; Ivanov *et al.*, 2018). Since MCM4 and MCM7 were found in the mass spectrometry analysis, CDYL2 may interact with the replication fork, possibly through the MCM complex, and collaborate for cohesion establishment. Interestingly, it was already reported that CDYL1 can interact with MCM4 and localizes at the replication fork (Y. Liu *et al.*, 2017b). It remains to be determined if CDYL2 is necessary at the replication fork for cohesin acetylation. If this is the case, a knock-down of CDYL2 would lead to a fragilized cohesion at pericentromeres. This fragilized cohesion would be enough to induce cohesion defects during metaphase but may not be sufficient to release completely cohesin from pericentromeres.

Cohesin subunits are also known to localize outside chromatin at the centrosome structures, where they regulate the cohesion between the two centrioles during the cell

cycle (Guan *et al.*, 2008; Schöckel *et al.*, 2011). Removal of cohesin at centrioles shows similarities with the removal of the complex at pericentromeres as it relies on RAD21 cleavage by separase and is protected by SGO1 activity (Schöckel *et al.*, 2011). Since CDYL2 is interacting with cohesin and localizes throughout the cell cycle at centrosomes, CDYL2 may be interacting with the complex at centrosomes as well, where it potentially could regulate its function or association with centrioles. If this is the case, a knock-down of CDYL2 might lead to a lack of centrioles cohesion defects. This would explain the multipolar metaphase plate and the multipolar spindle observed in mitotic HeLa cells after CDYL2 knock-down. In interphase and MCF7 cells, a difference in the pericentrin signal after CDYL2 RNAi transfection could not be observed in the number nor the appearance. However, it remains to be determined, through knock-down of cohesin proteins, if a defect in centrioles cohesion would be visible with a pericentrin staining. Indeed, in previous literature, centriole disengagement upon cohesin defects can be put in evidence using staining against C-NAP1, a centrosome associated protein (Schöckel 2011). Performing the same staining in cells depleted for CDYL2 would indicate if a centriole disengagement is present in interphase cells and would explain the defects in mitosis observed.

Finally, as described in the next section, CDYL2 might regulate mitosis through the expression regulation of genes necessary for a faithful mitosis.

10.3 CDYL2 AS A TRANSCRIPTION REPRESSOR?

As described previously CDYL2 is interacting with various epigenetic silencing proteins, including EHMT1 and EHMT2. Still, even if CDYL2 is confirmed to be dispensable to control satellite expression, we previously described its function for transcription repression for the miRNA mir124 (Siouda *et al.*, 2020). Using EHMT2 inhibitors, I demonstrated that the catalytic activity of EHMT2, recruited to the promoter region by CDYL2, is necessary for the silencing mechanism. As EHMT2 was reported by others to interact with EZH2 (Mozzetta *et al.*, 2014), I tested in our publication (Siouda *et al.*, 2020), whether I could observe EZH2 interaction with the CDYL2 – EHMT2 complex. Despite having an efficient coimmunoprecipitation of EZH2 and EHMT2 as can attest the presence in the precipitates of SUZ12 and EHMT1 respectively, an interaction between the two proteins could not be

observed in my hands, nor could a strong interaction between CDYL2 and EZH2 be found. Of note, the coimmunoprecipitation presented by Mozetta *et al.* reported an EZH2-EHMT2 interaction that seemed much weaker compared to the interactions with SUZ12 and EHMT1 respectively (Mozetta *et al.*, 2014). Additionally, the coimmunoprecipitation of Mozetta and colleagues in human HeLa cell line was performed using ectopically expressed EHMT2 proteins which may induce aberrant interactions due to overexpression of the protein. They confirmed the interaction in endogenous conditions using mouse embryonic stem cells, it remains therefore possible that EHMT2 and EZH2 interaction is species or cell line dependent and can therefore not be observed in our MCF7 and HeLa S3 cell lines models.

Interestingly, CDYL1 was found to regulate transcription, including genes expression, through a very similar repression mechanism (Abu-Zhayia *et al.*, 2018; Mulligan *et al.*, 2008) as the one we described for miRNA124 silencing by CDYL2. It is, therefore, likely that CDYL2 also has several other gene targets. Interestingly, WIZ a protein recruiting EHMT1/EHMT2 complex to the chromatin (Bian *et al.*, 2015), was found in the mass spectrometry analysis, further suggesting a function of CDYL2 in gene transcription repression. An RNA-seq analysis might reveal genes regulated by CDYL2 which are involved in mitosis progression.

Finally, cohesin does not only regulate sister chromatid cohesion during metaphase but is also responsible for transcription loops (Hagstrom and Meyer, 2003b). It could be interesting to see if CDYL2 is implicated in cohesin loading or localization on heterochromatin in the gene transcription context. To better understand CDYL2 and cohesin interactions, it would be of great interest to compare ChIP-seq analyses from CDYL2 and proteins implicated in the cohesin complex or its loading and see if they are concordant.

10.4 FURTHER CDYL2 CHARACTERIZATION

Potentially interesting, but not addressed in the present study, is the potential collaboration or redundancy between CDYL1 and CDYL2 proteins. From the mass spectrometry analysis, the proteins are interacting. Additionally, many of CDYL1 known

interactors, as EHMT2, WIZ, HDAC1 or SETDB1 (Mulligan *et al.*, 2008) were found in my analysis as well. When revealing the structure of CDYL proteins ECH domain, Wu *et al.* described a capacity of the proteins to trimerize and suggests, a possible trimers dimerization as this happens with proteins showing a conserved ECH domain (Wu *et al.*, 2009). As the ECH domain between CDYL proteins is highly conserved, it seems possible that CDYL proteins may also be able to form heterotrimers or heterohexamers. My results suggest CDYL2 and CDYL1 interact together. If both CDYL1 and CDYL2 work together, and if this putative heterotrimerization is necessary for CDYL protein functions *in vivo* is not yet elucidated. CDYL1 and CDYL2 seems also to have a similar mode of action for transcription repression as they were both found to recruit EHMT2 to promote H3K9 methylation (Y. Liu *et al.*, 2017b; Siouda *et al.*, 2020). It is therefore possible, that CDYL1 and CDYL2 are working together and are both necessary for faithful mitosis. Still, due to the high mitotic defects observed after CDYL2 knock-down (Fig. 11) and the poor viability of *cdyl1*^{-/-} mice (Wan *et al.*, 2013), it seems that at least some effects are non-redundant between the two proteins. It would be interesting to investigate mitosis phenotypes after CDYL1 knock-down and compare them with the one observed after CDYL2 knock-down.

Interestingly, CDYL2 was found to interact with components from the replication fork as well, notably MCM proteins. An interaction between CDYL1 and MCM complex was also previously described and linked with transmission of repressive marks by CDYL1 (Y. Liu *et al.*, 2017b). The transmission of histone marks happens during DNA replication and consists of reproducing the epigenetic marks from the original to the newly synthesized strand to maintain cellular identity. It raises therefore the possibility from my observation, that CDYL2 could also be linked with repressive histone marks transmission.

Finally, I observed that CDYL2 runs at two different weight when fractionating the nucleus into the nuclear extract and tight chromatin fractions, suggesting a difference in the structure or in post-transcriptional modifications when CDYL2 is loaded on the chromatin. From the Phosphosite database (Fig. 33) (Hornbeck *et al.*, 2004), it seems that CDYL2 indeed carries several sites that can potentially be phosphorylated or ubiquitylated. Another possibility would be that *CDYL2* gives rise to more than one transcript, that has a different molecular weight, similar to *CDYL1* which has three different splice forms. On

the Ensembl database (Zerbino *et al.*, 2018), the described CDYL2 transcripts differ in size with only one amino acid, which seems unlikely to match the difference I observed. However, the NCBI database (O'Leary *et al.*, 2016) computationally predicted three transcripts with a molecular weight of 60,2 kDa, 56,1 kDa, and 49,3 kDa, while the molecular weight of canonical CDYL2 is 56,5 kDa. When comparing the bands I observe with the molecular weight, I obtain a variant of 54,6 kDa on the chromatin fractions and 56,9 kDa in the nuclear fraction (Fig. 14, calculated using Image Lab Software, BioRad), which does not fit with the molecular weight of the predicted splice variants. It could be of interest to study the exact reason behind the molecular weight difference, as it could allow us to gain further insights into the CDYL2 loading mechanism on the chromatin and potentially use this information to control CDYL2 function in the cell.

10.5 CDYL2 AS A POTENTIAL THERAPEUTIC TARGET?

In the last decades, CDYL family proteins have been revealed to be implicated in various cellular processes and deregulated in many diseases, including cancer (Kim *et al.*, 2014; Siouda *et al.*, 2020; Wu *et al.*, 2013). CDYL2, the less studied proteins from the two autosomal genes, was never until recently, studied from a functional and mechanistic point of view. My thesis aimed at characterizing CDYL2 function in the cells. It appears from the work detailed here that CDYL2 knock-down induces mitotic defects and genomic instability. Even though the mechanism is not fully characterized yet, it could be interesting to see the effect of this induced genomic instability in cancer cells. During my thesis, I also participated in the study of CDYL2 function in breast cancer and how its overexpression is linked with a worse prognosis in breast cancer patients. In this study, a knock-down of CDYL2 was able to reduce the invasion potential of MDA-MB-231, an aggressive breast cancer cell line. A knock-down of CDYL2 in aggressive breast cancer tumors could thus both potentially decrease the metastatic potential of the tumor and induce cell death through genomic instability, conjugating both effects to fight cancer.

Interestingly, CDYL1 knock-down was shown to increase the efficiency of chemotherapy inducing DNA damage and Abu-Zhayia and colleagues suggested using it in combination with cisplatin therapy (Abu-Zhayia *et al.*, 2018). CDYL1 was shown in the same study to be involved in the deposition of repressive histone marks at DNA damage sites, by interacting

with the replication fork and recruiting EZH2 to the damaged site. From my analysis, I observed that CDYL2 and CDYL1 probably interact together, that CDYL2 possibly interacts with the MCM complex and that CDYL2 is able to recruit EHMT2 and EZH2 and promote methylation of both H3K9 and H3K27. It would be therefore interesting to study CDYL2 function in DNA damage repair context and analyze the effect of CDYL2 knock-down on cisplatin treatment.

The advantage of targeting proteins involved in histone methylation is that they are more specific to particular histone residues and are thus expected to have fewer side-effects than acetylation transferases or deacetylases inhibitors (Mohammad *et al.*, 2019). Chromodomain inhibitors were already designed (Barnash *et al.*, 2016; Stuckey *et al.*, 2016; Yang *et al.*, 2019). Their efficiency and specificity on CDYL2 would still need to be determine. It would then be interesting to test the effect of this drug both in cancerous cell lines and in tumors *in vivo*. This would reveal if, as expected, targeting CDYL2 would reduce cancer aggressiveness and increases chemotherapy efficiencies either by promoting genomic instability, increasing DNA damage, or avoiding epithelial to mesenchymal transition. A parameter to take into account, when analyzing the effects of CDYL2 inhibition in cancer cells, is the presence of wild-type p53. Indeed, we observed differences in HeLa and MCF7 response to CDYL2 knock-down which could potentially be explained by p53 effects. HeLa thus showed defects even during interphase, possibly because their lack of competent p53 reduces cell death as a response to genomic instability, while MCF7 had more subtle effects as the cells with the strongest response may have died after induction of the p53 pathway.

In conclusion, CDYL2 seems, in theory, a promising target for chemotherapies. Its mode of function may however need to be further analyzed before starting to study if it is adapted for chemotherapy. Indeed, the drugs already designed are targeting CDYL2 chromodomain, however, until now, it was never studied if CDYL2 chromodomain is necessary for its function in mitosis or to increase cancer aggressiveness. It is therefore possible that targeting its chromodomain might not be the strategy of choice. Additionally, we could during my thesis observe an interaction of CDYL2 with various proteins and at least two localization in the cell. It seems therefore likely that CDYL2 is

implicated in several other functions that were not detected here. If this is the case, targeting CDYL2 would most likely induce side-effects. To bypass this limitation, deeper analyses of CDYL2 functions and the implication of its different domains would be needed and allow us to consider other strategies to inhibit its action.

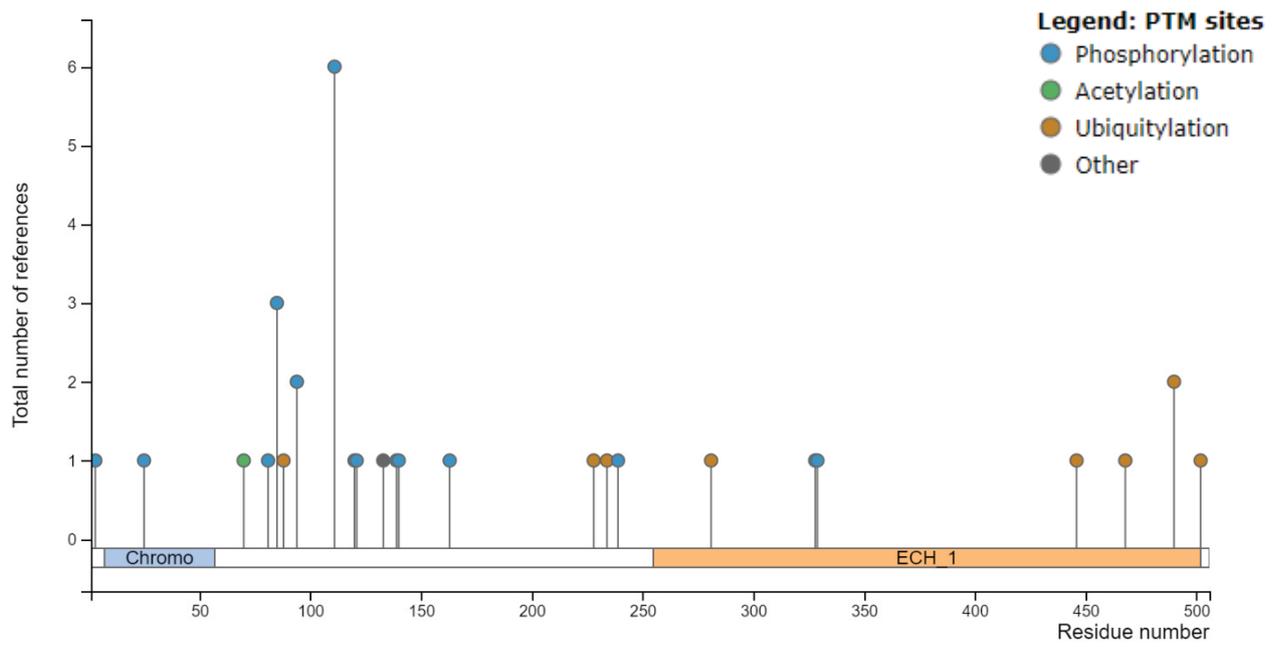


Figure 33: Potential post-transcriptional modifications on CDYL2 as predicted by Phosphosite database (Hornbeck et al. 2004).

Conclusion

11 CONCLUSION

To recapitulate, my thesis aimed broadly at characterizing CDYL2 cellular function in the cell. Indeed, while CDYL1, its ortholog, was found to be involved in several cellular functions including transcription regulation and DNA repair, CDYL2 function remains mostly unknown. Preliminary data from the team indicated a function of CDYL2 in mitosis regulation. I performed CDYL2 characterization with the aim to elucidate the mechanism behind mitosis regulation through CDYL2.

First, I performed a characterization of CDYL2 localization and levels during the cell cycle. I observed that CDYL2 is a nuclear protein, tightly bound to chromatin and relocalizing at least partially during mitosis. Using costaining techniques, I observed that CDYL2 binds to pericentromeres in a H3K9me3 dependent manner. It also localizes outside the nucleus at centrosomes throughout the cell cycle. Secondly, I revealed, through Mass Spectrometry Analysis and validated with either Immunoprecipitation or Proximity Ligation Assay, several interactors of CDYL2 that could potentially explain its function in mitosis regulation. From these interactors and the precedent characterization, three hypothesis were formulated and tested in the work I present here. Through its interaction with EHMT2 and DNMT1, it was proposed that CDYL2 could regulate centromeric repeats repression. However, from our experiments this could not be observed for satellite I, satellite II or retroviral repeat elements K111 and K222. Through its interaction with the cohesin complex, it was hypothesized that CDYL2 could regulate its loading at pericentromeric regions. This could however not be observed using confocal imaging. Our third hypothesis suggested that, since CDYL2 is interacting with CHAMP1, both proteins could work together in order to regulate microtubules-centromeres interaction. I, however could not reveal any microtubules attachment defects after CDYL2 knock-down, suggesting that CDYL2 is dispensable for this CHAMP1 described function.

Even though the mechanism linking CDYL2 and mitosis could not be elucidated during my thesis, I participated to the elucidation of CDYL2 action as a transcription repressor. Indeed, we observed that CDYL2 is interacting with EHMT2 and through this interaction, recruits EHMT2 to the chromatin and indirectly regulates histone trimethylation upstream of the *MIR124* gene. This can be considered as a proof of principle that CDYL2 can act as a gene transcription repressor.

To conclude, I could observe that CDYL2 is necessary both for mitosis and transcription repression, two key processes of cellular life. Similar to CDYL1, CDYL2 therefore seems to be a multi-functional regulator, whose mechanism still has to be investigated in future work.

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