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Aurora kinase-induced phosphorylation excludes RUNX from the chromatin to facilitate proper mitotic progression

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Abstract

RUNX transcription factors are master regulators of development, and major players in tumorigenesis. Interestingly, unlike most transcription factors, RUNX proteins are detected on the mitotic chromatin and apparatus, suggesting that they are functionally active in mitosis. Here, we identify key sites of RUNX phosphorylation in mitosis. We show that the phosphorylation of threonine 173 (T173) residue within the Runt domain of RUNX3 disrupts RUNX DNA binding activity during mitotic entry, and this in turn facilitates the recruitment of RUNX proteins to mitotic structures. Moreover, knockdown of RUNX3 delays mitotic entry. RUNX3 phosphorylation is therefore a regulatory mechanism for mitotic entry. Cancer-associated mutations of RUNX3 T173 and its equivalent in RUNX1 further corroborate the role of RUNX phosphorylation in regulating proper mitotic progression and genomic integrity.
Significance Statement

The *RUNX* family of transcription factors are critical regulators of development. Mutation or dysregulation of *RUNX* genes have been associated with diverse cancer types. Phosphorylation of the strictly conserved threonine 173 (T173) residue within the Runt domain of RUNX3 disrupts DNA binding activity, which facilitates the recruitment of RUNX proteins to mitotic structures. Moreover, *RUNX3* deficiency is associated with delayed mitotic entry. The tight conservation of T173 and its flanking residues from unicellular organism to human, and the fact that mitosis is indispensable to all metazoans, strongly indicate that T173 phosphorylation is fundamental to the role of RUNX in these divergent organisms. Cancer-associated mutation T173I further corroborates the critical role of RUNX phosphorylation in mitosis.
Introduction

Cell division is a highly ordered process comprising multiple steps that lead to dramatic changes to the cell architecture. Events such as cell rounding, chromatin condensation, spindle assembly, nuclear envelope disassembly and cytokinesis involve numerous proteins, whose activities are tightly coordinated by mitotic kinases and proteasome-mediated degradation (1). Given that most transcription has stopped (2), the roles of transcription factors during mitosis are ill-explored.

Runt-related transcription factors (RUNX) are master regulators of cell-fate decisions (3). Mutation or dysregulation of RUNX genes have been associated with diverse cancer types (3). Unlike many transcription factors (e.g. Ets-1, Oct-1) (4), RUNX proteins are retained at the condensed mitotic chromatin, where they maintain epigenetic memory and ensure proper transmission of gene expression patterns to progeny cells; RUNX2 binds to the promoters of various cell cycle and cell fate-related genes and regulates histone modifications during mitosis (5); RUNX2 and RUNX3 bind to regulatory regions of rRNA genes and are associated with their repression (6, 7). RUNX1 positively regulate the transcription of various spindle assembly checkpoint genes, such as BUB1 and NEK6 (8). These findings suggest that RUNX proteins are important for the accurate transmission of genetic information during mitosis and that defects in RUNX genes might contribute to aneuploidy and loss of cell identity.

Aside from binding to the chromatin, RUNX proteins also associate with microtubules (9, 10). RUNX3 molecules are detected at key mitotic structures such as the centrosome, mitotic spindle and midbody (11). Likewise, RUNX-binding partner CBFβ was found at the midbody and implicated in cytokinesis (12). The reason why RUNX proteins are present at non-DNA sites (i.e. mitotic apparatus) during mitosis is unknown. An intriguing observation is the hyperphosphorylation of RUNX proteins during mitosis (13, 14). RUNX2 is phosphorylated by mitotic kinase CDK1-cyclin B1 (14, 15), and dephosphorylated at mitotic exit by the PP1/PP2A phosphatase (14). CDK1-mediated phosphorylation of RUNX2 enhanced DNA binding activity, suggesting a role for RUNX2 in G2/M progression (15). In addition, CDK1/2 phosphorylates RUNX1, promoting its degradation by CDC20-associated anaphase promoting complex during
the late stages of mitosis (13, 16). However, despite these findings, the significance of RUNX hyperphosphorylation in mitosis remains unclear.

**Results**

**RUNX proteins are hyperphosphorylated at mitosis**

The localization of RUNX proteins at mitotic structures suggests direct involvement of RUNX proteins in mitosis. Since mitosis is regulated by phosphorylation events, we investigated RUNX phosphorylation using Phos-tag gel electrophoresis. In asynchronously growing cells, different migration patterns of phosphorylated RUNX1, 2 and 3 suggest unique phosphorylation signatures for each RUNX protein (Figure 1A, lane Asyn). Cells were then arrested with nocodazole, released from nocodazole block and analyzed for RUNX phosphorylation. During prometaphase, which was associated with high levels of cyclin B1 and activated mitotic kinases (Figure 1B, lane Noc), we detected marked reduction of RUNX mobility (Figure 1A, lane Noc). This indicates multiple phosphorylation sites in RUNX during mitosis. The removal of nocodazole resulted in rapid dephosphorylation of RUNX proteins (Figure 1A, lane 1 h) as cells progressed to telophase (Figure S1).

**T173 and T14 are critical for mitotic-specific hyperphosphorylation of RUNX3**

Because RUNX3 is believed to be the evolutionary founder of human RUNX family (17), we focus on RUNX3 phosphorylation. To identify phosphorylation sites of RUNX3 in mitosis, we transiently expressed truncated forms of RUNX3 in COS7 cells and examined their phosphorylation state in the presence of nocodazole. In asynchronously growing cells, both the N- and C-terminus of RUNX3 were phosphorylated, as indicated by slower migrating forms (Figure 1C). In mitotic cells, there was an increase in the phosphorylation of the N-terminus of RUNX3 (amino acids 1-234) (Figure 1C, lane 4 from left). Phosphatase treatment led to the collapse of the two prominent slower migrating bands, while the remaining rapidly migrating band indicated completely dephosphorylated RUNX3 (Figure 1D). Thus, there are multiple phosphorylation sites in the N-terminus of RUNX3, where differences in mobility reflected the degree of phosphorylation. For untreated cells, the C-terminus (amino acids 182-415) separated into two main bands, suggesting that approximately 50% of the C-terminus construct was phosphorylated (Figure 1C, lanes 5 & 6). Nocodazole treatment resulted in an additional upward
shift in mobility, indicating mitosis-specific phosphorylation of the C-terminus domain (Figure 1C, lanes 7 & 8). This is consistent with studies which showed that the C-termini of RUNX1 and RUNX2 harbor mitotic kinase CDK1 phosphorylation sites (13-16).

To identify phosphorylation sites in the RUNX3 N-terminus, we introduced individual point mutations to abolish putative phosphorylation sites in the truncated RUNX3 protein (amino acids 1-234). Two mutants, threonine 14 alanine (T14A) and threonine 173 alanine (T173A), exhibited reduced phosphorylation during nocodazole treatment – instead of the two slower migrating bands, there was only one of intermediate mobility, aside from the unphosphorylated RUNX3 (Figure 1D, lanes T14A and T173A). The double mutant T14A/T173A showed severe dephosphorylation (Figure 1D, lane T14A/T173A), indicating that residues T14 and T173 are major phosphorylation sites in early mitosis. To eliminate any artifact arising from truncation of the RUNX3 protein, these point mutations, either individually or in combination, were introduced into the full length RUNX3 cDNA (Figure 1E). In exponentially growing cells, the intensities of the two fastest migrating bands are reminiscent of the migration patterns of the RUNX3 C-terminus truncation (compare Figure 1C and Figure 1E, Noc negative lanes), indicating that approximately 50% of the RUNX3 protein is phosphorylated at the C-terminus domain (Figure 1E). Moreover, the multiple bands in the wild-type and T173A mutant reflected multiple phosphorylation sites at the N-terminus in untreated cells. By contrast, phosphorylation of the T14A mutant was notably diminished (Figure 1E, second lane from left). Prior phosphorylation at T14 is therefore important for subsequent phosphorylation events at the N-terminus, even in non-mitotic cells. Upon nocodazole treatment, wild-type RUNX3 was hyperphosphorylated, relative to T14A and T173A mutants (Figure 1E, compare lanes WT, T13A and T173A). The double mutant T14A/T173A (AA) showed a significant loss of phosphorylation. This highlights the importance of both phospho-T173 and phospho-T14 for RUNX3 hyperphosphorylation during mitosis. The T14 residue is conserved in RUNX1 but present as serine in RUNX2 (Figure 1F). Both were previously shown to be phosphorylated (18-20). T173 phosphorylation has not been reported. The T173 residue is conserved in all RUNX family members, including Runx1 and 2 of the unicellular holozoan Capsaspora owczarzaki and RNT-1 of the simple metazoan Caenorhabditis elegans (Figure 1G).

Aurora kinases are involved in RUNX phosphorylation
Computational prediction based on the Phosida algorithm (21) suggests that T173 is a putative Aurora kinase phosphorylation site. Immunoprecipitation of cells co-transfected with RUNX and Aurora kinases showed that all RUNX proteins interacted with Aurora kinases (Figure 2A). While both the N- and the C-terminus of RUNX3 interacted with Aurora kinases, the C-terminus bound activated Aurora kinases with higher affinity (Figure 2B). Therefore, the C-terminus of RUNX3 is (1) either a possible docking site for active Aurora kinases, or (2) promotes activation of Aurora kinases. Notably, co-expression of RUNX3 with Aurora A or B kinase led to a marked increase in RUNX3 phosphorylation (Figure 2C). Adding small molecule inhibitors of Aurora A or B kinases during nocodazole treatment resulted in decreased phosphorylation of RUNX3 (amino acids 1-234) (Figure 2D). Finally, in vitro kinase assays using recombinant proteins showed that Aurora A and B directly phosphorylated RUNX3 (Figure 2E).

**Phosphorylated T173 RUNX is located at mitotic structures**

To ascertain if T173 is phosphorylated in vivo, we generated an antibody that specifically recognizes phosphorylated T173. Phospho-T173 antibody immunoprecipitated wild-type RUNX3 (amino acids 1-234), but not the non-phosphorylatable T173A mutant (Figure 3A). Moreover, the phospho-T173 antibody binds preferentially to full-length RUNX3 from mitotic cells (Figure 3B). The antibody was unfortunately not suitable for immunoblot. Nevertheless, these results indicate that T173 is preferentially phosphorylated in vivo during mitosis. We determine the cellular distribution of T173-phosphorylated RUNX by performing immunostaining with phospho-T173 antibody. Because the sequence of the synthetic phosphopeptide used as antigen is highly homologous for all RUNX proteins with only one amino acid difference in RUNX1 (Figure 1G), the antibody staining likely reflects the subcellular localization of all RUNX proteins. siRNA-mediated knockdown of RUNX1, RUNX2 and RUNX3 (Figure 3C), as well as competition with excess phospho-T173 peptide (Figure 3D), significantly reduced staining intensity, thus validating the antibody for immunostaining. Cell lines COLO201, DLD1, and SNU5, with predominant RUNX1, RUNX2 or RUNX3 expression respectively (Figure S2A), were also prominently stained by the phospho-T173 antibody during mitosis, as compared to neighboring interphase cells, thus indicating that the antibody detects all three RUNX proteins specifically in mitosis (Figure S2B). Cells at early mitosis were strongly stained by phospho-T173 antibody (Figure 3E for COS7; Figure S2C for DLD1 cells). Staining
intensity was strongest at non-DNA regions (Figure 3C), suggesting that T173 phosphorylation might inhibit RUNX association with chromatin. Moreover, we detected phospho-T173 staining at the centrosome (indicated by γ-tubulin in Figure 3D) and midbody (indicated by α-tubulin in Figure 3F). Phospho-T173 staining was detected at the centrosomes early in mitosis; as the cells progress to telophase, the phospho-T173 staining diminished and was mainly detected at the midbody (Figure 3F). The elevated levels of phospho-T173 at the centrosome, midzone and midbody are reminiscent of the respective locations of Aurora A and B (22).

**Mutation of T173 results in impairment of DNA binding and transcription regulation**

We assessed the effects of T14 and T173 mutations on RUNX3 DNA binding ability. In electrophoretic mobility assay, the T14A and T14D mutations did not affect DNA binding. By contrast, T173A and T173D mutants were defective in DNA binding (Figure 4A). Interestingly, the T173I mutation was found in RUNX3 in the colon carcinoma cell line LS411 while its equivalent mutation in RUNXI (T196I) was detected in chronic myelomonocytic leukaemia (http://cancer.sanger.ac.uk) (23-25); the equivalent residue in RUNX2 was found to be mutated, also to isoleucine, in the disease cleidocranial dysplasia (CCD) (26). This indicates that the T173 residue is important for the function of the Runt domain. Crystallography studies showed the T173 residue at the Runt-DNA interface, where it contacts the phosphate backbone of the DNA helix through polar interactions (27, 28). Replacing threonine with a negatively charged (i.e. mimicking phosphorylation) or a neutral amino acid led to defective DNA binding. We next evaluated the transactivation potential of the mutants. Consistent with their DNA binding properties, T14 mutants showed comparable abilities to induce a RUNX responsive promoter (p14ARF) fused to firefly luciferase reporter (Figure 4B). Mutation at T173 resulted in impairment of transcriptional activation of the p14ARF promoter, similar to other cancer-derived mutations of the Runt domain (Figure 4C).

The strong conservation of T173 and its flanking residues suggest that T173 phosphorylation altered the role of the Runt domain. Subcellular fractionation studies showed increased accumulation of T173 mutants in the cytosolic fraction, while wild-type RUNX3 was predominantly nuclear (Figure 4D). The R143Q and G145E mutations, which affect DNA binding (29), also showed cytoplasmic accumulation, indicating that the cytoplasmic localization of the T173 mutants could be due to their inability to bind DNA. We observed pronounced
localization of T173D mutants at the centrosome, relative to the wild-type (Figure 4E). Moreover, immunoprecipitation of cytoplasmic RUNX3 revealed increased binding of the T173 mutants to the centrosomal marker γ-tubulin, compared to the wild type (Figure 4F). Together, our data indicate that T173 phosphorylation is a molecular switch that dissociates RUNX3 from DNA, inhibits its transcriptional regulatory role, and promotes relocalization to the cytoplasm and centrosome during early mitosis.

RUNX3 suppresses tumor formation through DNA-binding and transactivation of growth inhibitory genes (30). To assess the tumorigenic potential of the T173 mutants, stable wild-type RUNX3 and T173 mutant expressing cells (Figure 4G, left panel) were subjected to colony formation assay. Wild-type RUNX3 inhibited colony formation, in contrast to the T173 mutants (Figure 4G, right panel). DNA binding and transcription activation are therefore critical for RUNX3-mediated tumor suppression, while mutation at T173 might promote cancer.

**RUNX3 is involved in regulating mitotic entry**

Since Aurora kinases promotes RUNX3 phosphorylation, we investigated the effects of inhibiting Aurora kinases on RUNX3. Inhibition of Aurora B activity by siRNA-mediated depletion (Figure 5A & B) or Aurora B specific inhibitor (Figure 5C) resulted in downregulation of endogenous RUNX3 protein levels, suggesting that Aurora B enhances RUNX3 protein stability. It is possible that Aurora B stabilizes RUNX3 to ensure proper mitotic progression. Indeed, RUNX3 depletion in three cell types, namely U2OS osteosarcoma, HeLa cervical and NCI-H1299 lung cancer cells, was associated with reduced cyclin B1 levels during nocodazole treatment (Figure 5D). We asked if abrogation of other RUNX family members affected cyclin B1 accumulation during nocodazole treatment. U2OS cells were depleted of RUNX proteins by siRNA and treated with nocodazole (Figure 5E; Figure S3). Compared to control siRNA-treated cells, individual depletion of RUNX1, RUNX2 or RUNX3 was associated with reduced accumulation of mitotic markers such as cyclin B1, phosphorylated Aurora and PLK1 kinases. Notably, the effects of individual RUNX depletion were not uniform. When compared to RUNX1 knockdown, RUNX3 knockdown showed a more severe reduction in cyclin B1 and phospho-PLK1 (Figure 5E; Figure S3). RUNX2 depletion also revealed higher accumulation of cyclin B1, relative to RUNX1 depletion. Because RUNX3 knockdown also reduced RUNX2 expression (Figure S3), we were unable to evaluate the relative contributions of individual
RUNX proteins to mitosis. Importantly, depletion of all three RUNX proteins (siR123) resulted in a more pronounced reduction of mitotic markers such as phospho-Aurora kinases and phospho-Histone 3 (Figure 5E; Figure S3), suggesting functional redundancy for RUNX proteins in mitosis.

RUNX deficiency is either associated with (1) failure to enter mitosis or (2) premature mitotic exit. We asked whether RUNX3 deficiency affects mitotic entry by knocking down RUNX3 in HeLa Fucci (fluorescent ubiquitination-based cell cycle indicator) cells (Figure 5F). Cells were synchronized at S-phase by double thymidine block. After release from the block, time-lapse microscopy was used to follow the progression of cells from early S phase (i.e. cells with green fluorescence) to mitosis. RUNX3 depletion delayed the onset of mitosis, suggesting that RUNX3 promotes mitotic entry. To determine whether T173 phosphorylation contributes to mitotic entry, we subjected cells stably expressing wild type RUNX3 and T173 mutants to thymidine block and monitored cell cycle progression following release from block (Figure S4A). Induction of wild type RUNX3 resulted in a drastic delay in cell cycle progression – a large population of cells remained at the G1 phase (Figure S4A, see WT). This is consistent with RUNX3’s transcriptional activity in suppressing cell growth. As expected, the T173 mutants did not delay cells in the G1 phase of the cell cycle and progressed to mitosis. Compared to the vector control and T173A mutant, the T173D mutant accelerated entry into mitosis, as shown by increased numbers of phospho-histone 3 (ser 10) positive cells at 9 h post thymidine release (Figure S4B & C). This effect of T173D indicates a role for T173 phosphorylation in facilitating mitotic entry.

Discussion

Here, we uncover a key phosphorylation site that endows RUNX3 with an unexpected role in the regulation of mitosis. Phosphorylation of T173 inhibits the DNA-binding function of the Runt domain, which in turn facilitates the redistribution of RUNX proteins to mitotic structures such as the centrosome and midbody. Crystallography studies previously showed that T173 and its flanking residues constitute one of the key structural elements integral to the function of the Runt domain (27, 28). The disease derived-mutation T173I (26, 31) (http://cancer.sanger.ac.uk) resulted in drastic destabilization of the Runt domain and impairment of DNA binding. Moreover, the ability of T173 mutants to form colonies on soft agar is indicative of a loss of
growth suppressive function. This suggests that T173 phosphorylation must be tightly confined to mitosis. The frequent overexpression of Aurora kinases in cancer might nullify RUNX3 transcription activity during non-mitotic phases and promote tumor growth.

Earlier, we identified an acetylation site at lysine 171 (K171), which promotes interaction between RUNX3 and BRD2 to mediate transcription (32). The evolutionary conservation of the phospho-site T173 and acetyl-site K171, coupled with the strong preference of Aurora kinases for a positively charged residue at the -2 position (20), suggest cross-regulation between both types of modifications. One might expect acetylation/deacetylation of K171 to influence T173 phosphorylation during G2-M transition.

The localization of RUNX proteins at the chromatin, centrosome and the midbody reflects multiple roles during mitosis. It is likely that only a subset of RUNX proteins is phosphorylated at T173 for recruitment to the mitotic structures, while another population of RUNX proteins (i.e. non-T173 phosphorylated) is retained at the mitotic chromatin for epigenetic regulation. The centrosome has been proposed to serve as a scaffold where regulatory proteins interact to determine cell cycle progression (33, 34). How RUNX3 interact with other centrosomal proteins is unclear. Nevertheless, the association of RUNX proteins with Aurora kinases indicates close relationships with regulators of mitotic progression and spindle assembly checkpoint.

The observation that Runx3+/− mice develop intestinal adenoma after long latencies of 16 months after birth (35) is reminiscent of other mice models with mitotic checkpoint deficiencies – Mad2 heterozygous mice develop lung adenocarcinomas at 18-19 months of age (36). Upon carcinogenic insult, Runx3+/− and Runx3−/− mice show accelerated tumor development when compared to wild-type mice (35). Increased incidence of tumor formation in Runx3+/− mice may, in part, be due to the role of RUNX proteins in regulating mitotic fidelity or DNA repair (37).

In conclusion, our work highlights the strong influence of post-translational modification on RUNX function. The tight conservation of T173 and K171 residues through divergent evolution indicates that all RUNX proteins possess non-DNA binding functions, which must be tightly regulated during cell cycle progression. More in-depth studies of RUNX post-
translational modification will enhance our understanding of RUNX’s pleiotropic activities in the cell cycle as well as cancer.

Materials and Methods

Cell lines, drug treatment, transfection and siRNA knockdown

Cell lines were obtained from ATCC. For generation of permanent Dox-inducible RUNX3 cell lines, RUNX3 and T173 mutants were cloned into pRetroX-Tight-puro-3 x Flag (38). HeLa Tet-On cell line (Clontech) was infected with the constructs and selected with 0.5 µg/ml puromycin. Flag-RUNX3 expression was induced by doxycycline (0.5 µg/ml). DNA transfection was performed with TransIT-LT1 reagent (Mirus) while siRNA knockdown was performed with jetPRIME (Polyplus) using 35 nM siRNA (Dharmacon) for 72 h. All protocols were as recommended by the manufacturers. Nocodazole (Sigma) was used at 0.1 µg /ml for U2OS and 1 µg/ml for COS7. MLN8054 (0.5µM), ZM447439 (2µM) and MG132 (20µM) were used. Double thymidine block was performed with 2 mM thymidine.

Phos-tag® gel electrophoresis and immunoblot

U2OS cells were synchronized by nocodazole for 18 h. Mitotic cells were collected by shake-off and re-plated in media without nocodazole. Cells were boiled in buffer containing 2% SDS, 10% glycerol, 100 mM NaCl, 10 mM Tris.HCl pH 7.5, 1 mM EDTA, 0.01mM DTT, protease inhibitor (Roche), phosphatase inhibitor (Thermo Scientific) and 0.4 mM PMSF. Protein concentrations were determined by the BCA method (Pierce). Cell lysates (30 µg) were analyzed by conventional SDS-PAGE or with 20µM Phos-tag reagent (Wako).

Detailed information regarding the antibodies, immunofluorescence, Electrophoretic mobility shift assay, flow cytometry and immunoprecipitation and other experimental procedures are provided in the SI appendix.

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Excellence initiative and also by the Singapore National Research Foundation under its Translational and Clinical Research Flagship Programme.

Legend

**Figure 1: T14 and T173 are important for RUNX3 hyperphosphorylation in mitosis.**

(A) U2OS cells were synchronized with nocodazole (Noc), collected by mitotic shake-off, replated in media without nocodazole and harvested at the indicated times. Lysates were electrophoresed in conventional SDS-PAGE (top) and Phos-tag gels (bottom) for immunoblotting with the indicated antibodies. Asyn, asynchronous. (B) Lysates from (A) were immunoblotted with the indicated antibodies. (C) Top, schematic of human RUNX3. Runt domain, DNA-binding domain; AD, activation domain; ID, inhibitory domains. Bottom, Flag-tagged truncation constructs of RUNX3 spanning amino acids 1-234 and 182-415 were transfected into COS7 cells. After 28 h, cells were cultured with (−) or without (+) nocodazole. Nocodazole-treated cells were harvested by mitotic shake-off. Lysates were immunoblotted with anti-Flag antibodies. Short and long exposure times were indicated. (D) Phos-tag electrophoresis of lysates containing Flag-RUNX3 (1-234). Left, lysates treated with lambda-protein phosphatase. Right, point mutants of Flag-RUNX3 (a.a. 1-234) were transfected into COS7 cells and treated with nocodazole. Immunoblots were probed with anti-Flag antibodies. WT, wildtype. (E) T14 and T173 mutations were introduced into full-length Flag-tagged RUNX3 and transfected into COS7 cells. Transfectants were treated as in (C) and immunoblotted with the indicated antibodies. (F & G) Alignments of the regions flanking T14 and T173 in human RUNX1, RUNX2, RUNX3, *Caenorhabditis elegans* RNT-1, *Capsaspora owczarzaki* RUNX1 and RUNX2. Protein sequence alignments were done with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2).

**Figure 2: RUNX proteins interact with Aurora kinases A and B**

(A) HEK293T cells were co-transfected with various combinations of RUNX1, RUNX2, RUNX3, Flag-AURKA and GFP-AURKB expression vectors. Immunoprecipitation (IP) was performed using anti-Flag M2 beads to enrich for AURKA (left panel) and GFP affinity beads to enrich for AURKB (right panel) respectively. RUNX proteins were detected with anti-pan-RUNX antibody. WCE, whole cell extract. (B) COS7 cells were transfected with Flag-RUNX3
truncation constructs and GST-AURKA or AURKB expression constructs. Flag-RUNX3 constructs were precipitated by M2 beads. Bound AURKA (left panel) or AURKB (right panel) proteins were detected by immunoblot. (C) Wildtype (WT) RUNX3 (amino acids 1-234) or double mutant AA (T14A/T173A) were transfected into COS7 with AURKA or AURKB expression plasmids. λppase, lambda phosphatase. Flag antibody was used to detect RUNX3 proteins. RUNX3, p-RUNX3 and pp-RUNX3 refers to the different degrees of phosphorylation. Absence of p refers to unphosphorylated RUNX3. (D) COS7 cells were transfected with Flag-RUNX3 (amino acids 1-234). Cells were treated with nocodazole, followed by inhibitors (inh) of AURKA (MLN8237, 5 µM) and AURKB (ZM4473439, 2 µM) kinase inhibitors for 4 h, and harvested by mitotic shake-off. (E) In vitro kinase reaction of recombinant RUNX3 with AURKA or AURKB in the presence of [γ³²P]ATP. Proteins were resolved by SDS-PAGE and visualized by autoradiography.

Figure 3: Dynamic distribution of T173-phosphorylated RUNX3 in mitotic cells.
(A) COS7 cells were transfected with Flag-tagged RUNX3 (amino acids 1-234) or T173A mutant and treated with nocodazole. Immunoprecipitation was done with phospho-T173 (pT173) antibodies. (B) Full-length Flag-tagged RUNX3 was immunoprecipitated from cells cultured with or without nocodazole. Normal rabbit IgG was used as control. (C) Left, immunoblot of U2OS cells simultaneously transfected with RUNX1, RUNX2 and RUNX3 siRNA for 72 h (indicated as siR123). siControl, control siRNA. Right, fluorescence microscopy of cells treated with siRNA targeting RUNX1, RUNX2 and RUNX3. Cells are stained with pT173 antibody (red) and DAPI (blue). Cells at prophase are shown; (D) Competition with peptides to validate pT173 antibody. Cells were co-stained with pT173 (red), α-tubulin antibody (green) and DAPI (blue) to visualize DNA. Arrows indicate centrosomes. (E) Asynchronous COS7 cells were co-stained with pT173 antibody and DAPI. White arrows indicate cells at various stages of mitosis (F) U2OS cells were stained with pT173 (red) and α-tubulin (green); α-tubulin staining was adjusted by Min/Max function of the Axiovision program to eliminate background staining. Scale bar, 10 µm.

Figure 4: T173 mutants are impaired in DNA binding and transactivation properties.
(A) DNA binding abilities of GFP-RUNX3 and phosphomutants. Nuclear extracts of the transfectants were analyzed by electrophoretic mobility assay using a DNA probe with the
RUNX binding site. RUNX3 antibody (Ab) and non-biotinylated probe (cold) were used for super-shift reaction and competition assay respectively. WT, wild-type; GFP, empty GFP vector control; AA, T14A/T173A; DD, T14D/T173D mutants; (B) HEK293T cells were transfected with GFP-RUNX3 constructs, p14<sup>ARF</sup> promoter fused to firefly luciferase and β-galactosidase normalization control. Mean values ± standard deviations are obtained from triplicate experiments. Comparison of WT and T173A, ****<i>P</i> = 0.00003 (Student’s t-test). (C) Luciferase reporter assay of Runt domain mutants with the p14<sup>ARF</sup> promoter. Comparison of WT and T173I, ****<i>P</i> = 0.0001. (D) COS7 cells transfected with GFP-RUNX3 constructs were subjected to biochemical fractionation to isolate cytosolic fraction (C) and nuclear fraction (N). Immunoblot was done with GFP, PARP and α-tubulin antibodies to detect RUNX3 and validate purity of the nuclear and cytosolic fractions respectively. (E) GFP-RUNX3 (WT) and GFP-T173D were transfected into COS7 cells for fluorescence microscopy. Rootletin antibody and DAPI stained centrosome (red) and DNA respectively (blue). White arrow indicates centrosome. Scale bar, 10 µm. (F) Left, immunoprecipitation (IP) of cytosolic fraction in (D) using GFP beads; Right, cytosolic fraction. RUNX3 and bound γ-tubulin were detected by immunoblot with GFP and γ-tubulin antibodies. * indicates GFP-linked degradation products. (G) Left, expression of pRetro-X-flag-RUNX3 constructs in HeLa Tet-On cells (induced with doxycycline) as detected by immunoblot with anti-flag antibodies; right, colony formation assay of the stable cells in left panel. Vector, empty vector control. Representative photographs are shown. The graph shows mean colony numbers ± standard deviations from three independent experiments.

**Figure 5: RUNX3 is involved in regulation of mitosis**

(A) U2OS cells were treated with siRNA to deplete AURKA or AURKB proteins. Nocodazole-treated cells (Noc) were harvested by mitotic shake-off. RUNX3 and Aurora proteins were detected by immunoblot. (B) Densitometric analysis of RUNX3 expression after AURKA or AURKB knockdown as in (A); three independent experiments were done. (C) U2OS cells were treated with nocodazole prior to addition of MG132 and Aurora kinase inhibitors (MLN8405 and ZM4473439) for 4 h. (D) Cells were treated with non-targeting siRNA (siControl) or RUNX3 siRNA (siRUNX3). Nocodazole was added for the last 16 h. Immunoblot was done with the indicated antibodies. (E) Immunoblot of U2OS cells treated with siRNA targeting RUNX1, RUNX2 and RUNX3. R123 indicates treatment with all 3 RUNX siRNA. (F) HeLa.Fucci cells were treated with RUNX3 siRNA and subjected to double thymidine block. Cells were
monitored by time-lapse microscopy following release from block. Metaphase cells (visualized as mitotic rounding up) were scored. Number of cells counted for siControl and siRUNX3 are 447 and 492 respectively. The data represented mean ± S.E.M of three independent experiments. *P = 0.03, **P = 0.005 (Student’s t-test).

References


Figure 1. T14 and T173 are important for RUNX3 hyperphosphorylation in mitosis.
Figure 2. RUNX proteins interact with Aurora kinases A and B. (A) HEK293T cells were cotransfected with various combinations of RUNX1, RUNX2, RUNX3, Flag-AURKA, and GFP-AURKB expression vectors.
Figure 3. Dynamic distribution of T173-phosphorylated RUNX3 in mitotic cells.
Figure 4. T173 mutants are impaired in DNA binding and transactivation properties.
Figure 5. RUNX3 is involved in regulation of mitosis.
Immunofluorescence staining of U2OS cells after release from nocodazole treatment.

**Figure S1.** Immunofluorescence staining of U2OS cells after release from nocodazole treatment. Cells from Fig. 1 were analyzed by immunofluorescence microscopy with α-tubulin antibody (green). DNA was stained with DAPI (blue). Asyn, asynchronous cells; Noc, nocodazole treatment. (Scale bar, 10 µm.)
pT173 antibody recognizes RUNX1, RUNX2, and RUNX3 during mitosis.

**Fig. S2.** pT173 antibody recognizes RUNX1, RUNX2, and RUNX3 during mitosis. (A) Immunoblot comparing RUNX1, RUNX2, and RUNX3 expression in COLO201, DLD1, and SNU5 cells. A total of 30 µg of total cell lysate was loaded per lane. (B) Immunofluorescence microscopy with pT173 antibody. Cells were imaged by confocal microscopy (Olympus FluoView FV1000). Red, pT173; green, γ-tubulin; blue, DNA. (C) Asynchronous DLD1 cells were stained as in B. Arrows indicate cells at different stages of mitosis. (Scale bars, 10 µm.)
Densitometric analysis of protein levels after siRNA knockdown of RUNX1, RUNX2, and RUNX3.

**Fig. S3.** Densitometric analysis of protein levels after siRNA knockdown of RUNX1, RUNX2, and RUNX3. The immunoblot in Fig. 5E was quantified for signal intensity with the ImageJ program. All graphs were plotted relative to GAPDH levels.
SI appendix

Supplementary material and methods

Commercial antibodies used for immunoblot

The antibodies are as follows: anti-RUNX1 (39000, Activemotif), anti-RUNX1 (ab35962, Abcam), anti-RUNX2 (D1H7, Cell Signaling Technology), anti-RUNX3 (#9647, Cell Signaling Technology), anti-RUNX3 (R3-5G4), anti-RUNX3-biotin (R3-5G4, eBioscience), anti-pan-RUNX (3D9, MBL), anti-cyclin B1 (Cell Signaling Technology and Santa Cruz), anti-AURKA-pT288:AURKB-pT232:AURKC-T198 (D13A11, Cell Signaling Technology), anti-β-actin (Sigma-Aldrich), α-tubulin (Sigma-Aldrich), anti-γ-tubulin (GTU-88, Sigma-Aldrich), anti-p-Histone H3 serine 10 (D2C8, Cell Signaling Technology), anti-AURKA (1G4, Cell Technology), anti-AURKB (#3094, Cell Signaling Technology), anti-PARP (C2-10, Santa Cruz), anti-Flag (F7425 rabbit polyclonal and M2 mouse monoclonal antibody, Sigma-Aldrich), anti-GAPDH (#2118, Cell Signaling Technology), anti-HSP90 (#4877, Cell Signaling Technology).

Generation of phospho-specific antibody

Phospho-T173 antibody was generated by Biomatik (Canada): Phospho-T173 peptide CTYHRAIKV(pT)VDGRE and its non-phosphorylated counterpart were synthesized for rabbit polyclonal antibody production; the antibodies were purified by phospho-peptide affinity column. Antibody specificity was verified by dot-blot and ELISA techniques (Biomatik).

Electrophoretic mobility shift assay

COS7 cells were transfected with 3 µg of the indicated pEGFP-RUNX3 constructs for 24 h. Reactions were done with LightShift Chemiluminescent EMSA Kit (Thermo Scientific). Nuclear extracts (1.6 µg) were pre-incubated with 2 µg poly(dI.dC) and recombinant CBFβ protein for 15 min before addition of 20 nM 3’-biotinylated double-stranded DNA probe with RUNX3 binding site (sense 5’ – GTCTGGCCTGTGGTCTGGCT GT –3’; antisense 5’ – ACAGCCAGACCACAGGCCA GAC – 3’). For competition or antibody addition, 50-fold excess of non-biotinylated probe or 0.8 µg of anti-RUNX3 antibody (R3-5G4) were added
respectively. After incubation for 30 min, the samples were resolved by acrylamide gel electrophoresis, transferred to nylon membrane (Amersham) and UV-crosslinked.

**In vitro kinase reaction**

Full length RUNX3 was cloned into pET21d plasmid and expressed in bacteria as a histidine-tagged protein. The protein was purified with Nickel column (Qiagen) and eluted with imidazole as recommended by the manufacturer. Kinase reactions with recombinant AURKA and AURKB kinases (MBL International) were performed using 1 µl kinase and 0.5 µl [γ\(^{32}\)P] ATP at 30°C for 30 min.

**Generation of point mutations of RUNX3**

All point mutations were generated by the QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by DNA sequencing.

**Immunofluorescence staining**

COS7 or U2OS cells were transfected with pEGFP-RUNX3 or its mutant counterparts. 28 h after transfection, cells were sequentially fixed with 4% paraformaldehyde (10 min at 37°C) and absolute methanol (10 min at -20°C). Cells were permeabilized with 0.2% Triton X100 and blocked with 5% bovine serum albumin before incubation with the indicated primary antibodies in 3% BSA for 45 min at 37°C. The following secondary antibodies were used: Alexa Fluor 488 anti-rabbit, Alexa Fluor 488 anti-mouse, Alexa Fluor 594 anti-rabbit, Alexa Fluor 594 anti-mouse IgG (Molecular Probes). The cover slips were mounted with ProLong® Gold antifade reagent with DAPI (Invitrogen). Images were acquired at 63 x magnification using Zeiss Observer Z1 inverted microscope and analyzed with the AxioVision 4.8 software. To verify the specificity of the phospho-T173 antibody by immunofluorescence, phospho-T173 antibodies were used at concentration of 0.135 µg/ml for siRNA knockdown and 0.54 µg/ml for peptide competition experiments respectively. Peptide competition was performed at 1 µg/ml.

**Immunoprecipitation**

To verify the specificity of the phospho-T173 antibody by immunoprecipitation, COS7 cells were seeded on 15 cm dishes and transfected with the indicated plasmids (3 µg) for 48 h and treated with nocodazole for the last 16 h. Prometaphase enriched cells were harvested by
trypsinization. For phospho-antibody immunoprecipitation, 2 mg of cell lysate were incubated with 5 µg of antibody for 4 h. Bound proteins were analyzed by 10% SDS-PAGE.

To ascertain binding of RUNX proteins with Aurora kinases, HEK293T cells were seeded on 10 cm dishes and transfected with Aurora kinase (7.5 µg) and EF-BOS RUNX (7.5 µg) expression vectors. After 48 h, cells were harvested and lysed for immunoprecipitation. For Flag immunoprecipitation or GFP immunoprecipitation, M2 sepharose beads (Sigma-Aldrich) and GFP Trap beads (Chromotek) were used respectively. Lysates (400 µg) were incubated 500 µl of binding buffer (25mM Tris.HCl pH 8.0, 0.25% NP-40, 2.5% glycerol, 0.5mM EDTA, 150mM NaCl, 1mM DTT, phosphatase inhibitor (Thermo Scientific) and protease inhibitor cocktail (Roche)) with the respective beads for 3 h at 4°C. The GFP beads were washed copiously with binding buffer and eluted by boiling in Laemmli buffer. To elute Flag-tagged proteins from M2 beads, 3 x Flag peptide (Sigma-Aldrich) was used. Bound proteins were analyzed by 10% SDS-PAGE and immunoblot.

For immunoprecipitation of the cytosolic extract, COS7 cells were transfected with the GFP-RUNX3 constructs. After 24 h, cell fractionation was performed and 200 µg of cytosolic extract was incubated with GFP beads in binding buffer (conditions as described above).

Flow cytometry

Cells were harvested by trypsinization and fixed in 80% ice cold ethanol. The cells were blocked with 0.5% bovine serum albumin in PBS (Sigma) before incubation with phospho-Histone 3 (ser 10) antibody (Alexa Fluor 488 conjugate) (#9708, Cell Signaling Technology) for 1 h at room temperature (dilution of 1:65 in blocking buffer). After two washes with blocking buffer, the cells were resuspended in PBS containing 50 µg/ml propidium iodide, 20 µg/ml RNase A and 0.1% triton X100 and incubated at room temperature for 30 min. Flow cytometry was conducted using LSR II flow cytometer (BD). The data was analysed by the FlowJo software.

Lambda phosphatase treatment

COS7 cells were transfected with Flag-RUNX3 (amino acids 1-187) or Flag-RUNX3 (amino acids 182-415). After 24 h, the cells were lysed in buffer containing 50 mM Tris.HCL pH 8.0, 0.3 M NaCl, 5% glycerol, 1 mM EDTA, 0.5% NP-40, 1 mM DTT, 0.4 mM PMSF, protease
inhibitor (Roche) and phosphatase inhibitor (Thermo Scientific). Cell lysates (30 µg) were
incubated with 20 units of lambda phosphatase (New England Biolabs) for 30 min at 30°C and
analyzed by 12% SDS-PAGE (supplemented with Phos-tag).

**Luciferase reporter assay**

HEK293T cells were seeded on a 24-well dish and co-transfected with 100 ng p14ARF promoter
linked to firefly luciferase and 500 ng pEGFP-RUNX3 constructs and 50 ng of β-gal vector as
internal control. After 48 h, luciferase activities were analyzed with Luciferase® Reporter Assay
(Promega). All reactions were performed in triplicate. The Beta-Glo assay (Promega) was used
for the quantification of β-galactosidase to normalize luciferase readings.

**Colony formation assay**

HeLa Tet-Off cells were infected with pRetro-Tight-puro 3xFlag RUNX3 constructs, selected
with puromycin for four days, and cultured in media containing 0.5 µg/ml of doxycycline and
0.25 µg/ml of puromycin for two weeks. Colonies were stained with 0.01% crystal violet (w/v)
and photographed. Colonies (at least 2 mm radius) were counted with the OpenCFU program
(39).

**Subcellular Fractionation**

Cell fractionation was performed as described (40). 15 µg of cytoplasmic, nuclear or total cell
extract were analyzed by 10% SDS-PAGE.

**Supplementary Figures**

**Figure S1: Immunofluorescence staining of U2OS cells after release from nocodazole
treatment.**

Cells from Figure 1 were analyzed by immunofluorescence microscopy with α-tubulin antibody
(green). DNA was stained with DAPI (blue). Asyn, asynchronous cells; Noc, nocodazole-
treatment. Scale bar, 10µm.

**Figure S2: pT173 antibody recognizes RUNX1, RUNX2 and RUNX3 during mitosis.**

(A) Immunoblot comparing RUNX1, RUNX2 and RUNX3 expression in COLO201, DLD1 and
SNU5 cells. 30 µg of total cell lysate were loaded per lane. (B) Immunofluorescence
microscopy with pT173 antibody. Cells were imaged by confocal microscopy (Olympus FluoView FV1000). Red, pT173; green γ-tubulin; blue, DNA. (C) Asynchronous DLD1 cells were stained as in (B). Arrows indicate cells at different stages of mitosis. Scale bars, 10 µm.

**Figure S3: Densitometric analysis of protein levels after siRNA knockdown of RUNX1, RUNX2 and RUNX3.**

The immunoblot in Figure 5E was quantified for signal intensity with the Image J program. All graphs were plotted relative to GAPDH levels.

**Figure S4: Flow cytometric analysis of cell cycle progression of pRetro-flag-RUNX3 stable HeLa Tet-On cell lines after release from thymidine block.**

(A) Histogram showing cell cycle profiles of the indicated p-RetroX-Flag-RUNX3 cell constructs at various time points after release from thymidine block. RUNX3 expression was induced with 0.5 µg/ml of doxycycline 16 h before release from block. Propidium iodide (PI) was used to quantify DNA content and the histogram of PI staining was plotted using FlowJo program. At time 0 h, cells were synchronized at G1/S phase with 2N DNA content. By 24 h, all cell lines except for wild-type RUNX3 (WT) have entered the next cell cycle. (B) Levels of mitotic cells of empty vector, T173A and T173D cells in (A) were quantified by phospho-Histone 3 (ser 10) (H3 pS10) staining. (C) Density plots of T173A and T173D at 9 h time point from (B). Percentages of G2 and M subpopulations are indicated by elevated PI and H3 pS10 staining respectively.