



Identification of TET1-positive nuclear bodies in colorectal cancer cells : how Vitamin C and herbicides could both impact DNA demethylation in solid tumors

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Biologie Santé

Sciences Chimique et Biologiques pour la Santé – CBS2

Institut de Recherche en Cancérologie de Montpellier (IRCM)

**Identification of TET1-positive nuclear bodies in
colorectal cancer cells:**

**How Vitamin C and herbicides could both impact
DNA demethylation in solid tumors**

Présentée par Nour EL OSMANI

Le 2 Février 2021

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ABSTRACT

Ten-eleven translocation proteins (TET1-3) are key regulators of the active DNA demethylation pathway via triggering a series of oxidation reactions. TET proteins play an essential role in controlling gene expression and in modulating multiple cellular mechanisms. These proteins are frequently downregulated in many cancers, including colorectal cancer (CRC).

Moreover, some dietary and environmental factors have been identified as capable of influencing the activity and/or expression of TETs. Vitamin C (VitC), a therapeutic adjuvant, is well known to actively induce demethylation, but the molecular mechanisms involved are not clearly elucidated. On the other hand, Linuron, a phenyl-urea herbicide, is known for its genotoxicity, yet its effects on TETs have not been studied.

The main objective of this thesis was to study the subcellular distribution of TETs, in particular TET1, in non-tumoral and tumoral colonic cells and to evaluate the impact of VitC and Linuron on this subcellular distribution. Immunofluorescence assays detected novel compartmentalization of TET1 and its demethylation mark, 5hydroxymethylcytosine (5hmC), characterized by their recruitment into coarse nuclear bodies (NBs) in the nucleoplasm of CRC cells. While studying the potential crosstalk between TET1/5hmC-NBs and nuclear-body forming proteins in CRC cells, we observed their colocalization with Cajal bodies (CBs) but not with those positively stained for the promyelocytic leukemia protein (PML). CRC cells treated with VitC revealed stimulation in the number and size of 5hmC-NBs, PML-NBs, and CBs. Interestingly, confocal imaging, revealed that VitC enhanced the interaction of 5hmC-NBs with both PML- and Cajal bodies. Conversely, confocal analysis of TET1 revealed that exposure to Linuron induced a significant decrease in the number of TET1-NBs in CRC associated with an increase in cellular proliferation and invasiveness.

This work identified new nuclear bodies concentrating TET1 and 5hmC in CRC cells and demonstrated that the number, size, and dynamic properties of these nuclear bodies are inversely modulated by VitC and Linuron. These results highlight new biological functions for TET1 and open new research perspectives in digestive oncology.

RÉSUMÉ

Les protéines Ten – Eleven Translocation (TET1-3) sont des régulateurs clés de la voie active de déméthylation de l'ADN. Ces enzymes déclenchent une série de réactions d'oxydation qui jouent un rôle essentiel dans le contrôle de l'expression des gènes et, de fait, dans le contrôle de multiple mécanismes cellulaires y compris la prolifération et la différenciation cellulaire. Dans de nombreux cancers y compris dans certains cancers solides tels que le cancer colorectal (CRC), l'expression et l'activité des TETs sont fréquemment diminuées. Certains facteurs alimentaires, métaboliques et environnementaux ont été identifiés comme capables d'influencer l'activité et/ou l'expression des TETs. La VitC est bien reconnue pour son rôle stimulant de la déméthylation de l'ADN mais les mécanismes moléculaires impliqués ne sont pas clairement élucidés. D'autre part, le Linuron, un herbicide à base de phénylurée, est connu pour sa génotoxicité, mais ses effets sur les TETs n'ont pas été étudiés.

L'objectif principal de cette thèse a été d'étudier la distribution subcellulaire des TETs et en particulier de TET1 dans les cellules coliques non tumorales et tumorales et d'évaluer l'impact de la VitC et du Linuron sur cette distribution subcellulaire. Une étude par immunofluorescence a mis en évidence une nouvelle compartimentation de TET1 et le marqueur de son activité de déméthylation, la 5hydroxyméthylcytosine (5hmC), dans des corps nucléaires des cellules de CRC. En étudiant l'interaction potentielle entre TET1/5hmC et les protéines formant des corps nucléaires dans les cellules CRC, nous avons observé leur colocalisation avec les corps de Cajal (CB) mais pas avec ceux marqués positivement pour la protéine de leucémie promyélocytaire (PML). De plus, les cellules CRC traitées à VitC ont révélé une augmentation du nombre et de la taille des corps nucléaires de 5hmC, PML et CBs. Fait intéressant, l'imagerie confocale a révélé que la VitC potentialisait l'interaction des corps de 5hmC avec les corps de PML et de Cajal. A l'inverse, l'analyse confocale de TET1 a révélé que l'exposition au Linuron induisait une diminution significative du nombre de corps nucléaires de TET1 ainsi qu'une augmentation de la prolifération et de l'invasivité des cellules tumorales coliques.

Ce travail a permis d'identifier de nouveaux corps nucléaires concentrant TET1 et 5hmC dans les cellules tumorales coliques et de démontrer que le nombre, la taille et les propriétés dynamiques de ces corps nucléaires sont inversement modulées par la VitC et le Linuron. Ces résultats mettent en évidence de nouvelles fonctions biologiques pour TET1 et ouvrent de nouvelles perspectives de recherche en oncologie digestive.

APERÇU DE LA THÈSE

Contexte

Les protéines Ten – Eleven Translocation (TET1-3) sont des régulateurs clés de la voie active de déméthylation de l'ADN. Ces enzymes déclenchent une série de réactions d'oxydation qui jouent un rôle essentiel dans le contrôle de l'expression des gènes et, de fait, dans le contrôle de multiples mécanismes cellulaires y compris la prolifération et la différenciation cellulaire. Plusieurs études ont révélé que l'abondance des TETs ainsi que leur marque active, 5-hydroxyméthylcytosine (5hmC), diffère notamment selon le type cellulaire : les tissus cérébraux comprennent les niveaux les plus élevés de 5hmC (0,67%), alors que les tissus cardiaques et mammaires présentent les niveaux les plus bas parmi les tissus normaux (0,05 %).

Par ailleurs, les protéines TET régulent l'activité d'un grand nombre de protéines partenaires (Nanog ; OGT) via des mécanismes indépendants de leur activité de déméthylation de l'ADN.

Dans de nombreux cancers y compris dans certains cancers solides tels que le cancer colorectal (CRC), l'expression et l'activité des TETs sont fréquemment diminuées même si une surexpression de TET1 pourtant été associée à des effets tumorigènes dans le cancer du sein et du poumon. Ces observations suggèrent des propriétés multifonctionnelles des TETs et des rôles modulateurs de ces enzymes vis-à-vis de différentes voies de signalisation y compris celles impliquées dans les processus de tumorigénèse.

Certains des facteurs alimentaires, métaboliques et environnementaux ont été identifiés comme capables d'influencer l'activité et/ou l'expression des TETs. C'est par exemple le cas de l'herbicide Diuron qui modulerait l'activité de surveillance des cellules immunitaires de manière dépendante de TET2. L'antioxydant Vitamine C (VitC), un adjuvant largement utilisé en chimiothérapie, restaure quant à lui l'activité de toutes les TETs et resensibilise les cellules tumorales aux traitements chimio-thérapeutiques.

Objectifs

D'autre part, la VitC est bien reconnue pour son rôle stimulant de la déméthylation de l'ADN via l'augmentation des niveaux d'ions ferreux et/ou via l'interaction, en tant que cofacteur, avec le domaine catalytique des TETs. Malgré les nombreuses découvertes révélant les effets anticancéreux de la VitC via l'activation des TET, les mécanismes moléculaires impliqués ne sont pas clairement élucidés. Cela pourrait être expliqué par le fait que les TETs s'associent à une pléthore de protéines, entraînant ainsi diverses voies cellulaires contre la tumorigénèse.

Par ailleurs, malgré toute l'attention portée par les chercheurs aux régulateurs de la déméthylation de l'ADN, notamment les enzymes TET, la question de l'impact de certains facteurs environnementaux, comme par exemple une exposition aux pesticides, sur l'activité des TETs reste ouverte. L'absence de données dans ce domaine est certainement liée à la découverte des propriétés de perturbateurs endocriniens de nombreux pesticides, y compris du Linuron sur les organismes aquatiques de cet herbicide, en particulier sur les organismes aquatiques. Alors il y a une nécessité d'établir une étude évaluant le lien entre le Linuron et les déméthylases TET dans les cancers humains, notamment dans le CRC.

Comment le Linuron – en tant que facteur environnemental – ou la VitC – en tant qu'agent thérapeutique – module l'activité de TETs reste encore mal établi.

L'objectif principal de cette thèse a été d'étudier la distribution subcellulaire des TETs et en particulier de TET1 dans les cellules coliques non tumorales et tumorales et d'évaluer l'impact de la VitC et du Linuron sur cette distribution subcellulaire.

Pour réaliser cet objectif, j'ai :

- Examiné de la localisation intracellulaire de protéine TET1 dans les cellules coliques tumorales et non-tumorales.
- Identifié l'association de TET1 avec des protéines partenaires nouvelles.
- Étudié les effets du VitC à la fois sur l'activité de TET1 et les protéines partenaires.
- Évalué l'impact du Linuron sur la distribution subcellulaire de TET1 dans les cellules tumorales coliques.

Résultats et discussion

Mon travail de thèse met en évidence une localisation subcellulaire très particulière de TET1 dans des corps nucléaires. Les résultats expérimentaux sont présentés sous forme de deux articles démontrant comment le profil de distribution intracellulaire de TET1 peut être modulé différemment par VitC (article 1) et l'herbicide Linuron (article 2).

1. Article 1 : identifier de nouveaux corps nucléaires concentrant les TETs dans les cellules tumorales coliques et de démontrer que l'interaction de ces corps nucléaires avec les corps nucléaires positifs pour PML ou les corps de Cajal est stimulée par la vitamine C (*El-Osmani et al. ; article en révision à Cancers*).

Nous avons observé que TET1 est présente une distribution périnucléaire diffuse dans les cellules non tumorales issues de la muqueuse épithéliale colique (NCM460). En revanche, tout comme son marqueur d'activité de déméthylation 5hmC, TET1 est concentrée dans des corps nucléaires (TET1-NBs et 5hmC-NBs) dans les cellules

tumorales coliques (HC116, Caco-2, HT-29). Ces résultats ont identifié un nouveau profil de la protéine TET1 la marque de déméthylation, 5hmC dans les cellules tumorales (**Fig.1-partie 1.2**).

Nous avons validé ce profil de TET1 et 5hmC dans les cellules d'adénocarcinome cervical (HeLa), ce qui indique que les corps nucléaires ne sont pas une particularité de la cellule tumorale colique (**Fig.12-partie 1.4.1**). Par ailleurs, De plus, nous avons observé que les paralogues de TET2 et TET3 se concentrent également dans des corps nucléaires dans les cellules tumorales coliques (**Fig.13 – partie 1.4.2**).

Étant donné ses résultats, nous nous sommes demandé si l'enzyme TET1 co-localise avec les protéines du corps nucléaires les plus importantes : la protéine proleucémique myéloïde (PML). Bien que la PML soit impliquée dans le recrutement de nombreuses protéines dans des corps nucléaires PML-NBs, nous démontrons que TET1 et PML présentent des distributions subcellulaires distinctes aussi bien dans les cellules coliques non-tumorales (NCM460) que tumorales (HCT116, Caco-2, HeLa) (**Fig.2 - partie 1.2 ; Fig.S2 - partie 1.3 ; Fig.15-16 partie 1.4.4**). De même, nous démontrons que les 5hmC-NBs sont des NBs distincts des PML-NBs dans les cellules tumorales coliques HCT116 (**Fig.14-partie 1.4.3**). Ces observations suggèrent des fonctions cellulaires spécifiques aux NBs dans les cellules CRC, mais nous ne pouvons pas exclure la possibilité qu'un stimulus cellulaire puisse déclencher leur interaction.

En revanche, nous démontrons que TET1 et 5hmC interagissent avec les corps de Cajal (CBs), des structures nucléaires dynamiques qui échangent continuellement leurs composants avec les nucléoles. Ces résultats indiquent ainsi un rôle moléculaire potentiel de TET1/5hmC dans les processus associés à la biogenèse des ARNs.

Comme certains composants essentiels du CBs orchestrent les protéines de réparation de l'ADN et que des données récentes indiquent que les TETs joueraient un rôle dans les mécanismes de réparation des dommages à l'ADN, nous avons cherché à déterminer si TET1 s'associe à Rad51, une protéine de recombinaison homologue qui stabilise l'intégrité génomique. Cependant, bien que TET1 et Rad51 se concentrent toutes les deux dans des corps nucléaires, ceux-ci ne sont pas colocalisés dans les cellules HCT116 (**Fig.17 – partie 1.4.5**). Même si nous n'avons pas identifié la co-localisation de ces derniers, nous ne pouvons pas exclure la possibilité que les protéines TETs puissent réguler indirectement la réparation de l'ADN.

En outre, comme TETs interagissent avec la voie Wnt/ β -caténine et SOX9 est à la fois une cible et un régulateur clé de cette voie de signalisation oncogénique, nous avons été intrigués de vérifier si TET1 s'associe à SOX9 et pourrait donc influencer la prolifération des cellules coliques. De plus, SOX9 contrôle la transcription et joue un rôle essentiel dans la régulation de la prolifération des cellules du côlon. Ainsi, un double marquage de TET1 et SOX9 dans les cellules tumorales coliques (HCT116) montre ce facteur transcriptionnel (SOX9) a conservé son profil intracellulaire diffus et ne s'est pas concentré dans les TET1-

NBs (**Fig.18-partie 1.4.6**) suggérant que les TET1-NBs ne parviennent pas à recruter SOX9 dans leur noyau interne ou qu'il n'y a pas d'interaction entre les deux protéines.

Étant donné l'impact de la VitC sur le déclenchement de la déméthylation de l'ADN en stimulant les niveaux de 5hmC, nous avons étudié les effets de cet adjuvant des chimiothérapies sur TET1-NBs dans les cellules tumorales coliques. Nous avons observé que la VitC augmente significativement la formation des corps nucléaires positifs pour 5hmC-, PML- et Cajal-NBs dans divers cellules tumorales (**Fig.4-partie 1.2; Fig.S4-partie 1.3 ; Fig.19-partie 1.4.7**). Ces résultats confirment les propriétés activatrices de la VitC vis-à-vis de la voie de déméthylation de l'ADN. Nous avons également observé que la VitC n'augmente pas seulement la taille et le nombre des corps nucléaires positifs pour 5hmC, mais favorise également l'interaction des 5hmC-NBs avec les PML-NBs et les Cajal-NBs (**Fig.4; Fig.S4; Fig.19**). Notre hypothèse alors est que VitC pourrait induire à une modification post-traductionnelle de TET1, possiblement médiée par SUMO1/2/3, pour initier des complexes actifs 5hmC-PML. Tous ces résultats révèlent de nouvelles fonctions cellulaires dépendantes de TET1 et potentiellement de nouvelles avenues dans la gestion du cancer du côlon.

2. Article 2 : les corps nucléaires concentrant TET1 (TET1-NBs) sont fortement perturbés lors d'une exposition à l'herbicide Linuron dans les cellules tumorales coliques (*El-Osmani et al ; article en préparation*)

Sachant que la cytotoxicité du Linuron est strictement évaluée dans le foie et les testicules, nous avons été intrigués d'examiner les effets de cet herbicide sur les cellules coliques cancéreuses. En utilisant le test de viabilité cellulaire MTT, nous avons observé une augmentation significative de la prolifération de cellules tumorales coliques HCT116 soumises à 24h d'exposition à concentrations croissantes de Linuron (**Fig.1-partie 2.2**). Pour examiner plus en détail les effets pro-cancérigènes potentiels du Linuron, un test de cicatrisation des plaies (wound healing assay) a révélé un pourcentage de récupération rapide en fonction de la concentration (**Fig.1**). Alors, Linuron stimule la prolifération et l'agressivité des cellules tumorales coliques.

Afin d'étudier le comportement des TET1-NBs exposés au Linuron, nous avons évalué ces corps nucléaires de TET1 dans des cellules HCT116 traitées à l'herbicide. Une analyse par imagerie confocale a révélé que contrairement à la vitamine C, le Linuron induisait une diminution du nombre de TET1-NBs en fonction de la concentration dans les cellules HCT116. (**Fig.2-partie 2.2**).

De fait, Uren Webster *et al* a démontré l'inhibition de la voie de biosynthèse du cholestérol après l'exposition au Linuron. D'un point de vue mécanique, cet herbicide réduit l'expression de la plupart des gènes codant pour la synthèse du cholestérol, y compris

les protéines de liaison aux éléments régulateurs des stérols (SREBP1/2) (Uren Webster et al., 2015). Phénotypiquement, les embryons de souris à double knock-out TET1/3 présentaient une expression réduite de SREBP2 associée à des distorsions de développement (Kang et al., 2015). Cependant, cette réduction n'est pas liée à un statut de méthylation altéré du gène SREBP2 dans ces embryons (Kang et al., 2015). Bien que la régulation de SREBP via les protéines TET n'est pas clairement étudiée, Yan *et al*, en 2020, ont mis en évidence SREBP1 comme cible en aval de l'enzyme de déméthylation TDG (Yan et al., 2020). On pense que l'accumulation de 5caC (5-carboxylcytosine) sur le promoteur SREBP1 due à la suppression du TDG diminue l'affinité de liaison de l'ARN polymérase II sur ce gène de synthèse du cholestérol ; ainsi, abaissant l'expression transcriptionnelle et traductionnelle de SREBP1 (Kellinger et al., 2012; Yan et al., 2020). Compte tenu de ces données, et de notre observation de la réduction du nombre de TET1-NBs induite par le Linuron, nous spéculons qu'une inhibition de la protéine TET1 pourrait être impliquée dans la régulation du mécanisme de synthèse du cholestérol par dans les cellules tumorales coliques.

En fin, cette étude a établi un nouveau lien entre cet herbicide et les enzymes de déméthylation de l'ADN, ce qui favorise une compréhension claire des voies mécanistiques ambiguës de ce polluant. Ces résultats peuvent présenter des données importantes à prendre en compte pour la prévention du cancer.

Conclusion

Ce travail de thèse permis d'identifier de nouveaux corps nucléaires concentrant TET1 et 5hmC dans les cellules tumorales coliques. De plus, ces travaux ont démontré que le nombre, la taille et les propriétés dynamiques de ces corps nucléaires sont inversement modulées par la VitC et le Linuron.

Ces résultats mettent en évidence de nouvelles fonctions biologiques pour TET1 et ouvrent de nouvelles perspectives de recherche en oncologie digestive.

Perspectives

Dans des études ultérieures, on vise à bien comprendre le rôle des TET1-NBs dans divers processus cellulaires du cancer colorectal comme réparation de l'ADN et la biogenèse des ARNs. De plus, on cherche à évaluer l'implication clinique de l'exposition au Linuron dans des modèles de souris in-vivo.

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LIST OF ABBREVIATIONS

2-HG: 2-hydroxyglutarate

5caC: 5-carboxylcytosine

5fC: 5-formylcytosine

5hmC: 5-hydroxymethylcytosine

5mC: 5-methylcytosine

A: Adenosine

AKT: Protein Kinase B

AML: Acute myeloid leukemia

AMPK: AMP-activated protein kinase

APC: Adenomatous polyposis coli

ARE: Antioxidant response element

BER: Base Excision Repair

BLCA: Bladder cancer

BRCA: Breast cancer adenocarcinoma

C: Cytosine

CBs: Cajal bodies

CDKN2a: Cyclin-dependent Kinase inhibitor 2 A gene

CircRNA: Circular RNAs

CMML: Chronic myelomonocytic leukemia

COAD: Colorectal adenocarcinoma

COSMIC: The Catalogue Of Somatic Mutations In Cancer

CRC: Colorectal cancer

CRL4^{VprBP}: Cullin-RING-Ubiquitin Ligase 4

CXXC: Cysteine-X-X-Cysteine

Cys: Cysteine-rich region

DDR: DNA damage repair genes

DNMT: DNA methyltransferase

DPPA3: Development pluripotency-associated 3

DSBH: Double-Stranded β -Helix

EMT: Epithelial-to-mesenchymal-transition

EZH2: Enhancer of zeste homolog 2

FAK: Focal Adhesion Kinase

Fe²⁺: Ferrous ion

FECR1: FLI exonic circular RNA 1

FENDRR: FOXF1 adjacent non-coding developmental regulatory RNA

FLI-1: Friend leukemia integration 1

FLRT2: Fibronectin Leucine Rich Transmembrane protein 2

GBM: Glioblastoma

GLOI: Glyoxalase I

h/mESC: human/mouse embryonic stem cells

HCC: Hepatocellular carcinoma

HDAC-2: Hstone deacetylase enzyme

HIF-1/2: Hypoxia-inducible factor 1/2

hmU: HydroxymethylUracil

HNSC: Head and neck squamous carcinoma

HOXA: Homeobox A cluster

HSC: Hematopoietic Stem cells

IDH1/2: Isocitrate dehydrogenase 1/2

IL-6 : Interleukin 6

KIRC: Kidney renal carcinoma

KRAS: Kristen Rat Sarcoma virus gene

LAML: Acute myeloid leukemia

LncRNA: long non-coding RNAs

LUAD: Lung adenocarcinoma

LUSC: Lung squamous carcinoma

MBD1: Methyl-CpG Binding Domain 1

MDS: Myelodysplastic disorders

miR: MicroRNA

MLL: Mixed-lineage leukemia

MPN: Myeloproliferative neoplasms

mtDNA: Mitochondrial DNA

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide

NBs: Nuclear bodies

ncRNAs: Non-coding RNA

Nrf2: Nuclear factor–erythroid-derived 2-like family

OGT: OGlcN-Acetyl Transferase

PARP: Poly-ADP-Ribose polymerase

PKC α : Protein Kinase C- α

PKM: Pyruvate kinase muscle

PML: Promyelocytic Leukemia protein

POP: Persistent organic pollutants

PTEN: Phosphatase and tensin homolog

PTENp1: PTEN pseudogene 1

RASSF1A: Ras association domain family 1 isoform A

READ: Rectal adenocarcinoma

REST: RE-1 silencing transcription factor

ROS: Reactive oxygen species

Snrpn: Small nuclear ribonucleoprotein polypeptide N

STAT3: Signal transducer and activator of transcription 3

SWI/SWF: SWItch/Sucrose Non-Fermentable

T: Thymine base

TCA: Tricarboxylic acid

TCGA: The Cancer Genome Atlas

TDG: Thymine DNA Glycosylase

TET: Ten-Eleven Translocation

TP53: Tumor protein p53

tRNA: transfer RNA

UCEC: Uterine corpus endometrial carcinoma

UHRF1: Ubiquitin-Like with PHD and Ring Finger Domains

VitC: Vitamin C

XIST: X-inactive specific transcript

α -KG: alpha-Ketoglutarate

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PREAMBLE

TP53 (Tumor protein p53; 65.8%), KRAS (Kristen Rat Sarcoma virus; 52.1%), APC (Adenomatous Polyposis Coli; 39.3%), CDKN2a (Cyclin-dependent Kinase inhibitor 2 A; 32.5%) genes are frequently mutated in colorectal cancer (CRC), and these mutations account for high mortality (Ashktorab & Brim, 2014; Sheaffer, Elliott, & Kaestner, 2016; Ye et al., 2020). However, mutation events in anti- or pro- tumor genes are not the exclusive drivers of carcinogenesis. Indeed, changes in epigenetic landscapes trigger all classical hallmark mechanisms of cancer (J. Fan et al., 2020; Flavahan, Gaskell, & Bernstein, 2017; Hanahan & Weinberg, 2011; Vymetalkova, Vodicka, Vodenkova, Alonso, & Schneider-Stock, 2019). Namely, DNA accessibility and signaling plasticity in cancer-(pre)initiation events are epigenetically regulated via alterations in chromatin structure and condensation (Maleszewska, Wojtas, & Kaminska, 2018; Nemtsova, Mikhaylenko, Kuznetsova, Bykov, & Zamyatnin, 2020). DNA methylation, histone modification, chromatin remodeling, and non-coding RNAs are key heritable epi-processes that interdependently control the genomic stability in healthy and cancerous cells (Flavahan et al., 2017; Maleszewska et al., 2018; Rodriguez-Casanova et al., 2021; Vymetalkova et al., 2019). In colorectal cancer (CRC), the prevalent deregulation of DNA methylation pattern is associated with tumor progression (Hsu et al., 2020; Nemtsova et al., 2020; J. M. Ng & Yu, 2015; Qi & Ding, 2017; H. Zhang, Sun, Lu, Wu, & Feng, 2020). For instance, the promoter of HOXA (Homeobox A cluster), APC, FLRT2 (Fibronectin Leucine Rich Transmembrane protein 2), and MBD1 (Methyl-CpG Binding Domain 1) is commonly hypermethylated in CRC whereas LINE1 hypomethylation is linked to metastasis and poor prognosis (Coppede et al., 2014; H. Guo, Zhu, Zhang, Wan, & Shen, 2019; Hur et al., 2014; D. Li et al., 2019; Puccini et al., 2017; Qi & Ding, 2017); this reflects that shifts in DNA methylation between stochastic activation of oncogenes and focal repression of tumor suppressors greatly stimulates carcinogenesis (Kaminska et al., 2019; Vymetalkova et al., 2019).

A decade ago, Ten-Eleven Translocation (TET) proteins were reported as erasers of DNA methylation mark through a series of oxidation reactions (Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). Several studies assessed the implication of these proteins in various cellular mechanisms (e.g. cellular differentiation). Moreover, TETs were identified to regulate a continuously expanding number of partner proteins (e.g. Nanog; OGT) (Arvinden, Rao, Rajkumar, & Mani, 2017; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015); this underscores the catalytic-independent functions of TETs alongside active DNA demethylation. Despite being recurrently downregulated in several solid cancers, particularly in CRC, overexpressed TET1 display some tumorigenic roles in breast and lung cancer; this suggests a complexity of TET dual roles in modulating cancer-associated cellular pathways (Filipczak et al., 2019; Good et al., 2018; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). Moreover, dietary, metabolic, and environmental factors influence

TET1 activity and/or expression (Arvinden et al., 2017). For example, the Diuron herbicide modulates the surveillance activity of immune cells in a TET2-dependent manner (Briand, Joalland, et al., 2019). On the other hand, the antioxidant Vitamin C (VitC), a widely used adjuvant in chemotherapy, restores the activity of all TET proteins which appeared to re-sensitize tumor cells to chemotherapeutic drugs (Giansanti, Karimi, Faraoni, & Graziani, 2021).

A fundamental question of how environmental factors – including herbicides such as Linuron – or therapeutic agents like VitC modulate TET activity is however still open. My thesis work focused on this specific issue by exploring the incidence of either VitC or Linuron, an extensively used herbicide in Lebanon, on TET1 function in the context of colorectal cancer.

The introduction section intends to give an overview of TET structure, function, as well as TET roles in physiological situations and in cancers with a particular focus on solid cancers.

My thesis work highlights a very particular subcellular localization of TET1 in nuclear bodies. Experimental results are presented as two articles demonstrating how the intracellular distribution profile of TET1 can be differently modulated by VitC (Article 1) and Linuron herbicide (Article 2).

The discussion section conceptualizes our findings to understand the potential novel functions of TET1 and to elucidate the possible mode of action of Linuron in regulating TET1 in cancer.

Finally, the perspective section suggests future research directions to this work.

INTRODUCTION

1. Ten-Eleven Translocation (TET): An overview

1.1. TET genes

Ten-Eleven translocation (TET) family – primarily discovered in 2003 by Lorsch *et al* – encodes three distinct genes located on chromosomes 10, 4, and 2 respectively (Ensembl-database) (**Table 1**). Their nomenclature was subsequent to the observed translocation of TET1 gene from chromosome ten to eleven, forming a fusion partner with mixed-lineage leukemia (MLL) gene in acute myeloid leukemia (AML) (Lorsch *et al.*, 2003). Although the mechanism for TET diversification is still unknown, scientists believed that these paralogs underwent gene duplication and adaptive evolution (Ohsawa, Umemura, Akahori, Terada, & Muto, 2018). Those key enzymes trigger active demethylation process characterized by oxidation of the methylation mark 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), the first derivative of cytosine nucleotide marking the removal of methylation (Arviden *et al.*, 2017; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015).

Table 1: Profile of TET genes characteristics and their cellular distribution

TET gene	Chromosomal location	Number of exons	Gene Length	Protein Length / weight	Subcellular localization
TET1	10q21.3	12	134 Kb	2136 aa / 235 kDa	Nuclear
TET2	4q24	11	133 Kb	2002 aa / 224 KDa	
TET3	2q13	9	124 Kb	1660 aa / 179 KDa	Predominantly nuclear

1.2. TET structure

As shown in **figure 1**, all TET members display a C-terminal catalytic domain characterized by a Double-Stranded β -Helix (DSBH) and a cysteine-rich region (Cys) (Iyer, Tahiliani, Rao, & Aravind, 2009; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). The DSBH domain itself is made of 2 conserved regions: **A**. Eight anti-parallel β -strands containing a low-complexity insert with very limited conservation and unknown

functions; and **B.** an iron-binding motif where interaction with the demethylation-indispensable co-factors like ferrous ion (Fe^{2+}) and α -Ketoglutarate (α -KG) occurs (L. Hu et al., 2013; L. Shen, Song, He, & Zhang, 2014). On the other hand, Cysteine-rich domain is composed of 2 sub-domains suspected to manage TET-DNA association (An, Rao, & Ko, 2017). To favor DNA recognition, 2 zinc finger motifs from the C-terminus tightly compact both DSBH and Cys domains (An et al., 2017).

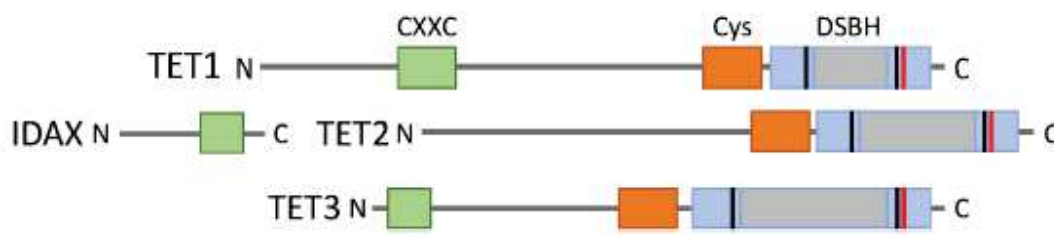


Figure 1: Structure of TET family of demethylases. (Tsiouplis, Bailey, Chiou, Wissink, & Tsagaratou, 2020). All 3 TET paralogs have the same structure except for TET2 that lacks a CXXC region in its N-terminal domain.

Moreover, TET1 and TET3 genes exhibit an N-terminal domain marked with a Cysteine-X-X-Cysteine (CXXC) zinc-chelating region responsible for *DNA recognition and binding* (Iyer et al., 2009; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). TET2 lacks the CXXC domain, but a distinct gene provides this domain for TET2; this gene is known as IDAX and precedes TET2 gene on chromosome 14 (**Fig.1**). According to Iyer *et al.*, an ancestral inversion of chromosome 14 is reasoned to yield this distinctive characteristic of TET2 genes (Iyer et al., 2009). The CXXC domain plays a critical role in TET activity since it favors specific chromatin targeting of TETs toward unmethylated sites. However, in absence of this domain, TET proteins retain their oxidative capacities over the methylation mark (5mC) regardless of DNA sequence methylation status (L. Shen et al., 2014). More precisely, TET1 and TET2 differentially interact with genomic regions of target genes: promoters, and gene bodies respectively; this implies a possible role of CXXC domain in modulating the genomic distribution of TET proteins (An et al., 2017).

1.3. TET isoforms

Each TET gene can give rise to several isoforms that are differentially expressed at cell- and stage-specific levels (W. Zhang et al., 2016). TET isoforms have distinctive distribution and variable effects on the DNA demethylation process (**Fig.2**) (Lou et al., 2019; Melamed, Yosefzon, David, Tsukerman, & Pnueli, 2018). Although factors giving rise to these splice variants are still not fully understood, evidence is increasing that TET genes are regulated by several promoters and distal enhancers (Melamed et al., 2018).

Table 2 summarizes all the reported isoforms of TET proteins in the literature as well as it displays their functions (**Table 2**).

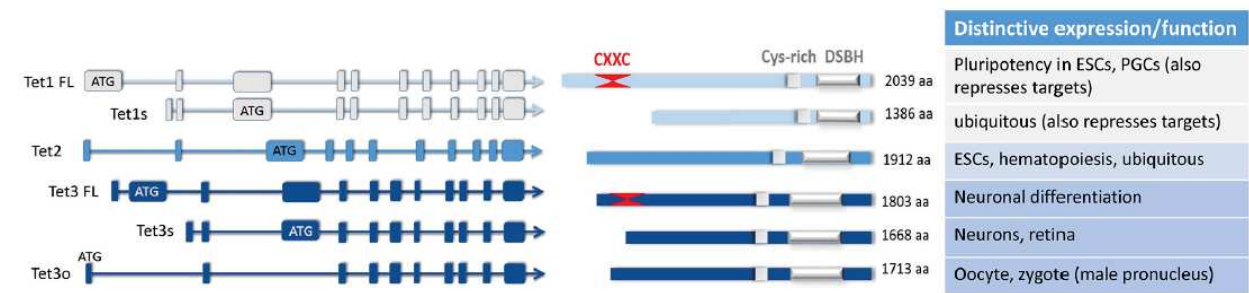


Figure 2: Structure, distribution and functions of TET splice variants (Melamed et al., 2018). Each TET gene gives rise to distinct isoforms that are differentially distributed and expressed in cell models.

Table 2: TET isoforms as well as their distribution and functions

Isoform	Catalytic domain	Expression	Functions	Reference(s)
TET1 full length	Present	Early embryos, embryonic stem cells, primordial germ cells Abundant in glial cells	Maintain pluripotency, genomic stability of neuronal genes Protect long-term memory formation Regulate a great number of target proteins Influence the activation of immune and cancer pathways	(Greer et al., 2021; W. Zhang et al., 2016)
TET1 short isoform (TET1s)	Absent	Mainly expressed in somatic cells Highly enriched in neurons Overexpressed in cancer	Weak affinity to DNA Functional but with weaker demethylation activity Target distinct sites than TET1 full length Inability to erase imprints in Primordial germ cells Leads to defects in development and memory erasure Negatively correlated with neuronal activity	(Good et al., 2017; Greer et al., 2021; W. Zhang et al., 2016)
TET2	Present	Widely expressed	Tumor suppressor; Controlled by IDAX gene Frequently mutated in hematological malignancies	(Lou et al., 2019)
TET2 short isoform (TET2s)	Absent	3 promoters distinctively controlling TET2 gene, they generate: Isoform 1: abundant in spleen Isoform 2: expressed only in progenitor cells	Isoform 1: not reported Isoform 2: act as demethylation inhibitor in progenitor cells	(Lou et al., 2019)

Table 2 continued

Isoform	Catalytic domain	Expression	Functions	Reference
TET3 full length	Present	Mainly expressed in germ cells	High affinity to 5caC* via its CXXC domain Present at transcription start sites genes Regulate genes implicated in lysosomes, mRNA processing, and BER* pathways	(S.-G. Jin et al., 2016)
TET3 oocyte isoform (TET3o)	Absent	Expressed in particularly oocytes and early preimplantation embryos	Regulated by a distinct promoter Expression decreases with differentiation	(S.-G. Jin et al., 2016; Krueger et al., 2017)
TET3-short isoform (TET3s)	Absent	Mainly found in retina Expressed in adult brain tissues	No affinity to DNA Upregulated during neuronal differentiation Recruited by REST*, a transcriptional repressor Partner with CXXC4 (IDAX) gene Interact with histone methyltransferase to trigger H3K36me3 levels and chromatin remodeling	(S.-G. Jin et al., 2016; Melamed et al., 2018; Perera et al., 2015)
TET3 variants	Not reported	Expressed in mouse adult tissues	Many isoforms with different N-terminal length resulting from alternative splicing Function regulated by several distinct CXXC domains	(N. Liu et al., 2013)

* **BER**: Base Excision Repair; **REST**: RE-1 silencing transcription factor; **5caC**:5-carboxylcytosine

1.4. Tissue distribution of TET proteins

Although only a few articles reported the subcellular localization of TET proteins in non-cancerous *in-vitro* cell models, TETs exhibited a diffused nucleoplasmic distribution. Interestingly, 5hmC displayed a similar nuclear profile to all 3 TET proteins in normal cells (Arioka, Watanabe, Saito, & Yamada, 2012; J. U. Guo, Su, Zhong, Ming, & Song, 2011; S.-G. Jin et al., 2016; Kong et al., 2016; L. Peng et al., 2016; M. J. Wu, Kim, Chen, Yang, & Chang, 2017).

The abundance of TET proteins as well as their 5hmC mark remarkably differs according to the cell type (Good et al., 2017; L. Alcaraz-Estrada et al., 2020). While brain tissues comprise the highest levels of 5hmC (0.67%), heart and breast tissues displayed the lowest levels amongst normal tissues (0.05%) (L. Alcaraz-Estrada et al., 2020). Similarly, few studies examined the distribution of endogenous TET in neurons (Gao et al., 2016; Mi et al., 2015). All of the 3 paralogs, implicated in promoting neuronal differentiation, were particularly present in the nucleus of *Mus musculus* brain neuroblastoma (Neuro-2a) cells. Moreover, Mi *et al* demonstrated a diffused intra-neuronal distribution pattern of TET2 protein with coarse nuclear granulation within the euchromatic region (Mi et al., 2015).

Knowing that the cortex and hippocampus brain tissues comprise the greatest expression level of TET proteins, this indicates TETs functions in brain activities like memory formation and learning plasticity (J. N. Ismail, Badini, Frey, Abou-Kheir, & Shirinian, 2019; Meng et al., 2014). Interestingly, the hypermethylation of adult neurogenesis-related genes resulted from the absence of TET1 in mice (Meng et al., 2014). All of these data highlight wide-spectrum functions of TET proteins in various cell types.

2. TET functions

2.1. Active DNA demethylation process

Following the discovery of TET proteins, Tahiliani *et al* demonstrated that this family is capable of converting 5mC into 5-hydroxymethylcytosine (5hmC) during active demethylation (Shinsuke Ito et al., 2010; Tahiliani et al., 2009). 5hmC is commonly considered as the 6th nucleotide base after the precursor 5mC (Münzel, Globisch, & Carell, 2011; C.-X. Song & He, 2011). In 2009, 5hmC was found to be an intermediate throughout this process. In other terms, the activation of TET family can further oxidize 5hmC into 5-formylcytosine (5fC) and -5caC, respectively (Shinsuke Ito et al., 2011) (**Fig.3**). Besides being of greater prevalence, 5hmC is stable up to 100 times greater than the oxidized bases: 5fC and 5caC (An et al., 2017; L. Hu et al., 2015; L. Alcaraz-Estrada et al., 2020). Moreover, Hu *et al* demonstrated that TET-5hmC complex is greatly stable to be further oxidized when compared to TET-5mC complex (L. Hu et al., 2013; L. Hu et al., 2015). Given this high stability, it is not serendipitous that TET demethylation frequently stops at

the 5hmC intermediate; this reflects that TET-mediated DNA demethylation is not restrictively a progressive process (An et al., 2017).

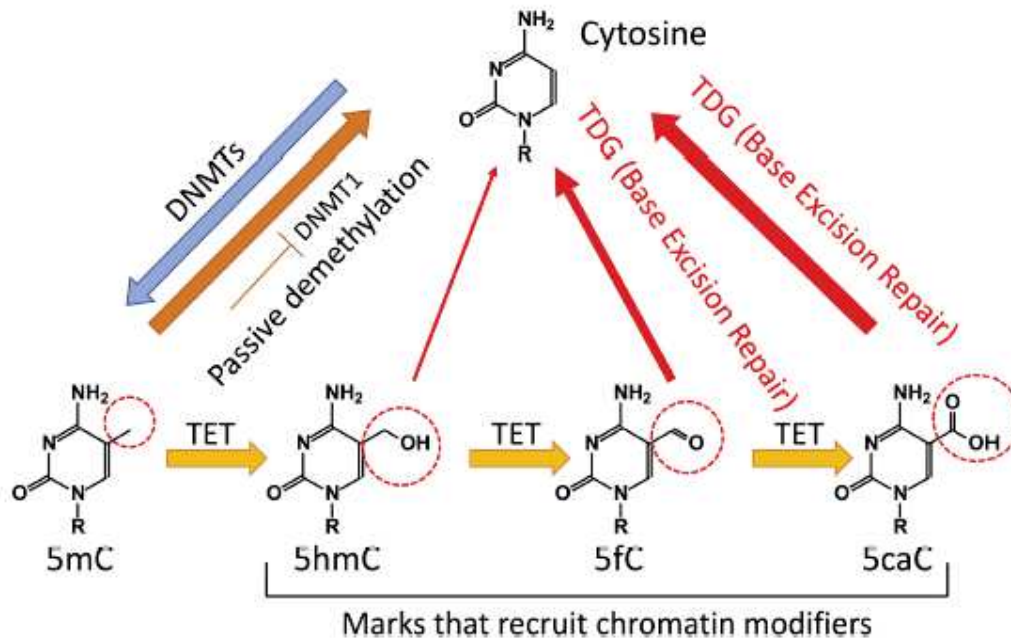


Figure 3: Active and passive DNA demethylation pathway (Tsiouplis et al., 2020). The restoration of the methylated Cytosine (C) base to an unmodified nucleotide is achieved by two distinct demethylation pathways: Active and Passive. While passive demethylation is a replication-dependent process that inhibits the expression of DNMT1, active demethylation is a TET-dependent enzymatic reaction that progressively oxidizes 5mC with and/or without TDG enzymes.

In addition to the high catalytic power, the demethylation pathway crucially requires the recruitment of co-factors (Fe^{2+} , 2-KG) as well as the presence of dioxygen (O_2) and energy (e.g. ATP) (Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). Therefore, 5hmC levels could be considered as a hallmark of TET activity, since its levels are highly influenced by TET expression (Ecsedi, Rodríguez-Aguilera, & Hernandez-Vargas, 2018; Yun Huang et al., 2014; Yotaro et al., 2012).

In concert with TET-demethylation, Thymine DNA Glycosylase (TDG) enzyme recognizes 5caC and 5fC as well as excises mismatched bases (X. Wu & Zhang, 2017) (**Fig.3**). Namely, TDG specifically targets 5caC and 5fC bases in high affinity as they disrupt the N-glycosidic bond (L. Shen et al., 2014). One common example of base mispairing during the demethylation processes is hmU-Adenosine (A) mismatch (Meng et al., 2014). In fact, TET1 frequently oxidizes the Thymine base (T) to hmU (hydroxymethylUracil) in mESC (mouse embryonic stem cells) (Meng et al., 2014). At the end of this process, BER restores the resultant abasic site into an unmethylated cytosine (X. Wu & Zhang, 2017) (**Fig.3**).

2.2. Passive DNA demethylation

Whereas active DNA demethylation is an enzymatic process depending on TETs as key actors, passive methylation is a replication-dependent process in which the methylation writers (DNA methyltransferase enzymes; DNMT) fail to sustain 5mC levels. More precisely, the reduced expression of DNMT enzymes during successive replication cycles dilutes the 5mC mark leading to its removal (Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015).

Besides its role in initiating active demethylation, TET proteins appeared to regulate passive DNA demethylation indirectly (Mulholland et al., 2020). In other terms, TET1/2 promote the expression of DPPA3 (development pluripotency-associated 3) gene which in turn interacts with and shifts the intracellular localization of UHRF1 (Ubiquitin-Like with PHD and Ring Finger Domains) – a cofactor of DNMT (Mulholland et al., 2020). Thus, the dissociation of UHRF1 from the chromatin and its export from the nucleus due to DDPA3 contribute to global demethylation in human embryonic stem cells (hESC) (Mulholland et al., 2020). This novel function of TET proteins might explain the presence of 5hmC as a passive demethylation intermediate (Ciccarone, Valentini, Zampieri, & Caiafa, 2015) (Fig.4).

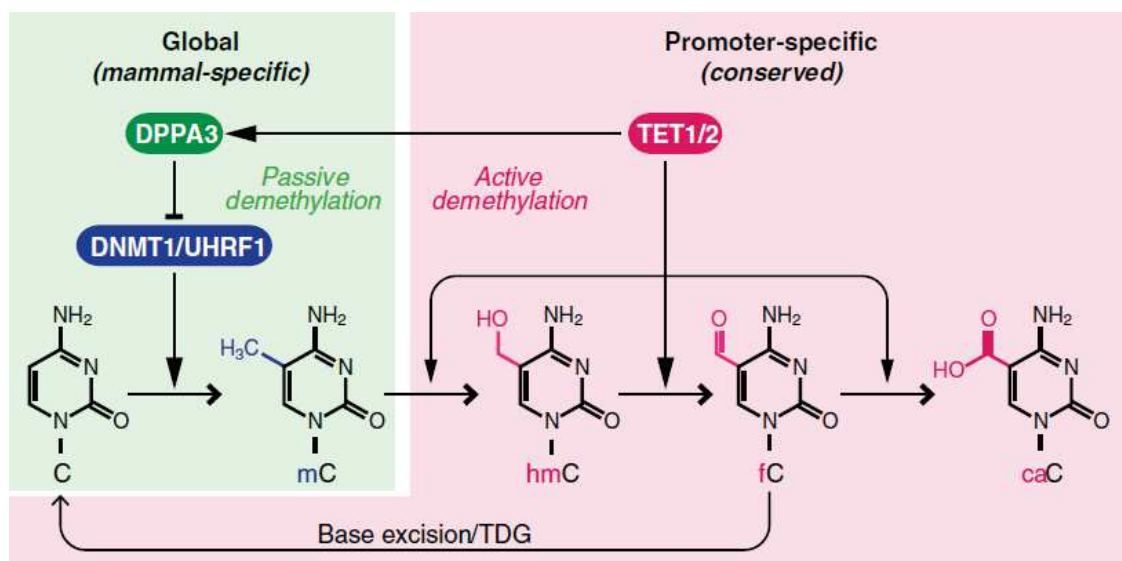


Figure 4: TETs indirectly regulate the passive DNA demethylation process via maintaining DPPA3 levels (Mulholland et al., 2020). The downregulation of DNMT levels during the passive demethylation appears to be mediated, at least in part, by TET1/2 enzymes. These methylation erasers actively enhance the expression of DPPA3 gene to favor 5mC dilution throughout the replication phase.

2.3. Mitochondrial DNA demethylation

Given the interplay between the nuclear and mitochondrial DNA, epigenetically modifying one of these genomes is evidenced to impact the activity of the other (Mohammed, Ambrosini, Luscher, Paneni, & Costantino, 2020; Sharma, Pasala, & Prakash, 2019). Moreover, any imbalance in oxygen availability, ATP levels, or mitochondrial metabolites directly controls TET-mediated DNA demethylation, especially since dioxygen and α -KG are crucial factors for TET activation (Mohammed et al., 2020).

Despite the lack of CpG islands in the mitochondrial DNA (mtDNA), a specific pattern of non-CpG methylation is reported (Mposhi, Van der Wijst, Faber, & Rots, 2017). Not only DNMT (1,3A/B) and TET(1-2) expression were detected in the mitochondria but also their relevant marks: 5mC and 5hmC, respectively; this provides further evidence that mtDNA is epigenetically modulated (Dzitoyeva, Chen, & Manev, 2012; F. C. Lopes, 2020; Mposhi et al., 2017).

Mitochondrial DNA harbors specific regions that cluster together forming mtDNA haplotypes (W. T. Lee et al., 2017). These haplotypes are evinced to control the expression of chromosomal genes (W. T. Lee et al., 2017). Practically, the activity of TET proteins is regulated by mtDNA haplotypes-mediated modulation of the mitochondrial respiratory cycle, tricarboxylic acid (TCA) (W. T. Lee et al., 2017). Furthermore, D-Loops are non-coding regions of mtDNA implicated in mitochondrial metabolism (Holt, 2019). In colorectal cancer tissues, the expression of the mitochondrial gene, MT-ND2, increased following the hypomethylation of D-loop, yet whether TETs mediate this hypomethylation in mitochondria is still unknown (Mposhi et al., 2017). Taken together, the crosstalk between the expression of mtDNA genes and TET demethylation requires to be experimentally validated in the future (F. C. Lopes, 2020).

2.4. Chromatin Targeting

Coding as well as regulatory sites - like promoters, enhancers, and sequences targeted by transcription factors - are greatly enriched with the transcriptional regulator 5hmC (Ciccarone et al., 2015). According to Battistini *et al*, 5hmC mark imposes greater DNA-binding affinity compared to the methylation mark 5mC (Battistini et al., 2020). This stiffness in DNA is negatively correlated with nucleosome compaction, which reflects TET-mediated de-condensation of chromatin (Battistini et al., 2020) (**Fig.5**). For instance, the deposition of 5hmC mark on enhancers favors open chromatin and subsequently recruits additional transcription factors for B cell maturation (C. W. Lio et al., 2016). Concomitantly, the interaction of TET3 with RNA polymerase II does not only enhance chromatin accessibility but also promotes global transcription levels (Krueger et al., 2017).

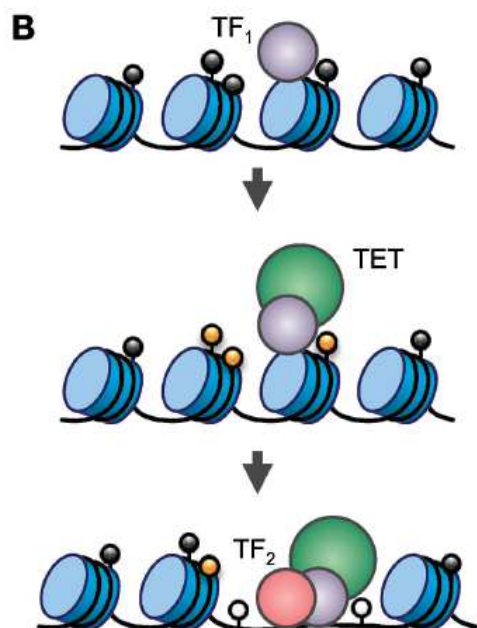


Figure 5: TETs remodel the chromatin to facilitate the activity of transcriptional factors (C. J. Lio & Rao, 2019). Practically, TET proteins shift the chromatin configuration to a loose structure which favors the enrichment of transcriptional regulators on targeted sequences.

Similarly to DNMT1 role in maintaining methylation levels, TET1 appeared to be the guardian of DNA demethylation (Good et al., 2017; C. Jin et al., 2014). Specifically, the CXXC domain of TET1 impedes aberrant methylation at the boundaries of CpG islands, thus controlling local 5mC levels in differentiated cells (Human embryonic kidney: HEK239T) (Good et al., 2017; C. Jin et al., 2014). Teif *et al* further indicated that TET1-mediated regulation of DNA methylation extends outside CpG island borders (Good et al., 2017; Teif et al., 2014). Yet in absence of this domain, TET1 failed to protect against uncontrolled local methylation (Good et al., 2017). Moreover, regulatory regions of genes (e.g promoters, enhancers, and DNase I hypersensitive sites) are directly targeted by TET proteins (X. Wu, Li, & Xie, 2018). In fact, the methylation levels of these regions significantly increase in absence of all 3 TETs (X. Wu et al., 2018). Taken altogether, these reports underline TET1 as an indispensable regulator of chromatin accessibility to favor demethylation as well as the binding of transcriptional factors (**Fig.5**).

3. Genetic-epigenetic interplay

Recently, cancer initiation emerges as a result of the accumulating interdependence among genetic mutations and deregulated DNA methylation (Cervena, Siskova, Buchler, Vodicka, & Vymetalkova, 2020). Mechanistically, this genetic-epigenetic crosstalk is well

exemplified by BRAF mutation-mediated global hypermethylation along with TET silencing in colon cancer patients (Noreen et al., 2019). Inversely, this dynamic interplay exhibit a remarkable increase in TP53 mutation rate (up to 40 folds) following the hypermethylation of its promoter (Molnar et al., 2018). Similarly, methylation-dependent inactivation of a DNA repair gene aggravates the mutational status of BRAF and TP53 in gliomas, suggesting that genetic mutations are associated with specific methylation signatures (Dobre et al., 2021; Esteller et al., 2000) (**Fig.6**). Unlike genetic mutations, epigenetic changes can be readily reversed which highlights epi-markers as promising diagnostic targets in cancer treatment (Cervena et al., 2020; Dobre et al., 2021).

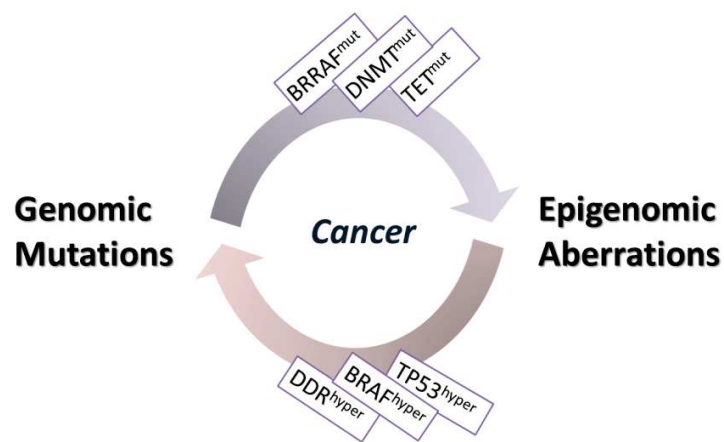


Figure 6: Cancer initiation results from the interplay between genomic mutations and epigenomic aberrations. The scheme exemplifies some mutations in (epi)genes reported to disturb the genome-wide methylation levels. On the other hand, hypermethylation of tumor suppressor genes, TP53 & BRAF, or DNA damage repair genes (DDR) increase their mutation rate.

4. TET interplay with epigenetic processes

Given that TETs bind to the chromatin to exert their functions, it is not surprising that these demethylases synergistically act with other epigenetic modifiers to dynamically orchestrate chromatin structure (Vymetalkova et al., 2019). The positive correlation between TET proteins and active histone marks underscores that transcriptional regulation is tightly governed by several layers of epigenetic control (Vymetalkova et al., 2019). For instance, TET2/3 interaction with OGT (OGlcN-Acetyl Transferase) activated hematopoietic genes expression via induced H3K4me3 levels (Deplus et al., 2013). Moreover, Dominguez *et al* reported diminished transcription due to an impaired acetylation of histone 3 at the 27th lysine residue in tissues harboring TET2 loss-of-function mutations (Dominguez et al., 2018). In concert with this, DNA methylation impedes the removal of H3K9me3 repressive mark on histones whereas it favors histone demethylation at H3K4 (Vymetalkova et al., 2019). TET and DNMT enzymes cooperatively act and/or

compete to regulate histone landscape and gene expression (Gu et al., 2018). Interestingly, losses in these two enzymatic families yield transcriptional perturbations and initiate cancer transformation (X. Zhang et al., 2016).

Furthermore, functional crosstalk is also evinced between TET demethylases and the non-coding RNAs, like microRNA (miR) (Gregorova, Vychytilova-Faltejskova, & Sevcikova, 2021). Although 12% of miR genes are susceptible to methylation-mediated inactivation, many miR target methylation writers/erasers; thus, indicating the bidirectional regulation between both epigenetic modifiers (Gregorova et al., 2021) (**Table 3 – on the next page**).

During the process of gene expression, TETs operate the chromatin configuration to rewire epigenetic modifiers via activating the chromatin remodeling complex SWI/SWF (SWItch/Sucrose Non-Fermentable) (Sepulveda, Villagra, & Montecino, 2017). Mechanistically, TET1/2 proteins trigger SWI/SWF-mediated nucleosomal reconstitution yielding the specific enrichment of histone active marks (e.g. H3 Acetylation; of H3K4me3) and the suppression of DNA/histone repressive marks (5mC; trimethylation of H3K9 and H3K27) (Sepulveda et al., 2017). Taken altogether, TET proteins dynamically control the enzymatic machineries involved in imposing DNA, histonal, and nucleosomal epigenetic signatures on the promoter of genes (Vymetalkova et al., 2019) (**Table 3 – on the next page**).

5. Non-enzymatic functions of TET

Accumulating evidence disclose unconventional roles of TET proteins characterized by their interaction with (epi)genetic factors (H. Lian, Li, & Jin, 2016; Tsiouplis et al., 2020). These roles include activating/repressing transcription, resolving inflammation, regulating stem/progenitor cell differentiation, and stimulating cancer invasiveness and migration (H. Lian et al., 2016; Tsiouplis et al., 2020). To exert these functions, TETs form complexes with chromatin remodelers as well as transcriptional regulators; regardless of neither their demethylation activity nor 5hmC distribution (H. Lian et al., 2016; Tsiouplis et al., 2020). Mechanistically, catalytic-dead mutants evinced to resolve the deleterious effects of TET silencing such as developmental abnormalities and hypoxia-associated epithelial-to-mesenchymal-transition (EMT) (Montalban-Loro et al., 2019; Tsai et al., 2014). **Table 4** summarizes some of TET catalytic-independent functions.

Table 3: TETs regulate non-coding RNAs

TET protein	ncRNA	Role	Reference(s)
TET1	miR-34-a	Control hepatocellular carcinoma progression and metastatic Regulate miR-34-a/BATCH1/p53 levels	(Sun, Zhu, Cao, Zhang, & Wang, 2021)
TET1/2/3	miR-200	Antagonize miR-22 Prevent metastasis and breast cancer aggressiveness	(S. J. Song et al., 2013)
TET3	miR-145	Suppress ovarian cancer Inhibit Warburg effect (high glucose uptake for fermentation regardless of aerobic conditions)	(J. Li, Zou, Pei, Zhang, & Jiang, 2021)
TET1 and TET3	miR-365-3p	Regulate nociceptive behavior	(Pan et al., 2016)

Table 4: An overview of some TET non-demethylation functions

TET	Cell type	Function	Reference(s)
TET2/3	Human and mice cardiac tissues	Reduce chromatin accessibility Diminish downstream heart development genes	(Fang et al., 2019)
TET 1/2/3	Neurons (Neuro2a cells)	Inhibit neuronal differentiation	(Gao et al., 2016)
TET1	Cervical cancer cells	Co-activates Hypoxia factor HIF-1/2* Stimulates EMT	(Tsai et al., 2014)

Table 4: continued

TET	Cell type	Function	Reference(s)
TET1	Bone marrow derived mesenchymal stem/stromal cells	Repress osteogenic and adipogenic differentiation Form a repressive complex with SIN3a and EZH2*	(Cakouros et al., 2019)
TET2	Dendritic cells and macrophages	Recruits HDAC-2* to repress IL-6*	(Q. Zhang et al., 2015)
TET2	Breast cancer cells	Recruits HDAC-1/2 Represses the chromatin structure Protects against immuno-evasion	(Y. Shen et al., 2021)
TET2	Nasopharyngeal carcinoma	Prevents PKM* translocation into the nucleus Suppresses glycolysis	(X. Zhang, Yang, Shi, & Cao, 2020)
TET3	Human embryonic kidney (HEK239) cells	Interacts with and stabilizes thyroid hormone nuclear receptor Sensitizes cells to secrete thyroid hormone	(W. Guan et al., 2017)
TET3	Trans-differentiation of ESC to trophoblast like cells	Global Hypertranscription (increase in RNA levels) Interaction with RNA pol II favors chromatin accessibility	(Krueger et al., 2017)

Table 4: continued

TET	Cell type	Function	Reference(s)
TET3	Neuronal stem cells	Prevents neurogenesis Directly binds to and repress <i>Snrpn</i> * protein	(Montalban-Loro et al., 2019)

* **Ezh2**: Enhancer of zeste homolog 2; **HIF-1/2**: Hypoxia-inducible factor 1/2; **HDAC-1/2**: Histone deacetylase enzyme 1/2; **PKM**: Pyruvate kinase muscle; **Snrpn**: Small nuclear ribonucleoprotein polypeptide N; **IL-6**: Interleukin 6

Furthermore, somatic-to-pluripotent cell transition can be exemplified by the binding of TET2 with PARP1 (poly-ADP-Ribose polymerase 1) (Doege et al., 2012). In fact, PARP1-induced post-translation PARylation of TET2 actively remodels/loosens chromatin architecture via enrichment of the active transcription marker, H3K4me2, at pluripotency loci (Doege et al., 2012). Another example shows the direct interaction of TET1-2 with Nanog to favor the establishment of induced-pluripotent stem cells; this reflects TETs properties in reprogramming somatic cells toward pluripotency (P. Wang et al., 2013).

On the other hand, Gao *et al* reported the inhibitory role of TET proteins during neuronal differentiation which can be restored, at least in part, by introducing catalytically inactive TET3 mutations in *Xenopus* frogs (Gao et al., 2016; Y. Xu et al., 2012). Moreover, during early heart development, silencing TET2/3 proteins lead to phenotypic cardiac distortions often associated with embryonic lethality (Fang et al., 2019). By perturbing the chromatin architecture, loss of TET proteins impedes the recruitment of cardiac differentiation-specific factors (Fang et al., 2019).

Although the catalytic domain favor, sometimes, the association of TETs with (epi)regulators, such as in the case of TET3-Thyroid hormone interaction, no changes in 5hmC levels were detected (W. Guan et al., 2017). It is worth noting that mutations, epimethylation as well as post-translational/transcriptional modifications can alter both catalytic and non-catalytic functions of TETs (H. Lian et al., 2016). Taken altogether, TET proteins are multi-level players in gene transcription and their catalytic domain may serve as a platform to rewire their demethylation-independent functions (**Table 4; Fig.7 on the next page**).

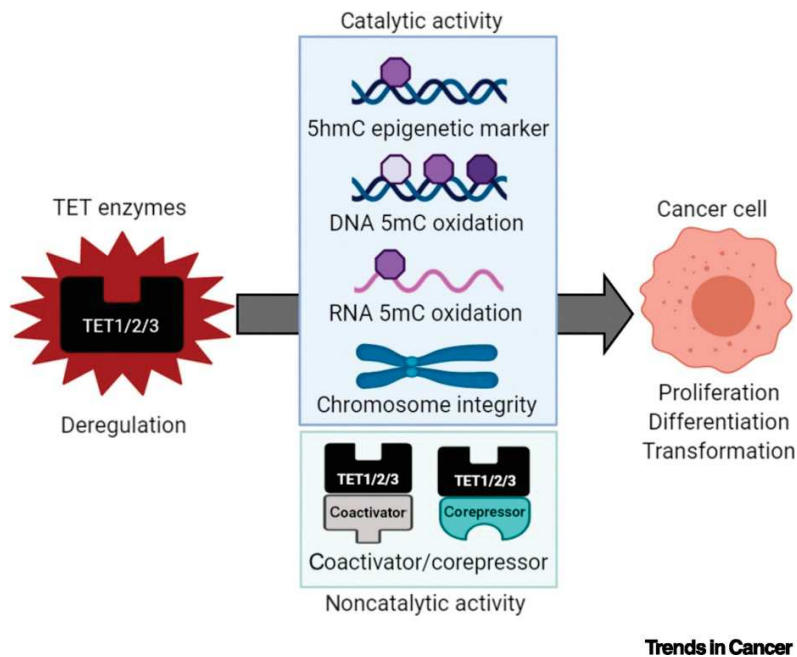


Figure 7: TET catalytic-dependent and -independent functions and their implication in carcinogenesis (Bray, Dawlaty, Verma, & Maitra, 2021). TET proteins are well known for their conventional role as erasers of methylation. Regardless of their catalytic activity, TET proteins emerge as novel regulators of gene expression via binding to co-activator or co-repressor complexes. Given the multi-faceted activity of these proteins, deregulation in TET enzymes is correlated with carcinogenesis.

6. Regulation of TET activity

It is worth noting that many factors can abate the expression of TET genes leading to a non-mutational phenotypic loss in solid cancers such as tumor microenvironment and cellular immunity regulators (Y.-P. Xu et al., 2019). In the following sections, we elaborate these non-mutational causes of TETs silencing with a special focus on the impact of cellular localization as well as post-transcriptional/translational modifications (**Fig.8 on the next page**).

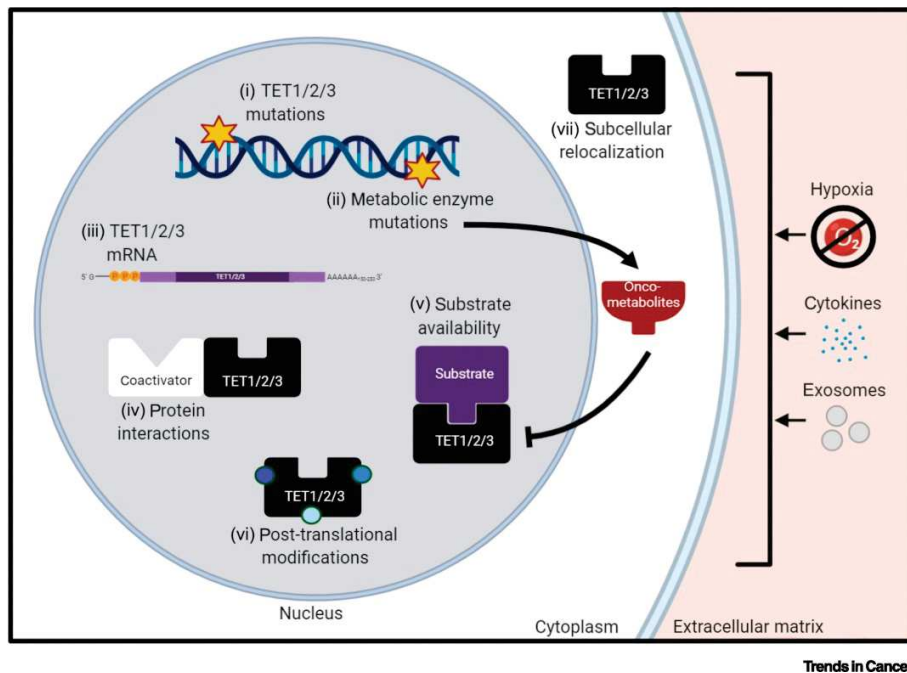


Figure 8: Mutational and non-mutational factors regulating the expression of TETs (Bray et al., 2021). A diagram showing that mutations in TET genes are not sole the players in their disrupted expression. TET levels is greatly influenced by various intracellular factors including: (ii) Mutations in metabolic enzymes (e.g. IDH), (iii) degradation of TET mRNAs (i.e. via microRNAs) (iv) interaction with proteins (i.e. transcription factors), (v) availability of indispensable co-factors of TETs (e.g. 2-OG) and/or methylated cytosine bases as substrates, (vi) post-translational modifications (e.g. acetylation); TET are influenced as well by extracellular factors such as hypoxia, cytokines and exosomes.

6.1. Epi-methylation

Interestingly, methylation of TET1/2 promoter is reasoned to predominantly cause its loss in various cancers; this implicates that TET1/2 constitutes a direct target of DNA methylation (Barazeghi et al., 2017; H. Q. Chen et al., 2021; L. Li et al., 2016). Besides, treating cancer cells with 5-AZA, a DNA methyltransferase inhibitor, significantly restored TET1 expression which subsequently inhibited carcinogenesis and can directly activate BER (Base-Excision Repair) pathway (Barazeghi et al., 2017; H. Q. Chen et al., 2021; L. Li et al., 2016). Unfortunately, it is still unclear whether DNMT alone drives TET1 silencing, or a repressive complex is responsible for maintaining DNA methylation levels on TET1 promoter. Furthermore, research groups have neither examined 5hmC levels to identify a possible re-compensation by TET2-3 nor investigated whether other TET proteins can also be methylated. Can TET proteins rescue themselves by feedback loop or do they require the intervention of yet-to-be-discovered partners or protein complexes? Indeed, revealing the ability of TET proteins to de-methylate each other's would be a great breakthrough in understanding TET mode of function

6.2. Subcellular localization

TETs are predominantly nuclear proteins and it is clear that a change in their subcellular localization disrupts their activity (Yuji Huang et al., 2016; Muller et al., 2012). In Isocitrate-dehydrogenase (IDH1)-wild type gliomas, for instance, loss of 5hmC is directly related to the cytosolic localization of TET1 (Muller et al., 2012). In parallel, restoration of 5hmC was observed post-treatment with the inhibitor of nuclear exclusion, leptomycin B, in LoVo cells; this implicates how the nuclear accumulation of TET2 is indispensable for initiating its activity (Yuji Huang et al., 2016).

6.3. Post-transcriptional modifications

According to the literature, TET expression can be regulated at the post-transcriptional level via various non-coding RNAs – including microRNA, long non-coding RNAs (lncRNA), and circular RNAs (circRNA) (Arvinden et al., 2017). These non-coding RNAs (ncRNA) target and modulate TET transcription thus inhibiting its catalytic activity (Arvinden et al., 2017). Here, **table 5** summarizes some of these ncRNAs.

Table 5: Non-coding RNAs regulating TET expression

ncRNA	TET proteins	Role(s)	Reference(s)
miR-22	TET1/2/3	Induce metastasis, EMT, tumorigenesis of breast cancer Poor survival outcome	(S. J. Song et al., 2013)
miR-21	TET1/2/3	Inhibit apoptosis Promoter HCC* progression Silence TETs to repress PTENp1* and PTEN*	(Cao et al., 2019)
miR-543	TET1/2/3	Global hypermethylation in myelofibrosis Decreased acetylation of Histone H3; of non-histone proteins: STAT3* & TP53	(Fuentes-Mattei et al., 2020)

Table 5: continued

ncRNA	TET proteins	Role(s)	Reference(s)
miR-191	TET1	Silence TET1 Hypermethylate APC tumor suppressive gene	(C. Yang et al., 2020)
XIST*	TET1	Inhibit TET1-p53 interaction Promoter bladder cancer progression	(B. Hu, Shi, Li, Li, & Zhou, 2019)
FECR1 circRNA	TET1	Breast tumor growth and metastasis via activating FLI* gene	(N. Chen et al., 2018)
miR200b/a	TET3	Regulation of olfactory male behavior Maintain a balance between proliferation and differentiation neuronal progenitor cells	(D. Yang, Wu, Zhou, Wang, & Wang, 2020)

* **HCC**: Hepatocellular carcinoma; **PTEN**: Phosphatase and tensin homolog; **PTENp1**: PTEN pseudogene 1; **FLI-1**: Friend leukemia integration 1; **XIST**: X-inactive specific transcript; **STAT3**: Signal transducer and activator of transcription 3; **FECR1**: FLI exonic circular RNA 1

6.4. Post-translational modifications

Furthermore, TET expression can be either enhanced or suppressed by various post-translational modifications. These modifications include PARylation mediated by PARP proteins; O-GlcNAcylation by OGT; Acetylation by p300; and mono-ubiquitination by CRL4^{VprBP} (Cullin-RING-Ubiquitin Ligase 4). Arvinden *et al* have previously summarized these modifications in a table showing their effects on 5hmC levels (Arvinden et al., 2017). Here, an adapted table is provided (**Table 6**).

Table 6: A summary of the post-translational modifications of TET proteins

Post-translational Modification	Regulatory protein	Function(s)	Effects on 5hmC	Model cell line	Reference
Acetylation of TET2	P300	Increases TET2 half-life Induces TET2 activity especially at hyper-methylated sites under oxidative stress	Increased	Ovarian (A2780) and Colorectal cancer (HCT116) cell lines	(Y. W. Zhang et al., 2017)
Monoubiquitination	CRL4 ^{VprBP}	Promotes TET-DNA binding	Increased	Mouse embryonic fibroblasts	(Nakagawa et al., 2015)
PARylation	PARP-1	<u>Dual activity</u> i. Increased TET1 activity ii. Decreases DNA binding	<u>Dual effect:</u> i. Increased ii. Decreased	HEK239 cells	(Ciccarone et al., 2015)
O-GlcNAcylation	OGT	Enables nuclear export of TET3 Enhance chromatin binding of TET	Decreased	Not cell type specific	(Q. Zhang et al., 2014)
Proteasomal degradation	Calpain-1/2	Decreases TET stability Enhances TET turnover	Decreased	Mouse embryonic stem cells; Neuronal progenitor cells	(Y. Wang & Zhang, 2014).

7. TET disruption in cancer

7.1. TET mutations

Given that epigenetic markers dictate the transcriptome, mutational variation in these regulators is associated with worse prognostic outcome (H. Shen & Laird, 2013). Moreover, mutations in methylation writers and/or erasers contribute to genome-wide aberrant methylation patterns (C. J. Lee et al., 2020). According to the *Catalogue Of Somatic Mutations In Cancer* (COSMIC) and *The Cancer Genome Atlas* (TCGA) databases, TET mutations in solid tumors occur at a lower frequency with respect to hematological cancers (Yun Huang & Rao, 2014; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). In prostate cancer, the reported increase in cancer aggressiveness is subsequent to rare TET2 mutations detected in 24.4% of cases (Koboldt et al., 2016). Recently, Yang et al disclosed novel deleterious TET2 mutations in renal, breast, colorectal, lung, and prostate cancer where the mutation frequency does not exceed 5%. However, neither the expression level, the activity nor the chromatin binding affinity of mutant TET2 were assessed by the authors. Interestingly, the same group evinced the anti-tumor activity of an epi-drug (T-dCyd) to efficiently treat patients carrying simultaneous TET2 and DNMT3a mutations (S. X. Yang et al., 2021). Among different solid tumors, colon adenocarcinoma, for example, harbors a dominant mutational profile of all TET proteins (C. J. Lee et al., 2020) (**Fig.9**). According to Yu *et al* cohort study, TET1 variants were observed in 4.75% of colorectal cancer patients (Yu, Zhang, & Chen, 2021). All of these findings confirm the low frequency of TET mutations in solid tumors and highlight the need for further investigations assessing the clinical implications of these epi-mutations.

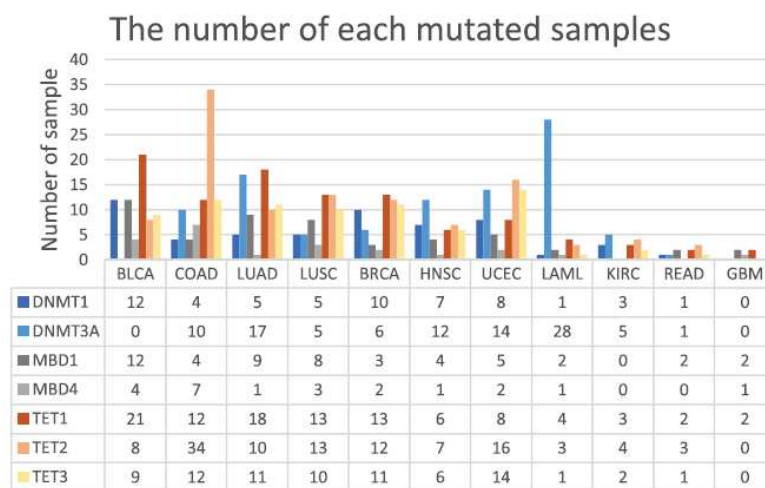


Figure 9: Frequency of TET mutations in myeloid and solid cancers (C. J. Lee et al., 2020).

According to the authors, a dominant TET mutation profile is observed in COAD. BLCA: Bladder cancer; COAD: Colorectal adenocarcinoma; LUAD: Lung adenocarcinoma; LUSC: Lung squamous carcinoma; BRCA: Breast cancer adenocarcinoma; HNSC: Head and neck squamous carcinoma; UCEC: Uterine corpus endometrial carcinoma; LAML: Acute myeloid leukemia; KIRC: Kidney renal carcinoma; READ: Rectal adenocarcinoma; GBM: Glioblastoma.

7.2. TET isoforms in cancer

Strikingly, a new TET1 isoform lacking its CXXC domain is found to be highly expressed in various solid cancers (e.g. Breast, uterine, glioblastoma) (Good et al., 2017). Although this truncated isoform retains its functional activity, it appeared to demethylate discrete target sites when compared to the full-length TET1 (Good et al., 2017). Besides, it is correlated with poor survival outcome (Good et al., 2017). Unfortunately, this research group has neither examined the intracellular distribution profile of this isoform nor identified its possible partner proteins in cancer. (Good et al., 2017). Yet, it is worth noting that overexpression of this isoform in mouse ESC displayed a diffused nuclear intracellular profile (W. Zhang et al., 2016).

7.3. Hematological cancers

The role of TET family in hematological malignancies has exhaustively been reported. TET1 was shown to be rarely translocated (0.3%) in MLL, while TET2 was observed to be frequently mutated in AML, MPN (myeloproliferative neoplasms), MDS (myelodysplastic disorders), and CMML (chronic myelomonocytic leukemia) (Abdel-Wahab et al., 2009; Delhommeau et al., 2009; Gaidzik et al., 2012; Kosmider et al., 2009; Langemeijer et al., 2009) (**Fig.10**). Interestingly, TET2 alterations are considered as an early mark for hematological cancers due to their occurrence in premalignant Hematopoietic Stem cells (HSC) (Rasmussen & Helin, 2016; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). Moreover, changes in TET2 levels regulate cancers progression (Baylin & Jones, 2011; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015; H. Wu & Zhang, 2011). Besides, other TET family members – TET1 and TET3 – do not show any loss-of-function mutations in these types of cancers, therefore suggesting that TET2 gene is the one that mainly plays a tumor-suppressive role in myeloid/lymphoid cancers (Abdel-Wahab et al., 2009; Ficzk & Gribben, 2014; Rasmussen & Helin, 2016).

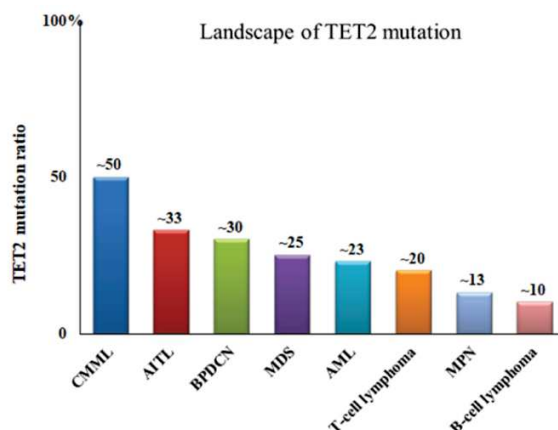


Figure 10: TET2 mutation frequency in several hematological cancers. (Feng, Li, Cassady, Zou, & Zhang, 2019). TET2 mutational rates appear to be highest in CMML and lowest B-cell lymphoma when compared to other hematological malignancies.

7.4. Solid cancers

TET expression as well as 5hmC levels are generally downregulated in different solid cancers (Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). This common feature highlights the role of TETs in diverse anti-oncogenesis pathways by regulating the expression of their target genes (L. Alcaraz-Estrada et al., 2020). TET1 is frequently reported to be silenced in solid cancers with respect to other family members and its restoration is directly associated with a better prognosis for patients with solid tumors (Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015).

7.4.1. Hepatocellular carcinoma

TET1 is commonly downregulated in HCC which is subsequently correlated with poor prognostic survival (Sun et al., 2021). Furthermore, TET1 exerts its tumor suppression functions by inducing microRNA demethylation and p53-pathway activation (Sun et al., 2021). According to Sun *et al*, HCC progression and metastasis are correlated with low TET1 levels, thus implicating the inhibition of TET1/microRNA/p53 machinery in carcinogenesis (Sun et al., 2021).

7.4.2. Breast cancer

In 2014, Song *et al* revealed a novel mechanism for silencing TET family of proteins in breast cancer that is mediated by microRNA antagonism (S. J. Song et al., 2013). TET silencing associates with decreased global demethylation and is negatively correlated with breast cancer aggressiveness (S. J. Song et al., 2013). More precisely, overexpression of

miR22 inactivates its anti-metastasis antagonist miR200 by directly blocking TET activity on its promoter region (S. J. Song et al., 2013).

Furthermore, TET depletion also impinges the expression of its downstream targets during carcinogenesis (X. Zhu & Li, 2018). For instance, caspase-4 downregulation is directly subsequent to hypomethylation loss on its promoter region (X. Zhu & Li, 2018). Strikingly, this enzyme appears to be indispensable in mediating TET2 tumor-suppressive functions in breast cancer cells (X. Zhu & Li, 2018).

While hypomethylation is most prominently observed in triple-negative breast cancer, Good *et al* in 2018 surprisingly reported the overexpression of TET1 in this breast cancer subtype (Good et al., 2018). This finding shed light on the oncogenic role of TET1 especially since PI3K mutations and tumor proliferation appeared to be mutual with increased hypomethylation (Good et al., 2018).

7.4.3. Lung cancer

Exposing normal lung cells to the chemical compound 3-methylcholanthrene has not only induced malignant transformation but has also contributed to promoter methylation-dependent silencing of TET1 (H. Q. Chen et al., 2021). As it directly induces the expression of BER enzymes, TET1 repression is associated with promoted migration and aggressiveness of lung cancer *in-vitro* and *in-vivo* (H. Q. Chen et al., 2021).

7.4.4. Renal cell carcinoma

As observed in other solid cancer, promoter demethylation of tumor suppressor genes greatly impedes renal tumor development and promotes the survival outcome (Gan et al., 2021). For example, the frequent silencing of the anti-oncogene, TRIM58, is due to its promoter hypermethylation in clear cell renal cell carcinoma as reported in lung, gastric, and colorectal carcinoma (Gan et al., 2021). Upon treatment with 5-AZA or using CRISPER-dCas9 system of genome editing, the levels of TRIM58 were restored along with a better prognosis; This highlights the indispensable implication of epigenetic demethylation processes in controlling carcinogenesis (Gan et al., 2021).

7.4.5. Gastric cancer

As in many solid cancers, TET2 and TET3 remain unchanged while TET1 decreases at its mRNA and protein levels (Pei et al., 2016). Mechanistically, Pei *et al* disclosed the activation of AKT (Protein Kinase B) and FAK (Focal Adhesion Kinase) pathways during oncogenesis (Pei et al., 2016). More precisely, PTEN expression, mediated by TET1-demethylation on its promoter, is indispensable to prevent cancer aggressiveness via the

suppression of AKT and FAK pathways; thus any deregulation in TET1 activity would indeed impede PTEN-driven control of tumor progression (Pei et al., 2016).

Moreover, hypermethylation of the tumor suppressor genes, RASSF1A (Ras association domain family 1 isoform A) and p16, is subsequent to the downregulation of the long non-coding RNA, FENDRR (FOXF1 adjacent non-coding developmental regulatory RNA) (Z. He et al., 2018). FENDRR inactivation does not only impede the recruitment of TET2 but also promotes gastric cancer progression (Z. He et al., 2018). In fact, miR-214-3p directly interacts with FENDRR to control its expression as well as its downstream targets (Z. He et al., 2018). According to the author, overexpression of FENDRR can sponge the effects of miR-214-3p and restore the activation of TET2 and RASSF1A (Z. He et al., 2018). Yet, it is still unknown whether the aberrant methylation of RASSF1A and p16 is restricted to FENDRR-mediated loss of TET2 or whether it encompasses the silencing of other TET proteins (Z. He et al., 2018).

7.4.6. Endometrial cancer

Despite the rising evidence regarding TET1 as a tumor suppressor gene that is frequently abated in solid tumors, few studies disclose the oncogenic functions of this protein (Good et al., 2018; M. Wu, Zhang, Tang, & Tian, 2016). In endometrial cancer, aberrant overexpression of IDH1 causes a proportional induction of α -KG metabolite and subsequently of TET1 levels (Bai et al., 2018). Interestingly, endometrial chemodrug resistance is reasoned to be caused by Nuclear-Factor-Erythroid 2-related factor-2 (NRF-2) activation as one of the downstream targets of IDH-1/ α -KG/TET1 pathway (Bai et al., 2018). Strikingly, Bai et al reported the direct binding of NRF-2 to the antioxidant response element (ARE) sequence in the promoter region of IDH1; this implicates a positive feedback loop regulation of this signaling pathway (Bai et al., 2018). On the other hand, metformin does not only sensitize endometrial cancer cells to chemotherapeutics via regulating IDH1, but can also inhibit Glyoxalase I (GLOI), another downstream target of IDH1/ α -KG/TET1 pathway (Bai et al., 2018; Jiang et al., 2019).

7.4.7. Colorectal cancer

TET genes are found to be frequently downregulated in various colorectal cancer (CRC) cell lines and tissues (Yuji Huang et al., 2016; Ichimura et al., 2015; Rawłuszko-Wieczorek, Siera, Horbacka, et al., 2015; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015; Y. Tian et al., 2017; Yotaro et al., 2012). Since 5hmC levels are highly influenced by TET family expression, low 5hmC levels are considered as a subsequent phenomenon to TET downregulation (Haffner et al., 2011; W. Li & Liu, 2011; H. Lu et al., 2016; Neri et al., 2014; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015; Y. Tian et al., 2017;

Yotaro et al., 2012). Yet, a decrease in 5hmC amount in CRC cell lines is not necessarily partnered with a simultaneous loss in 5mC which suggests that TET enzymes do not equally hydroxylate 5mC bases (Ciesielski et al., 2017; Haffner et al., 2011; Neri et al., 2015; Yotaro et al., 2012). In other terms, this underscores that TET proteins act in a catalytic-dependent and -independent manner to regulate target genes (Yotaro et al., 2012).

Although TET1 reported the highest reduction in comparison with other TET members (Yuji Huang et al., 2016; Yotaro et al., 2012), it is undeniable that TET2 and TET3 down-regulation is also implicated in CRC, which implies that each TET gene is involved differently in colorectal cancer (Rawłuszek-Wieczorek, Siera, Horbacka, et al., 2015; Rawłuszek-Wieczorek, Siera, & Jagodziński, 2015).

Nowadays, researchers seek to elaborate the reasons correlated with TET reduced expression. COSMIC database revealed that 0.75% of colon cancers present detectable somatic mutations in TET1 gene – 80% of which are synonymous substitutions (Lili Li et al., 2016). However, a recent cohort study conducted by Yu *et al* disclosed that TET1 mutation level reaches up to 4.75% in CRC patients (Yu et al., 2021). Although TET1 mutations do not seem the primary cause for solid tumors initiation and progression, this increase in TET1 mutation rate could be attributed to changes in dietary factors and/or to exposure to environmental modifiers.

Besides age/lifestyle factors, aberrant hypermethylation in CRC has been linked to BRAF^{V600E} oncogenic mutation known to initiate cancer and to repress TET1-2 levels (Noreen et al., 2019). Again, high TET2 levels implicate a better prognosis independently of its partner protein AMPK (AMP-activated protein kinase), implying the tumor-suppressive role of TET demethylation enzymes in colorectal cancer (D. H. Kang et al., 2021).

Very recently, Wong *et al* depicted the detailed mechanism of CRC progression and aggressiveness in KRAS-mutant in-vitro models (Wong et al., 2020). According to this study, CRC cells with KRAS mutation favor the accumulation of succinate due to their addiction to glutaminolysis (Wong et al., 2020). In fact, the increase in the expression level of glutaminase – *catalyzer of glutamine conversion into glutamate* - and of SLC25A22 - *importer of glutamine into the mitochondrion* – promotes succinate-mediated competitive inhibition of α -KG dependent demethylases (Wong et al., 2020). As a result, DNA and histone methylation significantly increases in these cells (Wong et al., 2020). Phenotypically, CRC cells with active KRAS strikingly displayed resistance to the chemotherapeutic drug, 5-Fluorouracil, and exhibited cancer stemness properties (Wong et al., 2020). Moreover, TET proteins are well-studied inhibitors of Wnt/ β -catenin pathway since the repression of TET demethylation stimulated CRC tumorigenesis by activating this pathway (Wong et al., 2020). Given that over-activation of Wnt/ β -catenin pathway is a hallmark of many cancers including CRC, targeting TET proteins may represent an attractive strategy for CRC treatment (Y. Zhang & Wang, 2020).

8. External modulators

8.1. Metabolic factors

As TET enzymes depend on Fe^{2+} ions and α -KG as co-factors to activate their catalytic domain, other enzymes and/or metabolites can negatively modulate TET activity (Meng et al., 2014). Practically, IDH family of enzymes produces α -KG from isocitrate, implying that any mutation in IDH family results in a high 2-hydroxyglutarate (2-HG) to α -KG ratio (Meng et al., 2014). 2-HG competitively antagonizes the binding of α -KG on the DSBH domain of TET demethylases. For example, phenotypic abnormalities in the central nervous system of mice were observed in IDH1 mutant gene (R132H) (Meng et al., 2014) (**Fig.11**).

Besides the inhibitory role of the 2-HG onco-metabolite, the accumulation of succinate and fumarate – the oxidation products of α -KG – also compete with α -KG to regulate TET-mediated DNA demethylation (Meng et al., 2014). Mechanistically, mutations in fumarate hydratase or succinate dehydrogenase enzymes yield high production levels of their substrates, fumarate and succinate respectively (M. Xiao et al., 2012). Thus, these onco-metabolites rewire deregulated epigenetic signaling during cancer progression due to their role in abrogating both DNA and histone demethylases (M. Xiao et al., 2012).

Interestingly, mutations in Krebs cycle enzymes are not the sole drivers of the metabolic regulation of demethylation, as KRAS activating-mutations also perturb α -KG production (Wong et al., 2020). Precisely, KRAS-mutant CRC cells stimulate excessive glutaminolysis, the enzymatic reaction producing α -KG from glutamine, which impinges TET activity via succinate accumulation (Wong et al., 2020). This process is directly associated with poor prognosis since aberrant hypermethylation promotes CRC tumorigenesis and therapeutic drug resistance (Wong et al., 2020).

Taken altogether, TET demethylation activity is critically modulated by metabolic factors, known to be readily influenced by lifestyle and dietary factors (Asadi Shahmirzadi et al., 2020) (**Fig.11**).

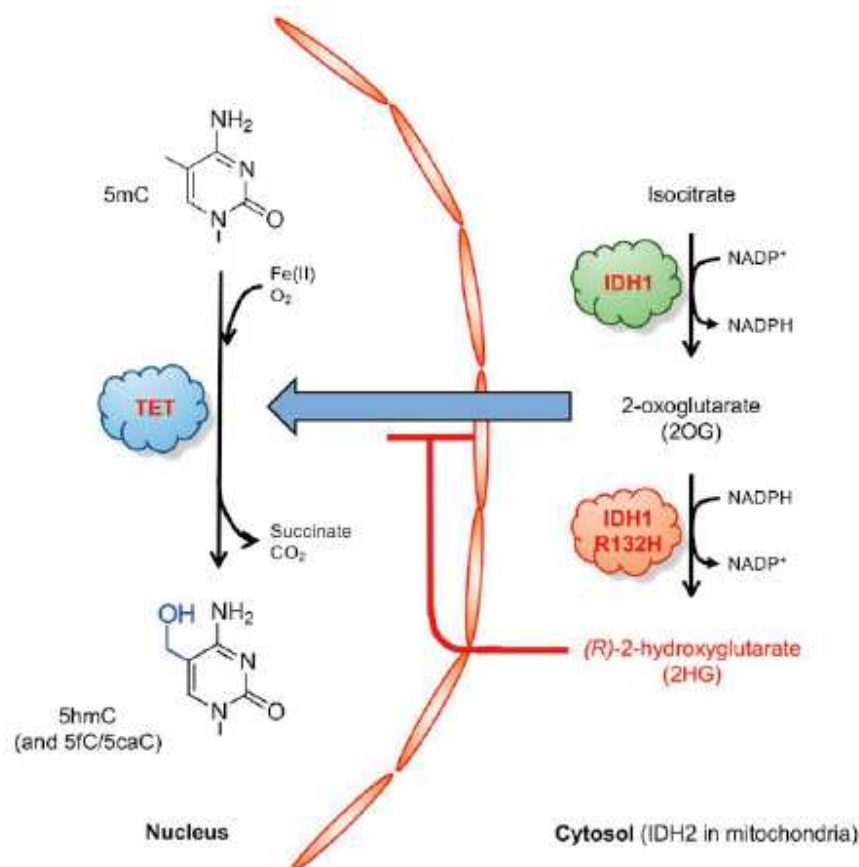


Figure 11: IDH mutations produce oncogenic metabolites that antagonize TET activity (An et al., 2017). The onco-metabolite 2-HG inhibits the activation of TET proteins via competitively antagonizing the key co-factor 2-OG.

8.2. Dietary/ Nutritional factors

The epigenome has extensively been studied for its sensitivity toward variations in dietary and environmental regulators (T. Zhu, Brown, & Ji, 2020). In fact, changes in nutritional factors are evidenced to modulate epigenetic markers (T. Zhu et al., 2020). For instance, the dietary flavonoid, 3,6-dihydroxyflavone, mediates its anti-oncogenic properties via inducing imbalances among DNA methylation writers and erasers: DNMT1 is repressed whereas TET1 is upregulated (X. Peng et al., 2017).

Other natural compounds were also reported for their role in regulating epi-proteins such as the honey's flavone, Chrysin, which abrogates tumorigenesis via triggering TET1 expression (X. Zhong et al., 2020). Additionally, Curcumin induces TET1-mediated hypomethylation of Wnt/ β -catenin inhibitors to circumvent drug resistance of colorectal cancer (Y. Lu, Zhang, Zhang, Zhang, & Yao, 2020).

Moreover, Vitamin C (VitC) micronutrient is well-known as a demethylation agent that restores TET activity and sensitizes cancer cells toward the chemotherapeutic drugs (Giansanti et al., 2021; Linowiecka, Foksinski, & Brozyna, 2020). Some studies disclosed VitC as an indispensable co-factor of TETs whereas others attribute the stimulation of TET activity to VitC capacity in reducing ferric ion (Mastrangelo, Pelosi, Castelli, Lo-Coco, & Testa, 2018). Remarkably, VitC retained its ability to stimulate 5hmC levels even in cells lacking or acquiring a mutation in TET proteins (Giansanti et al., 2021). Furthermore, the activity of this anti-oxidant extends to enhance 5hmC levels on mRNA transcripts (Lan et al., 2020).

All of these findings underscore the susceptibility of TET enzymes toward nutritional factors. Finally, it remains noteworthy to highlight VitC protective roles against chemical genotoxicity; this can be exemplified by its capacity to sponge deleterious effects on liver and kidney tissues exposed to Paraquat herbicide or to rescue exposed patients from oxidative and inflammatory responses (Awadalla, 2012; S. Hu et al., 2018).

8.3. Environmental factors

Exposure to pollutants is reported to influence TET expression, 5hmC levels, as well as the non-enzymatic functions mediated by TETs. In fact, some factors may promote hydroxymethylation levels to mediate their effects. For example, the phenyl-urea herbicide, Diuron, impinges the killing activity of plasmacytoid dendritic cells toward solid tumors in a TET2-dependent manner (Briand, Joalland, et al., 2019). Similarly, the benzene metabolite – hydroquinone – drives demethylation by stimulating iron levels in a reactive-oxygen species (ROS)-dependent manner (Coulter, O'Driscoll, & Bressler, 2013; Zhao et al., 2014).

However, other chemical pollutants exert opposing effects on TET demethylation pathway. Bisphenol A, for instance, enhances breast carcinogenesis via epi-methylation of TET2 promoter (Z. Li, Lyu, Ren, & Wang, 2020). Mechanistically, this endocrine disruptor activates estrogen receptors which in turn maintain DNMT expression during cancer development (Z. Li et al., 2020). Moreover, arsenite (As^{3+}) directly interacts with all 3 TET proteins, thus, diminishing the levels of oxidized cytosine bases (i.e. 5hmC, 5fC, and 5caC) (S. Liu et al., 2015). Cadmium, another heavy metal with multi-organ carcinogenicity, is believed to favor global hypermethylation subsequently to TET1 silencing by ROS-sensitive pathways (Hirao-Suzuki et al., 2021).

Taken altogether, environmental factors mediate their (epi)genotoxic via differentially reprogramming TET expression and activity.

THESIS OBJECTIVES

Epigenetic and environmental modifiers – as single or interdependent players – emerge as key regulators influencing our susceptibility to malignant pathologies, like cancer. Epigenetic pathways, particularly DNA (de)methylation, are readily deregulated during the early onsets of chemical-mediated carcinogenesis. However, a full comprehension regarding the impact of Linuron herbicide, a chemical pollutant extensively used in Lebanon, on DNA methylation erasers, namely TET enzymes, is required. The lack of such knowledge is attributed to the extensive research focus on the endocrine-disrupting properties of this herbicide, particularly on aquatic organisms. Hence, there is a need to establish a study assessing the link between Linuron and TET demethylases in human cancers.

On the other hand, Vitamin C (VitC) is a well-known inducer of TET demethylation via increasing ferrous ion levels and/or binding, as a co-factor, to the catalytic domain of TETs. Despite many discoveries revealing VitC-associated anti-cancer events through TET activation, the exact molecular mechanism of VitC is not completely understood. This could be explained by the fact that TETs partner with a plethora of proteins, thus, driving various cellular pathways against tumorigenesis.

How Linuron – as an environmental factor – or VitC – as a therapeutic agent – modulate TET activity is however still open.

In this research project, the principal aim is to assess, *in-vitro*, the impact of each compound on regulating TET1 function in the context of colorectal cancer.

Global aims

- Examine the intracellular localization profile of TET1 protein in non-tumoral colon and tumoral colorectal cancer (CRC) cells
- Identify novel partner proteins of TET1
- Assess the effects of VitC on the activity and the partners of TET1
- Investigate the impact of Linuron exposure on CRC cells
- Evaluate the impact of Linuron on the intracellular localization of TET1 in CRC cells

RESULTS

Article 1: Co-localization of novel TET1-nuclear bodies with Cajal-or PML-bodies is modulated by Vitamin C in colorectal cancer cells (under revision)

1.1. Scientific relevance

Novel functions of TET proteins are recently emerging especially those related to cancer regulation. TET proteins, along with their role as erasers of DNA methylation, appeared to recruit a plethora of partner proteins (e.g., Nanog; OGT) and to modulate various cellular mechanisms (e.g. cellular differentiation, immune response, and gene expression). Despite the growing evidence elucidating TETs' mode of function, these multifaceted proteins remain underestimated in cancer pathways. In this research study, we aim to unravel the functions of TET1 in colorectal cancer via identifying its partner proteins. Since Vitamin C (VitC) warrants the response to chemotherapy and is known to stimulate TET activity, we wanted to decipher the mechanisms involved in the regulatory control of TET activity by vitamin C.

In this study, we showed the condensation of TET1 and the first demethylation mark, 5hmC, into new nuclear bodies in colorectal cancer cells, whereas TET1 shows diffuse cytoplasmic localization in non-tumor colon cells. Using immunofluorescence assay and confocal analysis, we aimed to identify whether TET1 enzyme co-localizes with the most prominent nuclear body proteins: Promyelocytic Leukemia protein (PML), and Cajal bodies (CB). We observed that TET1 and 5hmC strikingly co-localized with CB, thus implicating a possible molecular role of TET1 in processes associated with RNA biogenesis/processing. Given that Vitamin C (VitC) is a conventional activator of DNA demethylation by stimulating 5hmC levels, we intended to examine VitC-mediated effects on these nuclear-body proteins. Interestingly, we showed that VitC treatment did not only induce the biogenesis of 5hmC, PML, and Cajal bodies, but also favored the interaction of 5hmC with these nuclear structures. Taken altogether, our data suggest a potential role of TET1 in non-coding RNA processing that is likely enhanced by VitC. Further studies are required to unravel the direct mechanistic effects of TET1-Cajal interaction on RNA processing as these findings disclose novel TET1-dependent cellular functions and potentially new avenues in colon cancer management.

1.2. Article

Article

Co-localization of novel TET1-nuclear bodies with Cajal- or PML-bodies is enhanced by Vitamin C in colorectal cancer cells

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Simple Summary: Given that TET1 is greatly modulated in cancer, and that nuclear bodies form central hubs for cellular processes, we aim to assess the function of newly identified TET1-nuclear bodies (TET1-NBs) in colorectal cancer cells. Using immunofluorescence assay, we observed that TET1-NBs together with the demethylation product (5hmC) frequently co-localized with Cajal bodies and not with promyelocytic leukemia protein (PML). Besides, using Vitamin C (VitC) as an active inducer of DNA demethylation, we further observed that 5hmC interaction with the common Cajal and PML nuclear body proteins was highly enhanced. These results suggest a role for TET1 in modulating additional cellular mechanisms including those related to the processing of non-coding RNA; this suggest novel functions for TET1 that are potentiated by exposure to VitC.

Abstract: Ten-eleven translocation protein 1 (TET1) deregulation is commonly reported to induce imbalances in gene expression and subsequently to colorectal cancer development (CRC). On the other hand, vitamin C (VitC) favors a better prognosis for colorectal cancer via re-programing the cancer epigenome and limiting the chemotherapeutic drug-resistance events. In this study, we aim to characterize TET1-specific subcellular compartments and evaluate the impact of VitC on TET1-compartmentalization in colonic tumor cells. Assessment of TET1 intracellular profile in the presence or

absence of VitC was performed by confocal analysis. Here, we demonstrate that TET1 concentrates in coarse nuclear bodies (NB) and 5-hydroxymethylcytosine (5hmC) into foci in colorectal cancer cells (HCT116; Caco-2; HT-29). To our knowledge, this is the first evidence reporting a novel intracellular localization profile of TET1 and its demethylation mark, 5hmC, in CRC cells. Strikingly, we found TET1-NBs frequently interacting with Cajal bodies but not with promyelocytic leukemia (PML) bodies. Besides, we report that VitC treatment induces the biogenesis of 5hmC foci and triggers 5hmC mark to form active complexes with nuclear body components including both Cajal and PML proteins. Our data highlights novel nuclear bodies concentrating TET1 in CRC cells and demonstrates that interactions of TET1-NBs with other nuclear structures are modulated by VitC. These findings unravel novel TET1-dependent cellular functions and potentially new insights for colon cancer management.

Keywords: TET1; nuclear bodies; vitamin C; Cajal bodies; PML bodies; partner proteins; CRC

1. Introduction

Colorectal cancer (CRC) ranks as the third most common cancer worldwide (Rawla, Sunkara, & Barsouk, 2019) and accounts for more than 10% of detected cancer cases (Rawla et al., 2019). Among epigenetic processes, active DNA demethylation has been evidenced to be aberrantly regulated in the onset and/or progression of cancers (Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). The epigenetic regulator Ten-Eleven Translocation (TET) is a family of proteins that initiates the demethylation process by removing the epigenetic mark 5-methylcytosine (5mC) through a series of oxidation reactions (Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). Interestingly, an increasing number of alterations in TET family were found to induce imbalances in DNA demethylation, leading subsequently to cancer development (Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015; H. Wu & Zhang, 2011). Some studies reported the tumor-suppressive roles of TET in cancers. Indeed, a significant increase of tumorigenesis was observed upon TET loss-of-function due to mutations or downregulation (Feng et al., 2019; Y.-P. Xu et al., 2019; S. Yu et al., 2020). Conversely, other studies reported that TET knockdown reduces cancer cell growth, thus highlighting oncogenic properties for TET proteins (Good et al., 2018; M. Wu et al., 2016).

According to the literature, TET1 mRNA and protein expression levels are greatly decreased in CRC cells and tissues (Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015; Y.-p. Tian et al., 2017). Consequently, the first oxidative product of the TET-induced active demethylation process, 5hmC, also showed a significant decrease (Y.-p. Tian et al., 2017). In addition, studies indicate that TET1 decrease does not only promote CRC cell proliferation *in vitro* but

also stimulates tumor volume and weight *in vivo*. (H. Guo et al., 2019).

On the other hand, patients with CRC have a low level of vitamin C (VitC) in their plasma when compared to healthy individuals (Saygili, Konukoglu, Papila, & Akcay, 2003). Recently, this antioxidant vitamin has been reported to improve CRC patients' quality of life by alleviating the side effects of chemotherapy (van Gorkom, Lookermans, Van Elssen, & Bos, 2019). According to Riordan *et al*, the combined VitC chemotherapy treatment remarkably promoted the survival outcome of stage IV colon adenocarcinoma patients (Riordan et al., 2004). VitC was also shown to suppress tumor growth in several animal models and tissue culture studies (Pawlowska, Szczepanska, & Blasiak, 2019). Interestingly, several recent reports revealed that VitC exerts its effect through the activation of TET proteins, as 5hmC levels remarkably increase after vitamin C treatment (Pawlowska et al., 2019). Although VitC is reported to upregulate the activity of TET proteins in an expression-independent process, the molecular mechanism by which VitC activates TET1 is still not fully understood (Dickson, Gustafson, Young, Züchner, & Wang, 2013; Yin et al., 2013).

In this study, we identified novel nuclear compartmentalization of TET1 protein in CRC cells as compared with normal colon cells. Although the exact process for TET1/5hmC concentration into coarse nuclear dots is unknown, we assessed their interaction with common nuclear bodies and identified Cajal bodies as specific partner protein of TET1-NBs in CRC cells. Furthermore, since, Vitamin C has been reported to stimulate TET1-demethylation activity and to restore 5hmC levels (Gillberg et al., 2018; Pawlowska et al., 2019), we studied the impact of Vit-C treatment on TET1 activity. We observed a VitC-mediated induction of PML and Cajal bodies, as well as their recruitment into active complexes with 5hmC. Thus, our results indicate a role of VitC in regulating the interdependent interplay between nuclear bodies in cancer cells as well as potential novel TET1-dependent cellular functions.

2. Materials and Methods

2.1. Cell culture and treatment

Normal human colon epithelial cell line (NCM460) (Moyer, Manzano, Merriman, Stauffer, & Tanzer, 1996) and cervical (HeLa) as well as colorectal adenocarcinoma cells (HCT116; Caco-2; HT-29), were generously obtained from Dr. Marwan El Sabban's lab. HT-29 were cultured in RPMI-1640 medium (Sigma SR8758), while Caco-2, HCT116 and HeLa cells were maintained in DMEM medium (Sigma SD5796). Both media were supplied with 10% Fetal Bovine Serum (FBS; Sigma SF9665) and 1% Penicillin/Streptomycin (P/S; Sigma L0022) solution at 37°C in 5% CO₂ humidified incubators. Cells were

seeded on 24-well plates with coverslips for 24h then treated with 500µM of Vitamin C (VitC, Sigma 47863) for 2h. Culture media, FBS, P/S and VitC were all purchased from Sigma Aldrich, Lebanon.

2.2. Immunofluorescence assay

Normal colon and CRC cell lines were fixed and permeabilized with ice-cold 100% methanol for at least 15 minutes at -20°C. Cells were blocked for 30 mins in a blocking buffer (0.25% BSA, 0.5% Gelatin), then incubated overnight with the primary antibodies: rabbit anti-TET1 (1:500; Genetex GTX124207), rabbit anti-5hmC (1:500; Abcam RM235), mouse anti-PML (1:500; Abcam ab96051), mouse anti-coilin (1:500; Abcam ab87913), SUMO1 (1:500; Santa Cruz Sc9060), SUMO2/3 (1:500; Santa Cruz Sc32873). Cells were then incubated for 1h with fluorochrome-labeled goat secondary antibodies: anti-rabbit Alexa Fluor 488 (1:500; Abcam ab150077), and/or anti-mouse Alexa Fluor 594 (1:500; Abcam ab150116). The nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride, Roche 10236276001) dye and mounted with Prolong Gold anti-fade reagent (Invitrogen, P36930). Images were processed and analyzed using *Confocal* microscopy linked to ZEN Imaging Software-LSM710. Post-confocal quantification and size measurement of nuclear bodies was conducted using ImageJ Software (NIH; <https://imagej.nih.gov/ij/>).

2.3. Statistical Analysis

Statistical analysis was carried out by using STATA software for statistics and data science. Mean values are presented with standard deviation error bars. One-way ANOVA statistical test was carried out to compare the number and size TET1/5hmC nuclear structures between normal colon and colorectal cancer cell lines. An independent Student's t-test was used for comparative analysis of different nuclear body proteins while paired t-test was applied to assess NBs before and after VitC treatment. Moreover, we used Pearson's correlation coefficients (r) to analyze the correlation between variables. All values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Identification of TET1-nuclear body (TET1-NBs) in CRC cells

To understand the role of TET1 in CRC, we assessed its intracellular localization in normal and colon cancer cell lines. Immunofluorescence showed random nuclear staining of TET1 along with a cytoplasmic perinuclear distribution in the normal colon epithelial cell line NCM460 (**Fig.1.A; Fig.S1.A**). Strikingly, TET1 concentrated in coarse nuclear bodies (NBs), or "nuclear hotspots", in HCT116 colon cells (**Fig.1.A; Fig.S1.A**). This profile was confirmed in two additional CRC cell lines (Caco-2, and HT-29) (**Fig.1.B; Fig.S1.A**). To further characterize these nuclear structures,

TET1-NBs were analyzed using ImageJ. Results revealed that TET1-NBs reach up to 16 condensates per nucleus and ranges from 0.3 to 0.5 μm in size (**Fig.1.C**). TET1-NBs number and size were not significantly different between NCM460 and different CRC cells ($p=0.805$ and $p=0.388$; for number and size, respectively) (**Fig.1.C**).

Furthermore, we examined the distribution profile of 5-hydroxymethylcytosine (5hmC), the direct TET demethylation mark on DNA. In NCM460 cells, 5hmC staining was diffused in the nucleus while completely shifted into nuclear foci in HCT116 cells (**Fig.1.D; Fig.S1.B**). 5hmC foci were also observed in Caco-2 and HT-29 cells (**Fig.1.E; Fig.S1.B**). Post-confocal quantification and size measurement of foci showed no significant difference between NCM460 and CRC cell lines ($p=0.385$ and $p=0.466$ for number and size, respectively) (**Fig.1.F**). However, while TET1-NBs and 5hmC foci were similar in size, TET1-NBs were approximately 10-fold more numerous than 5hmC foci. Taken together, these data indicate a novel nuclear localization profile of TET1 and 5hmC in CRC cells.

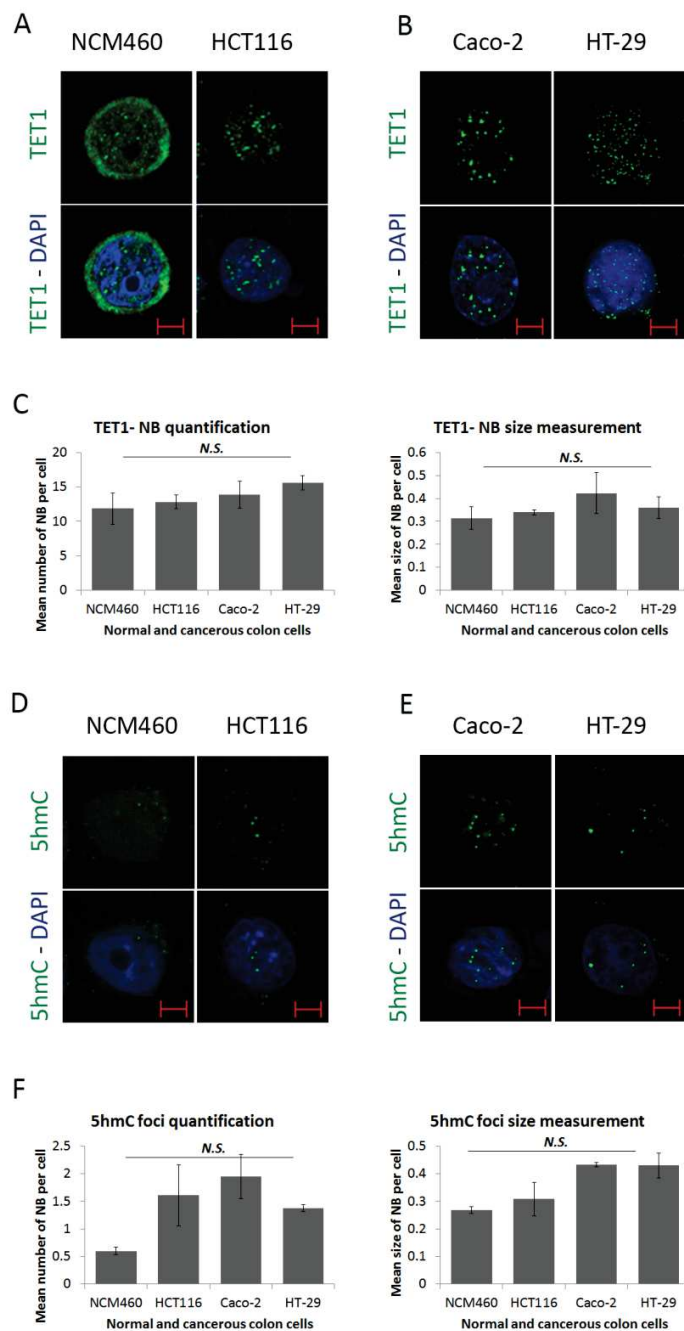


Figure 1. Formation of Ten-eleven translocation-nuclear bodies (TET1-NBs) and 5-hydroxymethylcytosine (5hmC) foci in CRC cells. Confocal images of A. TET1 showing diffused distribution with few subtle nuclear structures as well as a cytoplasmic perinuclear profile in normal colon (NCM460) cells (n=1). This intracellular distribution of TET1 shifted into coarse nuclear concentrates in HCT116 cells and B. is confirmed in other CRC cell lines (Caco-2, and HT-29) (n=3). C. For the comparative quantification and size measurement of TET1-NBs, we counted 50 cells from each cell line (NCM460, and CRC cells) (n=3). D. 5hmC, the demethylation mark of TET enzymes, displayed a comparable profile in NCM460 cells characterized by a randomly diffused distribution. However, 5hmC condensates into nuclear foci in D. HCT116 cells, and in E.

Caco-2 and HT-29 cells. Nuclei are stained with DAPI (blue) with a scale bar: 5µm. F. We counted 50 cells from NCM460 and from each CRC cell line for 5hmC foci comparative post-confocal analysis, respectively (n=3). Error bars here represent the standard deviation of means (N.S.: non-significant).

3.2. TET1-NBs are distinct from PML-NBs

Nuclear bodies are robust sub-nuclear structures that are actively involved in various molecular processes (Sawyer & Dundr, 2016; Staněk & Fox, 2017). Promyelocytic Leukemia (PML) is a well-characterized tumor suppressor protein that recruits and orchestrates many proteins in PML-NBs (D. Guan & Kao, 2015). Thus, we hypothesized that PML could confine TET1-NBs activity in CRC cells. To test this hypothesis, we performed a co-immuno-labeling assay to compare TET1- and PML-NBs distribution in HCT116 cells. Confocal images revealed that the majority of TET1-NBs have independent localization from the master organizer PML protein (**Fig.2.A; Fig.S2.A**).

Therefore, we wondered whether this outcome could reflect a defect in the ability of PML-NBs to recruit other partner proteins in CRC cells. To test this hypothesis, we assessed the intracellular profile of PML along with SUMO proteins, given that SUMOylation of PML is a prerequisite post-translational modification to confine target proteins in NB (Sahin, Thé, & Lallemand-Breitenbach, 2015). Indeed, SUMO1 and SUMO2/3 isoforms, are indispensable components favoring the dynamic recruitment of various partner proteins into PML-NBs after the formation of the NB outer shell (Sahin et al., 2015). Confocal images displayed frequent co-localization of PML-NBs with SUMO1, and with SUMO2/3 (**Fig.2.B-C; Fig.S2.B-C**). In addition, a co-localization profile analysis validated the overlay of PML-NBs with SUMO1/2/3 suggesting that HCT116 cells might still contain a functional PML able to recruit other partner proteins (**Fig.2.D-E; Fig.S2.B-C**).

Finally, comparative quantification of TET and PML nuclear body proteins indicated significant higher frequency for TET1-NBs ($p=0.0008$) (**Fig.2.F**). Besides, PML-NBs were significantly greater in size than TET1-NBs ($p=0.0286$) (**Fig.2.F**). Finally, a Pearson's statistical test indicated that TET1 and PML staining are not significantly correlated when analyzed in HCT116 cells (562 cells; $r=0.6597$; $p=0.5414$; **Fig.2.G**). Collectively, these observations suggest that TET1-NBs and PML bodies are independent nuclear hotspots.

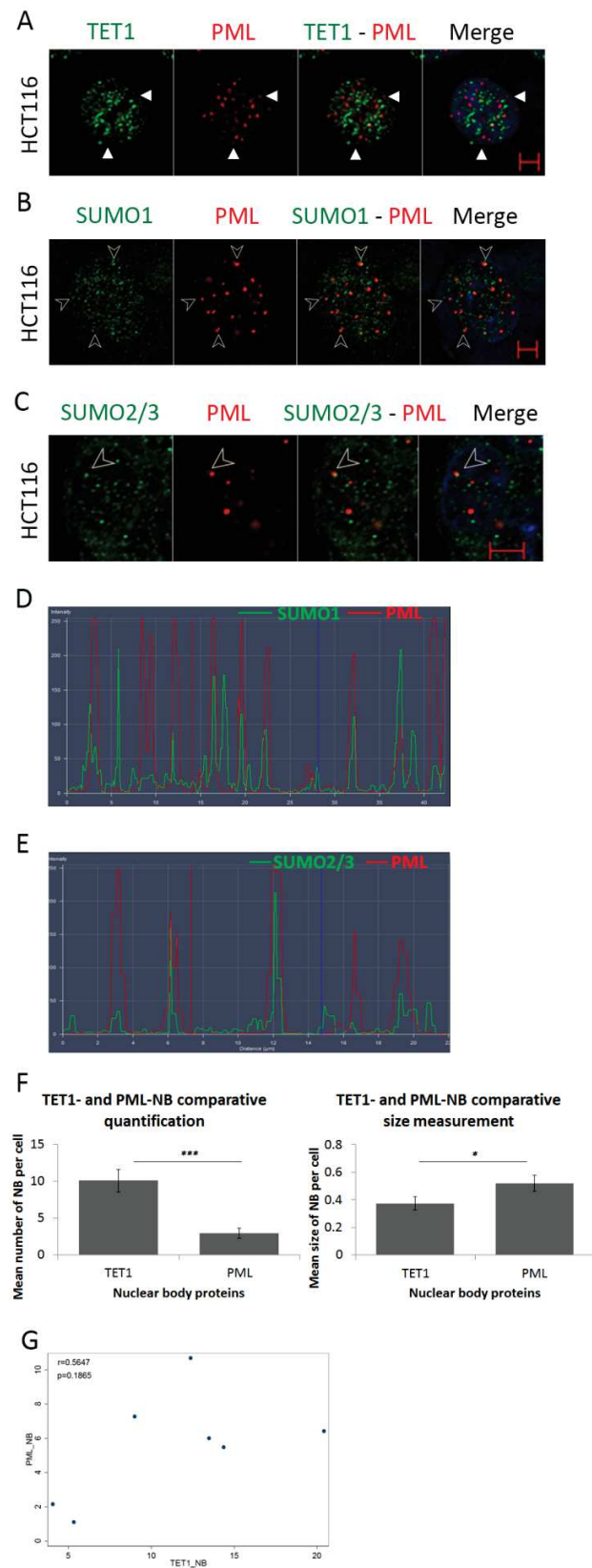
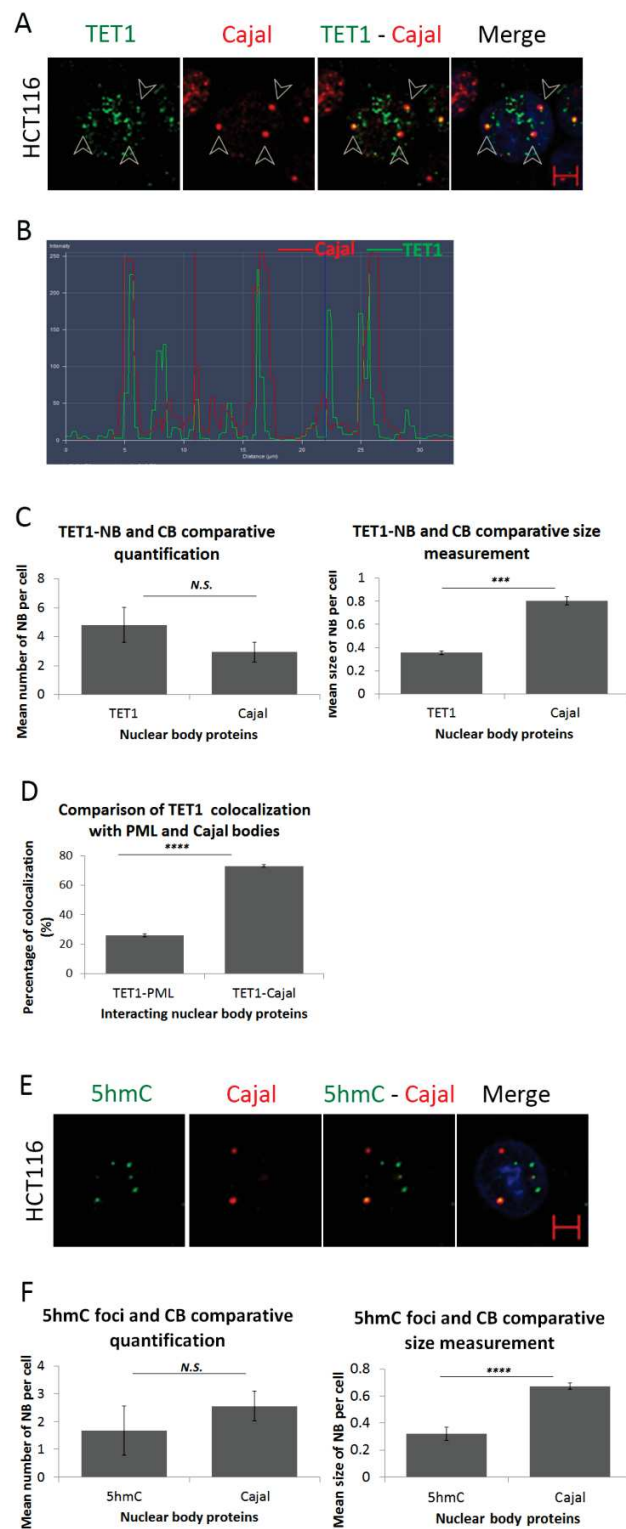


Figure 2. TET1 and promyelocytic leukemia protein (PML) are independent nuclear body proteins. A. TET1 and PML show no co-localization as indicated by white arrowheads proteins in HCT116 cells (n=3). To assess the ability of PML-NBs to recruit partner proteins, we co-immunostained PML-NBs with B. SUMO1, and C. SUMO2/3 (n=3). The overlay of PML and SUMO isoforms is indicated by arrowheads and is confirmed by co-localization profile analysis in D. and E. (Scale bar: 5µm). F. Comparative post-confocal analysis of count and size in 274 cells showing that PML-NBs are significantly fewer but larger in size with respect to the novel TET1-NBs in HCT116 cells. Error bars here represent the standard deviation of means (*p<0.05; ***p<0.001). G. Pearson's scatter plot shows no correlation between PML-and TET1-NBs in 562 cells.

3.3. TET1-NBs co-localize with Cajal bodies

Since TET1-NBs were distinct from PML-NBs, we examined other prominent nuclear structures that could potentially characterize TET1-NBs. For this purpose, we selected Cajal Bodies (CBs), another prototypical nuclear organelle involved in RNA processing (Henriksson & Farnebo, 2015). We targeted coilin-p80, a common marker protein of CBs, along with TET1 in HCT116 cells. Interestingly, 73% of coilin p-80 positive NBs co-localized with TET1 protein (**Fig.3.A; Fig.S3.A**). Analysis of the co-localization profile further confirmed the overlay of nuclear TET1 and CBs (**Fig.3.B**). Post-confocal analysis revealed no significant difference between the number of TET1-NBs and CBs (p=0.0969). Since TET1-NBs were significantly smaller in size when compared to CBs (p=0.0003) (**Fig.3.C**), TET1-NBs are most likely partners of Cajal bodies rather than identical nuclear structures. However, the statistically significant difference between the co-localization percentage of TET1-PML (26%) and TET1-Cajal (73%) suggests that Cajal bodies are frequent nuclear partners of TET1-NBs (p= 0.00001; **Fig.3.D**). Moreover, 16% of TET1-NBs positive cells co-localize with total CBs whereas 12% of CBs-positive cells appeared to co-localize with TET1 in HCT116 cells suggesting TET1 protein has a predominant function in colorectal cancer cells (**Supplementary Table 1**).

To assess whether 5hmC also concentrated in CBs, we performed immunostainings of coilin-p80 along with 5hmC in HCT116 cells. As observed for TET1, 5hmC co-localized with CBs (**Fig.3.E; Fig.S3.B**). Again, CBs were significantly greater in size with respect to 5hmC foci (p=0.00001) (**Fig.3.F**) and significantly correlated with 5hmC and CBs in HCT116 cells (264 cells; r= 0.8555; p=0.0298; **Fig.3.G**). To our knowledge, this is the first evidence of 5hmC and CBs association.



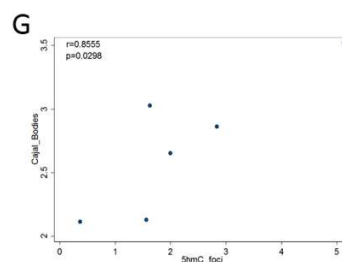


Figure 3. TET1 and its demethylation mark, 5hmC, interact with Cajal bodies . A. Confocal images showing co-localization of TET1-NBs and CBs in HCT116 cells as indicated by arrowheads (n=3; Scale bar: 5µm). **B.** Co-localization profile analysis of nuclear TET1-NBs and CBs further demonstrating the overlay of the two structures. **C.** Comparative quantification and size measurement of TET1-NBs and CBs in 308 cells. **D.** Comparative analysis showing significant difference between the co-localization percentage of TET1-PML and TET1-Cajal. **E.** Similarly, the direct demethylation mark, 5hmC, also co-localized with CBs. **F.** Post-confocal analysis in 241 cells showed that 5hmC foci were not significantly fewer than Cajal bodies while, as observed for TET1-NBs, 5hmC foci were significantly smaller than CBs. Error bars represent the standard deviation of means (**p<0.001; ***p<0.0001; N.S: non-significant) **G.** Pearson's scatter plot shows positive correlation between Cajal bodies and 5hmC foci in 264 cells.

3.4. Vitamin C favors demethylation, promotes NB biogenesis, and enhances the association of 5hmC with partner proteins

Aside from its clinical usage as an adjuvant in the treatment of various types of cancer, VitC has been reported to induce activation of TET proteins (Pawlowska et al., 2019; van Gorkom et al., 2019). To investigate whether VitC may impact TET1-NBs, we exposed HCT116 colorectal cancer cells to 500µM VitC. Immunostaining and post-confocal analysis revealed that VitC treatment associates with a significant induction in the number and size of 5hmC foci (p=0.0135, p=0.0357 respectively) (**Fig.4.A**), thus, our findings suggesting that VitC may stimulate the formation of 5hmC foci in CRC cells.

To address the question of whether the effects of VitC are or not restricted to 5hmC foci, we examined the impact of VitC on other nuclear body proteins, including PML-NBs and CBs. A significant increase in PML-NBs (p=0.0099, and p=0.0445) and CBs number (p=0.0352) was observed in HCT116 cells upon VitC exposure (**Fig.4.B-C**), thus indicating that VitC is a general inducer of the biogenesis of nuclear structures.

Since VitC induced both 5hmC and CBs, we suspected that VitC might enhance the interaction between both structures. Upon treatment with VitC, the percentage of 5hmC-Cajal co-localization significantly increased from 31% to 36% (p=0.0142; **Fig.4.D-E**; **Fig.S4.A-B**). Similarly, VitC significantly enhanced 5hmC-PML overlap by 7% in HCT116 cells (p=0.00001; **Fig.4.F**). Hence, VitC stimulates the association of 5hmC with PML and CBs. Moreover, to identify which nuclear structure functionally predominates in HCT116 cells, we assessed the co-localization percentage of 5hmC foci with Cajal bodies and vice versa. The percentage of 5hmC foci



positive cells co-localizing with total CBs significantly increases from 11% to 20% upon Vitamin C treatment ($p < 0.0001$). On the other hand, CBs-positive cells co-localization percentage with total 5hmC foci significantly decreased by 3% with treatment ($p < 0.0001$), which might be explained by the higher VitC-mediated induction of 5hmC foci with respect to CBs (**Fig.4.G-H; Supplementary Table 1**). Together, our data suggest that VitC has multiple impacts on both the biogenesis and dynamic of intra-nuclear organelles in CRC.

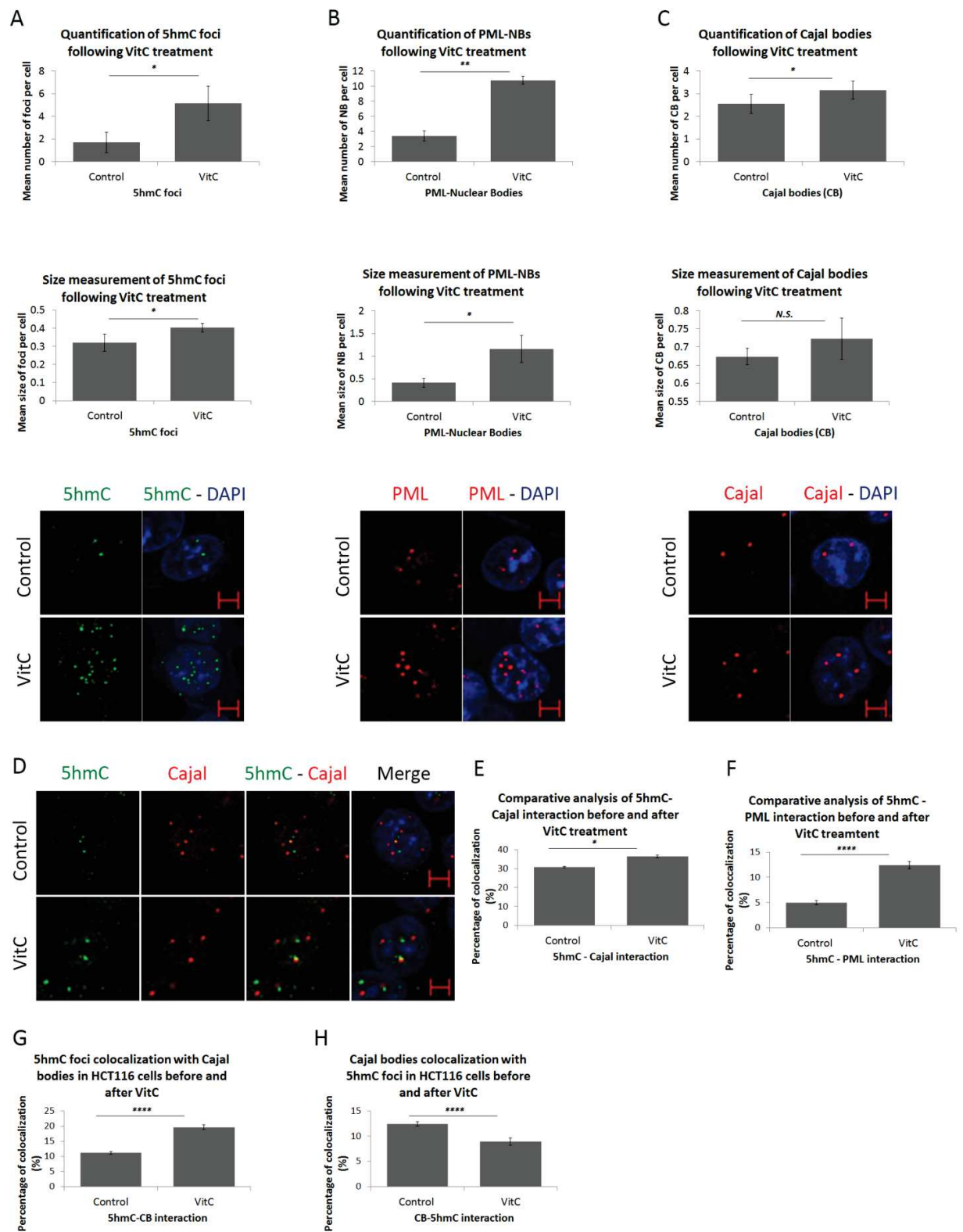


Figure 4. Vitamin C promotes nuclear body biogenesis and enhances co-localization in HCT116 cells. Comparative confocal and post confocal analysis showing a significant increase, upon VitC treatment, in the number and size of A. 5hmC foci in 241 cells, B. PML-NBs in 152 cells, C. CBs in 241 cells (n=3). D. Immunofluorescence images showing the intracellular distribution pattern of 5hmC foci and Cajal bodies following 500 μ M of Vitamin C (n=3; Scale bar: 5 μ m). VitC treatment significantly increased the co-localization of 5hmC with PML-NBs and Cajal bodies in E. and F., respectively. G. The co-localization percentage of 5hmC foci with CBs increased by 9% upon VitC treatment however, in H., the inverse co-localization percentage showed a 3% decrease in CBs co-localization with 5hmC foci in 195 cells. Error bars here represent the standard deviation of means (*p<0.05; **p<0.01; ****p<0.0001; N.S: non-significant).

4. Discussion

TET demethylases are highly deregulated in cancer (Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). TET1 and TET3 were found to be uncommonly mutated in hematological malignancies with respect to their paralog, TET2 (Blecua, Martinez-Verbo, & Esteller, 2020; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). Although the frequency of TET1 inactivation mutations does not exceed 14% in T-cell Acute Lymphoblastic Leukemia (T-ALL), recent findings revealed that TET1 favors the activation of several oncogenes to promote carcinogenesis in Mixed Lineage Leukemia (MLL) (Blecua et al., 2020). On the other hand the Catalogue Of Somatic Mutations In Cancer (COSMIC) and The Cancer Genome Atlas (TCGA) databases state that TET mutations in solid tumors occur at a lower frequency compared to blood cancers (Yun Huang & Rao, 2014; Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). Besides, TET expression is reduced in many cancers, including breast, liver, prostate, and colorectal cancers, (Rawłuszko-Wieczorek, Siera, & Jagodziński, 2015). Both TET(1-3) and its 5hmC product exhibit a diffused nuclear distribution pattern in solid cancer cell lines (Arioka et al., 2012; Shinsuke Ito et al., 2010; Christine G. Lian et al., 2012; M. J. Wu et al., 2017), but very little is known on how TET activity is modulated.

In the present study, we report that TET1 is distributed into subtle nuclear structures randomly diffused in the nucleoplasm along with a perinuclear distribution in normal epithelial colon mucosa (NCM460) cells. This profile concentrates into nucleoplasmic coarse nuclear bodies (TET1-NBs) in CRC cells thus, indicating that TET1 is a newly characterized nuclear body protein in CRC (**Fig.1; Fig.S1**). Over the past decade, few studies showed the nuclear diffusion of TET1 protein in normal mammalian cell lines: particularly in human embryonic kidney (HEK293T), and mammary epithelial (MCF-12a) cells (Arioka et al., 2012; L. Peng et al., 2016; M. J. Wu et al., 2017). Although it is localized into the nucleus at baseline conditions, our unprecedented finding of TET1 cytoplasmic perinuclear profile in NCM460 cells is intriguing. According to Arioka et al, changes in the expression levels of Activation-induced cytidine deaminase (AID) enzyme regulate the intracellular localization of TET1 (Arioka et al., 2012). Namely, AID promotes the

cytoplasmic translocation of TET1 in HEK239T cells (Arioka et al., 2012). Another study by Wu et al disclosed that cells lacking retinoic acid receptor beta (RAR β) displayed cytoplasmic TET2; a profile remarkably restored TET2 to the nuclear compartment with RAR β re-expression (M. J. Wu et al., 2017). Moreover, O₂ levels also influence TET1 localization in mouse trophoblast stem cells (Senner et al., 2020) thus indicating that multiple intra and extra-cellular factors may influence TET1 profile. Furthermore; Yu et al. (2021) recently reported that on a cohort of 876 patients with colorectal cancer, 40 (4.75%) harbored TET1 mutations (including nonsynonymous, frameshift and stop/gain mutations) and identified 66 TET1 single nucleotide mutations (Yu et al., 2021). Thus, even if the percentage of TET1 mutations is low in colorectal cancer, we cannot exclude the possibility of TET1 mutation in HCT116 cells that might affect its subcellular localization.

Given that our results indicate the concentration of diffused 5hmC profile into coarse nuclear foci in CRC cells (**Fig.1; Fig.S1**), it remains quite possible that catalytic-inactivation mutations are absent or that TET paralogs also drives this profile of the 5hmC mark. We suspect that the recruitment of TET1 and 5hmC into nuclear structures may implicate a novel compensatory mechanism to activate TET-mediated demethylation process in CRC. Future studies are required to validate this hypothesis. In addition toTET-interacting factors (e.g. AID, and RAR β), O-linked β -N-Acetylglucosamine (OGT) – mediated post-translational modification appeared to export TET3 and disrupt 5hmC formation in HEK239T cells (Q. Zhang et al., 2014). Moreover, TET3 – which displays a minor cytoplasmic distribution – might be conveyed from the oocyte cytoplasm to paternal nucleus directly after fertilization (H. Wu & Zhang, 2011). On the other hand, TET1 and TET2 are expelled from the nucleoplasm via their nuclear export signals in gastric cancer and small intestinal neuroendocrine tumor cells, respectively (Barazeghi et al., 2018; K.-C. Wang et al., 2018). By using a series of green fluorescent protein-tagged and mutation constructs, Xiao et al (2013) identified a conserved nuclear localization signal (KKRK) in mouse TET1/3; this indicates that the nuclear localization and/or translocation of these proteins into the nuclear compartment might be favored by importin- α/β (P. Xiao et al., 2013). Finally, an abnormal nuclear translocation of TET2 from cytosol has recently been reported in a non-tumoral but chronic stress situation through an Abelson helper integration site-1 (Ahi1)-dependent process in neuronal cells (Q. Zhang et al., 2021); these findings thus suggesting several nuclear transport mechanisms involving nuclear export/import signals, partner proteins, and/or post-translational modifications (Arioka et al., 2012; Barazeghi et al., 2018; M. J. Wu et al., 2017; P. Xiao et al., 2013; Q. Zhang et al., 2021; Q. Zhang et al., 2014). The prominent cytoplasmic TET1, detected in NCM460 cells may point towards TET1 forming a reserve pool bridging different cellular compartments to recompense the accumulating need of

global hypomethylation during carcinogenesis and/or trigger TET1 catalytic dependent and independent functions throughout malignant cell transformation. Taken together, we believe that this novel intracellular profile of TET1 would be the steppingstone to unravel the molecular functions of TET1 in cancer.

NBs are robust structures that confine many components involved in various mechanisms in the nucleoplasm (Sawyer & Dundr, 2016; Staněk & Fox, 2017). It is believed that interactions and processes within the restricted space of NBs are of higher efficiency and of greater speed rate (Sawyer & Dundr, 2016; Sawyer, Sturgill, Sung, Hager, & Dundr, 2016). Since TET1-NBs had never been described, we were intrigued to identify whether these nuclear hotspots interact with the commonly studied nuclear structures such as the Promyelocytic Leukemia nuclear bodies (PML-NBs) and/or the Cajal bodies (CB).

PML self-assembles into discrete nuclear structures in a process mediated by the recruitment of many partner proteins (like SP100 and DAXX, SUMO1/2/3 (Neerukonda, 2021). The ubiquitous PML-NBs sequester and release a wide range of substrates in order to regulate a number of crucial cellular processes (DNA repair, tumor suppression, apoptosis, antiviral responses, etc...) (D. Guan & Kao, 2015; Lallemand-Breitenbach & de Thé, 2010). Although PML is the master organizer of an ever-expanding number of proteins, we reveal a great distinctiveness in TET1- and PML-NBs (**Fig.2; Fig.S2**). This outcome suggests NBs-specific cellular functions in CRC cells.

CBs are distinct frequently studied dynamic nuclear structures that continuously exchange components with the nucleoli (Cioce & Lamond, 2005; Staněk & Fox, 2017). CBs are nuclear domains encountered in transcriptionally active cells (Staněk & Fox, 2017). They are involved in RNA splicing, telomere maintenance, and several other vital processes (Henriksson & Farnebo, 2015). The structural conformation of CBs is tightly maintained by coilin p80 (the molecular marker of CBs) and WRAP53β (an antisense transcript of the p53 gene) (Henriksson & Farnebo, 2015). When HCT116 cells were co-stained for TET1 and p80, we observed a co-localization by 73% (**Fig.3; Fig.S3**). Notably, immunostaining of 5hmC displayed a similar intracellular localization profile to that of TET1 characterized by its condensation into nuclear foci in CRC cells. Our findings also revealed a significant positive correlation between 5hmC and CBs (**Fig.3; Fig.S3**). These findings indicate that TET1 could constitute a frequent functional partner of CBs and might potentially play a role in RNA biogenesis and/or processing. Indeed, Fu *et al* revealed that TET-mediated demethylation is not limited to DNA and that TET proteins are capable of efficiently hydroxymethylating 5mC in RNA to 5hmC both *in vitro* and *in vivo* (Fu et al., 2014). In *Drosophila melanogaster*, a single TET protein – dTET – has been shown to induce demethylation of 5mC on both DNA and RNA (Delatte et al., 2016; Joy N. Ismail, Ghannam, Al Outa, Frey, & Shirinian, 2020). Besides, a recent finding reported dTET demethylation of N6-methyladenosine

(m6A) on DNA (Joy N. Ismail et al., 2020). RNA 5hmC mark (5hmC) has gained a lot of interest and its functions are still under investigation. Several studies implicated the enrichment of this mark in exons and in a specific sequence context (UCCUC repeat). Moreover, a possible role for 5mC in mRNA export was suggested (Joy N. Ismail et al., 2020). Another set of studies disclosed the implication of 5hmC mark in regulating the stability of mRNA in mouse embryonic stem cells (mESC) (Guallar et al., 2018; C. He et al., 2021; Lan et al., 2020). The diminished translational efficiency of oxidized mRNA transcripts is subsequent to their shorter half-life and faster degradation (Guallar et al., 2018; C. He et al., 2021; Lan et al., 2020). These destabilization effects of 5hmC demethylation mark is also applied on non-coding RNA (ncRNA). According to He *et al.*, transfer RNA – evidenced as a major target of TET2 – is greatly regulated in terms of biogenesis and expression levels in mESC (C. He et al., 2021). Moreover, it is worth noting that cytosine methylation of non-coding microRNA contributes to the loss of its repressive functions (Cheray et al., 2020). Subsequently, microRNA methylation inhibits the formation of duplexes with mRNA transcripts to suppress their translation (Cheray et al., 2020). This discovery shed light on the epigenetic regulation of non-coding RNA. Given that small nuclear ribonucleoproteins (snRNP) and small nucleolar ribonucleoproteins (snoRNP) are processed in Cajal bodies, a synergistic interaction between other ncRNAs and CBs is recently identified. In fact, the biogenesis and the maturation of microRNA appeared to take place in CBs (Logan, McLaurin, & Hebert, 2020). The disruption of Cajal bodies by knocking out one of its major constituents significantly reduced the microRNA levels in human choriocarcinoma cells (Logan et al., 2020). Additionally, the activity of ncRNA is deregulated with aberrant DNA hypermethylation (Ying & Chen, 2018). Collectively, these observations together with our own findings could indicate a link between TET epigenetic functions and RNA-mediated regulation of gene expression. Indeed, we suspect that TET1-Cajal co-localization might be an indispensable step toward activation of non-coding RNA to control cellular processes (**Fig.5**). Thus, TET-Cajal machinery should now be examined to assess its direct effects on RNA modulation and its implication in cancer progression.

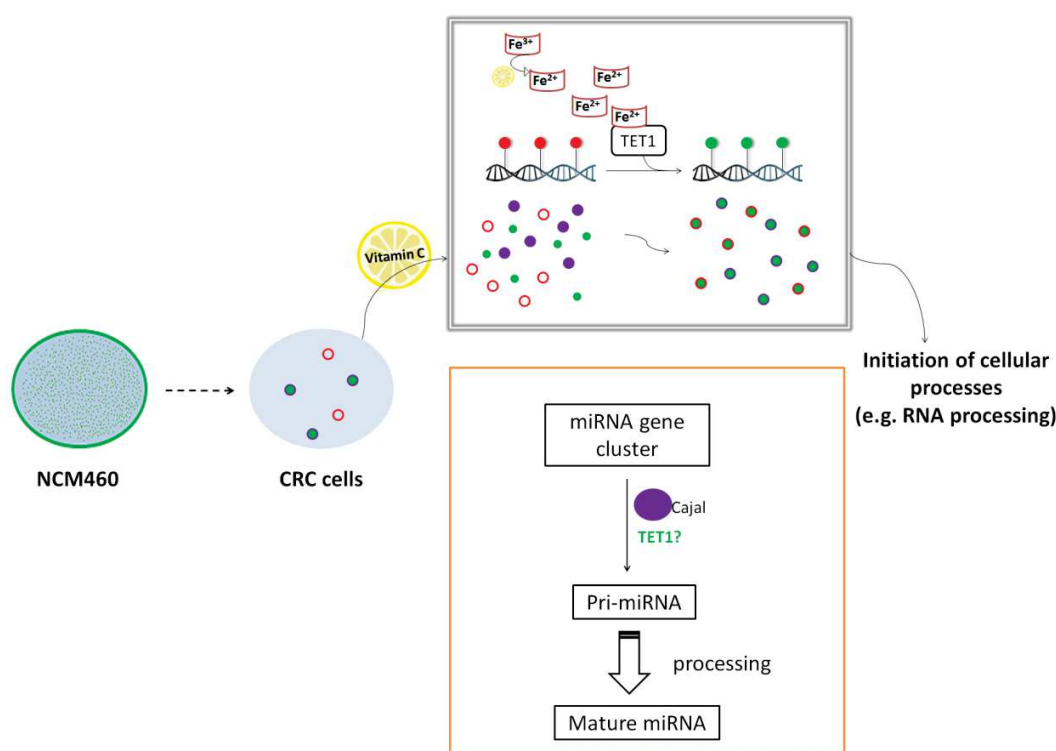
Our analysis of TET1-Cajal interdependent percentage of co-localization in CRC cells revealed the functional predominance of TET1 over CBs (**Table S1**). These percentages were not assessed following VitC treatment since VitC upregulates the hydroxymethylation activity of TET proteins in an expression-independent process (Dickson et al., 2013; Yin et al., 2013). In fact, it is not surprising to detect TET1-devoided CBs, as CBs have been implicated in multiple RNA-related metabolic processes including non-coding RNA maturation and telomere maintenance (Henriksson & Farnebo, 2015; Logan et al., 2020). Thus, it is expected that CBs may

have TET-independent roles. Furthermore, CBs may also possibly recruit different TET proteins depending on RNA processing.

CBs are also known for their role in telomere homeostasis (Henriksson & Farnebo, 2015). Indeed, the WRAP53 β protein does not only favor the accumulation of the telomerase complex in CBs but also promotes the recruitment of this enzyme to telomeres for elongation (Henriksson & Farnebo, 2015; Stern, Zyner, Pickett, Cohen, & Bryan, 2012). On the other hand, the methylation status of sub-telomeres modulates the telomeric length (H. Hu, Li, & Duan, 2019; L. J. Ng, Cropley, Pickett, Reddel, & Suter, 2009). Besides, telomerase activity increases with sub-telomeric methylation in telomerase-positive cells (like HCT116) (L. J. Ng et al., 2009). The levels of Telomeric-repeat-containing-RNA (TERRA), a telomerase inhibitor transcribed from the sub-telomeres, are highly upregulated after hypomethylation of the sub-telomeric region in HCT116 cells (Le Berre, Hossard, Riou, & Guieysse-Peugeot, 2019). Thus, we speculate that the sequestration of TET1 by CBs might (1) maintain telomerase levels/activity and (2) subsequently avoid its recruitment to TERRA transcription regions. Many observations support this hypothesis **1.** TET1 directly targets sub-telomeres in the telomere elongation process (J. Yang et al., 2016), **2.** TET1 induces the methyl-sensitive nuclear respiratory factor 1 (NRF1), a positive regulator of TERRA levels (Le Berre et al., 2019), and **3.** AMP-activated protein kinase pathway (AMPK) does not only activate NRF1 and TERRA but also increases TET1 levels (Le Berre et al., 2019; M. Zhu, Sun, & Du, 2019).

Finally, NBs proteins dynamically respond to external modulators, particularly chemotherapeutic drugs. Arsenic, for instance, is reported to stimulate the biogenesis of PML-NBs following the degradation of PML-RAR α fusion oncoprotein in acute promyelocytic leukemia cells (de Thé, Le Bras, & Lallemand-Breitenbach, 2012). Similarly, doxorubicin treatment drives the formation of PML-NBs and triggers the direct interaction of PML with TET2 (C. Song et al., 2018). As both drugs and VitC modulate oxidative stress to exert their effects, we studied the impact of VitC on different NB proteins, including the newly characterized 5hmC foci. Our results demonstrated that VitC significantly promotes the formation of PML- and Cajal-NBs (**Fig.4; Fig.S4**). Besides, VitC does not only increase 5hmC foci in terms of size and number but also favors the recruitment of 5hmC into PML- and Cajal-NBs (**Fig.4; Fig.S4**). Assessment of 5hmC-Cajal interdependent co-localization percentage, in presence of VitC, revealed the functional predominance of 5hmC over its nuclear counterpart (CBs) in HCT116 cells (**Fig.4; Table S1**) Thus, we hypothesize that VitC-induced TET1 activation mediates the formation of active 5hmC complexes with the PML and Cajal nuclear body proteins. In the literature, two mechanisms for TET activation by vitamin C are proposed. The first highlights the role of VitC as an indispensable co-factor of TETs. However, the second mechanism demonstrates that VitC-mediated

induction of TET enzymatic activity is based on the reduction of ferric ion (Fe^{3+}) into ferrous ion (Fe^{2+}) (Mastrangelo et al., 2018). Furthermore, 5hmC levels are remarkably stimulated after VitC treatment in various *in vitro* and *in vivo* models (Pawlowska et al., 2019). Concomitantly, vitamin C also induced 5hmC levels on mRNA transcripts of mESC (Lan et al., 2020). However, the outcome of this increase on mRNA transcription and stability is not examined. Taken together, our findings evidence the VitC ability to promote the biogenesis of NB and to enhance interactions among distinct nuclear components (Fig.5). We believe that TET1-Cajal machinery would likely initiate specific intracellular mechanisms like RNA processing and control the regulation and maturation of microRNA as well as other ncRNA.



Legend

• TET1/ 5hmC ○ PML • Cajal → Activation ↩ Reduction • 5mC mark • 5hmC mark ➡ Processing

Figure 5. Proposed model of TET1 and 5hmC activation in CRC cells. The intracellular profile of TET1 and 5hmC shifted from a diffused distribution in colon cells (NCM460) to the formation of coarse nuclear structures in CRC cells. Our data reveal the particular association TET1 and 5hmC nuclear condensates with Cajal bodies. VitC-mediated activation of TET1 promoted both the biogenesis of nuclear structures as well as the interaction of 5hmC with nuclear body proteins (PML

and Cajal). These findings suggest the implication of TET1 in initiating several cellular mechanisms, particularly RNA processing. Thus, this study sets the stage to elaborate the mechanism of Cajal-TET1 machinery in the regulation and maturation of microRNA and other non-coding RNAs.

5. Conclusions

Here, we report the shift of TET1 and 5hmC from a diffused nuclear distribution in control NCM460 cells to the formation of TET1-nuclear bodies and 5hmC foci in CRC cells. Based on this novel profile, we speculate that TET1/5hmC-CBs interaction is a starting point to initiate new studies to deciphering possible novel functions for TET1 including RNA processing or telomerase elongation. Further studies are however required to unravel the biological impact of TET1-Cajal interaction.

Supplementary Materials: Figure S1: Formation of TET1-nuclear bodies (TET1-NBs) and 5hmC foci in CRC cells; Figure S2: TET1 and PML are independent nuclear body proteins; Figure S3: TET1 and 5hmC interact with Cajal bodies (CBs); Figure S4: Vitamin C enhances the co-localization of nuclear condensates in HCT116 cells. TABLE S1: Co-localization percentage of the interdependent interaction between TET1/5hmC and Cajal bodies in HCT116 cells.

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1.3. Supplementary data

- **Figure S1-4**
- **Table S1**

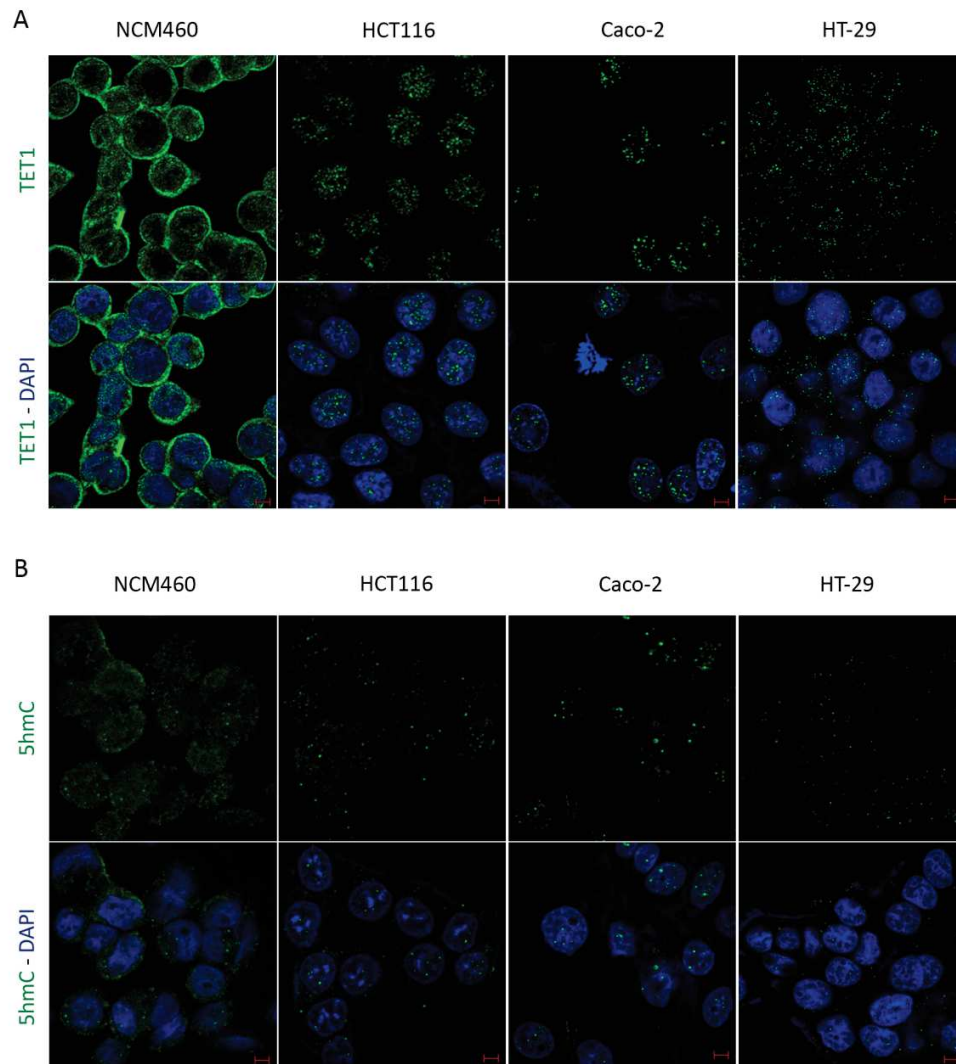


Figure S 1: Formation of TET1-nuclear bodies (TET1-NBs) and 5hmC foci in CRC cells. **A.** Whole field confocal images of showing diffused distribution of TET1 in normal colon (NCM460) cells (n=1). This profile shifted into coarse nuclear concentrates in all tested CRC cell lines (HCT116; Caco-2, and HT-29) (n=3). **B.** Similarly to TET1, 5hmC also condensates into nuclear foci in CRC cell lines when compared to its diffused profile in NCM460 cells. Nuclei are stained with DAPI (blue) with a scale bar: 5µm.

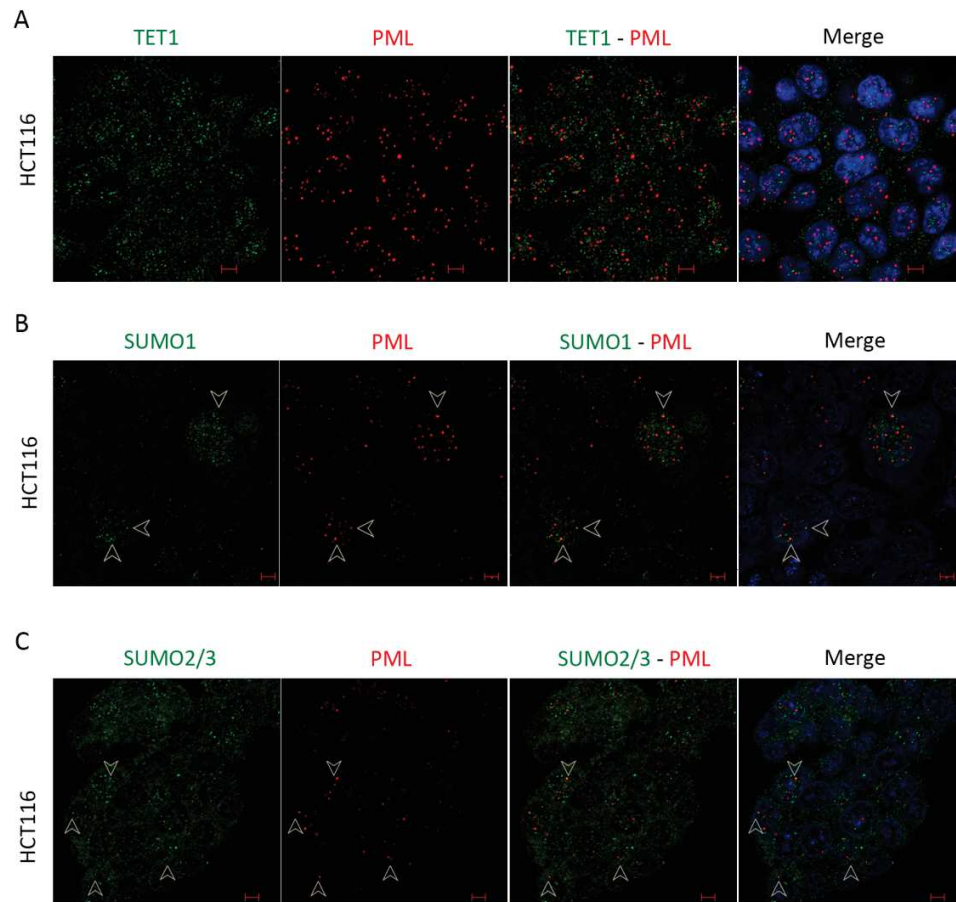


Figure S 2: TET1 and PML are independent nuclear body proteins. **A.** Whole field confocal images showing no co-localization between TET1 and PML in HCT116 cells (n=3). No defect in PML-NBs ability to recruit partner proteins is detected since PML-NBs normally co-localized with SUMO1 and SUMO2/3 as indicated by arrowheads in **B.** and **C.**, respectively (n=3). Scale bar: 5 μ m.

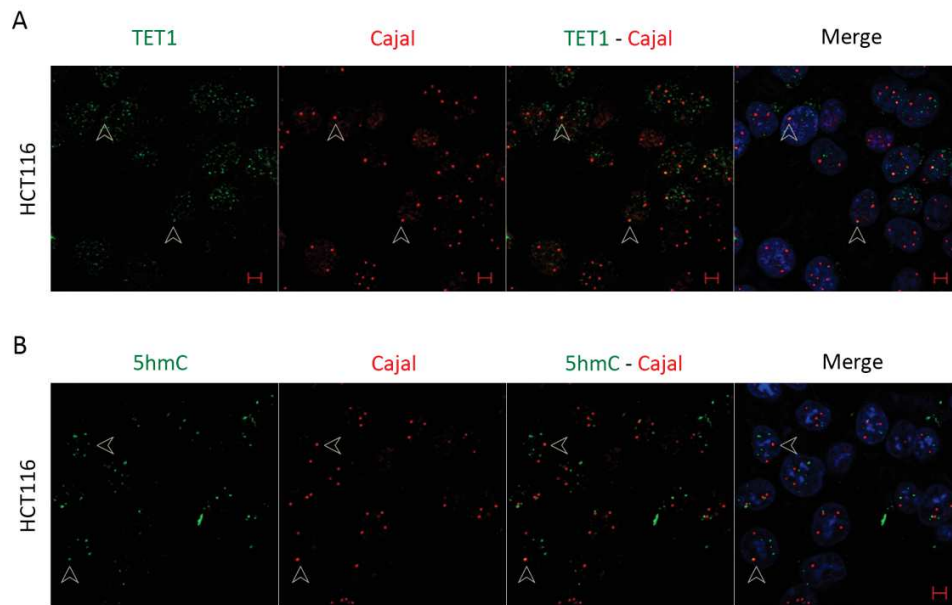


Figure S 3: TET1 and 5hmC interact with Cajal bodies (CBs). **A.** Whole field confocal images showing the co-localization of TET1-NBs and CBs in HCT116 cells as indicated by arrowheads (n=3; Scale bar: 5μm). **B.** Concomitantly with TET1, the co-immunostaining of the demethylation mark, 5hmC, and CBs further demonstrate the overlay of the two structures (n=3). Scale bar: 5μm.

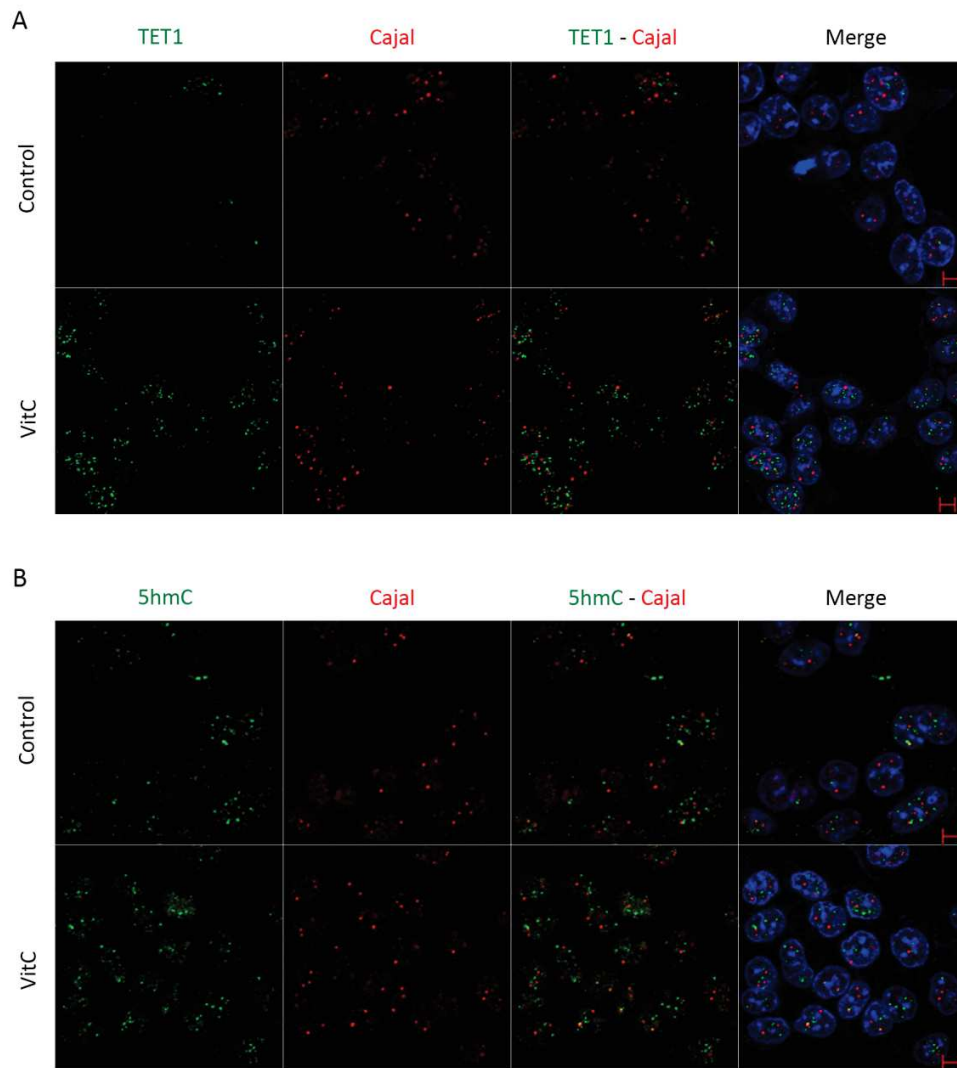


Figure S 4: Vitamin C enhances the co-localization of nuclear condensates in HCT116 cells. **A.** Co-immunolabeling 5hmC and PML-NBs in HCT116 cells revealed their enhanced co-localization upon vitamin C treatment (n=3). **B.** Whole field confocal images displaying the VitC-favored interaction between 5hmC foci and Cajal bodies (n=3). Scale bar: 5µm.

Table S 1: Co-localization percentage of the interdependent interaction between TET1/5hmC and Cajal bodies in HCT116 cells.

Percentage of Co-localization	Untreated cells	VitC-treated cells
%TET1-NBs and CBs positive cells out of total TET1 -NBs co-localized with CB	15.89% ~ 16%	Not assessed
% TET1-NBs and CBs positive cells out of total Cajal bodies	11.592% ~12%	
%5hmC-foci and CBs positive cells out of total CBs	11.22% ~ 11%	19.633% ~ 20%
%CBs and 5hmC-foci positive cells out of total 5hmC foci	12.418% ~ 12%	8.939% ~ 9%

1.4. Critical analysis of results

1.4.1. Are TET nuclear bodies a feature of cancer cells?

We showed randomly diffused subtle nuclear granulations of TET1 along with a perinuclear cytoplasmic profile in non-tumoral colon cells (NCM460). We revealed the nuclear condensation of TET1 into coarse bodies in HCT116 colorectal cancer cells. Using Caco-2 and HT-29 as alternative in-vitro models, we validated that TET1 nuclear body formation is not a HCT116-specific intracellular profile. Similarly, 5hmC demethylation mark also shifted from a diffused nuclear distribution to the formation of condensed foci in all tested CRC cells.

To determine whether TET1 exhibits a similar nuclear body formation in other solid tumor cells, we examined TET1 in human cervical adenocarcinoma (HeLa) cells. As shown in **Figure.12 (A-B)**, immuno-labeling revealed endogenous TET1 nuclear bodies with a mean of 14 dots per cell and measuring 0.3 μ m in size Furthermore, 5hmC nuclear foci were also observed by confocal imaging of HeLa cells (**Fig.12.B-C**); this highly suggests a specific nuclear pattern of both TET1 and 5hmC that is neither spontaneously formed nor restricted to a particular solid cancer cell.

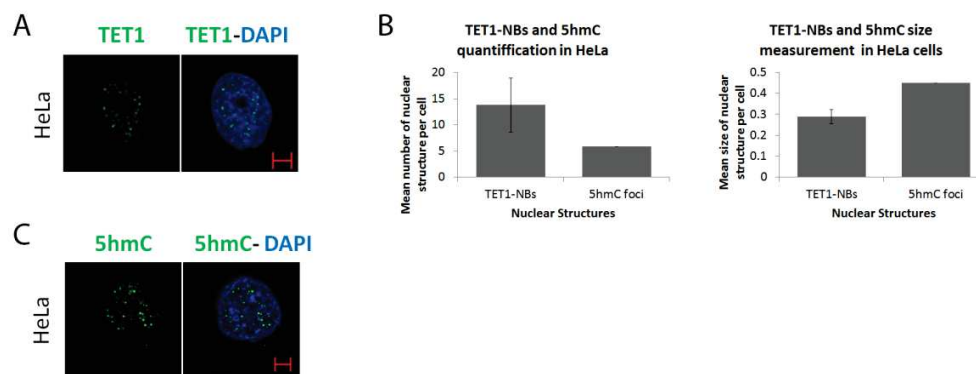


Figure 12: TET1 and 5hmC concentrate into nuclear structures in human cervical adenocarcinoma (HeLa) cells. **A.** Formation of TET1 into coarse nuclear bodies in HeLa cells (33 counted cells; n=2). **B.** Post-confocal analysis assessing the count and size of the TET1-NBs and 5hmC foci in cervical cancer. **C.** Similarly to TET1, 5hmC forms nuclear foci in HeLa cells (38 counted cells; n=1).

1.4.2. TET paralogs (TET2 and TET3) also condense into nuclear bodies

We checked TET2 and TET3 intracellular distribution profile in CRC cells. Using immunofluorescence assay, we identified coarse nuclear-bodies immuno-stained for TET2 and TET3 very similar to those labeled for their TET1 paralog (**Fig.13**). Thus, all 3 TET proteins concentrate into condensed nuclear structures in colon cancer cells.

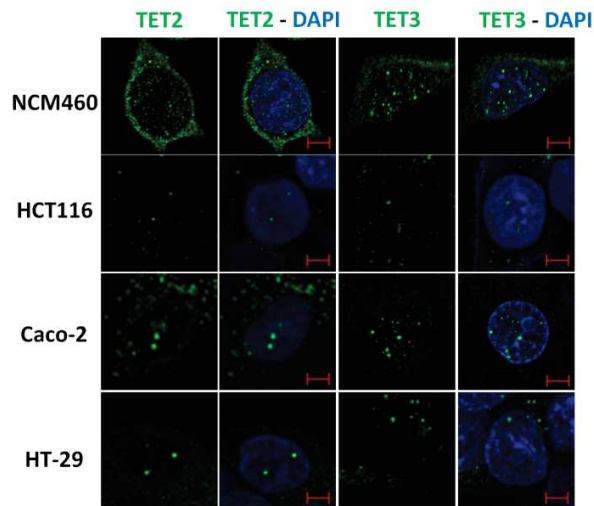


Figure 13: Panel showing the intracellular distribution profile of TET proteins in normal colon and CRC cells (HCT116; Caco-2; HT-29). TET2-3 distribution is diffused with few subtle nuclear structures as well as a cytoplasmic perinuclear profile in normal colon (NCM460) cells (n=1). This profile into coarse nuclear concentrates in CRC cells (n=1).

1.4.3. TET1-nuclear bodies are distinct from PML-Nuclear bodies

As PML bodies are well-known to recruit an ever-expanding number of partner proteins, we were intrigued to assess whether TET1 and PML co-localize. Using double immunostaining we observed that these two nuclear body-forming proteins are distinct and that this outcome is not subsequent to a dysfunctional PML. Our finding was further confirmed by the independent distribution of 5hmC foci and PML-NBs in HCT116 cells (**Fig.14**). Moreover, PML-NBs were also significantly greater in number and size when compared with 5hmC foci (**Fig.14**).

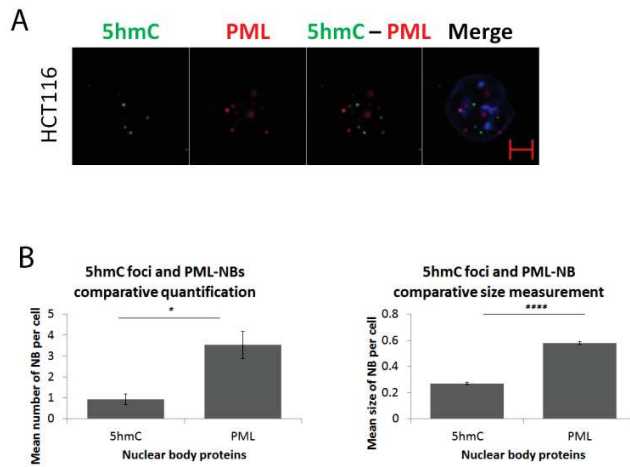


Figure 14: Independent distribution profile of 5hmC and PML in HCT116 colorectal cancer cells. **A.** Confocal image shows the distinctiveness in the intracellular profile of 5hmC and PML in HCT116 cells. **B.** Comparative post-confocal analysis of count and size displaying that PML-NBs are significantly greater than 5hmC foci (106 counted cells; n=1). * p<0.05; ****p<0.0001

1.4.4. TET1 and PML do not co-localize in non-tumoral colon cells

Confocal imaging of NCM460 cells co-immunostained for both TET-1 and PML disclose the absence of co-localization between TET1- and PML-nuclear bodies in normal colon epithelial cells (**Fig.15**). Furthermore, TET1- and PML-nuclear structures also showed predominant distinctiveness when examined in Caco-2 and HeLa cells (**Fig.16 A-B**). These results indicate that TET1 does not interact with PML in both non-cancerous and cancerous cells. Nevertheless, we speculate that a cellular stimulus could possibly trigger their interaction.

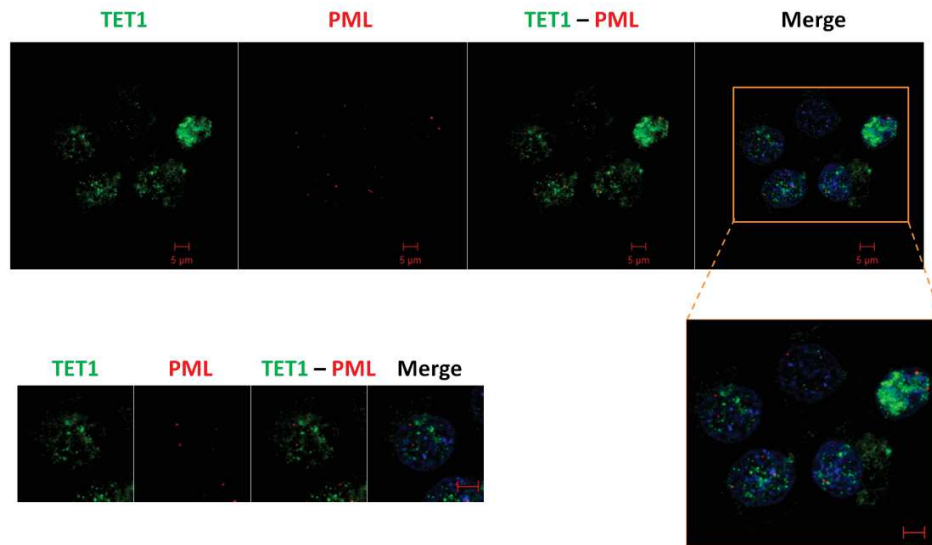


Figure 15: Confocal images showing distinct distribution profile of TET1 and PML in NCM460 normal colon epithelial cells (n=1).

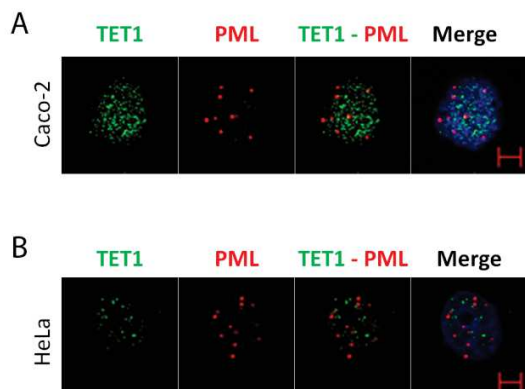


Figure 16: Confocal images of TET1- and PML-NBs in colorectal cancer (Caco-2) and cervical adenocarcinoma (HeLa) cells. Independent intracellular distribution of TET1- and PML-NBs in **A.** Caco-2 cells (n=1) and **B.** HeLa cells (n=2).

1.4.5. TET1-nuclear bodies are distinct from the DNA repair protein, Rad51

Unlike what we observed for PML-NBs, we evidenced the co-localization of TET1 and 5hmC with Cajal bodies in HCT116 cells; this finding suggests a potential role of active demethylation in the processing and the hydroxymethylation of RNAs (Delatte et al., 2016; Joy N. Ismail et al., 2020; Lan et al., 2020). Given that CBs-indispensable components

orchestrate DNA repair proteins and that TET displays emerging functions in DNA damage repair mechanisms, we aimed to examine whether TET1 associates with Rad51, a homologous recombination protein that stabilizes the genomic integrity (Henriksson & Farnebo, 2015; Lejart, Salbert, & Huet, 2018). Strikingly, TET1 and Rad51 form nuclear bodies that were independently distributed in HCT116 cells (**Fig.17.A**). Post-confocal analysis showed a significantly lower count of Rad51 nuclear bodies when compared with TET1-NBs (281 cells; $p=0.0171$) (**Fig.17.B**). Precisely, Rad51 can reach up to 2 dots per cell with a mean size of $0.38\mu\text{m}$. Although we have not identified TET1-Rad51 co-localization, we cannot rule out the possibility that TET proteins may indirectly regulate DNA repair pathway.

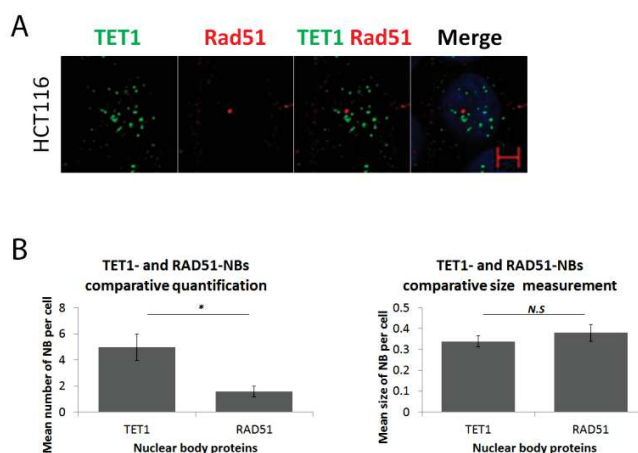


Figure 17: Subcellular distribution profile of TET1-NBs and Rad51 in HCT116 colorectal cancer cells. **A.** Co-immunostaining of TET1 and Rad51 exhibited their distinctive profile in HCT116. **B.** Comparative post confocal analysis indicating the TET1-NBs are greater in number with respect to Rad51 (281 counted cells; $n=1$). * $p < 0.05$. N.S. not significant.

1.4.6. TET1 does not co-localize with the Wnt/ β -catenin target and regulator SOX9

Since TET interacts with the Wnt/ β -catenin pathway and since SOX9 is both a target and a regulator of this oncogenic signaling pathway, we were intrigued to check whether TET1 partners with SOX9 and might therefore influence intestinal cell proliferation (Blache et al., 2004; H. Guo et al., 2019; Prevostel & Blache, 2017; Prévostel et al., 2016; Zalzal et al., 2008). In addition, SOX9 is involved in post-transcriptional events and plays a critical role in the control of colon cell proliferation (Penrad-Mobayed et al., 2018; Prevostel & Blache, 2017; Prévostel et al., 2016). Thus, we conducted a co-immunofluorescence assay of TET1 and SOX9 in HCT116 colorectal cancer cells. Confocal images indicated that this transcriptional factor retained its diffused intracellular

profile and did not concentrate into TET1-NBs (**Fig.18**) suggesting that either TET1-NBs fail to recruit SOX9 into their inner core or that there is no interplay between both proteins. Thus, there is a need to examine whether TET1 co-localize with other regulators of Wnt/ β -catenin signaling pathway such as Protein Kinase C- α (PKC α) (Dupasquier et al., 2019).

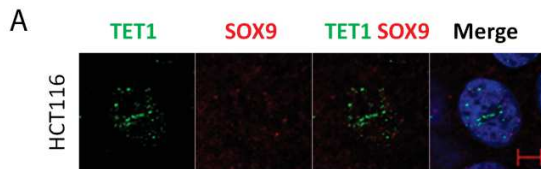


Figure 18: Co-immunofluorescence of TET1 and SOX9 in HCT116 cells (n=1).

1.4.7. Vitamin C increases the size and number of 5hmC-, PML- and Cajal- nuclear bodies and enhances the interaction of 5hmC-foci with both PML- and Cajal-NBs in all colon cancer cells tested

By treating HCT116 cells with the antioxidant micro-nutrient Vitamin C, we detected a significant stimulation in the size and count of 5hmC foci, PML- and Cajal-nuclear bodies. Similarly, VitC treatment remarkably increased 5hmC foci in Caco-2 which supports VitC-mediated activation of the DNA demethylation pathway (**Fig.19.A-B**). We further confirmed the observed induction of CBs in Caco-2 and HT-29 cells (**Fig.19.A-B**). Interestingly, this treatment has not only induced the biogenesis of nuclear structures but has also significantly triggered 5hmC co-localization with PML and CBs. Thus, we speculate that 5hmC-PML interaction might be subsequent to an activated PML-SUMO1/2/3-TET1/5hmC axis; namely, we hypothesize that VitC possibly drives SUMO-mediated post-translational modification of TET to initiate 5hmC-PML active complexes.

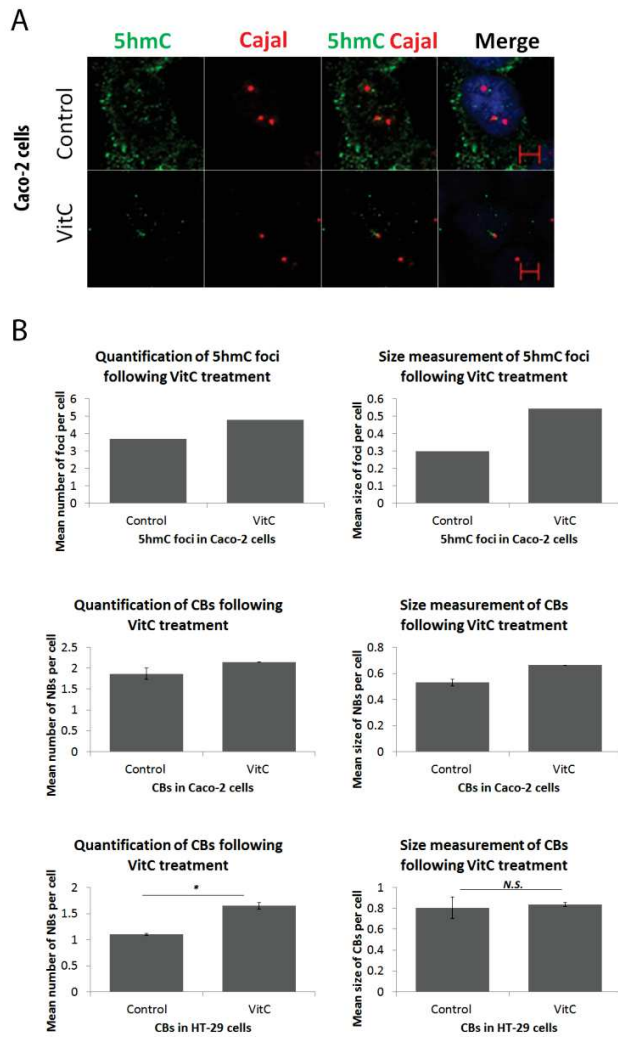


Figure 19: Formation of 5hmC foci, Cajal bodies is stimulated in Caco-2 and HT-29 colorectal cancer cells. A. Confocal images showing the co-localization of 5hmC foci and CBs in Caco-2 cells after VitC exposure. **B.** Post-confocal analysis displaying the enhancement in the count and size of 5hmC foci (in 73 cells), and Cajal bodies in Caco-2 (in 67 cells; n=1) and HT-29 cells (120 counted cells; n=1). * $p < 0.05$; N.S. not significant

1.5. Conclusion

In this study, we have characterized TET1 in colorectal cancer cells as a steppingstone to understand how this protein could modulate cellular processes in carcinogenesis.

- TET proteins condense into coarse nuclear bodies in CRC cells whereas it has a diffused nuclear distribution with subtle granulations and a perinuclear profile in NCM460 non-tumoral colon cells.
- 5hmC also concentrates into nuclear foci in CRC cells compared to a diffused profile in NCM460 cells.
- TET1-PML distribution profiles show major distinctiveness in solid cancer cells and in non-tumoral NCM460 cells.
- TET1-Cajal bodies are novel partner proteins suggesting a potential role of TET1 in RNA processing.
- VitC enhances the nuclear body biogenesis of 5hmC, PML, CBs in CRC cells and favors 5hmC interaction with both PML- and Cajal bodies.

Article 2: Exposure to Linuron herbicide disrupts TET1-Nuclear Bodies in colorectal cancer cells (in preparation)

2.1. Scientific relevance

This research study highlights the correlation between the environmental pollutants – Linuron herbicide - and the epigenetic regulation of genes. We aim to disclose how Linuron herbicide, commonly used in Lebanon, might modulate the activity of TET1 protein in colorectal cancer cells. As far as our knowledge, no research article has yet examined the effects of this herbicide on epigenetic processes in colorectal cancer. Thus, establishing a link between Linuron and DNA demethylation enzymes may promote a perspicuous understanding of the ambiguous mechanistic pathways of this pollutant. These findings may present important data to consider for cancer prevention.

2.2. Article

TET1-NBs are disrupted by Linuron herbicide in colorectal cancer cells

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ABSTRACT

Background: Environmental pollutants may greatly modulate colorectal cancer (CRC) incidence and prognosis. This study aims to investigate the incidence of Linuron exposure on TET1 activity and subcellular distribution in CRC cells.

Methods: MTT and wound healing were conducted to examine the Linuron-mediated impact on the growth and migration feature of CRC cells. Using immunofluorescence assays, we assessed the intracellular distribution of TET1-NBs following exposure to Linuron.

Results: Linuron treatment significantly stimulated both viability and migration of CRC cells. Confocal analysis revealed that Linuron can remarkably decrease in number of TET1-NBs.

Conclusion: We report pro-oncogenic effects of Linuron on exposed CRC cells. Moreover, we establish a possible TET1-dependent link between Linuron and epigenetic modifications.

Key words: TET1; nuclear bodies; Linuron; herbicide; CRC.

INTRODUCTION

Increased colorectal cancer (CRC) incidence rates are strongly linked to genetic and lifestyle factors (El-Tawil, 2010; Hagggar & Boushey, 2009). Moreover, environmental pollutants – like chlorinated chemicals, heavy metals, and persistent organic pollutants (POP) – have been associated with an elevated risk of CRC (El-Tawil, 2010; Y.-M. Lee et al., 2018; Rogala, Marchwińska-Wyrwał, Spychała, & Hajok, 2019). Lee *et al*, for instance, revealed that POP serum levels are significantly correlated with colorectal polyps and

cancer risk (Y.-M. Lee et al., 2018). Carcinogenic and non-carcinogenic chemical pollutants drive genomic instability and accumulate mutation rate via epigenetic deregulation in cancer (Pogribny & Beland, 2013). Thus, these findings highlight an environmental-genetic-epigenetic interplay in cancer.

Given that environmental factors genetically regulate disease pathogenesis, understanding the epigenetic mechanisms is indispensable to elucidate environmental-genetic interplay (Ayers, Boughanem, & Macías-González, 2019). In fact, identifying the carcinogenic effects of environmental modulators, namely chemicals, is directly linked to exposure-mediated reprogramming of DNA methylation (H. Q. Chen et al., 2021; Tabish, Poels, Hoet, & Godderis, 2012). According to Chen *et al*, treatment with the chemical 3-methylcholanthrene (3-MCA) impinges the expression of the demethylation protein, TET1, in-vitro and in-vivo (H. Q. Chen et al., 2021). Another study shows that Diuron herbicide regulates TET2 levels to abate the activity of plasmacytoid dendritic cells toward cancer (Briand, Joalland, et al., 2019). Taken together, epigenetic proteins emerge as molecular targets for assessing chemical tumorigenesis in cancer treatment and potentially in early diagnosis (Pogribny & Beland, 2013).

Linuron is a phenyl-urea herbicide reported to induce hormonal imbalances in fishes and in humans (Uren Webster, Perry, & Santos, 2015; USEPA, 2015). Besides several studies showing the impact of Linuron on aquatic organisms and amphibians, little is known about its underlying effects in mammals, particularly on the processes of tumorigenesis. Linuron is evidenced to exhibit cytotoxic effects in rat liver and testes (Scassellati-Sforzolini et al., 1997). In addition, Linuron exposure induced testicular hyperplasia and hepatocellular adenomas, but DNA damage was strictly detected in rat liver cells (Scassellati-Sforzolini et al., 1997). Besides, Linuron is still commonly used for treating cultures such as for Soyabean production including in USA as an example.(G. Chen, 2014)

On the other hand silenced TET1 represses Base-Excision Repair (BER) pathway in chemically-induced lung cancer but the impact of chemicals exposure on TET1 activity and subcellular distribution is still poorly investigated in solid tumors (H. Q. Chen et al., 2021). In a recent study, we identified TET1-Nuclear Bodies (NBs) in CRC cells and demonstrated that the size, number and interactions of 5hmC-foci with PML- and CB-NBs were significantly enhanced upon exposure to Vitamin C (VitC), a common adjuvant of chemotherapy for CRC treatments (El-Osmani et al. 2021). In the present study, we investigate whether potential environmental risk factors for health such as the Linuron herbicide could also impact TET1 activity and subcellular distribution in CRC cells.

METHODS

Cell culture and immunofluorescence assay were performed as described in El-Osmani et al. 2021.

Cellular Viability was evaluated by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cat #: M5655, Sigma-Aldrich, Beirut Lebanon). 7×10^4 CRC cells per well were plated in 24-well plates. Next, the cells were treated with increasing concentrations (0.05; 0.1; 0.4; 1 $\mu\text{g/mL}$) of Linuron (PESTANAL[®], Cat #: 36141, Sigma-Aldrich, Beirut Lebanon) for 24h. Cells were then washed twice with phosphate-buffered saline (PBS) solution and incubated with a 5mg/mL MTT dye solution for 4h. Formazan crystals were solubilized with Dimethyl sulfoxide (DMSO) and absorbance was measured at 570 nm by using BioTek ELx800 microplate reader.

Wound Healing Assay was conducted in a 24-well plate seeded with 7×10^4 cells /well for overnight in DMEM medium. The confluent monolayer of cells was scratched with a sterile 200 μL tip, and debris was washed out with PBS. Next, we exposed cells to different concentrations of Linuron for 24h. Wound images were taken at 0h and 24h after incubation and wound areas were calculated using ImageJ software.

Statistical Analysis was carried out by using STATA software for statistics and data science. All values of $p < 0.05$ were considered as statistically significant.

RESULTS

1. *Linuron herbicide stimulates the viability and aggressiveness of CRC cells.*

The cytotoxicity of Linuron has strictly been assessed in the liver and testes, and since Linuron is still used to maintain food crops (G. Chen, 2014; Scassellati-Sforzolini et al., 1997). Hence, we were intrigued to examine whether this herbicide might possibly impact CRC cells properties such as growth and migration potencies. Using MTT cellular viability assay, we detected a significantly stimulated the viability of HCT116 cells exposed for 24h of increasing concentrations of Linuron treatment (up to 0.4 $\mu\text{g/mL}$) (**Fig.1.A**). The concentration 1 $\mu\text{g/mL}$ did not show significant difference with untreated control cells, thus probably reflecting a balance between Linuron induced cell viability and death due to Linuron cytotoxicity (**Fig.1.A**). Wound healing assay revealed a concentration-dependent increase in the migration rate of CRC cells exposed up to 0.4 $\mu\text{g/mL}$ of Linuron (**Fig.1.B**). Thereupon, Linuron stimulates both the viability and migration potencies of CRC cells.

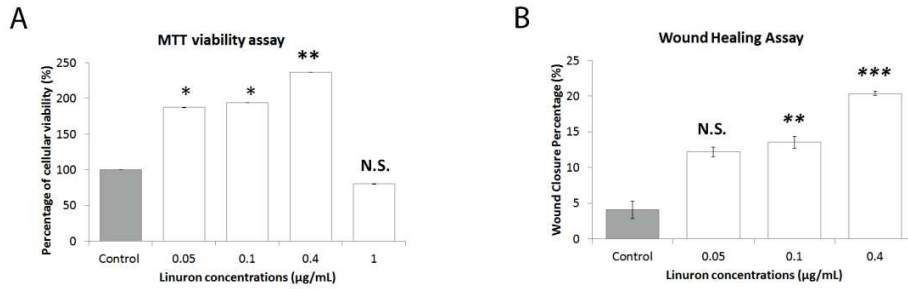


Figure. 1: Linuron exhibits pro-cancerous effects in HCT116 colorectal cancer cells. A. MTT assay indicating a significant increase in cellular viability post-Linuron exposure up to the concentration of 0.4µg/ (n=2). **B.** Wound healing assay showing that Linuron significantly stimulates the migration properties of HCT116 cells (n=1). Error bars represent the standard deviation of means. *p<0.05; **p<0.01; ***p<0.001

2. TET1-NBs number is reduced in Linuron-treated cells

We recently observed that VitC enhanced 5hmC-foci in CRC cells which could explain, at least in part, the beneficial impact of this vitamin in treatment of CRC (El-Osmeni et al 2021). In order to investigate how TET1-NBs behave in response to Linuron exposure, we assessed this nuclear-body protein in herbicide-treated HCT116 cells. Confocal analysis revealed that, unlike vitamin C, Linuron induced a decrease in the number of TET1-NBs in a concentration-dependent manner (**Fig.2.A-C**). Moreover, PML-NBs similarly decrease following exposure to increasing concentrations of Linuron (up to 0.4µg/mL) (**Fig.3.A-C**). However, the concentration 1µg/mL shows an upregulation of PML-NBs in terms of their number and size, which could suggest that Linuron may activate apoptosis via inducing cellular stress at high concentrations.

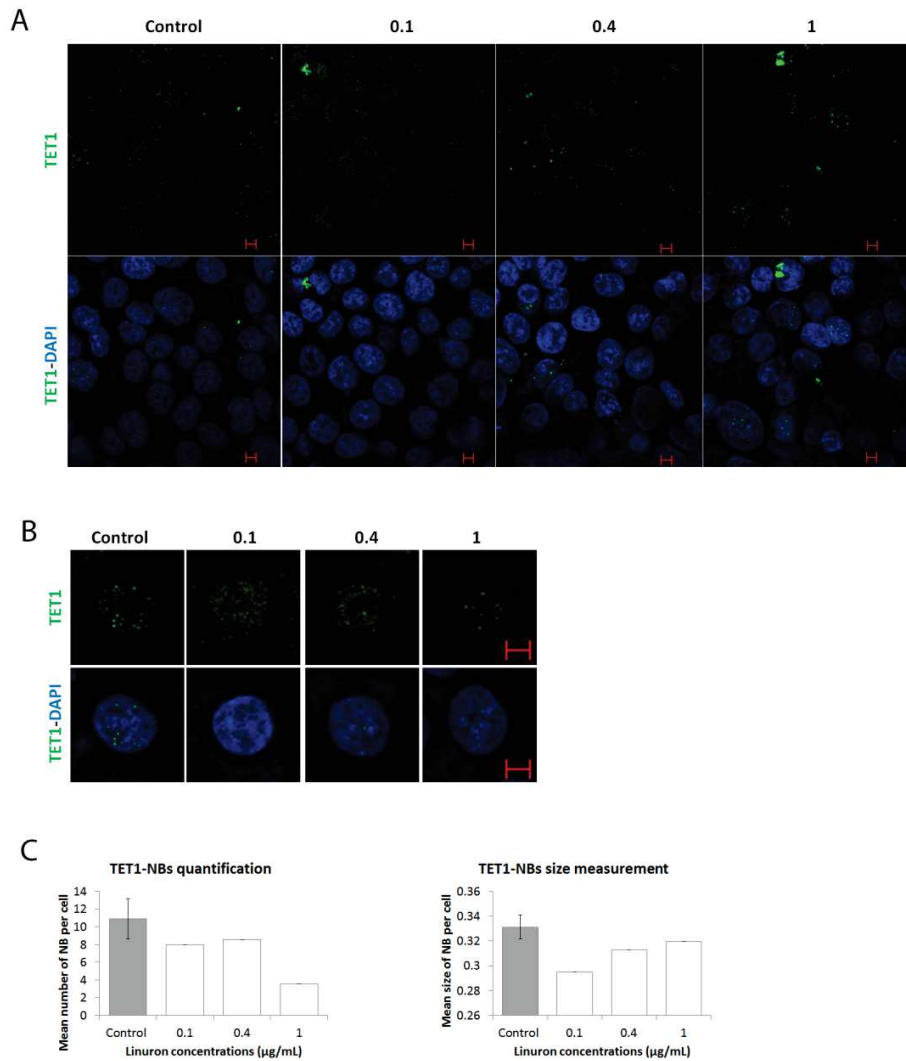


Figure. 2: Linuron downregulates the biogenesis of TET1-NBs in HCT116 cells. Confocal images showing TET1-NBs intracellular profile post-Linuron exposure in **A**. Whole field panel and in **B**. Single cell panel (n=1). **C**. Post-confocal analysis revealing a reduction in the count and size of TET1-NBs in HCT116 cells (126 counted cells; n=1).

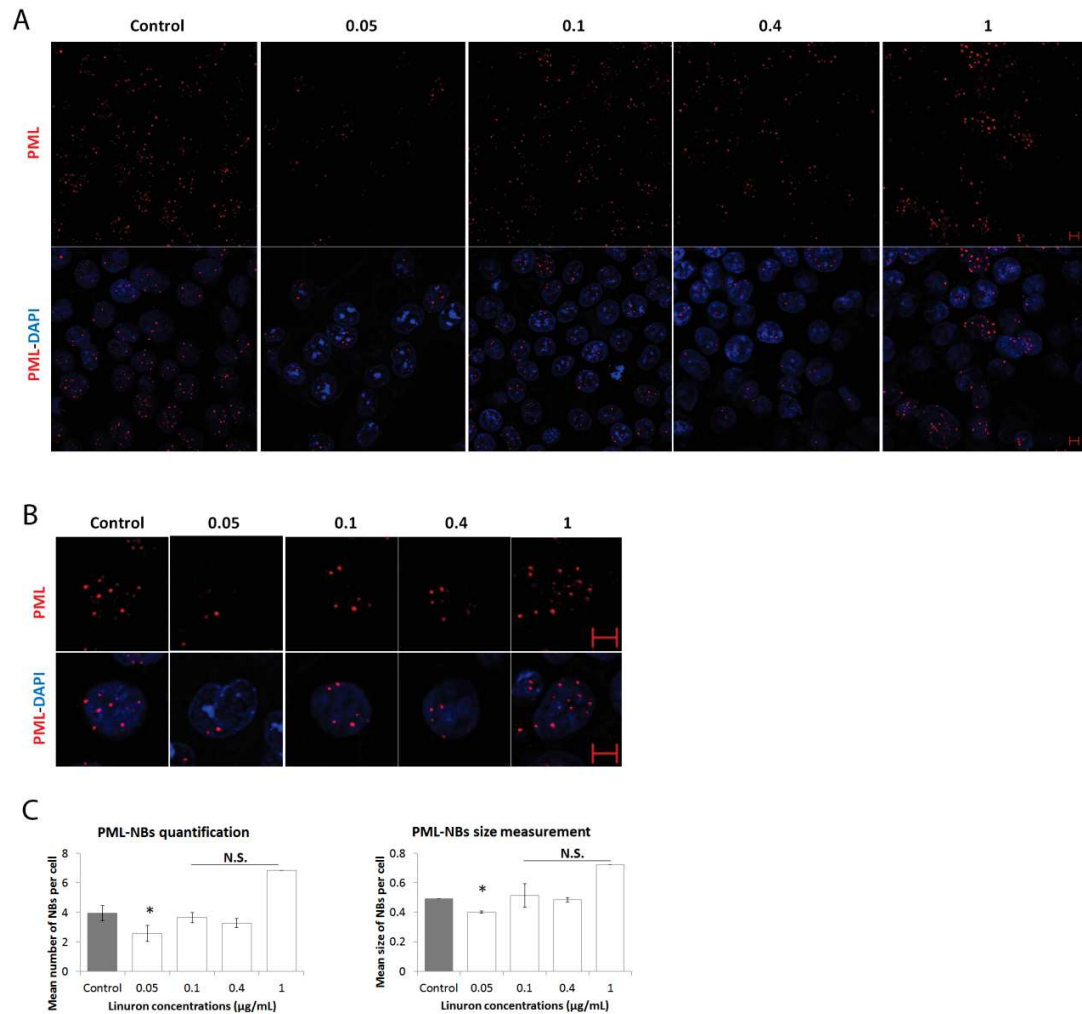


Figure 3: Linuron controls the biogenesis of PML-NBs in HCT116 cells. Confocal images showing PML-NBs intracellular profile post-Linuron exposure in **A**. Whole field panel and in **B**. Single cell panel (n=2). **C**. Post-confocal analysis revealing a reduction in the count and size of PML-NBs at all Linuron's concentrations except for 1 µg/mL at which there is an increase in both number and size of dots in HCT116 cells (78 counted cells; n=2). *p<0.05; N.S. not significant

DISCUSSION

Environmental and lifestyle risk factors such as obesity, smoking, alcohol, and environmental pollutants regulate the epigenome and impose unique signatures (Ayers et al., 2019; T. Zhu et al., 2020). Environmental–Epigenetic interplay emerges for its potential in clarifying the pro-carcinogenic effects of external factors (T. Zhu et al., 2020). Thus, what are the targeted molecular mechanisms and how they are modulated need to be specified for both cancer prevention and hopefully designing novel therapeutic strategies (Briand, Nadaradjane, et al., 2019; T. Zhu et al., 2020).

Linuron is a chlorinated urea-base herbicide commonly studied for its anti-androgenic effects (Boccard et al., 2019; Marlatt & Martyniuk, 2017; Stoker & Kavlock, 2010). In fact, Linuron binds to the androgen receptors (AR) and competitively inhibits testosterone production (Boccard et al., 2019; Marlatt & Martyniuk, 2017; Stoker & Kavlock, 2010). Yet, the correlation between androgens and colorectal cancer is still poorly understood (Farahmandlou, Oryan, Ahmadi, & Eidi, 2017; Lin et al., 2013; Roshan, Tambo, & Pace, 2016). It is worth noting that CRC risk is modulated via AR-methylation status; namely, hypomethylation of AR is proportionally associated with an increased CRC incidence (Xia et al., 2019).

Given that cholesterol processing is a substantial prerequisite for androgen production, the downregulation of the cholesterol biosynthesis pathway was not serendipitously reported post-Linuron treatment (Ornostay, Cowie, Hindle, Baker, & Martyniuk, 2013; Orton et al., 2018; Uren Webster et al., 2015). Mechanistically, this herbicide abates the expression of most cholesterol synthesis-encoding genes, including sterol regulatory element-binding proteins (SREBP1/2) (Uren Webster et al., 2015). Phenotypically, TET1/3 double knockout mouse embryos displayed reduced SREBP2 expression associated with developmental distortions (J. Kang et al., 2015). However, this reduction is unrelated to altered methylation status of SREBP2 gene in these embryos (J. Kang et al., 2015). Although SREBP regulation via TET proteins is still not investigated, Yan *et al.*, in 2020, evidenced SREBP1 as a downstream target of the TDG demethylation enzyme (Yan et al., 2020). 5caC accumulation on SREBP1 promoter due to TDG silencing is believed to decrease the binding affinity of RNA polymerase II on this cholesterol-synthetizing gene; thus, lowering the transcriptional and translational expression of SREBP1 (Kellinger et al., 2012; Yan et al., 2020). These findings, together with our observation of Linuron-mediated reduction in TET1-NBs' count may suggest that Linuron herbicide could downregulate the cholesterol synthesis pathway through TET1 protein inhibition in CRC cells. Figure 4 illustrates a schematic representation of Linuron's hypothesized mode of function.

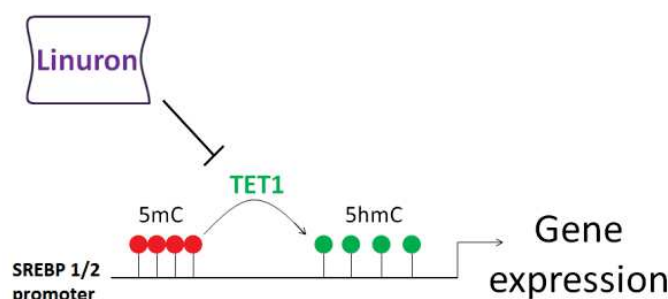


Figure. 4: Model of showing the hypothesized mode of action of Linuron in CRC cells. Linuron is believed to impede the activity of TET1 protein on the promoter region of SREBP1/2 proteins. Following blocking TET1 function, the absence of 5mC demethylation would repress the expression of SREBP proteins

Moreover, Diuron is another phenyl-urea herbicide poorly studied for its cytotoxic and pro-tumoral effects. Briand *et al* were the first to correlate this compound with the epigenetic DNA demethylation process (Briand, Joalland, et al., 2019). According to the authors, induced TET2 expression impinged the immuno-surveillance activity of plasmacytoid dendritic cells treated with Diuron (Briand, Joalland, et al., 2019). Later, the same group indicated the inability of Diuron *per se* to initiate glioma development. Interestingly, this Linuron's analog along with other non-oncogenic events synergistically contribute to gliomagenesis with characteristic signatures (Briand, Nadaradjane, et al., 2019). Combined Akt overexpression and Diuron treatment reprogram active and passive DNA demethylation processes and enhance the tumor volume with chemo-resistance properties (Briand, Nadaradjane, et al., 2019). However, the authors have not assessed the mechanistic functions of TET family in response to Diuron. Moreover, our results highlight the pro-oncogenic effects of Linuron exposure via stimulating the viability and migration of CRC cells. Taken altogether, elucidating the interplay between this herbicide and the epigenome would certainly be an added value to determine whether phenyl-urea herbicide could potentially represent risk factors for CRC occurrence.

Finally, we show that Linuron herbicide exerts pro-oncogenic activities in CRC cells and that it modulates the biogenesis of TET1-NBs. Linking Linuron with epigenetic modifications may present future research directions towards the design of therapeutic targets against CRC.

Declarations

Author Contributions Conceptualization: C.P., and Z.D.; Formal analysis & Investigation: N.E.O.; Project administration & Supervision: C.P. and Z.D; Original draft preparation: N.E.O.; Review & editing: C.P. All authors have read and agreed to the published version of the manuscript.

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GENERAL DISCUSSION & CONCLUSION

My thesis project unravels a novel profile of TET enzymes – particularly TET1 – and 5hmC – the demethylation mark – characterized by their condensation into coarse nuclear bodies in colorectal cancer cells. This novel intra-nuclear distribution recalled the nuclear structures formed by other PML and Cajal bodies. Using confocal imaging, we demonstrated that TET1-NBs and 5hmC- foci are distinct from PML- and Cajal Bodies but do interact with Cajal bodies which suggests an epigenetic role for TET1 in RNA processing. Although a 5hmC-PML co-localization was not detected in control conditions, the interaction between 5hmC- and PML-nuclear bodies was significantly enhanced by Vitamin C. Besides, even though we failed to identify an overlap in the profiles of either TET1-Rad51 or TET1-SOX9 in colorectal cancer cells, we cannot rule out the possibility that their interaction may occur following multiple extra-cellular stimuli.

Conversely, we show that Linuron decreases the number of TET1 nuclear bodies which highlights the impact of this pesticide on TET1-mediated epigenetic mechanisms.

A. Do TETs regulate RNA biogenesis and processing?

In this research project, we revealed an interaction between TET1/5hmC with Cajal bodies in colorectal cancer cells which suggests a probable role of TET1 in RNA processing. Our results support and explain the previously reported cross-talk between DNA demethylation and RNAs, particularly those non-coding (Arviden et al., 2017; Cheray et al., 2020; Fu et al., 2014; C. He et al., 2021). Mechanistically, TET demethylation activity is not restricted to DNA since the methylation mark (5mC) on RNA also undergoes TET-dependent hydroxymethylation (Fu et al., 2014; Joy N. Ismail et al., 2020). Although the role of 5hmC in RNA is still poorly delineated, TETs appeared to drive RNA splicing through imposing their demethylation mark on pre-mRNA; hence, suggesting new insights elucidating how TETs mediate gene expression (Lan et al., 2020). Furthermore, the stability of both coding and non-coding RNAs is readily influenced by (de)methylation levels (Cheray et al., 2020; Guallar et al., 2018; C. He et al., 2021; Lan et al., 2020). Whereas mRNA and transfer RNA (tRNA) exhibit short half-life and fast turnover via TET2-mediated demethylation, loss of 5hmC repressed miR-mRNA duplex formation and inhibited miR activity (C. He et al., 2021; Lan et al., 2020). Thereupon, these observations further highlight the crosstalk between the functions of TET demethylases and RNA-mediated regulation of gene expression.

Despite the fact that miR are well-reported to regulate TETs expression and activity, it remains noteworthy to underscore TETs roles either in sponging or activating some miR

(**Table 3 and 5**). For example, in hepatocellular carcinoma, miR-21 physically hinders TETs expression, resulting in cancer progression-related features: enhanced cellular proliferation and repressed levels of the tumor-suppressive gene, PTEN (Cao et al., 2019). On the other hand, TET3 activates miR-145 via restoring the demethylation levels at the promoter of miR-145 precursor gene, suggesting a role of TETs in the biogenesis of microRNAs (J. Li et al., 2021). Studies also established the interplay between methylation erasers and two distinct subsets of non-coding RNAs: lncRNA and circRNA (**Table 5**) (N. Chen et al., 2018; B. Hu et al., 2019; L. Yu et al., 2020). For instance, the circRNA FECR1 promotes breast cancer aggressiveness via TET1-mediated demethylation of FLI1 gene (N. Chen et al., 2018). Similarly, bladder carcinogenesis is directly linked to the disrupted TET1-p53 interaction in presence of the lncRNA XIST (B. Hu et al., 2019).

Taken altogether, our findings come in line with the previously reported cross-regulation between TET demethylation enzymes and non-coding RNAs. We believe that this study forms the steppingstone toward establishing an in-depth understanding of the targeted molecular mechanisms regulated during this crosstalk.

B. Do TET1-NBs mediate DNA damage repair?

It is not surprising that TET-mediated active demethylation drives DNA damage repair pathway, especially since the oxidized cytosine bases – 5fC and 5caC – impinge the integrity of the genome (S. Ito & Kuraoka, 2015; C. Jin, Qin, Barton, Jelinek, & Issa, 2015). Mechanistically, these modified bases were not only reported to block RNA polymerase II transcriptional activity but also to increase the mutation rate and genomic instability (S. Ito & Kuraoka, 2015; Kellinger et al., 2012). Moreover, TET1 silencing sensitizes tumorous cells to DNA double-strand breaks and represses the repair mechanism; thus, underscoring the cytoprotective role of TET1 (Coulter et al., 2017).

Kafer *et al* disclosed 5hmC as a novel epigenetic mark for DNA lesions following its accumulation in the endogenous damage foci (Kafer et al., 2016). Apart from its co-localization with DNA repair biomarkers (G1-specific 53Binding Protein 1 (53BP1) and the histone variant γ H2Ax) in cancer cells, 5hmC foci appeared to overlap with Rad51, a homologous recombination protein (Kafer et al., 2016) (**Fig.20**). Of note, the DNA damage repair foci comprising 53BP1 and/or Rad51 remained unaffected by total knockout of TET proteins in mESC; thus, indicating that low 5hmC levels do not influence 53BP1/Rad51 foci formation (Kafer et al., 2016). In our study, we report the absence of co-localization among TET1 and Rad51, yet we have not co-immunostained Rad51 and 5hmC in HCT116 cells. According to Zhong *et al*, the mRNA and protein expression levels of Rad51, 53BP1, and other repair genes are directly downregulated in TET1-deficient cells (J. Zhong et al., 2017). On the other hand, TET3 abrogation promoted Rad51 expression but impaired its

recruitment to DNA lesions in mouse cardiac fibroblasts (Rath, 2019). Hence, more studies are needed to unravel the controversial ambiguity regarding TET-Rad51 crosstalk.

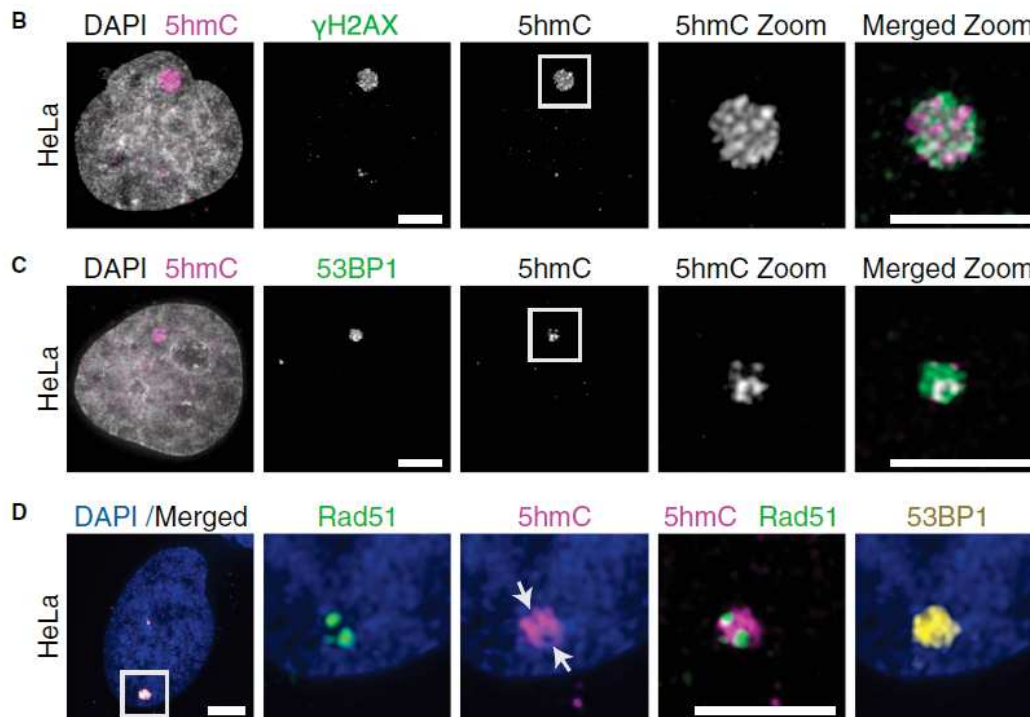


Figure 20: 5hmC co-localizes with DNA repair proteins γ H2Ax, 53BP1, and Rad51 (Kafer et al., 2016). Immunofluorescence images highlighting the role of 5hmC as a player in the process of DNA damage repair.

Based on our observation and the previously published reports, two possible speculations should be addressed: 1. TETs as well as 5hmC are potentially dispensable for the recruitment of repair proteins to damaged sites; 2. TET/5hmC role in managing impaired DNA may take place after the recognition and binding of early repair response elements. Furthermore, whether TETs signal the recruitment of partner proteins and transcription factors into the repair complex still require delineation. Precisely, we wonder whether the efficient restoration of the disrupted DNA strand could be governed by TET demethylation of repair components at the damaged site. One finding in favor of these hypotheses is the interdependent regulation evidenced between TET1 and PARP1, an essential DNA damage repair protein (Ciccarone, Zampieri, & Caiafa, 2017). Intriguingly, our novel identification of TET1-Cajal association could also support these hypotheses as coilin is implicated as well in the repair mechanism (Henriksson & Farnebo, 2015). Moreover, WRAP53 β - another major component of Cajal bodies – appeared as a substantial regulator of an efficient response to DNA

damage (Henriksson & Farnebo, 2015) (**Fig.21**). The fact that numerous repair factors are either implicated in RNA processing and/or enriched in Cajal bodies is fascinating (Henriksson & Farnebo, 2015); hence, future research is believed to open promising findings to elucidate the complexity of the DNA repair pathway.

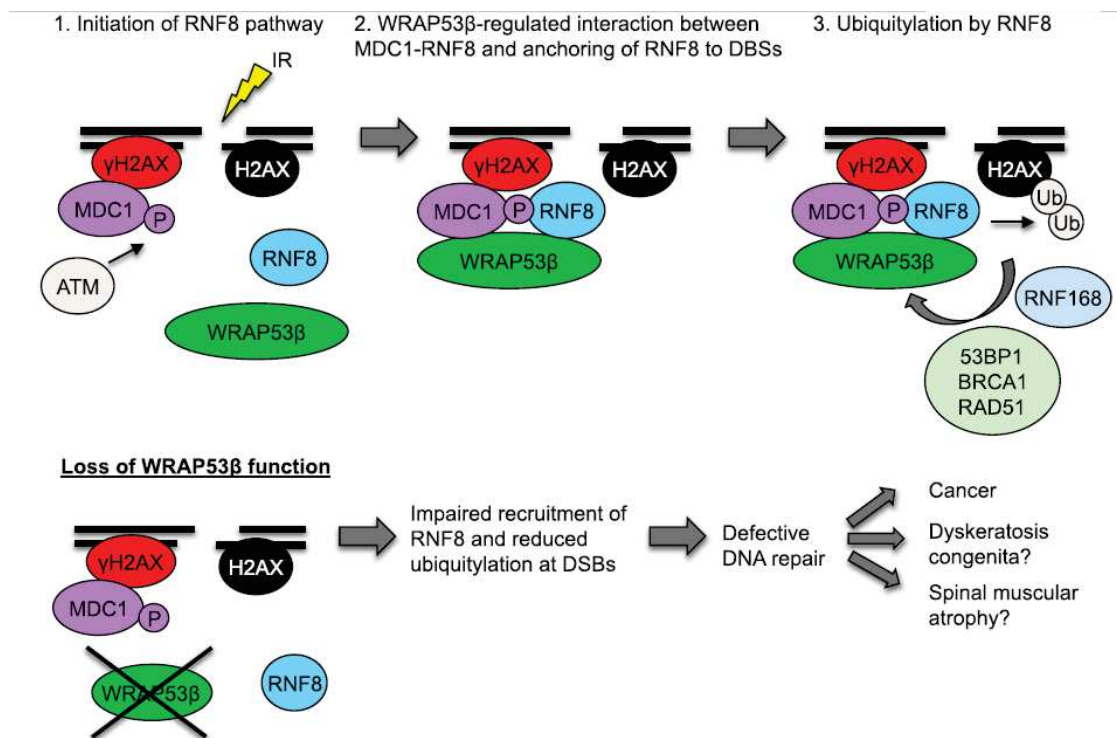


Figure 21: Model showing the implication of WRAP53, a core component protein of CBs, in DNA repair mechanism. WRAP53 is a crucial in DNA damage response as it favors the recruitment of repair proteins to initiate the repair pathway either by homologous recombination (e.g Rad51) or by non-homologous end-joining (e.g. 53BP1) (Henriksson & Farnebo, 2015).

C. Are there other molecular mechanisms possibly disrupted by Linuron?

i. Wnt/ β -catenin signaling?

KEGG pathway analysis of Linuron and Diuron – a less stable structural analog – revealed that both herbicides are implicated in pathways related to cancer (Marlatt & Martyniuk, 2017). Additionally, Ornostay *et al* disclosed the role of Linuron in modulating Wnt-signaling transcripts in minnow fishes (Ornostay et al., 2013). Two opposing mechanisms of action were evidenced while studying SREBP- Wnt interaction. First, research groups have identified a direct role of SREBP enzymes in activating Wnt/ β -catenin pathway in solid cancers (Y. Liu et al., 2020; J. Wang et al., 2020). However, another set of studies showed an inverse correlation characterized by SREBP ability to impede the recruitment of β -catenin to the nucleus and therefore inhibiting Wnt pathway

(Abiola et al., 2010; Ahn, Lee, Kim, & Ha, 2010). Thereupon, whether Linuron controls Wnt/ β -catenin signaling via SREBP enzymes still requires investigation.

Of note, TET1 is known to sustain demethylation at the promoters of Wnt inhibitors: DKK, DACT2, SRFP genes (Jiangxia Fan et al., 2018; Neri et al., 2015; J. Wu et al., 2019). Given that TET1 silencing is commonly reported in various solid cancers, Wnt pathway restored its proliferation-associated properties following the repression of its antagonists (Jiangxia Fan et al., 2018; Neri et al., 2015; J. Wu et al., 2019) (**Fig.22**). Our results indicate a Linuron-mediated increase in the proliferation and aggressiveness of HCT116 cells. One intriguing finding is the observed downregulation in TET1-NBs biogenesis following treatment. Taken together, more experimental evidence is needed to unravel how Linuron controls TET1 epigenetic protein and how this herbicide could impact the activity of Wnt/ β -catenin pathway. Furthermore, it is crucial to validate whether Wnt/ β -catenin pathway is particularly responsible for inducing the proliferation of colorectal cancer cells. Is there other underlying mechanisms mediating the pro-tumoral effects of Linuron? The PI3K/AKT/mTOR signaling pathway is one possibility given its role to control SREBP enzymes (Mohamed, Viveiros, Williams, & Posse de Chaves, 2018; B. Zhang et al., 2020).

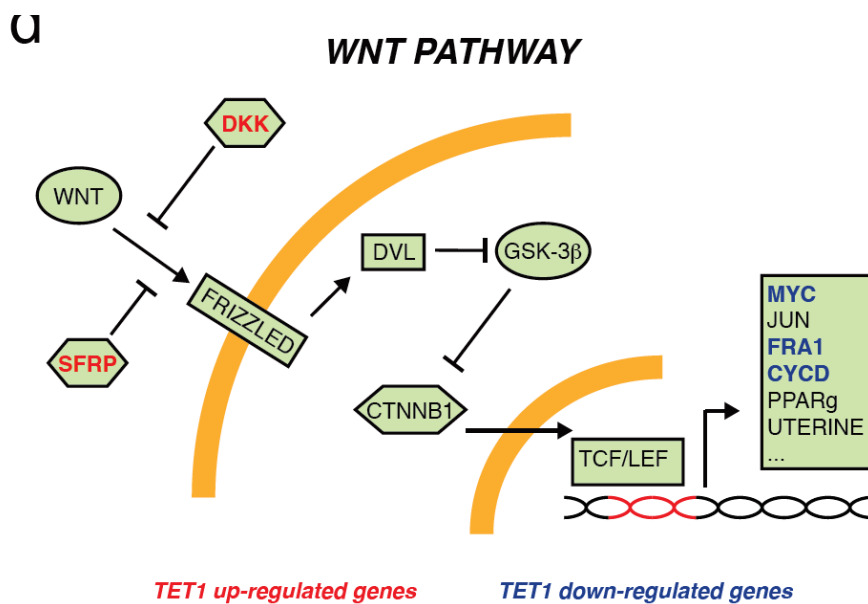


Figure 22: TET proteins activate Wnt inhibitors to block Wnt/ β -catenin pathway (Neri et al., 2015). A diagram elucidates how TET1 antagonize Wnt/ β -catenin pathway via upregulating Wnt inhibitors such as SFRB and DKK enzymes while downregulating Wnt-activating proteins including Myc.

ii. ROS production?

Xenobiotics are well known to promote carcinogenesis by stimulating ROS production (Henkler, Brinkmann, & Luch, 2010). Yet, studies investigating how Linuron generates ROS are very limited. Webster *et al* revealed the ability of this compound to trigger cell stress responses in brown trout (Uren Webster et al., 2015). According to the authors, this herbicide significantly increased the mRNA levels of many antioxidant enzymes as well as the key transcriptional factor Nuclear factor–erythroid-derived 2-like family (Nrf2) implicated in regulating cellular redox homeostasis (Uren Webster et al., 2015). Another report stated the multifaceted effects of Linuron on the antioxidant system of rat prostate tissues (Sang-Yoon et al., 2012). Using RT-PCR analysis, some antioxidant enzymes were shown to be upregulated by Linuron (SePP; SOD2; PHGPx) while others were reduced (GPx1; SOD1). In zebrafish larvae, Diuron exposure stimulated the expression of the antioxidant, glutathione; this suggests the scavenging activity against oxidative stress even though the authors have not detected an increase in ROS levels (Velki et al., 2019). Contrarily, several other studies evidenced significant ROS production in pacific oysters as well as in human cancer cell lines exposed to Diuron (Behrens, Rouxel, Burgeot, & Akcha, 2016; Huovinen, Loikkanen, Naarala, & Vahakangas, 2015). Thus, more evidence is certainly required to better understand the mechanism of this herbicide in humans.

Knowing that pesticide-mediated cellular toxicity is subsequent to the accumulation of free radicals, DNA methylation status is also influenced by ROS levels (Q. Wu & Ni, 2015). Even though upregulated ROS production is associated with global hypomethylation, tumor suppressor genes are downregulated in a site-specific dependent hypermethylation (Q. Wu & Ni, 2015). For instance, hydroquinone, a benzene metabolite, enhances TET1 expression and promotes the active DNA demethylation pathway via elevated iron II levels (Coulter et al., 2013; Zhao et al., 2014). Interestingly, Coulter *et al* showed that TET1 blocks cell cycle progression in hydroquinone-treated cells (Coulter et al., 2013). In CRC cells, chemo-drug resistance is evidenced following ROS-favored activation of TET1 enzyme (K. A. Kang et al., 2014). These findings potentially highlight TET1 enzyme as a primary target of chemicals to mediate their toxic/pro-cancerous effects, thus the need to reveal how Linuron influences cellular processes via TET1.

D. Could vitamin C counteract the oncogenic properties of Linuron?

Even though no study has yet addressed this question, Behrens *et al* revealed that the antioxidant effects of VitC can abrogate Diuron-induced abnormalities in pacific oysters (Behrens et al., 2016). Interestingly, this is not the first evidence highlighting the role of VitC against chemical-mediated synthetic lethality. For instance, VitC detoxifies the oxidative effects of the organophosphate pesticide, Dichlorvos, in human erythrocytes (Eroglu, Pandir, Uzun, & Bas, 2013). Moreover, neurotoxicity was reversed, at least in

part, by VitC supplementation to juvenile zebrafish subjected to a mixture of insecticides (Robea et al., 2020). Another study disclosed the cytoprotective properties of VitC against Paraquat via sponging the herbicide's deleterious effects on liver and kidney tissues derived from rats (Awadalla, 2012).

Furthermore, long-term treatment with VitC effectively reduced oxidative stress and the subsequent inflammatory response in patients with severe Paraquat poisoning (S. Hu et al., 2018). This anti-oxidative therapy warrants a good prognosis via protecting patients against progressive lung fibrosis (S. Hu et al., 2018). Given that VitC and Linuron reversely modulate TET1 in colorectal cancer, we believe it is indispensable to experimentally and clinically examine the impact of VitC on Linuron's oncogenic activities. This antioxidant vitamin is well-known to re-program the cancer epigenome and to limit the chemotherapeutic drug-resistance events in solid cancers (Gillberg et al., 2018; Pawlowska et al., 2019). Thereupon, we think that mixing the antioxidant vitamin with the herbicide formulation would limit and alleviate the damaging effects of Linuron; thus, this could potentially favor a better prognostic tool for treating colorectal cancer patients.

Finally, this research study uncovered a differential response of the newly characterized TET1-NBs/5hmC-foci to external modulators in colorectal cancer. In light of our findings, addressing the clinical significance of Linuron exposure on carcinogenesis, in murine CRC models, is now needed to investigate *in-vivo* whether VitC could possible counteract Linuron's effects on TET1 and hopefully on CRC cells growth and migration potencies.

PERSPECTIVES

A. Short-term perspectives

1. Understand the role of TET1-NBs in various cellular processes in colorectal cancer:

- Clarify the interdependence between TET1-NBs and Cajal bodies. Given that CBs are disrupted in absence of their core components (Coilin and WRAP53), it is essential to examine the structural integrity of CBs following TET1 silencing.
- Investigate TET1 expression level in CRC cells by western blot assay, and check its activity following repression of PML or CBs.
- Confirm the genetic integrity of TET1 by sequencing.
- Assess how the dynamic interaction of TET1 and CBs affects microRNA regulation. We propose miR-34-a as a potential candidate especially since it is frequently repressed via promoter hypermethylation in various solid tumors (Sun et al., 2021). Two important questions should be addressed to tackle this objective: Could TET1 retain its ability to control this miR in absence of CBs? Would CBs knockdown impinge the maturation of miR-34-a and subsequently CRC progression?
- Examine whether TET1 requires a post-translational modification to be recruited into the inner core of PML-nuclear bodies. Since PML SUMOylate its partner proteins, we believe the best way to assess the necessity of such a modification is via altering the expression levels of SUMO1-3 proteins, either by knockout or by over-expression assays.
- Identify whether exogenous stimuli could drive TET1-NBs and Rad51 interaction. VitC is one possible stimulus given its role in rescuing DNA double-strand breaks (Tao et al., 2019).

2. Elaborate the molecular mechanism of Linuron-mediated alterations in TET1-NBs.

- Examine the protein levels of TET1 post-Linuron treatment. Given that the biogenesis of TET1-NBs is impinged due to Linuron, it is indispensable to assess

whether this herbicide imposes dysfunctional defects on TET1 nuclear body formation or that this downregulation is subsequent to a decrease in TET1 protein levels.

- Confirm the cellular process mediated by Linuron to promote carcinogenesis via influencing TET1-NBs. Given that our results show a Linuron-mediated induction of CRC proliferation, two possible pathways should be investigated: the first is Wnt/ β -catenin signaling pathway that is known for its cross-talk with SREBP and TET enzymes (Abiola et al., 2010; Neri et al., 2015; J. Wang et al., 2020). The second is PI3K/Akt/mTOR pathway which has been reported to regulate its downstream target SREBP (Mohamed et al., 2018; Sekita et al., 2021; B. Zhang et al., 2020). Given that both pathways are regulated by TET and SREBP, this might reflect an indirect interplay between both of these enzymes particularly following Linuron treatment.
- Investigate the effects of ROS production on the biogenesis of TET1-NBs as well as on their interaction with Cajal bodies in cancer.

B. Long-term perspectives

- Inject mouse models with colorectal cancer cells subjected to various concentrations of Linuron in order to:
 - Confirm the in-vitro results in terms of Linuron-mediated cancer invasiveness and aggressiveness.
 - Examine the effects VitC on tumor size and volume in control and Linuron-exposed mice.

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