様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

平成　３０　年　　９　月　１１　日現在

機関番号：　１３３０１
研究種目：　基盤研究(C)（一般）
研究期間：　2015～2017
課題番号：　１５Ｋ０６８３４

研究課題名（和文）Identification of global epigenetic alterations indispensable for malignant transformation and mediated by the RB-ATM pathway

研究課題名（英文）Identification of global epigenetic alterations indispensable for malignant transformation and mediated by the RB-ATM pathway

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交付決定額（研究期間全体）：（直接経費）　　3,900,000円

研究成果の概要（和文）：コアリプログラミングタンパク質が分解される過程において、これらがどのように認識され、しかるべき'場'にリクルートされるかは不明である。本研究では、RBとATMの相互作用が、リジンアセチルトランスフェラーゼ-3b（Kat3b）のリクルート、アセチル化したコアリプログラミングタンパク質Oct3 / 4、Sox2、Klf4、NanongおよびC-Myc（OSKNM）の認識、ユビキチン化複合体への組み込みと、その後のプロテアソーム分解を介して、幹細胞様集団の多能性および自己再生能を抑制することを実証した。これらの知見は、再生医療、神経変性疾患および癌において重要な意味を有すると考えられる。

研究成果の概要（英文）：How the core reprogramming proteins are identified and recruited for degradation is unknown. Here, we demonstrate that functions of the retinoblastoma (RB) and the ataxia telangiectasia mutated (ATM) repress the pluripotency and self-renewal ability of the stem cell-like cells included in genetically modified mouse embryonic fibroblasts (MEFs) and A-T human adult fibroblasts (A-T HAFs) through acetylation-driven ubiquitination and subsequent proteasomal degradation of Oct3/4, Sox2, Klf4, Nanog and c-Myc (OSKNM) proteins. We discovered that RB recruits lysine acetyltransferase-3b (Kat3b) and inhibits the transcription of histone deacetylase-5 (Hdac5) whereas, ATM shuttles Hdac5 into the nucleus and serve as adaptor protein, which identify and assemble the acetylated-OSKNM proteins into ubiquitination complexes with the E3 ubiquitin ligase Uhrf1 or Fbxw7. These novel findings have important implications in regenerative medicine, neurodegenerative diseases and cancer.

研究分野：　Stem cell biology

キーワード：　RB　ATM　KAT3B　HDAC5　Proteasome　Reprogramming proteins

１．研究開始当初の背景

Several experimental methods have been successfully established for the generation of induced pluripotent stem (iPS) cells (Takahashi and Yamanaka, 2006; Wernig et al., 2007). However, genomic integration and low efficiency (0.01% ~ 0.1%) are major hurdles in clinical trials. Although adenoviral (Zhou and Freed, 2009) and episomal (Stadtfeld et al, 2008) vectors facilitate transient expression of the reprogramming genes without genomic integration, the efficiency is very low (~ 0.0006%) and they are not completely free from disruption of the host genome. The proteasome-mediated degradation of the core reprogramming proteins directly impacts pluripotency (Buckleyet al., 2012; Strikoudis et al., 2014; Suresh et al., 2016); and the protein-based iPS (p-iPS) cells are thought to be safe resources in regenerative medicine. However, the core reprogramming proteins are unstable and have very short half-life (90 minutes for Oct3/4; 120 minutes for Klf4 and Nanog; and 20 minutes for c-Myc). Therefore, identification of the molecular switch targeting the core reprogramming proteins for degradation is necessary for developing novel p-iPS technology for clinical trials.

On the other hand, it is widely believed that cancer is initiated and sustained by a tiny subset of tumor cells (0.1 ~ 1%) called cancer stem cells (CSCs) (Bonnet and Dick, 1997; Lapidot et al., 1994). The CSCs exhibit the ability of self-renewal and differentiation potential similar to normal adult stem cells; and are associated with drug resistance and relapse in cancer patients (Visvader and Lindeman, 2012; Beck and Blanpain, 2013). In addition, neural stem cells (NSCs) depletion has been considered a major mechanism of the impaired neurogenesis in neurodegen- erative diseases (Encinasa and Sierra, 2012). Hence, understanding the molecular pathology of the stem cell protein turnover in cancer and neurodegenerative diseases is also important for establishing new therapeutic rationales for these incurable diseases.

Previous studies have linked Rb and ATM functions to the *Ubiquitin- Proteasome-System* (*UPS*) (Binne et al., 2007; Assoian and Yung, 2008; Matsuoka et al., 2007; Mu et al., 2007). Indeed, we previously reported that the ATM-mediated DNA damage response (DDR) is a prerequisite for the Rb loss-induced senescence (Shamma et al., 2009); and Rb/ATM-deficient cells escape cellular senescence through DNA methyltran- sferase1 (DNMT1) protein stabilization and DNA methylation silencing of tumor suppressor genes (Shamma et al., 2013). In the current paper, we extended these findings; and screened proteins that are known as or predicted to be UPS substrates; and found that mutual functions of RB and ATM are crucial for the proteasome-mediated degradation of several UPS substrates; and interestingly the OSKNM proteins were the most dynamically regulated substrates by this pathway. Therefore, we focused on the RB/ATM functions in proteasomal degradation of the OSKNM proteins; and determined the significance of these novel functions of RB and ATM in pluripotency and self-renewal ability.

２．研究の目的

The main objective of this study was Identification of global epigenetic alterations indispensable for malignant transformation and mediated by the RB-ATM pathway; however due to the high cost of WGBS-Seq and RNA-Seq, we focused on the UPS pathway and tried to identify important substrates targeted by the RB/ATM functions for the proteasome- mediated degradation and determine the significance of these novel functions of RB and ATM in pluripotency and self-renewal ability.

３．研究の方法

- Immunohistochemistry
- Immunofluorescence
- BrdU incorporation
- Subcellular fractionation
- Cycloheximide-chase assay
- Immunoprecipitation
- Immunoblotting
- Sphere formation assay
- Sphere limiting dilution analysis
- Differentiation in xenograft
- MALDI-TOF/TOF mass spectrometry
- RT-qPCR

４．研究成果

Mutual loss of Rb and ATM stabilizes the OSKNM proteins

We determined the rate of protein degradation and the half-life ($t_{1/2}$) of several UPS substrate proteins by the cyclohexamide (CHX) chase assay analysis of *Rb*/*ATM* double knockout (DKO) MEFs stably expressing empty vector or vectors expressing pRB, ATM or both ATM and pRB. The OSKNM proteins were highly abundant, relatively stable and readily detectable in Rb/ATM-deficient MEFs. The Oct3/4, Klf4, Nanog and c-Myc protein half-life was significantly shortened in the RB reconstituted MEFs (2.3 ~ 1.0hr, 5.2 ~ 0.8hr, 4.7 ~ 0.7hr and 1.7 ~ 0.4hr); whereas the Sox2 protein half-life was unexpectedly prolonged in these MEFs (1.5 ~ 20.0hr) compared with the control. However, the half-life of the OSKNM proteins was significantly shortened in the ATM reconstituted MEFs (2.3 ~ 0.7hr, 1.5 ~ 0.7hr, 5.2 ~ 2.1hr, 4.7 ~ 1.3hr and 1.7 ~ 0.3hr) compared with the control. Interestingly, the half-life of the OSKNM proteins was not significantly changed in MEFs reconstituted with both ATM and RB compared with MEFs reconstituted with ATM alone except the Klf4 half-life was significantly shortened in MEFs reconstituted with both ATM and RB (2.1 ~ 1.0hr) similar to MEFs reconstituted with RB alone (5.2 ~ 0.8hr) indicating that Klf4 degradation is largely mediated by RB functions. We also observed that RB or ATM reconstitution into Rb/ATM DKO MEFs significantly increased the protein degradation rate of cyclin D1 and cyclin E. Taken together, these data indicate that RB and ATM are major regulators of the UPS pathway and suggest that degradation of the core reprogramming proteins and oncogenes might be a novel mechanism of the RB/ATM tumor suppressor functions.

Inhibition of the proteasome cancels the RB/ATM functions in the UPS

We next performed proteasome block-release analysis; and determined the amounts of protein rescued after inhibition of the proteasome and the protein degradation resume after release from proteasomal inhibition. The abundances of the OSKNM proteins were significantly decreased in Rb/ATM DKO MEFs reconstituted with RB, ATM or both ATM and RB compared with the control; and inhibition of the proteasome with MG132 recused the OSKNM proteins in these MEFs to levels comparable

with the controls. Notably the amounts of the rescued Klf4 and c-Myc proteins were significantly higher in the MG132-treated control than the untreated control MEFs suggesting that factors other than RB and ATM also mediate the Klf4 and c-Myc proteins degradation. Importantly, release of the RB reconstituted MEFs from proteasomal inhibition allowed moderate degradation of KLF4 and c-Myc proteins; however, the Oct3/4, Sox2 and Nanog proteins degradation was not resumed in these cells within 6 hours after release from proteasomal inhibition. On the other hand, release of the MEFs reconstituted with ATM alone or both ATM and RB from proteasomal inhibition provoked complete degradation of the Oct3/4, Sox2 and Nanog proteins as well as moderate degradation of the Klf4 and c-Myc proteins within the same period of time after release from proteasomal inhibition, suggesting a quick and strong effect of ATM in proteasomal degradation of the Oct3/4, Sox2 and Nanog proteins. We observed similar results of the proteasome block-release analysis of cyclin D1 and cyclin E. Immunofluorescence (IF) studies also indicated that the frequency of the Oct3/4-, Klf4-, Nanog- or c-Myc-positive cells was significantly decreased in the RB reconstituted MEFs whereas the frequency of Sox2-positive cells was not significantly changed in these MEFs compared with the control. On the other hand, the frequency of the cells stained positive for any of the OSKNM proteins was markedly decreased in the ATM reconstituted MEFs compared with the control. Treatment of the RB or ATM reconstituted MEFs with MG132 rescued the OSKNM proteins; and importantly, the rescued proteins were mainly localized in the cytosol of these MEFs; however, the subcellular localization of the OSKNM proteins was not significantly changed in the MG132-treated control MEFs compared with the untreated control. Consistent with the immunoblotting (IB) results, Klf4 and c-Myc proteins were further accumulated in the MG132-treated control MEFs compared with the untreated control; however, this was not the case with Oct3/4, Sox2 or Nanog proteins. These data indicate that ATM is a major regulator of proteasomal degradation of the Oct3/4, Sox2 and Nanog proteins, whereas RB cooperates with ATM and other unknown factors in targeting KLF4 and c-Myc proteins for proteasomeal degradation.

RB is well known as a transcriptional co-regulator through suppression of E2F transcription factors (Dick and Rubin, 2013). On the other hand, ATM is known as a major component of the DDR pathway; however, ATM can also function as a transcriptional regulator through phosphorylation and stabilization of the transcription factor Trp53 (Kastan et al., 1992); and has an important role in protein synthesis through phosphorylation of eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) (Alexander et al., 2010). To explore the roles of Rb and ATM in transcriptional regulation of the OSKNM genes, we performed real-time quantitative reverse transcription PCR (RT-qPCR) analysis of primary MEFs (passage #2) depleted from Rb or ATM with specific shRNA. We found that Rb depletion significantly increased the relative expression levels of the OSN genes as previously described (Kareta et al. 2015); whereas depletion of ATM significantly increased the relative expression levels of OSK genes in these MEFs indicating novel ATM functions in transcriptional repression of the core reprogramming factors. Furthermore, we used *in vivo* global protein stability (GPS) reporter system (pGPS-LP) (Emanuele et al., 2011) expressing a single transcript encoding DsRed and EGFP-ORFs separated by an internal ribosome entry site (IRES). Using this reporter system, we expressed DsRed and EGFP-KLF4 or DsRed and EGFP-c-MYC in human adult fibroblasts derived from patient with A-T syndrome (A-T HAFs; ECB); and determined the GPS (EGFP/DsRed ratio) of the KLF4 and c-MYC proteins in live cells. We found that RB depletion from ECB cells significantly increased the GPS of KLF4 and c-MYC proteins; however, re-expression of ATM into the RB-depleted ECB cells markedly decreased the GPS of the KLF4 and c-MYC proteins. Taken together, these results indicate that RB and ATM regulate the OSKNM proteins at transcriptional and posttranslational levels and suggest that these mutual functions of RB and ATM particularly in the proteasome-mediated degradation of the OSKNM proteins might be a major limiting mechanism of the pluripotency and self-renewal ability.

Mutual loss of RB and ATM promotes pluripotency and self-renewal ability

We next tried to determine the significance of the RB/ATM-mediated proteasomal degradation of the OSKNM proteins in pluripotency and self-renewal ability in normal MEFs, genetically modified MEFs, A-T HAFs and cancer lines. We developed a three-dimensional (3-D) stem cell culture system for *in vitro* enrichment and expansion of the stem-like populations included in these cells. We found that cells simultaneously lacking RB and ATM are highly competent with sphere formation; and further investigations indicated that simultaneous germ-line inactivation of Rb and ATM in MEFs enabled unlimited sphere formation ability under 3-D or 2-D stem cell culture conditions; however, re-expression of pRB, ATM or both ATM and pRB in these MEFs markedly attenuated the sphere formation ability after 4 cycles of sphere dissociation and sub-culturing under stem cell culture condition. In addition, the cells contained in the primary or secondary spheres generated from Rb/ATM-deficient MEFs express pluripotency markers including Oct3/4, Sox2, Klf4, Nanog, c-Myc and stage specific embryonic antigen 1 (Ssea1) even after 4 cycles of dissociation and sub-culturing. The frequency of the pluripotency markers was significantly increased in the cells contained in the spheres compared with the parental MEFs cultured under normal culture condition indicating that the *Rb*/*ATM*–deficient MEFs contain stem cell-like populations that have the ability of self-renewal; and hence became enriched under our stem cell culture conditions. Interestingly, inhibition of the proteasome with MG132 bypassed these functions of RB and ATM; and rescued the sphere formation abilities of Rb/ATM DKO MEFs reconstituted with pRB or ATM.

The pluripotent stem cells have unlimited capacity of self-renewal (Evans and Kaufman, 1981), and theoretically a single stem cell should be able to generate a sphere-like structure under stem cell culture conditions. To test this hypothesis, we performed sphere limitation dilution analysis (SLDA); and found that a single cell derived from the secondary spheres that were generated from Rb/ATM-deficient MEFs is able to form a sphere-like structure under stem cell culture conditions with a frequency of about 1 in 250 cultured cells ($y = -1$ intercept $x = 250$); and the number or the size of these

spheres is tightly correlated with the number of the plated cells. In addition, xenograft models indicated that stem cells are capable of differentiating into derivatives of all the three primary germ layers: ectoderm, mesoderm, and endoderm (Martin, 1981; Thomson et al., 1998). We also tested this possibility and found that Rb/ATM-deficient MEFs expressing EGFP as well as secondary spheres prepared from these MEFs generate tumors with no evidence of metastasis within 4 weeks after subcutaneous transplantation into NSG (NOD.Cg-Prkd-cscid-Il2rgtm1Wjl/SzJ) mice. Analysis of serial tissue sections from these tumors with hematoxylin and eosin staining (H & E) and immunohistochemistry (IHC) indicated that these tumors contain several structures of all the three embryonic germ layers including neurons stained positive with bIII tubulin, smooth muscles stained positive with anti-smooth muscle actin (aSMA) and glandular epithelium stained positive with anti-cytokeratin18 (KRT18) without histological signs of malignancy indicating that these tumors have histological features similar to teratoma. Notably, the EGFP signal was detected in the structures of all the three embryonic germ layers indicating that these teratomas are originated from the transplanted cells. Interestingly, the size and the frequency of terataoma formation from spheres were significantly higher (5 out of 5 trials) than the parental MEFs (2 out of 5 trials). Importantly, restoration of pRB, ATM or both ATM and pRB into Rb/ATM-deficient MEFs markedly attenuated teratoma formation by these MEFs (0 out of 5 trials).
To exclude the possibility of clonal defects in Rb/ATM DKO MEFs, we analyzed normal MEFs depleted from Rb, ATM or both Rb and ATM with specific shRNA. We found that simultaneous depletion of Rb and ATM markedly and mutually increased the abundances of OSKM proteins and enabled sphere formation in two different lines of MEFs. However, the OSKM proteins were not mutually detected and the spheres formation was not observed in these two lines of MEFs after depletion of either Rb or ATM alone. These consistent cellular responses to restoration or depletion of Rb and ATM exclude the possibility of clonal defects in the genetically modified MEFs and support our hypothesis that Rb and ATM exert important mutual functions in antagonizing pluripotency and self-renewal ability. Furthermore, depletion of ATM from *Rb* KO MEFs or depletion of RB from A-T HAFs with specific shRNA enabled sphere formation in these MEFs under 2-D or 3-D stem cell culture conditions. Interestingly, re-expression of ATM in the RB-depleted A-T HAFs markedly attenuated the sphere formation ability of these MEFs. Taken together, these data indicate that the RB/ATM-deficient mouse and human fibroblasts contain stem cell-like pools that have the ability of self-renewal under stem cell culture conditions owing to stabilization of the OSKNM proteins that maintain the undifferentiated state induced by simultaneous inactivation of RB and ATM; and these stem cell-like pools have *in vivo* developmental potential of differentiation into teratoma-like structures in xenografts; and hence achieved the state of pluripotency.

Cell cycle checkpoint activation and growth inhibition is one of the fundamental functions of RB (Weinberg, 1995) and ATM (Kastan et al., 1992); therefore, we tested whether the RB/ATM-mediated inhibition of sphere formation ability was due to cell cycle checkpoint activation and acute cell growth arrest. Our results indicated that despite some morphological changes of the cells, the percentage of the BrdU positive cells in the Rb/ATM-deficient MEFs reconstituted with pRB, ATM or both ATM and pRB was insignificantly changed compared with the control for at least 2 weeks after re-expression of RB or ATM, suggesting that acute cell growth arrest due to cell cycle checkpoint activation is an unlikely cause of the RB/ATM-mediated inhibition of sphere formation which supports our idea that RB and ATM have crucial functions in depletion of the stem cell-like pool through promotion of the proteasome-mediated degradation of the OSKNM proteins.

RB and ATM induce acetylation-driven ubiquitination of the OSKNM proteins

We next tried to understand how ATM and RB recruit the OSKNM proteins for degradation. We searched for the ATM- and pRB-binding proteins that might mediate these novel functions of ATM and RB (https://www.predictprotein.org/home); and found several proteins related to the UPS pathway are predicted as ATM- or pRB-binding partners (Table S1 and Table S2). Thereafter, we tried to identify these ATM- and pRB-binding partners in protein lysates prepared from *Rb*/*ATM* DKO MEFs stably expressing human ATM fused at the amino (N)-terminus with Flag and 6-histidines (6-His) tags (Abraham, 2001) or human pRB fused at the amino (N)-terminus with HA tag (Shamma et al., 2009; Shamma et al., 2013) by coimmunoprecipitation (coIP) with anti-Flag or anti-HA antibodies; and analysis of the protein bands by mass spectrometry (MS). Gene ontology analysis (http://www.pantherdb.org/) of the MS results identified 53 ATM- and 44 pRB-binding partner proteins. Among the ATM-binding partners, Hdac5 was classified as protein deacetylation process and five proteins (RNF181, Fbxl20, Fbxw4, Lrcc1 and Dtl) were classified as SCF-dependent proteasomal ubiquitin-dependent protein catabolic process; and five proteins (Apex2 and Dtl, Stk36, Dclk1 and Ulk2) were classified as DNA repair process by the GO term analysis. The pRB-binding partners included Hdac5 and two E3 ligases (Fbxl20 and RNF181). Interestingly, among these GO terms, only the E3 ligases-dependent protein catabolic process was statistically significantly overrepresented as ATM-partner proteins in the GO enrichment analysis ($p=0.05$). Notably, all the KDACs, E3 ligases and DDR proteins that were identified by MS as ATM- or pRB-partners were identified in the cytosolic fractions except RNF181 was identified only in total cell lysates (Table S5) suggesting that cytosolic functions of ATM that are linked to the UPS might be segregated from the canonical DDR pathway in the nucleus. We next focused on Hdac5, two representatives of the E3 ligases that were identified by MS (Lrrc1 and Rnf181) and another three E3 ligases (Fbxw7, Uhrf1 and Skp2) that were predicted (Table S1 and Table S2) or previously reported as ATM or pRB binding partners (Matsuoka et al., 2007; Shamma et al., 2013; Ji et al., 2004); and then validated the physical bindings of ATM or pRB with these proteins in association with the OSKNM proteins. We detected ATM-Hdac5 binding significantly higher in the cytosol than the nucleus; however, the pRB-Hdac5 binding was fairly detected only in the cytosol. On the other hand, pRB-Kat3b binding was detected equally in the cytosol and the nucleus. Interestingly, reconstitution of RB or ATM into Rb/ATM-deficient MEFs significantly decreased the Hdac5-OSKNM proteins

bindings in these MEFs suggesting that Hdac5 associates better with the OSKNM proteins in absence of pRb and ATM; and this association might safeguard against the OSKNM proteins degradation. Intriguingly, ATM but not pRB was detected in the protein complex containing the OSKNM proteins together with Lrrc1, Rnf181, Uhrf1 and Fbxw7; however, Skp2 was not a component of this complex. Although the HA-tagged pRB existed in protein complex containing Lrrc1, Rnf181 and Skp2, our anti-HA antibody failed to pull down significant amounts of the OSKNM proteins in this complex. These observations suggest that Uhrf1 and Fbxw7 might be important for the ATM-mediated degradation of the OSKNM proteins. Furthermore, we found that ATM physically binds with Uhrf1 and Fbxw7 but not with Skp2; and pRB enhances the ATM bindings with these E3 ligases. In addition, coIP with E3 ligases specific antibodies identified Uhrf1-Oct3/4, Uhrf1-Sox2, Uhrf1-Klf4, Uhrf1-c-Myc, Fbxw7-Klf4 and Fbxw7-Nanog bindings. Notably, the levels of the OSKNM proteins bound with Uhrf1 or Fbxw7 were significantly higher in the presence of pRB or ATM compared with the control. Importantly, depletion of Uhrf1 from Rb/ATM DKO MEFs reconstituted with ATM rescued Oct3/4, Sox2, Klf4 and c-Myc; and depletion of Fbxw7 from these MEFs rescued Sox2, Klf4, Nanog and c-Myc proteins; however, depletion of Skp2 from these MEFs caused insignificant changes in the abundance of the OSKNM proteins. Taken together, these data suggest that pRB might mediates epigenetic modification of the OSKNM proteins through recruitment of Kat3b; and ATM coordinates the assembly of the modified OSKNM proteins into E3 ligase ubiquitination complexes. These results also indicate that Uhrf1 and Fbxw7 are major ATM partner E3 ligases for degradation of the OSKNM proteins; and either pRB or Skp2 are not directly involved in assembly of the OSKNM proteins ubiquitination complexes.

The acetylation status of lysine residues in histone and non-histone proteins is dynamically regulated by the lysine acetyltransferases (KATs) and the lysine deacetylasees (KDACs). Therefore, we hypothesized that deregulation of Kat3b and Hdac5 by RB and ATM functions might disrupt the OSKNM proteins acetylation/deacetylation dynamics leading to acetylation labeling of the OSKNM proteins; and the acetylated OSKNM proteins become identified and recruited for proteasomal degradation. To test this hypothesis we first tried to determine how RB/ATM functions deregulate Hdac5. We performed RT-qPCR and found that reconstitution of RB into Rb/ATM DKO MEFs significantly decreased the relative mRNA and protein levels of Hdac5; however, ATM reconstitution caused insignificant changes in the relative mRNA or the protein levels of Hdac5 in these MEFs. In addition, subcellular fractionation and IF studies indicated that RB reconstitution into Rb/ATM DKO MEFs markedly decreased Hdac5 both in the nuclei and the cytosol of these MEFs whereas ATM reconstitution caused significant nuclear retention of Hdac5 in these MEFs compared with the control. Furthermore, treatment of Rb/ATM-deficient MEFs with LMK235 (class IIa HDACs inhibitor) markedly decreased the abundance of the OSKNM proteins; and importantly, additional treatment of these MEFs with MG132 rescued the OSKNM proteins to levels comparable with the DMSO-treated control. Furthermore, depletion of Hdac5 form Rb/ATM-deficient MEFs with specific shRNA moderately decreased the abundances of the OSKNM proteins; suggesting that not only Hdac5 but other members of class IIa HDACs might also regulate the proteasome-mediated degradation of the OSKNM proteins. On the other hand, treatment of RB-reconstituted Rb/ATM DKO MEFs with C646 (Kat3b specific inhibitor) antagonized RB functions and rescued the OSKNM proteins indicating that Kat3b functions are also important for the Rb-mediated degradation of the OSKNM proteins. However, C646 treatment of Rb/ATM DKO MEFs reconstituted with ATM caused insignificant changes in the abundances of the OSKNM proteins probably due to inability of Kat3b inhibition to counteract the passive lysine acetylation induced by the ATM-mediated inhibition of Hdac5 in these cells; which might also suggest that lysine deacetylation is a prevalent mechanism of the OSKNM proteins stabilization. These results clearly indicate that Hdac5 and perhaps other members of class IIa HDACs as well as Kat3b are major mediators of the RB/ATM mutual functions in proteasomal degradation of the OSKNM proteins probably through regulation of the lysine acetylation status of the OSKNM proteins.

We next searched for the predicted acetylated (http://bioinfo.bjmu.edu.cn/huac/predict_p/) or ubiquitinated (http://www.ubpred.org/) lysine residues of the mice OSKNM proteins; and found that the OSKNM proteins have several lysine residues predicted to be targets of KATs or KDACs or targets of ubiquitination in humans (Suresh et al., 2016) and mice. We further studied the acetylation and the ubiquitination statuses of the OSKNM proteins by CoIP and IB with specific antibodies; and found that restoration of pRB or ATM into Rb/ATM-deficient MEFs significantly increased the levels of the acetylated and the ubiquitinated Oct3/4, Klf4, Nanog and c-Myc proteins. In addition, reconstitution of ATM into Rb/ATM DKO MEFs significantly increased the levels of the acetylated and the ubiquitinated Sox2; however, reconstitution of RB caused insignificant changes in the levels of the acetylated and the ubiquitinated Sox2 protein in these MEFs compared with the control. Interestingly, the global proteins ubiquitination levels were also significantly increased in Rb/ATM-deficient MEFs reconstituted with pRB or ATM compared with the control. Furthermore, treatment of Rb/ATM-deficient MEFs with LMK235 significantly increased the acetylation and the ubiquitination levels of the OSKNM proteins; and these levels of acetylation and ubiquitination were enough to provoke proteasomal degradation of the OSKNM proteins. Taken together, these data suggest that mutual deregulation of Hdac5 and perhaps other members of class IIa HDACs by RB and ATM induces acetylation-driven ubiquitination and subsequent proteasomal degradation of the OSKNM proteins. Notably, treatment of Rb/ATM-deficient MEFs with LMK235 significantly increased the global proteins ubiquitination levels. In addition, Kat3b inhibition with C646 moderately decreased the acetylation and the ubiquitination levels of the OSKNM proteins in Rb/ATM-deficient MEFs reconstituted with pRB compared with the control; and this decrease in the protein acetylation and ubiquitination was enough to rescue the OSKNM proteins in these MEFs. On the other hand, Kat3b inhibition with C646 caused slight reduction of the acetylation and the ubiquitination levels of the OSKNM proteins in Rb/ATM-deficient MEFs

reconstituted with ATM compared with the control; however, this reduction in the protein acetylation and ubiquitination was not enough to significantly rescue the OSKNM proteins in these MEFs, probably due to the prevalent passive acetylation induced by the ATM-mediated inhibition of Hdac5 in these cells as we discussed earlier. Furthermore, C646 treatment of Rb/ATM-deficient MEFs reconstituted with RB or ATM slightly decreased the global proteins ubiquitination levels. Taken together, these results indicate that the passive lysine acetylation induced by the RB/ATM-mediated inhibition of Hdac5 as well as the active lysine acetylation induced by the RB-mediated recruitment of Kat3b provokes acetylation-driven ubiquitination and subsequent proteasomal degradation of the OSKNM proteins.

ATM kinase activity in the OSKNM proteins degradation

We next asked what the molecular signals initiating these protein-protein interactions are and whether the ATM kinase activity has a role in this process. Our results indicated that contrary to the wild-type ATM (ATM-Wt), expression of the ATM-kinase dead (ATM-Kd) mutant allele D2870A/N2875k in Rb/ATM DKO MEFs caused insignificant changes in the abundances of the OSKNM proteins or the sphere formation ability of these MEFs; and interestingly, treatment of the ATM reconstituted Rb/ATM DKO MEFs with KU55933 (specific ATM kinase inhibitor) bypassed ATM functions and rescued the OSKNM proteins as well as sphere formation ability in these MEFs. Furthermore, the ATM-Kd mutant binding affinity to Hdac5 or Uhrf1 was greatly impaired compared with the ATM-Wt; indicating that the ATM kinase activity is necessary for these protein-protein interactions that leads to Hdac5 sequestration and acetylation labeling of the OSKNM proteins that become identified by ATM and recruited for ubiquitination and subsequent proteasomal degradation. In order to determine the physiological relevance of these findings, we treated wild type MEFs and Rb KO MEFs with KU55933 and evaluated the abundances of OSKNM proteins and sphere formation abilities in these MEFs. We found the ATM kinase activity assessed by ATM^{pS1981} and p53^{pS15} was significantly higher in Rb KO MEFs than wild type MEFs as we previously reported (Shamma et al. 2013); and importantly, treatment of Rb KO MEFs with KU55933 decreased the ATM kinase activity, significantly and mutually increased the abundances of the OSKNM proteins and enabled sphere formation in these MEFs whereas treatment of wild type MEFs with KU55933 although significantly increased the abundances of the OKM proteins but this was not enough to enable sphere formation in these MEFs. These data indicate that the ATM kinase activity plays important roles in cellular reprogramming; however, we do not exclude other ATM functions especially in regulation of the Sox2, which was significantly increased after ATM knockdown but not after ATM kinase inhibition; and indeed we do not undermine the important role of RB in transcriptional regulation of Sox2 (Kareta et al. 2015 and this study). These data together suggest that germ-line inactivation of Rb and ATM, knockdown of Rb and ATM, and ATM kinase inhibition in Rb-negative context are functionally equal in MEFs reprogramming; and hence mutual chemical inhibition of RB and ATM might improve the p-iPS generation efficiency.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　0　件）

〔学会発表〕（計　1　件）
Shamma A. ATM and RB functions regulate stemness by targeting the core reprogramming proteins for proteasomal degradation. 76th Annual Meeting of the Japanese Cancer Association, Yokohama/Japan (2017).

〔図書〕（計　0 件）

〔産業財産権〕

○出願状況（計　0　件）
名称：
発明者：
権利者：
種類：
番号：
出願年：
国内外の別：

○取得状況（計　0　件）
名称：
発明者：
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