

genes during G_2 phase, whereas Sep1 binds there during M/G_1 phase.⁸³ It is likely that Fkh2 in combination with Plol1 represses expression of the target genes during G_2 , while Sep1 activates them during M/G_1 phase (Fig. 4).

FoxM1 is activated through phosphorylation by the Cdk2-Cyclin E and Cdk2-cyclin A complexes during S phase and its phosphorylation levels increase during G_2 . This results in the relief of inhibition by the N-terminal domain, allowing induction of FoxM1-mediated gene activation (Fig. 4).^{30,86} The C-terminal half of FoxM1 harbors a trans-activation domain in which the cyclin-binding motif (LXL) is found, and it is here that Cdk1 and Cdk2 bind. In addition, phosphorylation of FoxM1 by Cdk1 also creates a binding site for the PBD domain of Plk1.⁸⁷ Subsequently, Plk1 binds and phosphorylates other sites of FoxM1 and activates FoxM1 as a transcription factor, which is required for expression of many mitotic regulators including *plk1*⁺ itself.⁸⁷ This fact suggests that regulation of the Cdk-Plk-FoxM1 pathway provides a positive-feedback loop, ensuring proper G_2/M transition. The precise molecular mechanisms of the Cdk-Plk-Fox pathway may differ substantially between yeast and mammals, but the outcome is similar. In addition, both Fox- and Plk-related proteins apparently exist in organisms from yeast to mammals, but not in plants. As the organisms using Fox and Plk are so divergent, the conservation of Plk transcriptional control by Fox from yeast to mammals is remarkable.

Cdk1 and Fox in Development

There are several critical control points needed in order to maintain the integrity of genetic information and cell size during the cell cycle: in late G_1 , these are termed the "Start" in yeast and the Restriction point in mammals. Once cells have passed Start, they cannot commence alternative developmental pathways until they go back to G_1 . Before passing Start, cells can determine whether they grow, arrest or differentiate. In fission yeast, nutrient starvation and pheromone signals induce cells in G_1 by inhibiting the activity of Cdk1.⁸⁸ Additionally, nutrient exhaustion leads to a decrease in the intracellular concentration of cyclic AMP, and consequent inactivation of protein kinase A. Inactivation of protein kinase A eventually activates *ste11*⁺ expression. Ste11, a master regulator of sexual development, induces many genes required for mating. Nutrient starvation also activates the mitogen-activated protein kinase pathway, which leads to *ste11*⁺ induction.

In fission yeast, Fkh2 is phosphorylated by Cdc2 and this phosphorylation blocks sexual development by regulating *ste11*⁺ expression (Fig. 5).⁸⁹ Fkh2 is required for efficient mating, in combination with Fhl1 and Mei4.⁸⁹ Partial sterility of the Fox

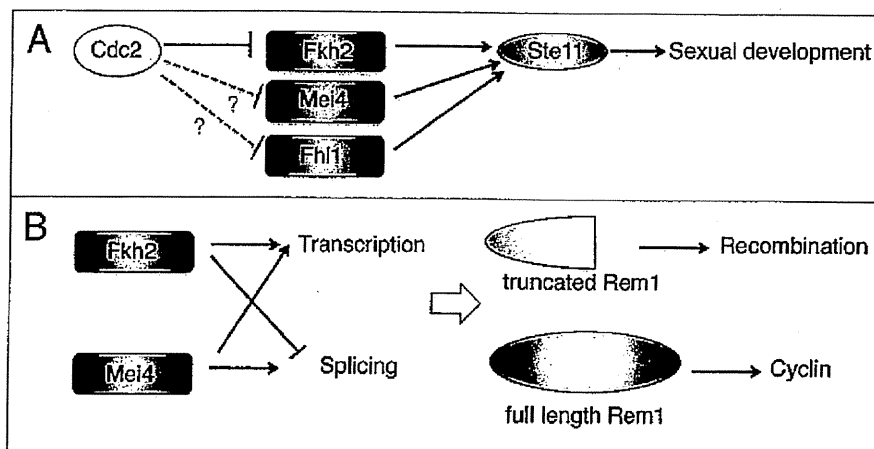


Figure 5. Diagram of Fox proteins in fission yeast regulating sexual differentiation and splicing. (A) In fission yeast, Cdc2 phosphorylates Fkh2 and inhibits transcriptional activity of Fkh2 during the mitotic cell cycle. Under nutrient starvation, Cdc2 kinase activity decreases, resulting in dephosphorylation of Fkh2. Dephosphorylated Fkh2 in combination with Fhl1 and Mei4 induces *ste11*⁺ expression. Ste11 drives cells to sexual differentiation by inducing many genes required for mating. (B) During meiotic prophase, Fkh2 activates expression but inhibits splicing of *rem1*⁺, producing a truncated Rem1 that is required for recombination. Mei4 induces both expression and splicing of *rem1*⁺, resulting in a full-length Rem1 functioning as a cyclin.

mutants (*fkh2*, *fhl1*, *mei4*, *fkh2 fhl1*, *fkh2 mei4* and *fkh2 fhl1 mei4*) is caused mainly by poor induction of *ste11*⁺, since *ste11*⁺ expression is low in the Fox mutants and ectopic *ste11*⁺ expression rescues the sterility of the Fox mutants.⁸⁹ Fkh2 regulates *ste11*⁺ transcription by its direct binding to the *ste11*⁺ promoter. Cdc2 directly phosphorylates at least the T314 and S462 residues of Fkh2. The sequence around T314 is located in the FKH domain and is conserved among the FoxN3 group, whereas S462 is conserved among the FoxJ2 group, although the functions of these residues are not known in other organisms. Fkh2 that is phosphorylated at these residues is deficient in ability to induce expression of *ste11*⁺ mRNA. Therefore, these results support a model in which during the mitotic cycle, Cdc2 is active and phosphorylates Fkh2, inhibiting *ste11*⁺ expression. Nutrient starvation lowers Cdc2 activity through cyclin degradation and upregulation of the Cdc2 inhibitor. This Cdc2 inactivation may allow dephosphorylation and consequent activation of Fkh2. This mechanism contributes to the prevention of sexual differentiation during the mitotic cell cycle.

Fox Regulates Pre-mRNA Processing

Recently, it has been reported that Mei4 also regulates meiosis-specific splicing⁹⁰ (Fig. 5). Meiosis-specific splicing is found in fission and budding yeasts, and is similar to, but at the same time differs from, alternative splicing in higher eukaryotes.^{91,92} In the final mRNA, introns may or may not be retained in yeast, whereas only exons remain in those of higher eukaryotes. For example, fission yeast Rem1 functions as a meiosis-specific cyclin when splicing is complete, whereas a Rem1 truncated protein is involved in proper recombination when the intron is not removed.⁹⁰ Ectopic expression of *rem1*⁺ during the mitotic cycle is highly toxic to the

cells, suggesting that a finely-controlled mechanism operates to ensure the absence of Rem1 during the mitotic cycle.⁹³ During meiotic prophase, Mei4 binds to the promoter of its target gene *rem1*⁺ and recruits the spliceosome to the *rem1*⁺ gene, in addition to inducing a burst induction of *rem1*⁺ expression.⁹⁰ Interestingly, the specificity of meiosis-specific splicing is determined by the promoter but not the transcript region. When the *rem1*⁺ promoter was fused to another gene, it was spliced only in meiotic cells. Conversely, when the *rem1*⁺ gene was driven by another promoter, *rem1*⁺ was spliced in both mitotic and meiotic cells. In addition, *rem1*⁺ splicing is negatively regulated by Fkh2, although Fkh2 is required for transcription of *rem1*⁺. These opposite effects of splicing by two Fox factors may be due to the specific binding of Mei4, but not Fkh2, to the spliceosome. Consistent with the role of Fox transcription factors in splicing, budding yeast Fkh1 and Fkh2 associate not only with the promoter region, but also with the coding region.⁴⁴ Budding yeast Fkh1 is required for transcriptional termination, whereas budding yeast Fkh2 is required for transcriptional elongation.⁴⁴ In addition, Ifh1 regulates pre-ribosomal RNA (rRNA) processing in association with the proteins involved in ribosome assembly.⁹⁴ Therefore, it may be generally considered that Fox proteins have additional roles in regulating pre-RNA processing, either alone or in association with accessory proteins.

Conclusions

Research to date on FoxM1 and yeast Fox proteins has revealed that, from yeast to mammals, they are key players in mitotic and

meiotic cell cycle progression during the G₂ and M phases. They execute their functions by regulating expression of Cdk1 activators and Plk proteins. Furthermore, Cdk1 regulates the activity of Fox proteins by direct phosphorylation. Although many cell cycle control mechanisms of FoxM1 and yeast Fox proteins are conserved, the molecular mechanisms operating between Cdk1 and these Fox proteins have not yet been clarified. In fission yeast, it has not been addressed whether Cdk1 phosphorylates Fox proteins or their associated factors, and whether Fox proteins regulate gene expression in complex with Ndd1-like proteins. In mammals, it is not known whether FoxM1 controls gene expression in collaboration with its associated proteins, like Ndd1 and MADS box protein in yeast. Besides cell cycle control of FoxM1-related proteins, fission yeast Fkh2 regulates sexual differentiation and its activity is negatively regulated by phosphorylation of Cdk1. It is not yet known whether FoxM1 or budding yeast Fox proteins also function in this way or whether other Fox proteins instead of FoxM1 perform this function in mammals. In addition to their roles in transcription, yeast Fox proteins have additional roles in regulating pre-mRNA processing including splicing, although it has not yet been shown whether mammalian Fox proteins perform similar functions. Future studies will aim to elucidate the precise mechanisms that couple transcription with pre-mRNA processing by Fox proteins. Yeast cells contain only four Fox proteins, and do not generally have redundant systems that may hinder analysis of the processes of Fox gene expression. Therefore, it appears that yeast cells will continue to be useful models for dissection of the mechanisms regulating Fox transcription factors in other organisms.

References

- Morgan DO. Cyclin-dependent kinases: engines, clocks and microprocessors. *Annu Rev Cell Dev Biol* 1997; 13:261-91.
- Nurse P. Universal control mechanism regulating onset of M-phase. *Nature* 1990; 344:503-8.
- Murakami H, Nurse P. DNA replication and damage checkpoints and meiotic cell cycle controls in the fission and budding yeasts. *Biochem J* 2000; 349:1-12.
- Bloom J, Cross FR. Multiple levels of cyclin specificity in cell cycle control. *Nat Rev Mol Cell Biol* 2007; 8:149-60.
- Fisher DL, Nurse P. A single fission yeast mitotic cyclin B p34^{cdc2} kinase promotes both S- phase and mitosis in the absence of G₁ cyclins. *EMBO J* 1996; 15:850-60.
- Satyanarayanan A, Kaldis P. Mammalian cell cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. *Oncogene* 2009; 28:2925-39.
- Santamaria D, Barriere C, Cerqueira A, Hunt S, Tardy C, Newton K, et al. Cdk1 is sufficient to drive the mammalian cell cycle. *Nature* 2007; 448:811-5.
- Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer* 2009; 9:153-66.
- Lee YM, Sicinski P. Targeting cyclins and cyclin-dependent kinases in cancer: lessons from mice, hopes for therapeutic applications in human. *Cell Cycle* 2006; 5:2110-4.
- Martin BT, Strebhardt K. Polo-like kinase 1: target and regulator of transcriptional control. *Cell Cycle* 2006; 5:2881-5.
- Archambault V, Glover DM. Polo-like kinases: conservation and divergence in their functions and regulation. *Nat Rev Mol Cell Biol* 2009; 10:265-75.
- van de Weerd BC, Medema RH. Polo-like kinases: a team in control of the division. *Cell Cycle* 2006; 5:853-64.
- Liu X, Erikson RL. Polo-like kinase 1 in the life and death of cancer cells. *Cell Cycle* 2003; 2:424-5.
- Benjamin KR, Zhang C, Shokat KM, Herskowitz I. Control of landmark events in meiosis by the CDK Cdc28 and the meiosis-specific kinase Imc2. *Genes Dev* 2003; 17:1524-39.
- Kishimoto T. Cell cycle control during meiotic maturation. *Curr Opin Cell Biol* 2003; 15:654-63.
- Chu S, DeRisi J, Eisen M, Mulholland J, Botstein D, Brown PO, et al. The transcriptional program of sporulation in budding yeast. *Science* 1998; 282:699-705.
- Mata J, Lyne R, Burns G, Bahler J. The transcriptional program of meiosis and sporulation in fission yeast. *Nat Genet* 2002; 32:143-7.
- Breedon LL. Periodic transcription: a cycle within a cycle. *Curr Biol* 2003; 13:31-8.
- Mata J, Wilbrey A, Bahler J. Transcriptional regulatory network for sexual differentiation in fission yeast. *Genome Biol* 2007; 8:217.
- Chu S, Herskowitz I. Gametogenesis in yeast is regulated by a transcriptional cascade dependent on Ndr80. *Mol Cell* 1998; 1:685-96.
- Cirillo LA, McPherson CE, Bossard P, Stevens K, Cherian S, Shim EY, et al. Binding of the winged-helix transcription factor HNF3 to a linker histone site on the nucleosome. *EMBO J* 1998; 17:244-54.
- Clark KL, Halay ED, Lai E, Burley SK. Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. *Nature* 1993; 364:412-20.
- Gajiwala KS, Chen H, Cornille F, Roques BE, Reith W, Mach B, et al. Structure of the winged-helix protein hREX1 reveals a new mode of DNA binding. *Nature* 2000; 403:916-21.
- Baldauf SL. A Search for the origins of animals and fungi: Comparing and combining molecular data. *Am Nat* 1999; 154:178-88.
- Tuteja G, Kaestner KH. Forkhead transcription factors II. *Cell* 2007; 131:192.
- Tuteja G, Kaestner KH. SnapShot: forkhead transcription factors I. *Cell* 2007; 130:1160.
- Partridge L, Bruning JC. Forkhead transcription factors and ageing. *Oncogene* 2008; 27:2351-63.
- Konopka G, Bomar JM, Winden K, Coppola G, Jonsson ZO, Gao F, et al. Human-specific transcriptional regulation of CNS development genes by FOXP2. *Nature* 2009; 462:213-7.
- Scott KL, Plon SE. Loss of Sin3/Rpd3 histone deacetylase restores the DNA damage response in checkpoint-deficient strains of *Saccharomyces cerevisiae*. *Mol Cell Biol* 2003; 23:4522-31.
- Major ML, Lepe R, Costa RH. Forkhead box M1B transcriptional activity requires binding of Cdk-cyclin complexes for phosphorylation-dependent recruitment of p300/CBP coactivators. *Mol Cell Biol* 2004; 24:2649-61.
- Pic A, Lim FL, Ross SJ, Veal EA, Johnson AL, Sultan MR, et al. The forkhead protein Fkh2 is a component of the yeast cell cycle transcription factor SFE. *EMBO J* 2000; 19:3750-61.
- Simon I, Barnett J, Hannett N, Harbison CT, Rinaldi NJ, Volkert TL, et al. Serial regulation of transcriptional regulators in the yeast cell cycle. *Cell* 2001; 106:697-708.
- Zhu G, Spellman PT, Volpe T, Brown PO, Botstein D, Davis TN, et al. Two yeast forkhead genes regulate the cell cycle and pseudohyphal growth. *Nature* 2000; 406:90-4.

34. Koranda M, Schleiffer A, Eandler L, Ammerer G. Forkhead-like transcription factors recruit Ndd1 to the chromatin of G₂/M-specific promoters. *Nature* 2000; 406:94-8.
35. Spellman PT, Sherlock G, Zhang MQ, Iyer VR, Anders K, Eisen MB, et al. Comprehensive identification of cell cycle-regulated genes of the yeast *Saccharomyces cerevisiae* by microarray hybridization. *Mol Biol Cell* 1998; 9:3273-97.
36. Kumar R, Reynolds DM, Shevchenko A, Goldstone SD, Dalton S. Forkhead transcription factors, Fkh1p and Fkh2p, collaborate with Mcm1p to control transcription required for M-phase. *Curr Biol* 2000; 10:896-906.
37. Loy CJ, Lydall D, Surana U. NDD1, a high-dosage suppressor of cdc28-1N, is essential for expression of a subset of late-S-phase-specific genes in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1999; 19:3312-27.
38. Shore P, Sharrocks AD. The MADS-box family of transcription factors. *Eur J Biochem* 1995; 229:1-13.
39. Boros J, Lim FL, Darieva Z, Pic-Taylor A, Harman R, Morgan BA, et al. Molecular determinants of the cell cycle regulated Mcm1p-Fkh2p transcription factor complex. *Nucleic Acids Res* 2003; 31:2279-88.
40. Lydall D, Ammerer G, Nasmyth K. A new role for MCM1 in yeast: cell cycle regulation of SWI5 transcription. *Genes Dev* 1991; 5:2405-19.
41. Reynolds D, Shi BJ, McLean C, Katsis F, Kemp B, Dalton S. Recruitment of Thr 319-phosphorylated Ndd1p to the FHA domain of Fkh2p requires Clb kinase activity: a mechanism for CLB cluster gene activation. *Genes Dev* 2003; 17:1789-802.
42. Hollenhorst PC, Pietz G, Fox CA. Mechanisms controlling differential promoter-occupancy by the yeast forkhead proteins Fkh1p and Fkh2p: implications for regulating the cell cycle and differentiation. *Genes Dev* 2001; 15:2445-56.
43. Darieva Z, Pic-Taylor A, Boros J, Spanos A, Geymonat M, Reece RJ, et al. Cell cycle-regulated transcription through the FHA domain of Fkh2p and the coactivator Ndd1p. *Curr Biol* 2003; 13:1740-5.
44. Morillon A, O'Sullivan J, Azad A, Proudfoot N, Mellor J. Regulation of elongating RNA polymerase II by forkhead transcription factors in yeast. *Science* 2003; 300:492-5.
45. Voth WP, Yu Y, Takahata S, Kretschmann KL, Lieb JD, Parker RL, et al. Forkhead proteins control the outcome of transcription factor binding by inactivation. *EMBO J* 2007; 26:4324-34.
46. Jorgensen P, Rupes I, Sharom JR, Schnepfer L, Broach JR, Tyers M. A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size. *Genes Dev* 2004; 18:2491-505.
47. Hermann-Le Denmat S, Werner M, Sentenac A, Thuriaux P. Suppression of yeast RNA polymerase III mutations by FHL1, a gene coding for a fork head protein involved in rRNA processing. *Mol Cell Biol* 1994; 14:2905-13.
48. Rudra D, Zhao Y, Warner JR. Central role of Ith1p-Fhl1p interaction in the synthesis of yeast ribosomal proteins. *EMBO J* 2005; 24:533-42.
49. Schawaldner SB, Kabani M, Howald I, Choudhury U, Werner M, Shore D. Growth-regulated recruitment of the essential yeast ribosomal protein gene activator Ith1. *Nature* 2004; 432:1058-61.
50. Wade JT, Hall DB, Struhl K. The transcription factor Ith1 is a key regulator of yeast ribosomal protein genes. *Nature* 2004; 432:1054-8.
51. Schmelele T, Beck T, Martin DE, Hall MN. Activation of the RAS/cyclic AMP pathway suppresses a TOR deficiency in yeast. *Mol Cell Biol* 2004; 24:338-51.
52. Zhu G, Muller EG, Amacher SL, Northrop JL, Davis TN. A dosage-dependent suppressor of a temperature-sensitive calmodulin mutant encodes a protein related to the fork head family of DNA-binding proteins. *Mol Cell Biol* 1993; 13:1779-87.
53. Pramila T, Wu W, Miles S, Noble WS, Breeden LL. The Forkhead transcription factor Hcm1 regulates chromosome segregation genes and fills the S-phase gap in the transcriptional circuitry of the cell cycle. *Genes Dev* 2006; 20:2266-78.
54. Young JA, Hyppa RW, Smith GR. Conserved and nonconserved proteins for meiotic DNA breakage and repair in yeasts. *Genetics* 2004; 167:593-605.
55. Horie S, Watanabe Y, Tanaka K, Nishiwaki S, Fujioka H, Abe H, et al. The *Schizosaccharomyces pombe* mei4⁺ gene encodes a meiosis-specific transcription factor containing a forkhead DNA-binding domain. *Mol Cell Biol* 1998; 18:2118-29.
56. Murakami-Tonami Y, Yamada-Namikawa C, Tochigi A, Hasegawa N, Kojima H, Kunimatsu M, et al. Mei4p coordinates the onset of meiosis I by regulating cdc25⁺ in fission yeast. *Proc Natl Acad Sci USA* 2007; 104:14688-93.
57. Harigaya Y, Tanaka H, Yamanaka S, Tanaka K, Watanabe Y, Tsutsumi C, et al. Selective elimination of messenger RNA prevents an incidence of untimely meiosis. *Nature* 2006; 442:45-50.
58. Borgne A, Murakami H, Ayte J, Nurse P. The G₂/S cyclin Cig2p during meiosis in fission yeast. *Mol Cell Biol* 2002; 22:2080-90.
59. Iino Y, Hiramane Y, Yamamoto M. The role of cdc2 and other genes in meiosis in *Schizosaccharomyces pombe*. *Genetics* 1995; 140:1235-45.
60. Murakami H, Nurse P. Meiotic DNA replication checkpoint control in fission yeast. *Genes Dev* 1999; 13:2581-93.
61. Bulmer R, Pic-Taylor A, Whitehall SK, Martin KA, Millar JB, Quinn J, et al. The forkhead transcription factor Fkh2 regulates the cell division cycle of *Schizosaccharomyces pombe*. *Eukaryot Cell* 2004; 3:944-54.
62. Szilagyi Z, Batra G, Enczi K, Sipiczki M. Characterisation of two novel fork-head gene homologues of *Schizosaccharomyces pombe*: their involvement in cell cycle and sexual differentiation. *Gene* 2005; 348:101-9.
63. Rustici G, Mata J, Kivinen K, Lio P, Penkett CJ, Burns G, et al. Periodic gene expression program of the fission yeast cell cycle. *Nat Genet* 2004; 36:809-17.
64. Buck V, Ng SS, Ruiz-Garcia AB, Papadopolou K, Bharti S, Samuel JM, et al. Fkh2p and Sep1p regulate mitotic gene transcription in fission yeast. *J Cell Sci* 2004; 117:5623-32.
65. Ribar B, Gallert A, Olah E, Szallasi Z. Deletion of the sep1(+) forkhead transcription factor homologue is not lethal but causes hyphal growth in *Schizosaccharomyces pombe*. *Biochem Biophys Res Commun* 1999; 263:465-74.
66. Ribar B, Banrevi A, Sipiczki M. sep1⁺ encodes a transcription-factor homologue of the HNF-3/forkhead DNA-binding-domain family in *Schizosaccharomyces pombe*. *Gene* 1997; 202:1-5.
67. Bahler J. A transcriptional pathway for cell separation in fission yeast. *Cell Cycle* 2005; 4:39-41.
68. Zitihi E, Salimova E, Simanis V, Sipiczki M. The *S. pombe* sep1 gene encodes a nuclear protein that is required for periodic expression of the cdc15 gene. *FEBS Lett* 2000; 481:105-8.
69. Wang Z, Ahmad A, Li Y, Banerjee S, Kong D, Sarkar FH. Forkhead box M1 transcription factor: A novel target for cancer therapy. *Cancer Treat Rev* 2009.
70. Laoukili J, Stahl M, Medema RH. FoxM1: at the crossroads of ageing and cancer. *Biochim Biophys Acta* 2007; 1775:92-102.
71. Pandit B, Halasi M, Garrel AL. p53 negatively regulates expression of FoxM1. *Cell Cycle* 2009; 8:3425-7.
72. Petrovic V, Costa RH, Lau LF, Raychaudhuri P, Tyner AL. Negative regulation of the oncogenic transcription factor FoxM1 by thiazolidinediones and mithramycin. *Cancer Biol Ther* 2010; 9:1008-16.
73. Tang SY, Jiao Y, Li LQ. [Significance of Forkhead Box m1b (Foxm1b) gene in cell proliferation and carcinogenesis]. *Ai Zheng* 2008; 27:894-6.
74. Korver W, Roose J, Wilson A, Clevers H. The winged-helix transcription factor Tridant is expressed in actively dividing lymphocytes. *Immunobiology* 1997; 198:157-61.
75. Korver W, Schilham MW, Moerter P, van den Hoff MJ, Dam K, Lamers WH, et al. Uncoupling of S phase and mitosis in cardiomyocytes and hepatocytes lacking the winged-helix transcription factor Tridant. *Curr Biol* 1998; 8:1327-30.
76. Laoukili J, Kooistra MR, Bras A, Kaur J, Kerkhoven RM, Morrison A, et al. FoxM1 is required for execution of the mitotic programme and chromosome stability. *Nat Cell Biol* 2005; 7:126-36.
77. Wang IC, Chen YJ, Hughes DE, Ackerson T, Major ML, Kalinichenko VV, et al. FoxM1 regulates transcription of JNK1 to promote the G₂/S transition and tumor cell invasiveness. *J Biol Chem* 2008; 283:20770-8.
78. Wang IC, Chen YJ, Hughes D, Petrovic V, Major ML, Park HJ, et al. Forkhead box M1 regulates the transcriptional network of genes essential for mitotic progression and genes encoding the SCF (Skp2-Cks1) ubiquitin ligase. *Mol Cell Biol* 2005; 25:10875-94.
79. Utsiyon V, Wang IC, Ren X, Zhang Y, Snyder J, Xu Y, et al. Forkhead box M1 transcriptional factor is required for smooth muscle cells during embryonic development of blood vessels and esophagus. *Dev Biol* 2009; 336:266-79.
80. Anon A, Surana U, Muroff J, Nasmyth K. Regulation of p34CDC28 tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. *Nature* 1992; 355:368-71.
81. Pic-Taylor A, Darieva Z, Morgan BA, Sharrocks AD. Regulation of cell cycle-specific gene expression through cyclin-dependent kinase-mediated phosphorylation of the forkhead transcription factor Fkh2p. *Mol Cell Biol* 2004; 24:10036-46.
82. Darieva Z, Bulmer R, Pic-Taylor A, Doris KS, Geymonat M, Sedgwick SG, et al. Polo kinase controls cell cycle-dependent transcription by targeting a coactivator protein. *Nature* 2006; 444:494-8.
83. Papadopolou K, Ng SS, Ohkura H, Geymonat M, Sedgwick SG, McInerney CJ. Regulation of gene expression during M-G₂-phase in fission yeast through Plo1p and forkhead transcription factors. *J Cell Sci* 2008; 121:38-47.
84. Veis J, Klug H, Koranda M, Ammerer G. Activation of the G₂/M-specific gene CLB2 requires multiple cell cycle signals. *Mol Cell Biol* 2007; 27:8364-73.
85. Sherriff JA, Kent NA, Mellor J. The Isw2 chromatin-remodeling ATPase cooperates with the Fkh2 transcription factor to repress transcription of the B-type cyclin gene CLB2. *Mol Cell Biol* 2007; 27:2848-60.
86. Laoukili J, Alvarez M, Meijer LA, Stahl M, Mohammed S, Kleij L, et al. Activation of FoxM1 during G₂ requires cyclin A/Cdk-dependent relief of autorepression by the FoxM1 N-terminal domain. *Mol Cell Biol* 2008; 28:3076-87.
87. Fu Z, Malureanu L, Huang J, Wang W, Li H, van Deursen JM, et al. Plk1-dependent phosphorylation of FoxM1 regulates a transcriptional programme required for mitotic progression. *Nat Cell Biol* 2008; 10:1076-82.
88. Yamamoto M, Imai Y, Watanabe Y. Mating and Sporulation in *Schizosaccharomyces pombe*. In: Pringle JR, Broach JR, Jones EW, Eds. *The Molecular and Cellular Biology of the Yeast Saccharomyces: Life Cycle and Cell Biology*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory 1997; 1037-106.
89. Shimada M, Yamada-Namikawa C, Murakami-Tonami Y, Yoshida T, Nakanishi M, Urano T, et al. Cdc2p controls the forkhead transcription factor Fkh2p by phosphorylation during sexual differentiation in fission yeast. *EMBO J* 2008; 27:132-42.

-
90. Moldon A, Malapcira J, Gabrielli N, Gogol M, Gomez-Escoda B, Ivanova T, et al. Promoter-driven splicing regulation in fission yeast. *Nature* 2008; 455:997-1000.
91. Kishida M, Nagai T, Nakaseko Y, Shimoda C. Meiosis-dependent mRNA splicing of the fission yeast *Schizosaccharomyces pombe* *msi1'* gene. *Curr Genet* 1994; 25:497-503.
92. Averbek N, Sunder S, Sample N, Wise JA, Leatherwood J. Negative control contributes to an extensive program of meiotic splicing in fission yeast. *Mol Cell* 2005; 18:491-8.
93. Malapcira J, Moldon A, Hidalgo E, Smith GR, Nurse P, Ayte J. A meiosis-specific cyclin regulated by splicing is required for proper progression through meiosis. *Mol Cell Biol* 2005; 25:6330-7.
94. Rudra D, Mallick J, Zhao Y, Warner JR. Potential interface between ribosomal protein production and pre-rRNA processing. *Mol Cell Biol* 2007; 27:4815-24.

Dual Mode of Regulation of Cell Division Cycle 25 A Protein by TRB3

Satoshi SAKAI,^a Nobumichi OHOKA,^{b,1)} Kikuo ONOZAKI,^b Masatoshi KITAGAWA,^c
Makoto NAKANISHI,^d and Hidetoshi HAYASHI^{*a}

^a Department of Drug Metabolism and Disposition, Graduate School of Pharmaceutical Sciences, Nagoya City University;

^b Department of Molecular Health Sciences, Graduate School of Pharmaceutical Sciences, Nagoya City University; Nagoya 467-8603, Japan; ^d Department of Cell Biology, Graduate School of Medical Sciences, Nagoya City University; Nagoya 467-8601, Japan; and ^c Department of Biochemistry 1, Hamamatsu University School of Medicine; Hamamatsu, Shizuoka 431-3192, Japan.

Received March 23, 2010; accepted March 25, 2010; published online April 23, 2010

We have recently demonstrated that TRB3, a novel stress-inducible protein, is an unstable protein regulated by the ubiquitin-proteasome system. The expression level of TRB3 protein is down-regulated by anaphase-promoting complex/cyclosome-cell division cycle division 20 homolog 1 (APC/C^{Cdh1}) through its D-box motif. Here we demonstrate that TRB3 regulates the stability of cell division cycle 25 A (Cdc25A), an essential activator of cyclin dependent kinases (CDKs). The expression level of Cdc25A protein is suppressed by over-expression of TRB3, while knockdown of TRB3 enhances the endogenous Cdc25A expression level. On the other hand, Cdc25A degradation induced by DNA damage is significantly rescued by TRB3. When serine residues in the DSG motif, which is the critical sequences for the degradation of Cdc25A induced by DNA damage, is mutated to alanine (Cdc25A^{DSG2X}), both stimulatory and protective effects of TRB3 on the Cdc25A degradation is disappeared. TRB3 protein interacts with both wild Cdc25A and mutant Cdc25A^{DSG2X}. Expression level of the endogenous TRB3 protein is down-regulated in a genotoxic condition. These results suggest TRB3 is a regulator for adjusting the expression level of Cdc25A both in a normal and a genotoxic conditions.

Key words TRB3; cell division cycle 25 A; DNA damage; degradation; ubiquitin

Cell division cycle 25 A (Cdc25A) is one of the highly conserved dual specificity phosphatases that activate cyclin dependent kinase (Cdk) complexes to regulate the cell cycle progression.^{2,3)} Cdc25A activates Cdk2–cyclin E and Cdk2–cyclin A complexes during G₁/S transition,^{3,4)} and contributes the G₂/M transition by activating Cdk1–cyclin B complexes.⁵⁾ Cdc25A expression is precisely regulated by ubiquitin-mediated proteolysis in both a normal cell cycle and a genotoxic stress condition.⁶⁾ Cdc25A ubiquitination is mainly mediated by two ubiquitin ligase complexes; anaphase promoting complex/cyclosome-Cdh1 (APC/C^{Cdh1}) for its destruction during mitotic exit and early G₁,⁷⁾ and S-phase kinase-associated protein 1 (Skp1)–Cul1–F-box–beta-transduction repeat-containing protein (β -TrCP) (SCF ^{β -TrCP}) for its proteolysis during G₁, S and G₂.⁸⁾ Binding of Cdc25A to APC/C^{Cdh1} is dependent on a KEN box without post-translational modifications,⁹⁾ while interaction with SCF ^{β -TrCP} requires the phosphorylation of serine residues within a so-called DSG motif.¹⁰⁾

Cdc25A plays a role in the checkpoint response to unrepliation or DNA damage.^{5,6,11)} Cdc25A is a phosphorylation target of checkpoint kinases (Chks) and is regulated by Chk kinases in response to DNA damage to degrade *via* the ubiquitin-proteasome pathway. Cells resistant to degradation of Cdc25A display defects in checkpoint arrest to lead the genomic instability.¹⁰⁾

In *Drosophila*, reinitiation of mitosis is controlled by regulated expression of Cdc25.¹²⁾ It has been reported that *tribbles*, an atypical member of the protein kinase superfamily, acts by specifically inducing degradation of *string*, one of the Cdc25A orthologs in *Drosophila*, *via* the proteasome pathway and delayed G₂/M transition.¹³⁾

We previously identified TRB3, as one of the human orthologs of *tribbles*, which induced by endoplasmic reticulum stress and contributed to cell growth inhibition.¹⁴⁾ *Trib-*

bles and TRB3 contain the classic substrate-binding domains of a protein kinase but not the ATP-binding and kinase-activating domains; therefore, they do not have a kinase activity.¹⁵⁾ Recently we have reported that TRB3 is an unstable protein regulated by the ubiquitin-proteasome system.¹⁶⁾ The expression level of TRB3 protein is down-regulated by anaphase-promoting complex/cyclosome Cdh1 (APC/C^{Cdh1}) which is a key ubiquitin ligase complex, which regulates the progression of the cell cycle.

In this study, we found that TRB3 interacts with Cdc25A to destabilize its protein in a normal condition, however prevented its degradation in response to DNA damage.

MATERIALS AND METHODS

Reagents Dulbecco's modified Eagle's medium, anti- β -actin monoclonal antibody (AC-15), and anti-FLAG monoclonal antibody (M2) were purchased from Sigma. Fetal bovine serum was from HyClone (Logan, UT, U.S.A.). MG132 was obtained from Peptide Institute (Osaka, Japan). Anti-Cdc25A polyclonal antibody (M-191) was from Santa Cruz (Santa Cruz, CA, U.S.A.). Anti-Myc monoclonal antibody (9E10) and anti-HA monoclonal antibody (12CA5) were from Roche (Indianapolis, IN, U.S.A.). Anti-green fluorescent protein (GFP) monoclonal antibody (JL8) was from Clontech (Mountain View, CA, U.S.A.). Anti-p21 monoclonal antibody (05-345) was from Upstate (Lake Placid, NY, U.S.A.). The antiserum against human TRB3 was prepared as described previously.¹⁵⁾

Cell Culture The embryonic kidney cell line 293, the human cervical carcinoma cell line HeLa and human melanoma cell line A375 were cultured as described previously.¹⁷⁾

Construction of Expression Plasmids The plasmids

* To whom correspondence should be addressed. e-mail: hhayashi@phar.nagoya-cu.ac.jp

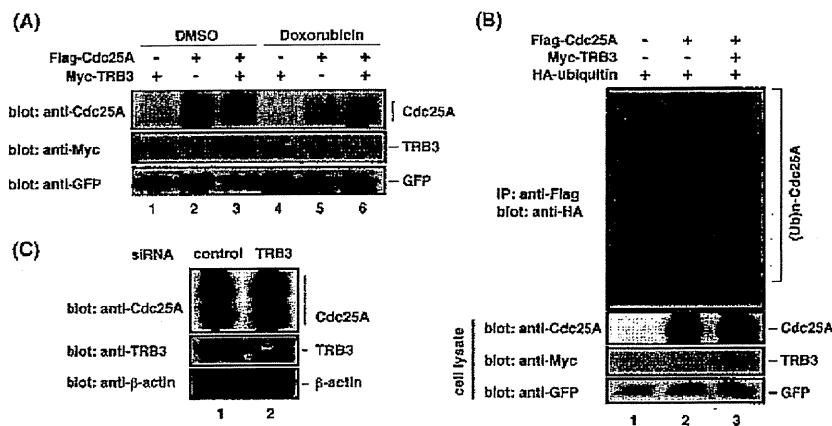


Fig. 1. TRB3 Positively and Negatively Regulates the Cdc25A Protein Stability

(A) HeLa cells were transiently transfected with indicated constructs. After 24 h, cells were treated with or without 0.5 μ M doxorubicin for 24 h. The cell lysates were analyzed by immunoblotting using indicated antibodies. The pEGFP-C1 expression plasmid was included in each transfection as a transfection efficiency control. (B) 293 cells were transiently transfected with indicated constructs. After 24 h, cells were treated with 10 μ M MG132 for 12 h. The cell lysates were immunoprecipitated (IP) with anti-Flag antibody and multi-ubiquitinated Cdc25A was detected by immunoblotting with anti-HA antibody. The expression level of each protein was assessed by immunoblotting using anti-Cdc25A, anti-Myc and anti-GFP antibodies. (C) HeLa cells were transiently transfected with control (scramble) siRNA or TRB3 siRNA twice every 24 h. The cell lysates were harvested and analyzed by immunoblotting using anti-Cdc25A, anti-TRB3 and anti- β -actin antibodies.

pCMV5-Flag-TRB3 was constructed as described previously.¹⁴ The plasmids pCMV5B-Flag-Cdc25A, pCMV5B-Myc-Cdc25A, pCMV5B-Flag-Cdc25A(1-260) lacking amino acids (aa) 261-525, pCMV5B-Flag-Cdc25A(261-525) lacking aa1-260, pCMV5B-Flag-Cdc25A(261-330) lacking aa1-260 and 331-525, pCMV5B-Flag-Cdc25A(331-460) lacking aa1-330 and 461-525, pCMV5B-Flag-Cdc25A(461-525) lacking aa1-460 or pCMV5B-Flag-Cdc25A(331-525) lacking aa1-330, pCMV5B-Flag-Cdc25A^{DSG2X}, replacing Ser⁸² and Ser⁸⁸ with Ala, pCMV5B-Flag-Cdc25A^{KEN2mt}, replacing Lys¹⁴¹, Glu¹⁴² and Asn¹⁴³ with Ala were generated by polymerase chain reaction (PCR). pMT-123 (HA-Ub)¹⁸ was kindly provided by Dr. D. Bohmann (University of Rochester Medical Center). All constructs were verified by sequencing.

Immunoprecipitation and Western Blot Analysis Cells were transiently transfected and treated as described in the Figure Legends. The cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate, and 1% Triton X-100) supplemented with protease inhibitors. The lysates were subjected to immunoprecipitation, and 1–2% of the lysate or co-immunoprecipitates was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (5–12.5%), transferred onto a polyvinylidene difluoride (PVDF) membrane and probed with the antibodies indicated in the Figure Legends. The immunoreactive proteins were visualized using ECL (Amersham Bioscience) or Immobilon (Millipore) Western blotting detection reagents, and light emission was quantified with a LAS1000 lumino image analyzer (FUJI, Japan).

RNA Interference Double stranded RNA duplexes corresponding to human TRB3 was obtained from Dharmacon Inc. (Chicago, IL, U.S.A.).

Transfection 293 and HeLa cells were transfected using the Chen-Okayama method as described previously.¹⁷ For RNA interference, HeLa cells were transfected using a lipofection method with Lipofectamine 2000 (Invitrogen).

RESULTS

TRB3 Negatively and Positively Regulates the Stability of Cdc25A Protein To determine the TRB3 effect on the Cdc25A stability, we first examined the co-transfection experiment in HeLa cells. Over-expression of TRB3 resulted in decreased Cdc25A steady-state protein levels (Fig. 1A, lane 3), which accompanied by accumulation of polyubiquitin-reactive signals in the Cdc25A immune-complexes (Fig. 1B). We also investigated whether TRB3 depletion affects the Cdc25A protein stability. As shown in Fig. 1C, endogenous TRB3 silencing by small interfering RNA (siRNA) in HeLa cells resulted in increased endogenous Cdc25A steady-state levels. Similar result has been observed when endogenous TRB3 was knock-downed in HepG2 cells.¹⁶

Cdc25A is one of the well-known targets of the DNA damage to induce cell cycle arrest.¹⁰ The expression level of Cdc25A protein is remarkably decreased, when cells are treated with doxorubicin, an anticancer drug that leads to DNA double-strand breaks (Fig. 1A, lane 5). Strikingly, TRB3 rescues the breakdown of Cdc25A protein induced in response to DNA damage (lane 6). These results indicate that TRB3 regulates the Cdc25A stability negatively in a normal condition and positively under the genotoxic stress.

Cdc25A Physically Interacts with TRB3 As TRB3 is considered to be a regulator of Cdc25A, we next examined whether TRB3 can interact with Cdc25A. Cell extract was prepared from 293 cells co-expressed with Myc-TRB3 and Flag-Cdc25A, followed by immunoprecipitation-Western blot analysis. As shown in Fig. 2A, TRB3 was found to interact with Cdc25A. We next assayed the interaction of various Cdc25A deletion mutants to map the region responsible for TRB3 binding. The deletion mutant with C-terminal half of Cdc25A (aa 261-525) is relatively stable and shown to be interacted with TRB3 (Fig. 2B). Further experiments demonstrated that the region aa 331-460 is crucial in TRB3 binding (Fig. 2C). On the other hand, the N-terminal half region of Cdc25A (aa 1-260) is not detected even in the presence of a

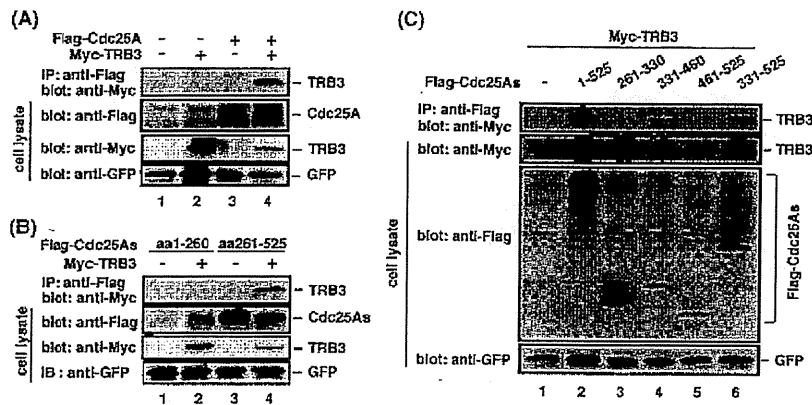


Fig. 2. TRB3 Physically Interacts with Cdc25A

(A, B) 293 cells were transiently transfected with indicated constructs. After 24 h, the cells were treated with 10 μ M MG132 for 12 h. The cell lysates were immunoprecipitated (IP) with anti-Flag antibody, and immunoblotting of cell lysates was performed with anti-Flag or anti-Myc antibodies. (C) 293 cells were transiently transfected with the expression plasmid for Myc-TRB3 in the presence of the expression plasmids for full length Flag-Cdc25A (1-525) or its deletion mutants. After 24 h, cells were treated with 10 μ M MG132 for 12 h. The cell lysates were immunoprecipitated (IP) with anti-Flag antibody and immunoblotted with anti-Myc antibody. The expression level of each protein was assessed by immunoblotting of cell lysates with anti-Flag, anti-Myc or anti-GFP antibodies.

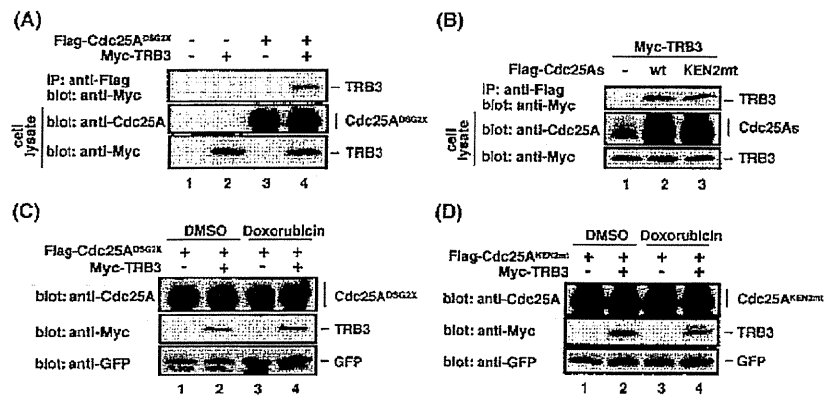


Fig. 3. DSG Motif and KEN Box in Cdc25A Are Not Necessary to Interact with TRB3, but Responsible for Its Unstabilizing Effect

(A, B) 293 cells were transiently transfected with the expression plasmids of Flag-Cdc25A^{DSG2X} (replacement of both Ser82 and Ser88 to Ala in Cdc25A) (A) or Flag-tagged wild-type Cdc25A or Flag-Cdc25A^{KEN2mt} (replacement of KEN (141-143) to AAA in Cdc25A) (B) in the presence of the expression plasmid Myc-TRB3. After 24 h, the cells were treated with 10 μ M MG132 for 12 h. The cell lysates were immunoprecipitated (IP) with anti-Flag antibody, and immunoblotted by anti-Myc antibody. The expression level of each protein was assessed by the immunoblotting of the cell lysates with anti-Cdc25A or anti-Myc antibodies. (C, D) HeLa cells were transiently transfected with the expression plasmids of Flag-Cdc25A^{DSG2X} (C) or Flag-Cdc25A^{KEN2mt} (D) in the presence of the expression plasmid Myc-TRB3. After 24 h, cells were treated with 0.5 μ M doxorubicin for another 24 h. The cell lysates were analyzed by immunoblotting using anti-Cdc25A or anti-Myc antibodies. The pEGFP-C1 expression vector was included in each transfection as a transfection efficiency control, and its level was detected with anti-GFP antibody.

proteasome inhibitor, MG132, so it is hard to consider whether N-terminal region is necessary for TRB3 association from this experiment.

Role of DSG Motif and KEN Box of Cdc25A in Its Down-Regulation by TRB3 Cdc25A is constantly tuned over in cycling cells. Two different ubiquitin ligases (SCF ^{β -TrCP} and APC/C^{Cdh1} complex) are known to be involved in Cdc25A turnover, and interaction with these complexes requires specific recognition motifs in Cdc25A. One is DSG motif for SCF ^{β -TrCP} binding,¹⁰ and the other is KEN box for APC/C^{Cdh1}.⁹ To examine the possible involvement of these regions in the unstabilizing effect of TRB3, we determined the effect of mutation of these motifs (Cdc25A^{DSG2X} and Cdc25A^{KEN2mt}). These mutants are quite stable and still interact with TRB3 in 293 cells (Figs. 3A, B). When Myc-TRB3 is co-expressed with Flag-Cdc25A^{DSG2X}, its expression level remained unaffected compared to that of single transfection

(Fig. 3C). Likewise, over-expressed TRB3 did not change the expression level of KEN2 mutated Cdc25A either (Fig. 3D). These results suggest that DSG and KEN motifs in Cdc25A are involved in the TRB3 effect of its unstabilizing.

The expression levels of these mutants are not different even after doxorubicin treatment (Fig. 3C, D, lane 3), indicating that both mutants are resistant to the proteolytic degradation induced in response to DNA damage (Figs. 3C, D, lane 3). When these mutants were used, the regulatory effect of TRB3 on Cdc25A expression was not observed (Figs. 3C, D, lane 4).

TRB3 Is Down-Regulated by Genotoxic Stress We have already shown that TRB3 is markedly induced by endoplasmic reticulum (ER) stress *via* induction of stress-related transcription factors, activating transcription factor (ATF) and CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP).¹⁴ To determine whether the expression

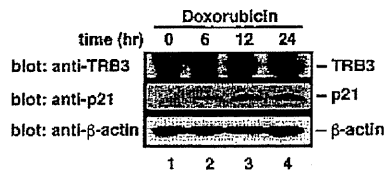


Fig. 4. TRB3 Is Down-Regulated in Response to DNA Damage

p53 wild-type human melanoma cell line, A375 cells were treated with 0.5 μ M doxorubicin for indicated periods of time. The cell lysates were analyzed by immunoblotting using anti-TRB3, anti-p21 and anti- β -actin antibodies.

level of TRB3 protein is also regulated by DNA damage stress, A375 cells, which are human melanoma cell lines with wild type p53, were treated with doxorubicin. As shown in Fig. 4, doxorubicin time-dependently down-regulates the expression of endogenous TRB3 protein. We also observed the obvious induction of endogenous p21 protein, which is a well-established downstream target of p53, by doxorubicin treatment.

DISCUSSION

As Cdc25A is an essential activator of cyclin-dependent kinase during normal cell-cycle progression, it is strictly regulated at the protein level, being periodically synthesized and degraded *via* ubiquitin-proteasome pathway.^{4,8,19} Cdc25A is also thought to be one of the proto-oncogenes because of its transformation ability²⁰ and its over-expression in many cancers, both at mRNA and protein levels.²¹ This deregulated expression may be due to anomalous E2F1/c-Myc transcriptional activity or alternatively to a reduced rate of protein degradation. Our results demonstrated that TRB3 down-regulates the Cdc25A expression level in a normal condition and recovers its degradation induced in response to DNA damage, suggesting that TRB3 could be a crucial regulator of Cdc25A for fine-tuning of its abundance.

In *Drosophila*, reinitiation of mitosis is regulated by Cdc25 expression level.¹² One of the Cdc25 orthologs in *Drosophila*, *string* is expressed zygotically in the embryo. It has been reported that *tribbles*, an atypical member of the protein kinase superfamily, acts by specifically inducing degradation of *string* *via* the proteasome pathway and delayed G₂/M transition.¹³ Our observation also demonstrates the Cdc25A unstabilizing effect of TRB3, one of the *tribbles* orthologs at the steady state. The TRB3 action to Cdc25A could be also shown and involved in the oocyte maturation.

We have previously showed that TRB3 is markedly induced by various ER stresses.¹⁴ However, genotoxic stress was reported to down-regulate TRB3 mRNA expression.²² Consistent with this, we demonstrate that the protein level of Cdc25A is also decreased in response to DNA damage induced by an anticancer drug, doxorubicin. This result indicates that under the genotoxic condition, the suppressive effect of TRB3 on the Cdc25A proteolysis is usually down-regulated due to the reduction of TRB3 expression itself. On the contrary, these finding support the idea that under the various stressful conditions (ER stress, hypoxia, amino acid deprivation, oxidative stress, *etc.*) to induce the TRB3 expression, Cdc25A protein would be stabilized even under the genotoxic conditions by up-regulated TRB3 and the

checkpoint function will be lost, thus resulting in genomic instability and cancer predisposition. Multiple primary human lung, colon, and breast tumors express high levels of TRB3 transcript.^{23,24} It is possible that the accumulation of Cdc25A protein by TRB3 over-expressed in multiple human tumors and tumor-derived cell lines is involved in the tumorigenesis and malignant alteration of cancer. Further study is necessary to clarify whether over-expressed TRB3 is contributed to tumorigenesis.

We show that TRB3 interacts with the region aa 331-460 of Cdc25A. This region is the part of catalytic domain of Cdc25A, which raises the possibility that TRB3 regulates the phosphatase activity of Cdc25A and its binding ability to CDK/cyclin complexes as well. TRB3 can also associate with KEN or DSG mutants of Cdc25A, indicating that TRB3 might not compete with β -TrCP or Cdh1 for binding to Cdc25A *via* these motifs. However, the unstabilizing activity of TRB3 to these two Cdc25A mutants are not observed at all, suggesting that these motifs are crucial to facilitate the unstabilizing of Cdc25A protein by TRB3 and that β -TrCP and/or Cdh1 are involved in this effect.

We have previously demonstrated that TRB3 is a short-lived protein and its steady-state level is balanced through proteasome-dependent degradation, which is facilitated by APC/C^{Cdh1}.¹⁶ APC/C is a key ubiquitin ligase complex, which regulates the progression of the cell cycle by control the ubiquitination and subsequent degradation of a number of core cell-cycle regulators. As previously mentioned, APC/C^{Cdh1} also regulates the Cdc25A stability in a normal condition at mitotic exit and in early G₁. Taken together, it is possible that the expression of TRB3 is periodically regulated in cell cycle, resulting the Cdc25A stability is also time-dependently modified.

Important question still remain unsolved. Why TRB3 regulates differently the Cdc25A stability in the cell conditions? A possibility is that the modification mode of Cdc25A protein is different from in a normal condition and under the genotoxic stress. Cdc25A is a well-characterized target of Chk1/Chk2 and other kinase(s) in response to DNA damage.¹² Phosphorylation could alter the interaction or response of TRB3 to Cdc25A, resulting in the different action of TRB3 to Cdc25A stability. A second possibility is that TRB3 may differently influence the function of Cdc25A kinase(s) and Cdh1. Steady state level of Cdc25A is mainly controlled by APC/C^{Cdh1} through its KEN box. In contrast, in the case of the genotoxic condition, SCF ^{β -TrCP} is mainly responsible for the breakdown of Cdc25A phosphorylated by Chk1 and a recently discovered kinase NEK11.^{10,25} We have previously demonstrated that TRB3 silencing caused the accumulation of Cdc25A and Cdc20, another well-known target of APC/C^{Cdh1}, in HepG2 cells without any change of Cdh1 expression level, suggesting that TRB3 has some influence on the Cdh1 dependent degradation.¹⁵ TRB3 is considered as a pseudokinase, which contains the typical substrate-binding domains, but lack the ATP binding and kinase-activation domains.¹⁵ It is possible that some kinase(s) or other modifying enzyme(s) cannot recruit to Cdc25A or upstream kinase(s) when they interact with TRB3, and that TRB3 could be an endogenous kinase inhibitor, acting as a decoy kinase-like protein for upstream kinase(s) for Cdc25A phosphorylation.

In summary, this study provides that the cell-cycle activator, Cdc25A is positively and negatively regulated by stress inducible pseudokinase TRB3 at the protein level. Important roles of TRB3 in Cdc25A implicate the additional function of TRB3 in cell cycle regulation besides the recently described roles in the stress response. These results allowed us to investigate the precise role of TRB3 in cell cycle to understand whether TRB3 contributes to deregulation of DNA damage checkpoints and tumorigenesis.

Acknowledgements We thank Dr. Yuka Itoh for their helpful discussions and advice with the manuscript. We are grateful to thank Dr. Dirk Bohmann for providing expression plasmids. This research was supported in part by a Grant-in-Aid for Scientific Research (C) from Japan Society for the Promotion of Science, and Grants-in-Aid for Scientific Research on Priority Areas from The Ministry of Education, Science, Sports and Culture, and Grants-in-Aid for Scientific Research from Nagoya City University.

REFERENCES

- 1) Present address: *Division of Biosignaling, National Institute of Health Sciences; Tokyo 158-8501, Japan.*
- 2) Galaktionov K., Beach D., *Cell*, **67**, 1181—1194 (1991).
- 3) Boutros R., Lobjois V., Ducommun B., *Nat. Rev. Cancer*, **7**, 495—507 (2007).
- 4) Hoffmann I., Draetta G., Karsenti E., *EMBO J.*, **13**, 4302—4310 (1994).
- 5) Zhao H., Watkins J. L., Piwnicka-Worms H., *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 14795—14800 (2002).
- 6) Mailand N., Falck J., Lukas C., Syljuåsen R. G., Welecker M., Bartek J., Lukas J., *Science*, **288**, 1425—1429 (2000).
- 7) Mainland N., Podtelejnikov A. V., Groth A., Mann M., Bartek J., Lukas J., *EMBO J.*, **21**, 5911—5920 (2002).
- 8) Blomberg I., Hoffmann I., *Mol. Cell. Biol.*, **19**, 6183—6194 (1999).
- 9) Donzelli M., Squatrito M., Ganoth D., Hershko A., Pagano M., Draetta G. F., *EMBO J.*, **21**, 4875—4884 (2002).
- 10) Busino L., Chiesa M., Draetta G. F., Donzelli M., *Oncogene*, **23**, 2050—2056 (2004).
- 11) Molinari M., Mecurio C., Dominguez J., Goubin F., Draetta G. F., *EMBO Rep.*, **1**, 71—79 (2000).
- 12) Edgar B. A., O'Farrell P. H., *Cell*, **62**, 469—480 (1990).
- 13) Mata J., Curado S., Ephrussi A., Rørth P., *Cell*, **101**, 511—522 (2000).
- 14) Ohoka N., Yoshii S., Hattori T., Onozaki K., Hayashi H., *EMBO J.*, **24**, 1243—1255 (2005).
- 15) Du K., Herzig S., Kulkarni R. N., Montminy M., *Science*, **300**, 1574—1577 (2003).
- 16) Ohoka N., Sakai S., Onozaki K., Nakanishi M., Hayashi H., *Biochem. Biophys. Res. Commun.*, **392**, 289—294 (2010).
- 17) Hattori T., Ohoka N., Inoue Y., Hayashi H., Onozaki K., *Oncogene*, **22**, 1273—1280 (2003).
- 18) Treier M., Staszewski L. M., Bohmann D., *Cell*, **78**, 787—798 (1994).
- 19) Jinno S., Suto K., Nagata A., Igarashi M., Kanaoka Y., Nojima H., Okayama H., *EMBO J.*, **13**, 1549—1556 (1994).
- 20) Galaktionov K., Lee A. K., Eckstein J., Draetta G., Meckler J., Loda M., Beach D., *Science*, **269**, 1575—1577 (1995).
- 21) Cangi M. G., Cukor B., Soung P., Signoretti S., Moreira Jr. G., Ranasinghe M., Cady B., Pagano M., Loda M., *J. Clin. Invest.*, **106**, 753—761 (2000).
- 22) Corcoran C. A., Luo X., He Q., Jiang C., Huang Y., Sheikh M. S., *Cancer Biol. Ther.*, **4**, 1063—1067 (2005).
- 23) Bowers A. J., Scully S., Boylan J. F., *Oncogene*, **22**, 2823—2835 (2003).
- 24) Xu J., Lv S., Qin Y., Shu F., Xu Y., Chen J., Xu B. E., Sun X., Wu J., *Biochim. Biophys. Acta*, **1770**, 273—278 (2007).
- 25) Meixetian M., Klein D. K., Sørensen C. S., Helin K., *Nature Cell Biol.*, **11**, 1247—1253 (2009).

Essential role of Tip60-dependent recruitment of ribonucleotide reductase at DNA damage sites in DNA repair during G1 phase

Hiroyuki Niida,¹ Yuko Katsuno,¹ Misuzu Sengoku,¹ Midori Shimada,¹ Megumi Yukawa,¹ Masae Ikura,² Tsuyoshi Ikura,² Kazuteru Kohno,³ Hiroki Shima,³ Hidekazu Suzuki,³ Satoshi Tashiro,³ and Makoto Nakanishi^{1,4}

¹Department of Cell Biology, Graduate School of Medical Sciences, Nagoya City University Medical School, Nagoya 467-8601, Japan; ²Radiation Biology Center, Kyoto University, Kyoto 606-8501, Japan; ³Department of Cell Biology, Research Institute for Radiation Biology and Medicine (RIRBM), Hiroshima University, Hiroshima 734-8553, Japan

A balanced deoxyribonucleotide (dNTP) supply is essential for DNA repair. Here, we found that ribonucleotide reductase (RNR) subunits RRM1 and RRM2 accumulated very rapidly at damage sites. RRM1 bound physically to Tip60. Chromatin immunoprecipitation analyses of cells with an I-SceI cassette revealed that RRM1 bound to a damage site in a Tip60-dependent manner. Active RRM1 mutants lacking Tip60 binding failed to rescue an impaired DNA repair in RRM1-depleted G1-phase cells. Inhibition of RNR recruitment by an RRM1 C-terminal fragment sensitized cells to DNA damage. We propose that Tip60-dependent recruitment of RNR plays an essential role in dNTP supply for DNA repair.

Supplemental material is available at <http://www.genesdev.org>.

Received September 15, 2009; revised version accepted December 22, 2009.

Maintenance of the optimal intracellular concentrations of deoxyribonucleotides (dNTPs) is critical not only for faithful DNA synthesis during DNA replication and repair, but also for the survival of all organisms. Ribonucleotide reductase (RNR), composed of a tetrameric complex of two large catalytic (RRM1) subunits and two small subunits (RRM2 or 53R2), catalyzes de novo synthesis of dNTPs from the corresponding ribonucleotides (Reichard 1993). This reaction is the rate-limiting process in DNA precursor synthesis and is regulated by multiple complex mechanisms, including transcriptional and subcellular localization regulation of RNR (Nordlund and Reichard 2006). In order to duplicate their chromosomal DNA,

mammalian S-phase cells possess 15–20 times more dNTP pools than resting quiescent cells, whereas whole dNTP pools were almost unchanged after DNA damage, suggesting the presence of a unique mechanism that supplies a sufficient quantity of dNTPs at repair sites (Hakansson et al. 2006). DNA synthesis must function properly in both repair and replication (dNTP concentrations in fibroblasts were estimated to be as follows: ~0.5 μ M in G0/G1-phase cells, and ~10 μ M in S-phase cells, given that the average volume of a fibroblast is 3.4 pL) (Imaizumi et al. 1996). Although the amount of dNTPs required for DNA repair is small, their concentration during DNA synthesis is critical because DNA polymerase involved in DNA repair (Kraynov et al. 2000; Johnson et al. 2003) has similar kinetic affinities for dNTPs (~10 μ M) to those involved in DNA replication (~10 μ M) (Dong and Wang 1995). Therefore, the dNTPs might be compartmentalized close to the damage sites during the DNA repair process. In this study, we show that, in mammals, both RRM1 and RRM2 rapidly accumulated at double-strand break (DSB) sites in a Tip60-binding-dependent manner.

Results and Discussion

In order to understand the mechanisms by which dNTPs are sufficiently supplied at DNA damage sites in mammals, we first examined changes in the subcellular localization of RRM1 and RRM2 subunits after ionizing irradiation (IR) irradiation. Although both RRM1 and RRM2 predominantly localized in the cytoplasm as reported previously (Pontarin et al. 2008), we also detected trace, but significant, signals of both proteins in chromatin fraction (see Fig. 1C; Supplemental Fig. S4A–D). After removing soluble RNR proteins by detergent extraction, we found that RRM1 and RRM2 proteins formed nuclear foci that colocalized with γ H2AX (Fig. 1A). RRM1 nuclear foci were not evident without DNA damage (Supplemental Fig. S1A) or after RRM1 depletion by siRNA (Supplemental Fig. S1B). Ultraviolet A (UVA) microirradiation resulted in the accumulation of RRM1 and RRM2 along microirradiated lines as early as 5 min after treatment (Fig. 1B). These accumulations were also observed when cells were not subjected to detergent extraction or preincubation with BrdU (Supplemental Fig. S2A,B), but were significantly compromised when R1 expression was knockdown by siRNA (Supplemental Figs. S2C, S4B), excluding the possibility that accumulated signals at DSB sites were artifacts during cell-staining processes. These results indicated that RNR, at least in part, was rapidly recruited to DSB sites.

In order to determine the molecular basis underlying RNR recruitment at the sites of DSBs, we performed yeast two-hybrid screening using RRM1 as a bait. Of a total of 5×10^6 transformants from a HeLa cell cDNA library, 45 positive colonies were confirmed to be lacZ-positive. They contained overlapping cDNAs derived from three genes: RRM2 and 53R2 (both encoding a small subunit of RNR), and another encoding Tip60 histone acetyltransferase (Tip60). Small C-terminal RRM1 deletion mutants (Δ 761-C and Δ 781-C) failed to bind Tip60, but retained the ability to bind to RRM2 (Supplemental Fig. S3A). In contrast, the N-terminal truncation mutant of Tip60

[**Keywords:** DNA repair; ribonucleotide reductase; Tip60; dNTPs; genomic instability; DNA double-strand breaks]

⁴Corresponding author.

E-MAIL mkt-naka@med.nagoya-cu.ac.jp, FAX 81-52-842-3955.

Article is online at <http://www.genesdev.org/cgi/doi/10.1101/gad.1863810>.

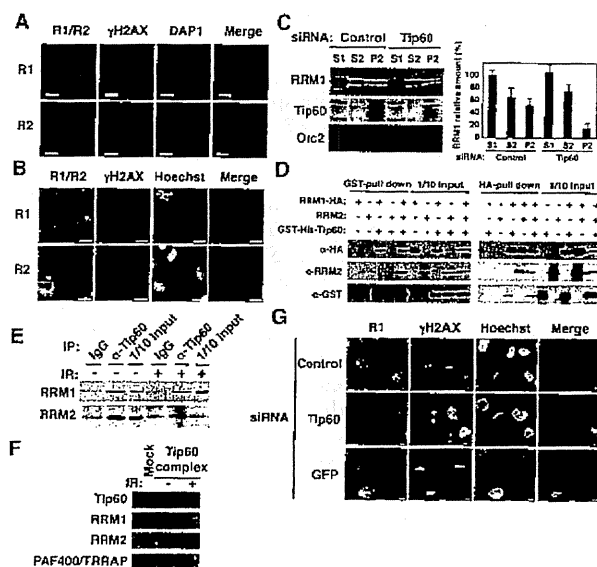


Figure 1. Tip60-dependent recruitment of RNR at DSB sites. **(A)** HeLa cells were exposed to IR at 1 Gy, subjected to in situ detergent extraction after 5 min, and immunostained with the indicated antibodies. Bars, 5 μ m. **(B)** GM02063 cells were subjected to UVA microirradiation and immunostained with the indicated antibodies after 5 min. RRM1 or RRM2 and γ H2AX signals are shown in green and red, respectively, in merged images. Bars, 10 μ m. **(C)** IR-irradiated HeLa cell lysates treated with the indicated siRNAs were fractionated as described in the Materials and Methods. (Left panels) The fractions were subjected to immunoblotting using the indicated antibodies. (Right panel) The RRM1 bands were quantitated, and the results are presented as percentages of S1 fraction. Data are mean \pm standard deviation ($n = 3$). **(D)** Sf9 lysates expressing RRM1-HA, RRM2, or GST-His-Tip60 were subjected to GST pull-down or HA pull-down assays using the indicated antibodies. **(E)** Chromatin fractions from IR- or mock-treated HeLa cells [after 5 min] were solubilized with micrococcal nuclease. The solubilized extracts were immunoprecipitated with anti-Tip60 antibodies or control IgG. The resulting precipitates and a 10% input [1/10 Input] were immunoblotted with the indicated antibodies. **(F)** The affinity-purified Tip60 complexes, as described in the Materials and Methods, were subjected to immunoblotting using the indicated antibodies. **(G)** GM02063 cells were treated with control, Tip60, or GFP siRNAs and then subjected to UVA microirradiation as in **B**.

[TC2] could interact with RRM1, but no mutant with any additional truncation of TC2 was able to do so (Supplemental Fig. S3B). Full-length Tip60 failed to bind full-length RRM2 (Supplemental Fig. S3C). We generated the C-terminal fragment of RRM1 [amino acids 701–792] with a SV40 nuclear localization signal (NLS-RC1-HA) and examined its ability to bind Tip60 in vivo and in vitro. NLS-RC1-HA, but not a control NL-GFP-HA fragment, was detected in the anti-Myc immunoprecipitates when transiently coexpressed with Tip60-Myc [Supplemental Fig. S3D]. Purified MBP-fused RC1 produced in *Escherichia coli* was capable of binding to GST-Tip60 expressed in insect cells (Supplemental Fig. S3E). Both Δ 761-C and Δ 781-C failed to bind chromatin, further confirming that the binding of RRM1 to chromatin required its interaction with Tip60 (Supplemental Fig. S3F).

Similarly to Chk1 [Niida et al. 2007; Shimada et al. 2008], endogenous RRM1 was present in cytosolic (S1), nucleoplasmic (S2), and chromatin-bound (P2) fractions (Supplemental Fig. S4A). Tip60 existed predominantly in

the chromatin-bound fraction (P2). Both RRM1 and Tip60 proteins in this fraction were partly solubilized by treatment with micrococcal nuclease (Mnase), suggesting that they associated with chromatin. RRM1 knockdown showed a significant decrease of RRM1 protein levels in both soluble and chromatin-bound fractions (Supplemental Fig. S4B). IKK α and Orc2 were detected predominantly in soluble and chromatin fractions, respectively, indicating that cell fractionation was done successfully. Ectopic RRM1-HA present in the chromatin fraction was increased when Tip60-Myc-His was coexpressed, although a low level of RRM1-HA was detected in the absence of Tip60-Myc-His, presumably due to the presence of endogenous Tip60 (Supplemental Fig. S4C). The amounts of RRM1 and Tip60 bound to the chromatin were not affected by DNA damage (Supplemental Fig. S4D). However, depletion of Tip60 resulted in a reduction in the amount of RRM1 on chromatin (Fig. 1C). Taken together, chromatin binding of RRM1 appeared to be Tip60-dependent. RRM1-HA, but not the RRM2 subunit alone, formed a complex with GST-His-Tip60 in insect cells (Fig. 1D, left panels). RRM2 also formed a complex with GST-His-Tip60 in a manner dependent on the presence of RRM1-HA. Consistently, accumulation of RRM2 at DSB sites was compromised when RRM1 was depleted (Supplemental Fig. S2D). Immunoprecipitations using anti-HA antibodies demonstrated that RRM1-HA bound to both RRM2 and GST-His-Tip60 (Fig. 1D, right panels). RRM1 and RRM2 were detected in the precipitates of anti-Tip60 antibodies from the solubilized chromatin, even in the absence of DNA damage (Fig. 1E). To further confirm the interaction between RNR and Tip60, we purified the Tip60 complex from HeLa cell nuclear extracts expressing Flag-HA Tip60 as reported previously [Ikura et al. 2000, 2007]. RRM1 and RRM2, as well as PAF400/TRRAP as a positive control [Murr et al. 2006], were detected in Tip60 complex from extracts with or without DNA damage (Fig. 1F). Tip60 knockdown by siRNA or shRNA abrogated accumulation of RRM1 along with microirradiated lines [Fig. 1G; Supplemental Fig. S2E]. These results suggested that RRM1 recruitment at DSB sites was Tip60-dependent.

To determine precisely whether RRM1 was recruited at the site of DNA damage, we generated *Ku*-deficient mouse embryonic fibroblasts (MEFs) in which a single DSB was introduced after infection with adenoviruses expressing I-SceI. This DSB was not rapidly repaired by nonhomologous end-joining, making it easy to detect proteins accumulating at this DSB site by chromatin immunoprecipitation (ChIP) analysis [*STEFKu70^{-/-}phprt-DR-GFP*] [Fig. 2A; Pierce et al. 2001]. Introduction of the DSB was confirmed by Southern blotting (Supplemental Fig. S5). ChIP analyses revealed a substantial increase in the binding of RRM1 as well as Rad51 and Tip60 to a DNA break site. An increase in acetylation of histone H4 was also observed at the damage site [Fig. 2B]. These were not seen on infection with control LacZ. Tip60 depletion by two independent siRNAs resulted in a loss of RRM1 binding to a DSB site, as well as a reduction in acetylation of histone H4 [Fig. 2C]. A mutant Tip60 lacking histone-acetylating activity could recruit RRM1 to the DSB site similarly to wild-type RRM1 (Supplemental Fig. S6A). Inhibition of ATM, ATR, and DNA-PK by caffeine did not affect RRM1 recruitment (Supplemental Fig. S6B). These results further supported the notion that complex

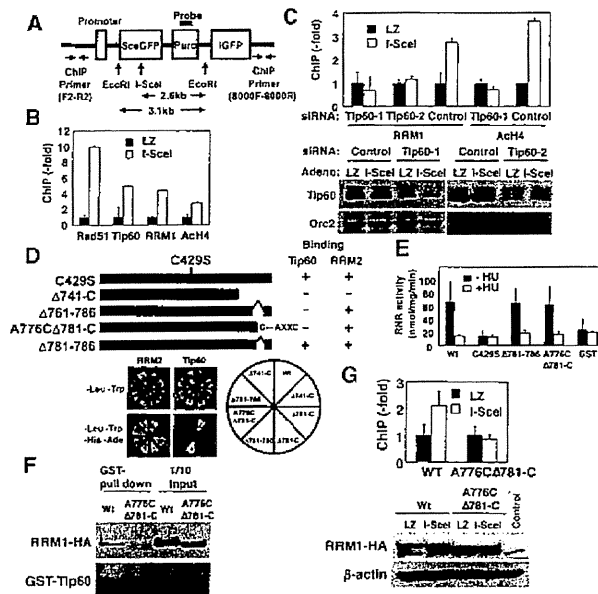


Figure 2. RRM1 is recruited at DSB sites in a Tip60-dependent manner. [A] Map of the I-SceI cassette construct containing the I-SceI site, the probe for Southern blotting, and a set of primers for the ChIP assay. [B] *STEFKu70*^{-/-}*phprt-DR-GFP* cells infected with I-SceI adenoviruses were subjected to ChIP analysis using the indicated antibodies as described in the supporting Materials and Methods. Data are shown as percentages of increases in PCR products from cells expressing I-SceI [I-SceI] relative to those from cells expressing Lac Z [LZ]. Data are mean \pm standard deviation ($n = 3$). [C] *STEFKu70*^{-/-}*phprt-DR-GFP* cells were transfected with two independent Tip60 siRNAs [Tip60-1 and Tip60-2] or control siRNA. ChIP analysis was performed as in B. [Bottom panels] Aliquots of cell lysates were subjected to immunoblotting using anti-Tip60 antibodies. [D] The constructs used are schematically represented, and the specific interaction between RRM1 mutants and Tip60 was assayed using yeast two-hybrid screening. [E] An in vitro RNR assay of complexes containing wild-type or various RRM1 mutants was performed as described in the Materials and Methods. (Black bars) -HU; (white bars) +HU (10 mM). Data are mean \pm standard deviation ($n = 3$). [F] S9 lysates expressing GST-His-Tip60 and the indicated RRM1-HA were subjected to GST pull-down assay using the indicated antibodies. [G] Knockout-knock-in *STEFKu70*^{-/-}*phprt-DR-GFP* cells expressing wild-type or A776CA781-C RRM1-HA were generated by transfection with vectors for either wild-type or A776CA781-C RRM1 and then with RRM1 siRNA. Expression vectors of wild type and A776CA781-C contain mutations in a specific sequence targeted by siRNA. (Top panel) Cells were subjected to ChIP analysis using anti-HA antibodies as in B. (Bottom panels) Aliquots of cell lysates were subjected to immunoblotting using the indicated antibodies.

formation between RNR and Tip60 is required for recruitment of RNR to sites of DNA damage.

We then examined if RNR recruitment at damage sites was required for effective DNA repair. We first generated RRM1 mutants that lack the ability to bind Tip60 but retain RNR activity. Given that the C-terminal CXXC motif of RRM1 is important for RNR function [Zhang et al. 2007], we constructed RRM1 mutants containing the CXXC motif but lacking Tip60-binding ability [Δ 761-786 and A776CA781-C] [Fig. 2D]. Wild-type RRM1 or its mutants were coexpressed with RRM2 in insect cells, and the resultant complexes were subjected to an in vitro RNR assay [Fukushima et al. 2001]. RNR complexes containing wild-type, Δ 761-786, and A776CA781-C RRM1

retained hydroxyurea [HU]-sensitive RNR activity [HU is a specific RNR inhibitor], whereas an inactive C429S mutant or GST protein as a negative control did not show RNR activity (Fig. 2E). The specific activity of RNR containing wild-type, Δ 761-787, and A776CA781-C RRM1 (\sim 50 nmol/mg per minute) was similar to that reported previously [Guittet et al. 2001], confirming the reliability of our results. The A776CA781-C mutant failed to form a complex with GST-Tip60 [Fig. 2F]. ChIP analysis using RRM1 knockout-knock-in *STEFKu70*^{-/-}*phprt-DR-GFP* cells revealed that the A776CA781-C mutant failed to accumulate at the DSB site (Fig. 2G). These results indicated that direct interaction of RRM1 to Tip60 is required for triggering its accumulation at the DSB site.

A comet assay revealed that DNA damage in cells was repaired efficiently within 1 h in the absence of HU. However, treatment with HU, and RRM1 or RRM2 depletion, resulted in an impairment of DNA repair (Fig. 3A,B). RNR activity was thus essential for effective repair. Ectopic expression of wild-type RRM1 with mutations in a specific sequence targeted by siRNA effectively rescued the impaired DNA repair in cells depleted of endogenous RRM1 [Fig. 3C]. In contrast, ectopic expression of C429S, Δ 761-786, and A776CA781-C RRM1 failed

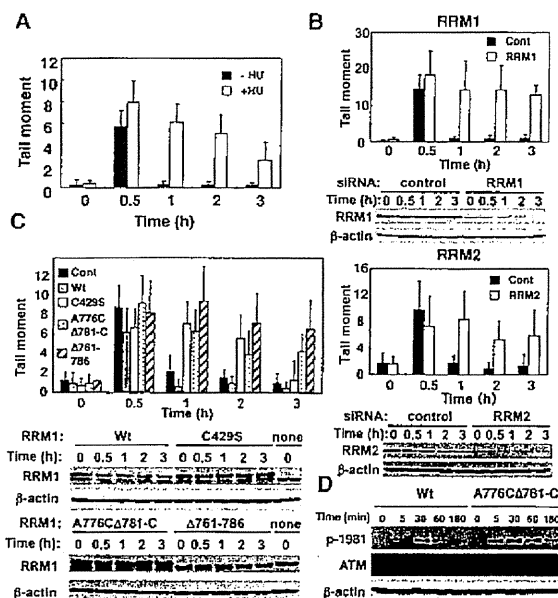


Figure 3. Recruitment of active RNR at DNA damage sites is a prerequisite for effective DNA repair. [A] HeLa cells were treated with (open bars) or without (filled bars) 2.5 mM HU, exposed to IR (4 Gy), and subjected to a comet assay as described in the Materials and Methods. The results were obtained by counting at least 50 cells per sample in three independent experiments. [B] HeLa cells were transfected with a control (open bars) or RRM1 siRNA (filled bars), and DNA repair was evaluated as in A. Cell lysates were subjected to immunoblotting using the indicated antibodies. [C] HeLa cells were transfected with or without (filled bars) either wild-type (gray bars), C429S (open bars), A776CA781-C (dotted), or Δ 761-786 (hatched) RRM1. RRM1-transfected cells were then transfected with RRM1 siRNA. Expression vectors of wild type and various RRM1 mutants contain mutations in a specific sequence targeted by siRNA. DNA repair activity and expression of RRM1 were examined as in B. [D] Knockout-knock-in HeLa cells expressing wild type or A776CA781-C RRM1-HA were exposed to IR, and cell lysates were subjected to immunoblotting as in C.

to do so. ATM was activated independently of Tip60 binding to RNR, but this activation was enhanced and prolonged in cells expressing A776CΔ781-C, presumably due to impaired DNA repair (Fig. 3D). It is therefore conceivable that recruitment of active RNR at DNA damage sites is a prerequisite for effective DSB repair, but not for activation of checkpoint signaling. Tip60 is also known to participate in transcriptional regulation of several genes. Neither RRM1 nor RRM2 proteins were affected by Tip60 depletion or overexpression (Supplemental Fig. S7), indicating that the effect of Tip60 did not result from changes in RRM1 and RRM2 expression.

ChIP analyses revealed that NLS-RC1-HA specifically inhibited RRM1 binding, but did not affect Rad51 or Tip60 binding, or increase H4 acetylation at the DSB site in *STEFKu70^{-/-}phprt-DR-GFP* cells (Fig. 4A). Expression of NLS-RC1-HA suppressed accumulation of endogenous RRM1 at DNA damage sites (Supplemental Fig. S8A,B), but did not affect the foci formation of 53BP1 at DSB sites (Fig. 4B), or complex formation and activity (Supplemental Fig. S9A,B) of endogenous RNR. However, cells expressing NLS-RC1-HA, but not NLS-GFP-HA, had

unrepaired DNA in the tail at 2 h (Fig. 4C). A quantitative colony formation assay was used to examine the DNA damage sensitivity of cells expressing NLS-RC1-HA. Induction of NLS-RC1-HA sensitized cells to IR (Fig. 4D).

Given that levels of dNTP pools are higher during S phase than during G1 phase (Hakansson et al. 2006), recruitment of RNR at damage sites may function at a specific phase of the cell cycle where dNTP pools are low. To address this issue, we synchronized cells at S phase or G1 phase by arrest and release of thymidine or nocodazole, respectively. Recruitment of wild-type RRM1 at a DSB site was observed at both G1 and S phase (Supplemental Fig. S10). However, a comet assay revealed that A776CΔ781-C failed to rescue the impaired DNA repair in RRM1-depleted cells at G1 phase, but not at S phase (Fig. 4E). Consistently, RRM1 mutation of Tip60 binding slightly sensitizes cells to Zeocin (Supplemental Fig. S11A), which causes DNA strand breaks, but not to MMC (Supplemental Fig. S11B), which can cause interstrand cross-linking repaired mainly at S-G2 phase. Intriguingly, this G1-phase-specific impairment of DNA repair was restored when excess amounts of dADP, dGDP, dCDP, and dUMP (250 μM) were supplied in the culture medium (Supplemental Fig. S12). These results suggested that recruitment of RNR was required specifically for effective DNA repair in cells with low levels of dNTPs.

The present study suggests that the RNR recruitment to DSB sites likely provides mechanistic insights into the regulatory events that ensure a balanced supply of dNTPs during mammalian DNA repair. RNR appears to form a complex with Tip60 independently of DNA damage. Thus, it is possible that the RNR–Tip60 complex might have an alternative function, such as regulation of transcription. In response to DNA damage, regulation of the RNR subunit by Wtm1 and Dif1 in budding yeast is radically different in terms of cellular localization (Lee and Elledge 2006; Lee et al. 2008) from that observed in the present study; however, important changes in the subcellular localization of RNR might be conserved. Given that Tip60 is a key regulator of DNA damage responses, the concomitant recruitment of RNR at damage sites suggests the presence of a synthetic regulatory mechanism for DNA repair in mammals.

Materials and methods

Antibodies

Antibodies used were as follows: α-Rad51 (Ab-1, Oncogene Research Products), α-RRM1 (sc-11733 and sc-11731, Santa Cruz Biotechnologies), α-HA (11 666 606 001, Roche Applied Sciences; and PM002, MBL), α-Myc (sc-40 and sc-789, Santa Cruz Biotechnologies), α-RRM2 (sc-10844, Santa Cruz Biotechnologies), α-GST (sc-459, Santa Cruz Biotechnologies), α-Chk1 (sc-8408, Santa Cruz Biotechnologies), α-IKKα (sc-7182, Santa Cruz Biotechnologies), α-Orc2 (sc-13238, Santa Cruz Biotechnologies), α-ATM (sc-23921, Santa Cruz Biotechnologies), α-ATMp1981 (no. 4526, Cell Signaling), α-acetylated histone H4 (no. 06-866, Upstate Biotechnologies), and α-phospho-histone H2AX (411-pc-020, TRVIGEN; and 05-636, Upstate Biotechnologies). Anti-Tip60 rabbit polyclonal antibodies were generated by immunization with recombinant GST-His-Tip60 produced in insect cells, and the serum obtained was affinity-purified using a GST-His-Tip60 column.

Two-hybrid interaction assays

The *pGBKT7-RRM1* plasmid was generated by insertion of the full-length human *RRM1*-encoding sequence. *pGBKT7-RRM1* was transformed into

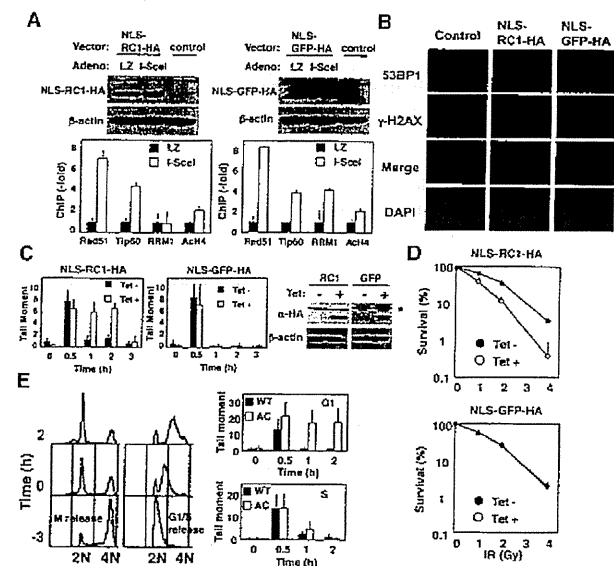


Figure 4. Inhibition of recruitment of RNR at DSB sites by ectopic expression of NLS-RC1-HA abrogates DNA repair and sensitizes cells to DNA damage. [A] *STEFKu70^{-/-}phprt-DR-GFP* cells expressing NLS-RC1-HA [SV40 NLS-RC1 fragment, 701–792 amino acids] or NLS-GFP-HA [GFP fragment, 1–93 amino acids] were subjected to ChIP analysis as in Figure 2B. [Top panels] Cell lysates were subjected to immunoblotting using the indicated antibodies. [B, left panels] Tet-on HeLa cells expressing NLS-RC1-HA or NLS-GFP-HA were treated with or without tetracycline (1 μg/mL), exposed to IR (4 Gy), and subjected to immunostaining with the indicated antibodies and a comet assay as in Figure 3A. [Right panels] IR-untreated lysates were subjected to immunoblotting using the indicated antibodies. [C] Asterisk (*) represents nonspecific bands. [D] These cells were exposed to the indicated dose of IR, and a quantitative colony formation assay was performed 8 d after treatment. Data are mean ± standard deviation ($n = 3$). [E] Knockout-knock-in HeLa cells expressing either wild-type (filled bars) or A776CΔ781-C (open bars) RRM1-HA were synchronized as described in the Materials and Methods. Synchronized cells were then released into G1 phase or S phase (time -3) and exposed to IR (4 Gy) 3 h after release (time 0). [Right panels] DNA repair was evaluated as in A. [Left panels] Cell cycle distributions are presented.

the yeast strain AH101 and mated with yeast Y187 pretransformed with a HeLa cell cDNA library (BD Biosciences). The deletion mutants of RRM1 and Tip60 were amplified by PCR using specific sets of primers. Primer sequences are supplied in the Supplemental Material.

Affinity purification of Tip60 complex

Affinity purification of Tip60 complex was performed as described previously [Ikura et al. 2000, 2007]. For the induction of DNA damage, cells were γ -irradiated (12 Gy) after centrifugation.

In situ detergent extraction and immunofluorescence analysis

Immunofluorescence on paraformaldehyde-fixed cells was performed according to a previous report [Green and Almouzni 2003], using the indicated antibodies.

Microirradiation

Microirradiation was performed as described previously [Ikura et al. 2007]. In brief, GM02063 cells were maintained on the microscope stage in a ChamSlide TC live-cell chamber system [Live Cell Instrument] at 37°C. Microirradiation was performed using an LSM510 confocal microscope [Carl Zeiss]. Sensitization of cells was performed by incubating the cells for 20 h in medium containing 2.5 μ M deoxyribosylthymine and 0.3 μ M bromodeoxyuridine [Sigma], and then staining with 2 μ g/mL Hoechst 33258 [Sigma] for 10 min before UVA microirradiation. The 364-nm line of the UVA laser was used for microirradiation (three pulses at 30 μ W). Samples were examined with a Zeiss Axioplan 2 equipped with a charge-coupled device camera AxioCam MRm controlled by Axiovision software [Zeiss].

Knockdown experiments

HeLa cells or STEFKu70^{-/-} *phprt-DR-GFP* cells were transfected with either control siRNA [Silencer Negative Control #1, Ambion 4611], siRNAs for human Tip60 [sc-37966, Santa Cruz Biotechnologies], mouse Tip60-1 [sc-37967, Santa Cruz Biotechnologies], mouse Tip60-2 [D-057795-02-0010, Dharmacon], or RRM1 [GGAUCGCUGUCUCUAA CUUtt] using Lipofectamine 2000 reagent [Invitrogen].

Subcellular fractionation and Mnase treatment

Subcellular fractionation was performed according to a previous report [Mendez and Stillman 2000]. The isolated chromatin fraction [1 \times 10⁶ cells] was treated with Mnase [15 U] for 30 min at 37°C.

Establishment of STEFKu70^{-/-} cells containing a *phprt-DR-GFP* cassette

The *phprt-DR-GFP* vector [10 μ g] was linearized with PvuI and transfected into STEFKu70^{-/-} cells. Cells were selected with 1.25 μ g/mL puromycin for 12 d, and single colonies were screened by Southern blotting using puromycin cDNA as a probe. Clones having only one copy of the *phprt-DR-GFP* cassette were used for experiments.

Establishment of Tet-on HeLa cells expressing NLS-RC1

pcDNA4/TO-NLS-RC1 [10 μ g] was linearized with XhoI and transfected into HeLa T-Rex cells [Invitrogen]. Positive clones were selected with Zeocin (250 μ g/mL) and Blastidin (5 μ g/mL) for 12 d and screened by immunoblotting using anti-HA antibodies for the detection of NLS-RC1 induction in the presence of tetracycline [1 μ g/mL].

Generation of adenoviruses expressing I-SceI endonuclease

The full-length *I-SceI* fragment harboring the CAG promoter and poly A signal was subcloned into *pAd/PL-DEST* [Invitrogen]. Adenoviruses expressing *I-SceI* were generated according to the manufacturer's protocol [Invitrogen].

ChIP assay

A population of STEFKu70^{-/-} cells (1 \times 10⁷) containing *phprt-DR-GFP* cells infected with adenoviruses expressing *I-SceI* was cross-linked with 1% formaldehyde for 10 min at 37°C. ChIP assays were performed essentially as described [Shimada et al. 2008]. Precipitated DNA was resuspended in 50 μ L of water and analyzed by quantitative real-time PCR with the ABI PRISM7000 system using Power SYBR Green PCR Master Mix [Applied Biosystems] as described [Katsuno et al. 2009]. Primers used for detection of the *I-SceI* break site were indicated in Figure 2A. As an internal control for normalization of the specific fragments amplified, mouse GAPDH locus was amplified using whole genomic DNAs with mGAPDH-F and mGAPDH-R. Primer sequences are supplied in the Supplemental Material.

Comet assay

Alkaline comet assays were performed using a Trevigen's Comet Assay kit [4250-050-k] according to the manufacturer's instructions. DNA was stained with SYBR Green, and slides were photographed digitally [Nikon Eclipse E800 lens and Fuji CCD camera]. Tail moments were analyzed as reported previously [Park et al. 2006] using TriTek Comet Score Freeware.

Measurement of DNA damage sensitivity

Tet-on HeLa cells expressing NLS-RC1-HA or NLS-GFP-HA were irradiated with varying doses of IR in the presence or absence of doxycycline [1 μ g/mL], and then washed with PBS. Eight days after an additional incubation, surviving colonies were counted, and their relative numbers were expressed as percentages of the untreated cells ($n = 3$).

RNR assay

Insect cells were coinfecting with baculoviruses expressing wild-type RRM1 or its mutants, and with those expressing wild-type RRM2. RNR complexes were immunopurified, and their activities were determined according to a method reported previously [Fukushima et al. 2001]. Amounts of wild-type RRM1 protein or its mutant proteins were determined by SDS-PAGE and used for calculating specific activities.

Cell cycle synchronization

For synchronization of cells at S phase, knockout-knock-in HeLa cells expressing wild-type or A776C Δ 781-C RRM1-HA were first synchronized at the G1/S boundary by exposure to 2.5 mM thymidine for 16 h, and then released into S phase by wash-out of thymidine with PBS and the addition of 20% FBS containing DMEM. Cells were then exposed to IR 3 h after release. For synchronization of cells at G1 phase, knockout-knock-in HeLa cells were synchronized at M phase by exposure to 100 ng/mL nocodazole for 16 h and released into G1 phase by wash-out of nocodazole with PBS and addition of 20% FBS containing DMEM. Cells were then exposed to IR 3 h after release.

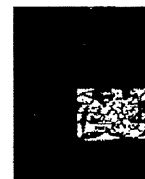
Acknowledgments

We thank M. Delhase for critical reading of the manuscript; M. Jasin for *hprt-DR-GFP* and *pCBASce* vectors; M. Fukushima for critical advice on the RNR assay; A. Kurimasa for STEFKu70^{-/-} MEFs; K. Murata, C. Namikawa-Yamada, and H. Kojima for technical assistance, and M. Inagaki and H. Goto for fluorescence microscopy. This work was supported in part by the Ministry of Education, Science, Sports, and Culture of Japan through Grants-in-Aid for Scientific Research [B] [to M.N.] and [C] [to H.N.], the YASUDA Medical Foundation [to M.N.], and the Sagawa Cancer Foundation [to M.N.].

References

- Dong Q, Wang TS. 1995. Mutational studies of human DNA polymerase α . Lysine 950 in the third most conserved region of α -like DNA polymerases is involved in binding the deoxynucleoside triphosphate. *J Biol Chem* 270: 21563–21570.

- Fukushima M, Fujioka A, Uchida J, Nakagawa F, Takeuchi T. 2001. Thymidylate synthase (TS) and ribonucleotide reductase (RNR) may be involved in acquired resistance to 5-fluorouracil (5-FU) in human cancer xenografts in vivo. *Eur J Cancer* 37: 1681-1687.
- Green CM, Almouzni G. 2003. Local action of the chromatin assembly factor CAF-1 at sites of nucleotide excision repair in vivo. *EMBO J* 22: 5163-5174.
- Gutter O, Hakansson P, Voevodskaya N, Fridt S, Graslund A, Arakawa H, Nakamura Y, Thelander L. 2001. Mammalian p53R2 protein forms an active ribonucleotide reductase in vitro with the R1 protein, which is expressed both in resting cells in response to DNA damage and in proliferating cells. *J Biol Chem* 276: 40647-40651.
- Hakansson P, Hofer A, Thelander L. 2006. Regulation of mammalian ribonucleotide reduction and dNTP pools after DNA damage and in resting cells. *J Biol Chem* 281: 7834-7841.
- Ikura T, Ogryzko VV, Grigoriev M, Croisman R, Wang J, Horikoshi M, Scully R, Qin J, Nakatani Y. 2000. Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. *Cell* 102: 463-473.
- Ikura T, Tashiro S, Kakino A, Shima H, Jacob N, Amunugama R, Yoder K, Izumi S, Kuraoka I, Tanaka K, et al. 2007. DNA damage-dependent acetylation and ubiquitination of H2AX enhances chromatin dynamics. *Mol Cell Biol* 27: 7028-7040.
- Imaizumi T, Jean-Louis F, Dubertret ML, Bailly C, Cicurel L, Petchot-Bacque JF, Dubertret L. 1996. Effect of human basic fibroblast growth factor on fibroblast proliferation, cell volume, collagen lattice contraction: In comparison with acidic type. *J Dermatol Sci* 11: 134-141.
- Johnson RE, Trincao J, Aggarwal AK, Prakash S, Prakash L. 2003. Deoxynucleotide triphosphate binding mode conserved in Y family DNA polymerases. *Mol Cell Biol* 23: 3008-3012.
- Katsuno Y, Suzuki A, Sugimura K, Okumura K, Zineldeen DH, Shimada M, Niida H, Mizuno T, Hanaoka F, Nakanishi M. 2009. Cyclin A-Cdk1 regulates the origin firing program in mammalian cells. *Proc Natl Acad Sci* 106: 3184-3189.
- Kraynov VS, Showalter AK, Liu J, Zhong X, Tsai MD. 2000. DNA polymerase β : Contributions of template-positioning and dNTP triphosphate-binding residues to catalysis and fidelity. *Biochemistry* 39: 16008-16015.
- Lee YD, Elledge SJ. 2006. Control of ribonucleotide reductase localization through an anchoring mechanism involving Wtm1. *Genes & Dev* 20: 334-344.
- Lee YD, Wang J, Stubbe J, Elledge SJ. 2008. Difi1 is a DNA-damage-regulated facilitator of nuclear import for ribonucleotide reductase. *Mol Cell* 32: 70-80.
- Mendez J, Stillman B. 2000. Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: Assembly of prereplication complexes in late mitosis. *Mol Cell Biol* 20: 8602-8612.
- Murr R, Loizou JI, Yang YG, Cuenin C, Li H, Wang ZQ, Herceg Z. 2006. Histone acetylation by Trp1-Tip60 modulates loading of repair proteins and repair of DNA double-strand breaks. *Nat Cell Biol* 8: 91-99.
- Niida H, Katsuno Y, Banerjee B, Hande MP, Nakanishi M. 2007. Specific role of Chk1 phosphorylations in cell survival and checkpoint activation. *Mol Cell Biol* 27: 2572-2581.
- Nordlund P, Reichard P. 2006. Ribonucleotide reductases. *Annu Rev Biochem* 75: 681-706.
- Park JH, Park EJ, Lee HS, Kim SJ, Hur SK, Imbalzano AN, Kwon J. 2006. Mammalian SWI/SNF complexes facilitate DNA double-strand break repair by promoting γ -H2AX induction. *EMBO J* 25: 3986-3997.
- Pierce AJ, Hu P, Han M, Ellis N, Jasin M. 2001. Ku DNA end-binding protein modulates homologous repair of double-strand breaks in mammalian cells. *Genes & Dev* 15: 3237-3242.
- Pontarin G, Fijolek A, Pizzo P, Ferraro P, Rampazzo C, Pozzan T, Thelander L, Reichard PA, Bianchi V. 2008. Ribonucleotide reduction is a cytosolic process in mammalian cells independently of DNA damage. *Proc Natl Acad Sci* 105: 17801-17806.
- Reichard P. 1993. From RNA to DNA, why so many ribonucleotide reductases? *Science* 260: 1773-1777.
- Shimada M, Niida H, Zineldeen DH, Tagami H, Tanaka M, Saito H, Nakanishi M. 2008. Chk1 is a histone H3 threonine 11 kinase that regulates DNA damage-induced transcriptional repression. *Cell* 132: 221-232.
- Zhang Z, Yang K, Chen CC, Feser J, Huang M. 2007. Role of the C terminus of the ribonucleotide reductase large subunit in enzyme regeneration and its inhibition by Sml1. *Proc Natl Acad Sci* 104: 2217-2222.



Anaphase-promoting complex/cyclosome-cdh1 mediates the ubiquitination and degradation of TRB3

Nobumichi Ohoka^{a,1}, Satoshi Sakai^a, Kikuo Onozaki^a, Makoto Nakanishi^b, Hidetoshi Hayashi^{a,*}

^a Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya 467-8603, Japan

^b Graduate School of Medical Sciences, Nagoya City University, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan

ARTICLE INFO

Article history:

Received 24 December 2009

Available online 11 January 2010

Keywords:

TRB3

APC/C

Cdh1

Proteasome

Degradation

ABSTRACT

We have recently demonstrated that TRB3, a novel endoplasmic reticulum (ER) stress-inducible protein, is induced by CHOP and ATF4 to regulate their function and ER stress-induced cell death; however, the regulation of TRB3 function has not been well characterized. Here we demonstrate that TRB3 is an unstable protein regulated by the ubiquitin–proteasome system. The carboxyl-terminal domain of TRB3 is necessary for protein degradation, and in this region, we found the typical D-box motif, which is a critical sequence for the anaphase-promoting complex/cyclosome (APC/C) dependent proteolysis. TRB3 proteins were stabilized by deletion of its D-box motif and interacted with APC/C coactivator proteins, Cdc20 and Cdh1. The expression level of TRB3 protein is down-regulated by over-expression of Cdh1 but not by that of Cdc20. In addition, knockdown of Cdh1 enhanced the endogenous TRB3 expression level and suppressed its ubiquitination level. These results suggest that APC/C^{Cdh1} is involved in ubiquitination and down-regulating the stability of TRB3 protein.

© 2010 Elsevier Inc. All rights reserved.

Introduction

The pseudokinase tribbles 3 (TRB3; also termed SINK, SKIP3, NIPK) is one of the mammalian orthologs of *tribbles*, a cell cycle regulator during development in *Drosophila* [1,2]. TRB3 and other *tribbles* family members (TRB1 and TRB2) contain the classic substrate-binding domains of a protein kinase but not the ATP-binding and kinase-activating domains; therefore, they do not have a kinase activity [3].

TRB3 does not possess a characteristic functional domain (possesses only an incomplete kinase like domain), however, it acts as a multifunctional molecule by interacting with the various proteins. The association of TRB3 to the kinases, such as Akt and MAPK, leads to inhibition of phosphorylation of them [4,5], and the interaction with the transcription factors, such as NF- κ B/p65, ATF4, CHOP, C/EBP β and PPAR γ , causes repression of these transcriptional activities [6–11]. On the other hand, TRB3 is also involved in the ubiquitin–proteasome pathway as well as *Drosophila* tribbles by functioning as an adaptor protein for the ubiquitin E3 ligases, thereby promoting the ubiquitination and degradation of acetyl-coenzyme A carboxylase (ACC) and Smurf1 [12,13]. Through these functions, TRB3 has been shown to play an important role in various phenomenon including gluconeogenesis, muscle and adipocyte differentiation, the stress response including hypoxia, anoxia and endoplasmic reticulum (ER) stress, and signal transduction of cytokines containing interleukins and tumor necrosis factor α (TNF α), and bone morphogenetic protein (BMP) [3–15].

It has been reported that the expression of TRB3 is regulated at transcriptional level and mRNA stabilization during fasting condition, differentiation and the stress responses [4,8,10–12,15]. Furthermore, in the past study, the treatment of proteasome inhibitors induced the increase of TRB3 protein expression, suggesting that TRB3 expression level is also modulated by a proteasome-dependent mechanism [13].

Some of the E3 ligases specifically degrade substrate proteins by recognizing the formal amino acid sequences in the target molecules. The D-box motif consists of a consensus sequence of RxxLxxxN and is recognized by the E3 ligase complexes APC/C containing Cdh1 or Cdc20, and APC/Cs degrade the cell cycle regulators possessing a D-box sequence, such as cyclinB1, Skp2, Aurora-A and Id2, as the target substrates [16–18]. In this study, we found that TRB3 protein contains a D-box motif and is ubiquitinated and degraded by Cdh1 via this motif.

Abbreviations: aa, amino acid; ACC, acetyl-coenzyme A carboxylase; APC/C, anaphase-promoting complex/cyclosome; ATF4, activating transcription factor 4; C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP homologous protein; E3, ubiquitin-protein isopeptidase; ER, endoplasmic reticulum; GFP, green fluorescent protein; PPAR, peroxisome proliferator-activated receptor.

* Corresponding author. Fax: +81 52 836 3484.

E-mail address: bhayashi@phar.nagoya-cu.ac.jp (H. Hayashi).

¹ Present address: Division of Biosignaling, National Institute of Health Sciences, Tokyo 158-8501, Japan.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium, anti- β -actin monoclonal antibody (AC-15), and anti-FLAG monoclonal antibody (M2) were purchased from Sigma. Fetal bovine serum was from HyClone (Logan, UT). MG132 was obtained from Peptide Institute (Osaka, Japan). Cycloheximide was obtained from Nacalai Tesque (Kyoto, Japan). Anti-Cdh1 monoclonal antibody (DH-01) was from Calbiochem (Darmstadt, Germany), and anti-Cdc20 polyclonal antibody (H-175) and anti-Cdc25A polyclonal antibody (M-191) were from Santa Cruz (Santa Cruz, CA). Anti-Myc monoclonal antibody (9E10) was from Roche (Indianapolis, IN). Anti-GFP monoclonal antibody (JL8) was from Clontech (Mountain View, CA). The antiserum against human TRB3 was prepared as described previously [8].

Cell culture. The embryonic kidney cell line 293 and human hepatocellular carcinoma cell line HepG2 were cultured as described previously [19].

Construction of expression plasmids. The plasmids pCMV5-Flag-TRB3, pCMV5-Flag-TRB3 Δ N lacking aa 1–127, pCMV5-Flag-TRB3 Δ C lacking aa 283–358, pCMV5-Flag-TRB3N179 lacking aa 180–358, pCMV5-Flag-TRB3C179 lacking aa 1–179 or pCMV5-Flag-TRB3 Δ Akt lacking aa 239–265, the region essential for the binding with Akt1 of human TRB3 were constructed as described previously [8]. pCMV5-Flag-TRB3 Δ D-box lacking aa 195–202 were generated by PCR. pMT-123 (HA-Ub) [20] was kindly provided by Dr. D. Bohmann (University of Rochester Medical Center). pcDNA3-Myc-Cdh1 and pcDNA3-Myc-Cdc20 [21] was kindly provided by Dr. J.M. Peters (Research Institute of Molecular Pathology, Austria). All constructs were verified by sequencing.

Immunoprecipitation and Western blot analysis. Cells were transiently transfected and treated as described in the figure legends. The cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, and 1% Triton X-100) supplemented with protease inhibitors. The lysates were subjected to immunoprecipitation, and 1–2% of the lysate or co-immunoprecipitates was subjected to SDS-PAGE (5–12.5%), transferred onto a PVDF membrane and probed with the antibodies indicated in the figure legends. The immunoreactive proteins were visualized using ECL (Amersham Bioscience) or Immobilon (Millipore) Western blotting detection reagents, and light emission was quantified with a LAS1000 lumino image analyzer (FUJI, Japan).

RNA interference. Double stranded RNA duplexes corresponding to human TRB3, Cdh1 and Cdc20 were obtained from Dharmacon Inc. (Chicago, IL).

Transfection. 293 and HepG2 cells were transfected using the Chen-Okayama method as described previously [19]. For RNA interference, HepG2 cells were transfected using a lipofection method with Lipofectamine RNAi MAX (Invitrogen).

Results

TRB3 protein is degraded by ubiquitin–proteasome pathway

To determine the stability of TRB3 protein, we first examined the effects of a proteasome inhibitor MG132 on its ectopic expression. Proteasome inhibition resulted in increased FLAG-TRB3 steady-state protein levels (Fig. 1A), which was accompanied by accumulation of polyubiquitin-reactive signals in the TRB3 immune-complexes (Fig. 1C). We determined the endogenous TRB3 stability in HepG2 cells by blocking *de novo* protein synthesis with cycloheximide and analyzing the remaining TRB3 protein amounts by immunoblotting. TRB3 had a short half-life of approximately 15 min, and this was increased markedly in the presence of MG132, as amounts remained virtually unchanged 60 min after

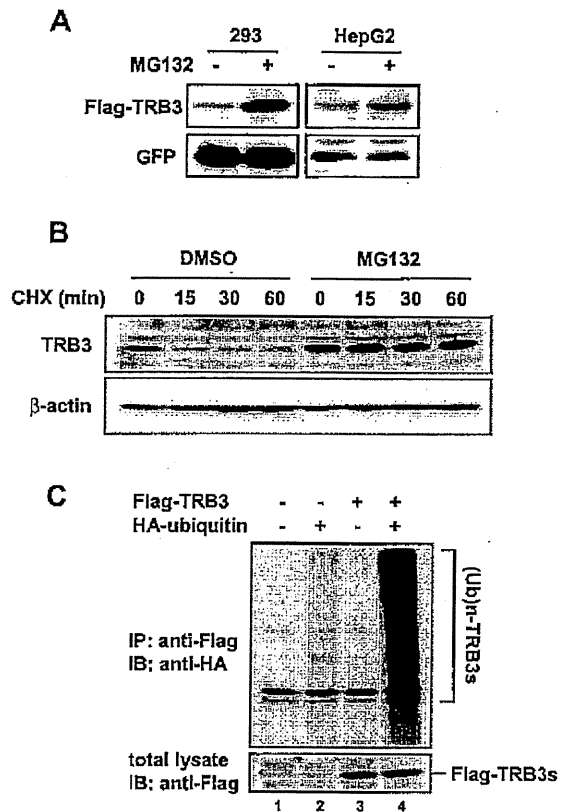


Fig. 1. TRB3 protein is degraded via the ubiquitin–proteasome pathway. (A) 293 and HepG2 cells were transiently transfected with expression vector for Flag-TRB3. After 24 h, the cells were treated with 10 μ M MG132 for 12 h. The cell lysates were analyzed by immunoblotting using anti-FLAG antibody. The pEGFP-C1 expression vector was included in each transfection as a transfection efficiency control, and its level was detected with anti-GFP antibody. (B) HepG2 cells were treated with 10 μ M MG132 for 6 h and then chased with or without 10 μ M MG132 and 10 μ g/ml of cycloheximide (CHX) for the indicated periods. The cell lysates were analyzed by immunoblotting using anti-TRB3 and anti- β -actin antibodies. (C) 293 Cells were transiently transfected with the indicated constructs. After 24 h, the cells were treated with 10 μ M MG132 for 12 h. The cell lysates were immunoprecipitated with anti-FLAG antibody, and multi-ubiquitinated TRB3 was detected by immunoblotting with anti-HA antibody. The expression level of each protein was assessed by immunoblotting with the indicated antibodies.

cycloheximide treatment (Fig. 1B). These data indicate that TRB3 is regulated by the ubiquitin–proteasome system.

Mapping of the TRB3 region required for its stability

Next we used various TRB3 deletion mutants to map the region responsible for TRB3 stability (Fig. 2A). This experiment demonstrated that the region aa 180–238 and aa 266–282 is crucial in TRB3 degradation. Within this region, a D-box motif was found in TRB3 between residues 195–202 that show a consensus of RxxLxxxxN (Fig. 2B). This motif, targeted by the anaphase-promoting complex/cyclosome (APC/C), is usually found in cell cycle-regulated proteins and mediates degradation of these proteins. To determine whether the D-box is required for the degradation of TRB3, the D-box was deleted, and the resulting TRB3 mutant (Δ D-box) was tested in the pulse-chase and the ubiquitination experiment. As shown in Fig. 2C, deletion of this D-box motif (TRB3 Δ D-box) increased the stability of TRB3 protein and no enhancement of its accumulation was observed with MG132 treatment. Consistent with this, deletion of the D-box prevented the

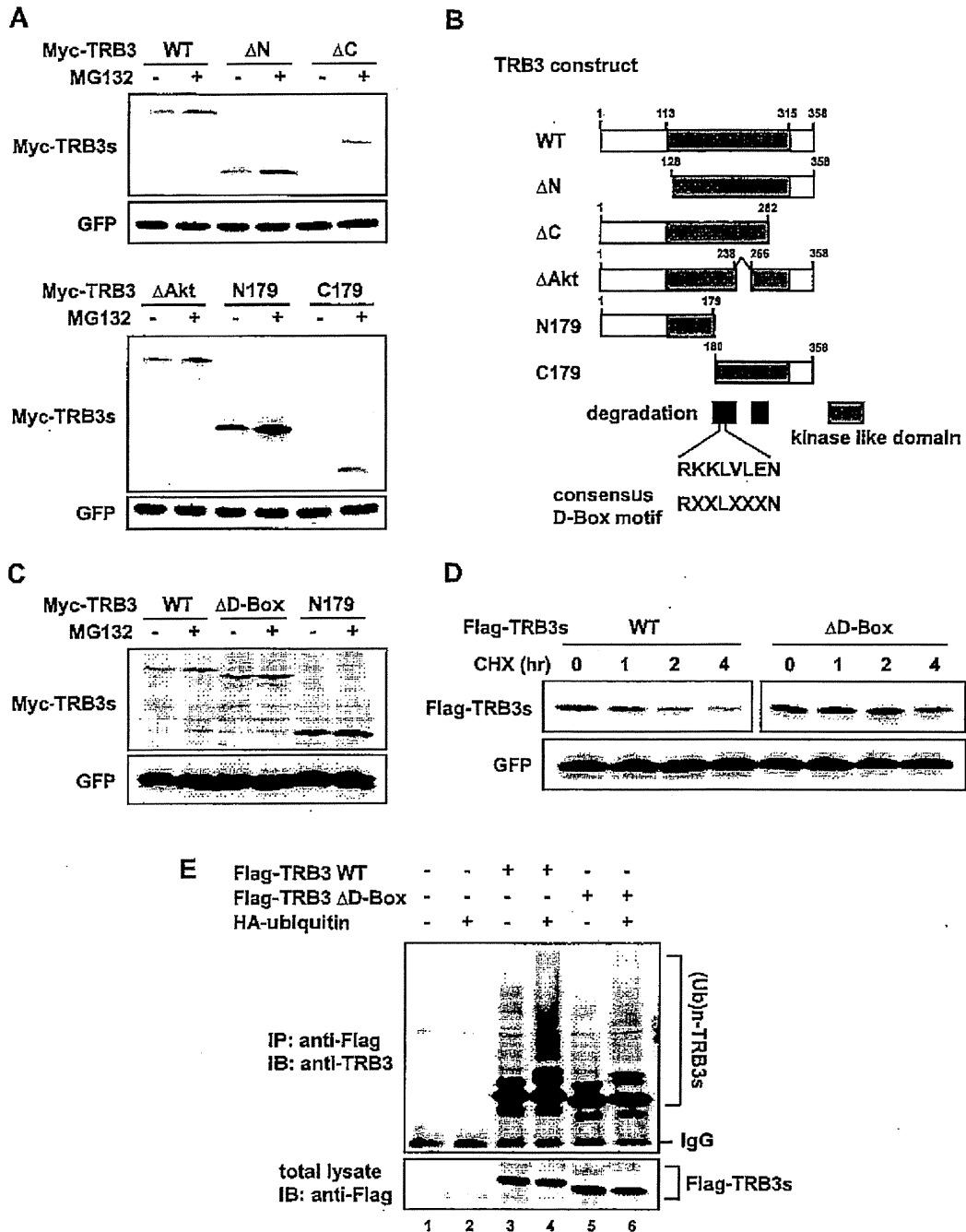


Fig. 2. The D-box motif of TRB3 is critical for its ubiquitination. (A, C) 293 Cells were transiently transfected with expression vector for wild-type Myc-TRB3 or its mutants. After 24 h, the cells were treated with or without 10 μ M MG132 for 12 h. The cell lysates were analyzed by immunoblotting using anti-Myc antibody. The pEGFP-C1 expression vector was included in each transfection as a transfection efficiency control, and its level was detected with anti-GFP antibody. (B) The constructs of TRB3 mutants and the amino acids sequence of the D-Box motif in TRB3. (D) 293 Cells were transiently transfected with expression vector for Flag-TRB3 wild type or Δ D-Box. After 36 h, the cells were chased with 10 μ g/ml of cycloheximide (CHX) for the indicated periods. The cell lysates were analyzed by immunoblotting using anti-FLAG and anti-GFP antibodies. (E) 293 Cells were transiently transfected with the indicated constructs. After 24 h, the cells were treated with 10 μ M MG132 for 12 h. The cell lysates were immunoprecipitated with anti-FLAG antibody, and multi-ubiquitinated TRB3s were detected by immunoblotting with anti-TRB3 antibody. The expression level of each protein was assessed by immunoblotting with the indicated antibodies.

ubiquitination and degradation of TRB3 (Fig. 2D and E). Thus, the D-box is essential for degradation of TRB3, and a ubiquitin ligase complex that targets the D-box, possibly the APC/C, is required for the degradation of TRB3.

TRB3 interacts with APC/C coactivators, Cdc20 and Cdh1

APC/C critically requires either one of two WD40-domain proteins, Cdc20 or Cdh1, as activators. These activators interact

dynamically with the APC/C and may either facilitate recruitment of substrates by their WD40 domains, or enhance the specific activity of the APC/C. To examine whether Cdc20 or Cdh1 can interact with TRB3 through its D-box, cell extract was prepared from 293 cells co-expressed with Flag-TRB3 and Myc-Cdc20 or Myc-Cdh1. TRB3 was found to interact with both Cdh1 and Cdc20 (Fig. 3A). We also found that endogenous TRB3 also co-immunoprecipitated efficiently with endogenous Cdh1 (Fig. 3B).

Cdh1 regulates TRB3 steady-state levels

To investigate whether two activators are involved in the TRB3 instability, FLAG-TRB3 was ectopically co-expressed with Cdh1 or Cdc20 in 293 cells. As shown in Fig. 4A, Cdh1 markedly decreased the TRB3 expression, but Cdc20 did not. This effect was fully recovered by MG132 (Fig. 4B). TRB3 protein lacking D-box motif was resistant to Cdh1-mediated degradation (Fig. 4C).

We also investigated whether Cdh1 depletion affects the TRB3 protein stability (Fig. 4D). Cdh1 silencing by small interfering RNA (siRNA) in HepG2 cells resulted in increased TRB3 steady-state levels. Cdh1 knockdown also induced the accumulation of Cdc20 and Cdc25A, known as targets of APC/C^{Cdh1}. When Cdc20 was knock down, no accumulation of TRB3 was observed. We have reported that endoplasmic reticulum stress induced the TRB3 expression in a CHOP dependent manner [8], however Cdh1 ablation caused the accumulation of TRB3 without any CHOP induction (Fig. 4E). Consistent with these data, Cdh1 depletion effectively suppressed the TRB3 ubiquitination at steady-state (Fig. 4F). Taken together, these findings indicate that APC/C^{Cdh1} regulates the TRB3 steady-state levels.

Interestingly, TRB3 silencing caused both Cdc20 and Cdc25A accumulation without any change of Cdh1 expression level (Fig. 4D, lane 4), suggesting that TRB3 has some influence on the Cdh1 dependent degradation.

Discussion

TRB3 is induced by an ATF4/CHOP dependent manner in the various stressful conditions and regulated the stabilities and functions of ATF4/CHOP [7–9]. However, the mechanism underlying TRB3 regulation has been unclear. Our results demonstrated that TRB3 is a short-lived protein and its steady-state levels are balanced through proteasome-dependent degradation, which is facilitated by Cdh1, one of the activators of ubiquitin ligase APC/C. APC/C is a key ubiquitin ligase complex, which regulates the progression of the cell cycle by control the ubiquitination and subsequent

degradation of a number of core cell cycle regulators. APC/C activity is controlled mainly through the regulated binding of two co-activators, Cdc20 and Cdh1, which target distinct substrates at specific stages during the cell cycle.

TRB3 has the typical D-box destruction motif, which is a critical sequence containing in substrates for the APC/C^{Cdc20} and APC/C^{Cdh1} dependent proteolysis. No KEN-box motif, which is additionally recognized by APC/C^{Cdh1} was existed in TRB3. We observed that both Cdh1 and Cdc20 interacted with TRB3, but that APC/C^{Cdh1} alone contributed the TRB3 stability.

During anaphase, Cdh1 was activated by interacting with APC/C complex to form APC/C^{Cdh1}, which continues to ubiquitinate mitotic cyclins along with new targets, including Cdc20, driving cells out of mitosis into G₀/G₁ phase. APC/C^{Cdh1} activity persists throughout G₁ phase until Cdh1 is inactivated at the G₁ to S transition through degradation, phosphorylation and binding of Emi1. TRB3 is considered as a pseudokinase, which contains the typical substrate-binding domains, but lack the ATP-binding and kinase-activation domains [3], and it could be a novel type of endogenous kinase inhibitor, acting as a decoy kinase-like protein for modulating the cell cycle. Expression level of TRB3 could be regulated in a cell cycle dependent manner according to the APC/C^{Cdh1} activity to modulate the activities of key kinases for the cell cycle.

We found that the Cdh1 expression level was not affected by TRB3 deletion (Fig. 4D) or over-expression (data not shown), however, TRB3 deletion up-regulated the expression levels of Cdc20 and Cdc25A, both of which are targets for APC/C^{Cdh1}. These results indicate that TRB3 may regulate the APC/C^{Cdh1} activity.

Multiple primary human lung, colon, and breast tumors express high levels of TRB3 transcript [3,22]. Consistently, several studies have reported reduced expression of Cdh1 in human tumors [23]. In fact, Cdh1 heterozygous animals show increased susceptibility to spontaneous tumors, suggesting that Cdh1 functions as a tumor suppressor by preventing genomic instability [24]. It is possible that TRB3 over-expressed in multiple human tumors and tumor-derived cell lines is caused by not only augmented transcription induced by various stresses but also the stabilization of its protein through the down-regulation of APC/C^{Cdh1}. Further study is necessary to clarify whether over-expressed TRB3 contributes to tumorigenesis.

Recently another type of E3 ubiquitin ligase, seven in absentia homolog 1 (SIAH1) was reported to be involved in the ubiquitination and degradation of TRB3 [25]. Siah is induced by p53, response to genotoxic stress to destruct β -catenin, contributing to cell cycle arrest [26]. Genotoxic stress was reported to downregulate TRB3 expression [27]. Stability of TRB3 is regulated by at least two kinds

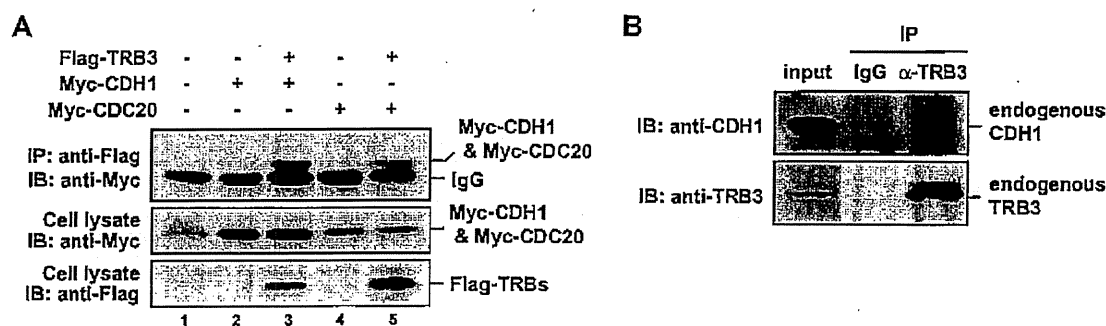


Fig. 3. TRB3 physically interacts with Cdh1. (A) 293 Cells were transiently transfected with the indicated constructs. After 24 h, the cells were treated with 10 μ M MG132 for 12 h. The cell lysates were immunoprecipitated with anti-FLAG antibody, and immunoblotting of cell lysates was performed with anti-FLAG or anti-Myc antibodies. The expression level of each protein was assessed by the immunoblotting of the cell lysates with the indicated antibodies. (B) HepG2 cells were treated with 10 μ M MG132 for 8 h. The cell lysates were immunoprecipitated with anti-TRB3 antibody or control IgG and immunoblotted with anti-Cdh1 or anti-TRB3 antibodies. The expression level of each protein was assessed by immunoblotting with the indicated antibodies.

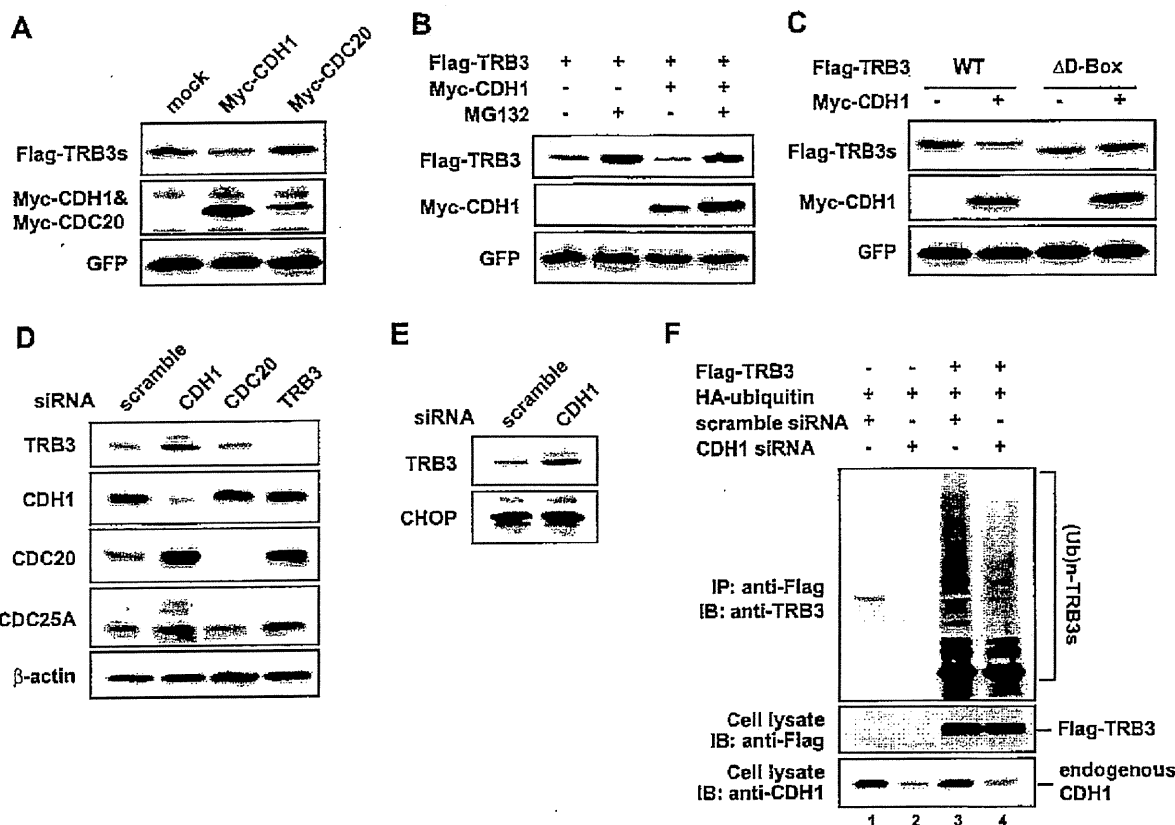


Fig. 4. Cdh1 ubiquitinates and degrades TRB3 protein via its D-box motif. (A–C) 293 Cells were transiently transfected with the indicated constructs. After 24 h, the cells were treated (B) or untreated (A, C) with 10 μ M MG132 for 12 h. The cell lysates were analyzed by immunoblotting using anti-FLAG or anti-Myc antibodies. The pEGFP-C1 expression vector was included in each transfection as a transfection efficiency control, and its level was detected with anti-GFP antibody. (D, E) HepG2 cells were transiently transfected with control (scramble) siRNA or indicated siRNA. After 48 h, the cell lysates were analyzed by immunoblotting using the indicated antibodies. (F) 293 Cells were transiently transfected with the indicated constructs and control (scramble) or Cdh1 siRNA. After 24 h, the cells were treated with 10 μ M MG132 for 12 h. The cell lysates were immunoprecipitated with anti-FLAG antibody, and multi-ubiquitinated TRB3s were detected by immunoblotting with anti-TRB3 antibody. The expression level of each protein was assessed by immunoblotting with the indicated antibodies.

of E3 ligases, APC/C^{Cdh1} and SIAH1, and these ligases would be used properly by the situation of cells; APC/C^{Cdh1} is used at the steady-state and SIAH1 response to genotoxic stress.

In summary, this study provides that the TRB3 expression is regulated by its protein degradation as well as at the transcriptional level. Important roles of the Cdh1-dependent form of the APC/C on the TRB3 stability implicate its additional function in cell cycle besides the recently described roles in and in the stress response. These results allowed us to investigate the role of cell cycle of TRB3.

Acknowledgments

We are grateful to thank Dr. Dirk Bohmann and Dr. Jan-Michael Peters for providing expression plasmids. This research was supported in part by a grant-in-aid for Scientific Research (C) from Japan Society for the Promotion of Science, and grants-in-aid for Scientific Research on Priority Areas from The Ministry of Education, Science, Sports and Culture, and Grants-in-Aid for Scientific Research from Nagoya City University.

References

[1] J. Grosshans, E. Wieschaus, A genetic link between morphogenesis and cell division during formation of the ventral furrow in *Drosophila*, *Cell* 101 (2000) 523–531.

[2] J. Mata, S. Curado, A. Ephrussi, P. Rorth, Tribbles coordinates mitosis and morphogenesis in *Drosophila* by regulating string/CDC25 proteolysis, *Cell* 101 (2000) 511–522.

[3] A.J. Bowers, S. Scully, J.F. Boylan, SKIP3, novel *Drosophila* tribbles ortholog, is overexpressed in human tumors and is regulated by hypoxia, *Oncogene* 22 (2003) 2823–2835.

[4] K. Du, S. Herzig, R.N. Kulkarni, M. Montminy, TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver, *Science* 300 (2003) 1574–1577.

[5] E. Kiss-Toth, S.M. Bagstaff, H.Y. Sung, V. Jozsa, C. Dempsey, J.C. Caunt, K.M. Oxley, D.H. Wyllie, T. Polgar, M. Harte, L.A. O'Neill, E.E. Qvarnstrom, S.K. Dowler, Human tribbles, a protein family controlling mitogen-activated protein kinase cascades, *J. Biol. Chem.* 279 (2004) 42703–42708.

[6] M. Wu, L.G. Xu, Z. Zhai, H.B. Shu, SINK is a p65-interacting negative regulator of NF- κ B-dependent transcription, *J. Biol. Chem.* 278 (2003) 27072–27079.

[7] D. Ord, T. Ord, Mouse NIPK interacts with ATF4 and affects its transcriptional activity, *Exp. Cell Res.* 286 (2003) 308–320.

[8] N. Ohoka, S. Yoshii, T. Hattori, K. Onozaki, H. Hayashi, TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death, *EMBO J.* 24 (2005) 1243–1255.

[9] N. Ohoka, T. Hattori, M. Kitagawa, K. Onozaki, H. Hayashi, Critical and functional regulation of CHOP (C/EBP homologous protein) through the N-terminal portion, *J. Biol. Chem.* 282 (2007) 35687–35694.

[10] O. Bezy, C. Vernochet, S. Gesta, S.R. Farmer, C.R. Kahn, TRB3 blocks adipocyte differentiation through the inhibition of C/EBP β transcriptional activity, *Mol. Cell Biol.* 27 (2007) 6818–6831.

[11] Y. Takahashi, N. Ohoka, H. Hayashi, R. Sato, TRB3 suppresses adipocyte differentiation by negatively regulating PPAR γ transcriptional activity, *J. Lipid Res.* 49 (2008) 880–892.

[12] L. Qi, J.E. Heredia, J.Y. Altarejos, R. Screaton, N. Goebel, S. Niessen, I.X. Macleod, C.W. Liew, R.N. Kulkarni, J. Bain, C. Newgard, M. Nelson, R.M. Evans, J. Yates, M. Montminy, TRB3 links the E3 ubiquitin ligase COP1 to lipid metabolism, *Science* 312 (2006) 1763–1766.