CNOT3 targets negative cell cycle regulators in non-small cell lung cancer development

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Abstract

Lung cancer is one of the major causes of cancer death and clarification of its molecular pathology is highly prioritized. The physiological importance of mRNA degradation through the CCR4-NOT deadenylase has recently been highlighted. For example, mutation in *CNOT3*, a gene coding for CNOT3 subunit of the CCR4-NOT complex, is found to be associated with T-cell acute lymphoblastic leukemia, T-ALL, though its contribution to other cancers has not been reported. Here, we provide evidence suggesting that CNOT3 is required for the growth of non-small cell lung cancer. Depletion of CNOT3 suppresses proliferation of A549 human non-small cell lung cancer cells with enhanced mRNA stability and subsequent elevated expression of p21. In addition, we identified the mRNA for Krüppel-like factor 2 transcription factor, an inducer of p21, as a novel mRNA degradation target of CNOT3 in non-small cell lung cancer cells. Aberrant up-regulation of Krüppel-like factor 2 by CNOT3 depletion leads to impairment in proliferation of A549 cells. Consistent with these findings, elevated mRNA expression of *CNOT3* in non-small cell lung cancer in comparison with the paired normal lung epithelium was confirmed through scrutinization of the RNA-sequencing datasets from The Cancer Genome Atlas. Moreover, we found an inverse correlation between *CNOT3* and *CDKN1A* (encoding p21) mRNA expression using the

combined datasets of normal lung epithelium and non-small cell lung cancer. Thus, we propose that up-regulation of CNOT3 facilitates the development of non-small cell lung cancer through down-regulation of Krüppel-like factor 2 and p21, contrary to tumor suppressive functions of CNOT3 in T-ALL. (251 words/300 words)

Introduction

Lung cancer is the top cause of cancer deaths for both men and women in the United States[1]. Hence, clarification of molecular pathology of lung cancer is highly prioritized. Lung cancer is classified into two major pathological types: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)[2]. NSCLC accounts for 85% of all lung cancer cases and is considered as typical type of lung cancer. Further, NSCLC is comprised of three main subtypes: lung adenocarcinoma (LADC), lung squamous cell carcinoma (LSqCC), and large-cell lung carcinoma, and LADC and LSqCC account for most of NSCLC[2, 3].

Cancer can be developed through the mutation or loss of genes regulating mRNA homeostasis because its deviation leads to aberrant protein expression that could cause unregulated cell proliferation. MicroRNA is one of the most essential factors for determining the fate of its target mRNA expression through mRNA degradation. In fact, a variety of microRNAs were identified as oncogenic factors (oncomiRs) or tumor suppressors by intense studies[4]. In addition, mRNA decay after shortening poly(A) tail by deadenylases is also a key factor in determining mRNA fate[5]. The CCR4-NOT complex is a major deadenylase in mammals[6]. Naturally, it is assumed that the CCR4-NOT also exerts similar functions as microRNAs that are relevant to lung

carcinogenesis[7].

Mammalian CCR4-NOT complex consists of eight subunits; CNOT1, CNOT2, CNOT3, CNOT6 or 6L, CNOT7 or 8, CNOT9, CNOT10 and CNOT11[8]. Of these subunits, CNOT6/6L/7/8 possess deadenylase activities[6]. CNOT1 is considered as a scaffold protein for this complex[9] and CNOT3 is also reported to be necessary for the integrity of the complex and deadenylase activities[10, 11]. Recent studies reveal that depletion of CNOT3 results in various abnormalities and diseases including impairment in embryonic development, leanness, and osteoporosis in mice[11-13]. Importantly, frameshift and missense mutations of the *CNOT3* gene have been identified in T-cell acute lymphoblastic leukemia (T-ALL) patients by exosome-sequencing[14]. Moreover, knockdown of CNOT3 induces tumor development using sensitized drosophila eye cancer model[14]. These findings suggest that CNOT3 functions as a tumor suppressor in T-ALL development. However, the role of CNOT3 in NSCLC development remains unknown.

In this study, we investigated the functions and target mRNAs of CNOT3 using human LADC and LSqCC cell lines. We have addressed the status of *CNOT3* expression through scrutinization of RNA-sequencing (RNA-seq) datasets of LADC and LSqCC patients from The Cancer Genome Atlas (TCGA). Importantly, we

identified the mRNA for KLF2 transcription factor as a novel target of CNOT3 in lungs. Our data suggest the possibility that up-regulation of CNOT3 is required for NSCLC development by controlling stability of *CDKN1A* and *KLF2* mRNAs, contrary to the supposed roles in T-ALL. Our findings would give novel insights into a relationship between cancer development and deadenylase-initiated mRNA decay.

Results

CNOT3 mRNA expression is up-regulated in NSCLC.

We first analyzed the RNA-seq datasets of TCGA database to examine if the expression of CNOT3 is altered in human NSCLC compared to normal lung epithelium. Since frequent frameshift and missense mutations of *CNOT3* were reported in T-ALL, and since knockdown of CNOT3 with enhanced Notch signaling resulted in development of eye cancer in Drosophila[14], CNOT3 was thought to be anti-oncogenic. However, the RNA-seq analysis revealed that mRNA expression of *CNOT3* is up-regulated in NSCLC, both LADC and LSqCC (Figures 1a and b). Because this up-regulation was less obvious in LADC than LSqCC, we also examined mRNA expression change of *CNOT3* in LADC using the microarray datasets downloaded from ONCOMINE database[15]. Up-regulation of *CNOT3* mRNA in LADC was commonly observed in all the datasets[16-20], with statistical significance except for one dataset using the minimum sample size in total[20](Supplementary Figure S1). Moreover, we examined *CNOT3* promoter activities in primary normal lung epithelial cells and NSCLC cell lines using the cap analysis of gene expression (CAGE)[21]-seq from the Functional ANnoTation Of the Mammalian genome 5 (FANTOM5) database[22]. Higher number of CAGE tag counts was detected at p1 promoter of *CNOT3* in NSCLC cell lines compared to human

primary normal lung epithelium, indicating that NSCLC cells have higher *CNOT3* promoter activity (Figure 1c). These findings indicate that CNOT3 expression is elevated in NSCLC compared to normal lung epithelium.

Proliferation of human NSCLC cells is attenuated by CNOT3 depletion.

According to the CAGE-seq data shown in Figure 1c, *CNOT3* promotor activity in A549 LADC cell line was about 2-fold higher than its average in normal lung epithelium (average; 35.4 and A549 cells; 68.5). Hence, in order to investigate the role of CNOT3 in NSCLC cells, we established A549 cells stably expressing tetracycline-inducible shRNA against CNOT3 targeting two different sequences within exons of CNOT3 using lentivirus (A549-T-shCNOT3-1 and -2 cells). About 70-80% knockdown of CNOT3 mRNA expression was achieved by their induction (Figure 2a). We found that CNOT3 depletion reduces the protein expression of some other CCR4-NOT subunits (CNOTs) without decreasing mRNA expression (Figure 2b and Supplementary Figure S2) as previously reported in murine embryonic fibroblasts (MEFs)[10]. CNOT3 overexpression restored this reduction in the expression of CNOTs (Figure 2c), indicating that CNOT3 is essential for the integrity of the CCR4-NOT complex in human NSCLC cells as well as MEFs. We also examined subcellular localization of

CNOT3 in A549 cells since CNOT3 was also reported to be involved in transcriptional activities[23] or chromatin modification[24]. In contrast with the finding that CNOT3 is expressed in the nucleus to the same extent with cytoplasm in colorectal cancer cells[25], CNOT3 expression in the cytoplasm was much higher than nucleus in A549 cells (Figure 2d), suggesting that CNOT3 is mainly involved in the regulation of mRNA degradation. Next, we checked the effects of CNOT3 knockdown on cell viability or proliferation because necroptosis or mitotic arrest was induced in CNOT3-depleted MEFs[10] or HeLa cells[26], respectively. As shown in Figure 2e, increase in dead cells or mitotic cells was hardly observed in CNOT3-depleted A549 cells. However, cell proliferation rate was reduced by about 50% by induction of two different CNOT3 shRNAs (Figure 2f). Although exogenous CNOT3 overexpression did not accelerate the proliferation of A549 cells (Figure 2g), this growth inhibition of A549-T-shCNOT3-1 cells was rescued by CNOT3 overexpression (Figure 2h). These findings indicate CNOT3 is necessary for the proper growth of A549 NSCLC cells.

CNOT3 depletion induces p21 expression and inhibits the cell cycle progression.

To know the mechanism by which CNOT3 depletion attenuates the proliferation of A549 cells, we examined the status of retinoblastoma (RB) protein, a master regulator

of cell cycle progression, when CNOT3 was knocked down. We found that the ratio of unphosphorylated form of RB (pRB) to hyperphosphorylated form (ppRB) was increased by CNOT3 depletion, while cleavage of poly(ADP-ribose) polymerase (PARP) which is essential for caspase-dependent apoptosis was not enhanced (Figure 3a). We further found the increase in the cell population in G0/G1 phase and no alteration in subG1 phase of A549-T-shCNOT3-1 cells by CNOT3 knockdown (Figure 3b), suggesting that CNOT3 depletion does not induce apoptosis but induces cell cycle arrest of A549 cells.

During G1-to-S transition of cell cycle, cyclin-dependent kinase (CDK) inhibitors, CIP/KIP proteins (p21, p27 and p57), function as a brake for cell cycle progression through inactivation of Cyclin D-CDK4, Cyclin D-CDK6, and Cyclin E-CDK2 complex[27]. We hypothesized that CNOT3 depletion aberrantly up-regulates the expression of CIP/KIP proteins via impairment of mRNA decay. Among the CIP/KIP proteins, the expressions of p21 was clearly elevated by CNOT3 depletion at both mRNA and protein level (Figures 3c and d). Less up-regulation of p27 compared to p21 at mRNA and protein level, especially in A549-T-shCNOT3-1 cells, was observed, and up-regulation of *CDKN1C* (coding p57) was not observed (Figures 3c and d). Further, to rule out a possible involvement of the other CDK inhibitors, the INK4 family, we also

checked expression change of *CDKN 2C* (coding p18) and *CDKN2D* (coding p19) by CNOT3 depletion and their up-regulation was not observed in common with induction of shCNOT3-1 and -2 (Figure 3c). *CDKN2A* (coding p16) and *CDKN2B* (coding p15) alleles are deficient in A549 cells[28, 29].

We next evaluated mRNA stability of *CDKN1A* (coding p21), *CDKN1B* (coding p27), and *CDKN2D* using Actinomycin D (ActD; Wako, Osaka, Japan), a transcription inhibitor. Quantitative real-time RT-PCR (qRT-PCR) revealed that mRNAs of *CDKN1A* and *CDKN1B*, but not *CDKN2D*, were stabilized by CNOT3 depletion in A549 cells (Figure 3e). These results suggest that CNOT3 depletion directly up-regulates the expression of *CDKN1A* and *CDKN1B* through their enhanced mRNA stabilization.

Identification of KLF2 transcription factor as a target of CNOT3

Because the expression of p21 was highly up-regulated, we assumed that up-regulation of p21 was most likely to be responsible for induction of cell cycle arrest by CNOT3 depletion. However, stabilization of *CDKN1B* mRNA was more obvious than *CDKN1A* mRNA, contrary to the results of expression change (Figures 3b and d). Then, we hypothesized that CNOT3 also suppresses the expression of p21 through Factor X

which induces p21 expression. In other words, we assumed that p21 expression is regulated by in direct and indirect ways, the latter being via Factor X (Figure 4a).

To identify Factor X, we performed microarray analysis and highlighted on up-regulated genes commonly observed in two types of shCNOT3-induced A549 cells. We narrowed down the candidate gene to 110 up-regulated probes based on our criteria and we found some transcription-related genes (Figure 4a and Supplementary Table S1, green color; transcription-related genes). Transcription factors directly regulate the expressions of target genes and are potent candidates. We found the only one transcription factor, Krüppel-like factor 2 (KLF2) among the 110 probes, which is also called as lung krüppel-like factor (LKLF)[30] since its expression is abundant in lungs and necessary for lung development[31]. KLF2 is known to induce p21 expression in Jurkat T-ALL cells via direct promoter regulation[30]. In addition, KLF2 was also reported to induce less preferentially p27 expression than p21 expression in mouse pre-B cells[32]. Thus, we hypothesized that up-regulation of KLF2 is responsible, at least partly, for p21 induction by CNOT3 depletion.

We confirmed up-regulation of KLF2 by CNOT3 depletion by qRT-PCR and this up-regulation was suppressed by CNOT3 overexpression (Figures 4b and c). We also found that *KLF2* mRNA was drastically stabilized by CNOT3 depletion while

mRNA of *KLF6*, another inducer of p21 and p27 in the same KLF family[33], was not up-regulated and was much less stabilized than *KLF2* (Supplementary Figure S3 and Figure 4d). Furthermore, the poly(A) tail length of *KLF2* mRNA was elongated in CNOT3-knocked down A549 cells while that of very stable *HPRT1* mRNA was not changed (Figures 4d and e). These findings indicate that KLF2 transcription factor is a bona fide direct target of CNOT3.

KLF2 is at least in part responsible for the growth inhibition by CNOT3 depletion

To confirm the involvement of KLF2 in the growth inhibition by CNOT3 depletion, we established A549-T-shCNOT3-1 cells stably expressing shRNA against KLF2 using lentivirus (Figure 5a). The mRNA expression level of *CDKN1A* under CNOT3 knockdown was lowered by knockdown of KLF2, in accord with the previous findings that KLF2 transcriptionally induces *CDKN1A* expression[34] (Figure 5b). In addition, cell proliferation assay revealed that the growth inhibition by CNOT3 depletion was significantly attenuated by knockdown of KLF2 (Figure 5c). These findings indicate that up-regulation of KLF2 mediates the induction of p21 and growth inhibition by CNOT3 depletion, at least partly.

p21 and KLF2 are common targets of CNOT3 in human NSCLC

To determine whether the regulation of KLF2 expression by CNOT3 is commonly observed in NSCLC cells or only in A549 cells which harbor KRAS mutation[35], we examined the expression of KLF2 using siRNA against CNOT3 (siCNOT3) in other human LADC and LSqCC cell lines with different mutation or amplification profiles of KRAS, TTF1, and EGFR; NCI-H441: KRAS mutation and TTF1 amplification[35], NCI-H520: wild-type, and NCI-H1975: EGFR mutation[36]. In all the NSCLC cell lines we examined, up-regulation of *KLF2* mRNA was commonly observed by two different siCNOT3 in good agreement with knockdown efficiency of CNOT3 (Figures 6a and Supplementary Figure 4), suggesting that KLF2 is a common target of CNOT3 in NSCLC, and that this regulation of KLF2 expression by CNOT3 is not limited to the NSCLC with some major specific mutations. Importantly, impairment of the proliferation by CNOT3 depletion was also observed in all these cell lines (Supplementary Figure 5).

Furthermore, we examined the correlation of CNOT3 and p21 or KLF2 using the TCGA datasets for both LADC and LSqCC in order to confirm if the expressions of these genes are regulated by CNOT3 in clinical samples *in vivo*. Since the expressions of p21 and KLF2 are supposed to be altered during carcinogenesis based on their tumor

suppressive functions, we used combined datasets of NSCLC and paired normal lung epithelium to get a more clear correlation, although the paired normal corresponded to just some of the cancer samples. In accord with the fact that KLF2 is a direct inducer of p21, we confirmed a positive correlation between KLF2 and p21 using these combined datasets (Figures 6d and e). Importantly, we found a negative correlation of CNOT3 and p21, with comparable extent of Pearson's correlation value with that of KLF2 and p21 (Figures 6d and e), suggesting that CNOT3 negatively regulates the expression of p21 in human lung epithelium. Furthermore, a negative correlation between CNOT3 and KLF2 was found in the LSqCC datasets (Figure 6e). These findings indicate that KLF2 and p21 are common targets of CNOT3 in NSCLC.

Discussion

The mammalian CCR4-NOT complex is a multi-functional protein assembly that regulates transcription, translational inhibition, and mRNA degradation[37]. Increasing reports have demonstrated that individual subunit possesses a unique role in cancer development and progression. For instance, CNOT2 was reported to inhibit metastasis of mouse breast cancer cells, while CNOT7 promotes it[38, 39]. In the study using MCF7 breast cancer cell lines, cell cycle arrest was induced by knockdown of

CNOT1, CNOT3[40], CNOT7, or CNOT8[41], while impaired cell survival was observed by knockdown of CNOT6 or CNOT6L in addition with cell cycle arrest[40]. Hence, the role of the CCR4-NOT in cancer needs to be clarified by focusing on each subunit, not a whole complex, to avoid complexity. Importantly, frequent frameshift and missense mutations of CNOT3 were reported in T-ALL recently and CNOT3 was found to be a tumor suppressor using sensitized drosophila eye cancer model[14]. These findings propelled us to examine the function of the CCR4-NOT, by focusing on CNOT3, in another major cancer, lung cancer.

CNOT3 is known to be essential for modulation of transcription activities[37]. Importantly, CNOT3 hetero-deficient mice developed impaired heart function through dysregulation of chromatin modification[24]. However, we found that CNOT3 is predominantly located in the cytoplasm in A549 cells, not nucleus (Figure 2d), different from colorectal cancer cells[25]. Thus, in the present study, we focused on mRNA degradation mediated by CNOT3 which is supposed to take place in the cytoplasm, and found stabilization of *CDKN1A* and *CDKN1B* mRNA (Figure 3e).

We also identified KLF2 as a novel common target of CNOT3 in NSCLC (Figure 4a and 6a). KLF2 is reported to induce p21 expression in NSCLC cells including A549 cells and its expression is associated with lung cancer progression or

prognosis [34, 42]. It should be noted that *KLF2* is included among the up-regulated genes by combined knockdown of CNOT7 and CNOT8 in MCF7 cells[41]. These indicate that the CCR4-NOT also participates in the regulation of tumor suppressor KLF2 as well as microRNAs[43] and long non-coding RNAs[44]. It is also worth mentioning that some important mRNAs such as *KLF2* are universally regulated by the CCR4-NOT complex in the various organs.

Tumor suppressor, p53 is another major regulator of p21 and its mRNA was reported to be directly regulated by CNOT3 in developing mouse B lymphocytes[45]. Up-regulation of *TP53* mRNA (encoding p53) by CNOT3 knockdown was not commonly observed in A549 cells (Supplementary Figure S6a). In addition, *TP53* mRNA was strikingly stable in lung cancer cells and was not stabilized by CNOT3 depletion (Supplementary Figure S6b), contrary to developing mouse B lymphosites[45]. These suggest that some target mRNAs of the CNOT3 are fairly cell- or tissue-type dependent, contrary to the finding regarding KLF2. However, we found that greater increase of p53 at protein level (average: 2.20 fold) than mRNA level (1.32 fold) by CNOT3 depletion in A549-T-shCNOT3-1 cells (Supplementary Figure S6c and d), suggesting the possibility that CNOT3 is also involved in the translation inhibition of p53. This increase in p53 protein might partly account for the growth inhibition by CNOT3, of

which knockdown of KLF2 showed only modest restoration.

A recent report showed that nuclear CNOT3 is important for colorectal cancer progression and highly linked to the prognosis[25]. We examined the prognosis of NSCLC patients by Kaplan-Meier plots with classifications based on the CNOT3 expression (top half versus bottom half, top third versus bottom third, and top quartile versus bottom quartile) using the Oncolnc (www.oncolnc.org)[46]. Unfortunately, however, we were not able to find significant difference in survival between the two groups (data not shown). This finding suggests that CNOT3 is most likely to be involved in the carcinogenesis step of NSCLC, rather than progression, through down-regulation of tumor suppressors.

We found up-regulation of CNOT3 in NSCLC (Figure 1 and Supplementary Figures S1), however how CNOT3 expression is regulated in body has been scarcely investigated. We examined if *CNOT3* gene is frequently amplified in NSCLC by analyzing the dataset from Campbell et al.[47]. Gene alteration frequency of *CNOT3* (0.96%, amplification) is much less than that of *MYC* (8.74%, amplification) or *CDKN2A* (21.07%, deletion) which is one of the most frequently altered gene in terms of copy number in this dataset[47] (Supplementary Figure S7), suggesting that gene amplification is not the cause of up-regulation of *CNOT3* in NSCLC. Therefore, it is

speculated that CNOT3 expression level is elevated through alteration of other genes or cancer microenvironment. Elucidation of the mechanism of CNOT3 up-regulation during lung carcinogenesis awaits further studies.

We also found the expression change of the other subunits commonly observed in the LADC and LSqCC: up-regulation of CNOT1, CNOT2, CNOT7, CNOT9, and CNOT11 and down-regulation of CNOT6L (Supplementary Figures S8 and 9). We further found that depletion of CNOT2 or both CNOT7 and CNOT8 using siRNA inhibits the cell proliferation of A549 cells, however, depletion of CNOT1 did not affect it unexpectedly (Supplementary Figures S10 and 11). Further studies are necessary for elucidation of how CNOT1 and CNOT3 regulate the functions of the CCR4-NOT complex or other interacting molecules in a specific way and cause this discrepancy in future.

In conclusion, we demonstrated that CNOT3 depletion attenuates the growth of NSCLC through direct and indirect regulation of p21 via mRNA degradation (Figure 7). The findings in this study suggest that CNOT3 facilitates development of NSCLC through modulation of mRNA decay machinery.

Materials and Methods

TCGA and CBio Cancer Genomics Portal Data

Level 3 RNA-seq data containing gene expression and clinical information for LADC and LSqCC were downloaded from TCGA data portal (https://tcga-data.nci.nih.gov/docs/publications/tcga/) by November 2015. The graphs for the mutation and copy number analysis of NSCLC were generated by the CBio Cancer Genomics Portal (http://www.cbioportal.org)[48, 49] using the dataset from Campbell et al.[47] which consists of the highest number of NSCLC patients (TCGA data together with the data from Imielinski et al.[50] : 660 LADC and 484 LSqCC) available on this website as of October 2016.

ONCOMINE Data

Microarray datasets for LADC (condition ; sample number>=20) were downloaded from ONCOMINE database (www.oncomine.org)[15] by December 2014. There are three probes for *CNOT3* mRNA expression (203239_s_at, 211141_s_at, and 229143_at) for Affymetrix (CA, USA) microarrays. 203239_s_at and 211141_s_at probes were used in common and the former one was used as a representative probe because of higher sensitivity. A probe for *CNOT3* mRNA for Illumina (CA, USA) microarray was ILMN_2207393.

CAGE Tag Count Analysis

CAGE data with raw read counts were obtained from FANTOM5 database and analyzed as previously reported[51, 52]. The data used in this study is composed of 16 primary normal lung epithelial cell samples (tracheal (TEC1-3), bronchial (BEC1-7), small airway (SAEC1-3), and alveolar (AEC1-3) epithelial cells) and 17 NSCLC cell lines (LADC(A549, PC-14, NCI-H441, NCI-H358, SW1573, and NCI-H650), LSqCC (EBC-1, LC-1F, REPF-LC-AI, and KNS-62), lung large cell carcinoma (IA-LM, NCI-H460, LU65, and Lu99B), and unclassified NSCLC (ChaGo-K-1 and NCI-H1385)). The CAGE tag counts between samples were normalized using edgeR package as described previously[53, 54].

Cell Culture and Reagents

A549 cells were obtained from RIKEN Cell Bank. NCI-H441, NCI-H520, and NCI-H1975 cells were purchased from ATCC. Cells were cultured in DMEM medium (Thermo Fisher Scientific, MA, USA) for A549 cell line or RPMI-1640 medium (Thermo Fisher Scientific) for NCI-H441, NCI-H520 and NCI-H1975 cell lines. Both of the media contained 10% fetal bovine serum (FBS), penicillin (50U/mL), and

streptomycin (50U/mL). All the cells were grown up in a 5% CO2 atmosphere at 37 degrees Celsius. Doxycycline (DOX; TaKaRa, Shiga, Japan) and ActD were used at a concentration of 1 or 3 ug/mL and 5 ug/mL, respectively.

Cell Proliferation Assay

A549-T-shNTC, CNOT3-1, and CNOT3-2 cells, or GFP or CNOT3-overexpressing cells (1 to 3 X 10^4 cells) were seeded in triplicate in 12-well plates (defined as Day0) and the cells were treated with DOX on the next day when necessary. Regarding the growth assay using siRNA, (5 to 25 X 10^4 cells) cells were seeded in duplicate or triplicate in 6-well or 12-well plates. The number of the cells was counted with hemocytometer or 4 or 5 days after DOX treatment or indicated Day (Some results using triplicates were obtained from duplicated wells because of technical error). Experiments were performed at least twice under similar conditions and representative results are shown in Figures.

Cell Cycle Analysis

A549-T-shCNOT3-1 cells were seeded in 10 cm dishes and the cell were treated with DOX on the next day. The cells were collected 3 days after DOX treatment, washed

with PBS, and fixed in an ice cold 70% EtOH and stored at -30 degrees Celsius until use. Cells were washed in 1xPBS twice, dissociated in staining buffer (PBS, 0.1% Triton X-100, 2%FBS) and labeled with 7-aminoactinomycin D (7-AAD; BD Biosciences, NJ, USA). FACS ARIA III (BD Biosciences) was used for acquisition of at least 30.000 events. Data was analyzed with FlowJo 10.3 software (Tree Star, OR, USA) using Dean-Jett-Fox algorithm. SubG1 fraction was calculated as 100-(G0/G1+S+G2/M). Experiments were performed twice under similar conditions and representative results (36 X 10^4 cells seeded) are shown in Figures.

Lentivirus Production

We used a lentiviral vector system to establish A549 cells stably expressing coding protein, shRNA or tetracycline-inducible shRNA, without sorting after infection. Entry vectors for shRNA and tetracycline-inducible shRNA (pENTR4-H1 and pENTR4-H1tetOx1), destination vectors (CSII-EF-RfA, CS-RfA-EG, and CS-RfA-ETV), GFP-expressing lentiviral vector (CS-CDF-EG-PRE), packaging vector (pCAV-HIVgp), and VSV-G and Rev-expressing vector (pCMV-VSV-G-RSV-Rev) were provided from Dr. Hiroyuki Miyoshi (RIKEN, Tsukuba, Japan). Lentivirus was produced basically according to the protocol made by Dr. Miyoshi (http://cfm.brc.riken.jp/lentiviral-

vectors/protocols/).

RNA Analysis

Total RNAs were extracted using RNeasy Mini Kit or RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA synthesis was performed as described previously[55]. qRT-PCR was performed using FastStart Universal SYBR Green Master (ROX; Roche, Basel, Switzerland) or TB Green™ Premix Ex Taq ™ II (Tli RNaseH Plus; TaKaRa) and the ABI PRISM 7900HT Sequence Detection System (Thermo Fisher Scientific). All samples were run in triplicate and the value for *GAPDH* mRNA was used for normalization. This *GAPDH* value was regarded as a fixed value without errors when calculating standard deviation of each objective mRNA expression. Regarding the measurement of remaining RNA, the control expression was also regarded as fixed value for statistics. Comparison of poly(A) tail length was performed as previously described[10]. Primers used in this study are listed in Supplementary Table S2. Experiments were performed at least twice under similar conditions and representative results are shown in Figures.

Microarray Analysis

Microarray analysis was performed using total RNAs extracted as described above and Gene Chip Human Genome U133 Plus 2.0 Array (Affymetrix) as described previously[10]. We used GeneSpring 12.6 (Agilent Technologies) to analyze the data using the MAS5 algorithm. We excluded the probes which showed Absent expression in all the six samples from the analysis. The complete data set has been submitted to the NCBI Gene Expression Omnibus[56] and can be accessible through GEO Series accession number GSE114694 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114694).

RNA Interference and Oligonucleotides

A549, NCI-H441, NCI-H520, and NCI-H1975 cells were transfected with siRNA for 3 days using RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's protocol. Targeting sequences of siRNA for NTC, CNOT3#1, CNOT3#2[26], CNOT1 (the same with CNOT1#1 in Ito et al[9].), CNOT2, and CNOT7[9] were previously described and the sequence for CNOT8 is as follows : 5'-GACCCUUCUCGAGGACAUUUG-3'. shRNA constructs were designed as previously reported[57] and targeting sequences are as follows : 5'-GCGCGCTTTGTAGGATTCG-3' (NTC), 5'-GGACCAGTTTGAGAGTGAAGT-3' (CNOT3-1), 5'-

GCCACATGGAGGATGAGATCT-3' (CNOT3-2), 5'-

ACCACGATCCTCCTTGACGAG-3' (KLF2).

Immunoblotting

Cell were lysed with TNE lysis buffer containing, 0.1% SDS, 50 mM Trs-HCl, 120 mM NaCl , 5 mM EDTA, 1% NP-40, and 10-20% Protease inhibitor (Nakalai Tesque, Kyoto, Japan or Wako). Western blotting was basically performed as previously reported[58]. TBS buffer containing 5% skim milk or BSA fraction V and 0.1% Tween-20 was used for blocking. For some blots, Can Get Signal® Immunoreaction Enhancer Solution (TOYOBO, Osaka, Japan) was used for enhancement of detection. We used an antibody against CNOT3 which is commercially available (H00004849-M01, Abnova, Taipei, Taiwan). Antibodies against CNOT1, CNOT2, CNOT6L, CNOT7 were described previously[10]. Rabbit CNOT9 antibody was obtained as described previously[59]. We purchased RB (554136) antibody from BD Biosciences and PARP antibody (#9542) from Cell Signaling Technology, MA, USA. Goat Lamin B antibody (sc-6217), mouse p53 (sc-126) and β-actin (sc-69879) antibodies were from Santa Cruz, TX, USA. p21 antibodies were from BD biosciences (556430) and Santa Cruz (sc-6246), and p27 antibodies were from Santa Cruz (sc-528) and abcam, Cambridge, England (ab32034).

α-tubulin (T9026) was purchased from Sigma-Aldrich, MO, USA. Quantification of the bands was performed using Image J in a vertical way. When it was impossible to measure in a vertical way because of the continuous band, it was performed in a horizontal way.

Subcellular Fractionation

Fractionation of cytoplasmic and soluble nuclear proteins of GFP or CNOT3-overexpressing A549-T-shCNOT3-1 cells with or without DOX treatment was performed using Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Fisher Scientific) according to the manufacturer's protocol.

Statistical Analysis

Two-tailed Student's t-tests or Welch's t-tests were used for the comparison of two samples. Tukey-Kramer post hoc tests were performed for the comparison of the multiple samples in Figure 6a and Supplementary Figures S4 and 10b. p values in Figure 6d and e were calculated using the Pearson's correlation value based on Student's t-distribution. p values in Supplementary Figure S1 was derived from ONCOMINE[15]. Results were considered to be statistically significant at $p<0.05$.

Supplementary information is available at *Oncogene*'s website.

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Figure Legends

Figure 1. *CNOT3* mRNA expression is up-regulated in NSCLC.

(a and b) Expression of *CNOT3* using the RNA-seq data from TCGA for LADC (a, N=57) and LSqCC (b, N=51). Red bar indicates NSCLC sample and blue bar indicates normal epithelium sample in the same patient (paired normal). RPKM ; reads per kilobase of exon model per million mapped reads. (c) Normalized CAGE tag counts for *CNOT3* p1 promoter from FANTOM5 database. Primary normal lung epithelium (N=16) and NSCLC cell lines (N=16) (Left). The averages of two groups were compared (Right). **p<0.01 by two-sided Student's paired t-test. Data are presented as mean ± SD.

Figure 2. Depletion of CNOT3 attenuates the proliferation of human NSCLC cells.

(a) qRT-PCR for *CNOT3* in A549 cells expressing tetracycline-inducible shRNA against non-target control (A549-T-shNTC), A549-T-shCNOT3-1, and -2 cells with or without DOX. Cells were treated with DOX for 3 days. (b) Cell lysate of each A549 stable with or without 3 days of DOX treatment was subjected to immunoblotting with antibodies, as indicated under *Materials and Methods*. (c) A549-T-shCNOT3-1 cells were infected with lentivirus expressing GFP or CNOT3. Immunoblotting was performed as described

in (b). (d) Subcellular fractionation of each A549 stable with or without 3 days of DOX treatment was performed as described under *Materials and Methods*. Soluble nuclear extracts and cytoplasmic extracts were subjected to immunoblotting. (e) Representative pictures of A549-T-ShCNOT3-1 cells with or without 4 days of DOX treatment. (f-h) Cell proliferation assay for each A549 stable. For (f) and (h), each A549 stable was treated with or without 4 days of DOX treatment. NS: not significant, ***$p<0.005$ compared to each (-) or GFP sample by two-sided Student's paired t-test or Welch's t-test. Data are presented as mean ± SD from three technical replicates, or two or three biological replicates.

Figure 3. Depletion of CNOT3 induces the expression of p21 in human NSCLC cells.

(a and d) Cell lysate of each A549 stable with or without 3 days of DOX treatment was subjected to immunoblotting with antibodies, as indicated under *Materials and Methods*. ppRB, pRB, and full-length form of PARP are shown with arrows. Quantification of pRB/ppRB, p21 or p27 /α-tubulin or β-actin was performed using Image J. The values were normalized to that of T-shCNOT3-1 without DOX. Average of the values obtained from four (p21 and p27) or three (pRB) independent sets of samples

are shown. (b) Cell cycle analysis for A549-T-shCNOT3-1 cells with or without 3 days of DOX treatment. (c) qRT-PCR for *CDKN1A*, *CDKN1B*, *CDKN1C*, *CDKN2C* and *CDKN2D* using the cDNA from the same samples with Figure 2a. (d) A549-T-shCNOT3-1 cells with or without 3 days of DOX treatment were treated with DMSO or ActD for 3 or 6 h. qRT-PCR for *CDKN1A*, *CDKN1B*, and *CDKN2D* using the cDNA from the cells with indicated time of treatment. NS: not significant, *$p<0.05$, **$p<0.01$, ***$p<0.005$ compared to each (-) sample by two-sided Student's paired t-test or Welch's t-test. Data are presented as mean ± SD from three technical replicates or individual samples.

Figure 4. Identification of KLF2 transcription factor as a target of CNOT3.

(a) Schematic model of regulation of p21 by CNOT3. We hypothesized that p21 expression is suppressed by a factor X which is a target of CNOT3, in addition with direct mRNA decay by CNOT3. We narrowed down the candidate X to up-regulated 110 probes with microarray based on the criteria as follows : 1. More than 2 fold increase by both shCNOT3-1 and -2 induction, 2. Less than 1.5 fold increase by shNTC induction (to exclude non-specific increase), 3. More than 1.5 fold higher expression both in A549-T-shCNOT3-1 and -2 induced cells than A549-T-shNTC cells without

DOX (to confirm the higher expression against A549-T-shNTC stable). We identified KLF2 as a factor X. (b) qRT-PCR for *KLF2* using the cDNA form the same samples with Figure 1a. (c) qRT-PCR for *KLF2* using the cDNA form A549-T-shCNOT3-1 cells expressing GFP or CNOT3 with or without 3 days of DOX treatment. (d) A549-T-shCNOT3-1 cells with or without 3 days of DOX treatment were treated with DMSO or ActD for 1 or 2 h. qRT-PCR for *KLF2* and *KLF6* using the cDNA from the cells with indicated time of treatment is shown. qRT-PCR for *HPRT1* using the cDNA from the same samples with Figure 2c is also shown. (e) Poly(A) tail assay for *HPRT1* and *KLF2* using the RNA from A549-T-shCNOT3-1 cells with or without 3 days of DOX treatment. Synthesized cDNA by reverse transcription was subjected to electrophoresis. NS: not significant, ***$p<0.005$ compared to each (-) or indicated sample by two-sided Student's paired t-test or Welch's t-test. Data are presented as mean ± SD from three technical replicates.

Figure 5. The growth inhibition by CNOT3 depletion is mediated by up-regulation of KLF2.

(a) Schematic model of establishment of A549 cells with stable double knockdown of both CNOT3 and KLF2 to examine the involvement of KLF2 in the growth inhibition

by CNOT3 depletion. (b) qRT-PCR for *KLF2* and *CDKN1A* using the cDNA form A549-T-shCNOT3-1 cells expressing shNTC or shKLF2 with or without 3 days of DOX treatment. (c) Cell proliferation assay for each A549 cells with or without 5 days of DOX treatment. The cell number of each A549 stable without DOX was standardized to 100%. *p<0.05, ** p<0.01, or ***p<0.005 by two-sided Student's paired t-test or Welch's t-test. Data are presented as mean ± SD from three technical or biological replicates.

Figure 6. KLF2 and p21 are common targets of CNOT3 in human NSCLC.

(a) qRT-PCR for *CNOT3* in NCI-H441, NCI-H520, or NCI-H1975 cells with siNTC, siCNOT3#1, or siCNOT3#2 transfection. ** p<0.01, ***p<0.005 vs siNTC by Tukey-Kramer post hoc test. Data are presented as mean ± SD from three technical replicates. (b and c) Scatter plot of *CNOT3* and *CDKN1A* (Left), *CNOT3* and *KLF2* (Middle), or *KLF2* and *CDKN1A* (Right) for LADC (b) or LSqCC (c) using RNA-seq datasets from TCGA database. These datasets include the data used in Figure 1. (b) Normal lung epithelium (Red, N=58) and LADC (Blue, N=518). (c) Normal lung epithelium (Red, N=51) and LSqCC (Blue, N=502). The r values indicate Pearson's correlation. The p values were calculated based on Student's t-distribution. RPKM ; reads per kilobase of

exon model per million mapped reads.

Figure 7. Schematic representation of the role of CNOT3 in NSCLC cells.

CNOT3, one key subunit of the CCR4-NOT complex, is highly expressed in NSCLC cells and is required for the proper expression of some other subunits including CNOT1, CNOT2, CNOT7, and CNOT9. CNOT3 regulates the expression of p21 through mRNA degradation. CNOT3 also specifically degrades the mRNA of *KLF2*, which regulates the expression of p21 through transcription. When CNOT3 is depleted in NSCLC cells, the expression levels of KLF2 and p21 are elevated, resulting in the impairment of cell proliferation.