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The role of chromatin and epigenetic mechanisms in nuclear architecture.

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Contents

Περίληψη

Το DNA περιέχει τη γενετική πληροφορία των κυττάρων και οργανώνεται σε νουκλεοσώματα καθώς περιτυλίσσεται γύρω από τα οκταμερή των ιστονών. Τα νουκλεοσώματα αποτελούν τη δομική μονάδα της χρωματίνης. Οι αμινοτελικές ουρές των ιστονών προεξέχουν από το νουκλεόσωμα και υφίστανται επιγενετικές τροποποιήσεις συμπεριλαμβανομένου την ακετυλίωση, μεθυλίωση, φωσφορυλίωση κ.λπ. σε συγκεκριμένα αμινοξικά κατάλοιπα. Ο συνδυασμός αυτών των επιγενετικών τροποποιήσεων κατά μήκους των γονιδίων οδηγούν σε διαφορετικό αποτέλεσμα όσον αφορά την κατάσταση της χρωματίνης και της γονιδιακής έκφρασης. Η μονομεθυλίωση της λυσίνης 20 στην ιστόνη H4 συνδέεται με ποικίλες κυτταρικές διεργασίες όπως ο διπλασιασμός του DNA, η επιδιόρθωση βλαβών στο DNA και η ανάπτυξη, και σχετίζεται θετικά με τη μεταγραφική ενεργοποίηση. Καταλύεται από τη μεθυλοτρανσφεράση λυσίνης Set8, η οποία ρυθμίζεται στενά από τον κυτταρικό κύκλο και συμμετέχει στην αδειοδότηση των αρχών αντιγραφής, τη ρύθμιση της γονιδιακής έκφρασης, τη συμπύκνωση χρωματίνης, την επιδιόρθωση βλαβών στο DNA καθώς και στην εξέλιξη του κυτταρικού κύκλου και την ανάπτυξη των οργανισμών. Η απώλεια λειτουργίας της πρωτεΐνης Set8 σε ποντίκια προκαλεί θνησιμότητα στα στάδια πριν την εμφύτευση του εμβρύου ενώ σε ποντίκια με ηπατοκυτταρική απώλεια της Set8 (*Set8^{loxp}/AlbCre*) οδήγησε στην αύξηση του μεγέθους των ηπατοκυττάρων και των πυρήνων τους, σε κυτταρικό θάνατο εξαιτίας εκτεταμένων βλαβών στο DNA και σταματήματος στη φάση G2 για μεγάλο χρονικό διάστημα και τελικά στην ανάπτυξη αυθόρμητου ηπατοκυτταρικού καρκινώματος σε ώριμο στάδιο. Ο στόχος αυτής της έρευνας ήταν η περαιτέρω εξέταση του νεκρωτικού φαινοτύπου των ηπατοκυττάρων *Set8^{loxp}/AlbCre* και της αύξησης του μεγέθους των πυρήνων τους με τη χρήση ανοσοϊστοχημείας σε κρυοτομές ήπατος και ηλεκτρονική μικροσκοπία διέλευσης. Δείχθηκε ότι η απώλεια της Set8 οδηγεί σε εγκολπώσεις του πυρηνικού φακέλου και στο σχηματισμό ενδοπυρηνικών κυστιδίων τα οποία περιέχουν κυτταροπλασματικούς παράγοντες ενώ αποκλείονται πυρηνικές πρωτεΐνες· αυτό το φαινόμενο μπορεί να ονομαστεί 'ενδονουκλέωση'. Εικόνες από ηλεκτρονικό μικροσκόπιο διέλευσης και η χρώση για H4K20me3 μέσω ανοσοφθορισμού έδειξαν ότι η ετεροχρωματίνη ίσως απομακρύνεται από την πυρηνική λαμίνη στα ποντίκια *Set8^{loxp}/AlbCre*. Συμπερασματικά, η πρωτεΐνη Set8 φαίνεται να συμμετέχει στη δομική ακεραιότητα του πυρήνα· η υπόθεση αυτή συμφωνεί με την ιδέα ότι το γονιδίωμα έχει μη γενετικές λειτουργίες όπως ως καθοριστής του μεγέθους και της ανθεκτικότητας του πυρήνα. Σε βάθος διερεύνηση της ενδονουκλέωσης ως χαρακτηριστικό κυτταρικού θανάτου και του ρόλου της Set8 στις διάφορες κυτταρικές διαδικασίες μπορεί να έχει βοηθητικό ρόλο στην αντιμετώπιση των παθολογικών καταστάσεων στις οποίες εμπλέκεται η Set8, καθώς ένας μεγάλος αριθμός μελετών συνδέουν αυτή την πρωτεΐνη με φυσιολογικές και παθοφυσιολογικές διεργασίες όπως η ωρίμανση των ερυθροκυττάρων, η διαφοροποίηση των λιποκυττάρων, η διατήρηση του δέρματος στους ενήλικες και η καρκινογένεση στον άνθρωπο.

Summary

The DNA contains the genetic information of cells and is organized in nucleosomes by being wrapped around histone octamers. Nucleosomes constitute the structural unit of chromatin. N-terminal histone tails protrude from the nucleosome and are subjected to epigenetic modifications including acetylation, methylation, phosphorylation etc. at specific amino acid residues. The combination of these epigenetic modifications across genes lead to different outcome regarding the chromatin state and gene activation status. Monomethylation of lysine 20 at histone H4 is linked to various cellular processes such as DNA replication, DNA damage repair and development, and is positively correlated to transcription activation. It is catalyzed by the lysine methyltransferase Set8, which is tightly regulated by the cell cycle and participates in licensing of replication origins, gene expression regulation, chromatin condensation, DNA damage repair as well as cell cycle progression and development of organisms. Set8 loss-of-function in mice causes lethality in preimplantation stages whereas a liver-specific knock out (*Set8^{loxp}/AlbCre* mice) resulted in enlargement of hepatocytes and their nuclei, cell death due to massive DNA damage and prolonged G2 arrest and eventually development of late-onset, spontaneous hepatocellular carcinoma. The purpose of this study was to further examine the necrotic phenotype of *Set8^{loxp}/AlbCre* hepatocytes and the enlargement of their nuclei by using immunohistochemistry on cryosections of liver tissue and transmission electron microscopy. It was revealed that Set8 depletion leads to invaginations of the nuclear envelope and the formation of intranuclear vacuoles that include cytoplasmic components but exclude nuclear proteins; this phenomenon can be called 'endonucleosis'. Transmission electron microscope images and immunostaining of trimethylation of H4K20me3 showed that heterochromatin might be dissociated from the nuclear lamina in *Set8^{loxp}/AlbCre* mice. In conclusion, Set8 protein seems to be involved in the structural integrity of the nucleus; this hypothesis is in line with the notion that the genome contains non-genetic functions e.g. as a determinant of nuclear size and robustness. In depth-investigation of endonucleosis as a characteristic of cell death and the role of Set8 in the various cellular processes could provide help in dealing with the pathological conditions mediated by Set8, given that a large number of studies link this protein to physiological and pathological processes e.g. erythroid maturation, adipogenesis, maintenance of adult skin and human carcinogenesis.

The role of chromatin and epigenetic mechanisms in nuclear architecture.

Introduction

Chromatin constitutes the organized form of DNA in cells. Nucleosome is the fundamental structural unit of chromatin and contains 146bp of DNA wrapped twice around the histone octamer. The histone octamer is highly conserved and consists of two molecules of the histones H2A, H2B, H3 and H4 (Luger *et al.*, 1997). The C-terminal histone fold domains are involved in histone-histone interactions within the nucleosome, whereas the highly basic, relatively unstructured N-terminal tails protrude from the nucleosome and interact with the nuclear environment (Luger *et al.*, 1997). Chromatin structure and architecture are not static; on the contrary, chromatin undergoes dynamic changes. These conformation changes play an essential role in a multitude of biological processes that require access of proteins to DNA such as DNA replication and repair, recombination and transcriptional regulation (Kouzarides, 2007; Bannister and Kouzarides, 2011). The compaction of chromatin into high-order structures and the recruitment of non-histone proteins to specific genomic regions are achieved through epigenetic mechanisms, specifically through the post-translational modifications of the N-terminal histone tails (Luger and Richmond, 1998; Wolffe and Hayes, 1999).

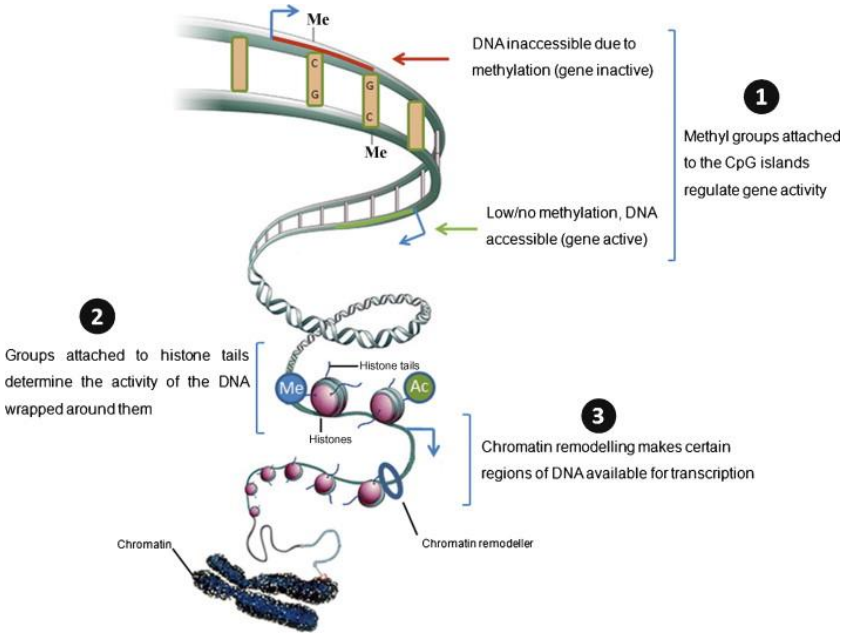

Fig. 1 Major epigenetic mechanisms affecting gene expression. Adapted from Rajender, Avery and Agarwal (2011).

A variety of histone modifications have been described which occur at different amino acids and encode epigenetically-transmitted information ('histone code') (Strahl and Allis, 2000) (fig. 1). These include lysine acetylation, lysine and arginine methylation, serine, threonine and tyrosine phosphorylation, ubiquitination, poly ADP-ribosylation, sumoylation etc (Strahl and Allis, 2000; Zhang and Reinberg, 2001; Kouzarides, 2007). Each histone modification and their combinations are associated with different chromatin states, either transcriptionally active or silenced. For example, histone acetylation in general correlates to active euchromatin and thus active transcription (Roth, Denu and Allis, 2001). Regarding histone methylation, it was first described by Murray in calf thymus histones in 1964 (Murray, 1964). This modification correlates with either transcriptional activation (H3K4, H3K36, H4R3) or repression (H3K9, H3K27, H4K20) depending on the modified residue and,

in some cases (e.g. X chromosome inactivation), its methylation status (mono-, di- or tri-methylation) (Martin and Zhang, 2005). A large amount of research focuses on these modifications as well as the identification and study of the enzymes that catalyze them.

Histone H4 lysine 20 methylation

One such modification is the methylation of lysine 20 on histone H4 (H4K20), which residue can be mono-, di- or tri- methylated. It was first observed during the chemical sequencing of calf and pea histone H4 by DeLange (DeLange *et al.*, 1969), followed by a number of reports supporting its universal nature. These studies also revealed that there is a difference in the relative proportions of H4K20 methylation states from simple to complex eukaryotes as well as between plants and animals (Balakrishnan and Milavetz, 2010). The first studies on H4K20 methylation, where antibodies against the dimethylated form of H4K20 were used, showed that H4K20me2 inhibits H4K16 acetylation and vice versa (Nishioka *et al.*, 2002; Rice *et al.*, 2002); furthermore, it was shown that this modification is not correlated with gene activity (Fang *et al.*, 2002). Instead, H4K20me2 is involved in the repair of double strand DNA breaks by guiding DNA repair proteins (e.g. Crb2, 53BP1) to euchromatin (Sanders *et al.*, 2004; Botuyan *et al.*, 2006; Du, Nakamura and Russell, 2006). On the other hand, H4K20me3 is a histone mark correlating to transcriptional repression and is enriched at the corresponding heterochromatin, as well as is associated with constitutive heterochromatin such as at centromeres and telomeres (Schotta *et al.*, 2004; Gonzalo *et al.*, 2005; Regha *et al.*, 2007). H4K20me2 and H4K20me3 are catalyzed by the enzymes Suv420h1/2 (Yang *et al.*, 2008).

The monomethylated state of H4K20, catalyzed by the lysine histone methyltransferase Set8 (Fang *et al.*, 2002; Nishioka *et al.*, 2002), has a critical role in DNA replication, chromatin condensation, DNA repair and development, as it is going to be discussed below. Even though H4K20 methylation has been implicated in heterochromatin formation and gene silencing, several reports suggest that H4K20me1 may be involved in transcriptional activation by being enriched in active promoters or transcribed regions (Talasz *et al.*, 2005; Vakoc *et al.*, 2006). According to a high-resolution mapping of several histone lysine and arginine methylations (Barski *et al.*, 2007), H4K20me1 is localized downstream of the transcription start site of active genes as well as throughout the transcribed region and is positively correlated with active transcription. On the contrary, inactive genes are characterized by low or negligible levels of H4K20me1 in gene-body regions. H4K20me1 levels oscillate during cell cycle with being almost absent during G1 and S phases and being robustly elevated during G2 phase and mitosis (Rice *et al.*, 2002). It also serves as a substrate for the catalysis of H4K20me2/3 modifications by the enzymes Suv420h1/2. This histone modification can be removed by the PHD and Jumonji C (JmjC) domain-containing protein 8, PHF8, a demethylase which is recruited to promoters by its PHD domain and controls G1/S transition (Liu *et al.*, 2010).

Lysine histone methyltransferases and Set8

An important breakthrough in the identification of lysine histone methyltransferases (HMTs) resulted from studies of suppressors of position effect variegation (PEV) in *Drosophila* (Reuter and Spierer, 1992). These suppressors, such as the Su(var)3-9, the polycomb-group protein Enhancer of zeste and the trithorax-group Trithorax, contain an evolutionarily conserved sequence motif named SET domain (Jenuwein *et al.*, 1998). The SET domain of the human homolog of *Drosophila* Su(var)3-9 (Suv39h1) was found to be similar in sequence to previously identified SET domain-containing methyltransferases from plants (Klein and Houtz, 1995; Zheng *et al.*, 1998). Thus, the mammalian Suv39h1 and its fission yeast homolog Clr4 were the first SET domain-containing lysine HMTs, which methylate the histone H3 on lysine 9 (Rea *et al.*, 2000). Plenty of lysine HMTs have been identified and characterized since then. However, it should be noted that not every SET

domain-containing protein is a lysine HMT and not every lysine HMT has a SET domain. For instance, H3K76 is methylated by Dot1, a methyltransferase which doesn't contain a SET domain (Ng *et al.*, 2002).

Among the SET domain-containing lysine HMTs, the protein Set8 (alternatively PR-Set7, Setd8, Kmt5a) is the only known lysine HMT that catalyzes the monomethylation of H4K20 (Fang *et al.*, 2002; Nishioka *et al.*, 2002), as mentioned above. Set8 recognizes the sequence RHRK^{20}VLRDN within the N-terminus of histone H4 for its monomethylation activity (Yin *et al.*, 2005). Set8 consists of 352 amino acids in human and 350 aa in mouse (approximately 40kDa) (fig. 2). It is highly conserved among multicellular organisms and supposedly acts as a homodimer in nucleosomal-specific context using S-adenosyl methionine as template. Its SET domain lies at the C-terminus (215-341aa in the human homolog). Set8 also contains two PIP (PCNA-interacting protein) domains; PIP box 1 lies at the N-terminus (Huen *et al.*, 2008) and PIP box 2 is upstream the SET domain (Jørgensen *et al.*, 2007). In a GST pull down assay done in U2OS cells, it was shown that GST-tagged Set8 with mutations in PIP box 2 completely abrogated Set8-PCNA binding (Abbas *et al.*, 2010). Thus, Set8 interacts with PCNA through PIP box 2 whereas the function of PIP box 1 remains unknown. There is a speculation that it could contribute to strengthen Set8-PCNA binding (Huen *et al.*, 2008; Oda *et al.*, 2010); however, this box is dispensable for this interaction and the methyltransferase function of Set8 (Tardat *et al.*, 2007; Abbas *et al.*, 2010; Centore *et al.*, 2010; Oda *et al.*, 2010; Jørgensen *et al.*, 2011). Moreover, it has been shown that Set8 monomethylates lysine 248 of PCNA, therefore stabilizing PCNA by inhibiting poly-ubiquitination and significantly enhancing the interaction of PCNA with flap endonuclease FEN1 (Takawa *et al.*, 2012).

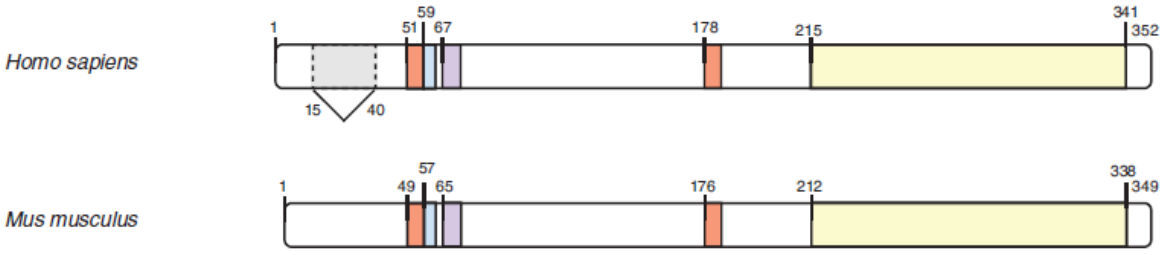

Fig. 2 Schematic representation of Set8 protein and its conserved domains. Red boxes represent the two PIP boxes whereas the yellow box represents the SET domain. Adapted from Brustel *et al.* (2011).

Set8 regulation during cell cycle and upon DNA damage

Set8 protein is tightly regulated by the cell cycle (Rice *et al.*, 2002) (fig. 3). Its protein levels are nearly absent during G1 and S phases; in addition, Northern blot analysis in HeLa cells showed that Set8 mRNA levels have the same pattern. Its expression reaches its peak during G2 phase and the beginning of mitosis (M phase). Set8 levels rapidly decline as cells exit mitosis. Proliferating cell nuclear antigen (PCNA) interacts with Set8 during S phase via its PIP box 2, as mentioned above. It has been shown in HeLa and 293T cells that this interaction results in the recruitment of Set8 to replication foci (Huen *et al.*, 2008). Since Set8 protein levels are almost undetectable during that phase despite its high mRNA levels, it means that Set8 must be rapidly degraded after its recruitment to replication foci. Indeed, several research reports show that the E3 ubiquitin ligase CRL4^{Cdt2} is responsible for the ubiquitination and subsequent targeting of chromatin-bound Set8 for degradation and that this interaction relies on PCNA binding (Abbas *et al.*, 2010; Centore *et al.*, 2010; Oda *et al.*, 2010; Jørgensen *et al.*, 2011).

Actually, in U2OS cells, the disruption of the CRL4-Cdt2-PCNA-Set8 degradation axis resulted in inhibition of cell proliferation, though without significantly disturbing the S phase progression (Abbas *et al.*, 2010, in contradiction to the studies of Tardat *et al.*, 2007 and Jørgensen *et al.*, 2011). In particular, stabilization of Set8 in cells led to an increase in

H4K20me2 and H4K20me3, chromatin decompaction, DNA damage, the activation of p53, its downstream target p21, some p53 pro-apoptotic genes such as *Fas*, *PUMA* and *PIG3* and the activation of G2 checkpoint pathways. This could explain the failure of the mitotic entry (G2 arrest) and, therefore the cell cycle completion, and the increased apoptosis. Furthermore, the CRL4/Cdt2/PCNA-mediated Set8 degradation during S phase is critical for preventing the repression of histone genes and many E2F1-related genes (e.g. *c-myc*, *cyclin E1*) during a normal cell cycle. The chromatin decompaction observed when Set8 was stabilized might have been the result of the loss of histone proteins, probably due to gene expression deregulation of histone genes through the accumulation of the heterochromatin mark H4K20me3 on their promoters. On the other hand, Centore *et al.* (2010) showed that stabilization of Set8 resulted in premature chromatin condensation due to aberrant accumulation of H4K20me1, proposing it as a histone mark important for chromatin compaction.

Prior to S phase, chromatin-unbound Set8 is ubiquitinated and targeted for proteasomal degradation by the E3 ubiquitin ligase SCF^{Skp2} (Yin *et al.*, 2008; Oda *et al.*, 2010). This E3 ubiquitin ligase also seems to interact with chromatin-unbound Set8 during S phase, in parallel with $CRL4^{Cdt2}$ which targets the chromatin-bound pool. Set8 is also a target of E3 ubiquitin ligase APC/C^{Cdh1} (Wu *et al.*, 2010). According to studies done on HeLa cells, Set8 is recognized and phosphorylated at serine 29 at the onset of mitosis by the Cdk1/cyclinB1 complex, a mitosis-specific complex. This phosphorylation stabilizes Set8 and inhibits its ubiquitination by APC/C^{Cdh1}. Furthermore, this phosphorylation causes the removal of Set8 from mitotic chromosomes and its relocation to the extrachromosomal space, following the global accumulation of H4K20me1 in G2. After the metaphase stage, Set8 is dephosphorylated by Cdc14 phosphatases, thus the ubiquitination by APC/C^{Cdh1} is permitted and Set8 is targeted for proteolysis. Recent studies revealed a new degradation mechanism of Set8 that specifically acts upon UV exposure (Wang *et al.*, 2015; Zheng *et al.*, 2016). In particular, UV irradiation triggers the phosphorylation of Set8 at serine 253 by casein kinase I (CKI) which subsequently results in its ubiquitination by the E3 ligase complex $SCF^{\beta\text{-}TRCP}$ and its targeting for proteolysis.

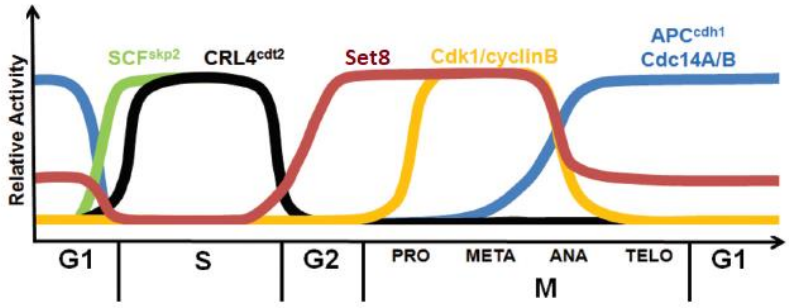

Fig. 3 Relative abundance of Set8 and activity of the various enzymes that regulate Set8 abundance during different cell cycle phases. Adapted from Wu and Rice (2011).

Set8 is also targeted to proteasomal degradation by $CRL4^{Cdt2}$ via PCNA interaction in case of DNA damage. Oda *et al.* (2010) proposed a model of Set8 regulation on DNA damage sites. Apart from DNA replication, PCNA has also a significant role in DNA damage repair. PCNA-Set8 interaction leads to the recruitment of Set8 at the DNA damage sites. The rapid recruitment of Set8 may depend also on the NHEJ Ku70 protein and is necessary for NHEJ-directed repair of double strand DNA breaks (DSBs) (Tuzon *et al.*, 2014). The resulting H4K20me1 histone mark is required for 53BP1 recruitment, which is a key regulator of DNA damage checkpoint response (Panier and Boulton, 2014). Tuzon *et al.* determined that H4K20me1 facilitates the recruitment of Suv420h1 which in turn catalyzes the dimethylation of H4K20, a necessary histone mark for 53BP1 binding. 53BP1 was previously reported to target H4K20me1/2 for binding via its tandem Tudor domains. After 53BP1 recruitment to

the damaged sites, Set8 is targeted for degradation by the E3 ubiquitin ligase $CRL4^{Cdt2}$. On the other hand, another research group showed that the recruitment of Set8 to and its role in promoting 53BP1's accumulation on DSBs can also take place independently of PCNA interaction (Dulev *et al.*, 2014). In that case, histone deacetylases (HDACs) seem to negatively regulate Set8's occupancy on DSBs. Collectively, it appeared that Set8 may have a role in DNA damage repair, particularly in non-homologous end-joining (NHEJ) pathway.

Regulation of p53 by Set8 was further studied by Shi *et al.* (2007). p53 is a transcriptional regulator that plays a central role in tumor suppression by regulating the cellular response to a variety of stresses. *In vitro* and *in vivo* experiments showed that monomethylation of p53 at lysine 382 strongly suppresses its function as transcriptional activator of highly responsive target genes (e.g. *p21*, *PUMA*), whereas expression of weak targets (e.g. *Bax*, *NOXA*) is not affected. p53K382me1 by Set8 inhibits the acetylation of the same residue, thus rendering p53 inert. It was shown that Set8 depletion increased the pro-apoptotic and checkpoint activation functions of p53 and that Set8 levels are reduced upon DNA damage. Taking these data into account, the researchers proposed a model where Set8 monomethylates p53 under normal conditions, thus suppressing it (fig. 4). Upon DNA damage, this inhibitory modification is reversed due to the degradation of Set8 and the stabilization of p53, either by its demethylation and subsequent acetylation at the same residue or by the up-methylation of p53K382me1 to me2/3 by an unknown methyltransferase (Shi *et al.*, 2007).

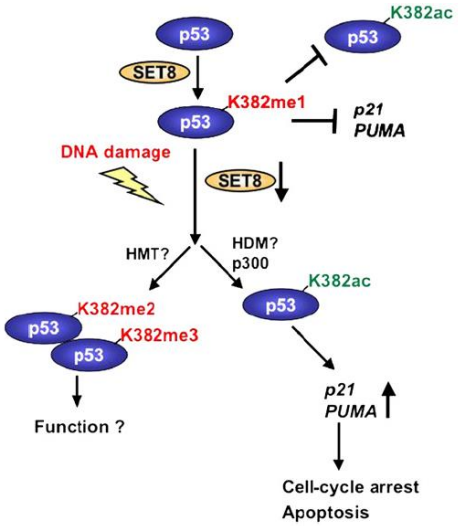

Fig. 4 Model for Set8 regulation of p53. Adapted from Shi *et al.* (2007).

The apoptotic pathway mediated by p53 is additionally regulated by Set8 through the methylation of Numb protein which promotes apoptosis in a p53-dependent manner. Specifically, its function is suppressed by Set8-mediated methylation of Numb on K158 and K163 residues. These modifications lead to the release of Numb from its interaction with p53, thus leading to increased p53 ubiquitination and degradation (Dhami *et al.*, 2013). Nevertheless, in a later investigation of Set8-mediated methylation of Numb, the researchers stated that more evidence is needed to support this claim (Weirich *et al.*, 2015).

Role of Set8 in DNA replication

In addition to the above functions of Set8, it has a significant role in DNA replication licensing. According to studies on mammalian cells, the onset of replication licensing coincides with an increase of H4K20me1 at the replication origins (Tardat *et al.*, 2010). The abolition of PCNA-dependent degradation of Set8 caused a G2 arrest in cells and replication re-licensing. Furthermore, Set8 methyltransferase activity was artificially directed to a locus which normally does not constitute a replication origin. This resulted in the appearance of

H4K20me1 and the loading of pre-RC proteins on the locus, generating the conditions for a replication origin. It has been also discovered that Set8 function in replication licensing depends on Suv420h1/2 activity (Beck *et al.*, 2012). Either Set8 stabilization or Cdt2 knockdown caused increased global levels of H4K20me3, making H4K20me3 the key effector of the re-replication pathway. The cellular phenotype of Set8 stabilization (cell cycle progression, DNA damage accumulation) seemed to be a consequence of Suv420h. Again, by using the artificially-directed recruitment of Set8 at a locus, loading of pre-RC proteins coincided with increased H4K20me3, whereas Suv420h1 depletion had the opposite result.

Beside its function in replication licensing, Set8 may regulate DNA replication by managing gene expression. As mentioned above, this lysine HMT is involved in the repression of E2F-related genes, which are required for DNA synthesis, and histone genes supposedly through the conversion of H4K20me1 into H4K20me3 (Abbas *et al.*, 2010). Alternatively, H4K20me1 seems to serve as a docking site for the transcriptional repressor L3MBTL1 (lethal 3-malignant brain tumor like protein 1) in the vicinity of the transcription start sites of E2F-target genes (Trojer *et al.*, 2007; Kalakonda *et al.*, 2008; Sims and Rice, 2008). L3MBTL1 association with chromatin reflects the progressive accumulation of H4K20me1 during cell cycle (Kalakonda *et al.*, 2008). Set8, and not Suv420h1, enhances L3MBTL1 repressive function and, also, knock down of Set8 decreases its chromatin association. During G1/S transition, L3MBTL1 repressive mechanism is suppressed by the recruitment of H4K20 demethylase PHF8 in combination with E2F factors and the transcriptional co-regulator HCF1 (Liu *et al.*, 2010). Collectively, the cell cycle oscillation of Set8 ensures that origin licensing occurs due to mitotic Set8 activity, but further progression is blocked by the repression of genes important for DNA synthesis. Afterwards, Set8 degradation during S phase and/or PHF8 activity results in the inactivation of origin licensing and the proper DNA synthesis, guaranteeing that replication licensing and activation are two separate events in the cell cycle.

Role of Set8 in chromatin compaction

There is compelling evidence that Set8 has an important role in chromatin compaction during G2/M transition (Oda *et al.*, 2009). As mentioned above, a degradation-resistant Set8 mutant expressed at levels high enough to generate H4K20me1 on total histones during S phase causes premature chromatin compaction, instead of DNA re-replication, accompanied by a checkpoint-mediated G2 arrest (Centore *et al.*, 2010; Jørgensen *et al.*, 2011). These data suggest that the high levels of H4K20me1 normally observed during G2/M transition may play a role in the conversion of relaxed interphase chromatin into condensed mitotic chromosomes (Brustel *et al.*, 2011). In addition, Set8 degradation via $CRL4^{Cdt2}$ ubiquitination during S phase could avail the restriction of chromatin compaction until DNA replication is completed. The way in which Set8 and H4K20me1 promote chromatin condensation remains to be elucidated. One hypothesis is that the monomethylated form of H4K20 is subsequently up-methylated to H4K20me2 and H4K20me3, with the latter being an important mark for heterochromatin.

Alternatively, H4K20me1 may be involved in high-order chromatin structure through inter-nucleosomal contacts e.g. with histone H2A of the adjacent nucleosome (Dorigo *et al.*, 2003). Another possibility is that H4K20me1 constitutes a binding site for chromatin condensation-related proteins, such as HDACs (Scharf *et al.*, 2009), condensin II subunits or condensin protein SMC4 (Liu *et al.*, 2010). Therefore, one can assume that Set8-mediated chromatin compaction contributes to the configuration of replication origins and vice versa.

Complementary to the above, Set8 has been shown to play a role at imprinting control regions (ICRs) in the mouse (Pannetier *et al.*, 2008). It was demonstrated that Set8 catalyzes the monomethylation of H4K20 on the ICRs of both alleles of the imprinted genes. Then, Suv420h is recruited to the DNA-methylated allele to promote trimethylation of H4K20. Furthermore, Set8 is implicated in the regulation of RNA polymerase II pausing

(Kapoor-Vazirani and Vertino, 2014). RNA pol II promoter-proximal pausing plays a critical role in post-initiation transcriptional regulation and is controlled by H4K16 acetylation and H4K20 trimethylation, which signifies release of paused RNA pol II or enforces pausing, respectively. Since Set8-mediated H4K20me1 inhibits MSL-mediated H4K16ac and also can be up-methylated to H4K20me3 by Suv420h enzymes, the local balance between H4K20me3 and H4K20me1/H4K16ac levels regulates pausing and, therefore, the transcriptional outcome. A recent work on liver-specific Set8-deficient mice revealed that decreased H4K20me1 levels resulted in reduced RNA pol II release from promoter-proximal pausing sites. This decreased RNA pol II release affected more the genes that regulate glucose and fatty acid metabolism, eventually leading to energy starvation, metabolic reprogramming, ROS overproduction, oxidative DNA damage and senescence (Nikolaou *et al.*, 2017). Finally, the stabilization of Set8 by CRL1/FBXO11-mediated degradation of Cdt2 results in the decrease of phospho-Smad2 and N-cadherin in response to TGF-β, as well as promotes cellular migration (Abbas *et al.*, 2013), a concept previously studied by Yang *et al.*, (2011).

Set8 loss-of-function phenotype

Loss-of-function studies in *Drosophila* and mouse models revealed that Set8 has an evolutionarily conserved role in cell cycle progression. This is consistent with its conservation during animal evolution and confirms the already determined importance of Set8 as well as the significance of its oscillation during cell cycle. Inactivation of *Drosophila* Set8 (dSet8) resulted in reduction of both S phase and mitotic indices in third instar larval brain cells and a progression delay through early mitosis, as well as lethality at the larval-to-pupal transition (probably at late second to third instar larval stage) (Nishioka *et al.*, 2002; Karachentsev *et al.*, 2005; Sakaguchi and Steward, 2007). The deficiency of Set8 had an extreme effect on mouse development, leading to improper transition from S to M phase and early embryonic lethality (Oda *et al.*, 2009). Since no Set8 null embryos were found having eight or more cells at 2.5-dpc, this phenotype indicated that Set8 is required for preimplantation development, most likely during the transition from the four-cell stage to the eight-cell stage.

Studies performed in mammalian cell lines (e.g. U2OS, HEK 293 cells) showed that Set8 depletion induces G2 arrest accompanied by improper high-order chromatin structures (Tardat *et al.*, 2007; Houston *et al.*, 2008; Huen *et al.*, 2008); similar studies in HeLa cells showed only a slight delay in cell cycle progression (Tardat *et al.*, 2007; Pesavento *et al.*, 2008). This G2 arrest is mainly caused by replicative stress in the preceding S phase. Specifically, this includes alteration in replication fork velocity, decrease in origin firing and a potent induction of DNA damage along with the activation of the DNA damage checkpoint (Jørgensen *et al.*, 2007; Tardat *et al.*, 2007). Conditionally Set8-knocked out ES cells at the G1/S transition which were then released into S phase, progressed normally through the first S and M phases with the defects occurring at the beginning of the subsequent S phase (Oda *et al.*, 2009). The replicative stress due to Set8 depletion could be the consequence of cells progressing through one cell division without Set8-mediated H4K20 monomethylation.

Recently, it was demonstrated that Set8 is downregulated in oncogene-induced and replicative senescent IMR-90 cells (Tanaka *et al.*, 2017). Conversely, loss of Set8 in IMR-90 cells by siRNA induced senescence. Further analysis of Set8 function in cellular senescence revealed its involvement in the transcriptional repression of ribosomal protein genes, *rRNA* genes and senescence-associated genes such as $p15^{INK4B}$, $p16^{INK4A}$, $p21^{CDKN1A}$ and *interleukin-1*. Also, it was found by ChIP-seq in adult human dermal fibroblasts (NHDF-Ad cells) that these genes are enriched in H4K20 monomethylation. Depletion of Set8 resulted in characteristic senescence-associated metabolic remodeling, such as activation of ribosome biogenesis and nucleolar stress during senescence. Remodeling of the energy metabolism balance was also observed upon Set8 depletion. Specifically, mitochondrial oxygen consumption rate, mitochondrial mass, membrane potential and intracellular levels of reactive oxygen species were increased. This increased OXPHOS activity depended on

Retinoblastoma protein (RB). Thus, under normal conditions Set8 acts to suppress nucleolar and mitochondrial activities to prevent cellular senescence.

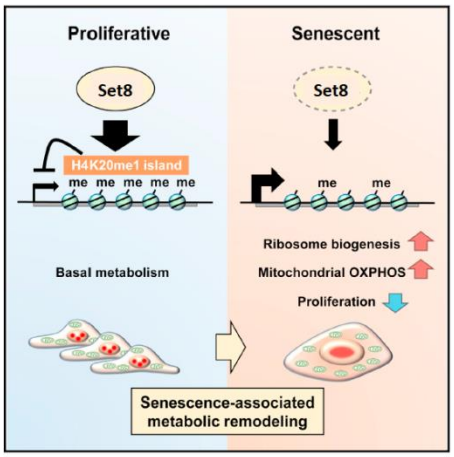

Fig. 5 Schematic role of Set8 in proliferative and senescent cells. Adapted from Tanaka *et al.* (2017).

In a quite recent work done by Nikolaou *et al.* (2015), the researchers focused on the role of Set8 in liver using mice carrying a hepatocyte-specific depletion in embryonic or adult life. In the first case, *Set8^{loxp}/AlfpCre* mice did not even reach birth and further investigation showed impaired liver organogenesis. Inactivation of Set8 in postnatal hepatocytes was achieved by crossing the *Set8^{loxp}* mice with *AlbCre* mice. Complete loss of Set8 was observed in postnatal day 20. No phenotype was observed in Set8-deficient liver of young mice (P45); however, at 4 months mouse livers displayed small regenerative foci with areas containing enlarged hepatocytes with enlarged nuclei undergoing cell death. These cells were not apoptotic as shown by TUNEL assay and electron microscopy, and were subjected to massive DNA damage and prolonged G2 arrest. H4K20me1 was completely lost in the enlarged hepatocytes. This profound phenotype is consistent with the previous observation that Set8-depleted cells are able to progress through one cell division without Set8-mediated H4K20me1 but display a variety of defects at the beginning of the next cell cycle (Oda *et al.*, 2009). Eventually, *Set8^{loxp}/AlbCre* mice develop late-onset, spontaneous hepatocellular carcinoma; the model proposed by the researchers is illustrated in fig. 6.

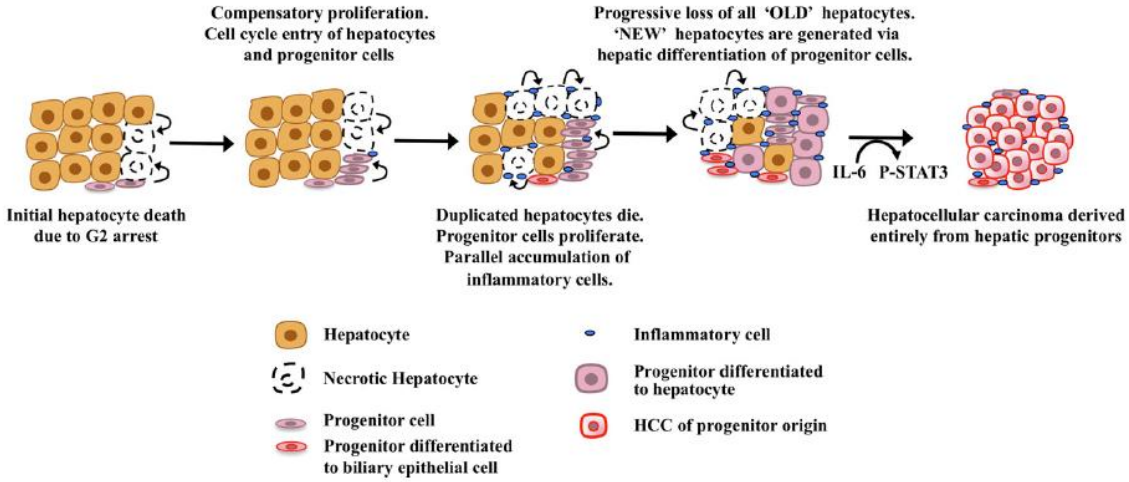

Fig. 6 Schematic presentation of the temporal events leading to spontaneous hepatocellular carcinoma development in *Set8^{loxp}/AlbCre* mice. Adapted from Nikolaou *et al.* (2015).

Purpose of the study

The objective of the present study was to further examine the necrotic phenotype of Set8 knock out hepatocytes. For the investigation of Set8 protein in a tissue-specific manner, *Set8^{loxp}/AlbCre* mice were used, in which *Cre* gene expression is regulated by *Albumin* promoter restricting its expression and activity in hepatocytes. Since hepatocytes are mainly quiescent, it takes time to complete one cell cycle and begin the next. As one and a half cell cycle is necessary for the emergence of cell defects, these mice develop hepatic cancer spontaneously approximately after 4 months (Nikolaou *et al.*, 2015). In order to reduce the time needed to examine the defected cells, *Set8^{loxp}/AlbCre* mice were injected with TCPOBOP, a mitogen that induces hepatocyte proliferation (Columbano and Ledda-Columbano, 2003). Specifically, liver sections of TCPOBOP-treated *Set8^{loxp}/AlbCre* mice were stained for different cytoplasmic and nuclear components using immunofluorescence in order to investigate the enlarged hepatocytes and gain insight into the potential mechanism that underlies this abnormal phenotype. In addition, tissue from TCPOBOP-treated *Set8^{loxp}/AlbCre* liver was fixed and processed for transmission electron microscopy to investigate in depth and greater magnification the morphology of the enlarged nuclei to gather information which could lead to the responsible mechanism. The above experiments could provide information and preliminary evidence on the role of Set8 protein and the corresponding histone methylation (H4K20me1) in the cellular and nuclear architecture.

Materials and Methods

Animal model and histological analysis.

The animals used in these experiments were *Set8^{loxp}/AlbCre* mice and wild type mice of the same genetic background (C57Bl6/CBA) as control. A stock solution of 10mg/mL TCPOBOP in DMSO was stored at 4°C, from which a 3% dilution in corn oil was prepared just before the treatment. *Set8^{loxp}/AlbCre* mice were injected with TCPOBOP in a concentration of 3μg per gr of mouse body weight. The isolation of tissue was performed after 24h.

For immunohistochemistry with fluorescent secondary antibodies, isolated liver tissues were embedded in O.C.T. (Optimal Cutting Temperature compound, VWR) without pre-fixation. The samples were frozen in liquid nitrogen and then stored at -80°C. The frozen tissues were cryosectioned (5μm sections) and fixed with 4% formaldehyde. Then, the cryosections were incubated with 0.2% Triton X-100 for cell membrane permeabilization, blocked with 5% BSA and 0.2% Triton X-100 and incubated with the primary antibodies overnight at 4°C. After washing, they were incubated with the secondary fluorescent antibodies and mounted with ProLong™ Gold antifade reagent with DAPI (Invitrogen). Visualization of immunostained tissue sections was done with a Leica TCS SP8 confocal microscope.

For electron microscopy, mice were perfused with a fixative solution containing 4% paraformaldehyde, 0.2% picric acid and 0.05% glutaraldehyde. Subsequently, isolated liver tissues were post-fixed with 2% osmium tetroxide for 1.5h. After 1.5h of 1% uranyl acetate treatment, the samples were dehydrated with ethanol series and incubated in 100% propylene oxide for 20min. Finally, the samples were embedded in resin. Sections of 70μm on copper grids were observed at 80kV with a JEOL JM2100 transmission electron microscope.

Antibodies

The primary antibodies used in these experiments were the following: Cell Signaling Technology: anti-LaminA/C (#4777), Abcam: anti-histone H4 mono methyl K20 (ab9051), anti-histone H4 tri methyl K20 (ab9053), anti-nucleolin (ab22758), Santa Cruz Biotechnology: anti-HNF4 (sc-8987), Bethyl laboratories: anti-albumin (A90-234), Invitrogen: Alexa Fluor 488 Phalloidin (A12379). As secondary antibodies, Alexa Fluor secondary antibodies (Invitrogen) were used.

Results

DAPI-stained nuclei from liver sections of *Set8^{loxp}/AlbCre* mice (post-natal day 45 - P45) were closely examined and compared to wild type (wt) by confocal microscopy. Large regions without DAPI staining were observed, thus showing absence of DNA and indicating the existence of 'holes' within the nuclei (fig. 1 – dotted arrows). These 'holes' could be considered as vacuoles. Evidence from different approaches is needed to clarify and confirm the manifestation of vesicle-like structures rather than just foldings of the nuclear envelope. Nuclear engulfments were visible as well (fig. 1 – solid arrows). The enlargement of Set8KO hepatocytes observed by Nikolaou *et al.* (2015) was also evident.

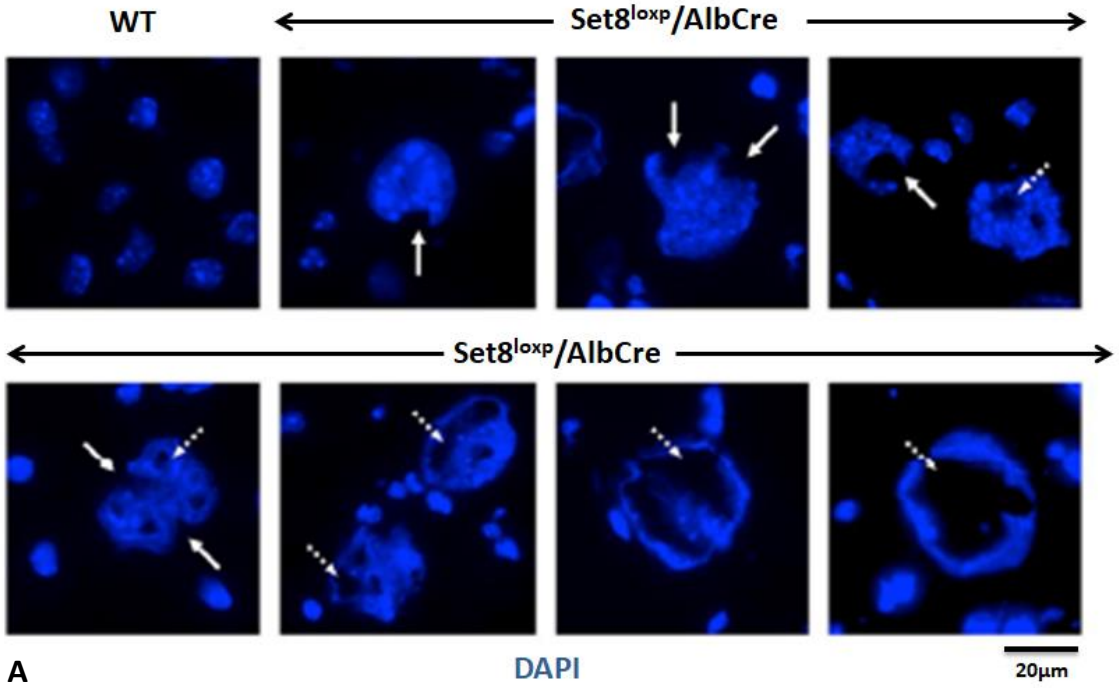

Fig. 1
DAPI-stained nuclei from liver cryosections of wt and *Set8^{loxp}/AlbCre* mice (P45). Nuclear engulfments are indicated by solid arrows and vacuoles by dotted arrows.

Hence, it was plausible to examine whether these vacuoles had derived from invagination of the nuclear membrane. Fluorescent antibody staining against the lamina component LaminB1 revealed that the regions lacking DAPI staining were surrounded by nuclear membrane (fig. 2). In addition, alterations of nuclear lamina structure were observed in Set8KO nuclei. Double staining of lamina component LaminA/C and monomethylation of H4K20 (H4K20me1) with fluorescent antibodies showed lack of H4K20me1 in Set8KO hepatocyte nuclei, as expected (fig. 3A). On the contrary, the pattern of the trimethylation of H4K20 (H4K20me3) was not altered, as depicted in fig. 3B; however, it appears that H4K20me3 staining pattern overlaps with LaminA/C staining in wt nuclei whereas this overlapping seems to disappear in Set8KO nuclei. The putative lamina/H4K20me3 overlapping could lead to significant progress towards the understanding of the underlying mechanism; nevertheless, more evidence is needed to support this hypothesis.

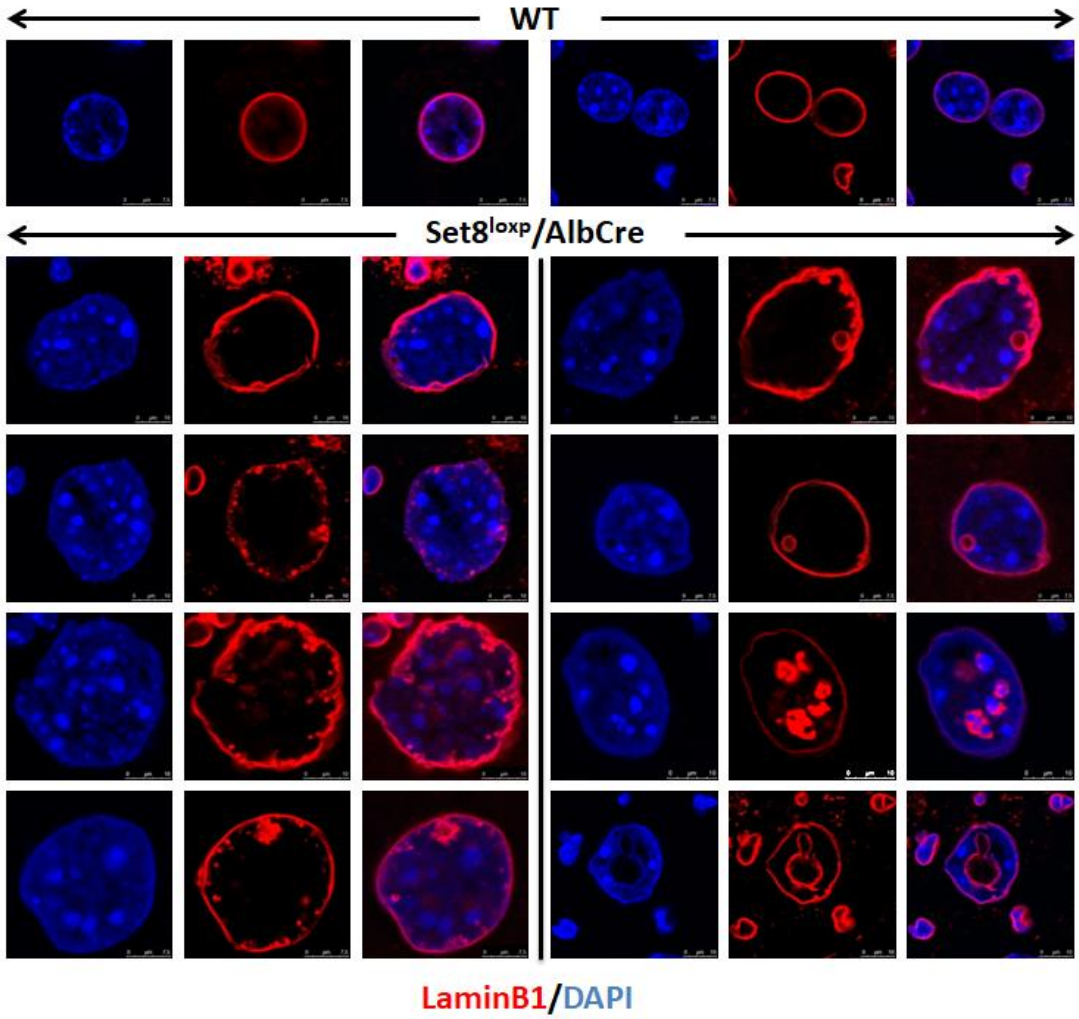

Fig. 2

LaminB1 staining in hepatocytes of *Set8^{loxp}/AlbCre* mice (P45) revealed structural alterations of nuclear lamina compared to wt. LaminB1 staining is fuzzy and surrounds the vacuoles.

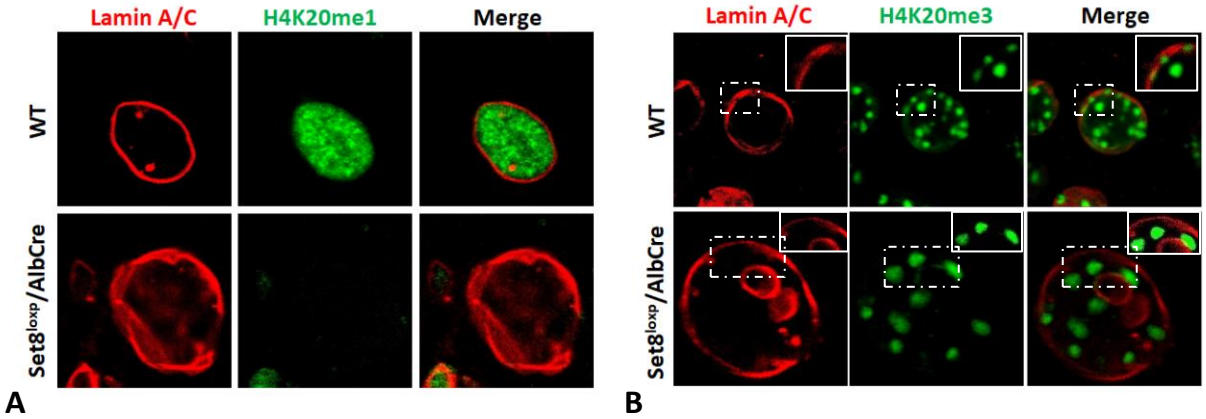

Fig. 3

A H4K20me1 is absent in hepatocyte nuclei of *Set8^{loxp}/AlbCre* mice (P45).

B H4K20me3 is still present in hepatocyte nuclei of *Set8^{loxp}/AlbCre* mice (P45); however, in wt liver sections LaminA/C and H4K20me3 staining seem to overlap but this overlapping is not visible in *Set8^{loxp}/AlbCre* liver sections. Emphasis of a specific region is displayed at the upper right corner of each image.

Additional stainings were performed in order to try to determine the content of these vacuoles. Use of HNF4α (transcription factor) and nucleolin (nucleolus marker) antibodies showed that these proteins are excluded from the vacuoles (fig. 4). Furthermore, staining of Albumin and F actin which are cytoplasmic proteins indicated that they are present in these structures. In conclusion, Set8 depletion from hepatocytes results in invaginations of the nuclear envelope presumably creating vacuoles that internalize cytoplasmic components and exclude nuclear proteins.

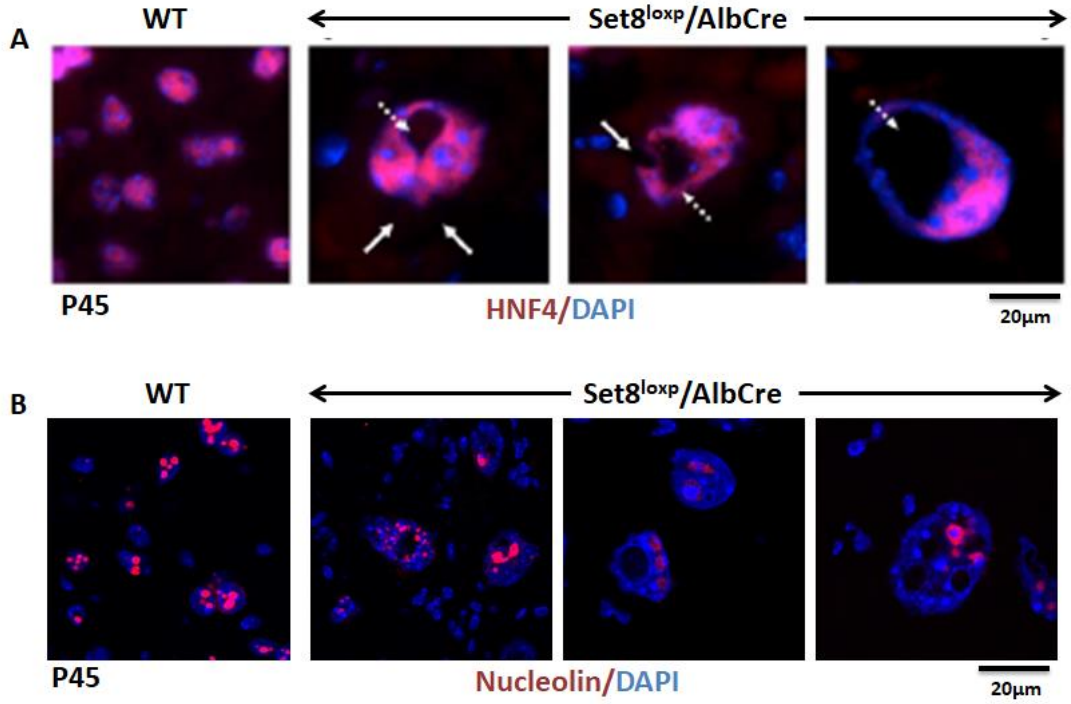

Fig. 4

A Exclusion of HNF4α protein from the nuclear vacuoles of *Set8^{loxp}/AlbCre* mice (P45).
B Exclusion of nucleolin from the nuclear vacuoles of *Set8^{loxp}/AlbCre* mice (P45).

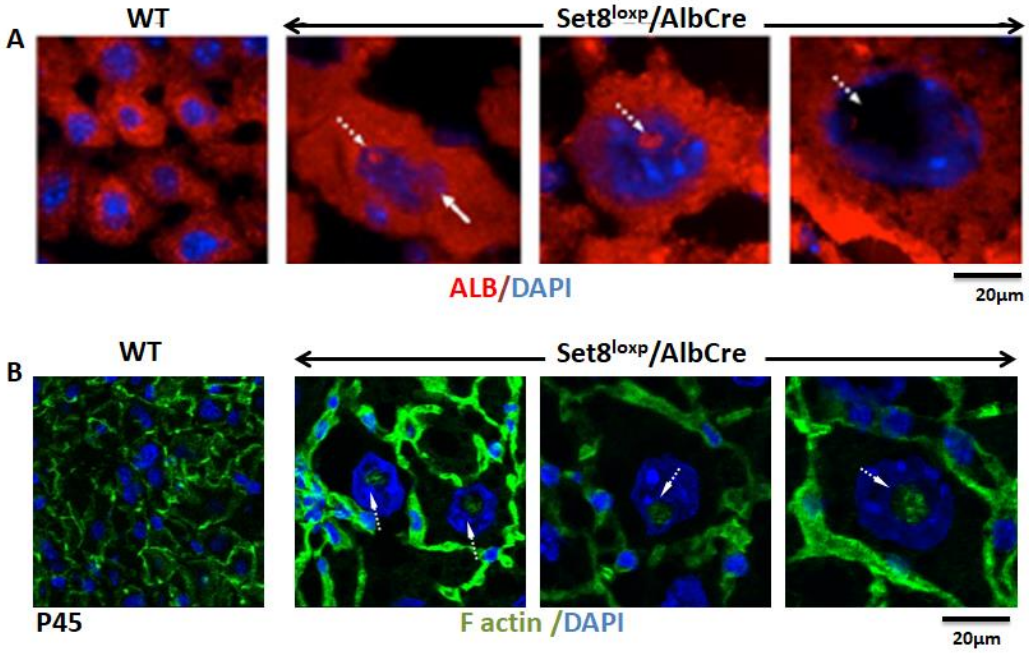

Fig. 5

A Albumin staining depicting its internalization in the nuclear vacuoles of *Set8^{loxp}/AlbCre* mice (P45).
B Phalloidin staining indicating the existence of F actin in the vacuoles.

To study the morphological changes of the nuclear lamina and the nuclear membrane invaginations in greater detail, liver sections were observed using transmission electron microscopy (TEM). Compared to wild type, enlargement of Set8KO hepatocyte nuclei and alteration of lamina structure are clearly depicted in TEM images, including nuclei in different stages of vacuolation (fig. 6). Heterochromatin seemed to be dissociated from the nuclear lamina and heterochromatin foci appeared to be disintegrated compared to wild type. These nuclei contained vacuoles of seemingly different composition either when compared among the different nuclei or within the same nucleus.

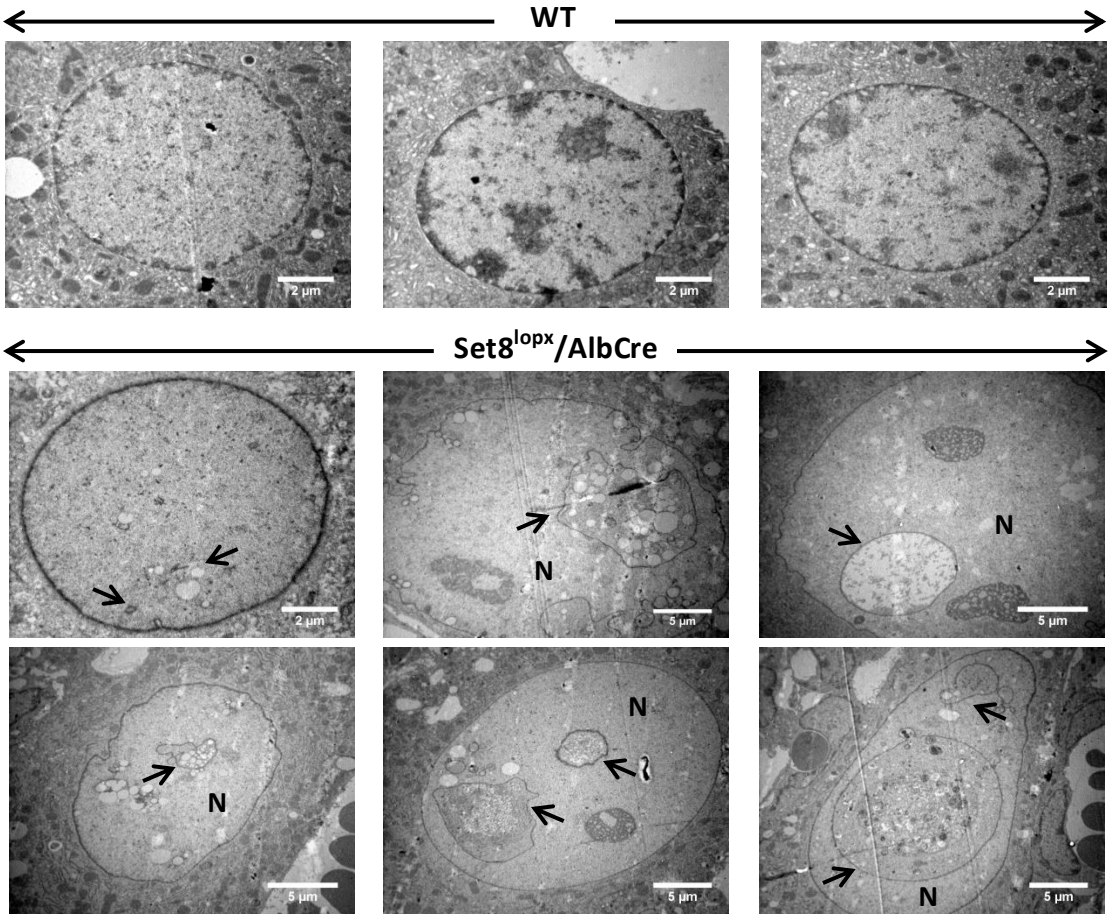

Fig. 6
Transmission electron microscope images of wt and Set8KO liver showing the difference in nuclear size and the existence of vacuoles (with difference in size, shape and content) in Set8KO nuclei. The first Set8KO nucleus seems to be in an early stage of the expected phenotype since its size is slightly larger than the wt and there are very few and small intranuclear vacuoles. N: nucleus, arrow: vacuole.

Taking into account all of the above, depletion of Set8 in hepatocytes leads to enlargement of the nucleus, invaginations of the lamina and development of intranuclear vacuoles in which cytoplasmic components are present and nuclear proteins are excluded.

Discussion

Traditionally, the genetic functions of the genome are to store, propagate, transmit and express the genetic information which is encoded in the DNA sequence. These functions rely on and are regulated by the level of chromatin compaction and its global organization; this includes intra- and interchromosomal interactions that result in higher-order chromatin structures (Bickmore, 2013; Cavalli and Misteli, 2013). The genome also shapes and manages the architectural and functional apparatus of the cell by gene expression. A great variety of proteins, such as remodeling complexes, transcription factors and the DNA repair machinery, are recruited to chromatin and their combination controls gene expression programs.

On the other hand, the genome also constitutes a major structural entity of the nucleus and affects cellular structure and function based on its physical properties. For example, the genome exerts significant mechanical forces within the nucleus and also towards the cytoplasm. This is attributed to the inherent motion of the chromatin fiber (Gorski, Dundr and Misteli, 2006; Pederson, King and Marko, 2015), the chromatin reorganization events and the dynamic interactions of intra- or interchromosomal domains. The genome also experiences mechanical forces deriving from the cytoplasm (Swift and Dischер, 2014; Maizels and Gerlitz, 2016). These forces, either from within the nucleus or the cytoplasm, are propagated either passively at the contact sites of chromatin with the nuclear envelope or actively through the linker of nucleoskeleton and cytoskeleton (LINC) protein complex. The LINC protein complex comprises a bridge extending across the nuclear envelope that physically connects the genome with the cytoskeleton (Tapley and Starr, 2013).

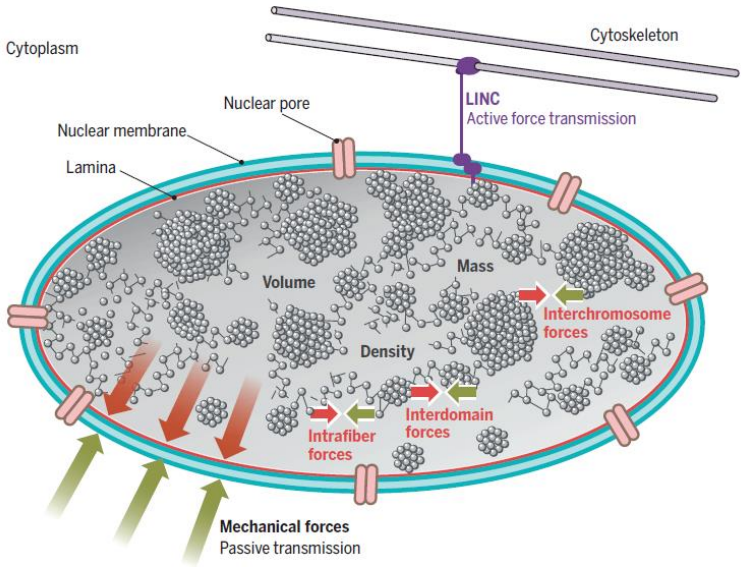

Fig. 1 The genome as a physical entity. Adapted from Bustin and Mistelli (2016).

Taken into account that a small fraction of the genome is transcribed in protein-coding or non-coding RNAs, it is plausible to anticipate that these non-transcribed genomic regions are not just remnants of evolutionary events but may also serve to strengthen the genome as a structural entity regulating the nuclear architecture and function. Therefore, the genome possesses non-genetic functions that rely on its physical properties rather than the encoded genetic information for their integration. These are present in a number of biological processes that include nuclear assembly, response to mechanical forces, cell migration, cell cycle progression, cellular signaling, and physiological functions such as vision in nocturnal animals (Bustin and Misteli, 2016).

Regarding the function of genome as a determinant of nuclear structure, it is conspicuous based on studies of the post-mitotic nuclear envelope reassembly (Güttinger, Laurell and Kutay, 2009). During mitosis, the nuclear envelope disassembles and its fragments are either spread over the dividing cell or integrated by the endoplasmic reticulum (ER). The reassembly of nucleus occurs during telophase and chromatin avails as a physical scaffold assisting the fusion of the fragments. Capture of nuclear membrane fragments involves several proteins of the inner nuclear membrane (INM) and can be achieved either directly by the genomic scaffold or via linker proteins. The reassembly occurs independently of the DNA sequence or the source of genetic material used. The genome also plays a significant role during the formation of nuclear pore complexes (NPCs) as well as the assembly of nuclear lamina.

In addition, the genome supports the determination of nuclear size. Although one mechanism appears to be the sensing of cytoplasmic/nuclear ratio of soluble factors (Jevtic *et al.*, 2014), evidence from different sources establishes the global genome architecture and especially chromatin compaction as regulator of nuclear size. For example, the linker histone H1 family is implicated in the chromatin compaction in eukaryotic cells (Hergeth and Schneider, 2015). *Tetrahymena* comprises a large macronucleus and a small micronucleus, each with a distinct isoform of histone H1. Depletion of each isoform results in nuclear enlargement of -only- the respective nucleus, supporting the above hypothesis. Also, knock down of condensins (proteins involved in chromosome condensation during mitosis) by RNA interference (RNAi) led to nuclear size increase in mouse embryonic stem cells and human cells (Bell and Straight, 2015). Collectively, these studies imply a significant role of chromatin compaction in nuclear size regulation via non-genetic mechanisms.

Targeting of condensed heterochromatin to the nuclear periphery is considered to promote or be the aftermath of gene silencing (Bickmore and van Steensel, 2013). Still, several lines of evidence suggest that the accumulation of heterochromatin to the nuclear periphery may also increase the structural durability of the nucleus and its ability to endure physical stresses such as mechanical forces, such as in migrating cells (Gerlitz and Bustin, 2011; Furusawa *et al.*, 2015). First observations of chromatin having a significant role in nuclear robustness derive from studies in the yeast *Schizosaccharomyces pombe* (Bustin and Misteli, 2016). The condensed centromeric chromatin is positioned opposite the microtubule organizing center (MTOC), which is subjected to the strongest mechanical forces from the cytoplasm. In addition, some nuclear proteins tethering chromatin to the INM, when mutated, decrease the nuclear resistance to mechanical forces originated from microtubules.

In vertebrate cells, the nuclear lamina has a role in supporting the nuclear membrane, thus providing mechanical sturdiness to the nucleus. Simultaneous depletion of Prdm3 and Prdm16 by RNAi in mouse embryonic fibroblasts (MEFs) led to disintegration of heterochromatin foci and global heterochromatin compaction, as well as to the disruption of nuclear lamina, invaginations of the nuclear envelope and changes in nuclear shape (Pinheiro *et al.*, 2012). Prdm3 and Prdm16 are lysine methyltransferases specific for the monomethylation of the lysine 9 of histone H3 (H3K9me1). Similar nuclear defects were observed in a HMGN5-overexpressing system (Furusawa *et al.*, 2015); HMGN5 is an architectural chromatin protein that promotes chromatin decompaction by interfering with the function of histone H1. Similar evidence emerged from analysis of transgenic mice overexpressing HMGN5 or lacking LaminA/C (Nikolova *et al.*, 2004; Furusawa *et al.*, 2015).

Taken together, it seems that chromatin decondensation changes the mechanical properties of the nucleus similarly to cells with defective lamina components. It also appears that an interplay exists between chromatin and nuclear lamina in determining nuclear robustness, since in *Lmna*$^{-/-}$ cells (which lack the nuclear proteins lamin A and C and have impaired lamina) chromatin decompaction aggravates the nuclear sturdiness (Furusawa *et al.*, 2015). In conclusion, the non-genetic functions of the genome affect several cellular

processes that are closely linked to mitosis, such as post-mitotic nuclear reassembly and chromatin compaction as well as chromatin-initiated spindle checkpoint signaling allowing the speculation that these genomic functions aim to ensure proper cell cycle progression.

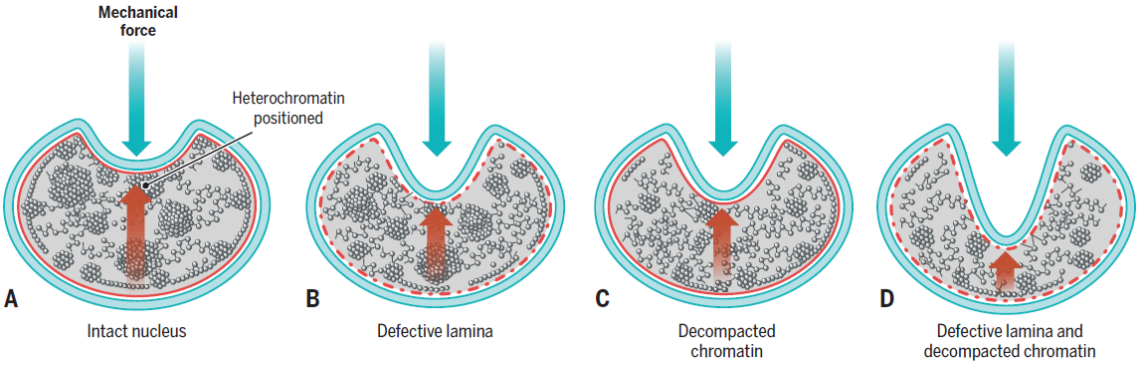

Fig. 2 Genome compactness enhances the sturdiness of the nucleus. Adapted from Bustin and Mistelli (2016).

According to the results, Set8 protein seems to be involved in the structural integrity of the nucleus. Collectively, the abolishment of its function in the hepatocytes of *Set8*^{loxp}*/Alb-Cre* mice leads to a significant increase of their nuclear size compared to the wild type. Large membrane invaginations or vacuoles are developed in the majority of these nuclei. Their membrane is derived from the nuclear envelope, since they consist of two lipid bilayers and are characterized by the presence of nuclear lamina. Cytoplasmic components are present (e.g. F actin, albumin) whereas nuclear components are excluded (e.g. HNF4α, nucleolin) from these structures. Examination of the enlarged nuclei with electron microscopy gives more information about the development of these nuclear folds. However, more investigation is needed in order to determine whether these structures are indeed some kind of vesicles.

So, Set8 loss-of-function seems to induce cell death by necrosis. This necrosis, however, is accompanied by nuclear envelope invagination, a phenomenon which could be called “endonucleosis” in correspondence with the cellular “endocytosis”. Endonucleosis appears to be a characteristic of cell death, representing a final step that destroys the nucleus before the destruction of the whole cell. However, it could also represent a mechanism of specialized necrosis occurring only in cases when cells experience prolonged G2 arrest, being unable to progress towards mitosis (as it happens in hepatocytes of *Set8*^{loxp}*/Alb-Cre* mice). The nuclear lamina gets disassembled when cells progress from G2 phase to prophase. Hypothetically, the nuclear lamina may become partially disassembled (yet not completely eliminated) when cells remain at G2 phase for an extended period of time, thereafter becoming more elastic and permitting intrusions from the cytoplasm. Thus, in cells unable to complete the cell cycle the nuclei may gradually become enlarged and after some time initiate endonucleosis.

This hypothesis complies with the above results and the notion that the chromatin state regulates the mechanical properties of the nucleus along with the nuclear lamina as well as regulates the integrity of the lamina itself. Moreover, another hypothesis could be that endonucleosis is a mechanism of necrosis when the mechanical forces that keep nuclear lamina stiff are damaged. Such forces can be lamina-bound heterochromatin. In case of *Set8*^{loxp}*/Alb-Cre* mice, H4K20me3-containing heterochromatin could possess that role since it is the up-methylated form of the Set8-mediated H4K20me1. H4K20me3 may be disrupted in those cells, leading to the destabilization of lamina. However, the hypotheses mentioned are not necessarily mutually exclusive. Regarding the role of Set8 protein in this phenomenon, endonucleosis could be Set8-specific implying that Set8 may participate in

nuclear membrane dynamics by methylating or interacting in general with lamina components.

To examine these hypotheses, the first step should be to determine the most comfortable but reliable model to study endonucleosis. Apart from liver of *Set8*^{loxp}/*Alb-Cre* mice, some experiments are more easily performed in cultured cells. Therefore, MEFs from *Set8*^{loxp} mice could be transfected with a vector carrying the Cre-ERT system in order to conditionally knock out Set8 protein with the use of tamoxifen. Since MEFs are viable for a limited number of passages and are not easily transfected, a similar approach could be to isolate primary hepatocytes from *Set8*^{loxp}/*Alb-Cre* mice of young age, so *Set8* gene will not be knocked out yet and the cells would be able to proliferate and expand before the G2 arrest and initiation of endonucleosis.

However, considering that Set8 depletion leads eventually to cell death, it may be difficult to perform experiments on Set8 knock out (Set8KO) cells. Hence, knocking down of Set8 by RNAi could be a better approach. Cell lines such as NIH3T3 cells (immortalized MEFs) could be transfected with Set8 siRNA and the conditions needed for the phenomenon to appear should be determined. In parallel, efforts should be made to simulate the conditions for endonucleosis in cells, including prolonged G2 arrest and DNA damage. Prolonged G2 arrest can be achieved using nocodazole or inhibitors of key regulators of G2 phase, such as Cdk1. DNA damage can be easily attained by exposing cells to UV irradiation or treat them with hydrogen peroxide. This can give insight to whether endonucleosis is a Set8-specific event; study of different animal models whose cells are also subjected to similar conditi on could be enlightening regarding this question.

Once the appropriate model is established for the study of endonucleosis, the appropriate stainings (e.g. nuclear lamina, cytoplasmic and other nuclear components) should be done in order to confirm the existence of endonucleosis. Additionally, the interaction of Set8 with lamina components (e.g. LaminA/C, LaminB1, LaminB receptor - LBR) and the interaction of lamina with chromatin (e.g. H4K20me1&3) should be investigated through different approaches. Indications can be collected by simultaneous staining of these proteins and histone modifications. Proximity Ligation Assay (PLA) is an efficient method to visualize these potential interactions and their disruption in the examined cells (either Set8-depleted cells or cells with simulated conditions for endonucleosis). Pull-down assay between Set8 and lamina components can also determine the existence of such interactions. Potential function of Set8 directly on lamina components could be addressed by *in vitro* methylation studies.

In addition, 3D reconstruction of the signal from LaminA/C or B1 staining in Set8-depleted hepatocytes and/or cultured cells should be done so as to confirm the occurrence of vesicle-like structures within the nucleus instead of just foldings of the nuclear membrane. In order to provide mechanistic evidence for the hypothetical chromatin-based mechanism underlying endonucleosis, a closer investigation of the nuclear topology of Set8, H4K20me1&3 and the disruption of nuclear lamina in terms of Set8 depletion must be carried out. Immunogold labeling of these components in wild type (WT) and Set8KO livers can be performed and analyzed by transmission electron microscopy, thus providing evidence in greater magnification and detail. Fluorescent or immunogold staining of ER markers could reveal the participation of ER in endonucleosis by providing the membrane extension needed for the enlargement of the nucleus. ChIP-sequencing of crosslinked WT and Set8KO liver extracts could give information on the alteration of lamina-associated domains (LADs) that could lead to changes in nuclear sturdiness. Lastly, FRAP experiments of GFP-fused Set8 protein could support the existence of different functional pools of the protein, one acting at the nuclear center and the other acting at the nuclear periphery aiding the mechanical properties of the nucleus.

During the last decade, an increasing number of studies indicate a significant role of Set8 protein in physiological and pathophysiological processes. As demonstrated by Takawa

et al. (2012), Set8 is overexpressed in various types of human cancer, including bladder cancer, non-small cell and small cell lung carcinoma, chronic myelogenous leukemia, hepatocellular carcinoma and pancreatic cancer, and regulates the growth of cancer cells by aberrantly methylating PCNA. In addition, Set8 is involved in cancer invasiveness and metastasis e.g. through its interaction with TWIST, a master regulator of epithelial-mesenchymal transition (EMT) (Yang *et al.*, 2012). Despite its role in carcinogenesis, Set8 is also implicated in physiological processes such as erythroid maturation (Malik, Getman and Steiner, 2015), adipogenesis (Wakabayashi *et al.*, 2009) and maintenance of adult skin, and is necessary for c-Myc-induced epidermal proliferation (Driskell *et al.*, 2012). Therefore, the dissection of the role of Set8 in the numerous cellular processes will provide assistance dealing with and finding treatment for the pathological conditions mediated by Set8.

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