The wisdom of Weismann: Epigenetic erasure mechanisms and germ line immortality

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Cell Cycle Features:

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Epigenetic erasure mechanisms and germ line immortality

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Comment on: A *C. elegans* LSD1 demethylase contributes to germline immortality by reprogramming epigenetic memory. Katz DJ, Edwards TM, Reinke V, Kelly WG. Cell 2009; 137:308-20.

As first proposed by August Weismann in 1893, the germline is both the repository and the source of heritable information that is passed between generations. In the ensuing 20th century, it was recognized that DNA is one component of information that is passed between generations. Towards the end of the 20th and now into the 21st century, it became clear that information surrounding the DNA sequence—or epigenetic information—is another component. Furthermore, as predicted by Weismann, the germline is becoming recognized as not only the guardian but perhaps also the editor of the information that is transferred. Indeed, the more we learn about the continuity or "immortality" of the germline, and the molecular nature of the *germplasm* described by Weismann, the more we have come to appreciate the insight of this amazingly creative thinker and founding father of developmental biology.

Recognition of the importance of transgenerational epigenetic information has not come from an understanding of the information content itself—of which we still have little understanding—but instead from the discovery of processes that are important for its careful *erasure* in the embryo. For example, there are successive waves of genome-wide DNA methylation removal that occur in the mammalian zygote accompanied by large scale changes in heterochromatin structure (reviewed in ref. 1). The importance of these events can be inferred from early cloning experiments, in which terminally differentiated nuclei regain pluripotency, with increasing efficiency upon repeated exposure to oocyte cytoplasm, the *germplasm* to which Weismann referred. This "reprogramming" process likely involves DNA demethylase and histone modifying activities that have only recently been identified and whose characterization is incomplete. Furthermore, the excitement caused by the recent development of induced pluripotent stem cells (iPS cells), in which forced expression of a remarkably minimal set of factors can restore pluripotency in adult somatic cells, is tempered by the inefficiency of the technique and the lack of mechanistic information about the processes that are induced.

At least two important concepts can be appreciated after several decades of cloning experiments: (a) the reversal of the differentiated state involves a reversal of the epigenetic information imposed by differentiation, and (b) the germplasm carries the tools to perform this reversal, and normally accomplishes this feat at each generation. The question remains: what are the endogenous targets of the epigenetic reprogramming mechanisms in the zygote? During somatic cell nuclear reprogramming, the epigenetic landscape that maintains the differentiation-specific phenotype has to be erased and reprogrammed to the pluripotent ground state. It is thus reasonable to assume that, at each generation, epigenetic information acquired during development of the highly differentiated gametes would also need to be reprogrammed by this process. There has been little direct evidence to date that this is the case; however, we propose that the recent results of experiments our lab performed in the nematode *C. elegans* may provide such evidence.[2]

There is no DNA methylation in *C. elegans*, and thus all epigenetic information is presumably encoded in histone modifications and their consequences. One particular histone modification, histone H3 di-methylated on lysine 4 (H3K4me2) is dramatically erased from the genome during primordial germ cell (PGC) specification.[3] This process may be analogous to epigenetic erasure mechanisms that are observed in PGCs of other organisms.[4,5] Histone demethylases, such as the mammalian co-repressor component Lsd1, have been shown to actively remove this mark in other systems.[6] We therefore tested whether worm homologs of Lsd1 were involved in the H3K4me2 removal in the PGCs. Mutants in all three homologs, alone and in all combinations, did not show a defect in this process; a result that was initially disappointing. However, it was noticed that as mutant lines were maintained, they produced fewer progeny, and that the progeny were often sterile. Careful generational analysis showed that continued passage of strains defective in one particular homolog, *spr-5*, yielded sterile animals with increasing frequency in successive generations; the hallmarks of a "germline mortality" phenotype. Furthermore, there was no evidence of accumulating genetic defects. Instead, the population seemed to be accumulating *epigenetic* defects, since the phenotypes could be reversed by transient exposure to SPR-5 demethylase activity.

Microarray experiments that compared expression profiles between multiple generations showed striking and coordinated expression changes in one particular class of genes—those expressed in spermatogenesis. The expression of these genes climbed in early generations until peaking near the generations in which fertility plummeted—at which point the expression of these genes also fell. Importantly, the level of H3K4me2 in the promoter chromatin of these genes continued to increase despite the loss of expression. This indicated that there was a persistence of aberrant epigenetic accumulation in successive generations without increased transcription. Furthermore, in these later stages there was an increased failure to efficiently erase H3K4me2 from PGC chromatin, which suggested that the SPR-5-independent erasure mechanism was being overwhelmed. We concluded from these studies that histone demethylases are required to remove epigenetic information acquired in the parental germline, and that defects in this erasure lead to its persistence and accumulation in successive generations. This ultimately causes a failure of proper gametogenesis—and an end to germline immortality. Over 100 years ago August Weismann proposed that germplasm endows a special property upon the germline—the ability to differentiate into gametes, yet restore totipotency following fertilization. Our studies in *C. elegans* suggest that H3K4me2 demethylation by the Lsd1 demethylase SPR-5 may be a part of this special property.

Important questions remain: Where in the "germline cycle" is the histone demethylase activity required? Antibodies to SPR-5 recognize the protein in the adult germ cells of both sexes as well as in the early embryo, so it could be functional in either or both stages. Why does the defect take multiple generations to unfold? One possibility is that the H3K4me2 erasure mechanisms in the PGCs provide an imperfect back-up erasure system, such that successive generations come with a stoichastically increasing level of H3K4me2 to be erased. Interestingly, in Drosophila PGC-specific H3K4me2 erasure doesn't appear to occur, and mutants in the fly Lsd1 ortholog are sterile in the first generation of homozygosity.[7,8] Another important question concerns the targeting of erasure mechanisms; how does any mechanism determine what information is to be erased and what is to be maintained?

It is interesting to note that a number of studies showing trans-generational epigenetic phenomena in mammals point out that the critical window of the initiating event falls within the period of fetal germline development (reviewed in refs. 9 and 10). This indicates that the consequences of epigenetic events occurring in the germline, if unchecked, have the capacity to unfold for many generations. Incredibly, this is consistent with Weismann's original hypothesis of over a century ago that modes of heredity are limited to the germline, and thus events in the germline can have consequences for multiple generations and impact the evolution of species.

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Cell Cycle Features:

Metadherin as a link between metastasis and chemoresistance

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Key words: breast cancer, metastasis, chemoresistance, metadherin, AEG-1, cancer genomics, poor prognosis

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Comment on: MTDH activation by 8q22 genomic gain promotes chemoresistance and metastasis of poor-prognosis breast cancer. Hu G, Chong RA, Yang Q, Wei Y, Blanco MA, Li F, et al. Cancer Cell 2009; 15:9-20.

Despite the recent steady decline of breast cancer mortality, 30–40% of breast cancer patients develop metastatic diseases, including ~5% in newly diagnosed patients and ~30% as systemic recurrence after successful surgical removal of early stage tumors.[1] Unfortunately for these patients, palliative chemotherapy is the only treatment option available and treatment failure because of drug resistance is common. As the result, patients with metastatic disease generally have poor prognosis with a median survival of 12–24 months.[1] Therefore, metastasis and chemoresistance remain major hurdles for curative therapy for breast cancer and most of adult solid tumors.

A close correlation between metastasis and chemoresistance was frequently observed in human cancer patients. Metastatic tumors are invariably more chemoresistant than primary tumors as evidenced by the marked decrease of chemotherapy response rate in metastatic settings as compared to neoadjuvant settings.[1] Conversely, poor response to neoadjuvant chemotherapy often correlates with earlier metastatic recurrence and shorter survival, indicating chemoresistant tumors are prone to metastasize.[1] However, the molecular mechanism underlying the association of metastasis and chemoresistance remains poorly understood. Both metastasis and chemoresistane are complex, multigenic traits of malignant tumors that are further modulated by the interactions between tumor cells and the stromal microenvironment.[2,3] Previous studies based on in vitro cell culture and even xenograft animal tumor models often cannot fully reflect the complexity of human cancers, resulting in research findings that are difficult to translate into clinical applications. For instance, decades of studies initiated by in vitro cell culture experiments have discovered several well-defined genetic mechanisms for multi-drug resistance including overexpression

of various drug efflux transporters, e.g., MDR1/P-glycoprotein, MRP1 and BRCP.[3] Although inhibitors of these transporters have been proven to be effective in preclinical models, clinical applications of these inhibitors remained largely unsuccessful.[4] To define clinically relevant mechanisms and identify novel therapeutic targets that are functionally important for breast cancer progression, a more systemic and unbiased strategy that integrate genome-level clinical data analysis with experimental functional characterization in vitro and in vivo is needed.

Following this strategy, we recently identified Metadherin (MTDH,[5] also called AEG-1,[6] or LYRIC[7,8]) as a major contributor to poor prognosis in breast cancer.[9] In this study, innovative computational analysis of three large gene expression datasets of breast cancer revealed that the copy number of a 2.9 Mb genomic region in 8q22 was frequently elevated in primary tumors of patients with poor prognosis (i.e., higher risk of metastasis and shorter survival). This observation was further validated in an independent collection of breast tumor samples, indicating that a mediator(s) of metastasis and/or treatment failure is likely to be present in this region. Further functional studies of candidate genes in this region pinpointed *MTDH* as the likely culprit. MTDH promotes metastasis through increasing the adhesion of tumor cells to endothelial cells, a function that is consistent with a previous study by Brown et al. that identified a lung-homing domain in MTDH.[5]

Pharmacogenomic analysis of the NCI60 panel of cancer cell lines also indicated MTDH as a possible mediator of broad-spectrum chemoresistance. This hypothesis was subsequently validated using in vitro and in vivo models of chemoresistance. As a dual functional mediator of breast cancer metastasis and drug resistance, MTDH may become a novel target for therapeutic inventions to simultaneously prevent metastatic spreading and improve chemotherapy efficacy. Interestingly, MTDH-induced chemoresistance is more pronounced when the tumor cells are co-cultured with endothelial cells, suggesting that endothelial involvement in tumor progression may be more than the well-established function in tumor angiogenesis. The chemoresistance function of MTDH may be mediated by a number of downstream drug resistance-related genes that were identified based on microarray study of MTDH-knockdown or -overexpressing cells,[9,10] although the mechanism of regulation of these genes by MTDH remains unknown. MTDH has also been shown to activate PI3K-Akt and NFκB signaling pathways in tumor cells,[11,12] which may contribute to increased survival of tumor cells after chemotherapeutic challenges.

Immunohistochemical analysis of breast tumor samples of patients in the US[9] and in China[13] consistently showed the overexpression of MTDH in more than 40% of breast tumors. For both breast cancer[9,13] and esophageal cancer,[14] MTDH is a poor prognosis marker independent of other common clinicopathological factors. MTDH overexpression has also been documented in glioma, melanoma, neuroblastoma, liver cancer and prostate cancer,[10-12] indicating that it may play a general role in solid tumor progression.

The discovery of MTDH as a dual functional gene in breast cancer metastasis and chemoresistance highlighted the necessity of using integrative and multidisciplinary approach when searching for functionally important and clinically relevant mediators of cancer progression. As a evolutionally highly conserved gene with no recognizable protein domains, the molecular and biochemical properties of MTDH remain poorly characterized.[11,12] Further studies of the functional mechanism of MTDH may help unravel the long-standing mystery of association between metastasis and chemoresistance in human cancer.

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Cell Cycle Features:

Transcription-blocking DNA damage in aging and longevity

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Key words: aging, longevity, transcription, IGF-1, progeroid syndromes, DNA damage

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Comment on: Persistent transcription-blocking DNA lesions trigger somatic growth attenuation associated with longevity. Garinis GA, Uittenboogaard LM, Stachelscheid H, Fousteri M, van Ijcken W, Breit TM, et al. Nat Cell Biol 2009; 11:604-15.

Aging is widely believed to be driven by the random accumulation of damages and regulated through genetic longevity pathways. Recent evidence has uncovered a link between those two principle components of aging through stalling of RNA polymerase II at persistent DNA damage that triggers longevity assurance programs.

DNA damage is not only a cause of cancer but also a driving force of aging. The genetic material is under constant attack from extrinsic genotoxic sources such as the sun's UV radiation and intrinsic damaging agents such as harmful metabolic byproducts. Cells have developed a host of repair mechanisms that are essential to maintain genome integrity.

The underlying role of DNA damage in aging becomes particularly apparent in a variety of progeroid (premature aging) syndromes that are linked to congenital defects in DNA repair systems.[1] Progeroid conditions such as Cockayne syndrome (CS), trichothiodystrophy (TTD), Werner syndrome (WS), Bloom's syndrome (BLS), Nijmegen breakage syndrome (NBS), Rothmund-Thomson (RTS) and Hutchison-Gilford progeria syndrome (HGPS) have all been linked to defects in the maintenance of nuclear DNA integrity.[2]

Persistent DNA damage that accumulates either rapidly as a result of defective DNA repair or increases over time as an organism ages, might interfere with two basic cellular functions: replication of DNA in dividing cells, and transcription of DNA in all cell types regardless of whether they are dividing, quiescent or terminally differentiated. Acute responses to DNA damage have been studied extensively in the past as they induce traceable cellular responses such as halting the division cycle to allow time for repair, permanently arresting cells as they enter senescence or driving cells into programmed cell death.[3]

Reponses to damages that are not sufficiently severe to permanently arrest cells or trigger apoptosis have been much less explored. These damage responses might, however, be of critical importance in aging as low amounts of damages might persist and thus accumulate during the organisms' lifespan. For once, it has been technically difficult to measure low amounts of the large variety of changes to the DNA that are inflicted by the various damage sources. In vitro systems for inducing non-toxic amounts of persistent DNA damage have been difficult to establish as for instance oxidative DNA lesions are either rapidly repaired or they are too severe and drive cells into apoptosis. A unique paradigm for persistent DNA lesions that in low amounts enable the cell to continue its functioning is the UV-induced cyclobutane pyrimidine dimer (CPD). CPDs persist in the DNA for days, are carried through replication and are repaired by transcription-coupled repair (TCR) when the RNA polymerase II (RNAPII) stalls at a CPD lesion.[4] Progeroid syndromes such as CS, TTD as well as XPF-ERCC1 progeria (XFE) are characterized by defects in the TCR machinery.[2] Also other progeroid syndromes such as WS, BLS and HGPS have been linked to defects in the transcriptional machinery,[5] suggesting that the consequences of unrepaired DNA damage on the transcriptional apparatus play an important role in (premature) aging.

Recently, we showed that cells in vitro react to even low amounts of such transcription-blocking lesions with a response program that is also seen in naturally aged mice.[6] Intriguingly, persistent DNA damage attenuated the expression of key regulators in the somatotropic axis and conferred IGF-1 resistance, which represent hallmarks of extended longevity.[7] The somatotropic axis regulates body growth through insulin-like growth factor 1 (IGF-1) receptor signaling. IGF-1 is secreted upon growth hormone receptor (GHR) activation by pituitary GH. Insulin/IGF signaling represents an evolutionary conserved genetic pathway of longevity regulation,[8] and IGF-1 receptor or GHR loss, pituitary dysfunction, or calorie restriction confer extended longevity in mice.[7] Moreover, the cellular response to transcription-blocking DNA lesions led to resistance to oxidative stress, a conserved feature of extended longevity mutants in worms, flies and mammals.[9,10] Thus, transcription-blocking lesions induce a shift from growth to somatic preservation characteristic of lifespan extension programs. This response program is specifically induced upon persistent DNA damage in active genes in dividing, quiescent and terminally differentiated cells. Neither transient damages nor lesions remaining outside of transcribed genes induced such longevity assuring stress resistance programs. This specific response to transcription-blocking lesions might also explain why TCR deficient patients remain tumor-free despite their repair deficiency but instead show growth defects and premature aging.[11] Intriguingly, IGF-1R can act oncogenic through its functioning in survival signaling.[12] Indeed, many tumors are critically dependent on IGF-1 signaling.[13] Attenuation of somatotropic signaling might thus serve as a tumor suppressor mechanism that antagonizes the oncogenic potential of accumulating DNA damage in aging. Consequently, somatotropic attenuation in response to persistent damages might confer a survival mechanism amid gradually increasing damage loads with age (Fig. 1).

Mechanistically, RNAPII has previously been suggested to function as damage dosimeter even in post-replicative cells.[5] The specific link between stalled RNAPII at persistent DNA lesions and the induction of growth suppressing longevity assurance mechanisms suggests that the transcriptional machinery plays a central role in surveying the damage load in the aging organism and orchestrating the age-related shift from growth to somatic maintenance. It will be of pivotal interest to identify

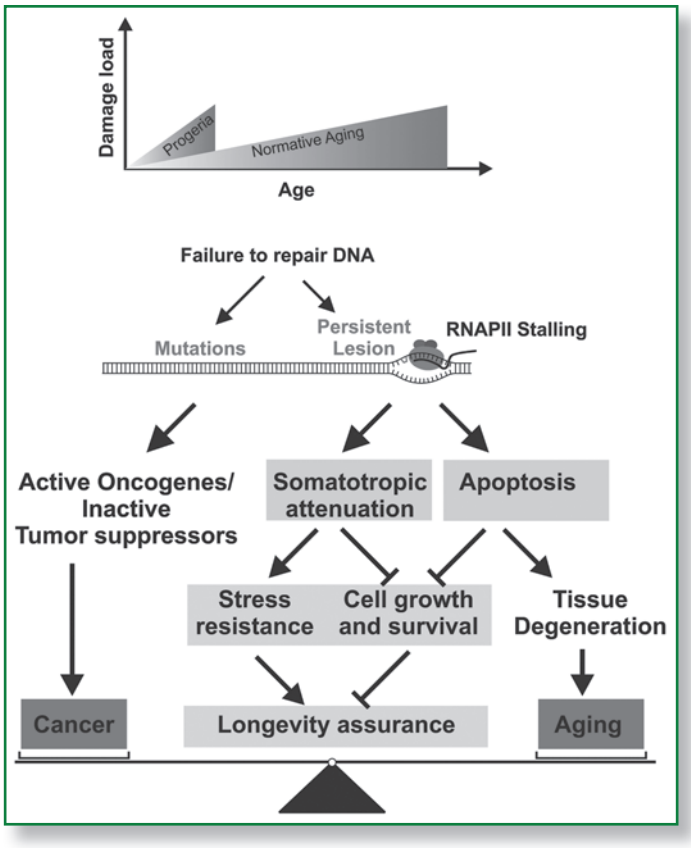

Figure 1. Balancing responses to DNA damage in aging. DNA damage accumulates during aging and prematurely in progeroid DNA repair-deficiencies. Incorrect repair can lead to mutations in oncogenes and tumor suppressor genes thus contributing to cancer. DNA lesions that persist lead to stalling of RNA polymerase II in active genes. Stalled RNAPII induces programmed cell death, contributing to the age-related decline in tissue integrity. Recently, stalled RNAPII was shown to also induce a longevity assurance program through somatotropic attenuation, which leads to tumor suppression and enhanced stress resistance thus enabling the organism to survive amid increasing damage loads with aging.

the mechanisms through which stalled RNAPII leads to the induction of longevity assurance programs.

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Cell Cycle Features:

To be or NOT to be demethylated

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Comment on: Polyubiquitination of the demethylase Jhd2 controls histone methylation and gene expression. Mersman DP, Du HN, Fingerman IM, South PF, Briggs SD. Genes Dev 2009; 23:951-62.

Histone methyltransferases and demethylases possess key chromatin remodeling activities that are needed to control proper gene expression in eukaryotes. Importantly, the recent discovery of histone demethylases has revealed that histone methylation is a dynamic process and is likely to be regulated. How histone demethylases are regulated is not thoroughly understood and is still currently being investigated. It has been suggested that post-translational modifications may play a role but how modifications regulate histone demethylase activity or how the balance of histone methyltransferases and demethylases is maintained have not been fully explored.[1] To address these issues, we turned to *S. cerevisiae* as our model organism of choice. In *S. cerevisiae*, it was recently discovered that deletion of *NOT4* leads to decreased global histone H3 lysine 4 (H3 K4) trimethylation. However, until our study, the mechanism by which Not4 was regulating histone H3 K4 trimethylation was not clear.[2,3] We determined that expression of Jhd2, a histone H3 K4 demethylase, in wild-type yeast cells is tightly regulated by Not4, which in turns allows for proper H3 K4 trimethylation and gene expression levels (Fig. 1A).[4] How, then, does Not4 control Jhd2 protein levels and histone methylation? We demonstrate for the first time that Not4, a known E3 ubiquitin ligase, can polyubiquitinate Jhd2 and target it for degradation by the proteasome.[4] Furthermore, in the absence of Not4 or upon proteasome inhibition, Jhd2 protein levels increase leading to demethylation of trimethylated H3 K4 even in the presence of the H3 K4 methyltransferase, Set1.[4] This, in turn, results in decreased gene expression of the GMP Synthetase, *GUA1*. We also show that the human homolog of Jhd2, JARID1C, is ubiquitinated by human NOT4 in vitro, suggesting that this may be a conserved mechanism by which histone demethylase activity is regulated.[4] Our study also raises additional questions to be considered and addressed.

What are other substrates for Not4? Besides Jhd2, Egd2, a member of the nascent-associated polypeptide (NAC-EGD) complex which is a complex associated with the ribosomal exit tunnel needed to ensure nascent protein targeting, is one of the few identified substrates for the

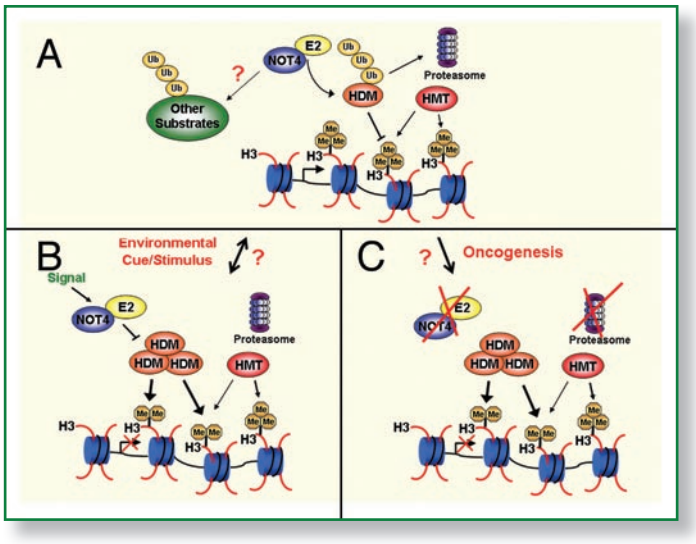

Figure 1. Regulation of histone demethylases. (A) The E3 ubiquitin ligase Not4 can ubiquitinate Jhd2, a histone H3 lysine 4 demethylase (HDM), tagging it for protein degradation by the proteasome. This prevents demethylation of H3 K4 and allows the Set1 H3 K4 methyltransferase (HMT) to maintain trimethylated histones which are needed for proper levels of gene expression. It is not yet clear if there are other transcriptional regulators that are targets of Not4's E3 activity, but if so, this may help explain the diverse roles of Not4 in regulating gene expression. (B) We propose that this seemingly futile cycle of production and degradation of Jhd2 is a rapid response system whose activity is modulated by an environmental cue/stimulus or progression through the cell cycle. This system would allow the cell to respond to an environmental condition, send a signal and rapidly modulate gene expression via histone demethylation. (C) Deregulation of this process, perhaps via changes in Not4 expression/activity or proteasome mutations, could lead to overexpression of HDM's such as JARID1B/PLU-1 resulting in unintended H3 K4 demethylation and consequently changes in gene expression. This inappropriate change in gene expression profiles could result in oncogenesis or, perhaps, the onset of other human diseases.

E3 activity of Not4.[5] Interestingly, ubiquitination of Egd2 appeared to only affect its cellular localization.[5] More recently, it was shown that Not4's ubiquitin ligase activity is needed for the degradation of nascent protein products arrested during translation.[6] Therefore it is likely that Not4 has additional targets. Given that our work and the work of others show that Not4 is also important in positively and negatively regulating gene expression, it would be interesting to determine if Not4 targets other transcriptional regulators such as activators/repressors, other histone modifying activities or elongation factors (Fig. 1A).[7] The identification of additional Not4 substrates might clarify why Not4 can act to promote or inhibit transcription.

Does Not4 influence H2B ubiquitination through regulating GMP Synthetase gene expression? It has been previously noted that deletion of Not4 leads to increased monoubiquitination of histone H2B.[2] H2B ubiquitination is important for wild-type levels of H3 K4 and H3 K79 methylation and it is known that, in Drosophila, GMP Synthetase, GMPS, can help stimulate deubiquitination of H2B via association with USP-7, an ubiquitin protease.[8,9] Intriguingly, we find that loss of *NOT4* results in a 2.5-fold decrease in expression of the yeast GMP Synthetase, *GUA1*.[4] Keeping in mind the observations found in Drosophila, this result suggests that the increase in H2B ubiquitination observed in a *not4Δ* strain could be due to lower expression of *GUA1* which would result in less ubiquitin protease activity. Therefore, it would be interesting to determine if yeast GMP Synthetase associates with and/or regulates known H2B ubiquitin proteases in yeast such as Ubp8 or Ubp10.[10,11] If this process is conserved, it raises the possibility that other basic metabolic enzymes are likely regulating chromatin-mediated processes.

Is control of Jhd2 protein levels by Not4 a housekeeping or a regulated process? The fact that Jhd2 protein is being transcriptionally produced and, at the same time, is kept at an extremely low level in the cell through proteasome-mediated degradation is not energy efficient for the cell. Therefore, we favor the idea that this is a regulated process rather than just a housekeeping function. Why would a cell maintain this futile cycle given the cost in cellular energy and materials? We propose that this futile cycle exists until a rapid response is needed for modulating gene repression via removing histone H3 K4 trimethylation and effector binding proteins. We would predict that Not4's activity and Jhd2 protein levels would most likely respond to different environmental queues/stimuli or as a result of progression through the cell cycle (Fig. 1B). Although it is unclear what these environmental conditions are, if similar mechanisms occur in humans, this could give vital insight into how human demethylases are regulated.

Why are histone demethylases deregulated in human cancers? The JARID1 family of histone demethylases contains human homologs of Jhd2, including JARID1A/RBP2, JARID1B/PLU-1, JARID1C/SMCX and JARID1D/SMCY, and has been linked to a wide variety of human disorders from X-linked mental retardation to breast cancer.[12] PLU-1/JARID1B shows restricted and low expression in most human adult tissues and is found to be highly upregulated in breast cancer.[13] The mechanism behind PLU-1 overexpression in breast cancer is not yet known, but our study may shed some light on this observation. We show that the human demethylase JARID1C/SMCX can be polyubiquitinated in vitro by human NOT4.[4] In addition, protein levels of human LSD1, a non-JARID1 family member histone demethylase, have been shown to be influenced by the proteasome, suggesting that ubiquitination and degradation of demethylases may be a common mechanism for regulating their activity.[14] Because of these observations and the conserved nature of these enzymes, we are currently determining if members of the JARID1 family are ubiquitinated in vitro and in vivo. If this is the case, we predict that defects in ubiquitination and degradation of PLU-1/JARID1B by human NOT4 and/or the proteasome could be a mechanism behind increased PLU-1 expression in breast cancer (Fig. 1C). It is also quite possible that NOT4 expression itself is affected in human cancers which could explain why PLU-1 is upregulated. Finally, if regulating histone demethylases by NOT4 is conserved and the reason for various human cancers, this could lead to a new therapeutic target.

Given the importance of the JARID1 family of histone demethylases in human diseases, the questions above will be exciting for us and others to explore. Ultimately, we hope our current and future studies will provide new insights for basic and cancer research.

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