

## **Cyclin D1 binding protein 1 responds to DNA damage through the ATM–CHK2 pathway**

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## Abstract

**Background:** Since CCNDBP1 is a cyclin D-binding dominantly-negative helix–loop–helix protein that is expressed in various tissues, it is considered a tumor suppressor, as it negatively regulates TGF- $\beta$  signal-induced cell migration depending on the surrounding condition. When expressed in tumor cells, CCNDBP1 can contribute to the viability of cancer cells by rescuing them from chemotherapy-induced DNA damage. Therefore, this study focused on investigating the function of CCNDBP1, which is directly related to survival from DNA damage and chemoresistance in cancer cells.

**Methods:** Molecular mechanisms of CCNDBP1 related to the recovery from DNA damage were examined *in vitro* and *in vivo*, using hepatocellular carcinoma (HCC) cells and tissues obtained from *Ccndbp1* knockout mice. X-ray irradiation was then used to induce DNA damage. Subsequently, gene and protein expression changes associated with CCNDBP1's upregulation, downregulation, and irradiation were assessed.

**Results:** As observed, CCNDBP1's overexpression in HCC cells stimulated cell growth and showed resistance to X-ray irradiation. Gene expression analyses of CCNDBP1-overexpressed cells and *Ccndbp1* knockout mice revealed that *Ccndbp1* inhibitory-dependently controlled the expression of *Ezh2*. However, irradiation *Ccndbp1*-dependently decreased *Ezh2*'s expression. Further analyses of DNA damage-related protein expressions *in vitro* and *in vivo* also showed that *Ccndbp1* activated the Atm–Chk2 pathway through the inhibition of *Ezh2* expression, thereby accounting the resistance to DNA damage.

**Conclusions:** Our study, therefore, demonstrated that by inhibiting EZH2, CCNDBP1 contributed to the activation of the ATM–CHK2 pathway to alleviate DNA damage, leading to chemoresistance.

**Keywords:** Ccndbp1, Atm, Ezh2, Chk2, DNA damage, hepatocellular carcinoma

**List of abbreviations**

Ccndbp1, Cyclin D1 binding protein 1

Atm, ataxia telangiectasia mutated

Chk2, checkpoint kinase 2

HCC, hepatocellular carcinoma

GAPDH, glyceraldehyde 3 phosphate dehydrogenase

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PCR, polymerase chain reaction

Ezh2, enhancer of zeste homolog 2

CDDP, Cisplatin

## Introduction

Cyclin D1 binding protein 1 (CCNDBP1, also known as GCIP and HHM) is a cyclin D-binding dominant-negative helix–loop–helix protein with no DNA binding region <sup>(1,2)</sup>. It is expressed in various tissues, including the thymus, spleen, liver, small intestine, colon, brain, muscle, heart, kidney, lung, and peripheral leukocytes <sup>(3, 4)</sup>. Furthermore, it correlates with various proteins, including cyclin D1 <sup>(5)</sup>, SYF2 <sup>(6-8)</sup>, E12 <sup>(4)</sup>, CT847 <sup>(9)</sup>, Jab1 <sup>(10)</sup>, Sirt6 <sup>(5)</sup>, MyoD <sup>(11)</sup>, and Olig1 <sup>(12)</sup>. The molecular mechanisms of the interaction of CCNDBP1 with these proteins involve G1/S cell cycle phase progression in hepatocytes <sup>(13)</sup>; tumor suppression in liver cancer <sup>(10, 14-16)</sup>, breast cancer <sup>(15, 17-19)</sup>, gastric cancer (GC) <sup>(19)</sup>, lung cancer <sup>(20)</sup>, prostate cancer <sup>(19)</sup>, and colon cancer <sup>(5)</sup>; regulation of TGF- $\beta$  target genes, including the Olig1-Smad synexpression group <sup>(12)</sup>; and regulation of skeletal myogenesis interacting with MyoD <sup>(11)</sup>. The significant downregulation of CCNDBP1 in nonimmortalized human cell lines has been recently reported to result in chromosomal imbalance <sup>(21)</sup>. In addition, Takami et al reported that CCNDBP1 expression in hepatocellular carcinoma (HCC) was associated with pathologic differentiation by demonstrating its expression upon the initiation of hepatocarcinogenesis and a more positive staining in well-differentiated HCC than in poorly differentiated HCC <sup>(10)</sup>. These results indicated the possibility that CCNDBP1 is expressed upon cell proliferation in the early stage of malignant transformation <sup>(22)</sup> and contribute to the maintenance of chromosomal stability as a tumor suppressor of malignant cell growth; suppression of its function may further enhance the malignant potential.

Furthermore, CCNDBP1 methylation in cancer cells was reported to be related with the chemosensitivity of colorectal cancer to 5-fluorouracil <sup>(23)</sup>, suggesting that CCNDBP1 expression in cancer cells might be related with tumor viability upon the initiation of antitumor agents. These results were supported by evidence that its interactor SYF2 induced apoptosis in neuronal <sup>(6)</sup> and retinal ganglion cells <sup>(8)</sup>; however, upregulation of SYF2 in esophageal cancer cells contributed to the chemoresistance and caused DNA damage in the cells. These results indicated that when expressed in tumor cells, CCNDBP1 may contribute to the viability of cancer cells by rescuing them from DNA damage <sup>(7)</sup>.

The molecular function of CCNDBP1 as a tumor suppressor has been reported to be through negative regulation of TGF- $\beta$  signaling-induced cell migration depend on its mode condition <sup>(24)</sup>. The direct interaction of MEK2 had been recently reported to phosphorylate CCNDBP1 at its Ser313 and Ser356 residues, thereby, promoting its turnover by ubiquitin-mediated proteasomal degradation, which led to cancer cell proliferation, migration, and invasion <sup>(25)</sup>. However, the function of CCNDBP1 that is directly related with the survival from DNA damage and chemoresistance in cancer cells has not been investigated. Therefore, this study aimed to investigate the molecular mechanisms of CCNDBP1, focusing on the recovery from DNA damage of liver cancer cells and in *Cndbp1* knockout mice.

## **Methods**

### *Plasmids*

The CCNDBP1-expressing plasmid, which contained a chicken  $\beta$ -actin promoter, cytomegalovirus enhancer, and an IRES, was generated through a multistep and ligation-based cloning procedure using the full-length complementary DNA of the human homologue of Ccndbp1. The plasmid was purified using a Plasmid Mega Kit (Qiagen, Hilde, Germany). The purity of the plasmid preparation was checked by absorbance at 260 and 280 nm and 1% agarose gel electrophoresis.

### *Cells*

Human hepatoma HLE and HepG2 cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank (National Institutes of Biomedical Innovation, Health and Nutrition, Ibaraki, Osaka) and were cultured in Dulbecco's Modified Eagle Medium (gibco, 11885-084, Thermo Fisher Scientific, Waltham, MA, USA), which contained 10% fetal bovine serum and 100 IU/mL of penicillin and streptomycin. Cells were placed in a 5% CO<sub>2</sub>-humidified incubator at 37°C. Either mock or CCNDBP1 cloned vectors were transfected into the HLE and HepG2 cells using FuGENE HD Transfection Reagent (Promega, Madison, WI), followed by G418 sulfate selection. From each of the four cell lines, three independent clones were isolated and used for assay.

The expressions of CCNDBP1 and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) in the cell lines were confirmed by reverse transcription polymerase chain reaction (PCR). For this analysis, the RNA Easy Mini kit (Qiagen, Valencia, CA) was used to prepare the total RNA from cells, according to the protocol recommended by the manufacturer. Using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA),

complementary DNA was synthesized from 1–5 mg of total RNA using an oligo (dT) primer; 1–2 aliquots of complementary DNA products PCR were used with the following primers:

- CCNDBP1 (forward): GCTGTGGAAGAATGTGACC
- CCNDBP1 (reverse): AGAGCCAAATCATCCACA
- GAPDH (forward): AGGTCGGTGTGAACGGATTTG
- GAPDH (reverse): TGTAGACCATGTAGTTGAGGTCA

Multiplex PCR was carried out similarly, and GAPDH primers were always included as the reference. The PCR products were separated by electrophoresis in 1% agarose gel and stained with ethidium bromide for visualization. The PCR protocol was as follows: 10 minutes at 95°C, followed by 35 cycles (30 seconds at 95°C, 30 seconds at 55°C and 1 minute at 72°C) and a 7-minute extension at 72°C.

### *Animals*

All animal experiments were approved by and conducted in full compliance with the regulations of the Institutional Animal Care and Use Committee at Niigata University, Niigata, Japan. Male BALB/c mice (n = 50, 8 weeks of age, 25–30 g) were purchased from CLEA Japan, Inc. (Tokyo, Japan). *Ccndbp1* knockout mice (n = 50, 8 weeks of age, and 25–30 g) were kindly provided by Yamaguchi University. The mice were housed in specific pathogen-free facilities under standard conditions at a temperature of 20°C–23°C and humidity of 45%–55% and were fed a standard diet.

### *Irradiation*



The cells and mice were irradiated with X-ray at 160.0 kV, 5.0 mA, 400 mm, 0.8 Gy/min for three minutes and at 160.0 kV, 5.0 mA, 400 mm, 1 Gy/min for 10 minutes using MBR-1605RA (Hitachi Power Solutions Co., Ltd., Hitachi, Ibaraki, Japan). The tissue samples from the cells were collected at appropriate time points, and animals were sacrificed at 12 hours after irradiation for tissue collection.

#### *Cell growth assay*

Normal-HLE, Normal-HepG2, CCNDBP1-HLE, and CCNDBP1-HepG2 cells were plated in 96-well tissue culture dishes at  $1 \times 10^4$  cells per well in 100  $\mu$ L of the aforementioned medium and were treated with and without X-ray (0.8 Gy/min, 3 minutes) or 20  $\mu$ M of cisplatin (CDDP). The reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cells at the indicated times after treatment, followed by counting with Premix WST-1 Cell Proliferation Assay System (Takara Inc., Kyoto, Japan), according to the instructions supplied.

#### *Western blotting*

The cells and tissues samples from the mice were collected for Western blotting at the appropriate time points; suspended in phosphate-buffered saline; and mixed with an equal volume of lysis buffer, 50-mM Tris-HCl (pH 8.0), 1.5% TritonX-100, 150-mM NaCl, 1-mM CaCl<sub>2</sub>, 1-mM MgCl<sub>2</sub>, and protease inhibitor; and homogenized with an electric homogenizer. The extract was subjected to gel electrophoresis using 8%–16% MINI-PROTEAN TGX Stain Free Gels (No. 456-8105; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blotted onto PVDF membranes (Transfer Pack, No. 1704156; Bio-Rad Laboratories, Inc., Hercules, CA, USA) by Trans-Blot Turbo (No. 1704150J1; Bio-Rad

Laboratories, Inc., Hercules, CA, USA) using either 10× Tris/ Glycine/ SDS buffer (No. 161-0732; Bio-Rad Laboratories, Inc., Hercules, CA, USA) or 10× Tris/ CAPS Buffer (No. 161-0778; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were then blocked by EzBlockChemi (AE-1475; ATTO Corporation Taito-ku, Tokyo, Japan). The following antibodies were used to detect the proteins:

anti-CCNDBP1 (ab220275, Abcam, Cambridge, UK) at 1:2000 dilution; anti-EZH2 antibody (ab186006, Abcam) at 1:1000 dilution; anti-ATM antibody (ab78, Abcam) at 1:2000 dilution; anti-phospho S1981 ATM antibody (ab36810, Abcam) at 1:1000 dilution, anti-Chk2 antibody (No. 2662, Cell Signaling Technology, Inc., Danvers, MA, USA) at 1:1000 dilution; anti-phospho Chk2 (Thr68) antibody (No. 2661, Cell Signaling Technology, Inc.) at 1:1000 dilution; anti-Chk1 antibody (ab47574, Abcam) at 1:1000 dilution; anti-phospho Chk1 (S345) antibody (ab58567, Abcam) at 1:1000 dilution; anti-ATR antibody (ab2905, Abcam) at 1:10000 dilution; anti-phospho ATR (T1989) antibody (ab227851, Abcam) at 1:3000 dilution; anti-Cdc25C antibody (ab226958, Abcam) at 1:1000 dilution; anti-CyclinD1 antibody (ab134175, Abcam) at 1:10000 dilution; anti-p53 antibody (ab131442, Abcam) at 1:1000 dilution; anti-p21 antibody (ab188224, Abcam) at 1:1000 dilution; antimouse immunoglobulin G horseradish peroxidase (NA931-1ML; GE Healthcare Life Sciences, Pittsburgh); and antirabbit immunoglobulin G horseradish peroxidase (NA934-1ML; GE Healthcare Life Sciences, Pittsburgh). Protein bands were visualized using EzWestLumi plus (WSE-7120L; ATTO Corporation, Taito-ku, Tokyo, Japan) and LuminoGraphI (WSE-6100; ATTO Corporation, Taito-ku, Tokyo, Japan).

### *Histological analysis*

Tissues of thymus, spleen, and liver were collected at appropriate time points after the procedures and fixed in 10% formalin before embedding in paraffin for hematoxylin and eosin and immunohistochemical staining. Immunohistochemical staining was conducted with an anti-ATM antibody (ab78, Abcam, Cambridge, UK) at 1:1000 dilution and an anti-phospho S1981 ATM antibody (ab36810, Abcam, Cambridge, UK) at 1:250 dilution using the Vectastain Elite ABC mouse IgG kit (PK-6102, Vector Laboratories, Burlingame, CA, USA); an anti-CHK2 antibody (No. 2662, Cell Signaling Technology, Inc., Danvers, MA, USA) at 1:200 dilution; an anti-phospho Thr68 CHK2 antibody (No. 2661, Cell Signaling Technology, Inc., Danvers, MA, USA) at 1:100 dilution; an anti-ATR antibody (ab222820, Abcam, Cambridge, UK) at 1:100 dilution; an anti-phospho T1989 ATR antibody (ab223258, Abcam, Cambridge, UK) at 1:100 dilution; an anti-CHK1 antibody (ab47574, Abcam, Cambridge, UK) at 1:250 dilution; an anti-phospho S345 Chk1 antibody (ab47318, Abcam, Cambridge, UK) at 1:100 dilution; and an anti-KMT6/EZH2 antibody (ab191080, Abcam, Cambridge, UK) at 1:250 dilution using the Vectastain Elite ABC rabbit IgG kit (PK-6101, Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine chromogen tablets (Muto Pure Chemicals, Tokyo, Japan). Thereafter, images from each tissue section were captured randomly, and quantitative analysis was performed with ImageJ software (version 1.6.0\_20; National Institutes of Health, Bethesda, MD, USA) with an RGB-based protocol, as reported previously <sup>(26)</sup>.

#### *Microarray and bioinformatic analyses*

The SurePrint G3 Human Gene Expression (v2) Microarray Kit (Agilent Technologies, Inc., Santa Clara, CA) and SurePrint G3 Mouse Gene Expression (v2) Microarray Kit

(Agilent Technologies, Inc., Santa Clara, CA) GeneSpring GX, version 14.5.1 (Agilent Technologies, Inc.), were used to compare the gene expression levels in mock-transfected cells and in hepatocytes from *CCNDBP1*-transfected HLE and *Ccndbp1* knockout mice and wild mice. There were 6,597 of the 26,083 genes in human and 7,530 of the 27,122 genes in mice that were clustered hierarchically according to the level of gene expression, with more than two-fold differences in expression. The gene ontology terms were selected on the basis of Fisher's exact test, followed by the Benjamini–Yekutieli correction method. The expressions in the genes were compared among the groups; genes with more than fivefold differences in expression were shown in the heat map.

#### *Statistical analyses*

The obtained data were analyzed with either one-way or two-way factor repeated measures analysis of variance, followed by Bonferroni's multiple comparison test or Student's t-test using GraphPad Prism7 software (version 8.4.3; GraphPad, San Diego, CA, USA). A p value of  $\leq 0.05$  was considered to indicate statistical significance.

## **Results**

#### *Effect of CCNDBP1 expression in HCC cells on X-ray irradiation*

To examine the molecular function of CCNDBP1 on liver cancer cells, we produced *CCNDBP1*-overexpressing cell lines by transfecting plasmid DNA-expressing human *CCNDBP1* into cancer cell lines of HLE and HepG2. **Fig. 1a** shows the *CCNDBP1* gene and CCNDBP1 protein expression in the transfected HLE and HepG2 cell lines. A slight endogenous level of CCNDBP1 expression can be seen in the cells, whereas significant overexpression of CCNDBP1 was achieved by the transfection. Compared with the

mock-transfected cells, the *CCNDBP1*-overexpressing cells showed increased growth ratio in the culture condition containing 10% FCS, as determined by the MTT assay (**Figs. 1b and 1c**). After irradiation, the *CCNDBP1*-expressing cell lines showed significantly higher growth rate than did the mock-transfected cells, in which cell growth was inhibited by X-ray (**Figs. 1d and 1e**); this difference was not seen when treated with 20  $\mu$ M of CDDP (**Figs. 1f and 1g**). These results indicated that *CCNDBP1* overexpression in HCC stimulated cell growth under 10% FCS and resistance to X-ray.

#### *Gene expression analyses in CCNDBP1 overexpressed cells and Ccndbp1 knockout mice*

To determine the molecular mechanism of *CCNDBP1*, the gene expression in mock-transfected and *CCNDBP1*-transfected HLE (**Fig. 2**) and *Ccndbp1* knockout mice and wild type mice (**Fig. 3**) were compared using DNA microarray analyses. The analysis of gene ontology terms after the hierarchical clustering of genes showed gene differences in terms of molecular function, catalytic activity, nucleotide, DNA, binding, negative regulation if transcribed from RNA polymerase II promoter, RNA polymerase II regulatory region sequence-specific DNA binding, and nuclear chromatin in both cell and mice (**Figs. 2c, 2d, 3c, and 3d**). Among the genes involved in these terms and those that showed more than two-fold difference in all comparisons of cells and mice with X-ray irradiation, we focused on enhancer of zeste homolog 2 (*EZH2*), which showed expression differences both *in vitro* and *in vivo* (**Figs. 2e and 3e**). *EZH2* is an epigenetic silencer of the polycomb repressor complex 2 (<sup>(27, 28)</sup>); a negative regulator of DNA damage-related proteins of ATM (<sup>(29)</sup>) involved in the X-ray-induced DNA damage; and has been associated with the prognosis of several cancer entities (<sup>(27-29)</sup>). The *EZH2* gene expression was lower in the *CCNDBP1*-overexpressed cell lines than in the mock-

transfected cell lines and was lower in the irradiated *CCNDBP1*-overexpressed cell lines than in the nonirradiated *CCNDBP1*-overexpressed cells. These results implied that *CCNDBP1* expression and irradiation inhibited EZH2 expression. These findings were confirmed in the *in vivo* gene expression analysis. **Fig. 3e** shows higher *Ezh2* in the *Ccndbp1* knockout mice under normal housing and after X-ray irradiation than in the wild type mice. Irradiation did not decrease the *Ezh2* expression in the *Ccndbp1* knockout mice (**Fig. 3e**). Overall, these results indicated that *Ccndbp1* controlled the expression of *Ezh2* in an inhibitory manner and that irradiation decreased *Ezh2* expression in a *Ccndbp1*-dependent manner.

#### *CCNDBP1 expression and DNA damage-related proteins*

Based on the results obtained from the cell growth assay and microarray analyses of the cell lines, we examined the DNA damage related proteins ATM and CHK2, which are involved in X-ray-induced DNA damage. **Fig. 4a** shows the representative results of the Western blotting of the HLE cell lines and **Fig. 4b** summarizes the results of protein expression in the HLE and HepG2 cell lines. The expression of CCNDBP1 was activated by irradiation in a time-dependent manner, and it inhibited the expression of EZH2 protein, which is a negative regulator of ATM (<sup>(29)</sup>). The X-ray irradiation induced the phosphorylation of ATM protein by inhibiting EZH2 expression after 24 hours (**Fig. 4**). In addition, overexpression of CCNDBP1 showed continuous inhibition of EZH2, followed by sustained phosphorylation of ATM for at least for 72 hours after irradiation. Following the activation of ATM, CHK2 was continuously activated by its phosphorylation in the CCNDBP1-overexpressed cells. In addition, the higher increase in p53 and p21, inhibition of cdc25C, and gradual increase of cyclin D1 expression in the

CCNDBP1-expressed cells than in the mock-transfected cells indicated cell cycle arrest upon irradiation and recovery of the tumor growth (**Fig. 4**). Based on these results obtained in the *in vitro* assay, we next examined the molecular mechanisms of *Ccndbp1* *in vivo* using *Ccndbp1* knockout mice.

#### *Effect of Ccndbp1 in vivo*

The effect of *Ccndbp1* gene in mice was examined using the tissues collected from wild and *Ccndbp1* knockout mice before and after X-ray irradiation. **Fig. 5a** shows the representative results of the Western blotting of thymus tissue. After irradiation, the expression levels of the Atm and Chk2 proteins were not different in both mice, but there was suppressed phosphorylation of the Atm and Chk2 proteins in the thymus cells, which are mostly lymphocytes, in the knockout mice, compared with that in the wild type mice (**Fig. 5a**). In addition, the expression of Ezh2 was higher in the knockout mice than in the wild type mice (**Fig. 5a**).

These results were confirmed on immunohistochemical staining of thymic and hepatic tissues. **Fig. 5b** summarizes the results of the analysis of the protein expressions in the thymus and liver based on immunohistochemical staining (representative images are shown in **Supplementary Figure 1**). The hepatocytes showed similar findings, with more clear differences and maintained expression of Ezh2 when irradiated (**Fig. 5b**). The thymic tissues showed a relatively mild difference in Ezh2 expression. These results suggested that *Ccndbp1* activated the Atm–Chk2 pathway through inhibition of *Ezh2* expression. Therefore, knockout of *Ccndbp1* caused Ezh2 activation, which led to

insufficiency of the Atm–Chk2 pathway and suppression of Chk2 phosphorylation upon irradiation in the knockout mice.

## **Discussion**

In this study, we aimed to assess the molecular mechanism of CCNDBP1 and focused on the recovery from DNA damage. Indeed, our results demonstrated that *CCNDBP1* overexpression in HCC contributed to higher cell growth and resistance to X-ray-induced DNA damage and that this mechanism was dependent on the activation of the ATM–CHK2 pathway in cancer cells. These results were supported by the fact that abnormal activation of the ATM–CHK2 pathway had been associated with resistance to chemoradiotherapy and poor prognosis in nasal extranodal NK/T cell lymphoma <sup>(30)</sup>. Moreover, our results demonstrated that CCNDBP1-overexpressed cell lines have gained resistance to X-ray irradiation but not to cisplatin. This difference was partly explained by Ziegler et al, who reported that ionizing radiation caused the formation of numerous DSB and triggered a substantial activation of ATM–CHK2 signaling; however, the DNA crosslinking agents of cisplatin triggered a substantial blockage of transcription, which was not reflected by an appreciable number of DSB and did not increase the expression of the ATM–CHK2 pathway <sup>(31)</sup>. In addition, we demonstrated that the suppressive effect of CCNDBP1 on EZH2, which is a histone H3K27 methyltransferase that was reported to be a negative regulator of ATM <sup>(29)</sup>, contributed to the activation of the ATM–CHK2 pathway. This result was supported by a previous report showing that decreased EZH2 expression increased ATM phosphorylation and induced resistance to anticancer treatment <sup>(28)</sup>. These results were further supported by our analyses, which showed weaker activation of the ATM–CHK2 pathway after X-ray irradiation on the liver,



spleen, and thymus in *Ccndbp1* knockout mice. Recently, it has been reported that the expression of *CCNDBP1* gene could be regulated by DNA methylation <sup>(32)</sup>. This mechanism might be related with the modification of *CCNDBP1* expression in normal cells to gain malignant potential in the early stage of malignant transformation <sup>(10)</sup> and in cancer cells to be chemoresistant <sup>(23)</sup>.

The limitations of our study included the lack of molecular-based analysis of the direct link between *CCNDBP1* and *EZH2* and of human HCC samples to enable application of the results to radiotherapy and chemotherapy resistance. In one report on progressive prostate cancer despite standard treatments, high response to chemotherapy was seen when the DNA repair system was inhibited by Olaparib, which is a poly adenosine diphosphate–ribose polymerase <sup>(33)</sup>. Similarly, *CCNDBP1* can be a therapeutic target in HCC with poor response to conventional therapy. Therefore, further basic research that will focus on the molecular mechanisms of *CCNDBP1* and *EZH2*, carcinogenic assay using *Ccndbp1* knockout mice with DNA damaging agents, and analyses of human samples will further bring important information.

## **Conclusions**

According to our results, *CCNDBP1* contributed to the activation of the ATM–CHK2 pathway by inhibiting *EZH2* to ameliorate DNA damage. Therefore, management of *CCNDBP1* expression in the tumor may recover the sensitivity to anticancer therapy.

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## **Declarations**

### *Ethics approval and consent to participate*

This basic and observational study protocol with the clinical samples was approved by the ethics committee and institutional review board of Niigata University School of Medicine (Nos. 751-716 and G2018-0023, respectively). All patients provided written informed consent for collection of the samples, and the study was conducted in accordance with the ethical guidance of the 1975 Declaration of Helsinki. All authors had access to the study data and have reviewed and approved the final manuscript.

### *Consent for publication*

All patients provided written informed consent to publish the results obtained from the samples and images.

### *Competing interests*

The authors declare that they have no competing interests.

### *Authors' contributions*

Yusuke Niwa, Kenya Kamimura, Kohei Ogawa, and Shuji Terai conceived and supervised the study. Yusuke Niwa, Kenya Kamimura, and Kohei Ogawa designed the experiments. Yusuke Niwa, Kohei Ogawa, Chiyumi Oda, Ryoko Horigome, Masato Ohtsuka, Hiromi Miura, Naoki Yamamoto, Taro Takami, Shujirou Okuda, Koichi Fujisawa, Masayoshi Ko, Takashi Owaki, Atsushi Kimura, Osamu Shibata, Shinichi Morita, Norihiro Sakai, Hiroyuki Abe, Takeshi Yokoo, Akira Sakamaki, and Isao Sakaida collected the samples and performed the experiments. Yusuke Niwa, Kohei Ogawa, Kenya Kamimura, and Shuji Terai analyzed the data. Kenya Kamimura and Shuji Terai wrote the first draft of the manuscript. All authors read and approved the final manuscript.

### **Figure legends**

#### **Fig. 1. Effect of CCNDBP1 expression on HCC cell growth**

(a) The development of CCNDBP1 overexpressing cell lines. Representative real-time polymerase chain reaction of CCNDBP1 and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) and Western blotting of CCNDBP1 and  $\beta$ -actin are shown. The cell growth of hepatocellular carcinoma (HCC) cell lines and the permanent clones overexpressing CCNDBP1 determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (b, c) Growth of cell lines in normal culture condition with 10% fetal bovine serum. (d, e) Cell growth after irradiation with 0.8 Gy of X-ray. (f, g) Cell

growth with 20  $\mu\text{M}$  of CDDP. The values represent mean  $\pm$  standard deviation ( $n = 3$  for each group at the time points). \*  $p < 0.05$  and no statistical significance (NS) on two-way analysis of variance followed by Bonferroni's multiple comparison test

**Fig. 2. Microarray analyses *in vitro***

(a) Microarray analyses were performed to compare the levels of gene expression in mock-transfected HLE and CCNDBP1-transfected HLE (a) and X-ray irradiated mock-transfected HLE and X-ray irradiated CCNDBP1-transfected HLE (b). A total of 6,597 genes with more than two-fold differences in expression were clustered hierarchically according to the level of gene expression. The color represents the expression level of the gene; green represents low degree of change in expression, whereas red represents high degree of change of expression. The color key and histogram are shown. (c) The gene ontology terms related with the genes that have more than two-fold differences between the mock-transfected HLE and CCNDBP1-transfected HLE are shown. (d) The number of genes included in each ontology are shown. (e) The expression level of genes showed more than two-fold changes in all comparisons between CCNDBP1-HLE and mock-transfected HLE, CCNDBP1-HepG2 and mock-transfected HepG2, Rx CCNDBP1-HLE and Rx mock-transfected HLE, Rx CCNDBP1-HepG2 and Rx mock-transfected HepG2, CCNDBP1-HLE and Rx CCNDBP1-HLE, and CCNDBP1-HepG2 and Rx CCNDBP1-HepG2.

Rx, X-ray irradiation

**Fig. 3. Microarray analyses *in vivo***

(a) Microarray analyses were performed to compare the levels of gene expression in WT and *Ccndbp1* KO mice (a) and X-ray irradiated WT and *Ccndbp1* KO mice (b). A total of 7,530 genes with more than two-fold differences in the expression were clustered hierarchically according to level of gene expression. The color represents the expression level of the gene; green represents low degree of change in expression, whereas red represents high degree of change of expression. The color key and histogram are shown.

(c) The gene ontology terms related with the genes that have more than two-fold differences between the WT and *Ccndbp1* KO mice are shown. (d) The number of genes included in each ontology are shown. (e) The expression level of genes showed more than two-fold changes in all comparisons between *Ccndbp1* KO and WT, Rx *Ccndbp1* KO and Rx WT, and *Ccndbp1* KO and Rx *Ccndbp1* KO

WT, wild type; KO, knockout; Rx, X-ray irradiation

**Fig. 4. Effect of CCNDBP1 on the changes in various proteins in HCC cells after X-ray irradiation**

(a) Western blotting of proteins related with the ATM–CHK2 pathway in the cells harvested at the indicated times after X-ray irradiation (immediately and at 24, 48, and 72 hours). (b) The relative expression ratios of the proteins are shown.

The values represent mean  $\pm$  standard deviations (n = 5), \* $p$  < 0.05, \*\* $p$  < 0.01 on one-way analysis of variance followed by Bonferroni's multiple comparison test

CCNDBP1, cyclin d1 binding protein 1; EZH2, enhancer of zeste homolog 2; ATM, ataxia telangiectasia mutated; pATM, phosphorylated ataxia telangiectasia mutated; CHK2, checkpoint kinase 2; pCHK2, phosphorylated checkpoint kinase 2; CDC25C, cell division cycle 25 homolog C

**Fig. 5. Effect of Ccndbp1 on the changes in various proteins in X-ray irradiated mice**

(a) Western blotting of proteins related with the ATM–CHK2 pathway in mice thymic tissue harvested 12 hours after X-ray irradiation. (b) Quantitative analyses of the immunohistochemical analyses of proteins in the thymus and liver of wild (WT) and Ccndbp1 knockout (KO) mice with or without X-ray irradiation. Representative images and a quantitative analysis of positively stained area are shown. The scale bar represents 100  $\mu\text{m}$ . The values represent mean  $\pm$  standard deviations ( $n = 5\text{--}6$ ),  $*p < 0.05$ ,  $***p < 0.001$  on one-way analysis of variance followed by Bonferroni's multiple comparison test

**Supplementary Figure 1**

Representative images of the immunohistochemical staining of the ATM–CHK2 pathway-related proteins in the thymus and liver of wild (WT) and Ccndbp1 knockout (KO) mice with or without irradiation of X-ray

## References

1. Seto A, Ikushima H, Suzuki T, Sato Y, Fukai S, Yuki K, Miyazawa K, Miyazono K, Ishitani R, Nureki O. Crystallization and preliminary X-ray diffraction analysis of GCIP/HHM transcriptional regulator. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **65**: 21-24, 2009.
2. Ishii R, Isogaya K, Seto A, Koinuma D, Watanabe Y, Arisaka F, Yaguchi S, Ikushima H, Dohmae N, Miyazono K, Miyazawa K, Ishitani R, Nureki O. Structure of a dominant-negative helix-loop-helix transcriptional regulator suggests mechanisms of autoinhibition. *EMBO J* **31**: 2541-2552, 2012.
3. Xia C, Bao Z, Tabassam F, Ma W, Qiu M, Hua S, Liu M. GCIP, a novel human *grap2* and cyclin D interacting protein, regulates E2F-mediated transcriptional activity. *J Biol Chem* **275**: 20942-20948, 2000.
4. Terai S, Aoki H, Ashida K, Thorgeirsson SS. Human homologue of maid: A dominant inhibitory helix-loop-helix protein associated with liver-specific gene expression. *Hepatology* **32**: 357-366, 2000.
5. Ma W, Stafford LJ, Li D, Luo J, Li X, Ning G, Liu M. GCIP/CCNDBP1, a helix-loop-helix protein, suppresses tumorigenesis. *J Cell Biochem* **100**: 1376-1386, 2007.
6. Xu W, Cao M, Zheng H, Tan X, Li L, Cui G, Xu J, Cao J, Ke K, Wu Q. Upregulation of SYF2 is associated with neuronal apoptosis caused by reactive astrogliosis to neuroinflammation. *J Neurosci Res* **92**: 318-328, 2014.
7. Zhu J, Ji L, Zhang J, Yang L, Guan C, Wang Y, Zhu J, Liang L, Ni R. Upregulation of SYF2 in esophageal squamous cell carcinoma promotes tumor cell proliferation and predicts poor prognosis. *Tumour Biol* **35**: 10275-10285, 2014.

8. Sang A, Yang X, Chen H, Qin B, Zhu M, Dai M, Zhu R, Liu X. Upregulation of SYF2 relates to retinal ganglion cell apoptosis and retinal glia cell proliferation after light-induced retinal damage. *J Mol Neurosci* **56**:480-490, 2015.
9. Chellas-Géry B, Linton CN, Fields KA. Human GCIP interacts with CT847, a novel *Chlamydia trachomatis* type III secretion substrate, and is degraded in a tissue-culture infection model. *Cell Microbiol* **9**: 2417-2430, 2007.
10. Takami T, Terai S, Yokoyama Y, Tanimoto H, Tajima K, Uchida K, Yamasaki T, Sakaida I, Nishina H, Thorgeirsson SS, Okita K. Human homologue of maid is a useful marker protein in hepatocarcinogenesis. *Gastroenterology* **128**: 1369-1380, 2005.
11. Huang Y, Chen B, Ye M, Liang P, Zhangfang Y, Huang J, Liu M, Songyang Z, Ma W. Ccndbp1 is a new positive regulator of skeletal myogenesis. *J Cell Sci.* **129**: 2767-2777, 2016.
12. Ikushima H, Komuro A, Isogaya K, Shinozaki M, Hellman U, Miyazawa K, Miyazono K. An Id-like molecule, HHM, is a synexpression group-restricted regulator of TGF-beta signalling. *EMBO J* **27**: 2955-2965, 2008.
13. Sonnenberg-Riethmacher E, Wustefeld T, Mieke M, Trautwein C, Riethmacher D. Maid (GCIP) is involved in cell cycle control of hepatocytes. *Hepatology* **45**: 404-411, 2007.
14. Ma W, Xia X, Stafford LJ, Yu C, Wang F, LeSage G, Liu M. Expression of GCIP in transgenic mice decreases susceptibility to chemical hepatocarcinogenesis. *Oncogene* **25**: 4207-4216, 2006.
15. Chang TW, Chen CC, Chen KY, Su JH, Chang JH, Chang MC. Ribosomal phosphoprotein P0 interacts with GCIP and overexpression of P0 is associated with

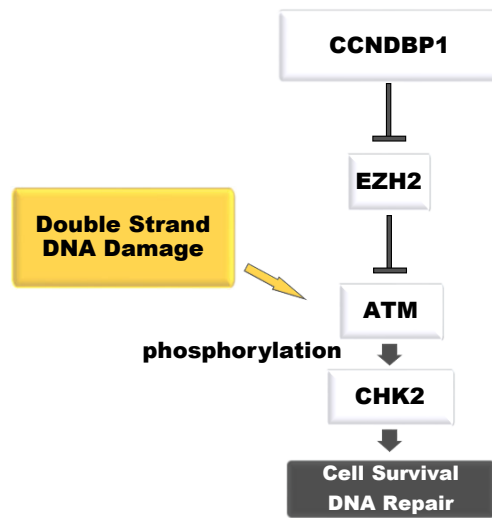


- cellular proliferation in breast and liver carcinoma cells. *Oncogene* **27**: 332-338, 2008.
16. Fujisawa K, Terai S, Matsumoto T, Takami T, Yamamoto N, Nishina H, Furutani-Seiki M, Sakaida I. Evidence for a role of the transcriptional regulator maid in tumorigenesis and aging. *PLoS One* **10**: e0129950, 2015.
  17. Su T, Deguchi A, Yao Y, Luo J, Weinstein IB. Dip1 inhibits growth and gene transcription in MCF-7 breast cancer cells. *J Exp Ther Oncol* **6**: 117-127, 2007.
  18. Chen WC, Su PF, Jin YT, Chang MC, Chang TW. Immunohistochemical expression of GCIP in breast carcinoma: Relationship with tumour grade, disease-free survival, mucinous differentiation and response to chemotherapy. *Histopathology* **53**: 554-560, 2008.
  19. Lee I, Yeom SY, Lee SJ, Kang WK, Park C. A novel senescence-evasion mechanism involving Grap2 and Cyclin D interacting protein inactivation by Ras associated with diabetes in cancer cells under doxorubicin treatment. *Cancer Res* **70**: 4357-4365, 2010.
  20. Chen KY, Chen CC, Tseng YL, Chang YC, Chang MC. GCIP functions as a tumor suppressor in non-small cell lung cancer by suppressing Id1-mediated tumor promotion. *Oncotarget* **5**: 5017-5028, 2014.
  21. Hélias-Rodzewicz Z, Lourenco N, Bakari M, Capron C, Emile JF. CDKN2A depletion causes aneuploidy and enhances cell proliferation in non-immortalized normal human cells. *Cancer Invest* **36**: 338-348, 2018.
  22. Gong H, Gao S, Yu C, Li M, Liu P, Zhang G, Song J, Zheng J. Effect and mechanism of YB-1 knockdown on glioma cell growth, migration, and apoptosis. *Acta Biochim Biophys Sin (Shanghai)* **52**: 168-179, 2020.

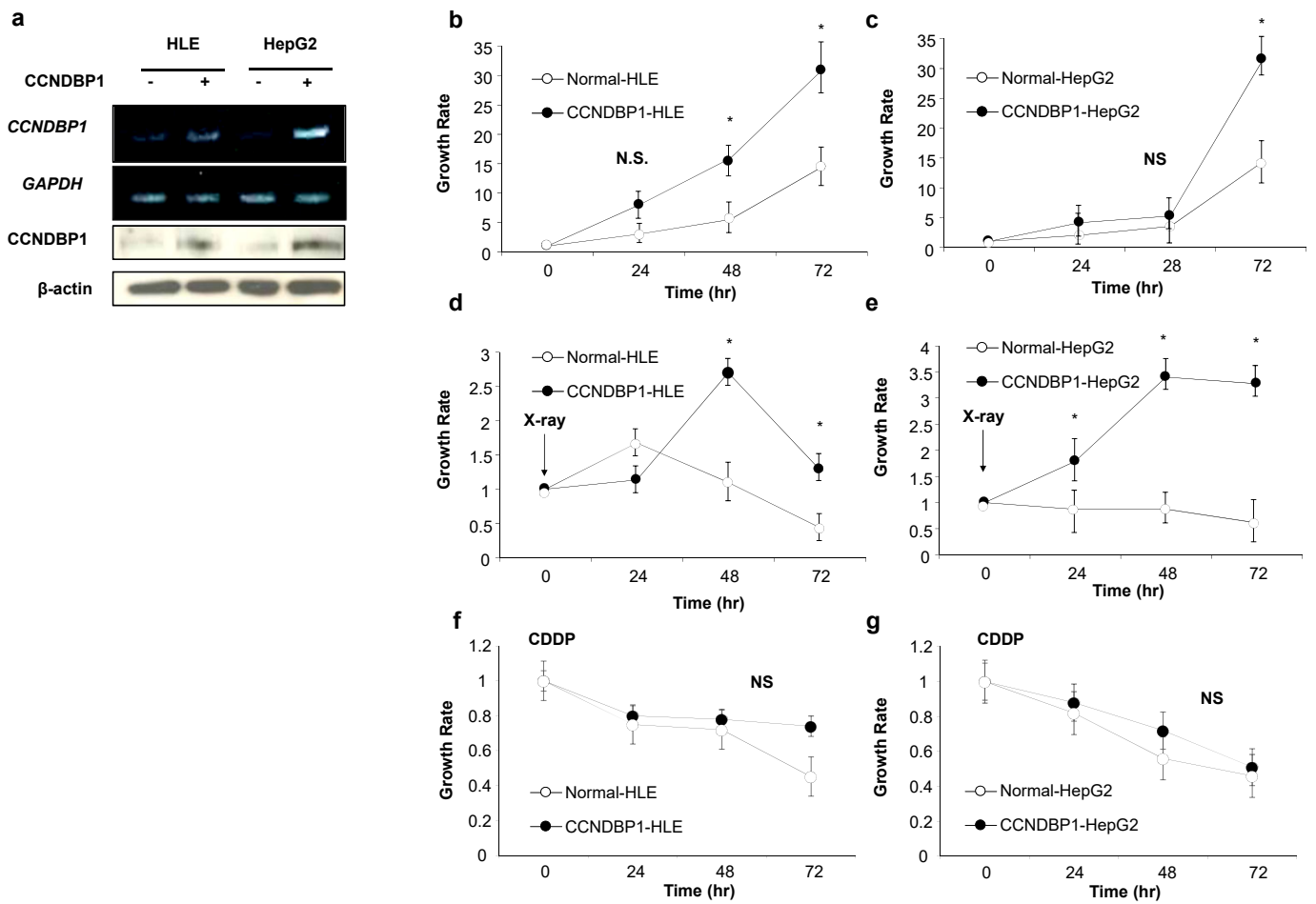
23. Baharudin R, Ab Mutalib NS, Othman SN, Sagap I, Rose IM, Mohd Mokhtar N, Jamal R. Identification of predictive DNA methylation biomarkers for chemotherapy response in colorectal cancer. *Front Pharmacol* **8**: 47, 2017.
24. Motizuki M, Saitoh M, Miyazawa K. Maid is a negative regulator of transforming growth factor-beta-induced cell migration. *J Biochem.* **158**: 435-444.
25. Liang RY, Liu BH, Huang CJ, Lin KT, Ko CC, Huang LL, Hsu B, Wu CY, Chuang SM. MEK2 is a critical modulating mechanism to down-regulate GCIP stability and function in cancer cells. *FASEB J* **34**: 1958-1969, 2020.
26. Vrekoussis T, Chaniotis V, Navrozoglou I, Dousias V, Pavlakis K, Stathopoulos EN, Zoras O. Image analysis of breast cancer immunohistochemistry-stained sections using ImageJ: An RGB-based model. *Anticancer Res* **29**: 4995-4998, 2009.
27. Bremer SCB, Conradi LC, Mechie NC, Amanzada A, Mavropoulou E, Kitz J, Ghadimi M, Ellenrieder V, Ströbel P, Hessmann E, Gaedcke J, Bohnenberger H. Enhancer of zeste homolog 2 in colorectal cancer development and progression. *Digestion* **102**: 227-235, 2021.
28. Naskou J, Beiter Y, van Rensburg R, Honisch E, Rudelius M, Schlenzog M, Gottstein J, Walter L, Braicu EI, Sehouli J, Darb-Esfahani S, Staebler A, Hartkopf AD, Brucker S, Wallwiener D, Beyer I, Niederacher D, Fehm T, Templin MF, Neubauer H. EZH2 loss drives resistance to carboplatin and paclitaxel in serious ovarian cancers expressing ATM. *Mol Cancer Res* **18**: 278-286, 2020.
29. Ahmad F, Patrick S, Sheikh T, Sharma V, Pathak P, Malgulwar PB, Kumar A, Joshi SD, Sarkar C, Sen E. Telomerase reverse transcriptase (TERT)-enhancer of zeste homolog 2 (EZH2) network regulates lipid metabolism and DNA damage responses in glioblastoma. *J Neurochem* **143**: 671-683, 2017.

30. Ye Q, Chen H, Wen Z, Guo W, Huang Y, Mo X. Abnormal expression of p-ATM/CHK2 in nasal extranodal NK/T cell lymphoma, nasal type, is correlated with poor prognosis. *J Clin Pathol*, **74**: 223-227, 2021.
31. Ziegler V, Deußen M, Schumacher L, Roos WP, Fritz G. Anticancer drug and ionizing radiation-induced DNA damage differently influences transcription activity and DDR-related stress responses of an endothelial monolayer. *Biochim Biophys Acta Mol Cell Res* **1867**: 118678, 2020.
32. Yang L, Wu Z, Sun W, Luo P, Chen S, Chen Y, Yan W, Li Y, Wang C. CCNDBP1, a prognostic marker regulated by DNA methylation, inhibits aggressive behavior in dedifferentiated liposarcoma via repressing epithelial mesenchymal transition. *Front Oncol* **11**: 687012, 2021.
33. Mateo J, Carreira S, Sandhu S, Miranda S, Mossop H, Perez-Lopez R, Nava Rodrigues D, Robinson D, Omlin A, Tunariu N, Boysen G, Porta N, Flohr P, Gillman A, Figueiredo I, Paulding C, Seed G, Jain S, Ralph C, Protheroe A, Hussain S, Jones R, Elliott T, McGovern U, Bianchini D, Goodall J, Zafeiriou Z, Williamson CT, Ferraldeschi R, Riisnaes R, Ebbs B, Fowler G, Roda D, Yuan W, Wu YM, Cao X, Brough R, Pemberton H, A'Hern R, Swain A, Kunju LP, Eeles R, Attard G, Lord CJ, Ashworth A, Rubin MA, Knudsen KE, Feng FY, Chinnaiyan AM, Hall E, de Bono JS. DNA-repair defects and olaparib in metastatic prostate cancer. *N Engl J Med* **373**: 1697-1708, 2015.

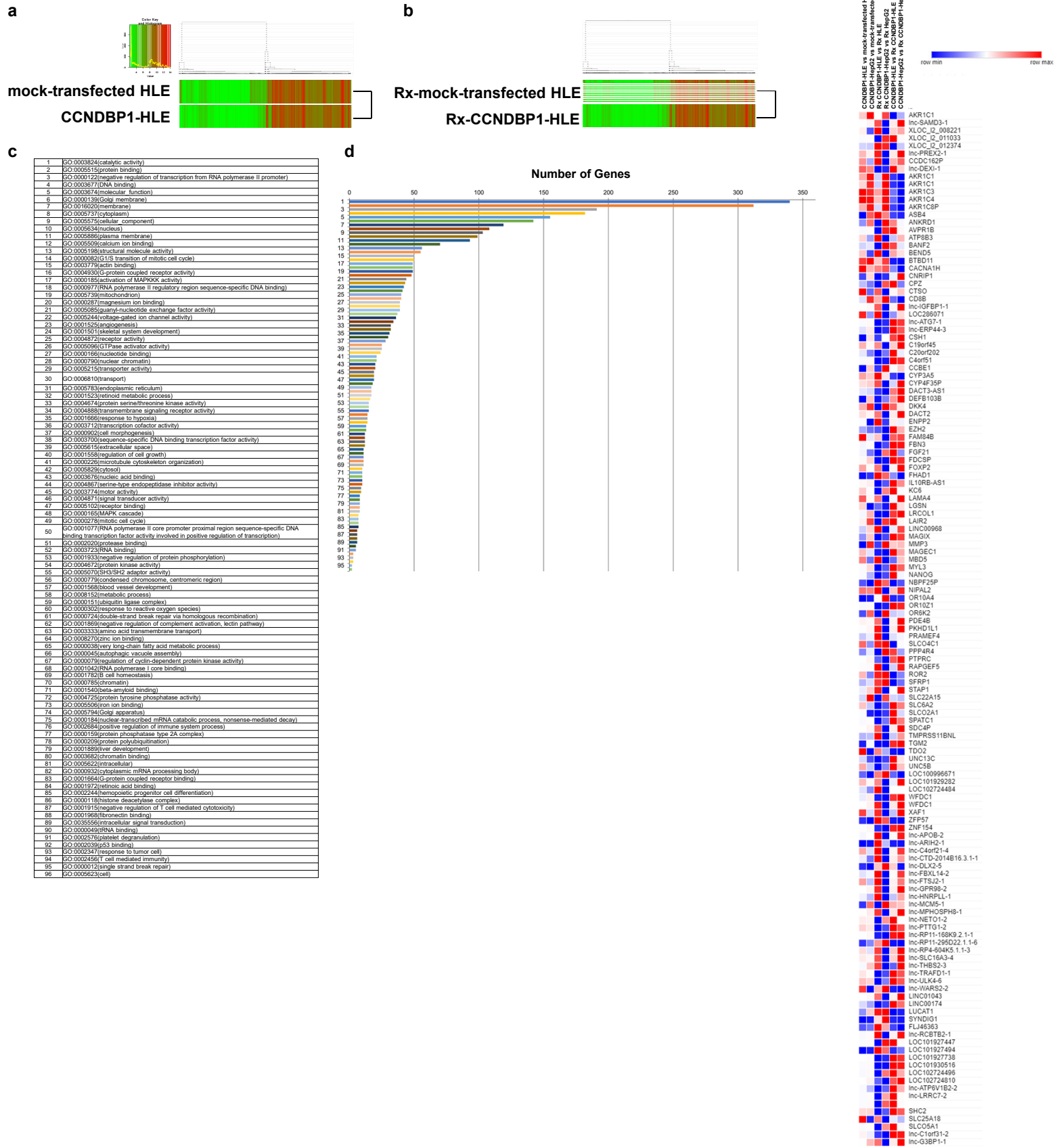
Graphical  
Abstract



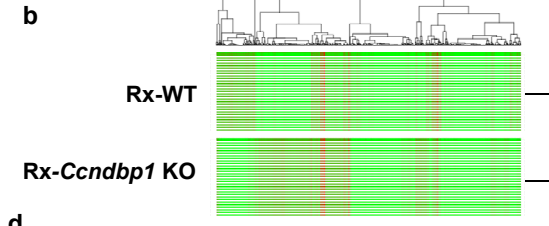
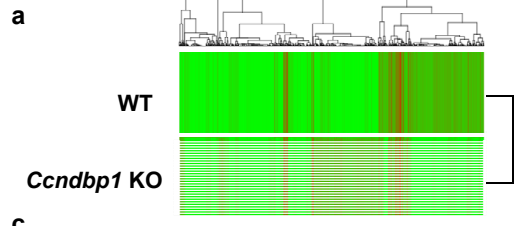
**Figure 1**



**Figure 2**

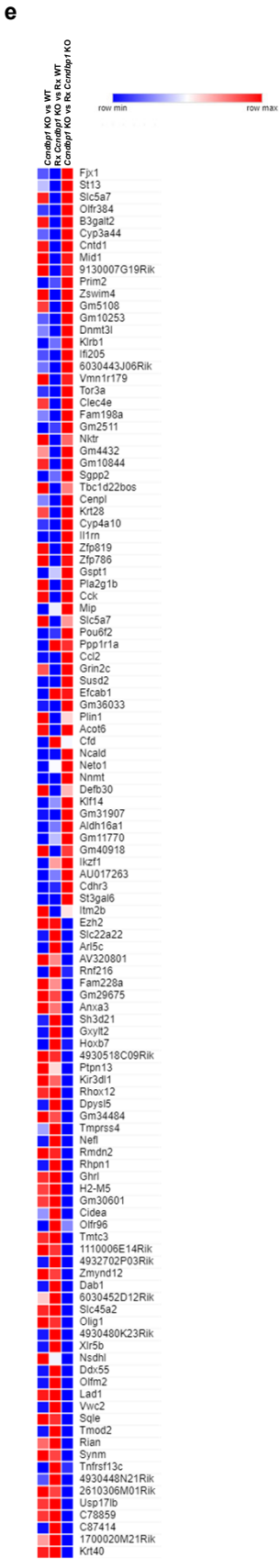
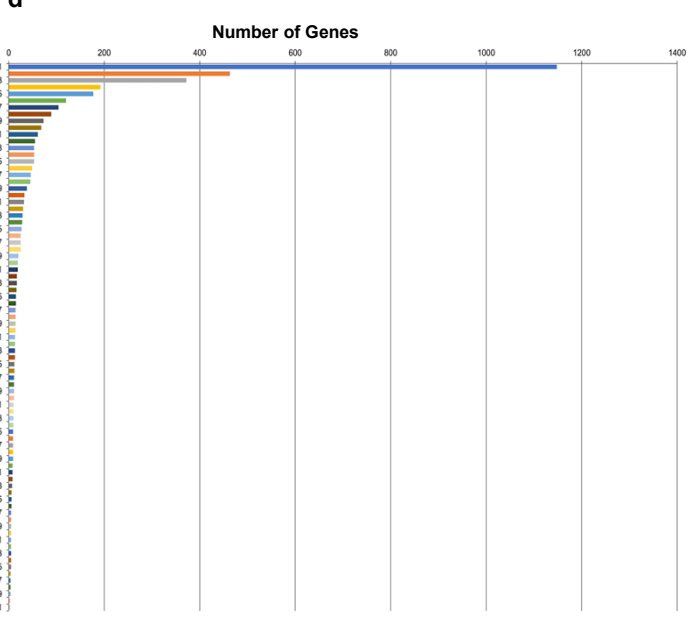


**Figure 3**



**c**

1	GO:0003674(molecular_function)
2	GO:0003824(catalytic_activity)
3	GO:000166(nucleotide_binding)
4	GO:0005515(protein_binding)
5	GO:000122(negative_regulation_of_transcription_from_RNA_polymerase_II_promoter)
6	GO:0004872(receptor_activity)
7	GO:0005737(cytoplasm)
8	GO:0005216(ion_channel_activity)
9	GO:0005634(nucleus)
10	GO:0003677(DNA_binding)
11	GO:000977(RNA_polymerase_II_regulatory_region_sequence-specific_DNA_binding)
12	GO:0005509(calcium_ion_binding)
13	GO:0001701(in_uterio_embryonic_development)
14	GO:0003676(nucleic_acid_binding)
15	GO:0005102(receptor_binding)
16	GO:0005576(cellular_component)
17	GO:0005576(extracellular_region)
18	GO:0003779(actin_binding)
19	GO:0002376(immune_system_process)
20	GO:0005096(GTPase_activator_activity)
21	GO:0005215(transporter_activity)
22	GO:0005085(quanyl-nucleotide_exchange_factor_activity)
23	GO:000775(chromosome_centromeric_region)
24	GO:0000082(G1/S_transition_of_mitotic_cell_cycle)
25	GO:0005739(mitochondrion)
26	GO:000185(activation_of_MAPKK_activity)
27	GO:0001525(angiogenesis)
28	GO:0005886(plasma_membrane)
29	GO:000790(nuclear_chromatin)
30	GO:000981(sequence-specific_DNA_binding_RNA_polymerase_II_transcription_factor_activity)
31	GO:0002244(hemopoietic_progenitor_cell_differentiation)
32	GO:0003700(sequence-specific_DNA_binding_transcription_factor_activity)
33	GO:0003723(RNA_binding)
34	GO:0002020(protease_binding)
35	GO:000312(transcription_cofactor_activity)
36	GO:0005198(structural_molecule_activity)
37	GO:0000287(magnesium_ion_binding)
38	GO:0001501(skeletal_system_development)
39	GO:0001558(regulation_of_cell_growth)
40	GO:0005622(nucleoside)
41	GO:000175(RNA_(adenine-N6-N6-)-dimethyltransferase_activity)
42	GO:0000278(mitotic_cell_cycle)
43	GO:0001933(negative_regulation_of_protein_phosphorylation)
44	GO:0016020(membrane)
45	GO:0001568(blood_vessel_development)
46	GO:0005178(integrin_binding)
47	GO:0000060(protein_import_into_nucleus_translocation)
48	GO:000724(double-strand_break_repair_via_homologous_recombination)
49	GO:0001517(N-acetylglucosamine_6-O-sulfotransferase_activity)
50	GO:0005125(cytokine_activity)
51	GO:0000077(DNA_damage_checkpoint)
52	GO:0000079(regulation_of_cyclin-dependent_protein_kinase_activity)
53	GO:0001684(G-protein_coupled_receptor_binding)
54	GO:0001764(neuron_migration)
55	GO:000228(microtubule_cytoskeleton_organization)
56	GO:000302(response_to_reactive_oxygen_species)
57	GO:0001659(temperature_homeostasis)
58	GO:0001666(response_to_hypoxia)
59	GO:000333(amino_acid_transmembrane_transport)
60	GO:0000009(alpha-1,6-mannosyltransferase_activity)
61	GO:000105(histidine_biosynthetic_process)
62	GO:0009022(cell_morphogenesis)
63	GO:000049(RNA_binding)
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65	GO:000228(nuclear_chromosome)
66	GO:000975(regulatory_region_DNA_binding)
67	GO:0000045(autophagic_vacuole_assembly)
68	GO:0000075(cell_cycle_checkpoint)
69	GO:000209(protein_polyubiquitination)
70	GO:000711(meiotic_DNA_repair_synthesis)
71	GO:0000910(cytokinesis)
72	GO:000209(s3_binding)
73	GO:0005506(iron_binding)
74	GO:0005507(copper_ion_binding)
75	GO:0005623(cell)
76	GO:0000052(citrulline_metabolic_process)
77	GO:0000123(histone_acetyltransferase_complex)
78	GO:0000375(RNA_splicing_via_transesterification_reactions)
79	GO:0005022(proteasome_complex)
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81	GO:0000062(tatyl-acyl-CoA_binding)



**Figure 4**

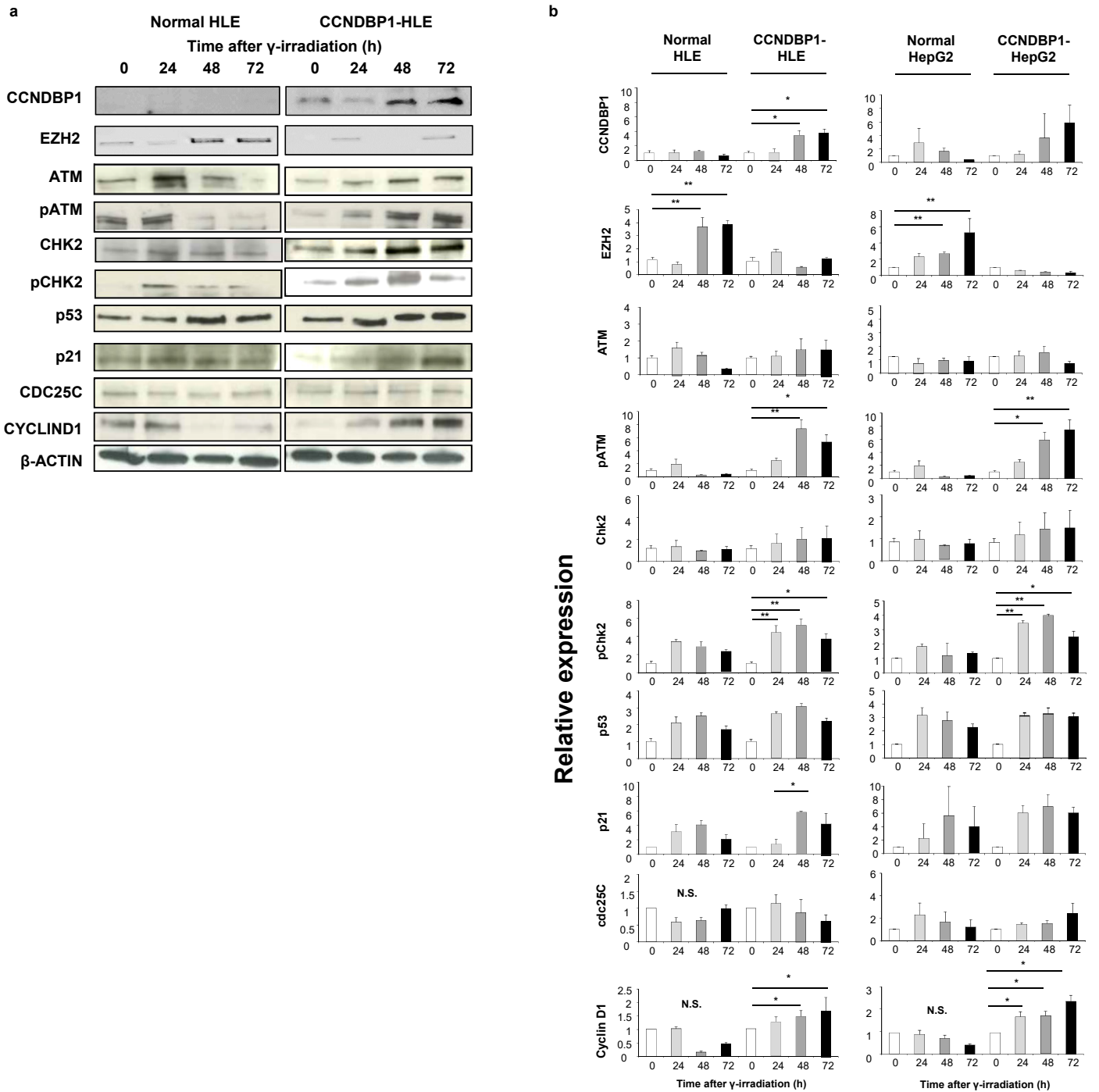
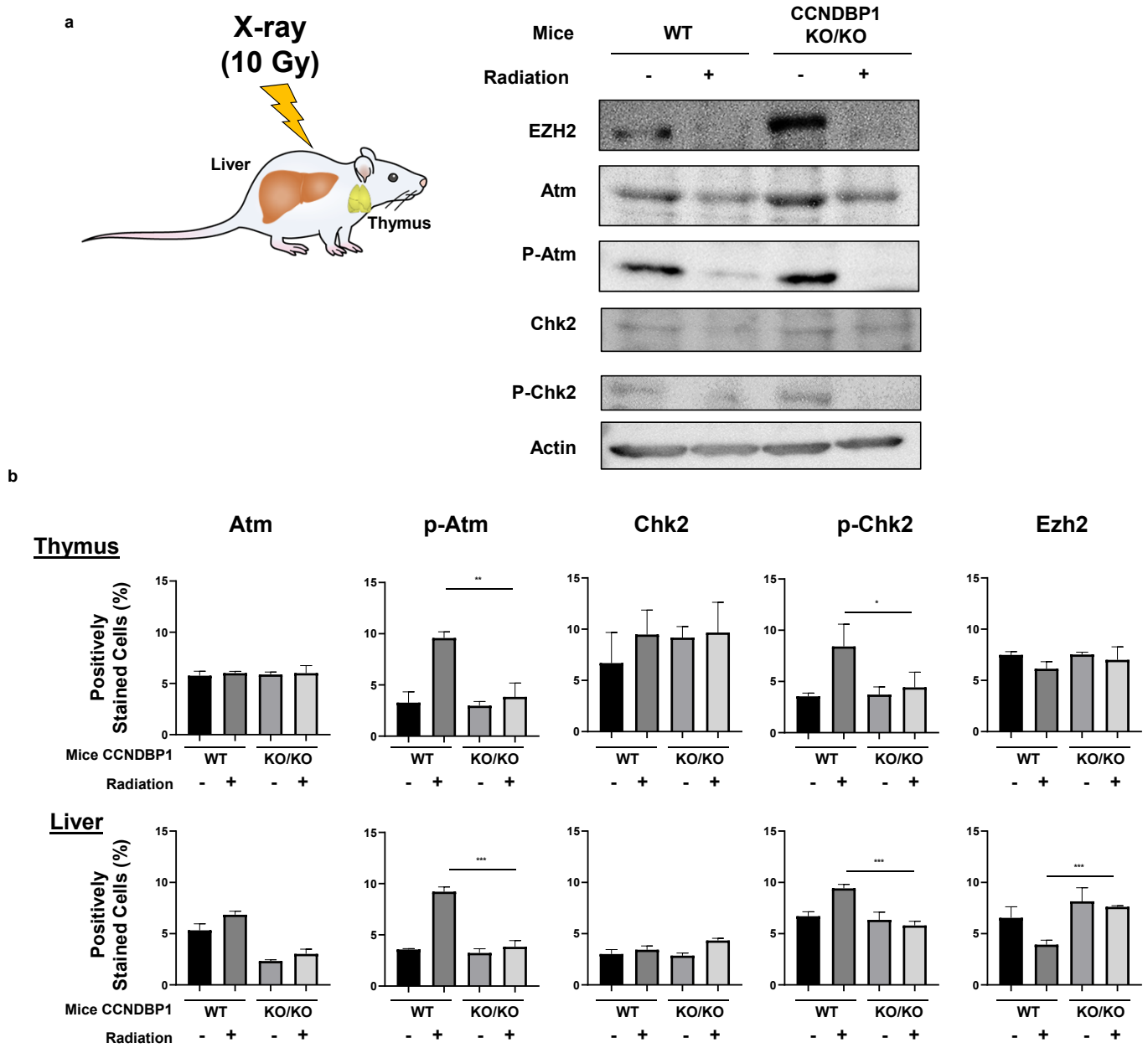


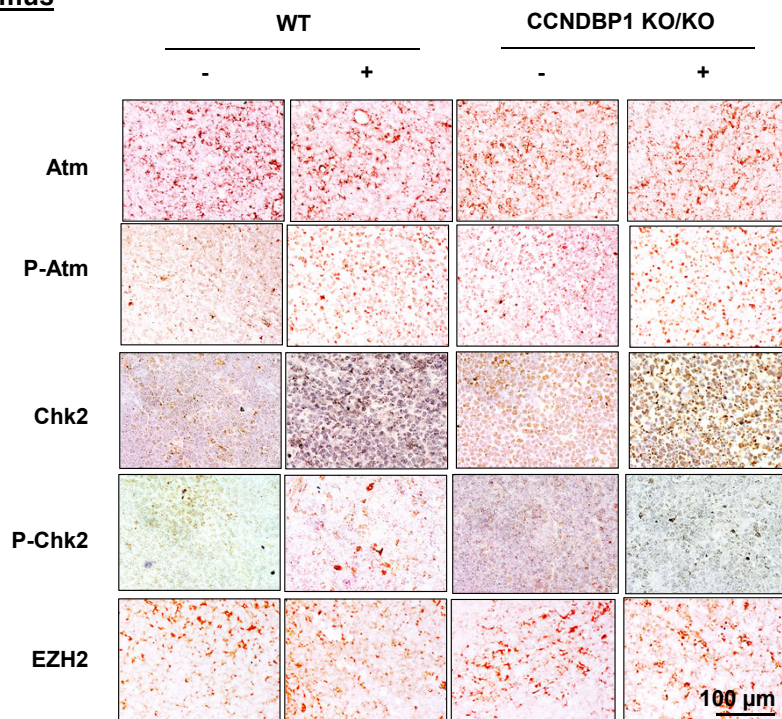


Figure 5



Supplementary  
Figure 1

Thymus



Liver

