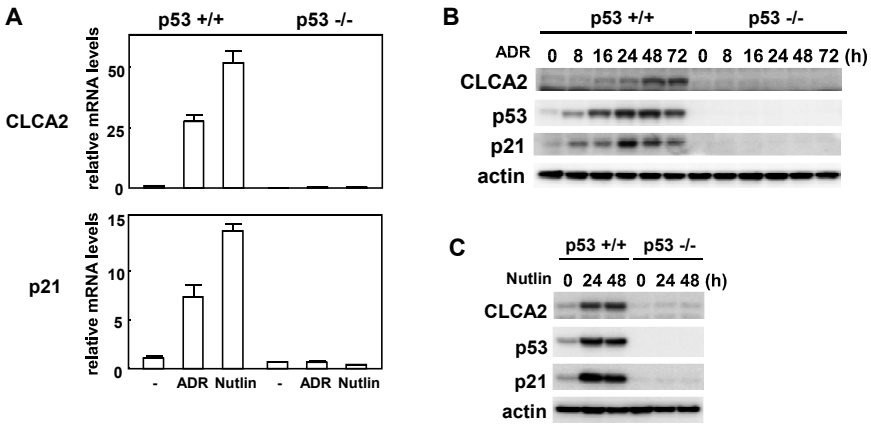
# Supplemental Material to:

Yasushi Sasaki, Ryota Koyama, Reo Maruyama, Takehiro Hirano, Miyuki Tamura, Jun Sugisaka, Hiromu Suzuki, Masashi Idogawa, Yasuhisa Shinomura and Takashi Tokino

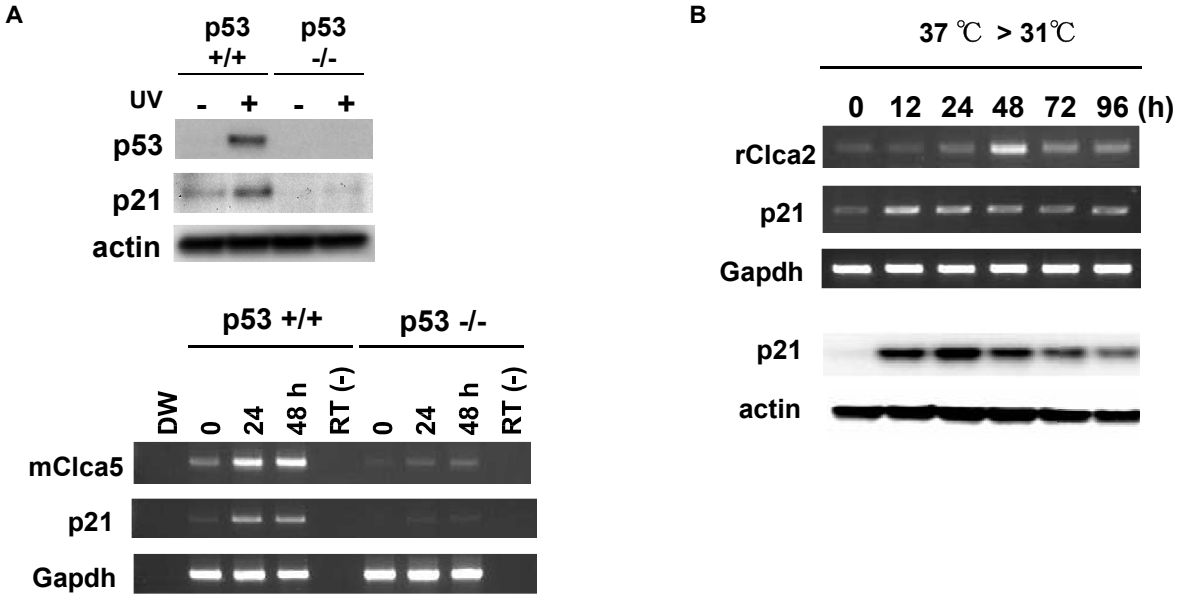
## CLCA2, a target of the p53 family, negatively regulates cancer cell migration and invasion

Cancer Biology & Therapy 2012; 13(14)
http://dx.doi.org/10.4161/cbt.22280

http://www.landesbioscience.com/journals/cbt/article/22280

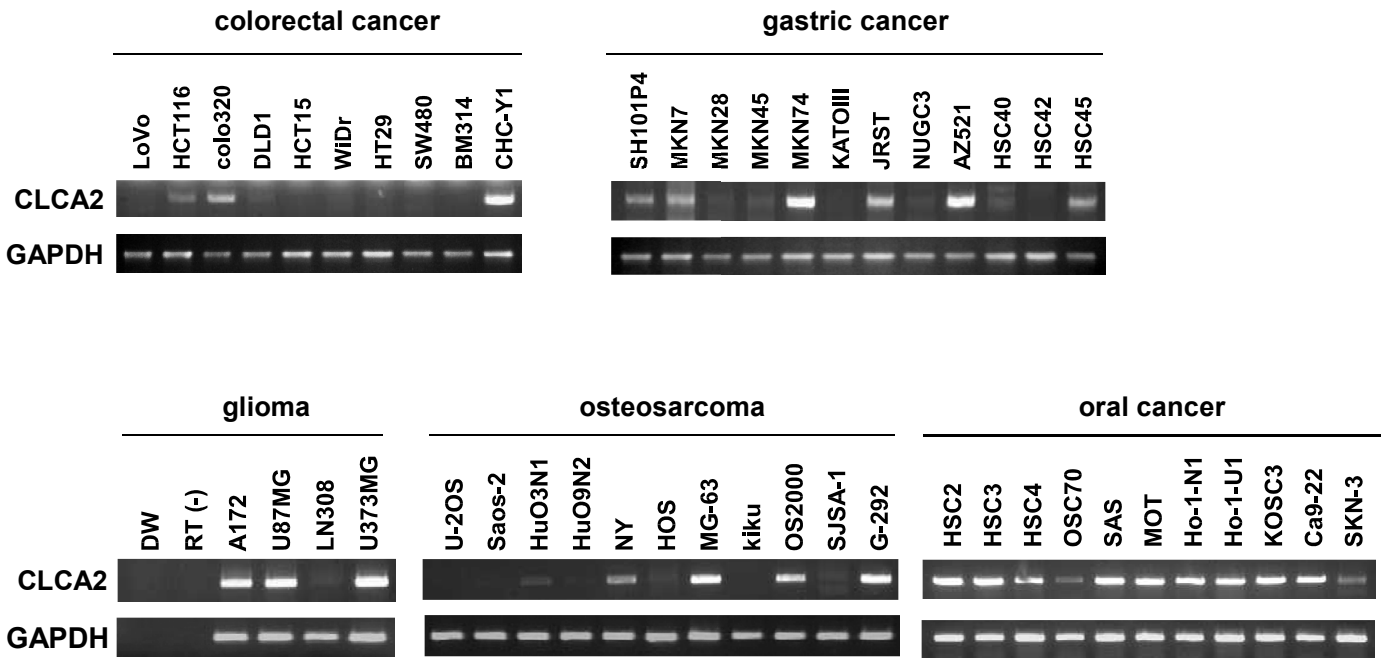



**Supplementary Fig. S1**
**Endogenous p53 up-regulates *CLCA2* mRNA and protein levels**
A) HCT116-p53(+/+) and HCT116-p53(-/-) cells were treated with ADR (Sigma, 0.2 $\mu$g/mL) or Nutlin-3 (Sigma, 10 $\mu$M) for 24 h. *CLCA2* and *p21* mRNA levels were assayed by real-time PCR. Values shown are the means ± standard errors of three independent experiments normalized to their respective controls as 1.
B, C) HCT116-p53(+/+) and HCT116-p53(-/-) cells were treated with 0.2 $\mu$g/ml ADR (B) or 10 $\mu$M Nutlin-3 for the indicated time (hours) , and immunoblot analysis of CLCA2, p53, p21 and $\beta$-actin was performed.

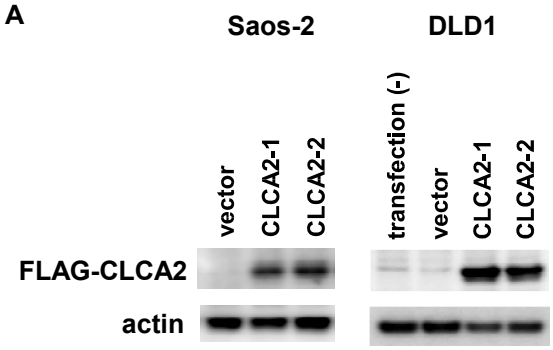


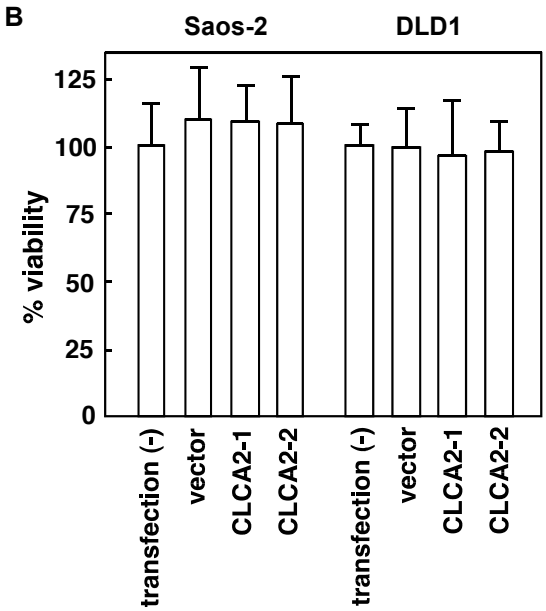

**Supplementary Fig. S2**
**Expression of *CLCA2* mRNA is induced in mouse and rat fibroblasts in a wild-type p53-dependent manner**

A) Upper panel, mouse embryonic fibroblasts (MEFs) from p53(+/+) mice and p53(-/-) mice were provided by Dr Motoya Katsuki (National Institute for Basic Biology, Japan). Cell lysates were prepared 24 h after the UV treatment (50 J/m$^2$). Immunoblot analysis was performed using antibodies against p53 (1C12, Cell Signaling), p21 (F-5, Santa Cruz Biotechnology) and β-actin. Lower panel, semi-quantitative RT-PCR analysis of *mClca5* mRNA following UV radiation in MEFs derived from p53(+/+) and p53(-/-) mice. Cells were treated with UV (50 J/m$^2$) for the indicated time and subjected to semi-quantitative RT-PCR analysis of mCLCA5. *Gapdh* mRNA levels were examined as a control for cDNA integrity. Distilled water (DW) and total RNA untreated with reverse transcriptase (RT (-)) were used as negative controls.
B) Semi-quantitative RT-PCR analysis of *rCLCA2* in REFp53vahCLCA25 cells following thermoshift. Cells were incubated at 31° C (wild-type p53 conformation). Total RNA was prepared from cells at 0, 12, 24, 48, 72, and 96 h following thermoshift and subjected to RT-PCR analysis of rClca2. In addition, immunoblot analysis was performed on a known p53-target, p21, and β-actin.
Oligonucleotide primer sequences were as follows:
mouse *Clca5* sense 5'- TTTTGGATGGCGGAGCAGGTG-3',
mouse *Clca5* antisense 5'- GGGTGACAAGCTTGGGTGAGAATG-3',
mouse and rat *p21* sense 5'- GCCCGAGAACGGTGGAACTTT-3',
mouse and rat *p21* antisense 5'- CCTCCTGACCCACAGCAGAA-3',
rat *Clca2* sense 5'- ACCCACCATTCTCCTCAAGCTCTG-3',
rat *Clca2* antisense 5'- AAGTCCCACCGCTCCTTCACA-3',
mouse and rat *Gapdh* sense 5'-ACCACAGTCCATGCCATCAC-3',
and mouse and rat *Gapdh* antisense 5'-TCCACCACCCTGTTGCTGTA-3'.

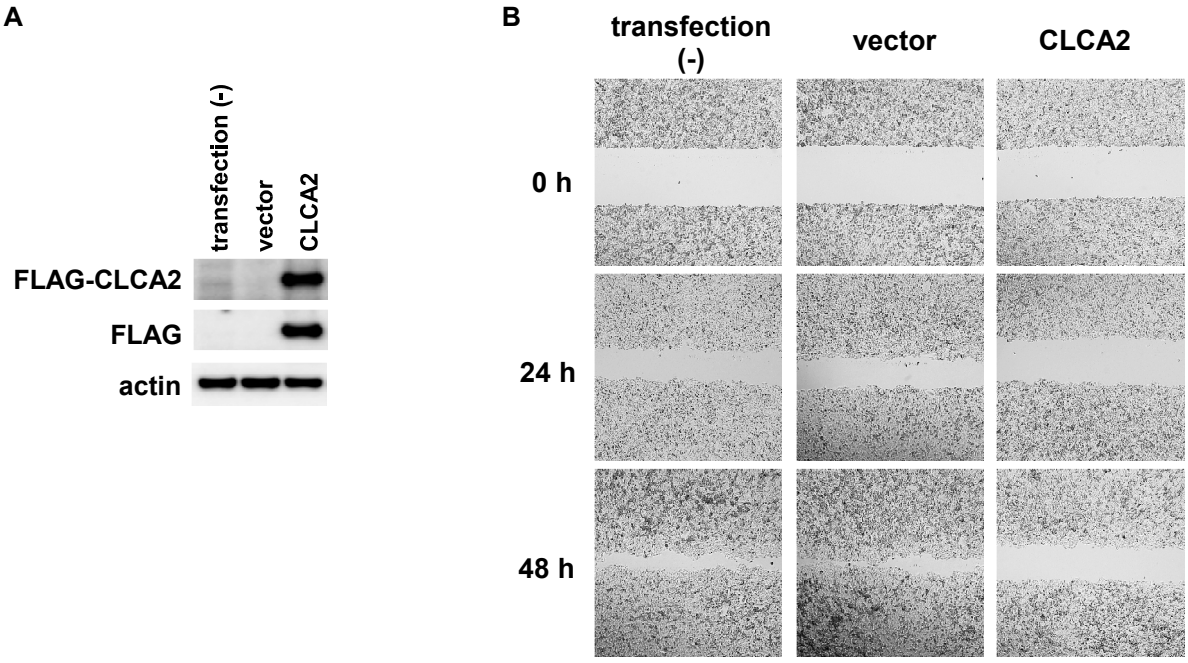



**Supplementary Fig. S3**
**Expression of endogenous *CLCA2* mRNA in human cancer cell lines**
Semi-quantitative RT-PCR analysis was performed using primers for *CLCA2* (5'-GGGCTTAAGAAAGGATTTGAGGTGGT-3' and 5'-ACAGGATGAGGAAAATGGAGGCTGT-3') and *GAPDH* (5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3').

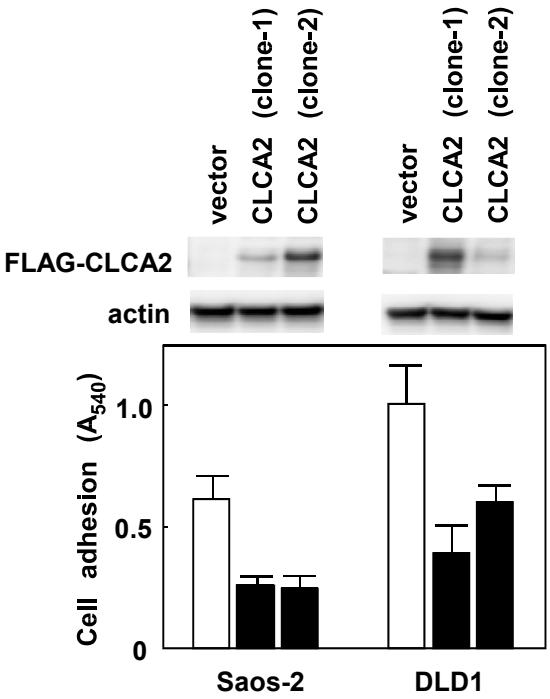



**Supplementary Fig. S4**
**Overexpression of CLCA2 does not inhibit cancer cell growth**
A) Saos-2 or DLD1 cells were transiently transfected with CLCA2 expression vector pReceiver-M11-CLCA2 containing a FLAG epitope tag (two populations) or empty vector plasmid (vector). At 24 h after transfection, cell lysates were prepared, and the expression of FLAG-CLCA2 was detected by Western blotting.
B) Saos-2 and DLD1 cells were transfected as described above. After 24 h, cell viability was assessed by the uptake of tritium thymidine using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The assay was performed in triplicate with a Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer's instructions. Error bars equal one standard error.




**Supplementary Fig. S5**
**Overexpression of CLCA2 inhibits cancer cell migration**
A) Western blot analysis after stable transfection of CLCA2 expression vector containing a FLAG epitope tag or an empty vector in Saos-2 cells.
B) Wound healing assay of Saos-2 cells following the stable transfection of CLCA2 expression vector. A transfected Saos-2 cell monolayer on collagen-coated coverslips was scratched manually with a plastic pipette tip. Cells were washed with fresh medium to remove floating cells, and phase contrast images were taken 0, 24 and 48 h after wounding. Representative images show decreased cell migration to the wounded area in CLCA2-overexpressing Saos-2 cells.




**Supplementary Fig. S6**
**Overexpression of CLCA2 inhibits cancer cell adhesion**
Tissue culture plates (96 wells) were coated for 2 h at 37° C with fibronectin at 10 $\mu$g/ml. Subsequently, an equal number of stably transfected cells (1 X $10^5$/ml) in a FBS-free medium were added per well and allowed to adhere for 30 min. The adherent cells were fixed with 200 $\mu$l of cold 70% ethanol, stained with 0.1% crystal violet, and washed three times with water. Finally, the cells were incubated for 1 h with 2% SDS to solubilize the crystal violet. Absorbance was measured at 540 nm.
Upper panels show the immunoblot analysis of FLAG-CLCA2 with β-actin as a loading control in cells after stable transfection of CLCA2 expression vector containing a FLAG epitope tag or empty vector. The results from pooled controls and two individual CLCA2 transfectant clones are shown.