Regulating the levels of key factors in cell cycle and DNA repair

New pathways revealed by lamins

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Spatial and temporal organization of the genome represents an additional step in the regulation of nuclear functions. The nuclear lamina, a polymeric meshwork formed by lamins (A/C and B type) and lamin-associated proteins, plays a key role in the maintenance of genome localization, structure and function. Specifically, mutations in the *LMNA* gene encoding lamins A/C or changes in its expression, either upregulation or silencing, are associated with defects in DNA replication, transcription and repair, as well as alterations in epigenetic modifications of chromatin. These data, together with the fact that defects in A-type lamins are associated with a whole variety of degenerative disorders, premature aging syndromes and cancer, support the notion that these proteins operate as caretakers of the genome. However, our understanding of their functions is limited due to the lack of well-defined mechanisms behind the genomic instability observed in lamin-related diseases. Here, we summarize our recent discovery of new pathways that are affected by the loss of A-type lamins. In particular, we found that A-type lamins control transcription and degradation of proteins with key roles in cell cycle regulation and DNA double-strand breaks (DSBs) repair by nonhomologous end-joining (NHEJ) and homologous-recombination (HR). Importantly, the proteins regulated by A-type lamins— Rb family members, 53BP1, BRCA1 and RAD51— exert tumor suppressor functions, with their loss being associated with cancer susceptibility. Moreover, our studies revealed novel pathways that contribute to genomic instability and that can be activated in disease states independent of the status of A-type lamins.

Introduction

Lamin-related diseases are characterized by the presence of nuclear deformation, epigenetic alterations of chromatin and chromosomal aberrations.[1,2] Most of the data on genomic instability has resulted from the study of cells from patients with Hutchinson-Gilford Progeria Syndrome (HGPS) and from mouse models of progeria. The progeria phenotype arises from mutations that alter the normal processing and maturation of lamin A.[3,4] Accumulation of unprocessed lamin A species at the nuclear lamina causes the characteristic nuclear defects that lead to cell toxicity.[5] Interestingly, progeria cells accumulate DNA DSBs,[6,7] similar to cells of aged individuals,[7,8] indicating a compromised DNA repair system. However, no clear defects in repair proteins themselves or in the activation of the DNA damage response (DDR) pathway have been observed in progeria cells.[9] One exception is the observed accumulation of the protein XPA (Xeroderma pigmentosum group A) at DSBs[10] which was associated with impaired recruitment of key DNA repair factors such as Rad50, Rad51 and 53BP1 to the breaks. Binding of XPA also activates ATM- and ATR-dependent signaling cascades that arrest the cell cycle. However, depletion of XPA in progeria cells only partially restored the recruitment of DNA repair factors to DSBs, indicating that additional mechanisms contribute to the DNA repair deficiencies

in these cells. Interestingly, a recent report demonstrated the absence of the nuclear DNA-PK holoenzyme in premature as well as physiological aging.[11] These studies have started to shed some light into putative molecular mechanisms that could be impacted upon by alterations in A-type lamins function. We have undertaken a loss-of-function approach to gain a deeper understanding of the role that A-type lamins play in the maintenance of genomic stability. We found that loss of A-type lamins leads to the downregulation of a number of factors with key roles in cell cycle regulation, e.g., Rb family members and DNA DSBs repair, e.g., 53BP1, BRCA1 and RAD51. We will summarize here the molecular mechanisms behind the regulation of these factors by A-type lamins and their significance for understanding aging-related diseases.

Mechanisms of DNA DSBs Repair

Repair of damaged DNA is critical for maintenance of genomic stability. Among the various types of DNA damage, DSBs are the most deleterious, leading to mutations, loss of genomic material and translocations if not properly repaired. The two major pathways of DSBs repair, homologous recombination (HR) and classic nonhomologous end-joining (C-NHEJ) are considered to compete for repair substrate and be mutually exclusive.[12-14] HR is error-free and requires both resection of the 5' DNA ends around the break and the presence of a homologous template. In contrast, C-NHEJ involves end ligation of damaged DNA and requires neither extensive resection nor homologous templates. C-NHEJ is a fast and error-prone mechanism which can cause translocations and/or loss of genetic material. While C-NHEJ is the predominant repair mechanism in G_0/G_1 stages of the cell cycle, when the lack of the sister chromatid prevents HR from being activated, the slower HR repair mechanism has traditionally been thought to dominate during S and G_2 phases of the cell cycle. However, recent evidence[15] has challenged the notion of HR dominance in S/G_2, suggesting that the need for rapid DNA damage repair makes NHEJ the preferred pathway even when HR is possible. According to this data, it is only

when the damage cannot be repaired by NHEJ that end-resection is promoted and additional mechanisms undertake DNA repair. Besides HR and C-NHEJ, a less understood pathway, alternative nonhomologous end-joining (A-NHEJ),[16,17] is sometimes used as a backup repair pathway. A-NHEJ involves processing of DNA by end-resection to reveal regions of short microhomology which are then ligated. In contrast to HR, resected DNA is not filled in during A-NHEJ, making it a potentially more deleterious process than both C-NHEJ and HR. In line with this notion, A-NHEJ is associated with high frequencies of chromosomal translocations and genomic instability.

Lamins Role in DNA DSBs Repair by NHEJ

Our studies have provided strong evidence for a role of A-type lamins in the maintenance of DNA repair mechanisms and telomere homeostasis. We found that loss of A-type lamins leads to sustained DNA damage signaling as measured by formation of γH2AX foci, as well as an increase in aneuploidy, chromosomal abnormalities and telomere shortening/loss.[18-21] Furthermore, loss of these structural proteins was associated with alterations in the nuclear organization of chromosomes, such that the distribution of telomeres was shifted toward the periphery of the nucleus.[18,21] Despite the increase in genomic instability, and similar to what was reported in progeria cells, loss of A-type lamins does not impair activation of the DNA damage response (DDR) when cells are exposed to ionizing radiation.[19] ATM-dependent phosphorylation of H2AX (γH2AX) and p53 at Ser15 was not affected in Lmna-KO mouse embryonic fibroblasts (MEFs), and the kinetics of formation and resolution of γH2AX ionizing radiation-induced foci (IRIF) was indistinguishable between lamins-deficient and -proficient cells. In contrast, lamins-deficient cells show defective accumulation of 53BP1 at IRIF at all post-irradiation times tested.[19] Importantly, we found that this deficiency is due to a marked decrease in the global levels of the 53BP1 protein, and not to failed recruitment, since 53BP1 IRIF formed although
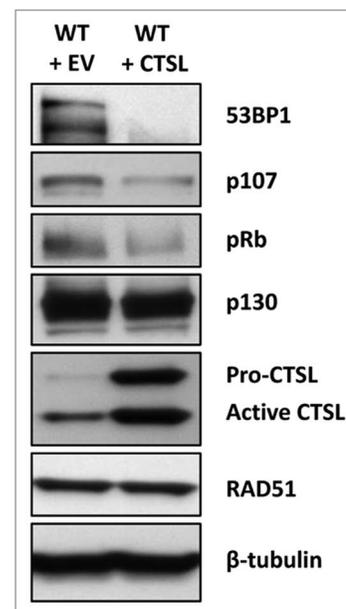

Figure 1. Regulation of Rb family members by CTSL. Blots showing that overexpression of CTSL via retroviral transduction of wild-type MEFs leads to downregulation of the pocket proteins pRb and p107 but has no effect in the levels of p130. As a control for increased CTSL activity, we show decreased levels of 53BP1 in the CTSL overexpressing cells.

at a much lower intensity. These results are highly relevant, since 53BP1 is an important player in long-range end-joining processes such as class-switch and V(D)J recombination, as well as in the joining of dysfunctional telomeres.[22-25] In addition, 53BP1 is thought to play a role in the repair of short-range DNA DSBs by binding to the breaks, inhibiting end-resection and facilitating the recruitment of the NHEJ DNA repair machinery.[26,27] These data, together with the fact that 53BP1-deficient cells exhibit increased genomic instability and radiosensitivity[28-31] suggested that the loss of 53BP1 could be responsible for the DNA repair deficiencies observed in Lmna-KO cells. Consistent with our hypothesis, we found that lamins-deficient cells treated with ionizing radiation exhibit profound defects in the fast phase of DNA DSBs repair.[19,32] Fast repair is traditionally associated with C-NHEJ, since similar defects are observed upon depletion or mutation of essential factors in this process such as DNA-PK, Ku80, XRCC4 and DNA ligase IV.[16] Furthermore, lamins-deficient cells are defective in the processing
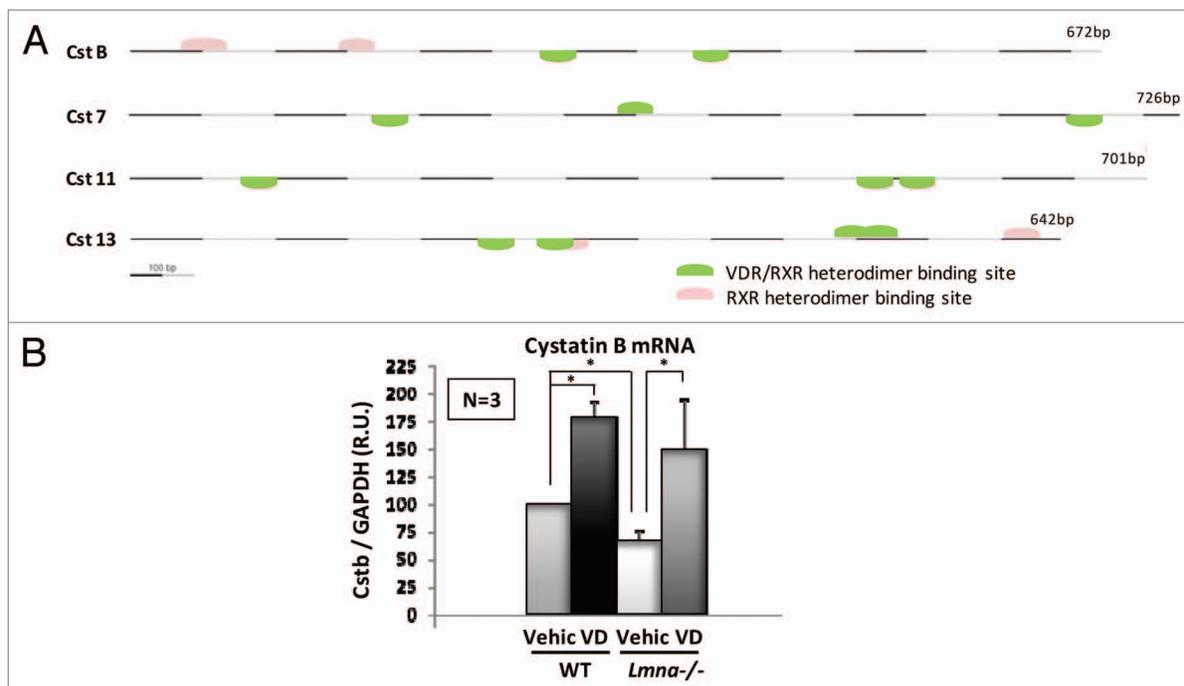
Figure 2. Vitamin D-dependent inhibition of CTSL activity may be mediated by cystatins. (A) Promoter analysis for vitamin D responding elements (VDREs) in the 13 cystatins encoded in the mouse genome resulted in four candidate genes containing at least two RXR/VDR heterodimer binding sites. (B) qRT-PCR experiments show that cystatin B expression is downregulated in lamins-deficient cells. Treatment with vitamin D 10^{-7} M for 48 h increases cystatin B expression in both wild-type and Lmna-KO MEFs. Values are expressed as mean ± SEM. N, the number of independent experiments; *, p value of statistical significance (p ≤ 0.05); R. U., relative units (normalization to GAPDH).

of dysfunctional telomeres by NHEJ. Importantly, we found that reconstitution of 53BP1 in lamins-deficient cells rescues the defects in NHEJ of DNA DSBs and dysfunctional telomeres.[19] Overall, these results revealed that 53BP1 deficiency is a major contributor of the DNA repair phenotype observed in lamins-deficient cells. This is a critical observation, since many studies rely on foci formation to determine whether a step in the DDR is functional. Our results indicate that it is important to monitor the levels of DDR proteins at DSBs when assessing deficiencies in DNA repair.

How are the Levels of 53BP1 Regulated by A-Type Lamins?

During our exploration of mechanisms by which A-type lamins affect DNA DSBs repair we discovered a role for cathepsin L (CTSL) in the stability of 53BP1 protein. CTSL is a cysteine protease from the papain family that is ubiquitously expressed in mouse and human tissues. Like many other proteases, it is synthesized as a zymogen which undergoes autoproteolytic processing within the lysosomal/endosomal compartment to release the mature active form.[33] Though its activity is enhanced by the low pH at the lysosome, CTSL can also be found in other cellular organelles, where it can selectively process other targets at less acidic or even neutral environments. CTSL can be secreted to the extracellular matrix where it is known to degrade some of its components under physiological conditions, i.e., favoring bone resorption in osteoclasts.[34] Increased extracellular CTSL has been reported in numerous types of cancer and is often associated with increased invasiveness and metastasis.[35-37] More recently, CTSL was found inside the nucleus, where in a more regulated fashion, it processes specific nuclear components such as histone H3 tails during stem cell differentiation and the transcription factor CDP/Cux during cell cycle progression.[38,39]

The first link between CTSL and A-type lamins was established in a mouse model of progeria. In particular, mice lacking Zmpste24, a metalloprotease that participates in the maturation of lamin A, exhibit a drastic increase in the levels of CTSL mRNA.[40] Although this suggested a relationship between CTSL and the aging phenotype, no association was established between CTSL and the increase in genomic instability displayed by these mice. Our studies showed that Lmna-KO cells exhibit a marked increase in the levels of CTSL mRNA and protein, indicating that loss of A-type lamins induces transcriptional upregulation of CTSL.[32] Furthermore, we demonstrated that the increase in CTSL is directly responsible for the downregulation of 53BP1 protein levels. Depletion of CTSL via lentiviral transduction with a specific shRNA restored 53BP1 protein levels and rescued NHEJ defects in lamins-deficient cells. Moreover, transduction with both 53BP1 and CTSL shRNAs in this context prevented restoration of NHEJ, demonstrating that the recovery is brought about by stabilization of 53BP1.

The regulatory effect of CTSL on 53BP1, and thus in DNA repair is not restricted to lamins-deficient cells. Overexpression of CTSL in wild-type cells is sufficient to lower the levels of 53BP1 and impair repair by NHEJ.[32] This is a
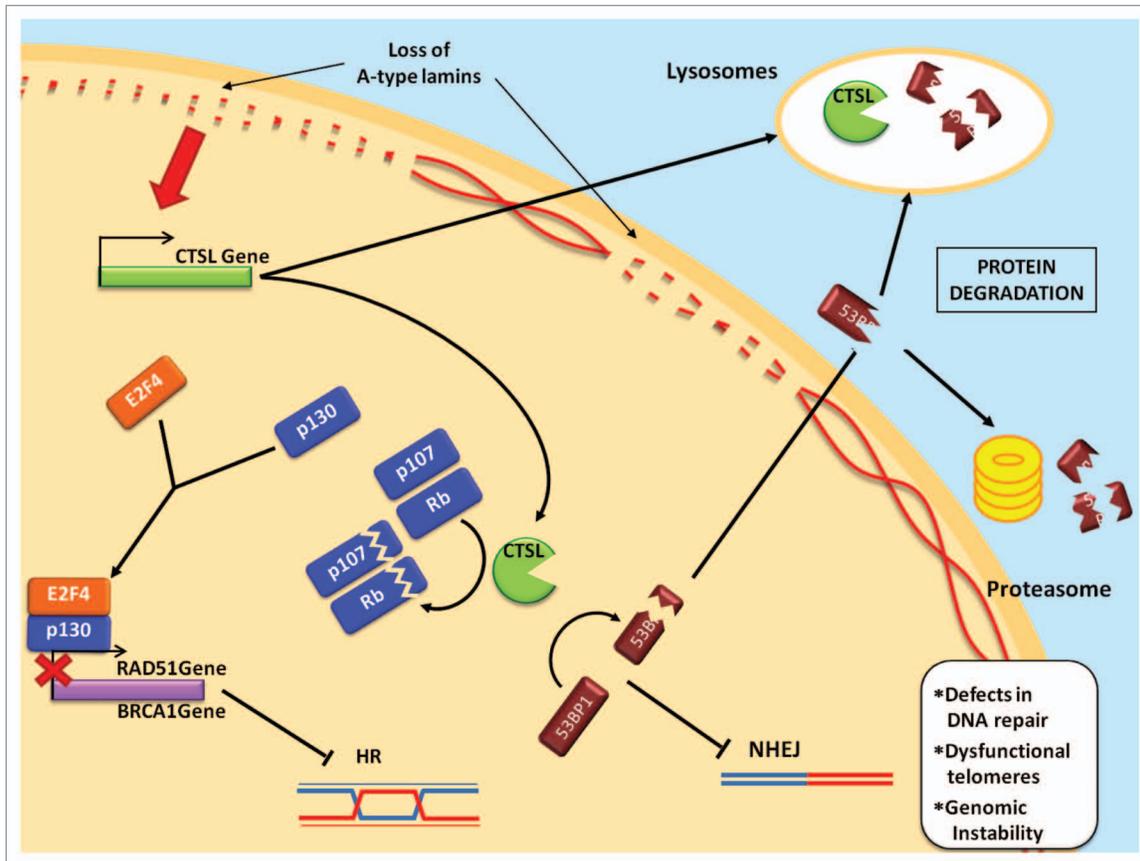
Figure 3. Proposed model for the regulation of DNA repair mechanisms by A-type lamins. The loss of A-type lamins upregulates CTSL expression, resulting in elevated protein levels both in the nucleus and in the lysosomes. CTSL processes 53BP1, which then accumulates in the cytoplasm and is targeted to degradation by the lysosomal pathway and/or the proteasome. Loss of 53BP1 impairs DNA repair by NHEJ. On the other hand, CTSL degrades the pocket family proteins pRB and p107, favoring the formation of p130/E2F4 repression complexes, which in turn inhibit RAD51 and BRCA1 gene expression and thereby impair DNA repair by HR.

very relevant result because a great variety of tumors present with high CTSL expression. Our studies suggest that in addition to the previously reported effects of CTSL upregulation on the degradation of extracellular matrix components and cell-adhesion molecules, CTSL upregulation in cancer could inhibit mechanisms of DNA repair. Future studies need to determine whether upregulation of CTSL activity is a novel mechanism contributing to genomic instability in cancer, which could be targeted with therapeutic purposes.

Lamins Role in DNA DSBs Repair by HR

Loss of 53BP1 favors repair of DNA DSBs by HR.[27,41] However, despite decreased 53BP1 levels, HR is suppressed upon depletion of A-type lamins.[19] This inhibition of HR is explained by the significant reduction in expression of two key factors in this process, BRCA1 and RAD51. In contrast to the CTSL-mediated degradation of 53BP1, decreased levels of BRCA1 and RAD51 are brought about by transcriptional gene repression.[19] Previous reports had demonstrated transcriptional repression of BRCA1 and RAD51 under certain stressful conditions, such as hypoxia or PARP inhibition, via formation of p130/E2F4 complexes at E2F sites within their promoters.[42,43] Interestingly, in the context of lamin A/C-deficiency, we also find that repression of BRCA1 and RAD51 genes is linked to the status of the Rb family of tumor suppressors, pRb, p107 and p130, such that repression of BRCA1 and RAD51 requires p130 and occurs in the context of pRb and p107 deficiency. Furthermore, co-immunoprecipitation studies in cells depleted of A-type lamins showed an increase in p130/E2F4

complexes.[19] These data suggest activation of a similar repressive mechanism in lamins-deficient cells, where altering the balance of the pocket proteins might favor association of p130 with E2F4, leading to transcriptional inhibition of responsive promoters. However, we cannot rule out the possibility that loss of A-type lamins leads to alterations in the nuclear localization of BRCA1 and RAD51 genes, which might contribute to their transcriptional repression.

It is well established that pocket proteins associate with lamins and that loss of A-type lamins leads to increased degradation of pRb and to a lesser extent p107.[44,45] This is thought to occur partly through the ability of A-type lamins to regulate the sub-nuclear localization of these proteins. However, the specific mechanism by which pRb and p107 are targeted for degradation remains quite elusive, being independent

of both MDM2 and gankyrin, a component of the 19S proteasome subunit which is overexpressed in Lmna-KO cells.[46] Given our recent findings that CTSL promotes the degradation of 53BP1, we speculated that this protease could be the missing link between A-type lamins and pRb/p107 degradation. We envisioned a scenario where CTSL-mediated degradation of pRb and p107 alters the balance between the pocket family proteins, leading to increased formation of p130/E2F4 complexes, which can in turn mediate transcriptional repression of BRCA1 and RAD51, inhibiting HR. To test our model we overexpressed CTSL in wild-type MEFs via retroviral transduction and monitored the levels of Rb family members, BRCA1 and RAD51. Indeed, we found that upregulation of CTSL is associated with a substantial decrease in pRb and p107, with little or no effect on p130, mirroring the phenotype observed in lamins-deficient cells (**Fig. 1**). These results demonstrate a novel role for CTSL in the regulation of the Rb family of tumor suppressors. However, altering the levels of these proteins was not sufficient to induce transcriptional repression of BRCA1 or RAD51 in MEFs (data not shown). These data suggest that lamins have additional roles in the regulation of transcription of these genes independently of the CTSL-mediated degradation of Rb family proteins.

Overall, our studies revealed that A-type lamins regulate HR indirectly by impacting on transcription of key players in this process. On one hand, loss of A-type lamins leads to transcriptional upregulation of CTSL, which in turns degrades pRb and p107. We speculate that the loss of these two proteins might favor the formation of p130/E2F4 complexes, which provide a permissive environment to repress transcription from BRCA1 and RAD51 gene promoters. However, loss of A-type lamins might induce some other changes, perhaps epigenetic modifications at the promoter, which result in shut down of gene expression.

Vitamin D Inhibition of CTSL Reduces Genomic Instability

Vitamin D is a liposoluble steroid prohormone present in certain foods but mainly obtained from the conversion in the skin of 7-dehydrocholecalciferol to vitamin D3 by the action of UV light. Vitamin D3 is then hydroxylated in the liver, producing 25OH vitamin D3, and later in the kidney, where the enzyme 1-α hydroxylase produces $1,25(OH)_2D3$ or calcitriol, the active vitamin D metabolite (hereinafter vitamin D). Vitamin D exerts its actions through the vitamin D receptor (VDR), a member of the nuclear receptor superfamily.[47] Ligand-bound VDR is translocated to the nucleus where it associates with the retinoic-X receptor (RXR). The VDR/RXR heterodimer can interact with a number of co-activators and/or co-repressors, regulating transcription of multiple target genes. Vitamin D is a pleiotropic hormone, with a major role in the regulation of bone and mineral metabolism. In addition, vitamin D regulates proliferation, inhibits invasion and promotes apoptosis or differentiation depending on the cellular context.[48]

Recently, it was shown that calcitriol inhibits invasion in colon cancer cells by activating the transcription of cystatin D, an endogenous cysteine protease inhibitor of cathepsins S, H and L.[49] Similarly, the vitamin D analog EB1089 upregulates the expression of cystatins E/M, other endogenous cathepsin L inhibitors, in squamous carcinoma cells.[50] These observations prompted us to investigate whether vitamin D could be used to counteract the effects of CTSL upregulation in lamins-deficient cells.

We found that treatment with vitamin D restored 53BP1 protein levels and DNA repair by NHEJ in both cells exogenously overexpressing CTSL and Lmna-KO cells.[32] Furthermore, vitamin D ameliorated genomic instability, decreasing the levels of unrepaired DNA damage (determined as γH2AX foci) as well as the nuclear abnormalities characteristic of lamins-deficient cells. Previous studies in MDA-MB-231 breast cancer cells have shown that the expression of CTSL is inhibited by vitamin D.[51] Interestingly, in our hands vitamin D did not alter the levels of CTSL, but rather inhibited its activity, suggesting a cystatin-mediated mechanism. Therefore, we decided to investigate whether inhibition of CTSL by vitamin D could be mediated by a cystatin

family member. Since there is no mouse ortholog for cystatin D, we performed a promoter analysis for vitamin D responsive elements (VDRE) in the 13 cystatin genes encoded by the mouse genome. We used the Genomatix software and ElDorado mouse genome database. In the initial analysis we selected 4 cystatin genes which contain at least two RXR/VDR heterodimer binding sites (**Fig. 2A**). One of them, cystatin B (also known as Stefin B) contains four VDR responding elements (human cystatin D contains five elements) and is downregulated in lamin deficient-cells. Moreover, treatment with vitamin D increases the expression of cystatin B (**Fig. 2B**), suggesting a role in the regulation of CTSL activity. Nevertheless, additional studies will be necessary to fully determine which cystatin(s) is responsible for the vitamin D-dependent inhibition of CTSL activity.

Conclusions

Our findings support a fundamental role for CTSL in the regulation of cell cycle progression and DNA repair, both in lamin-deficient and -proficient cells. Upregulation of CTSL, a hallmark of a variety of cancers, is directly linked to degradation of Rb family members and 53BP1 and possibly indirectly to transcriptional downregulation of BRCA1 and RAD51 (**Fig. 3**). Thus, inhibition of CTSL activity via treatment with vitamin D or specific inhibitors could represent a novel approach in the management of cancer and other age-related diseases that course with increased genomic instability and defects in DNA repair.

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