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The Truncated Isoform of the Receptor Tyrosine Kinase ALK Generated by Alternative Transcription Initiation (ALK\textsuperscript{ATI}) Induces Chromatin Structural Changes in the Nucleus in a Kinase Activity-Dependent Manner

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Received July 8, 2017; accepted August 20, 2017

Anaplastic lymphoma kinase (ALK) is a receptor-type tyrosine kinase that promotes cell growth upon stimulation with ligands such as midkine and pleiotrophin. Recently, a truncated isoform of ALK was identified in a variety of tumors. This isoform is expressed from a novel ALK transcript initiated from a de novo alternative transcription initiation (ATI) site in ALK intron 19 (referred to as ALK\textsuperscript{ATI}), ALK\textsuperscript{ATI}, which consists of only the intracellular kinase domain, localizes to the nucleus as well as the cytoplasm. However, its nuclear role is unknown. In this study, we determined that ALK\textsuperscript{ATI} promoted chromatin structural changes in the nucleus in a kinase activity-dependent manner. We found that expression of ALK\textsuperscript{ATI} increased the level of the heterochromatin marker Lys9 tri-methylated histone H3. In addition, we demonstrated that ALK\textsuperscript{ATI} phosphorylated the nuclear protein A-kinase anchoring protein 8 (AKAP8) and altered its subcellular localization from the insoluble fraction to the soluble fraction. These results suggest that ALK\textsuperscript{ATI} induces chromatin structural changes and heterochromatinization through phosphorylation of AKAP8 in the nucleus.

**Key words** anaplastic lymphoma kinase; nuclear tyrosine kinase; A-kinase anchoring protein 8; heterochromatinization; alternative transcription initiation; chromatin structural change

Chromatin structure and organization are dynamically changed during cellular processes, such as proliferation, differentiation, DNA damage responses, and tumorigenesis.\textsuperscript{1,2} Chromatin structural changes, which are controlled by various chromatin modifying enzymes, regulate gene expression, DNA replication, DNA repair, and mitotic progression.\textsuperscript{3–6} Anaplastic lymphoma kinase (ALK) is a receptor-type tyrosine kinase that consists of an extracellular domain, a transmembrane domain, and an intracellular kinase domain. The extracellular domain contains two MAM (meprin/A5/protein tyrosine phosphatase M\textsubscript{IP}) segments and a glycine-rich region.\textsuperscript{7} Stimulation with midkine and pleiotrophin, ligands for ALK, activates ALK kinase activity and subsequent growth signaling.\textsuperscript{8,9} ALK was originally identified as a fusion gene in anaplastic large cell lymphomas.\textsuperscript{10} The identification of the fusion gene echinoderm microtubule associated protein like 4 (EML4)-ALK in lung tumors further established the role of ALK in tumorigenesis.\textsuperscript{11} EML4-ALK is caused by a chromosomal translocation in chromosome 2q, which encodes a protein consisting of an N-terminal region of EML4 fused to the intracellular tyrosine kinase domain of ALK. The ALK kinase is constitutively active in EML4-ALK due to dimerization of the coiled-coil domain of EML4.\textsuperscript{11} Because the kinase activity of ALK is critically involved in progression of EML4-ALK-positive lung tumors, the ALK inhibitor crizotinib is used for treatment of these types of tumors.\textsuperscript{12} Recently, ALK\textsuperscript{ATI}, a truncated isoform of ALK, was identified in a variety of tumors.\textsuperscript{13} This isoform is expressed from a transcript that is initiated from an alternative transcription initiation (ATI) site in ALK intron 19. ALK\textsuperscript{ATI}, which consists of only the intracellular kinase domain, displays constitutive activation similar to EML4-ALK. As both EML4-ALK and ALK\textsuperscript{ATI} are critically involved in tumor progression, understanding the roles of these “intracellular kinase domain-only mutants of ALK” is crucial for understanding tumorigenesis.

ALK is reported to mainly promote cell growth by activating the RAS-mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR), and Janus kinase (JAK)-signal transducers and activator of transcription (STAT) pathways.\textsuperscript{7} Given that the kinase domain of the full-length isoform of ALK localizes just under the plasma membrane, activation of ALK targets is expected to occur in the cytoplasm. The ALK\textsuperscript{ATI} also predominantly localizes in the cytoplasm where it is involved in the activation of these three signaling pathways,\textsuperscript{13} so its major targets are likely cytoplasmic proteins. However, unlike the full-length isoform, ALK\textsuperscript{ATI} is reported to localize to the nucleus as well as the cytoplasm, and its nuclear role remains unclear.

Tyrosine kinases mainly localize to the cytoplasm or the plasma membrane, so most studies have focused on their roles in cytoplasmic signal transduction.\textsuperscript{14,15} However, emerging evidence has revealed that tyrosine kinases also localize to the nucleus where they regulate events such as DNA damage responses, gene expression, and nuclear actin structure formation.\textsuperscript{1,2,16–24} Therefore, we designed the current study to investigate the role of ALK\textsuperscript{ATI} in the nucleus.

**MATERIALS AND METHODS**

**Plasmids** cDNAs encoding human ALK full-length (ALK-FL) and ALK\textsuperscript{ATI} were generated by PCR from pENTR223.1/ALK (I.M.A.G.E. Consortium [LLNL] cDNA Clones) and subcloned into the pcDNA4/T0 vector (Thermo

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Fisher Scientific, Gaithersburg, MD, U.S.A.). The primers used for PCR were as follows: ALK-FL, 5'-AGTGAATCGA ATCC ACC ATGG GAG ACC ATC GGG TCT GTG GTC CTA TGG C3 (sense) and 5'-CCAATGCTGAGTCAAGGC CAGGGTCGTGTACATGTCTACCTT3 (antisense); and ALKATI, 5'-TGGC ATC AGA ACC ACC ACC ATG CAG GAC TGG CAG AAG CGG TCT AGG TCT CGT AC AGG AACT GAC CGG CACC AGC CTG TTG TTG CTA TGC CAG GTCT GTC AAT TAT TCT T3 (antisense). The ALKATI kinase-dead K1150A mutant (ALKATIKD) was generated by inverse PCR with the following primers: 5'-CCAGGCCCATGCAAGTGCTG TGG CAA CTT CTT AGT GCT TCT AGT GGT CAG ACC ACC ATG CAG GAC TGG CAG AAG CGG TCT AGG TCT CGT AC AGG AACT GAC CGG CACC AGC CTG TTG TTG CTA TGC CAG GTCT GTC AAT TAT TCT T3 (antisense). The ALKATI cDNA and pcDNA4/TO/FH-NLS-Lyn plasmid 20) were seeded in a 35-mm (or 60-mm) culture dish and then transiently transfected with Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer’s instructions. The primers used for PCR were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-ACC ACA GTC CAT GCC ATC AC-3' (sense) and 5'-TCC ACC CTT GCT TCA T GA-3' (antisense); and RASSF1A, 5'-ACG AAC TGG-3' (sense) and 5'-GGT CGT TGG GCC ATC TT-3' (antisense). The amplified RASSF1A cDNA was digested with XhoI, and then the Lyn cDNA was replaced with the ALKATI cDNA. Plasmids for A-kinase anchoring protein 8 (A-kinase) were prepared as described previously. 21)

**Antibodies**

The following antibodies were used: ALK (3633; Cell Signaling Technology, Beverly, MA, U.S.A.), myc (ab9106; Abcam, Cambridge, MA, U.S.A.), phosphotyrosine (pTyr) (4G10; Merck Millipore, Bedford, MA, U.S.A.), lamin A/C (N-18; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), cytosolic phospholipase A2 (cPLA2) (4G4-3C; Santa Cruz Biotechnology), FLAG (M2; Sigma-Aldrich, St. Louis, MO, U.S.A.), H3K9me3 (Lys9 tri-methylated histone H3) (ab8989; Abcam), and actin (C4; Merck Millipore, Guyancourt, France). Horseradish peroxidase (HRP)-F(ab')2 secondary antibodies were purchased from GE Healthcare (Waukeha, WI, U.S.A.).

**Cells and Transfection**

HeLa S3 cells (Japanese Collection of Research Bioresources, Osaka, Japan) were cultured in Dulbecco’s modified Eagle’s medium (Nissui, Tokyo, Japan) containing 5% bovine serum. To transfect plasmids, cells were seeded in a 35-mm (or 60-mm) culture dish and then transiently transfected with 1 µg (or 3 µg) of plasmid DNA using PEI (25KDa) (Polysciences, Inc., Warrington, PA, U.S.A.) or Lipofectamine 2000 (Thermo Fisher Scientific), according to the manufacturer’s instructions.

**Immunoprecipitation, Subcellular Fractionation, and Western Blotting**

Cells were suspended in TNE buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 mM Na2VO4, 1 mM NaF, 4 µg/mL aprotinin, 1.6 µg/mL peptatin A, 4 µg/mL leupeptin, 2 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride). The suspension was then centrifuged at 2130×g for 15 min, and the resulting lysate was subjected to immunoprecipitation using anti-myc antibody-precoated protein G beads, as described previously. 20) Cell fractionation and Western blotting were performed as described previously. 16, 20, 21)

**Immunofluorescence**

Confocal images were obtained with a Fluoview FV500 confocal laser scanning microscope (Olympus, Tokyo, Japan), as described. 23) In brief, cells were fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized in phosphate-buffered saline (PBS) containing 0.1% saponin and 3% bovine serum albumin at room temperature. Cells were subsequently incubated with antibodies for 1 h, washed with PBS containing 0.1% saponin, stained with Alexa Fluor 488- or 546-conjugated secondary antibody for 1 h, and mounted with ProLong antifade reagent (Thermo Fisher Scientific). For nuclear staining, cells were treated with 200 µg/mL ribonuclease (RNase) A for 1 h and then stained with propidium iodide (Sigma-Aldrich) or TO-PRO-3 (Thermo Fisher Scientific). Quantitation of chromatin structural changes was performed as described previously. 21)

**Quantitative Real-Time PCR (qPCR) Analysis**

qPCR was performed as described previously. 23) Briefly, total RNA was isolated from cells with RNAiso plus reagent (TaKaRa, Shiga, Japan), and cDNA was synthesized from 0.5 µg of each RNA preparation using a ReverTra Ace qPCR RT Kit (TOYOBO, Tokyo, Japan), according to the manufacturer’s instructions. The primers used for PCR were as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-ACC ACA GTC CAT GCC ATC AC-3' (sense) and 5'-TCC ACC ACC GTC TTG GTA-3' (antisense); and ras association domain family 1 isoform A (RAASF1A), 5'-GGCTGGAAACCRCGGTG-3' (sense) and 5'-TCTCAGGACAGGTGCTTCT-3' (antisense). After initial denaturation at 95°C for one minute, PCR was performed for 40 cycles (15 s at 95°C and 45 s at 60°C) using a Thunderbird SYBR Green Polymerase Kit (TOYOBO) and an Eco Real-Time PCR System ( Illumina, San Diego, CA, U.S.A.).

**RESULTS**

**ALKATI Induces Chromatin Structural Changes in the Nucleus**

Recent studies have reported that ALKATI localizes in the nucleus as well as the cytoplasm. 23) In order to confirm this finding, we transiently expressed ALK-FL and ALKATI in HeLa S3 cells (Figs. 1A, B). The cells were then fixed and stained with anti-ALK antibody to analyze the subcellular localization of each isoform (Fig. 1C). Consistent with the previous study, although ALK-FL showed cytoplasmic or membrane localization, ALKATI localized both in the nucleus and the cytoplasm (Fig. 1C).

We previously found that other kinases, including non-receptor Src-family tyrosine kinases (SFKs) and c-Abl tyrosine kinase, induce chromatin structural changes in the nucleus. 1, 2, 20, 21) To assess the potential effects of ALKATI on chromatin structural changes, we used the quantitative pixel imaging method, which we developed previously. 1, 2) In this method, level of chromatin structural changes can be detected as standard deviation (S.D.) value of propidium iodide (PI) intensity. Although ALK-FL-expressing cells showed only minor changes, ALKATI-expressing cells exhibited a greater degree of chromatin structural changes than ALK-FL-expressing cells (Fig. 1D). To further evaluate the effect of the nuclear localization of ALKATI on chromatin structural changes, we generated a nuclear-targeted form of ALKATI by introducing the SV40 NLS to its N-terminal region (NLS-ALKATI) (Figs. 1A, 2A). The extent of chromatin structural changes in NLS-ALKATI-expressing cells was greater than those in ALKATI-expressing cells (Figs. 2B, C). Notably, the kinase-dead mutant of nuclear-targeted ALKATI (NLS-ALKATIKD) did not induce chromatin structural changes (Figs. 1A, 2D–F). These results suggest that the nuclear localization
of ALK^ATI promotes chromatin structural changes in a kinase activity-dependent manner.

**ALK^ATI Promotes Heterochromatinization in the Nucleus**

Given that the chromatin structural changes promoted by SFKs and c-Abl are involved in heterochromatinization, we analyzed the effect of ALK^ATI on this process. We assessed the levels of Lys9 tri-methylated histone H3 (H3K9me3), a heterochromatin marker, in HeLa S3 cells transfected with ALK^ATI or the control plasmid (pcDNA4/TO). After 24h, cells were fixed and subjected to immunofluorescence analysis with the indicated antibodies. Bars: 10 µm. (D) The levels of chromatin structural changes were assessed using standard deviation (S.D.) values of propidium iodide (PI) intensity per pixel. The plot represents the S.D. value of PI intensity per pixel in each cell, and bars represent mean±S.D. from a representative experiment. Numbers in parentheses indicate mean values, and asterisks indicate the statistical significance (**p<0.01; ***p<0.001) calculated by Student’s t-test. n, cell number.
These findings indicate that ALK ATI may induce heterochromatinization in a kinase activity-dependent manner.

**ALK ATI Phosphorylates the Nuclear Protein AKAP8**

Our recent studies showed that tyrosine phosphorylation of AKAP8 is involved in SFK-induced chromatin structural changes. AKAP8 is a nuclear protein that binds to chromatin and the nuclear matrix, and it contains 11 tyrosine residues that contribute to SFK-induced phosphorylation. Given the similar activities of nuclear ALK ATI and SFKs, we examined the possibility that ALK ATI also phosphorylates AKAP8 in the
nucleus. Co-expression of myc-tagged AKAP8 (myc-AKAP8) with ALK\(^{\text{ATI}}\) promoted tyrosine phosphorylation of AKAP8 (Figs. 4A, B). However, ALK\(^{\text{ATI-KD}}\) failed to promote phosphorylation of AKAP8, and ALK\(^{\text{ATI}}\)-induced phosphorylation of AKAP8 was suppressed by the ALK inhibitor crizotinib. These findings suggest that ALK\(^{\text{ATI}}\) phosphorylates AKAP8 in a kinase activity-dependent manner.

To identify the phosphorylation sites in AKAP8 that are targeted by ALK\(^{\text{ATI}}\), we analyzed phosphorylation of AKAP8 mutants that have multiple phenylalanine substitutions at the tyrosine residues (4CYF, 5NYF, and 11YF\(^21\)) (Fig. 4A). ALK\(^{\text{ATI}}\)-induced phosphorylation levels were not affected in the 4CYF and 5NYF mutants (Fig. 4C). However, phosphorylation was almost completely suppressed in the 11YF mutant (Fig. 4D). These results suggest that the three tyrosine residues in the N-terminal region of AKAP8 (Tyr51, Tyr53, and Tyr80), which have been substituted in only the 11YF mutant, are major phosphorylation sites for ALK\(^{\text{ATI}}\).

ALK\(^{\text{ATI}}\) Promotes Dissociation of AKAP8 from Nuclear Structures through Its Phosphorylation Phosphorylation of AKAP8 by nuclear SFKs promotes its dissociation from nuclear structures, which can be detected as a change in the subcellular localization of AKAP8 from the Triton X-100-insoluble fraction to the Triton X-100-soluble fraction.\(^1\) The Triton X-100-soluble fraction is enriched in cytoplasmic proteins, such as cPLA2, whereas the Triton X-100-insoluble fraction is enriched in nuclear structures, such as Lamin A/C\(^21\) (Figs. 5A, B). We examined the effect of nuclear ALK\(^{\text{ATI}}\) on the subcellular localization of AKAP8. Expression of NLS-AKAP8\(^{\text{ATI}}\), but not NLS-AKAP8\(^{\text{ATI-KD}}\), promoted the shift of AKAP8 from the Triton X-100-insoluble fraction to the Triton X-100-soluble fraction (Fig. 5A). Notably, the subcellular localization of the 11YF mutant was not affected by the expression of NLS-AKAP8\(^{\text{ATI}}\) (Fig. 5B). Taken together, these results suggest that ALK\(^{\text{ATI}}\)-mediated phosphorylation of AKAP8 promotes its dissociation from nuclear structures, which may contribute to chromatin structural changes.

Finally, we assessed the effect of NLS-AKAP8\(^{\text{ATI}}\) on expression of the tumor suppressor gene RASSF1A, whose expression was significantly suppressed by c-Abl through chromatin structural changes in the nucleus.\(^3\) RASSF1A expression was slightly but significantly repressed by NLS-AKAP8\(^{\text{ATI}}\) (Fig. 5C), suggesting that, like c-Abl, ALK\(^{\text{ATI}}\) represses expression of RASSF1A via chromatin structural changes in the nucleus.

**DISCUSSION**

Non-receptor type tyrosine kinases mainly localize to the cytoplasm, and most studies have focused on their roles as cytoplasmic signal mediators.\(^1,4,5\) However, we have focused on their nuclear roles for more than a decade because we found that they can localize to the nucleus as well as the cytoplasm. Lyn, a member of SFKs, localizes to the nucleus despite the fact that it lacks a defined NLS.\(^2,18,22\) c-Abl, which is known to have three NLSs and one nuclear exports signal (NES),\(^27\) translocates to the nucleus upon DNA damage stimulation.\(^1\) Nuclear Lyn regulates DNA-damage response through phosphorylation of the heterochromatin protein KAP1 (Krüppel-associated box-associated protein-1).\(^20\) Nuclear Lyn also phosphorylates the nuclear matrix-binding protein AKAP8 upon oxidative stress.\(^23\) Nuclear c-Abl regulates estrogen signaling through phosphorylation of the pioneer transcription factor FOXA1\(^23\) and DNA damage response through phosphorylation of the transcription factor JunB.\(^24\) Nuclear c-Abl also induces chromatin structural changes and heterochromatinization and regulates expression of genes, such as the tumor suppressor RASSF1A.\(^1\)

Recently, the truncated isoform of ALK (ALK\(^{\text{ATI}}\)) was reported to localize to the nucleus as well as cytoplasm, but its nuclear role was not clarified.\(^13\) Therefore, in the present study, we analyzed its role in the nucleus. We found that nuclear ALK\(^{\text{ATI}}\) induces chromatin structural changes and heterochromatinization. In addition, ALK\(^{\text{ATI}}\) phosphorylated the nuclear protein AKAP8, thereby changing its subcellular localization from the insoluble fraction to the soluble fraction. Finally, ALK\(^{\text{ATI}}\) was shown to suppress expression of...
RASSF1A. These results suggest that ALK<sup>ATI</sup> induces chromatin structural changes by promoting dissociation of AKAP8 from nuclear structures via its phosphorylation and regulates gene expression. Because ALK is a proto-oncogene and its activation mutant ALK<sup>ATI</sup> promotes tumorigenesis, nuclear role of ALK<sup>ATI</sup> may be involved in tumor formation.<sup>7</sup> In this study, we found that, like c-Abl, ALK<sup>ATI</sup> promotes chromatin structural changes and represses expression of RASSF1A. RASSF1A is a tumor suppressor gene, whose expression is frequently suppressed by promoter methylation in various tumors.<sup>26</sup> Because c-Abl is also a proto-oncogene and its aberrant activation is involved in tumorigenesis,<sup>27</sup> ALK<sup>ATI</sup>- or c-Abl-mediated repression of RASSF1A expression via chromatin structural changes may be one of the causes of RASSF1A silencing in tumors.

Although precise molecular mechanisms of ALK<sup>ATI</sup>-
mediated chromatin structural changes are unclear, our present results suggest that tyrosine phosphorylation of the nuclear matrix protein AKAP8 is involved in this phenomenon. We previously found that AKAP8 is a nuclear target of the SFK member Lyn and that AKAP8 is phosphorylated by c-Abl as well as Lyn, suggesting that nuclear tyrosine kinases similarly phosphorylate AKAP8. However, major phosphorylation sites of AKAP8 for ALKATI were suggested to be different from those for Lyn: Lyn-mediated phosphorylation of AKAP8 was reduced in the 4CYF and 5NYF mutants, but ALKATI-mediated phosphorylation was not (Fig. 4C). Although major phosphorylation sites of AKAP8 for c-Abl is not yet determined, we speculate that ALKATI and c-Abl similarly phosphorylate AKAP8 because these kinases have similar nuclear roles: chromatin structural changes, heterochromatinization, and repression of RASSF1A expression.

In conclusion, our present results suggest that ALKATI promotes chromatin structural changes and heterochromatinization in the nucleus and that phosphorylation of AKAP8 is involved in these phenomena. Our results shed light on the previously uncharacterized nuclear roles of ALK and their involvement in tumorigenesis.

Acknowledgments This work was supported in part by Grants-in-Aid for Scientific Research C [Grant numbers 16K08227 (to Noritaka Y.) and 15K07922 (to Naoto Y.)] and
Program for Leading Graduate Schools (Leading Graduate School Chiba University Nurture of Creative Research Leaders in Immune System Regulation and Innovative Therapeutics) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Grants for Scientific Research from the Promotion and Mutual Aid Corporation for Private Schools of Japan (Kyoto Pharmaceutical University and Chiba University); a Grant from the Hamaguchi Foundation for the Advancement of Biochemistry (to Noritaka Y.); and a Grant from the Japan Foundation for Applied Enzymology (to Naoto Y.). M.M. and T.H. are Research Assistants of Program for Leading Graduate Schools and Research Fellows of the Japan Society for the Promotion of Science.

Conflict of Interest The authors declare no conflict of interest.

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