Chek2ing out the p53 pathway: Can Puma lead the way?

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Regulation of DNA licensing by targeted chromatin remodeling

Comment on: Wong PG, et al. Cell Cycle 2010; 9:4351–63

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In eukaryotic cells, the initiation of DNA replication is regulated by the formation of the pre-replication complex (pre-RC) on replication origins. Pre-RC is formed by the sequential loading of the origin recognition complex (ORC), licensing factors Cdt1 and Cdc6 and, eventually, the mini-chromosome maintenance (MCM) complex, the presumptive DNA replication helicase.[1] Replication factor Cdt1 accumulates in the nucleus in G_1 phase and plays an essential role in pre-RC assembly by directly recruiting the MCM complex. In early S phase, when replication is initiated, two independent pathways contribute to Cdt1 downregulation. First, Cdt1 is degraded by the ubiquitin-proteasome pathway in a replication-dependent manner.[2] Second, in metazoan, Cdt1 interacts with Geminin, a protein that accumulates in S phase and inhibits the loading of the MCM complex in a dose-dependent manner.[3] This tight regulation of Cdt1 ensures that MCM complex is loaded only during the G_1 phase and that replication occurs only once per cell cycle.

Investigations are currently ongoing to decipher the molecular mechanisms by which mammalian Cdt1 loads the MCM complex. On the one side, Cdt1 physically interacts with several subunits of the MCM complex.[4,5] On the other side, Cdt1 interacts with enzymes involved in the regulation of the chromatin structure, such as, in human cells, histone acetylase HBO1 and deacetylase HDAC11.[6,7] Recent genome-wide analyses also indicate that replication origins are located in a specific chromatin environment in mammalian cells.[8] Yet, whether Cdt1 is involved in chromatin remodeling at origins and whether chromatin remodeling favors MCM loading needs to be addressed.

In a previous issue of *Cell Cycle*, P.G. Wong and colleagues addressed this important issue.[9] Using a powerful in vivo engineered system to monitor targeted chromatin remodeling events, Wong et al. assessed the ability of Cdt1 to induce chromatin remodeling. In brief, a reporter composed of a repeat of Lac-operator (LacO) was used to monitor the impact of LacI-fusion proteins on the surrounding chromatin structure. The fusion protein LacI-Cdt1 is tethered on LacO and induces a strong decondensation of the chromatin, while a fusion between LacI and Cdc6 does not. Importantly, overexpression of Geminin suppresses LacI-Cdt1-induced chromatin decondensation, providing strong evidence that the reporter system can recapitulate Cdt1 function and its regulation by Geminin. In addition, when using cell cycle markers, Wong et al. observed that LacI-Cdt1 foci are decondensed only when cells are in G_1 phase, at the time of pre-RC assembly and condensed in all other phases of the cycle. These observations are the first demonstration that Cdt1 induces chromatin remodeling and enhances DNA accessibility when tethered to the chromatin in G_1. A mutant approach identifies a central domain of human Cdt1 encompassing residues 201-355 as the region necessary for chromatin remodeling. This domain overlaps with the region previously described as essential for cell proliferation, further supporting the idea that chromatin remodelling activity is important for Cdt1 function.[4]

How is Cdt1 controlling chromatin decondensation in G_1? Using their reporter system, Wong et al. demonstrated that histone acetylase HBO1 and deacetylase HDAC11 regulate Cdt1- and cell cycle-dependent chromatin remodeling. In their assay, overexpression of HDAC11 or expression of an HAT-deficient HBO1 suppresses LacI-Cdt1 ability to decondense the chromatin. Thus, HBO1 functions as a coactivator of Cdt1, and HDAC11 functions as a repressor of Cdt1 activity. Consistent with this conclusion, recent reports demonstrated that HBO1 is recruited onto origins during the G_1 phase and that HBO1 acetylase activity is essential to load the MCM complex.[6,10] In contrast, HDAC11 interacts with Cdt1 specifically during S phase when pre-RC formation is prevented.[7] Thus, Wong et al. proposed an appealing molecular model where the cell cycle regulation of the chromatin structure at replication origins relies on the sequential interaction between Cdt1 and HBO1 during G_1 phase and Cdt1 and HDAC11 during S phase. Nevertheless, there is still no evidence that HBO1 and HDAC11 binding are mutually exclusive on Cdt1. Indeed, whether both proteins competitively interact with the same region on Cdt1 or whether post-traductional modifications, such as Cyclin-dependent kinase phosphorylation, regulates their interaction or enzymatic activities during the cycle should be investigated.

Is chromatin decondensation required for MCM loading? Wong et al. observed that LacI-Cdt1 decondensed foci are bound by the MCM complex. On the contrary, in a few cases, LacI-Cdt1, even if present on LacO, could not decondense the chromatin structure, and MCM complex binding could not be detected by immunofluorescence. These important observations indicate that Cdt1 tethering alone is not sufficient to target the MCM complex, and that chromatin decondensation is necessary to load the MCM complex. Nevertheless, decondensation induced by fusion proteins such as LacI-VP16, LacI-p53 or LacI-E2F is not sufficient to recruit the MCM complex. Thus, Cdt1 is somehow necessary to attract the MCM complex onto the chromatin. Whether it is Cdt1 itself or a Cdt1-associated factor remains elusive. Indeed, Cdt1 directly interacts with the MCM complex in cells,[4,5] while HBO1 interacts with Cdt1 and the MCM complex and may physically bridge them together.[6]

Lastly, using their reporter system, Wong et al. investigated the role of Cdt1 repressor Geminin. As already mentioned, overexpression of Geminin suppresses LacI-Cdt1 remodelling activity, providing a possible mechanistic support for Geminin inhibitory function. Wong et al. made two additional observations to reinforce this conclusion. Firstly, Geminin stregthens the physical interaction between Cdt1 and co-repressor HDAC11. Secondly, the Cdt1 region necessary for chromatin remodeling (residues 201–355) is also a part of the Geminin interaction domain.[4]

Therefore, in S phase, Geminin blocks Cdt1-dependent chromatin decondensation by masking residues important for this activity.

P.G. Wong and colleagues established that Cdt1-dependent chromatin remodeling is essential prior to MCM complex loading in G_1 and that Geminin prevents Cdt1-dependent chromatin remodeling. Future work should assess the role of chromatin in regulating MCM loading. Is DNA accessibility sufficient for efficient MCM loading? Alternatively, are there specific histone marks deposited by Cdt1 coactivators and read by specific DNA replication factors? Finally, one should investigate whether the interaction between MCM and Cdt1 is controlled by post-translational events triggered by Cdt1 and its co-activators. The elegant cell culture system described by Wong et al. should make these investigations easier. It also opens new avenues for researchers to design genome-wide screens aimed at the characterization of new regulators of pre-RC assembly.

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Targeting Plk1 in cutaneous T-cell lymphomas (CTCLs)

Comment on: Nihal M, et al. Cell Cycle 2011; 10:1303–11

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Cutaneous T-cell lymphoma (CTCL) is a rare type of non-Hodgkin lymphoma, which is a cancer of lymphatic system. CTCLs are characterized by the uncontrolled growth of a type of white blood cells called T cells. Mycosis fungoides (MF) and Sezary syndrome (SS) are two common CTCLs, and both patients suffer with skin defects. The 5-year survival rate of the patients diagnosed as CTCL in early stage is 97%, but it was decreased to 41% in patients with an advanced stage of the disease.[1] Different therapies, including chemotherapy and ionizing radiation, were applied but with limited success. Target-based approaches are urgently needed to develop novel diagnostic markers and therapeutic methods. One recent study by Nihal et al. showed that Polo-like kinase (Plk1), a critical regulator in many cell cycle-related events, is overexperessed in the CTCL patients. Moreover, genetic knowdown of Plk1 by RNAi or enzymatic inhibition of Plk1 by a small molecule inhibitor was shown to result in a significant inhibition of CTCL cell growth.[2] This groundbreaking work suggested that overexpression of Plk1 might be a novel prognostic marker and that targeting Plk1 in CTCLs could be a promising therapeutic strategy for CTCL treatment.[2]

Plk1 is a serine-threonine kinase, which has been characterized as a critical player in many cell cycle-related events, such as mitotic entry, bipolar spindle formation, sister chromatid segregation and cytokinesis.[3] Accumulating data suggest that Plk1 acts as a coordinator to couple cell cycle progression and the DNA damage checkpoint pathways, specifically, by silencing the checkpoint signal to promote cell proliferation.[3] Interestingly, Plk1 overexpression is found not only in various carcinomas, such as melanomas, ovarian cancer, non-small cell lung cancer, head and neck carcinoma and gastric cancer, but also in several neoplasms including lymphomas/leukemias.[4] It has been proposed that Plk1 is an attractive target for treatments of carcinomas and neoplasms, but further validation is urgently needed.

In the study reported by Nihal et al., the expression of Plk1 both in protein and transcript levels were shown to increase in multiple CTCL cell lines, including HH, Hut78, MyLa, SeAX and SZ4. This finding is consistent with their immunohistochemical study showing that Plk1 expression was elevated in the advanced lesions from patients with CTCL.[5] More significantly, the authors evaluated the effects of Plk1 inhibition on cell viability, growth and proliferation of CTCL cells. They found that Plk1 inhibition via RNAi or GW843682X (a small molecule Plk1 inhibitor) treatment dramatically reduces the cell viability, slows down the growth and decreases the proliferation activity of these cell lines. Mechanistically, the authors demonstrated that Plk1 inhibition in these cell lines results in a G_2/M cell cycle arrest, which is consistent with the critical function of Plk1 for mitotic entry. Further, they found that the levels of Cyclin B1 and Cdc25C proteins are increased upon Plk1 inhibition, supporting the G_2/M arrest phenotype. Mitotic abnormalities were also observed in Plk1-inhibited CTCL cells, namely, the formation of monopolar spindle. These phenotypes provide a solid mechanism to support the notion that reduced cell viability, slow growth and decreased proliferation of CTCL cells are due to the inhibition of Plk1. Being cautious enough, the authors followed the fate of G_2/M arrested cells by Plk1 inhibition, and they found that these cells underwent apoptosis by FACS analysis and cleaved PARP immunoblotting analysis.

In summary, Plk1 was predicted to be a novel prognostic marker for CTCL, and Plk1 inhibition might be a good strategy to treat CTCL.

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Chek2ing out the p53 pathway: Can Puma lead the way?

Comment on: Kabacik S, et al. Cell Cycle 2011; 10:1152–61

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Three decades of intensive research on the p53 tumor suppressor have simplified some fundamental issues regarding human cancer. Cancer is a general term referring to many distinct neoplastic diseases, yet, remarkably, it has become clear that most if not all tumors harbor disturbances in the p53 pathway.[1] The price of discovering this unifying defect is the ever more complex picture we are acquiring on the biological processes that p53 has a hand in.[2] Some of these activities are likely to have a direct impact on protecting us from tumorigenesis, such as p53's role in inducing senescence, apoptosis or cell cycle arrest, whereas the potential impact on cancer of other more recently discovered biological processes governed by p53, such as autophagy, immune response and metabolism may be more difficult to pinpoint.

Given the importance of p53 in protecting us from cancer, it comes as no surprise that a germline mutation in *TP53* or the genes controlling expression or activity of the protein increases an individual's risk of cancer if the mutation causes loss of function. Copy number alteration in major genes of the pathway, or intragenic deletions and nonsense mutations are predictably detrimental, but other variants or mutations that could debilitate the p53 pathway, such as SNPs in promoter regions of *TP53* or its regulators (**Fig. 1**), may be more cumbersome to find or more difficult to distinguish from innocuous sequence variation. On the researcher's wish list is surely a crystal ball that would simply tell us straightaway whether an individual has a properly functioning p53 pathway, without resorting to extensive DNA sequencing or, in the case of newly identified mutations, conducting experiments to assess phenotypic consequences of the sequence variant. A simple measure of p53 pathway functional integrity despite complexities of its regulatory network and the numerous activities that p53 performs would have potential as a biomarker of risk, and as a research tool for geneticists.

Since p53 is a transcription factor, its ability to transactivate or repress the p53 downstream genes is a logical starting point for developing an assay of p53 pathway status overall, yet here too, the complexity is

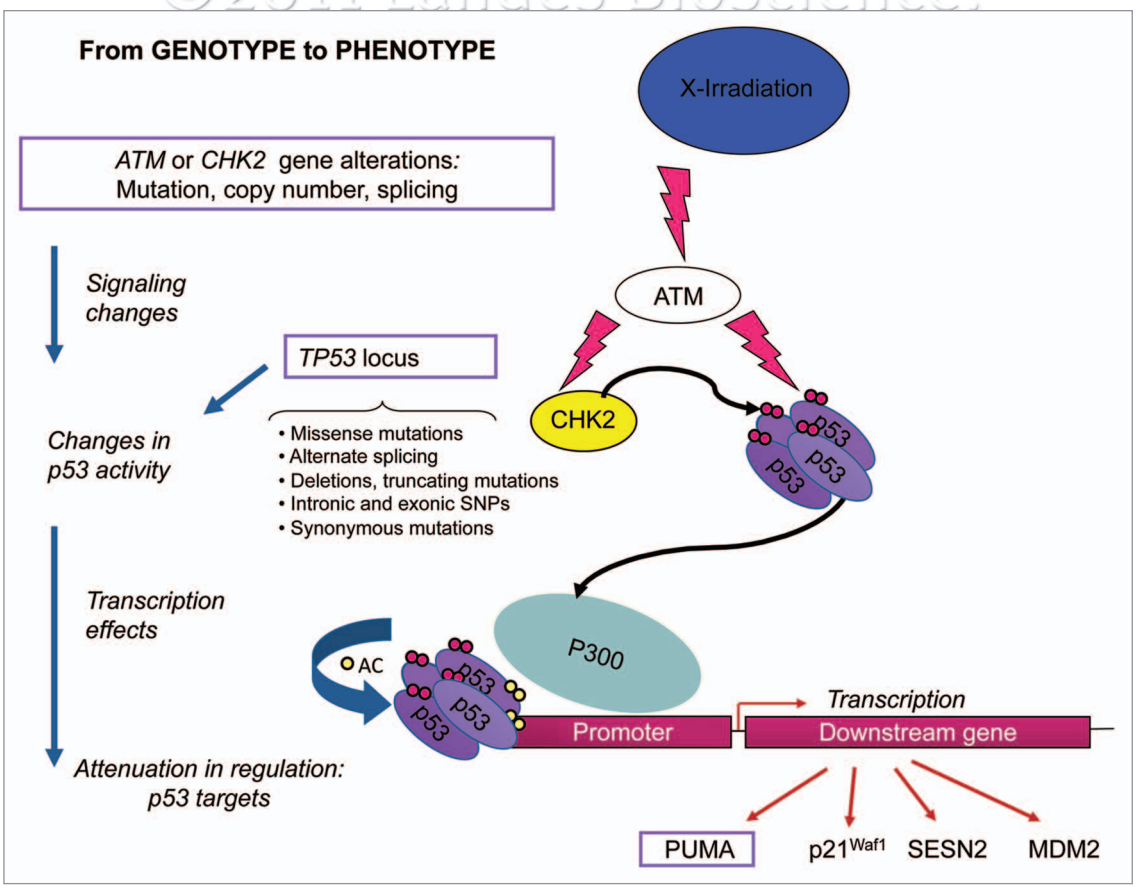

Figure 1. Various genetic alterations in the p53 pathway can result in attenuation of transcriptional responses to DNA damage.

daunting. The repertoire of p53-modulated genes is large, so which of the several hundred downstream genes is the best monitor? Or should we assess the whole transcriptome with high throughput technology? P53 influence on the transcriptome is affected by the tissue and cell type,[3] so what cell type should we use to identify individuals at higher risk of cancer because of genetic alterations in p53 pathway genes?

In a recent issue of *Cell Cycle*, Kabacik and colleagues addressed these challenges.[4] They used a multiplex RT-PCR-based procedure to assess the ability of ex vivo ionizing radiation (IR) to induce expression of three known p53 target genes, *CDKN1A (p21)*, *BBC3 (PUMA)* and *SESN2*, in blood samples, and showed that the transcriptional response to IR, particularly of *PUMA*, is severely compromised in blood samples from mice lacking both copies of either Atm, Chek2 or Trp53 itself. Haploinsufficiency results in an intermediate induction of *PUMA* that is still considerably lower than in samples from wild-type mice. The authors propose that the blood test can be used as a simple assay to identify individuals with deficient p53 pathway activity and support this by testing blood samples from cancer-prone individuals with inherited alterations in the *ATM* or *TP53* genes. As anticipated, samples from AT patients and AT carriers (who harbor *ATM* mutations) as well as from patients with the Li-Fraumeni cancer syndrome (carrying *TP53* mutations) showed an attenuated expression of PUMA in response to ex vivo irradiation.

Amongst the hundreds of genes upregulated by p53, the *BBC3* (BCL2-homology domain-3 BH3 only) gene, more commonly referred to as *PUMA*, is a pro-apoptotic gene that appears to be of central importance in executing p53's role as a tumor suppressor. There is a tight correlation between frequency in human tumors of a given p53 mutant and loss of the mutant's ability to induce *PUMA*.[5] The ability of cells to program their own extinction under conditions that threaten genetic integrity depends on Puma[6] and its molecular collaborators. If the p53 pathway function can be conveniently gauged by IR induction of *PUMA*, it will be interesting to see whether this simplified screening approach will help to reveal p53 pathway deficiencies conferred by certain somatic tumor mutations, naturally occurring intronic polymorphic mutations,[7] or germline SNPs previously thought to be phenotypically silent (**Fig. 1**).

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RB pathway and therapeutic sensitivity: New insights in breast cancer and Tamoxifen therapy

Comment on: Lehn S, et al. Cell Cycle 2011; 10:956–62

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The treatment of approximately 70% of all breast cancers is influenced by the dependence of such tumors on estrogen receptor (ER) activity.[1-3] ER-dependent cancers are currently treated by modulation of estrogen levels (e.g., letrazole), the use of selective estrogen receptor modulators (e.g., tamoxifen) or estrogen receptor antagonists (e.g., fulvestrant). These therapies are generally effective for ER-positive breast cancers, but recurrence and ultimately therapeutic failure are major clinical problems.[1,4] The recent study by Lehn and colleagues demonstrates that disruption of the retinoblastoma tumor suppressor (RB) pathway is associated with resistance to tamoxifen.[5]

The retinoblastoma tumor suppressor is a critical negative regulator of cell cycle that is functionally inactivated in a significant fraction of human cancers.[6,7] Inactivation of RB can occur via multiple independent events in breast cancer; however, such inactivation is reliant on two simple principles. First, RB growth-suppressive functions are attenuated by phosphorylation. Consonantly, RB phosphorylation is requisite for efficient cellular proliferation in all studied tissues. In tumors, aberrant phosphorylation of RB is a consequence of deregulated CDK/cyclin activity and is frequently driven by CDK4/cyclin D1. Cyclin D1 amplifications occur in approximately 15% of breast cancers, and overexpression of cyclin D1 is a particularly common feature of ER-positive disease.[8] Second, RB function has been observed to be compromised in a subset via mutation, genetic loss, epigenetic silencing and perhaps disruption of downstream signaling.[6] In such contexts, failure to phosphorylate the RB protein is typically observed and is attributed in part to the fact that common, tumor-associated point mutations of RB compromise its ability to serve as a CDK/cyclin substrate.[9] Additionally, tumors with RB loss typically express very high levels of the CDK4/6 inhibitor p16ink4a.[10,11]

A recent study published in *Cell Cycle* exploited RB phosphorylation status as a means to define tumors that have lost normal RB-pathway control. By focusing on tumors that are phospho-RB-negative yet retain high proliferation rate, the study defined a specific subtype of tumor that either lacks RB or exhibits some other dysregulation in the RB pathway that enables proliferation in the absence of RB phosphorylation. The ability of this approach to define RB-pathway status is complementary to total RB staining or other surrogates of RB-pathway status, such as p16ink4a levels.[10] In each context, the proliferation marker (Ki67) is used for discriminating indolent tumors from those with inactivation.[5,10]

Consistent with prior studies, the method used here by Leah and colleagues finds that disruption of the RB pathway is predominant amongst ER-negative breast cancers. Specifically, loss of RB function is associated with ~40% of ER-negative cancer as opposed to ~5% of ER-positive cases. In ER-positive disease, the loss of RB-pathway function was associated with a lack of clinical benefit with tamoxifen. Although the overall size of the patient population was small, the results were significant in multivariate analyses, thus reinforcing the concept that RB loss portends

an altered therapeutic response to tamoxifen. Interestingly, in this cohort RB status was not associated with prognosis in either ER-positive or ER-negative cancers, thus suggesting that the role of RB could be relevant for outcomes associated with therapy yet holds less significance in predicting overall disease course.

This study represents an important extension of prior work in this area by showing that direct effects on RB status impact the efficacy of tamoxifen therapy. This concept is supportive of preclinical studies and the analyses of gene expression signatures indicative of RB deficiency.[11-14] Furthermore, recent work in mouse models showed that RB loss can contribute to the development of ER-positive breast cancers that harbor molecular characteristics of the luminal B subtype.[15] Such models could be particularly relevant for determining how alterations in RB-pathway function contribute to ER-positive breast cancer and tamoxifen resistance.

Now that specific dysfunction in the RB-pathway has been identified in select patient populations, the key question remains: what is the underpinning mechanism that leads to RB pathway disruption in ER-positive tumors? Work from Perou and colleagues suggests that LOH in the Rb1 gene is of likely consequence in luminal B breast cancers that exhibit poor response to endocrine therapy.[13] Similarly, recent studies demonstrated that genetic loss at 13q14 was associated with lack of RB protein and progression to resistance to hormone therapy in prostate cancer.[16] Therefore, whether tumors that harbor pathway dysregulation, as identified by Lehn and colleagues, actually harbor genetic Rb1 inactivation should be determined. Such analyses in ER-positive breast cancer could close the loop and demonstrate a specific pathological impact of RB genetic inactivation on the response to tamoxifen therapy.

The important findings of Lehn and colleagues related to RB-pathway dysfunction and tamoxifen response will yield a renewed interest in determining how cell cycle deregulation modulates therapeutic response in ER-positive breast cancer and provide the seed for novel approaches of intervention to yield more efficacious treatment.

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Targeting expression or function of Plk1 in CTCL, that is a question

Comment on: Nihal M, et al. Cell Cycle 2011; 10:1303–11

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Cutaneous T-cell lymphoma (CTCL) is a class of non-Hodgkin lymphoma that forms skin lesions due to the accumulation of malignant T-helper cells in the skin.[1] The main drugs that currently approved by FDA for CTCL treatment target retinoid X receptors (Bexarotene)[2] or histone deacetylase (Vorinostat, Romidepsin).[3] In a previous issue of *Cell Cycle*, M. Nihal et al. reported a new potential target, polo-like kinase 1 (Plk1), for treating CTCL.[4] Plk1 plays a key role in regulation of mitotic entry of proliferating cells. An increased expression of Plk1 was found in many cancer cells,[5-7] including CTCL cells,[8] which was believed to lead to an accelerated proliferation of cancer cells. In this study, M. Nihal et al. systematically analyzed the expression and function of Plk1 in regulating the fate of CTCL. They demonstrated that Plk1 was dramatically increased at both the mRNA and protein levels in CTCL. The genetic knockdown or functional inhibition of Plk1 led to an increase of cell cycle regulators cyclin B1 and cdc25c. The reduction of Plk1 also results in an increase of mitotic error accompanied with G_2/M phase cell cycle arrest and apoptosis of CTCL. These results suggest that targeted inhibition of Plk1 can potentially be used to treat CTCL.

In normal cells, Plk1 expression is tightly regulated at transcriptional and translational levels during the cell cycle progression.[9] Interestingly, M. Nihal et al. showed that the increase of Plk1 protein was not always proportionally correlated to the increase of its mRNA in different CTCL cell lines. Genetic knockdown of Plk1 had only minor effects on Plk1 protein level, cell proliferation and/or apoptosis of certain CTCL cell lines. These results indicate that the accumulation of Plk1 in CTCL could be a result of coordinative regulation of Plk1 expression at transcription, translation and/or post-translation levels. All tested CTCL cell lines showed an increased apoptotic death in a dose-dependent manner in response to a potent ATP-competitive inhibitor of Plk family members (Plk1 and Plk3). However, the sensitivity towards the inhibitor varied from cell line to cell line. In addition, side effects for using an ATP-competitive inhibitor is often a problem due to non-specificity of the drug. These findings lead to a conclusion that, while the inhibition of Plk1 can be potentially used to treat CTCL, how to target it could be a question to ask before treatment. An analysis of the mechanism for Plk1 accumulation for each case of CTCL may provide guidance and improve efficacy for treating CTCL using Plk1 as a target.

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CDK4: Regulatory functions related to lymphocytes

Comment on: Chow YH, et al. Cell Cycle 2010; 9:4922–30

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Cyclin-dependent kinase 4 (Cdk4) is an important component of cell cycle activation, with critical roles in embryogenesis, homeostasis and cancer. In cancer and cardiovascular disease, Cdks, including Cdk4, are considered potential therapeutic targets that function independent of cell cycle regulation.[1,2] Chow et al. demonstrate that Cdk4$^{-/-}$ mice have a hypoplastic thymus and an increase in immature CD4/CD8 double negative thymocytes.[3] Thymocytes from Cdk4$^{-/-}$ mice, but not splenocytes, exhibit decreased adhesion to the endothelial cell matrix and fibronectin. The authors speculate that lack of Cdk4 impairs the ability of thymocytes to migrate to fibronectin and affects maturation of thymocyte, since T-cell development in the thymus requires migration from the cortex to the fibronectin-rich thymic medulla (**Fig. 1**). They also address the role of Cdk4 in a model of pulmonary allergic (ovalbumin) inflammation. In a previous study, analysis of a bleomycin-induced acute lung injury model indicates that Cdk4$^{-/-}$ mice exhibit impaired recruitment of lymphocytes to the lung.[4] In contrast, in this study, analysis of an allergic model suggests that Cdk4 is not critical for lymphocyte recruitment to the lung. The authors suggest that the different findings may be due to the different cell types in recruitment mediated by Cdk4. For example, the cellular response in acute lung injury may be due to hematopoietic cells, and, in an allergic model, it may be due to structural cells. Differences in rate of exposure and timing are also important considerations. These data support the concept that Cdk4 may play important roles in thymocyte maturation and in innate (acute lung injury), but not necessarily adaptive (allergen), models of inflammation.

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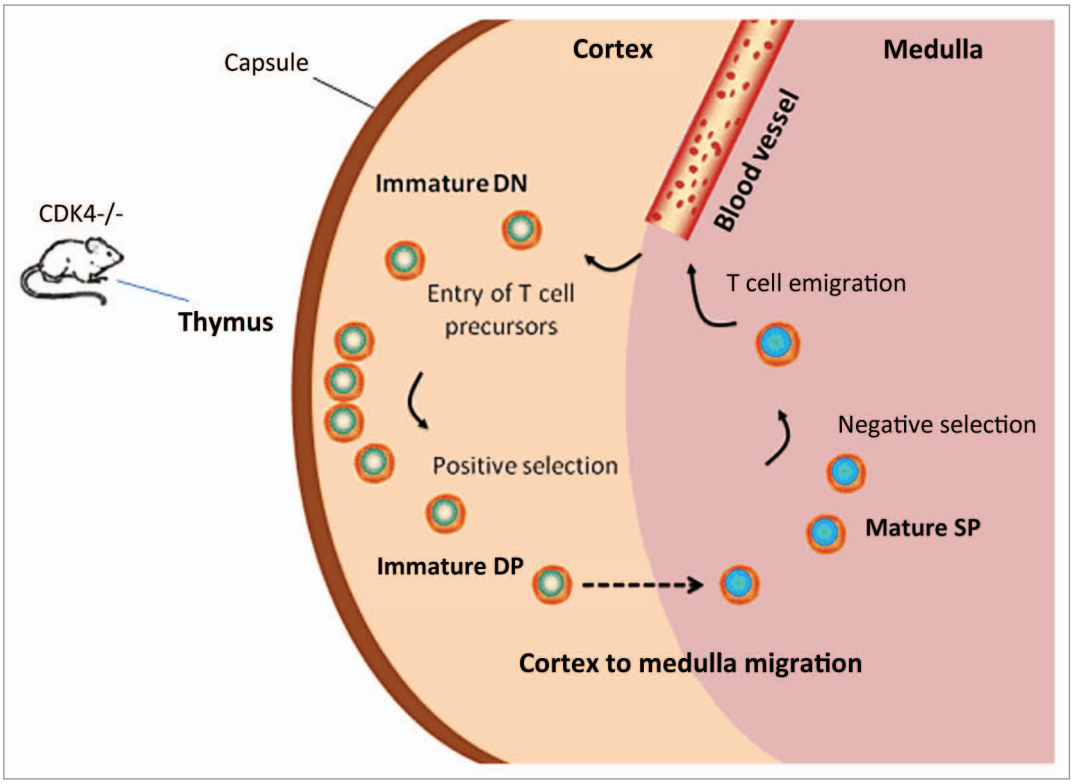

Figure 1. Maturing thymocytes. The thymus is broadly divided into two histologically defined regions, the cortex and the medulla. T cell precursors enter the thymus at the cortico-medullary junction to begin a differentiation program. The cortex contains immature CD4/CD8 double negative (DN) T cells and CD4/CD8 double positive (DP) T cells. The T cells must migrate through the cortex into the medulla to differentiate into mature either CD4 or CD8 single positive (SP) T cells. Positive selection occurs in the cortex whereas negative selection occurs in the medulla. SP cells that have completed the differentiation program emigrate from the medulla to the periphery. Thymocytes from Cdk4$^{-/-}$ mice exhibit inability to migrate from the cortex to the fibronectin-rich medulla and thus to differentiate into mature T cells.