Master's Thesis

# Transcriptional regulation of TRAP1 and Anti-cancer drug development targeting TRAP1

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Department of Biological Sciences

Graduate School of UNIST

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# Transcriptional regulation of TRAP1 and Anti-cancer drug development targeting TRAP1

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# Abstract

TNF receptor associated protein 1 (TRAP1) is a Heat shock protein 90 (Hsp90) homolog protein and mitochondrial chaperone that performs a critical role for maintaining mitochondrial homeostasis. TRAP1 is highly overexpressed in variety of cancers and protects cancer cells. Protective functions of the TRAP1 to cancer cells include regulating reactive oxidative species, affecting energy metabolism, and preventing cell death which leads to drug resistance.

Heat shock protein 90 (Hsp90) is a molecular chaperone whose clients participate in important cellular signaling in cancer. Many researchers studied the characteristics of Hsp90 and developed its inhibitor. Unlike to expect, Hsp90 inhibitors showed modest function as a cancer drug in vivo. However, if Hsp90 inhibitors were forced to accumulate in mitochondria, Hsp90 inhibitors inhibit both mitochondrial Hsp90 and TRAP1, and showed tremendous potential as cancer drugs. Then, TRAP1 specific inhibitors introduced with high cancer targeting whereas showing the low cytotoxic effect on normal cells, indicating TRAP1 as a novel cancer drug target protein.

Previously, it has been discovered that TRAP1 is overexpressed in glioblastoma cancer stem cell (GSC) compared to differentiated glioblastoma. Upregulated TRAP1 helps stem cells to maintain their stemness. Despite the importance of TRAP1, the underlying mechanism of TRAP1 overexpression remained unclear.

In study 1, we investigated the transcription mechanism of TRAP1 in cancer, based on that TRAP1 is upregulated in the transcriptional level on GSC. To identify the key transcriptional regulatory element, we researched on TRAP1 promoter characteristics. By using luciferase reporter assay, we specified the TRAP1 promoter and identified that c-AMP responsive element (CRE) is a significant regulatory element for TRAP1 expression. Since TRAP1 is a mitochondrial key surveillant for homeostasis, we can understand communication between cellular transcription signal and mitochondrial stress response by finding out the regulatory mechanism of TRAP1.

In study 2, we developed an anti-cancer drug named SJ-T140 targeting TRAP1. We analyzed specific binding to TRAP1 in vitro through the fluorescence polarization assay and ability to inhibit TRAP1 function in cancer cells by observing client protein stability. We checked the cytotoxic activity of SJ-T140 to cancer cells and normal cells. Through the xenograft assay, we recognized SJ-T140 has improved in vivo activity compared with the positive control, gamitrinib.

**Key word**: TRAP1, promoter, mitochondria, transcriptional regulation
TRAP1 inhibitors, anti-cancer drug

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## [Chapter II. Development of TRAP1 inhibitor for future cancer drug]

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# Abbreviation

Hsp90: Heat shock protein 90

TRAP1: TNF receptor-associated protein 1

ROS: reactive oxygen species

ETC: electron transport chain

GSC: glioblastoma stem cell

DHS: DNase I hypersensitive site

ENCODE: Encyclopedia of DNA elements

BRE: TFIIB recognition element

TBP: TATA box binding proteins

TSS: transcription start site

DPE: downstream promoter element

Aryl hydrocarbon receptor nuclear translocator-like protein 1 (ARNTL)

E-box: enhancer box

CRE: cAMP response element

GTF: General transcription factor

RLU: Relative Luciferase Unit

UCSC: University of California, Santa Cruz

# [Chapter1. Transcription regulation of mitochondrial chaperone TRAP1]

## Introduction

**Mitochondrial function in cancer and role of TRAP1**

It had been known that cancer metabolism shifted to aerobic glycolysis which is known as the "Warburg effect". (Ward & Thompson, 2012) Mitochondria had been considered functionally degraded in cancer, however, it had discovered that in many cancers, mitochondrial have an intact function. Even, in some cancer, cells depend on mitochondria for energy generation. (Gao et al., 2009) Mitochondria produce and regulate reactive oxidative species (ROS), help adapt cells to their microenvironment by responding to stress, and regulate cell death. (Vyas, Zaganjor, & Haigis, 2016) Generating oncometabolites in mitochondrial mutation lead to tumor development or tumor malignancy. Also, they flexibly handle energy depletion from excessive proliferation by generating energy not only from glucose but also glutamine. (Vyas et al., 2016) Due to this flexible mitochondrial function, mitochondria are considered to have a pro-cancerous function.

TNF-receptor associated protein 1 (TRAP1) is heat shock protein 90 (Hsp90) homolog protein mainly in the mitochondrial matrix. (Cechetto & Gupta, 2000; Felts et al., 2000) TRAP1 participates in mitochondrial fusion and fission, and apoptotic cell death along with cyclophilin D. (B. H. Kang et al., 2007; Takamura et al., 2012) In cancer, TRAP1 is upregulated, and maintains mitochondrial homeostasis by regulating ROS(Masuda et al., 2004) and affecting energy metabolism by orchestrating electron transport chain (ETC) complex. (Chae et al., 2013) Protective functions of TRAP1 in cancer cells prevent cell death and induce drug resistance. (Altieri, Stein, Lian, & Languino, 2012; B.-H. Kang, 2012; Landriscina, Amoroso, Piscazzi, & Esposito, 2010) With the listed properties, TRAP1 is considered an essential protein to cope with the stress caused by cancer development and growth, however the mechanism of how TRAP1 is overexpressed in cancer remained unclear.

In this research, we studied on transcriptional regulation of TRAP1, based on previous research that TRAP1 is upregulated in the transcriptional level in glioblastoma cancer stem cell (GSC). GSC model can easily differentiate or de-differentiate cells, showing TRAP1 expression difference according to cell's stemness. We measured the TRAP1 promoter activity in both conditions and observed transcription factor candidate expression differences as well.

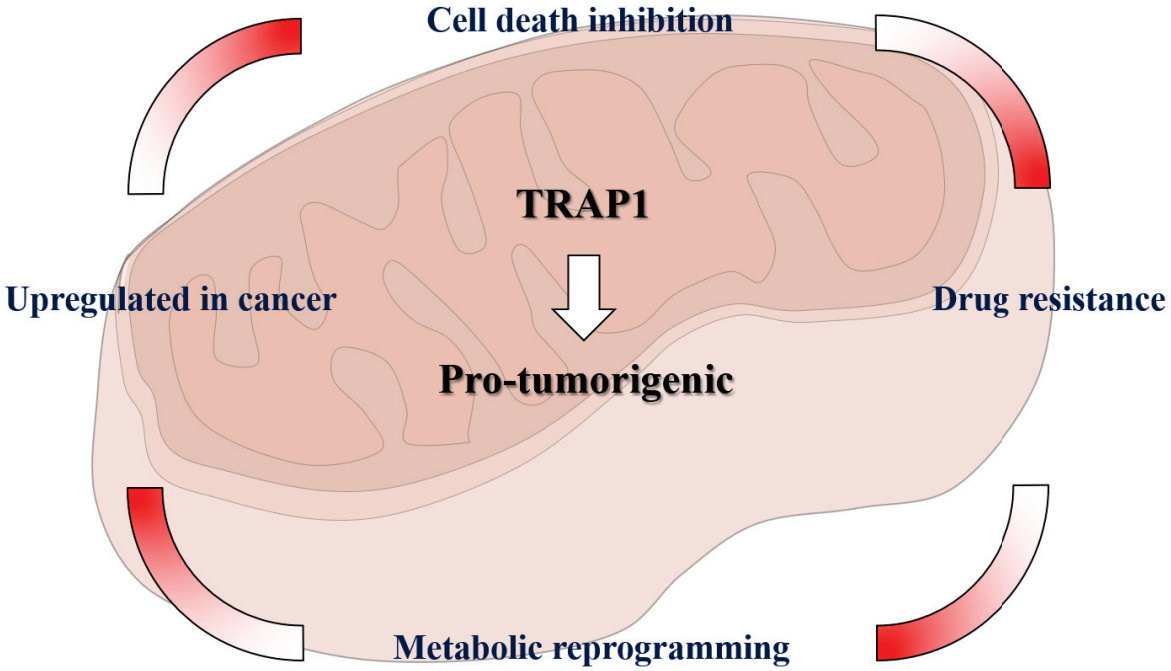



**Figure A. Pro-tumorigenic function of TRAP1 in cancer cell mitochondria.**

**Transcriptional gene regulation**

Transcriptional gene regulation is affected by several regulatory elements including promoter, enhancer, and silencer. Active regulatory elements (enhancers and promoters) can be distinguished by chromatin markers. Typical markers are histone post-translational modification and DNase I hypersensitivity clusters. Histone modification patterns such as H3K4me1, H3K4me3, and H3K27ac are observed in gene regulatory elements. These kinds of modifications maintain transcriptional activity and euchromatin status thus physically accessible for transcription initiation machinery. DNase I hypersensitivity cluster of DNase I hypersensitive site (DHS) is considered as DNA region that can be cut by DNase I, due to its open structure. Thus, these characteristics indicate that the chromatin construct is physically unwound. (Haberle & Stark, 2018)

An enhancer is a regulatory element adjacent or/and far from the TSS. This element can bind with specific transcription factors (activators) and affect transcriptional gene regulation communicating with transcriptional preinitiation complex with mediators. (Roy & Singer, 2015) Silencer is the gene regulatory element bound by repressors, to downregulate target genes. (Maston, Evans, & Green, 2006)

Promoter is the genomic region that consists of core promoter and proximal promoter that has the ability to transcribe certain genes. (Haberle & Stark, 2018) Core promoters are short sequences adjacent to the transcription start site (TSS) that can assemble the transcription machinery including RNA polymerase II. Proximal promoter contains the transcription factor binding site located proximal to the core promoter. Typically, the proximal promoter is located within 250bp from the TSS. (Haberle & Stark, 2018) Core promoter can be categorized into canonical core promoter and non-canonical core promoter. Canonical promoter contains general transcription factor binding motifs such as TATA box, initiator, and TFIIB recognition element (BRE). (Garraway, Semple, & Smale, 1996; Mathis & Chambon, 1981; Smale & Baltimore, 1989; Smale & Kadonaga, 2003) TATA box (consensus TATAAA) binds with TATA Box binding Proteins (TBP), subunit of TFIID, at the 30-31 base pair upstream from the TSS. (Ponjavic et al., 2006) Despite TATA box highly conserved its consensus, only few eukaryotic promoters contain it. Initiator which is considered as TFIID binding element like TATA box (Roeder, 1996), has consensus YYA(+1)NT / AYY. Initiator sequences penetrate TSS. Typically, a promoter with TATA box serves the genes that are expressed in tissue-specific manner with sharp TSS distribution. In contrast, a promoter with the initiator appears in ubiquitously expressed gene or housekeeping gene. (Gershenzon & Ioshikhes, 2004; Lenhard, Sandelin, & Carninci, 2012; Sandelin et al., 2007) Downstream promoter element (DPE), one of the core promoter element, is found with initiator located in +28 to +32 from TSS and support TFIID binding with the initiator. (Smale & Kadonaga, 2003) B recognition element (BRE) is found in the TATA box containing core promoter, which aligns upstream or downstream of the TATA box. (Smale & Kadonaga, 2003) However, many protein-coding genes do

not have consensus sequences for the general transcription factors (GTF). These promoters are called non-canonical core promoters. The most significant characteristics of the non-canonical promoters are CpG island and ATG desert. (Roy & Singer, 2015) CpG island is a genomic region where the concentration of CG dinucleotides is higher than other genomic regions. (Gardiner-Garden & Frommer, 1987) The molecular function of CpG island in transcription remains unknown. The other non-canonical promoter characteristic, ATG desert is the phenomenon that ATG absent near TSS. This feature is expected to appear due to dispersed TSS, whose genes can produce a variety of RNA transcript. The problem is a few combinations of transcripts with abundant ATG induces high error. To avoid those mistakes, DNA sequences of those genes lack the ATG sequence from upstream 1kb to downstream 1kb. (M. P. Lee et al., 2005)

**ENCODE project and UCSC genome browser**

ENCODE is an abbreviation of Encyclopedia of DNA elements. This project is a follow-up study of Human genome project, aim to identify the functional element of human genome. Protein-coding gene account only 1.5% of DNA in human genome(Consortium, 2001), and the remained DNA's function is unclear. ENCODE project started to identify the function of DNA other than protein-coding genes. It started in 2003, and the fourth phase was initiated in February 2017. University of California Santa Cruz (UCSC) genome browser is website-based database that researchers can be accessible to ENCODE project result. UCSC genome browser provides the transcription factor binding sites, histone marks, chromatin accessibility, DNA methylation, RNA expression, RNA binding and cell-state indicators(Raney et al., 2010). Especially transcription factor binding site is organized in a web-based repository "factor book". Information was produced based on chromatin immunoprecipitation sequencing of 119 transcription factors in various cell lines. We utilized the genome browser to identify the rough outline of promoter region. Even accurate transcription factors and regulatory elements can be different depending on cell types, information provided by genome browser is very helpful for determining gene regulatory elements and putative transcription factors.

# Material and method

## 1. Cell culture

Glioblastoma stem cell MT and 528NS were kindly generated by institute the national cancer research center. Glioblastoma cell line LN229 was purchased from American Type Culture Collection (ATCC). MT and 528NS were cultured in DMEM/F12 (Wellgene) supplemented with EGF (10ng/ml, R&D system), FGF (5ng/ml, R&D system), B27(Invitrogen), and 1% penicillin/streptomycin (Gibco). LN229 was cultured in DMEM(Gibco) supplemented with 5% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Differentiated cancer stem cells were cultured in DMEM/F12 with 5%FBS. LN229 were cultured in DMEM/F12 media supplemented with EGF, FGF, B27, and 1% penicillin/streptomycin for stem enrichment.

## 2. Total RNA extraction and Reverse Transcriptional – PCR (RT-PCR)

Total RNA was extracted from the cultured cell using the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesized using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs). cDNA was amplified by thermal cycle PCR machine (life technology). Primer for RT-PCR is listed in **Table 1.**

## 3. promoter construct cloning

For the construction of TRAP1 promoter-luciferase reporter plasmids, genomic DNA was extracted from MT cell and purified with QiAamp DNA Blood Mini kit (QIAGEN) according to manufacturer’s instruction. Using extracted genomic DNA as a template, the TRAP1 promoter region -2306~+50 was amplified by PCR. The amplified -2306/+50 region of TRAP1 promoter was cloned into pGL3-basic vector (Promega) with restriction enzyme KpnI and XhoI (New England Biolabs). Other reporter constructs (-900/+50, -700/+50, -299/+50, -176/+50, -77/+50, -10/+50, CRE deletion, CRE mutant) were generated by PCR from -2306/+50 construct as a template. For site-directed mutagenesis of mutant E-Box, E-Box and CRE containing sequences were generated using -176/+50 construct as a template. Primer for promoter construct is listed in **table 1.**

**4. Immunoblotting analysis**

Whole lysate from cells was resuspended in RIPA with protease inhibitor cocktail and phosphatase inhibitor cocktail (R&D system). Lysates were centrifuged at 15000rpm for 10minute at 4℃. Protein concentrations were measured by protein assay dye reagent concentrate (Bio-Rad). 20㎍ of protein was loaded for immunoblotting analysis. Listed antibodies were used: TRAP1 (BD bioscience, 612344), GAPDH (Santa Cruz, sc-25778), GFAP (Abcam, ab7260), SOX2 (R&D system, AF2018), Nestin (R&D system, MAB1259), Horseradish peroxidase-conjugated mouse or rabbit secondary antibodies (KLP Inc.) and goat secondary antibody (R&D system). Membranes were detected by LAS 4000 (GE Healthcare) with clarity western ECL substrate (Bio-Rad)

**5. Promoter reporter assay**

All luciferase reporter constructs were generated by PCR using the described primer sets from **Table 1.** Cells were plated in 6mm$^2$ plate 12 hours prior to transfection. Promoter constructs were transfected with jet prime transfection reagent (polyplus) according to manufacturer's instruction. 1000ng of promoter construct DNAs were transfected, and 100ng of pRL-SV40 vector were co-transfected for internal control of transfection efficiency. Cells were harvested after 36 hours with passive lysis buffer enclosed in Dual-Luciferase Reporter Assay System (Promega). Luciferase assays were performed with the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instruction. Promoter activity is measured by the Relative light unit (RLU); Firefly (Promoter construct) / Renilla (pRL-SV40).

**6. siRNA treatment**

For the RNA interference following siRNA is applied: TCF3 5'-GCGGAGAAAUGGAAACAUA -3', CREB1 5'- AACAGTATTCTGTAGGATCTA -3', CREB5 5'- ACGGTGCCAACTCCAATTTAC - 3', PAX2 5'-GAAGUCAAGUCGAGUCUAU-3', control 5'- ACUCUAUCUGCACGCUGAC -3' Cells were plated in 6mm$^2$ dish and incubated 12 hours until si RNA treatment, G-fectin (Genolution) is mixed with siRNA 1:1, injected to the cell. 48 hours later, cells were harvested for the following experiments.

**7. Software for analyzing TRAP1 promoter**

Information of Histone posttranslational modification and DNase I hypersensitive site is referred to UCSC genome browser (http://genome.ucsc.edu, Feb. 2009, GRch37/hg19). Putative transcription factor binding region identified with “factor book” motif.

**Table 1. Primer list**

| **TRAP1 promoter construct** | | |
|---|---|---|
| -2306 forward | CGGGGTACCATTACACCCTCCACCCCTTG | |
| -900 forward | CGGGGTACCTGATGGGTTTTCACCATG | |
| -703 forward | CGGGGTACCGTCCTGCCTTTCCACACG | |
| -397 forward | CGGGGTACCAATCCCCTTCCTCATTCGCT | |
| -176 forward | CGGGGTACCCCACCTCCCCCACCTCC | |
| -77 forward | CGGGGTACCTAGTACCCCGCCACG | |
| -10 forward | CGGGGTACCCCCCGCGCCCGAGGAAG | |
| +50 reverse | CCGCTCGAGGTACACGATGGGAAGG | |
| **Site directed mutagenesis of TRAP1 promoter** | | |
| | Forward primer (5'→ 3') | Reverse primer (5'→ 3') |
| C-Myc binding site | CCCGAGCAGGCAGCTGCC | GCGGGGTACTACCCGTCTCGT |
| TCF3 binding site | CCCGGACAGCCCGGCGC | CCACGCGCCCGAGCAGG |
| CREB1, PAX2 binding site | CCCGGCGCCCGCCGTGA | CGAGGAAGCCCCGCCCCG |
| CRE deletion | CCGCCCCGCGCCCGA | CGGCGGGCGCCGGGCT |
| CRE mutation | TGAATTCACCGCCCCGCGCCC | CGGCGGGCGCCGGGCT |
| **RT-PCR primers** | | |
| | Forward primer (5'à3') | Reverse primer (5'à3') |
| TRAP1 | AGCGCACTCATCAGGAAACT | TCAAACTCACGAAGGTGCAG |
| GAPDH | CGGGAAGCTTGTCATCAATGG | GGCAGTGATGGCATGGACTG |
| ATF1 | CAACCTGGTTCAGCAGTTCA | TTTCTGCCCCGTGTATCTTC |
| ATF2 | CAGCTATTGTTCGTCCAGCA | CCTGGAACACTAGGCACCAT |
| ATF3 | TCGGAGAAGCTGGAAAGTGT | TCTGGAGTCCTCCCATTCTG |
| ATF4 | TCAAACCTCATGGGTTCTCC | GTGTCATCCAACGTGGTCAG |
| ATF5 | ACCGCAAGCAAAAGAAGAGA | GGCCTTGTAAACCTCGATGA |
| ATF6 | GCACCATGGAGTCACCTTTT | GAAAGTGGCTGAGGTTCTGC |
| CREB1 | GTGTTACGTGGGGGAGAGAA | GGGCTAATGTGGCAATCTGT |
| CREB3 | TTCTGAGGTACCGAGCGACT | ATAAGCCCCTCCTTCTCCAA |
| CREB5 | GGGAAAGGAAACAACCCATT | GAGCAAAGGCAAGTTTGAGG |
| C-jun | GAGGGGGTTACAAACTGCAA | ACAACACTGGGCAGGATACC |
| junB | TGGAACAGCCCTTCTACCAC | GAAGAGGCGAGCTTGAGAGA |
| junD | CGCCTGGAAGAGAAAGTGAA | GTTGACGTGGCTGAGGACTT |

# Result

### TRAP1 is upregulated in cancer stem cell regardless of growth factors

Through the previous studies, TRAP1 is revealed as a crucial mitochondrial chaperone functioning in the cancer cells. TRAP1 is upregulated in cancer, and increased TRAP1 level leads to apoptosis resistance and drug resistance. Abnormal TRAP1 expression in cancer and organelle-specific expression made TRAP1 as a promising cancer drug target because it can reduce the off-target effect. TRAP1 is also upregulated in patient-derived glioblastoma stem cell (GSC) **(Figure 1A)** and it shows a critical role in maintaining the stemness. (Park et al., 2019) Interestingly, TRAP1 was diminished in the transcriptional level after GSC differentiation and it increased after glioblastoma cell undergone stem enrichment. **(Figure 1B, C, D)** Two growth factors are supplied to cancer stem cell media for selecting cancer stem cells and maintaining stemness (Lathia, Mack, Mulkearns-Hubert, Valentim, & Rich, 2015), and we wondered whether the growth factors can affect the TRAP1 expression level. To investigate the effect of the growth factors, differentiated GSC and LN229 were incubated with 10ng/ml of EGF and 5ng/ml of FGF for 2days. As shown in Figure2, EGF and FGF did not affect TRAP1 expression. **(Figure 2B)** These results indicate that TRAP1 changed throughout stem cell differentiation, but it is not mediated by the extracellular growth factors directly and solely. Since the TRAP1 expression is regulated in transcriptional level, understanding of the TRAP1 transcription mechanism can reveal what causes TRAP1 expression difference during GCS differentiation.

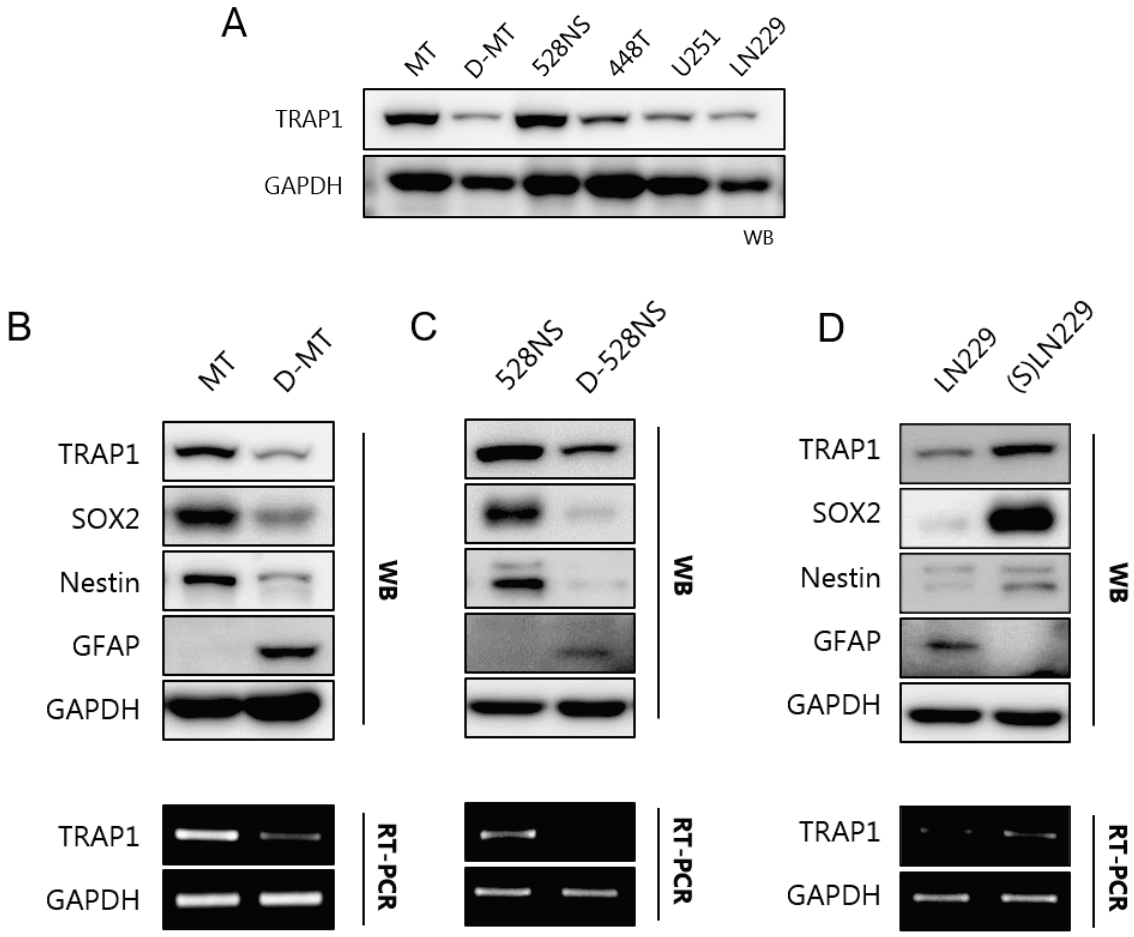



**Figure 1. TRAP1 is upregulated in cancer stem cell**

(A) TRAP1 expression comparison in GSC cells (MT, 528NS, 448T), glioblastoma cell line (U251, LN229) and differentiated GSC (D-MT). (B, C) TRAP1 expression difference in GSC and differentiated GSC in transcriptional level and protein level. (D) TRAP1 expression difference in glioblastoma cell line and stem enrichment glioblastoma cell. SOX2 and Nestin represent stem markers, and GFAP represents differentiation marker.

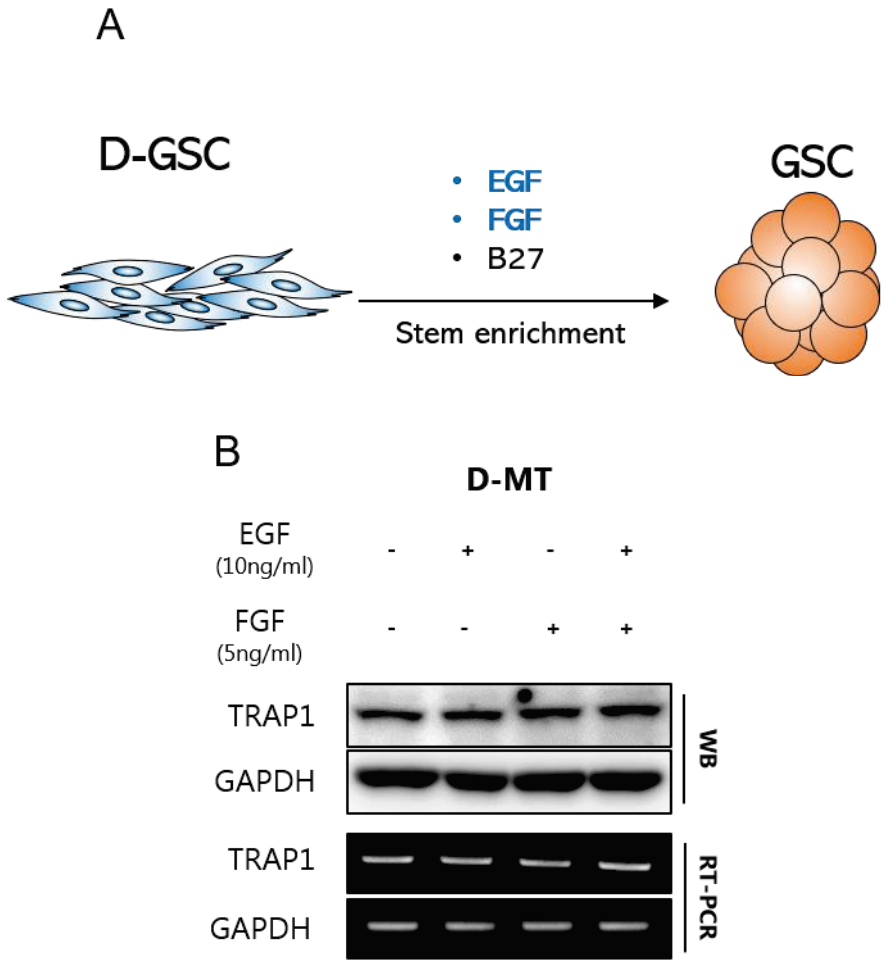



**Figure 2. Growth factor does not affect TRAP1 expression**

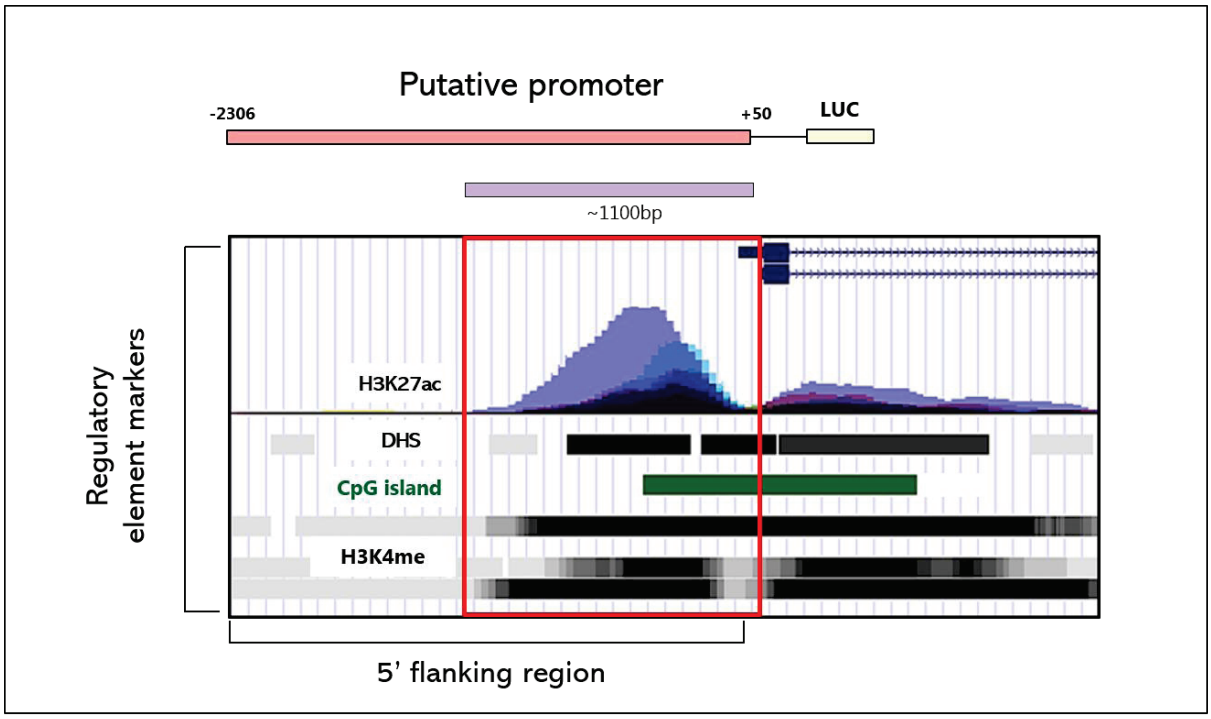
(A) Graphical image of stem enrichment process. EGF, FGF, and B27 are supplemented for stem enrichment. (B)TRAP1 expression after growth factor treatment. 10ng/ml of EGF and 5ng/ml FGF was supplemented for EGF, FGF treatment group.

**Putative TRAP1 promoter is a non-canonical promoter**

In GSC and differentiated GSC, changing of TRAP1 occurs at the transcription level, however the underlying mechanism of TRAP1 transcription regulation was not studied. Understanding TRAP1 transcriptional regulation can give a clue of understanding which cellular signals dominate the stress response of mitochondria. To understand the transcriptional regulation of TRAP1, firstly we studied about the TRAP1 gene regulatory elements near TSS.

We began with finding the TRAP1 gene regulatory element by defining the TRAP1 promoter. TRAP1 gene is locus on chromosome 16p13. 5' flanking region of the TRAP1 gene is 7500base pairs. To find gene regulatory elements in the 5' flanking region, information was referred from the University of California, Santa Cruz (UCSC) genome browser. Histone post-translation modification and DNase I hypersensitive clusters were used as markers for gene regulatory elements. According to the H3K4me, H3K27ac, and DNase I hypersensitivity site (DHS), 5' flanking region of the TRAP1 gene regulatory element adjacent to the transcriptional start site (TSS) has putative regulatory elements. Considering the histone post-translational modification pattern and other gene regulatory markers, this site is expected as a promoter. The markers representing putative promoter cover approximately 1100 base pairs from the TSS. We decided to begin the TRAP1 promoter research from -2306 to fully include the desired region. Thus, the TRAP1 putative promoter reporter construct was produced including 2306 base pairs upstream to TSS, and 50base pair downstream to TSS. **(Figure 3)**

Next, we analyzed the TRAP1 promoter to distinguish its characteristics. At first, we analyzed whether TRAP1 has a canonical promoter. Canonical promoter preserved the core promoter elements which are binding partners for GTF. We searched general transcription factor binding consensus on the TRAP1 putative promoter. **(Table 2)** GTFs bind to specific DNA consensus, and the consensus location is also crucial for recruiting the transcription preinitiation complex (PIC) for accurate transcription initiation. However, the TRAP1 promoter lacks the consensus or predicted consensus is located on irrelevant locus. Putative promoter has CpG island near TSS and without ATG desert. Throughout this analysis, TRAP1 is expected to have a non-canonical promoter.




Human assembly: hg19, chr16:3,765,497-3,769,963

**Figure 3. TRAP1 putative promoter according to UCSC genome browser**

Schematic image of the TRAP1 gene regulatory element. Reference human assembly is GRCh37/hg19. Among the TRAP1 gene and 5' flanking region, partial TRAP1 5' flanking region, exon 1 and partial intron 1 appear in the enlarged image. Gene regulatory elements are aligned; H3K27 acetylation pattern, DNase I hypersensitivity cluster, CpG island, and H3K4 methylation from the top.

**Table 2. Core promoter elements in TRAP1 promoter**

| Motif | Consensus sequence | Position related to TSS | Binding partner | Position in TRAP1 promoter |
|---|---|---|---|---|
| TATA box | TATAAA or TATATA | ~-31 | TBP | -625~-631 |
| Initiator | YYA$^{+}$NT/AYY | ±3~4 | TAF1 and TAF2 | - |
| CCAAT box | GGCCAATCT | -50~-100 | CTF | -753~-760 |

**Putative TRAP1 promoter has promoter activity**

In order to confirm that putative TRAP1 promoter functions properly, we performed luciferase reporter assay with various cancer cell lines. Comparing with the empty vector, TRAP1 promoter showed higher promoter activity, inferring that -2306/+50 construct includes the actual TRAP1 promoter. Also, promoter activity indirectly represents the TRAP1 expression level in different cancer cell lines**. (Figure 4)**

Since the putative promoter encloses vast 5' flanking area, we narrowed down the promoter by deleting sequence remote from TSS to find the most significant element in the promoter. In MT cell removing 1406base pairs between -2306/+50 and -900/+50 did not change the TRAP1 promoter activity, indicating that this region is not functioning as a promoter. However, by removing additional 200 base pairs, promoter activity increased, referring the possibility of presence of negative regulatory element. Promoter activity increased slightly until the promoter narrowed down to construct -176/+50. Subsequent deletion showed a feeble decrease of promoter activity, but still -77/+50 construct maintained 76% of the highest promoter activity. This may infer that the minimal promoter element is included in -77/+50. However, TRAP1 promoter activity was diminished in -10/+50 region. Thus, we concluded that sequences between -77 and -10 contain cis-acting elements that are crucial for TRAP1 expression. **(Figure 5 A, B)**

Comparing the result of luciferase reporter assay in MT and D-MT, in both cells, transcription activity increased from -703/+50 construct, and maintained until -77/+50. Dramatic promoter activity diminish is observed in -10/+50 construct. In both cells, the most decisive element for TRAP1 transcription is located between -77 and -10. However, quantitative promoter activity was much higher in MT, proportional to TRAP1 expression in both cells. This result represents that TRAP1 expression difference is determined in the promoter activity, especially between -77 and -10. To sum up, it is expected that transcription activators regulating TRAP1 expression intimately work on the same region in promoter in both cells and those transcription factors affect differently according to their expression and activation. **(Figure 5B)** Thus, if we find out the trans-acting element working on the region between -77 and -10, TRAP1 expression can be tightly regulated with expected TRAP1 transcription factors.

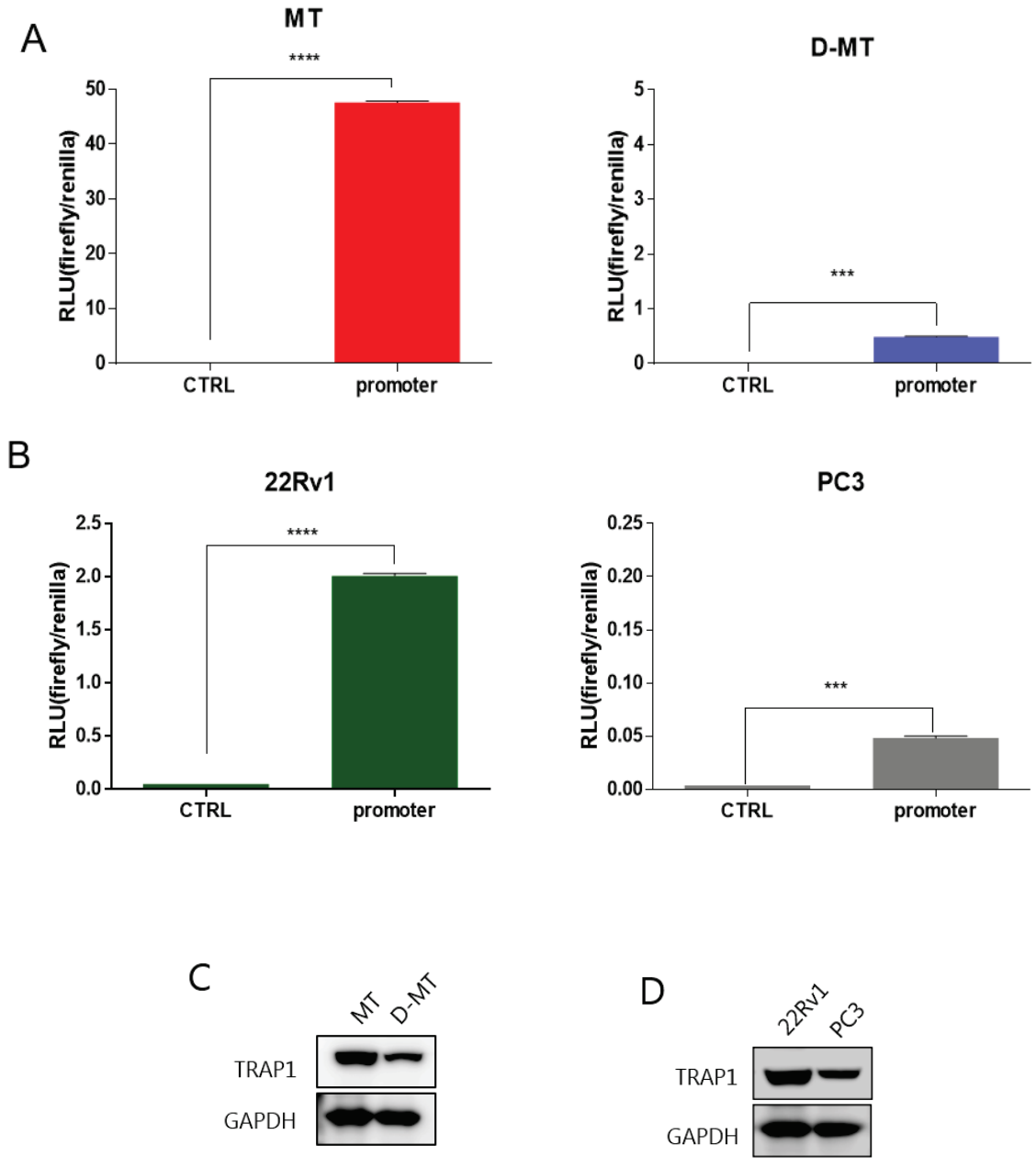



**Figure 4. TRAP1 promoter activities in cancer cell lines**

(A, B) TRAP1 putative promoter functional activity in several cancer cell lines (PC3, 22Rv1: human prostate cancer cell lines). Promoter activity measured relative luciferase unit (RLU). Firefly luciferase activity / Renillia luciferase. Values represent mean ± SD of two replicates (C, D) TRAP1 expression in cancer cell lines.

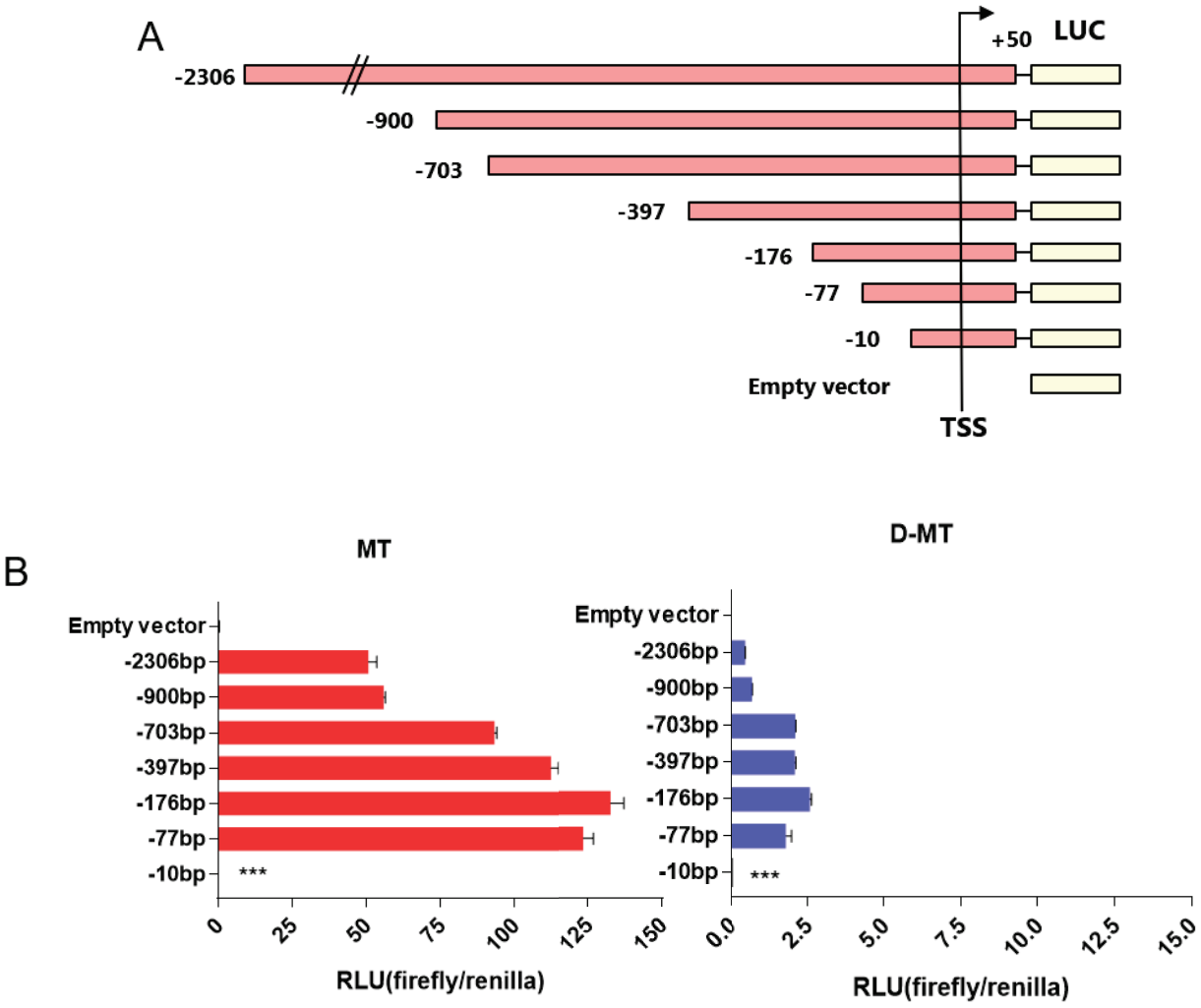



**Figure 5. Promoter deletion assay**

(A) Schematic image of the TRAP1 promoter deletion construct. Promoter construct includes transcription start site and downstream 50bp. Firefly luciferase gene inserted downstream of the promoter construct. (B) Deletion reporter assay in MT and D-MT cell by narrowing down from -2306 to -10. Data represent mean ± SD of two replicates.

**Deleting promoter region including putative transcription factor binding sites showed a significant reduction in promoter activity**

To find the most significant element for regulating TRAP1 expression, we analyzed the sequence between -77 and -10. According to the UCSC factor book, three positions are capable to be bound by transcription factors. The first position is C-Myc binding site, and the second position is TCF3 binding site, and the last transcription binding sequence is CREB1 and PAX2 binding site. **(Figure 6A)** To study which regulatory element is the most significant to regulate TRAP1 transcription, we generated site direct mutation for 3 elements. **(Figure 6B)** Each regulatory element sequences are deleted from the -176/+50 construct which shows the highest promoter activity. Deleting the C-Myc binding site showed 25% higher promoter activity compared to -176/+50 construct while deleting the TCF3 binding site reduced to 61% of the original construct. Promoter activity almost wiped out in the third construct, only showing 3.7% of the original construct. Of three candidates, CREB1 and PAX2 binding site affected to the promoter activity most significantly.

Since the TCF3 binding site and the CREB1 and PAX2 binding site showed significant decrease in promoter activity, we knockdown TCF3, CREB1 and PAX2 genes to test this gene involved in TRAP1 expression in GSC. However, after the genes were diminished, the TRAP1 expression did not change. **(Figure 7)**

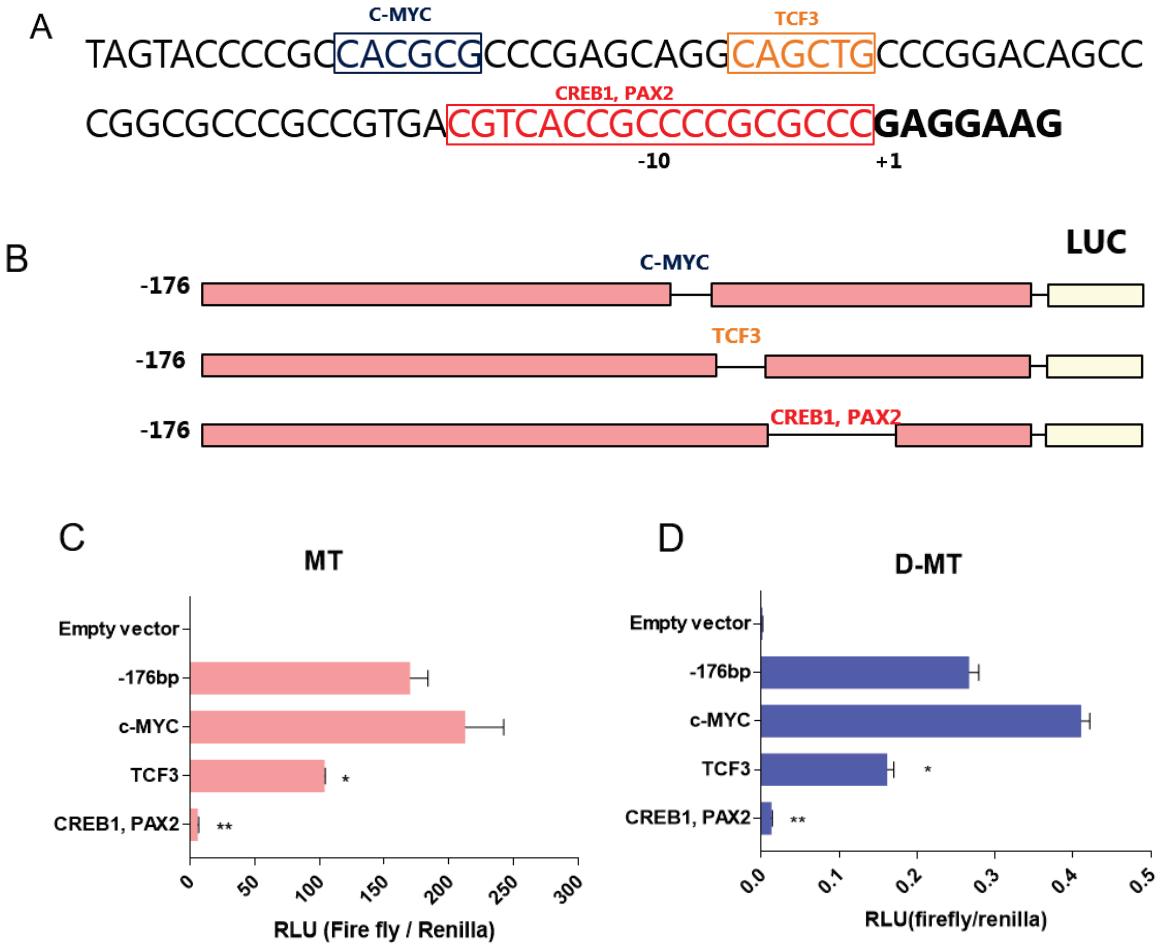



**Figure 6. E-box and CRE-CS is significant TRAP1 regulatory element**

(A) Sequence information of TRAP1 promoter between -77 and +7. According to the factor book, three elements are expected to role in transcription factor binding consensus. (B) Schematic image of site direct mutant construct for further luciferase reporter assay. (C) Result of luciferase reporter assay. Deleting the C-Myc binding site increased transcription activity, whereas deleting the TCF3 binding site and CREB1 and PAX2 binding site reduced the promoter activity. Data represent mean ± SD of two replicates

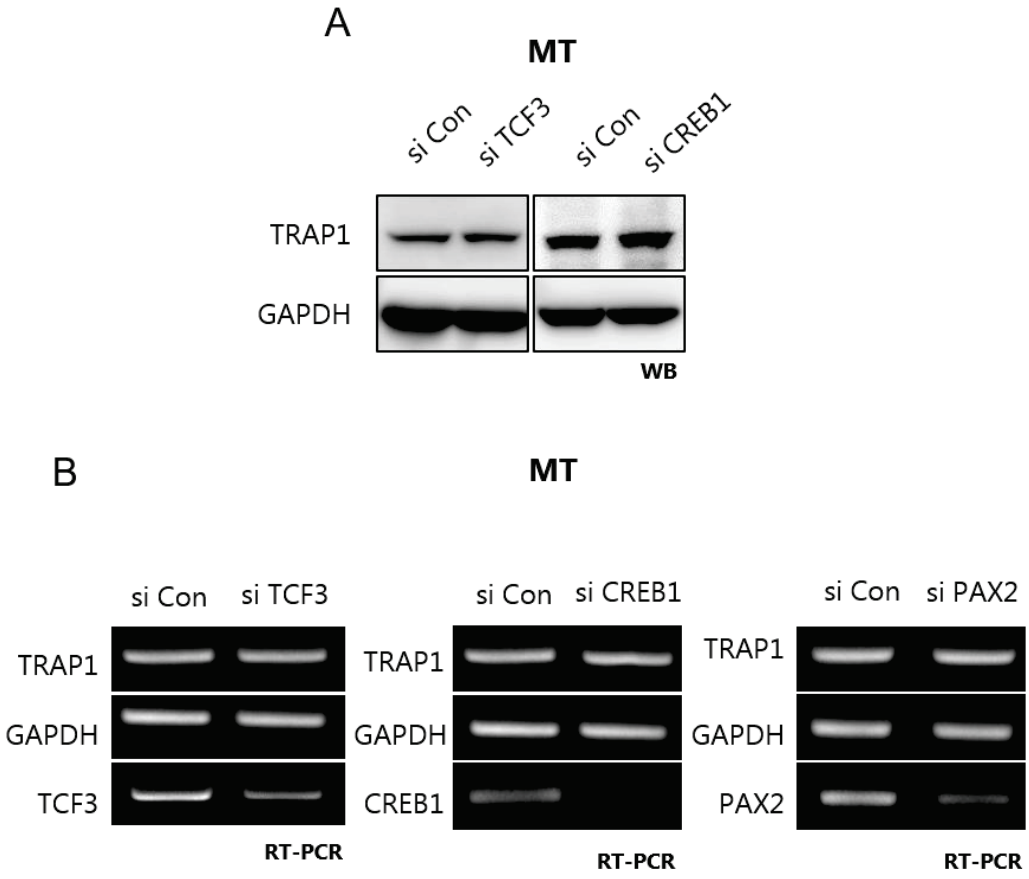



**Figure 7. Knock down of TCF3 and CREB1 in GSC**

(A) Protein expression of TRAP1 after TCF3 and CREB1 knockdown. (B) TRAP1 RNA level after Knock down of TCF1, CREB1 and PAX2

**c-AMP responsive element (CRE) is crucial for TRAP1 regulation**

To specify the most decisive element in the TRAP1 promoter, we compared the homology of TRAP1 promoter through the species. As a result, in mammalian TRAP1, CRE is highly conserved in the promoter, and every CRE is located close to TSS. **(figure 8A)** Conserved CRE consensus is completely maintain the sequences comparing the consensus logo provided by factor book **(figure 8B).**

CREB1and PAX2 binding site contains 5 base pairs of CRE consensus (CGTCA) and 13 base pairs of DNA sequence between CRE and TSS. **(Figure 6A)** CREB1 and PAX2 binding site-specific deletion mutant showed a dramatic reduction of promoter activity. **(Figure 6C)** We assumed that CRE is critical for TRAP1 transcription regulation. Thus, the activity reduction of the CREB1 and PAX2 binding site mutation is due to partial deletion of CRE consensus. We generated CRE specific deletion mutant and CRE mutation mutant by changing two middle nucleotides [CG] into [AT]. (Zhou et al., 2013) **(Figure 9A)** As a result, both CRE specific deletion and mutation showed a dramatic reduction in promoter activity. **(Figure 9B)** We concluded that CRE is the most powerful cis-acting element for TRAP1 transcription.

Next, we wondered what trans-acting element binds with CRE. As mentioned above, the result of luciferase reporter assay pattern was not different from GSC and differentiated GSC. **(Figure 5B, C)** However, MT showed much higher promoter activity quantitatively, referring that overall trans-acting element taking charge of TRAP1 expression would activate in GSC. We first checked the expression level of transcription factors those known as binding partners for CRE. **(Figure 10)** (Gazon, Barbeau, Mesnard, & Peloponese Jr, 2018) (Montminy & Bilezikjian, 1987) Among the transcription factors, only CREB1 and CREB5 showed reduced transcription levels after GSC differentiation. We knock down the CREB1 and CREB5 to confirm that elevated two transcription factors in GSC lead to TRAP1 upregulation. However, TRAP1 remained unchanged after CREB1 or/and CREB5 knockdown. **(Figure 11)**

A

**Homology through species**

```
Human          CCGGGGGCGGCGGGGCCACCTCCCCCACCTCCGCGCGCAAGGCCCGTCGCCCTTGACAAC   120
mouse          CCGGG----GACACTATAACCCTCACACGCACAATGCAAGGGATCAGCGACCTTGTCACT    72
Rat            CCGGG----GACACTAGAACCATCACACACTCCAGGCAAGGGGTCAGCGTCCTTGTCATT    71
Chimpanzee     CCGGGAGCGGCGGGGCCACCTCCCCCACCTCCGCGCGCAAGGCCCGTCGCCCTTGACAAC   97
Dog            GCCGCCGCCTCCCTCTCACGCCCTCCCGGGCCCGCGCACCCGCCCGTCGTCCTTGACAAC   84
Elephant       CCTAAGGAACTCAGCCCATCTACCCCAGCTCTGCGCGCACGGCCCGTCGCCCTTGACAAT   82
                *                *       *                 *  *  ** ***** **

Human          GCGATCCAAGGTCACGGAGGTCCCACCCCCAGGCCCGACCGTCCACGAGACGGGTAGTAC   180
mouse          GAGATCCAAGGTCAAAAAGTACAGTCTTCAGGGC-CCCCGCGCTGCGGTTTGCT------   125
Rat            GAGATCCAAGGTCAAAAAGTACGGTCTTTTGGCTCTCCCGCGCAGCAGTTTGCT------   125
Chimpanzee     GCGATCCAAGGTCACGGAGGTCCCACCCCCAGGCCCGACCGTCCACGAGACGGGTAGTAC   157
Dog            GCGACACAAGGTCACGGAGGGCCCGTCCCCAGGCCCGACGCTCCCCGCGCGGCGCGGGCC   144
Elephant       GCGACACAGGGTCACGGGAGTCCCGCCCCCAGGCCCGACGGACCCCGCGCCACAGAGACC   142
               * **  ** *****       *         *      *   *  *

Human          CCCG---------CCACGCGCCCGAGCAGGCAGCTGCC----------------------   209
mouse          ---------GCGTTCCCGTTCTCGCACAGGCAACGCCTTGGACAGCCCGTC---------   167
Rat            ---------GCGTTCGTGTTCTCGCACAGGCATCGCGTTGGACAGGCCGGCGCCTCCGTG   176
Chimpanzee     CCCG---------CCACGCGCCCGAGCAGGCAGCTGCC----------------------   186
Dog            ACCAGGGCCCCGCCCGCTCTCCCGCGCAGGCAGCGGGC----------------------   182
Elephant       TCGCAGGGACGCTCCCACCGCCTGCGCAGGCTACTGGC----------------------   180
                        *     *  *  *****  *

Human          --CGGACAGCCCGGCGCCCGCCGTGACGTCACCGCCCCGCGCCCGAGGAAGCCC-CGCCC   266
mouse          ---------------GCGCGCCATGACGTCACTGAGCCGCCCCCGCGGAAGGCCCCCTCA   212
Rat            ACGTCACAACCCGGCGCGCGCGATGACGTCATTGAGCCGCCCCTGCGGAAGGCCCCCTCG   236
Chimpanzee     --CGGACAGCCCGGCGCCCGCCGTGACGTCACCGCCCCGCGCCCGAGGAAGGCCCCGCCC   244
Dog            --CGGGCAGCCCTGCGCTCGGCGTGACGTCACCGCGCCGCGCCCGAGGAAGGCCCCGCCC   240
Elephant       --CGGGCAGCCCGGCGCGTTCCGTGACGTCACAGAGCCGCGTCCCAAGGGAGGCCCGCTC   238
                        **      ********  *  ****  *    *     * *
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B

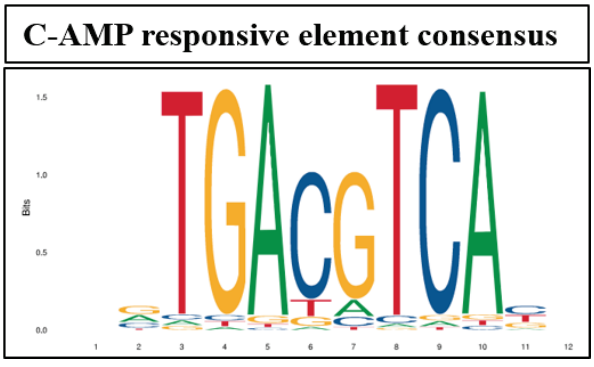



**Figure 8. Sequence homology of CRE in several mammalian.**

(A) CRE is highly conserved in mammalian. TRAP1 promoter. Genomic DNA sequence is extracted from ensembl genome browser. (B) CRE consensus logo from the factor book motif.

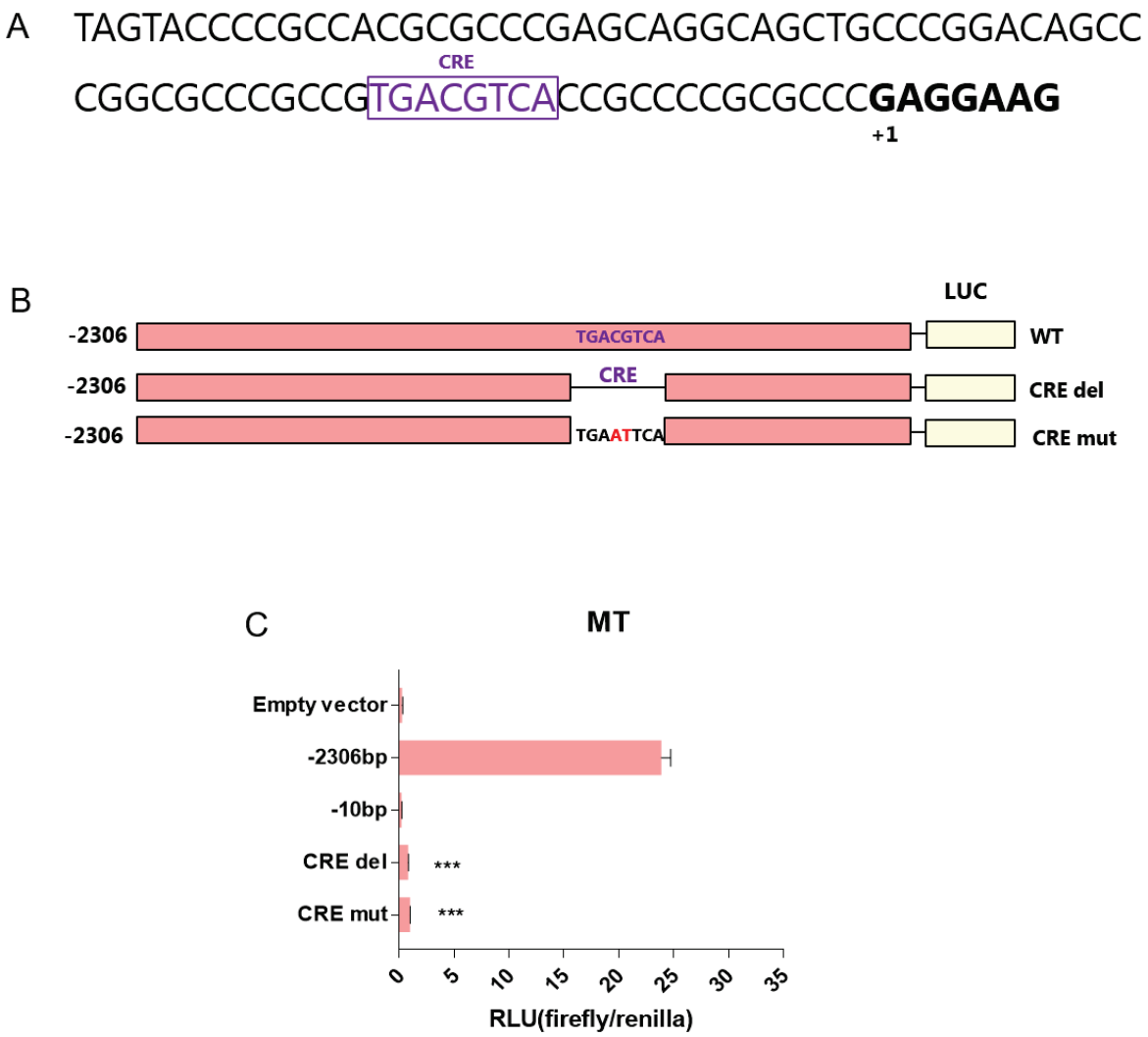



**Figure 9. Luciferase reporter assay in GSC of CRE deletion and mutation construct**

(A) DNA sequence of TRAP1 promoter including CRE consensus. (B) Schematic image of CRE deleted (CRE del) and CRE mutated (CRE mut) construct. Two nucleotide middle of CRE mutated. (C) CRE deleted and mutated promoter constructs showed decreased promoter activity in GSC. Data represent mean ± SD of two replicates

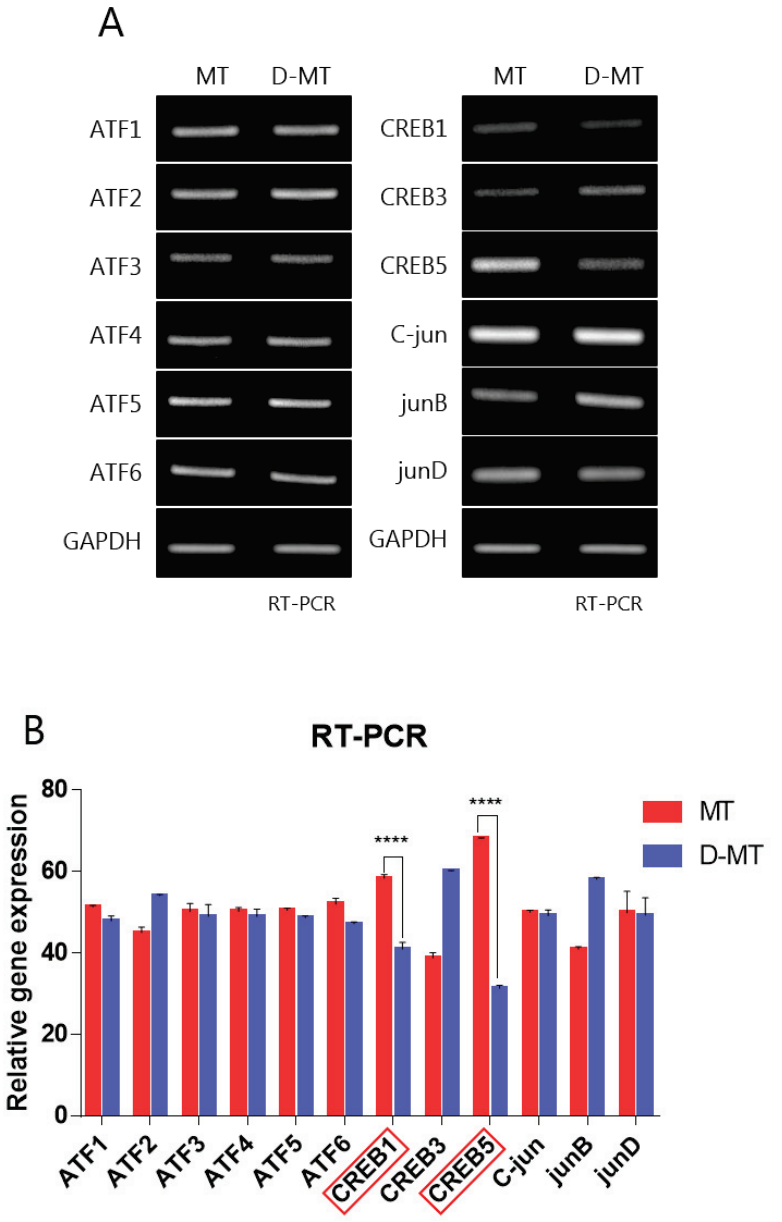



**Figure 10. CRE binding partner expression in cancer stem cell and differentiated cancer stem cell**

(A) Expression level of CRE binding partners in GSC and differentiated GSC. (B) Quantification of RT-PCR. Data represent mean ± SD of two replicates

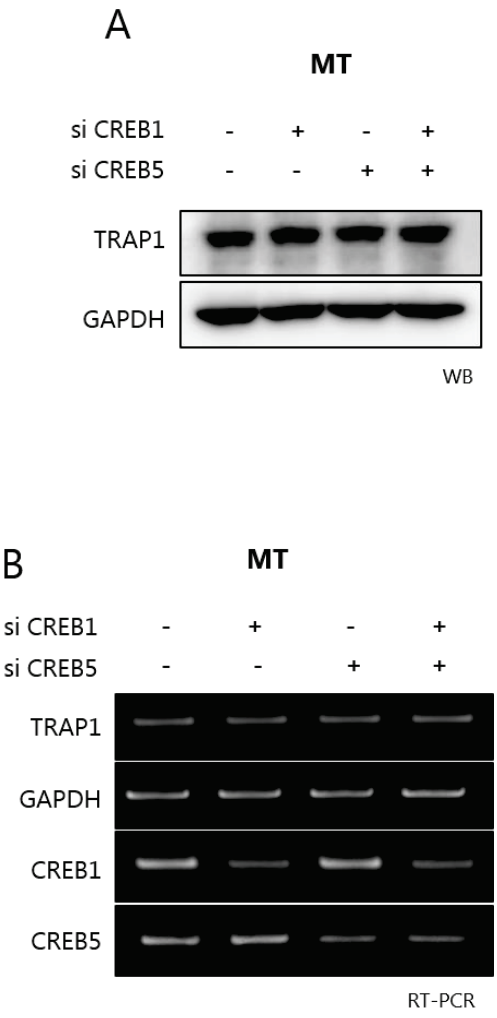



**Figure 11. CREB1 and CREB5 knockdown in MT cell**

TRAP1 expression after treating CREB1 and CREB5 si RNA in protein level (A) and transcriptional level(B).

## Discussion

TRAP1 is a molecular chaperone located in mitochondria. TRAP1 regulates the mitochondrial integrity by control the protein quality and response to mitochondrial stress. (B.-H. Kang, 2012) The protective role of TRAP1 makes it possible for cells to prevent death. This Hsp90 homolog molecular chaperone is highly expressed in cancer cells and powerful mitigation of cellular stress inducing from high proliferation and high mitochondrial function. It leads to drug resistance and apoptosis resistance. (B. H. Kang et al., 2007) TRAP1 abnormal high expression is characteristics of cancer cells and patients with high TRAP1 level appeared to have a poor prognosis. (Park et al., 2019)

Despite the importance of TRAP1 in cancer and GSC, the mechanism of how TRAP1 is upregulated in cancer and GSC remained unknown. The previous study about TRAP1 function in GSC showed that TRAP1 is regulated in the transcription level.

In this study, to elucidate the transcriptional regulatory mechanism of TRAP1, we analyzed the TRAP1 promoter. First, we utilized the database from genome browser to identify the gene regulatory element adjacent from TRAP1 TSS. Then, we defined the putative promoter, and by the luciferase reporter assay, we confirmed the putative TRAP1 promoter functions as expected. We narrowed down the promoter area to specify the key regulatory element. We concluded that DNA sequences between -77 and -10 contain the most significant regulatory element. Information from the factor book and the promoter homology throughout the species, we predicted that the c-AMP responsive element is the most significant candidate as TRAP1 key regulatory element and this hypothesis confirmed through the site-specific mutant. Throughout the 2kb promoter, by deleting the CRE consensus or mutating two nucleotides of the CRE consensus, TRAP1 promoter activity crushed down. This means that TRAP1 transcription is highly depending on this sequence.

In this study, we could not find out the key trans-acting element. CRE is expected to serve several transcription factors, such as CREB/ATF family and AP1 family. We found out the expression difference of transcription factors in MT and D-MT cells and CREB1 and CREB5 are maintaining high expression level in MT. We knock down the CREB1 and CREB5 and observe the consequence. Down-regulation of two genes did not change TRAP1 expression. Other transcription factors are possible to participate in transcription regulation according to their activation state. Even though they maintain the low expression level, with the high activation status, it can functionally regulate the transcription activity. Furthermore, identifying the signaling pathway driving transcription factor activation and translocation to the nucleus, we can track the connection between signaling pathways responding to mitochondrial stress signal and how those pathways interact with each other.

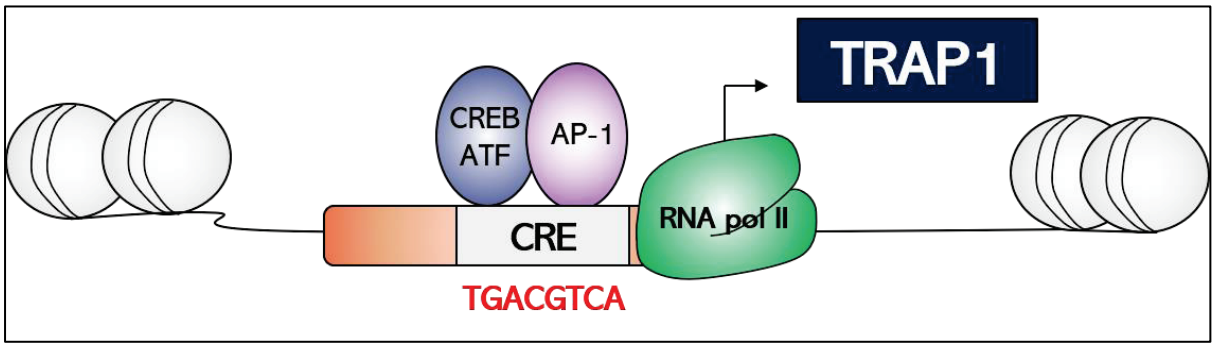



**Figure 12. Hypothetical image of TRAP1 transcription regulation on the promoter**

TRAP1 is expected to be controlled precisely by the CRE-partner transcription factor direct binding with transcriptional preinitiation complex adjacent to TSS.

# [Chapter 2. Development of TRAP1 inhibitor for future cancer drug]

## Introduction

Heat shock protein 90 (Hsp90) is a molecular chaperone known as a key component of a multichaperone complex with crucial functions in the development and progression of pathogenic cellular transformation. Client proteins have been revealed performing a central role in cancer development and procession. (Chiosis, 2006; Neckers & Neckers, 2005; Powers & Workman, 2006) With those properties, Hsp90 became a fascinating drug target, and several Hsp90 inhibitors have been developed. Hsp90 inhibitors showed anti-cancer drug efficiencies, but not effectively in vivo. (B. H. Kang & Altieri, 2009; B. H. Kang et al., 2009)

TNF-receptor associated protein 1 (TRAP1) is mitochondrial chaperone homolog to Hsp90. TRAP1 is considered as the dominant chaperone in mitochondria rather than Hsp90. (C. Lee et al., 2015) Additionally, TRAP1 plays critical roles in maintaining mitochondrial homeostasis and inducing drug resistance. Also, TRAP1 is exclusively expressed in cancer cells, and organelle-specific, which is mitochondria. These properties can reduce off-target side effects, thus make TRAP1 as a novel anti-cancer drug target protein.

Many Hsp90 inhibitors showed in vitro binding affinity with TRAP1, mitochondrial Hsp90 homolog protein. (B. H. Kang & Altieri, 2009; Patel et al., 2013) However, inhibitors showed low TRAP1 inhibition in cell level, because of the limitation of binding that most of the inhibitor cannot accumulate into mitochondria. This phenomenon caused to lower drug efficiency. (B. H. Kang & Altieri, 2009; B. H. Kang et al., 2009; C. Lee et al., 2015) Anti-cancer drug efficiency of Hsp90 inhibitors has increased by accumulating inhibitors in mitochondria. (B.-H. Kang, 2012; B. H. Kang et al., 2010) Mechanism of mitochondrial accumulated Hsp90 inhibitors mediates inhibition of Hsp90 and TRAP1. Further research has been performed on developing TRAP1specific inhibitors. (Park et al., 2017) It revealed TRAP1 specific inhibitors or mitochondrial accumulated Hsp90 inhibitors which effectively bind with TRAP1 showed highly improved anti-cancer efficiency. (C. Lee et al., 2015; Park et al., 2017) In this study, we found new TRAP1 inhibitor, SJ-T140, much improved in vivo activity compared to mitochondrial Hsp90 inhibitor Gamitrinib which used as a positive control.

# Material and method

## 1. Cell culture

Human glioblastoma cell line LN229 and human prostate cancer cell line PC3 were purchased from American Type Culture Collection (ATCC). LN229 was cultured in DMEM(Gibco) supplemented with 5% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). PC3 was cultured in RPMI (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco).

## 2. Immunoblotting analysis

Whole lysate from cells was resuspended in RIPA with protease inhibitor cocktail and phosphatase inhibitor cocktail (R&D system). Lysates were centrifuged at 15000rpm for 10minute at 4℃. Protein concentrations were measured by protein assay dye reagent concentrate (Bio-Rad). 20-25㎍ of protein was loaded for immunoblotting analysis. Listed antibodies were used: ACTIN (Millipore, MP691001), SDHB (Santa Cruz, SC-271548), Sorcin (Santa Cruz, SC-100859) Horseradish peroxidase-conjugated mouse or rabbit secondary antibodies (KLP Inc.) Membranes were detected by LAS 4000 (GE Healthcare) with clarity western ECL substrate (Bio-Rad)

## 3. MTT assay

Cells were seeded on 96well plates and attached for 24hr before drug treatment. Drugs were dissolved in DMSO and the final DMSO proportion in media was 0.5%. Cells were incubated with the drugs for 24hr and at 20hr, 3-(4,5-dimethyl-thyzoyl-2-yl)-2,5- diphenyltetrazolium bromide (MTT) treated and incubated for 4hr to generate insoluble formazan. Cytotoxic effect of the anti-cancer drug was determined using MTT assays and was calculated by measuring the absorbance of the tetrazolium at 595 nm. Absorbance value was normalized to the solvent control and data were shown as percent viability.

**4. Fluorescence polarization**

Recombinant TRAP1 is prepared described in (C. Lee et al., 2015). For fluorescence polarization experiments, the fluorescence probe 1- FITC3 preparation is described in (Taldone et al., 2013). 10 nM of 1-FITC3 and 400 nM of protein were incubated for 1 h at 25 °C with described concentrations of inhibitors in FP buffer consisting with 135 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, 1 mM DTT, 2 mM MgCl2, 0.1 mg/mL BSA, and 0.05% NP40. Fluorescence polarization was measured using a SYNERGY NEO microplate reader (BioTek Instruments, Inc.).

**5. Analysis of mitochondrial ROS and mitochondrial membrane potential**

To measure mitochondrial membrane potential and mitochondrial ROS induced by drugs, PC3 cells were treated with SJ-T140 and gamitrinib 10 μmol for 6h and incubated with 100nM of Mito-Sox (Invitrogen, M36008) or 100nM of TMRM with sytox for 20 min at 25 °C. Subsequently, the cells were diluted with PBS and analyzed using the FACS Calibur system (BD Bioscience).

**6. Tumor Xenograft Experiments.**

PC3 cells ($1 \times 10^7$) were suspended in 100 μL of PBS and were injected subcutaneously into both flanks of 8-week-old BALB/c nu/nu male mice (Charles River Laboratories). Tumor grew until the average volume of approximately 80 $mm^3$ and animals were randomly grouped. Subsequently, DMSO (vehicle), Gamitrinib (Positive control) and SJ-T140 dissolved in DMSO were each mixed with polyethylene glycol 400 (PEG 400) and PBS at a 1:1:3 ratio and was generated to mice as intraperitoneal injection (5 mg/ kg) every day. Tumors were measured using calipers, and tumor volumes were calculated using the following formula: $V = 0.5 \times (\text{width})^2 \times \text{length}$. At the end of the experiment, animals were euthanized, and organs including heart, kidney, liver, lung, spleen, stomach, and tumor were collected.

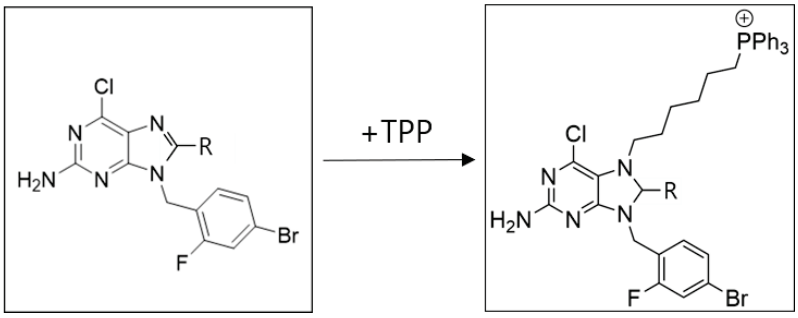
# Result

**TRAP1 binding compound SJ-T104 improved cancer-specific cytotoxic activity after modification to accumulate in mitochondria**

SJ-T104 compound had been selected as TRAP1 binding inhibitor through chemical screening. This compound binds with TRAP1 protein in vitro very specifically **(Figure 1B)**, however showed low cytotoxic effect on the cancer cell and hepatocyte **(Figure 1D)**. We hypothesized that even SJ-T104 has a unique binding affinity with TRAP1, this compound is not able to accumulate in mitochondria. Thus, lowered the TRAP1 inhibition activity in cancer cells showing poor cytotoxic effect. To accumulate drugs in mitochondria, we conjugate SJ-T104 with mitochondrial targeting molecule, triphenylphosphonium (TPP), and this compound is SJ-T140. **(Figure 1A)**

TPP conjugation with SJ-T104 (SJ-T140) reduced TRAP1 specificity in vitro, however comparing the gamitrinib, mitochondrial Hsp90 inhibitor, SJ-T140 still showed high TRAP1 binding affinity **(Figure 1B)**. Interestingly, SJ-T140 had much higher cancer cytotoxicity than SJ-T104, because TRAP1 specific binding part SJ-T140 inhibit TRAP1 directly after drug accumulation in mitochondria. SJ-T140 showed a comparative higher anti-cancer effect than gamitrinib in both prostate cancer and glioblastoma cancer cell lines. **(Figure 1D)** However, SJ-T140, SJ-T104, and gamitrinib showed low cytotoxicity on normal cells in respective concentrations, referring that in those dose conditions SJ-T140 remove cancer cells exclusively **(Figure 1D).** IC50 value of SJ-T140 and gamitrinib in cancer inhibition on PC3 and LN229 cell lines are 3.23 μmol, 1.35 μmol,5.239 μmol, and 4.193 μmol in respective, showing that SJ-T140 is more effective in inducing cancer cell death.

A

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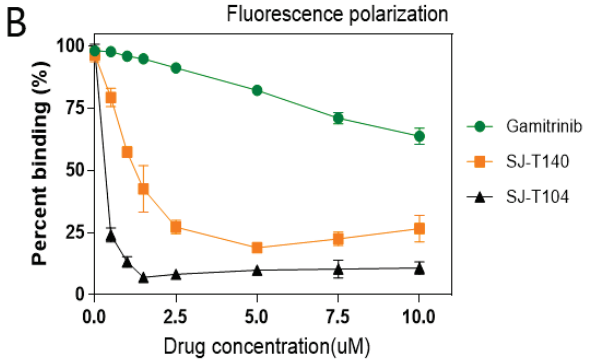
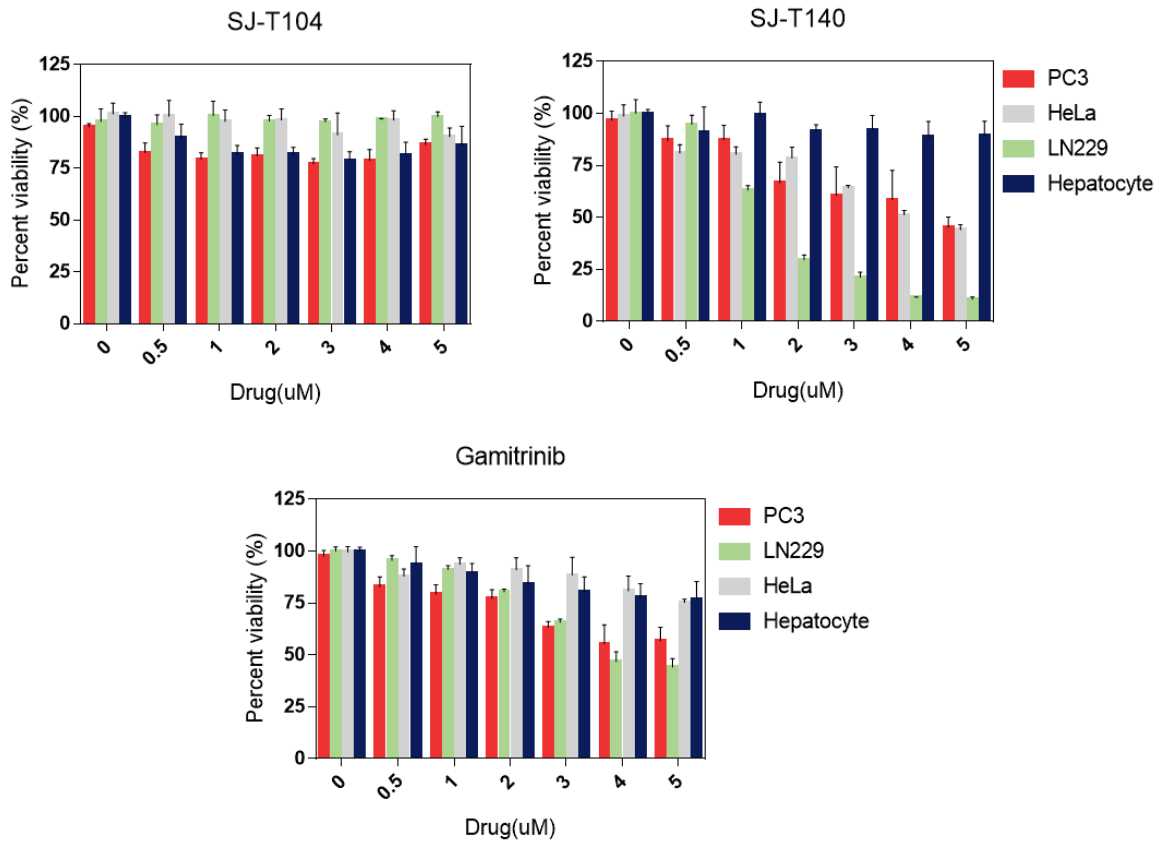



C

| Drug | IC50 (uM) |
|---|---|
| Gamitrinib | 4.782 |
| SJ-T140 | 0.8622 |
| SJ-T104 | 0.3225 |

D




E

| Drug\cell line | IC50 (uM) | | |
|---|---|---|---|
| | PC3 | LN229 | Hepatocyte |
| Gamitrinib | 5.239 | 4.193 | - |
| SJ-T140 | 3.25 | 1.35 | - |
| SJ-T104 | - | - | - |

**Figure 1. Mitochondrial accumulation of TRAP1 specific inhibitor showed increased cytotoxic ability in cancer-specific manner.**

(A) Molecular structure of TRAP1 specific inhibitor SJ-T104 and SJ-T140. TPP represents triphenylphosphonium. (B) Fluorescence polarization of SJ-T104, SJ-T140, and gamitrinib. Binding affinity measured in described concentration, 0 values were set by adding 10 micromoles of PU-H71, a competitive inhibitor of TRAP1, to 10 micromoles of the drug's maximum concentration. Data represent mean ± SD of three replicates. (C) IC50 value of TRAP1 binding affinity of each drug. Values were calculated in using the software program Prism 7.0 (GraphPad). (D) Cytotoxic activity of SJ-T140, SJ-T104, and gamitrinib. Cell viability is measured after incubating cells with chemicals for 24hr. values represent mean ± SD of three replicates. (E) IC 50 value of cytotoxic activity on caner cells and normal cell. Three chemicals showed innoxious toxicity to hepatocyte in represented dose, whereas SJ-T140 and gamitrinib showed cytotoxic effect on cancer cells.

**SJ-T140 inhibits TRAP1 results in TRAP1 client degradation and induces mitochondrial stress.**

TRAP1 regulates cellular oxidative stress through managing mitochondrial ROS and regulates energy metabolism by orchestrating with electron transport chain complex. TRAP1 inhibitors suppress chaperoning function, then they inhibit protein quality control maintained through protein structure retention and folding refolding of clients. TRAP1 clients, SDHB and Sorcin disrupted their stability after 6hr exposure to inhibitors in dose-dependent manner. **(Figure 2A)**

After cell incubated with 10μmol of chemicals for 6hr, we observed the impact on mitochondria. We made sure to analyze live cells only by co-staining with sytox with TMRM and Mitosox. Using the flow cytometer, we measured mitochondrial membrane potential and mitochondrial ROS level. Compared to DMSO, both gamitrinib and SJ-T140 reduce membrane potential, considering severe energy depletion occurs due to mitochondrial dysfunction. Additionally, both chemicals enhanced mitochondrial ROS, meaning that TRAP1 functional failure induces excessive oxidative stress that induces apoptosis. Thus, we refer that cytotoxic activity after TRAP1 inhibitors treatment occurs apoptotic cell death mediated by mitochondrial stress. **(Figure 2B)**

**SJ-T140 dramatically improved in vivo activity compare to mitochondrial Hsp90 inhibitor gamitrinib**

We had discovered TRAP1 inhibitor SJ-T140 that specifically binds with TRAP1 in vitro and inhibits TRAP1 function in cancer cells. As a result, SJ-T140 induces cell death which is mediated by mitochondrial stress. This pro-apoptotic process occurs in the cancer cell-specific manner which shows high TRAP1 expression. Thus, we recognized SJ-T140 as an effective TRAP1 inhibitor and a potential anti-cancer drug.

We performed xenograft assay in order to find out SJ-T140 inhibition ability to tumor progression in vivo. Cancer cells injected to mice flank and 5mg/kg of chemicals and DMSO (vehicle) were injected every day for 24days. As a result, SJ-T140 treated mice group showed dramatic growth inhibition, however gamitrinib which showed similar cell toxicity and mitochondrial dysfunction was not able to repress tumor growth. **(Figure 3A)** Tumor mass showed consistent result. **(Figure 3B)** Thus, we concluded that new anti-cancer drug candidate, SJ-T140 showed improved cellular anti-cancer effect, and showed a surpassing effect on cancer in vivo by specifically targeting mitochondrial chaperone TRAP1.

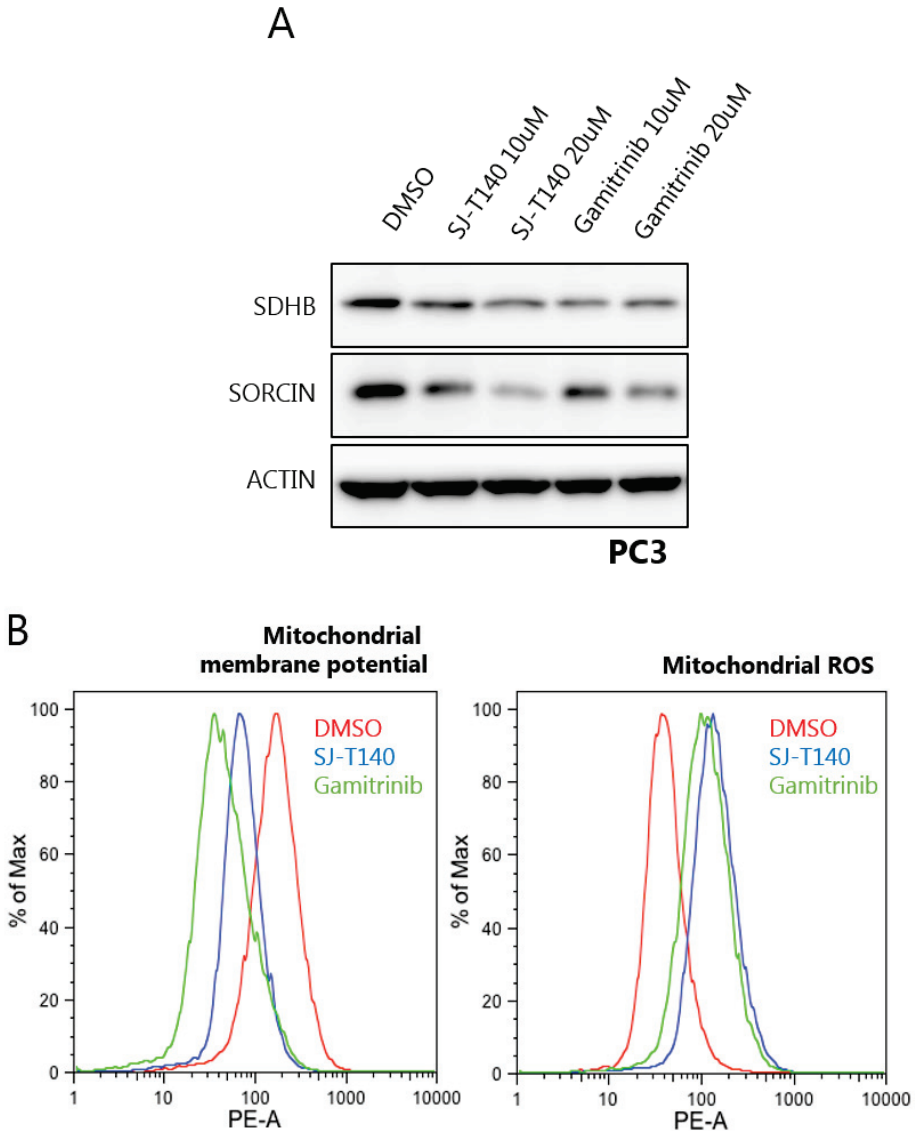



**Figure2. Cytoplasmic analysis of TRAP1 inhibition.**

**(**A) TRAP1 client stability after 6hr exposure to SJ-T140 and gamitrinib. SDHB and Sorcin was degraded dose-dependent manner on PC3 cell. Actin was used as a loading control. (B) Analysis of mitochondrial function after inhibitor treatment. Mitochondrial membrane potential and mitochondrial ROS were measured in PC3 cell line after 6hr exposure to 10μmol of TRAP1 inhibitor SJ-T140 and positive control gamibrinib. Red peak represents negative control DMSO, blue peak represents SJ-T140, and green peak represents gamitrinib. Cells were co-stained with sytox and gated only sytox negative cells. All groups showed at least 97% cell viability.

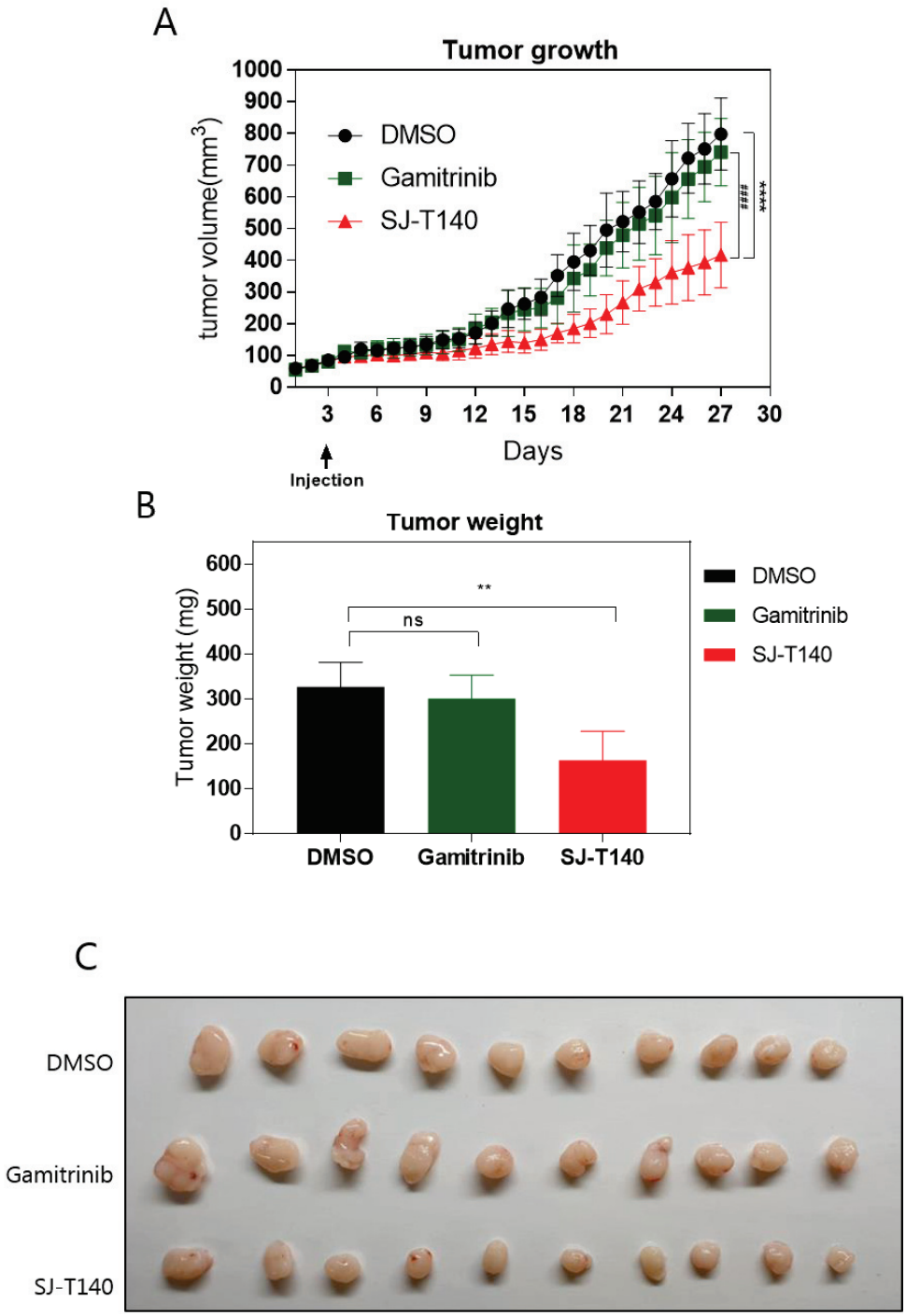



**Figure 3. SJ-T140 showed dramatic tumor repression in vivo.**

(A) Tumor growth in the xenograft model. Cells were subcutaneously injected on the mice, on day 3, chemicals begun to inject to randomly grouped mice every day. Mice were sacrificed on day 27. Tumor volume and mice weight were measured every day. All group n=5. (B) Tumor weight after tumor harvest (C) Gross image of tumor after harvest. Statistical analysis performed on software program Prism 7.0

## Discussion

Hsp90 is overexpressed in cancer cells and aggravates through its client protein signaling. Many Hsp90 inhibitors have been developed as anti- cancer drug, but they showed disappointing anti-cancer abilities. TRAP1 is a mitochondrial chaperone, structurally resemble Hsp90. Hsp90 inhibitors are possible to bind with TRAP1 in vitro, however they cannot inhibit TRAP1 in the cellular level because Hsp90 inhibitors hardly approach and accumulate mitochondria. Conjugating Hsp90 inhibitors with mitochondrial targeting chemical, TPP, these inhibitors showed higher anti-cancer effect. Previously, TRAP1 is revealed dominant chaperone in cancer mitochondria, not Hsp90, and realized that TRAP1 specific chemicals showed improved drug effect than mitochondrial Hsp90 inhibitor.

In this study, SJ-T104, the precursor of SJ-T140, was discovered as TRAP1 inhibitor through chemical screening and by adding TPP, SJ-T140 acquire cancer cell toxicity. Compared to gamitrinib, mitochondrial Hsp90 inhibitor, SJ-T140 took a slight improved cytotoxic effect with similar mitochondrial dysfunction. However, SJ-T140 showed significantly improved in vivo activity. Thus, we identified new TRAP1 inhibitor SJ-T140 as improved in vivo activity anti-cancer drug candidate.

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