Human mismatch repair gene, *MLH1*, is transcriptionally repressed by the hypoxia-inducible transcription factors, DEC1 and DEC2

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Abstract

Tumor hypoxia has been reported to cause a functional loss in DNA mismatch repair (MMR) system as a result of down-regulation of MMR genes, although the precise molecular mechanisms remain unclear. In this study, we focused on the down-regulation of a key MMR gene, *MLH1*, and demonstrated that hypoxia-inducible transcription repressors, DEC1 and DEC2, participated in its transcriptional regulation *via* their bindings to E-box-like motif(s) in *MLH1* promoter region. In all cancer cell lines examined, hypoxia increased expression of *DEC1* and *DEC2*, known as hypoxia-inducible genes, but decreased *MLH1* expression in an exposure time-dependent manner at both the mRNA and protein levels. Co-transfection reporter assay revealed that DEC1 and, to greater extent, DEC2 as well as hypoxia repressed *MLH1* promoter activity. We further found that the action was remarkably inhibited by trichostatin A, and identified a possible DEC-response element in the *MLH1* promoter. *In vitro* electrophoretic gel mobility shift and chromatin immunoprecipitation assays demonstrated that DEC1 or DEC2 directly bounds to the suggested element, and transient transfection assay revealed that overexpression of DEC2 repressed endogenous MLH1 expression in the cells. Hypoxia-induced DEC may impair MMR function through repression of *MLH1* expression, possibly *via* the histone deacethylase (HDAC)-mediated mechanism in cancer cells.

Introduction

Hypoxia is a common feature in many solid tumors and the microenvironment is now recognized as a key factor linked to the biologically aggressive phenotypes and their resistance to chemotherapeutic agents and irradiation therapies (Teicher, 1994; Brown *et al*, 1998; Cairns *et al*, 2006). Extensive studies of molecular mechanisms have shown that transcription factor hypoxia-inducible factor-1α (HIF-1α) is a key regulator of hypoxic reaction; these studies have led to a better understanding of the mechanisms of HIF-1α activation and the subsequent alteration of gene expressions under hypoxic conditions (Harris, 2002; Denko *et al*, 2003; Semenza, 2003; Poellinger *et al*, 2004).

Recently, revealing findings have reported that hypoxia can reduce expression of several DNA repair genes - *MLH1*, *RAD51*, *BRCA1* and *MSH2* - resulting in genomic instability in several cancer cell lines (Mihaylova *et al*, 2003; Bindra *et al*, 2004; Bindra *et al*, 2005; Koshiji *et al*, 2005; Bindra *et al*, 2006; Bindra *et al*, 2007). Since the human mismatch repair (MMR) system plays a critical role in the maintenance of genomic integrity, the mechanisms of transcriptional repression, especially in *MLH1* and *MSH2* genes, are of key importance in tumor biology: Germline mutations in *MLH1* (~50%) and *MSH2* (~40%) exist in approximately half of all hereditary non-polyposis colorectal cancer patients (Peltomäki, 2001; Hoeijmakers, 2001). Under hypoxic conditions, the cellular DNA repair function becomes impaired, which causes hypermutability to DNA damage (Reynolds *et al*, 1996; Yuan *et al*, 2000). These findings strongly suggest that tumor hypoxia probably causes loss of genomic stability through suppression of MMR functions, and that defects of MMR function may dramatically increase mutation rates.

These studies have also suggested that a transcription factor, E2Fs, p130, HIF-1α, SP-1, or Myc/Max system may participate in the mechanisms of down-regulation of *BRCA1*, *RAD51*, *MSH2* or *MLH1*, but details remain unclear. Among numerous hypoxia-inducible genes, differentiated embryo chondrocyte (DEC) 1 and 2 may be the most likely candidates (Ivanova *et al*, 2001; Miyazaki *et al*, 2002). DEC1 and DEC2 have been reported to participate in the transcriptional repression of *PPARG*, *PER*, *STAT1* and themselves *via* E-box or other motifs in their promoter regions, which results in the regulation of adipogenesis, circadian rhythm, immune system or carcinogenesis (Yun *et al*, 2002; Honma *et al*, 2002; Ivanova *et al*, 2007). *DEC1* (also known as *BHLHB2* / *STRA13*) was originally identified as the gene expressed in cAMP-dependently differentiated embryo chondrocytes that encodes a basic helix-loop-helix (bHLH) transcription factor (Shen *et al*, 1997); *DEC2* (also known as *BHLHB3* / *SHARP1*) was identified from a human expression sequence tag (EST) database as a member of DEC subfamily (Fujimoto *et al*, 2001).

In the present study, we focused on the mechanisms of down-regulation of *MLH1*, and demonstrated for the first time that the hypoxia-inducible transcription repressors DEC1 and DEC2 participated in the transcriptional regulation through their bindings to E-box-like motif(s) in *MLH1* promoter region. These findings may contribute to a better understanding of the biological functions of tumor hypoxia, based on the novel proposal that hypoxia-inducible DEC can impair MMR function through repression of *MLH1* expression, and may subsequently cause genomic instability in cancer cells.

Results

MLH1 expression at both protein and mRNA levels under hypoxic conditions

We first examined whether hypoxia decreased MLH1 expression in cancer cells. HepG2 cells were collected after incubation under normoxic or hypoxic conditions for various periods. Immunoblotting using whole cell extracts revealed that hypoxia decreased MLH1 protein up to 48 hours in an exposure-time dependent manner, unlike the stable expression levels of β-actin (Figure 1a). The hypoxic induction of HIF-1α was confirmed at well-detected protein levels as well as that of DEC1, a known hypoxia inducible transcriptional repressor, whereas aryl hydrocarbon receptor nuclear translocator (Arnt), also known as HIF-1β, and β-actin constitutively expressed (Figure 1a). Next, *MLH1* mRNA levels were evaluated along with hypoxia-inducible genes, *DEC1* and *DEC2*. Real-time RT-PCR analyses demonstrated that *MLH1* mRNA level alone decreased from 6 to 48 hours with hypoxic treatment (Figure 1b). In contrast to *MLH1*, expressions of *DEC1* and *DEC2* increased under hypoxic conditions, despite of the relatively short duration of *DEC2* up-regulation (Figure 1b). We further examined mRNA expression of these genes in the other cell lines and found the similar expression patterns (Figure 1c).

Promoter activities of MLH1 and DEC

To clarify the mechanisms of the decreased *MLH1* mRNA level, we next subcloned the 5' region of human *MLH1* (from -1653 to -4) into a luciferase reporter plasmid, pGL3-Basic vector, designated as a pGL-MLH1Pro1.65 (Figure 2a). Transient transfection into HepG2 revealed that pGL-MLH1Pro1.65 has strong promoter activity in comparison with an empty plasmid vector pGL3-basic under normoxic conditions. Since the pGL3-basic vector itself has a lot of hypoxia response element (HRE) consensus sequences (according to Promega), background reporter activities were increased under hypoxic conditions (data not shown). Therefore, *MLH1* promoter was swapped into pGL4.10 plasmid vector, in which consensus sequences

for transcription factors were reduced from backbone sequences (according to Promega), and transient transfection experiments were performed. As we expected, *MLH1* promoter reporter was down-regulated under hypoxic conditions, suggesting that the promoter region contained hypoxia response repression sequences (Figure 2b). Interestingly, this promoter activity was repressed by co-transfection with DEC1 or DEC2 expression plasmid vector in a dose-dependent manner, and the repression of *MLH1* promoter activity was notable when DEC2 was co-transfected (Figure 2c).

As histon deacetylase (HDAC)-dependent mechanisms had been previously suggested (Sun *et al*, 2000), TSA treatments remarkably canceled the repression of *MLH1* promoter activity by DEC in a treatment-dose dependent manner without any detectable cytotoxicity (Figure 2d). Moreover, mutant-type of DEC1 which had DNA binding domain but lacked most of functional domains (Li *et al*, 2003; Sato *et al*, 2004) failed to repress *MLH1* promoter activities, even enhanced them, suggesting that just a competitive occupancy on the promoter was not sufficient to explain the repression (Figure 2e).

Response element to DEC on MLH1 promoter region

To identify a response element to DEC in the *MLH1* promoter region, we constructed a series of deletion mutants of *MLH1* promoter reporter (Figure 3a). The luciferase reporter assays for co-transfection with pcDNA (vector only) revealed that *MLH1* promoter had several putative positive (from -556 to -274)- and negative (from -893 to -557)-regulatory regions. We also found that promoter activity of pGL-MLH1Pro0.27 was almost identical to that of pGL-MLH1Pro1.65, indicating that the region from -273 to -4 probably contains critical regulatory regions. Furthermore, co-transfection with DEC-expressing pcDNAs showed that all of the reporter activities

were significantly repressed by DEC, suggesting that the region from -273 to -4 is the most likely site containing the DEC-response element (Figure 3a).

We therefore constructed four mutant reporters in which several nucleotides were substituted in the putative E-box motifs (Figure 3b): Three mutants (MT1-3) showed stronger activity than that of the wild-type promoter reporter (Figure 3c). Co-transfection experiments with DEC indicated that MT1 and MT2 showed resistance to the repression caused by DEC, whereas MT3 and MT4 were significantly repressed, as strongly as the wild type was (Figure 3c). These results suggested that DEC might repress *MLH1* expression through their bindings to the indicated region containing putative E-box motifs.

Direct binding of DEC to the response element containing E-box motif on MLH1 promoter

To demonstrate that DEC directly binds to the response elements, we performed an electrophoretic gel mobility shift assay (EMSA) with ^{32}P-labeled probes containing DEC-response elements in the *MLH1* promoter from -69 to -47. DEC1 and DEC2 were synthesized using *in vitro* transcription/translation system, and protein amounts were equally adjusted by calculation of incorporated ^{35}S-labeled methionines. A ^{32}P-labeled probe was incubated with synthesized proteins and subjected to electrophoresis. EMSA showed that both DEC1 and DEC2 specifically bound to these elements (Figure 4a), DEC1 or DEC2 probe-specific DNA binding complexes had shifted, and the complexes formed were competed out by pre-incubation with the non-labeled probes or specific antibodies for DEC1 or DEC2. The observed intensities of shifted bands indicated that the binding activity of DEC2 to this probe was much stronger than that of DEC1.

Chromatin immunoprecipitation (ChIP) assay was then performed after incubation of HepG2 cells in normoxia or hypoxia for 24 hours to examine the binding of endogenous DEC to response elements in *MLH1* promoter. Real-time PCR clearly demonstrated that immunoprecipitation of the chromatin fragment containing the DEC-response element in *MLH1* promoter was increased in the hypoxic samples pre-incubated with anti-DEC1 antibody, indicating that DEC1 specifically bound to the elements (Figure 4b).

Expression of DEC and endogenous MLH1 protein

To confirm the function of DEC on MLH1 at the cellular level, we investigated endogenous MLH1 protein in cells overexpressing DEC. Immunoblotting analysis using whole cell extract prepared from HepG2 cells transiently transfected with DEC1 or DEC2 demonstrated that MLH1 protein decreased and inversely associated with the expression levels of DEC (Figure 5a). To confirm the suggested function of DEC on MLH1 at each cellular level, we then performed immunostaining in HepG2 transiently transfected with DEC2. Double staining with anti-MLH1 and -DEC2 showed that MLH1 and DEC2 were compensatively expressed in each cell: MLH1 expression was significantly decreased in the DEC2 overexpressed cells, while high expression levels of MLH1 were maintained in cells without DEC2 expression (Figure 5b). Next, we performed knock-down assay for *HIF1A*, *DEC1*, or *DEC2*, to estimate how HIF-1-DEC pathway contribute to the *MLH1* expressions. As results, transient transfection of specific siRNA for *HIF1A* in HSC-2 represented more than 80% reduction of *HIF1A* expression compared to that of non-specific (NS) siRNA as well as significant repression of *DEC1* and *DEC2*, and hypoxic repression of *MLH1* disappeared (H/N ratios of siNS : si*HIF1A* = 0.62 : 0.94) (Figure 5c). Interestingly, *DEC1* knock-down represented a little increased expression of *MLH1* under both

normoxic and hypoxic conditions. Since DEC1 represses *DEC2* expression (Li *et al*, 2003), *DEC1* knock-down resulted in increased *DEC2* expression and persistence of the hypoxic repression of *MLH1* (H/N ratios of si*DEC1* = 0.69). On the other hands, *DEC2* knock-down strikingly increased expression of *MLH1* under hypoxic condition, indicating complete attenuation of hypoxic repression of *MLH1* (H/N ratios of si*DEC2* = 1.01).

Discussion

Hypoxic reaction has been clearly shown to involve alterations in gene transcription (Harris, 2002; Denko *et al*, 2003; Semenza, 2003; Poellinger *et al*, 2004), and hypoxia-inducible factor-1 (HIF-1) is well known as the pivotal factor that regulates cellular responses to hypoxia *via* transactivation of a variety of genes. We previously demonstrated that DEC1 and DEC2 were transcriptionally activated by HIF-1, suggesting their crucial roles in HIF-1 mediated cellular hypoxic reaction (Miyazaki *et al*, 2002). The mechanisms of the activation of HIF-1 and the subsequent transactivation of various genes have also been intensively studied, which has promoted a better understanding of the genetic and molecular basis underlying intricate hypoxic reactions of cells (Harris, 2002; Denko *et al*, 2003; Semenza, 2003; Poellinger *et al*, 2004). However, little is known about the precise mechanisms and the factors causing transcriptional repression under hypoxia, despite their critical roles in cellular hypoxic reaction. In fact, decreased expression of DNA repair genes under hypoxia and a possible association with genomic instability were recently shown (Mihaylova *et al*, 2003; Bindra *et al*, 2004; Bindra *et al*, 2005; Koshiji *et al*, 2005; Bindra *et al*, 2006; Bindra *et al*, 2007). The analysis of molecular mechanisms is of key importance in understanding cellular hypoxic reaction and its role in tumor

biology, so we attempted to clarify the molecular mechanisms: we found that DEC1 and DEC2 strongly repress the promoter activity of *MLH1*, possibly *via* a histone deacetylase (HDAC)-dependent mechanism but not by just a competitive occupancy on the promoter. We further identified a possible DEC-response element on the *MLH1* promoter region, and confirmed the direct binding of DEC to that element. Forced expressions of both DEC1 and 2 efficiently repressed *MLH1* promoter and expression, and knock-down of *DEC2* by siRNA significantly attenuated hypoxic repression of the *MLH1* expression. On the other hands, while knock-down of *HIF1A* also caused disappearance of hypoxic repression of *MLH1*, *DEC1* knock-down failed to attenuate the *MLH1* repression under hypoxic conditions, since decreased expression of *DEC1* resulted in increased *DEC2* expression as previously reported (Li *et al*, 2003). Taken together, these results suggested that HIF-1-DEC pathway was one of the important mechanisms. Very recently, several mechanisms were suggested to participate in regulation of DNA repair genes, including E2F4/p130, HIF-1α/SP-1, and Myc/Max system. Bindra RS and Glazer PM (2007) demonstrated a dynamic shift in occupancy from activating c-Myc/Max to repressive Mid/Max and Mnt/Max complexes at the proximal promoters of *MLH1* and *MSH2* by using series of ChIP assays, but did not determine repressive activities of those complexes on the promoters. Although it is well known that both Myc/Max and DEC bind to E-box motif to regulate gene transcription, our experiments using mutant-type of DEC1 which had DNA binding domain but lacked most of functional domains failed to repress *MLH1* promoter activities, even enhanced them, suggesting that just a competitive occupancy on the promoter was not sufficient to explain the repression, but HDAC-dependent repressive activities of DEC transcription factors were important. Since the loss of functions of *MLH1* is thought to be a significant cause of

the complete inactivation of MMR (Peltomäki, 2001; Hoeijmakers, 2001) - which may lead to carcinogenesis, tumor progression and emergence of resistance to anticancer therapies - these new findings, we believe, could contribute to a better understanding of the functional roles of hypoxia in malignant phenotypes of various tumors.

Our data also suggested that DEC2 might repress *MLH1* stronger than DEC1 does, which would be an important evidence of diversification of DEC functions. It has been suggested that DEC participates also in adipogenesis, circadian rhythm, immune system or carcinogenesis through transcriptional repressions of several genes *via* E-box or other motifs in their promoter regions (Yun *et al*, 2002; Honma *et al*, 2002; Ivanova *et al*, 2007). Their differential effects on *MLH1* could be explained in part by varying specificity to the element sequence identified as the binding site, which contains a sequence motif of AACGTG with one nucleotide difference from canonical E-box motif (CACGTG). In this study, we found that mRNA expression of *DEC1* increased for more than 72 h under hypoxia, while that of *DEC2* only temporarily increased. Even so, DEC2 was shown to have much stronger affinity to the *MLH1* promoter. These findings led us to hypothesize that DEC2 could be the initiator of the event, whereas DEC1 might act on the maintenance of the down-regulated level of *MLH1* expression. This hypothesis may be supported in a part by one report showing that DEC1 transcriptionally repressed *DEC2* expression in an autofeedback system, suggesting their hierarchical functions (Li *et al*, 2003). In the present study, we did not detect an endogenous DEC2 protein induction as well as other investigators, and did not observe DEC2 binding to *MLH1* promoter *in vivo* using ChIP assay. On the other hands, knock-down experiments clearly showed a significant role of DEC2 in regulation of the *MLH1*. Taken together, it might be tough

to detect endogenous DEC2 protein in both experiments due to an antibody activity, but DEC2 protein actually functions on *MLH1* regulations. The diverse roles of DEC1 and DEC2 are now being intensively investigated in our laboratory.

In conclusion, we demonstrated here that the hypoxia-inducible transcription repressors DEC1 and DEC2 participate in transcriptional regulation of the *MLH1 via* their bindings to an E-box-like motif in the *MLH1* promoter region. Hypoxia-induced DEC1 or DEC2, we think, probably play very important roles in the transcriptional down-regulation of genes under hypoxia, and the HIF-1-DEC pathway as well as other pathways may impair MMR function through the repression of *MLH1* expression, subsequently causing genomic instability in cancer cells (Figure 5d).

Materials and methods

Chemicals

All chemicals were analytical grade and were purchased from Wako Pure Chemicals (Osaka, Japan) or Sigma (St. Louis, MO).

Cell lines and RNA preparation

Human cancer cell lines used were as follows: a hepatoma line, HepG2 and an oral squamous cell carcinoma line, HSC-2 (The Japanese Cancer Research Resource Bank); a cervical adenocarcinoma line, HeLa and a breast adenocarcinoma line, MCF-7 (American Type Culture Collection). For gene expression analyses, cells (2-4 x 10^5/10 cm diameter dish) were cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions for various incubation-times (6, 12, 24, 48, or 72 h) in a hypoxic chamber (Hirosay Corp., Hiroshima, Japan). For knock-down analyses, *HIF1A*, *DEC1*, *DEC2*, or nonspecific (NS) siRNA (QIAGEN, Inc., Valencia, CA) was transfected with TransIT®-siQUEST™ Transfection Reagent (Mirus Corporation, Madison, WI)

in HSC-2 (1 x 10^6/10 cm diameter dish) for 12 h, and then the cells were incubated under normoxic or hypoxic conditions for 24 h. Cells were then harvested and stored at -80˚C until use. Total RNA was prepared from frozen cell pellets by using QIAGEN RNeasy® mini kit (QIAGEN) according to the manufacturer's instruction.

Reverse transcription-polymerase chain reaction (RT-PCR).

Two-micrograms of total RNA extracted from each cell line were reverse-transcribed using High-Capacity cDNA Archive™ Kit (Applied Biosystems, Foster City, CA). Two-hundredth aliquot of the cDNA was subjected to real-time RT-PCR using TaqMan™ Gene Expression Assays (Applied Biosystems) for *HIF1A*, *BHLHB2* (*DEC1*), *BHLHB3* (*DEC2*), and *MLH1*, and Pre-Developed TaqMan™ Assay Reagents (Applied Biosystems) for *ACTB* as an internal control. More than three independent measurements were averaged and relative gene expression levels were calculated as a ratio to *ACTB* expression of each cell line.

Immunoblot analysis

To analyze protein expression, whole cell extracts were prepared from cultured cells with or without hypoxic treatment as previously described (Tanimoto *et al*, 2000). Twenty-five μg of protein was blotted onto nitrocellulose filters following SDS-polyacrylamide gel electrophoresis. Anti-FLAG (Sigma), anti-MLH1, anti-HIF-1α, anti-Arnt (BD Pharmingen, San Diego, CA), or anti-β-actin (Sigma) were used as primary antibodies, diluted 1:5000, 1:2000, 1:1000, 1:2000 or 1:5000, respectively. A 1:2000 dilution of anti-mouse IgG horseradish peroxidase conjugate (Amersham Life Science) was used as a secondary antibody. Immunocomplexes were visualized using the enhanced chemiluminiscence reagent ECL Plus (Amersham Life Science).

Plasmid Constructions

The 1.65-kb DNA fragment (nucleotide positions from -1653 to -4 when transcriptional start site is designated as at +1) including the 5' region of MLH1 gene was amplified by PCR from a HepG2 genomic DNA and subcloned into *Nhe* I and *Xho* I sites of a luciferase reporter plasmid pGL3-Basic® or pGL4.10 (Promega, Madison, WI) and the construct was designated as pGL-MLH1 Pro1.65. A series of 5' deletion mutant of pGL-MLH1 Pro was constructed by PCR method using internal specific primer sets with pGL-MLH1 Pro1.65 as a template. Base-exchanged mutants of putative E-box sites in pGL-MLH1 Pro0.27 were generated by PCR-based site-directed mutagenesis as previously reported (Tanimoto *et al*, 2003). Details of expression plasmid vectors of DEC1 (pcDNA-DEC1, p3xFLAG-CMV-DEC1, or pcDNA-DEC1 1-139) and DEC2 (pcDNA-DEC2) were previously described (Sato *et al*, 2004; Kawamoto *et al*, 2004). pcDNA-FLAG-DEC2 was constructed by swapping DEC2 cDNA fragment of pcDNA-DEC2 with the pcDNA-FLAG (kindly provided by Dr. Igarashi).

Luciferase Reporter Assay

Transient transfection was performed as follows: pGL-MLH1 Pro (0.3 μg/15-mm well) with pcDNA-FLAG, p3xFLAG-CMV-DEC1 or pcDNA-FLAG-DEC2 (0.001-0.1 μg/15-mm well) were mixed with 0.8 μl of Trans-IT LT1® Transfection Reagent (Mirus). Renilla-luciferase vector (pRL-SV40, 1.0 ng/15-mm well) (Promega) was used as a transfection efficacy control. Cells were incubated under normoxic or hypoxic conditions for 36 - 48 h after transfection prior to analysis of luciferase reporter activity. Using the HDAC inhibitor, trichostatin A (TSA), treatments were started (final concentrations: 10 or 100 ng/ml) 24 h before harvesting cells. Luciferase luminescence was measured as previously described (Tanimoto *et al*, 2003)

Electrophoretic gel mobility shift assay (EMSA)

Double-stranded oligoprobes containing consensus DEC binding sequences in the *MLH1* promoter from -69 to -47 were synthesized as follows: sense, 5'-AAGAACGTGAGCACGAGGCACTGGG-3' and antisense, 5'-CAGTGCCTCGTGCTCACGTTCTTGG-3', and labeled with [α-^{32}P]-dCTP. Adjusted equal amounts of *in vitro* translated DEC1 or DEC2 were incubated with 200 pmol of labeled probe in 20 μl of reaction mixture for 30 min at room temperature. A hundred-fold excess amounts of unlabeled probes for competition or 2.5 μl of anti-DEC1 or anti-DEC2 polyclonal antibody (Kawamoto *et al*, 2004) for supershift was pre-incubated for 30 min at room temperature before the addition of hot-labeled probes. The reaction mixtures were then loaded onto 5% polyacrylamide gels and were run for 4 h at 4˚C. Resulting gels were dried and visualized using BAS2000.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using EZ ChIP™ Chromatin Immunoprecipitation Kit (UPSTATE USA, Inc., Charlottesville, VA) according to the manufacturer's instruction. Anti-DEC1 or anti-DEC2 rabbit polyclonal antibody (Kawamoto *et al*, 2004) was used for a specific precipitation, and anti-IgG mouse monoclonal antibody was used as a negative control for an immunoprecipitation. The PCR primer set was synthesized to encompass the candidate DEC-binding sites in *MLH1* promoter as follows: forward, 5'-ATCAATAGCTGCCGCTGAA-3' and reverse, 5'-CTCGTGCTCACGTTCTTCCT-3', and the probe (#42) was selected from Universal Probe Library (UPL, Roche Diagnostics, Tokyo, Japan). Real-time PCR was performed using the 1/30 volume of precipitates. Three independent measurements

were averaged and relative amounts were calculated as a ratio to amplicons using HepG2 genomic DNA.

Immunostaining

HepG2 cells grown on cover slips were transiently transfected with DEC2 expression plasmid, pcDNA-DEC2. After incubation for 24 h, immunostaining was performed with anti-DEC2 (1:100) or anti-MLH1 (1:100) as primary antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit Ig's (1:100) (BioSource, Camarillo, CA) or Rhodamine-conjugated sheep anti-mouse Ig's (1:100) (Chemicon, Temecula, CA) as secondary antibody. Nuclei were stained with DAPI. Subcellular distribution of fluorescence was examined using a Zeiss Axiovert 135 microscope with an FITC-filter set, epifluorescence with illumination from a Gixenon burner (Carl Zeiss Jena GmbH, Jena, Germany).

Statistical analysis

All of the statistical tests were performed using StatView® version 5.0 software (SAS Institute Inc., Cary, NC, USA), and Student *t* test was used to determine the *P*-value.

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Figure Legends

Figure 1 Hypoxia decreased MLH1 expression and increased HIF-1α, DEC1 and DEC2 expression in cancer cell lines. Protein levels of MLH1, HIF-1α, Arnt, DEC1, and β-actin (**a**), and mRNA levels of *MLH1*, *DEC1*, and *DEC2* expressed in HepG2 (**b**), and HeLa, MCF-7 and HSC-2 (**c**) cells after indicated periods of hypoxic treatment analyzed by immunoblotting or real-time RT-PCR method. Relative mRNA levels were calculated as the ratio to that of *ACTB*, and each bar represents the mean + SD for at least three independent experiments. *: $P < 0.05$ and ≥ 0.01, **: $P < 0.01$.

Figure 2 Hypoxia or DEC transcription factors repressed promoter activities of *MLH1* in HepG2 cells. (**a**) The 5' region (nt -1653~-4) of *MLH1* was subcloned into pGL3 basic plasmid vector. (**b**) The *MLH1* promoter reporter was transiently transfected into HepG2 cells, and promoter activities were evaluated under normoxic or hypoxic conditions (**c**) Various amounts of *DEC1* (hatched bar) or *DEC2* (closed bar) expression vectors were co-transfected with *MLH1* promoter luciferase reporter. Effects of trichostatin A (TSA) treatment (**d**) or *DEC1* mutant (**e**, striped bar) on *MLH1* promoter were evaluated by co-transfection assay. Relative luciferase activities were calculated as the ratio to activity of pRL-SV40. Each bar represents the mean + SD for at least three independent experiments. *: $P < 0.05$ and ≥ 0.01, **: $P < 0.01$.

Figure 3. DEC repress promoter activity of *MLH1 via* the E-box motifs on its promoter region. (**a**) Comparative analysis of transcriptional activity using 5' deletion mutants of *MLH1* promoter. A series of deletion mutants of *MLH1* promoter is shown in the schematic (left). Transcriptional activities of the deletion mutants of *MLH1*

promoter were evaluated by luciferase assay after cotransfection with or without DEC-expressing vectors (right). (**b**) Nucleotide sequence of detailed DEC-response elements near the transcription start site. Substituted nucleotides in mutants are indicated above the wild-type sequence. In the open box, consensus E-box like motif is indicated. The lower bar shows the sequence of oligo-probe for EMSA. (**c**) Comparative analysis of transcriptional activity using nucleotide substituted mutants of *MLH1* promoter. Transcriptional activities of *MLH1* promoter mutants were evaluated as described above. Each bar represents the mean + SD for at least three independent experiments. **: $P < 0.01$.

Figure 4. DEC directly bound to the DEC-response elements containing E-box motif on *MLH1* promoter. (**a**) The EMSA was performed as described in materials and methods. Specificities of their bindings (* for DEC1 complex) were confirmed by pre-incubation with non-labeled probes or specific antibodies for DEC1 or DEC2. NS: non-specific band. (**b**) The ChIP assay was performed as described in materials and methods using anti-DEC1, anti-DEC2 or anti-IgG. Relative amounts of precipitated DNA fragments were evaluated by real-time PCR, and calculated using HepG2 genomic DNA as a standard. Each bar represents the mean + SD for at least three independent experiments. *: $P < 0.05$ and ≥ 0.01

Figure 5. DEC decreased endogenous MLH1 expression. (**a**) Immunoblotting analysis was performed using whole cell extract prepared from HepG2 cells transiently transfected with DEC1 or DEC2. Anti-MLH1, anti-FLAG, or anti-β-actin was used for specific detection of each protein. (**b**) Immunostaining analysis with anti-MLH1 and anti-DEC2 was performed using HepG2 transiently transfected with

DEC2. (i) DAPI, (ii) FITC (anti-DEC2), (iii) Rhodamine-red (anti-MLH1), (iv) merged pictures. Bar: 10 μm. (**c**) Knock-down assays for *HIF1A*, *DEC1*, and *DEC2* were performed using HSC-2 cells. Expression levels of *HIF1A*, *MLH1*, *DEC1*, and *DEC2* were evaluated as Figure 1. Statistical significances were calculated by student *t* test between the non-specific and each knocked-down cells under normoxic or hypoxic conditions respectively. Each bar represents the mean + SD for at least three independent experiments. *: $P < 0.05$ and ≥ 0.01, **: $P < 0.01$. (**d**) Hypothetical model of hypoxic malignant cycles.

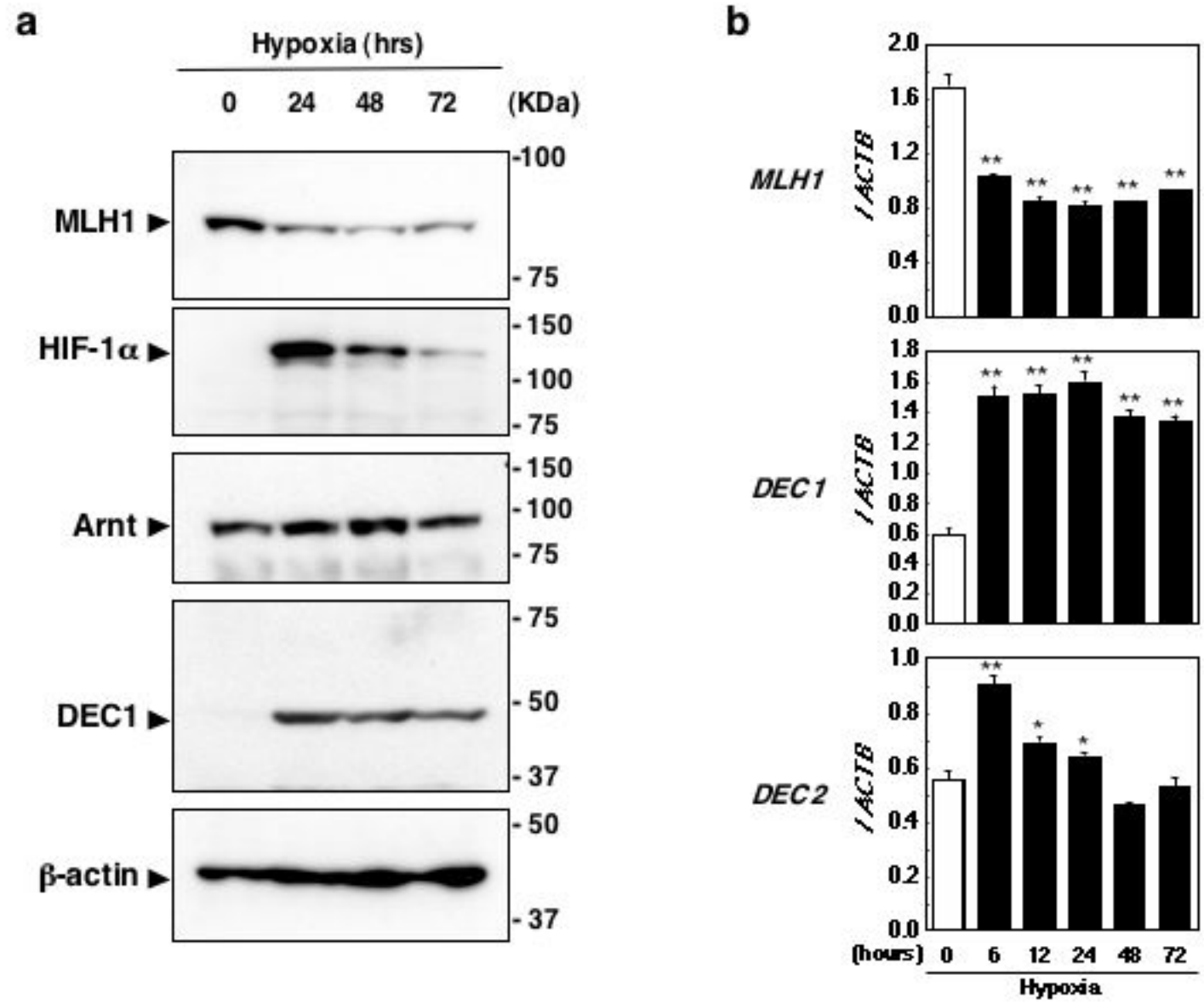

a
Hypoxia (hrs)
0
24
48
72
(KDa)
MLH1
HIF-1α
Arnt
DEC1
β-actin
-100
-75
-150
-100
-75
-150
-100
-75
-75
-50
-37
-50
-37
b
MLH1
DEC1
DEC2
/ACTB
2.0
1.6
1.2
0.8
0.4
0.0
1.8
1.4
1.0
0.2
**
*
(hours)
0
6
12
24
48
72
Hypoxia

Figure 1ab

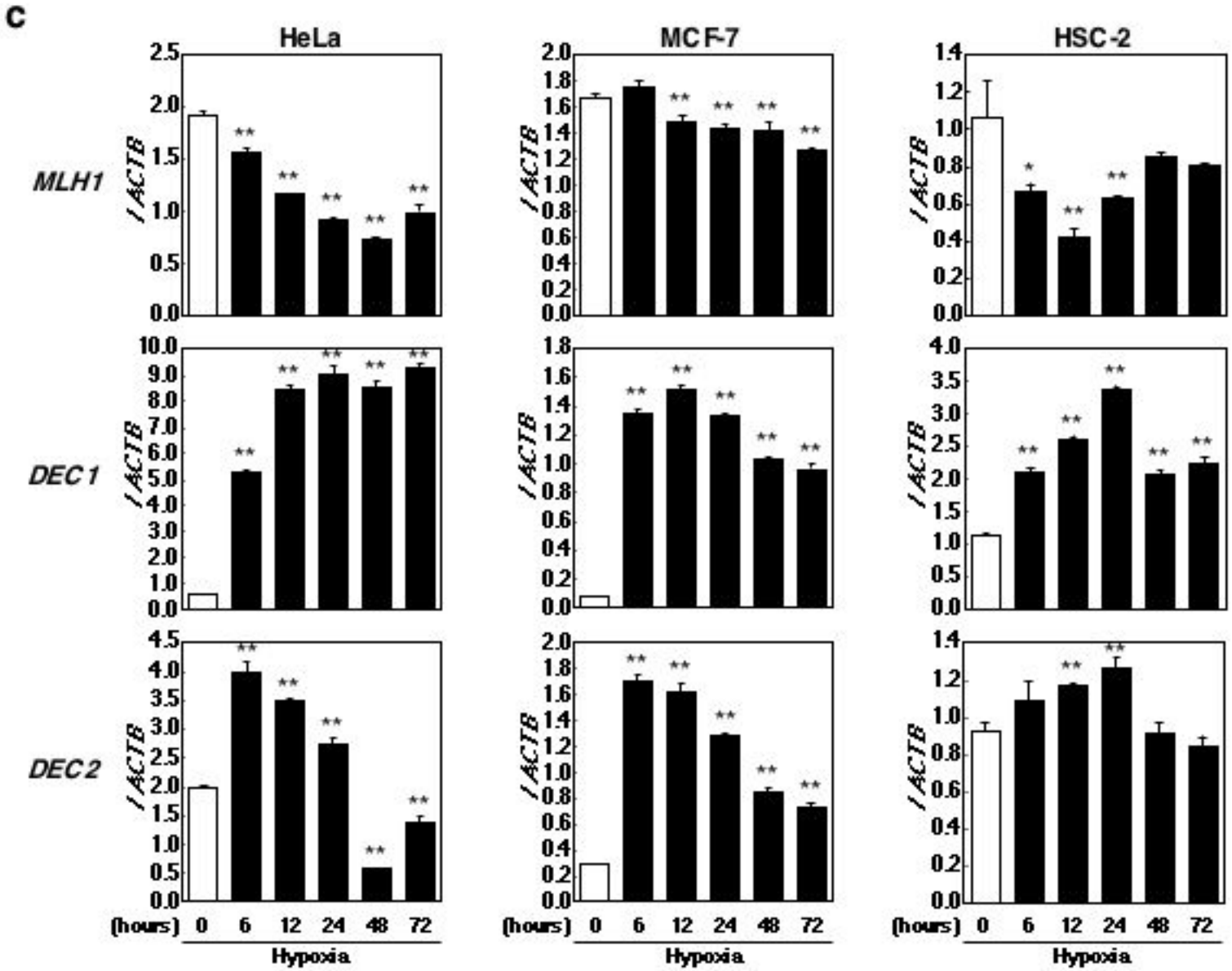

C
HeLa
MCF-7
HSC-2
MLH1
DEC1
DEC2
/ACTB
(hours) 0 6 12 24 48 72
Hypoxia

Figure 1c

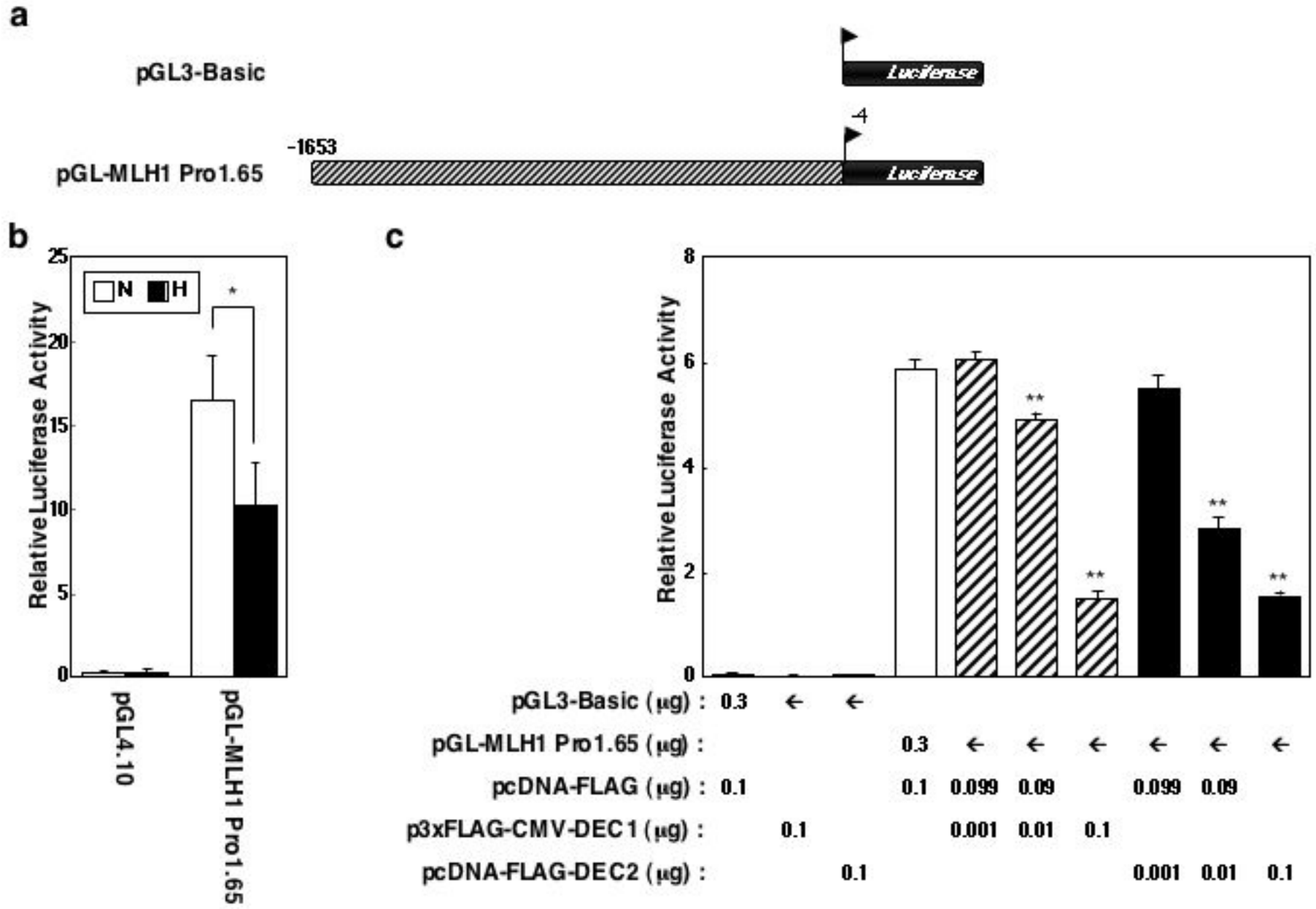

a
pGL3-Basic
Luciferase
-4
-1653
pGL-MLH1 Pro1.65
Luciferase
b
N
H
*
Relative Luciferase Activity
25
20
15
10
5
0
pGL4.10
pGL-MLH1 Pro1.65
c
Relative Luciferase Activity
8
6
4
2
0
**
pGL3-Basic (μg) : 0.3 ← ←
pGL-MLH1 Pro1.65 (μg) : 0.3 ← ← ← ← ← ← ←
pcDNA-FLAG (μg) : 0.1 0.1 0.099 0.09 0.099 0.09
p3xFLAG-CMV-DEC1 (μg) : 0.1 0.001 0.01 0.1
pcDNA-FLAG-DEC2 (μg) : 0.1 0.001 0.01 0.1

Figure 2abc

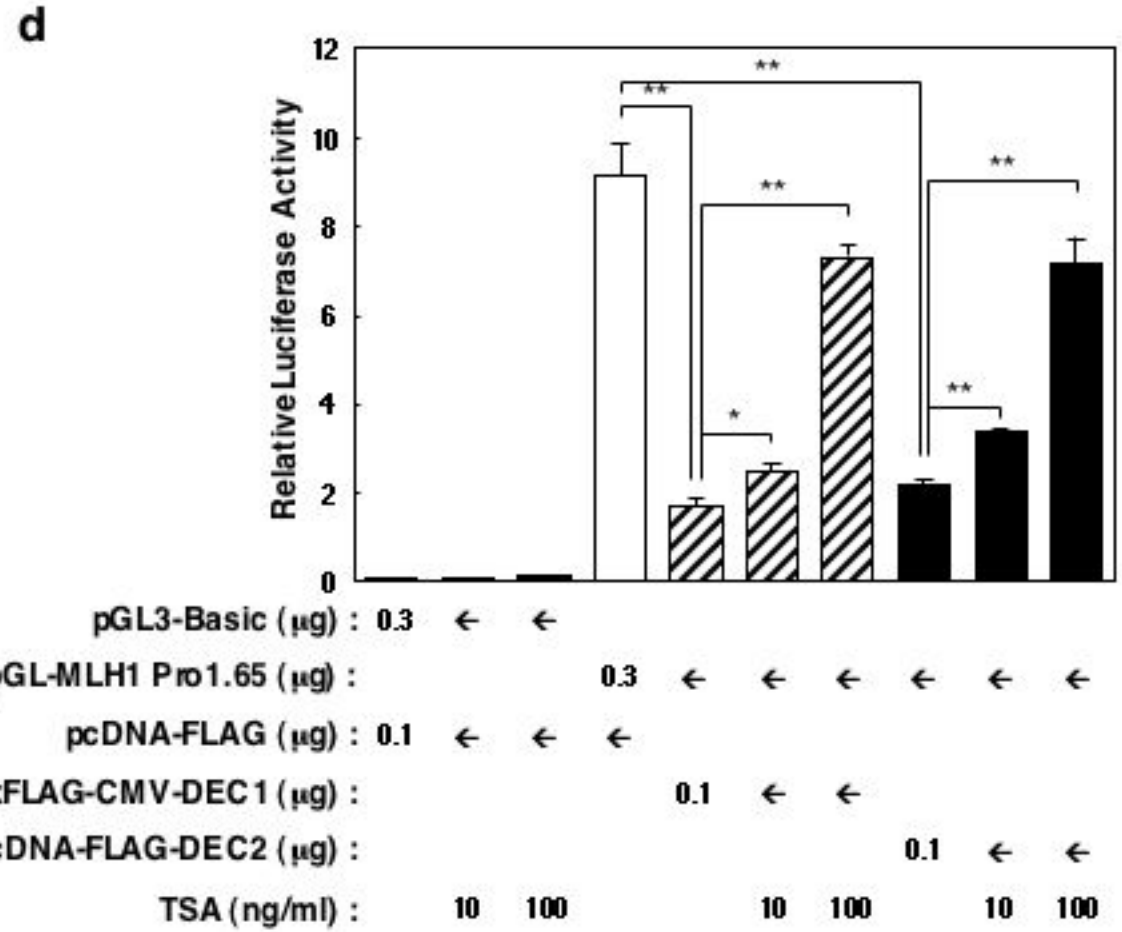
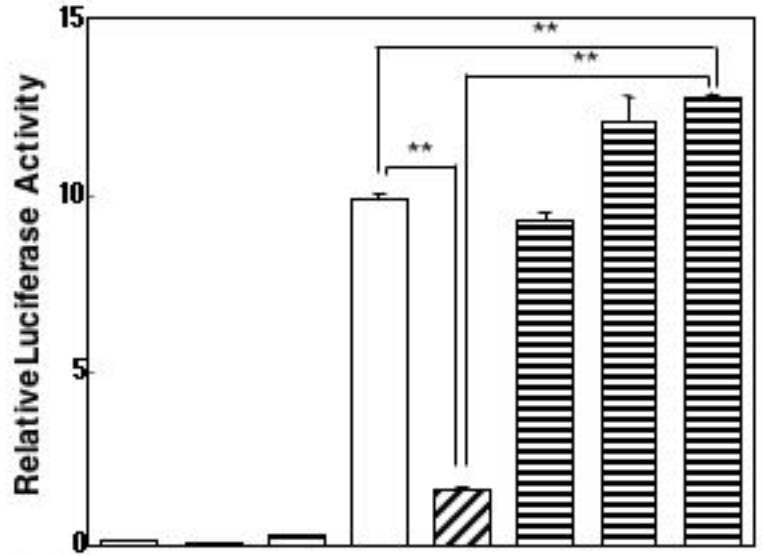

d
Relative Luciferase Activity
0
2
4
6
8
10
12
**
**
**
**
**
*
pGL3-Basic (μg) : 0.3 ← ←
pGL-MLH1 Pro1.65 (μg) : 0.3 ← ← ← ← ← ←
pcDNA-FLAG (μg) : 0.1 ← ← ←
p3xFLAG-CMV-DEC1 (μg) : 0.1 ← ←
pcDNA-FLAG-DEC2 (μg) : 0.1 ← ←
TSA (ng/ml) : 10 100 10 100 10 100

Figure 2d

e



pGL3-Basic (μg) :	0.3	←	←					
pGL-MLH1 Pro1.65 (μg) :				0.3	←	←	←	←
pcDNA-FLAG (μg) :	0.1			0.1		0.09	0.05	
p3xFLAG-CMV-DEC1 (μg) :		0.1			0.1			
pcDNA-DEC1 (1-139) (μg) :			0.1			0.01	0.05	0.1

Figure 2e

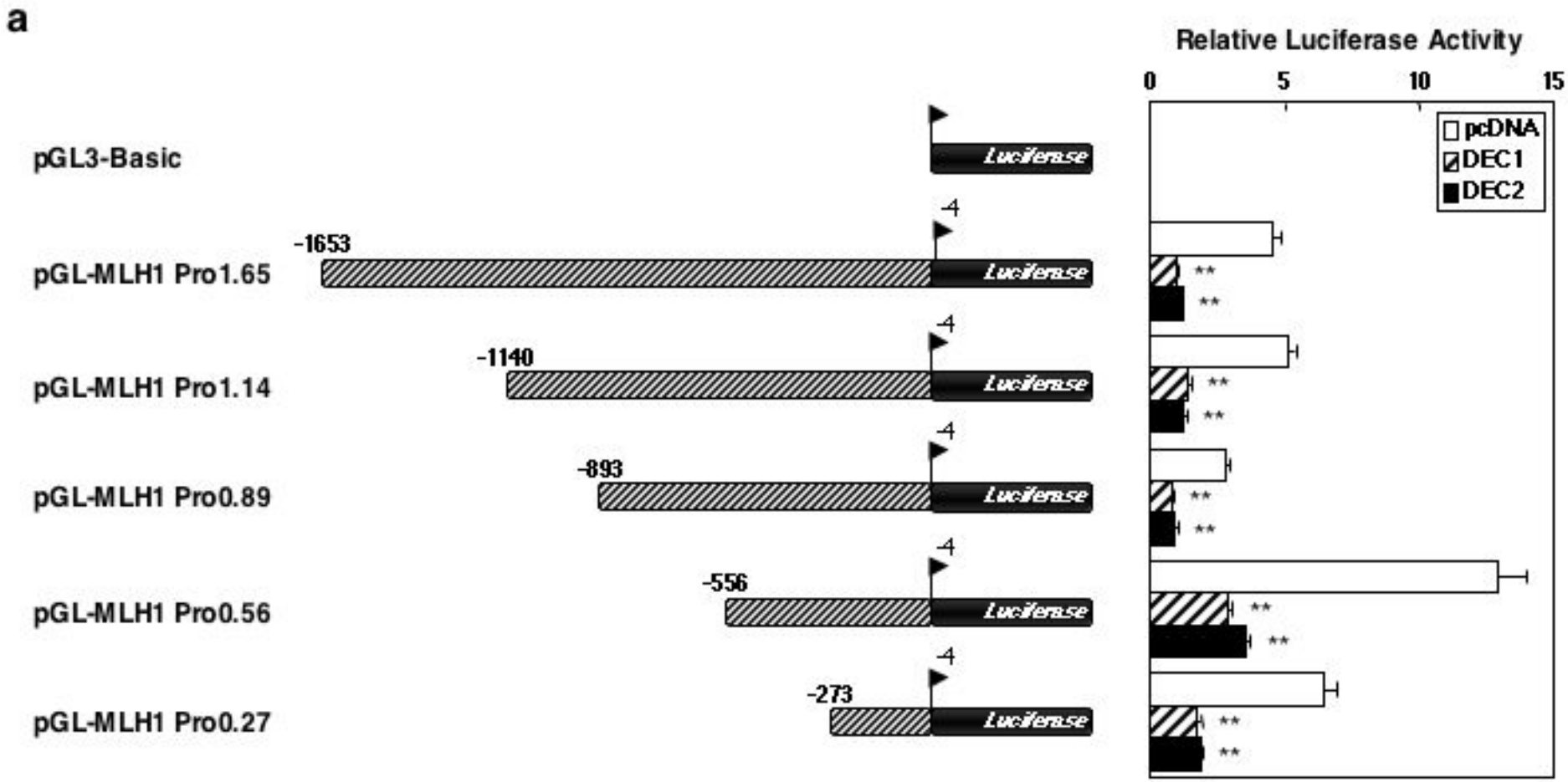

a
pGL3-Basic
pGL-MLH1 Pro1.65
pGL-MLH1 Pro1.14
pGL-MLH1 Pro0.89
pGL-MLH1 Pro0.56
pGL-MLH1 Pro0.27
-1653
-1140
-893
-556
-273
-4
Luciferase
Relative Luciferase Activity
0
5
10
15
pcDNA
DEC1
DEC2
**

Figure 3a

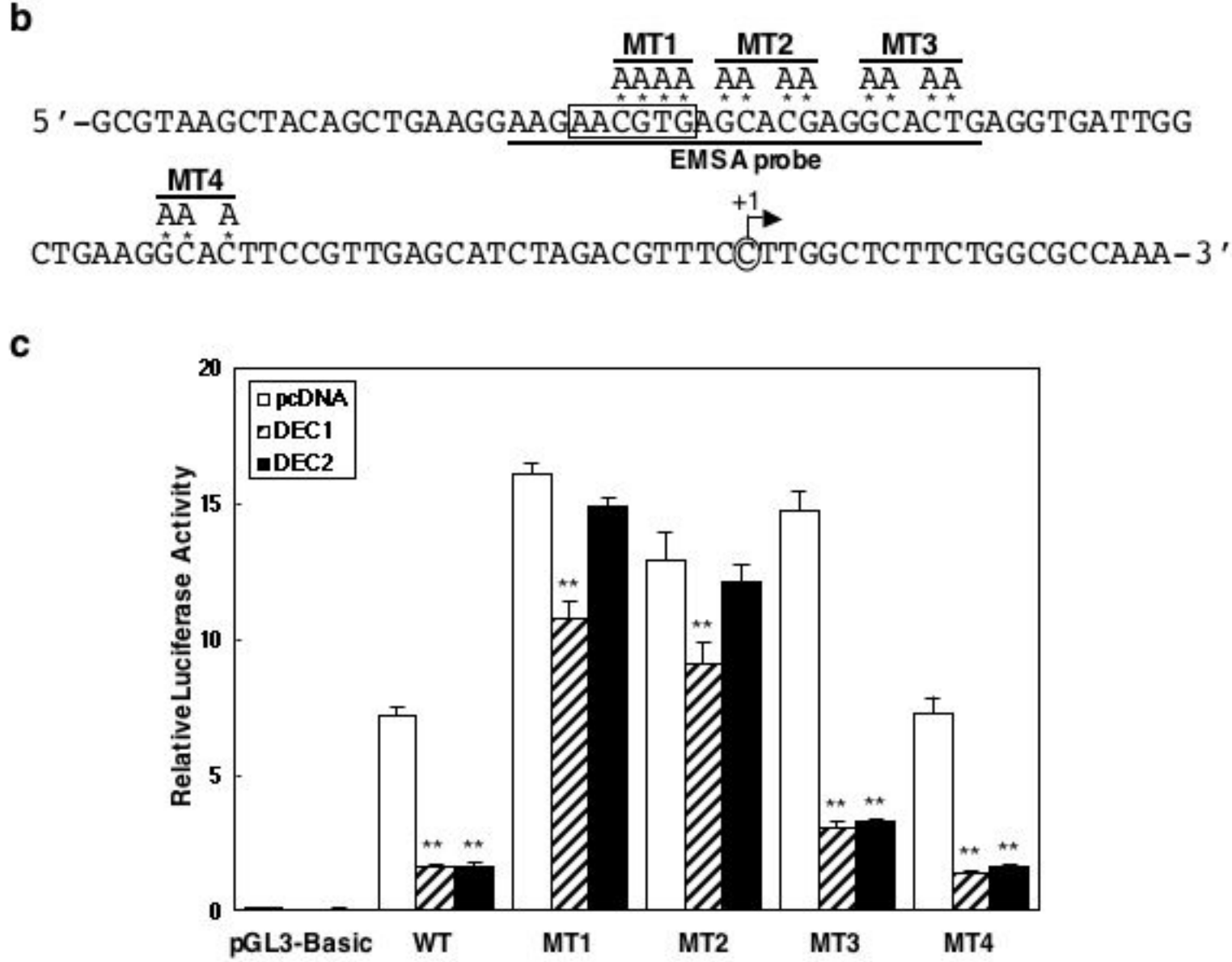

b
MT1
MT2
MT3
AAAA
AA AA
AA AA
5′-GCGTAAGCTACAGCTGAAGGAAGAACGTGAGCACGAGGCACTGAGGTGATTGG
EMSA probe
MT4
AA A
+1
CTGAAGGCACTTCCGTTGAGCATCTAGACGTTTCCTTGGCTCTTCTGGCGCCAAA-3′
c
pcDNA
DEC1
DEC2
Relative Luciferase Activity
20
15
10
5
0
**
pGL3-Basic
WT
MT1
MT2
MT3
MT4

Figure 3bc

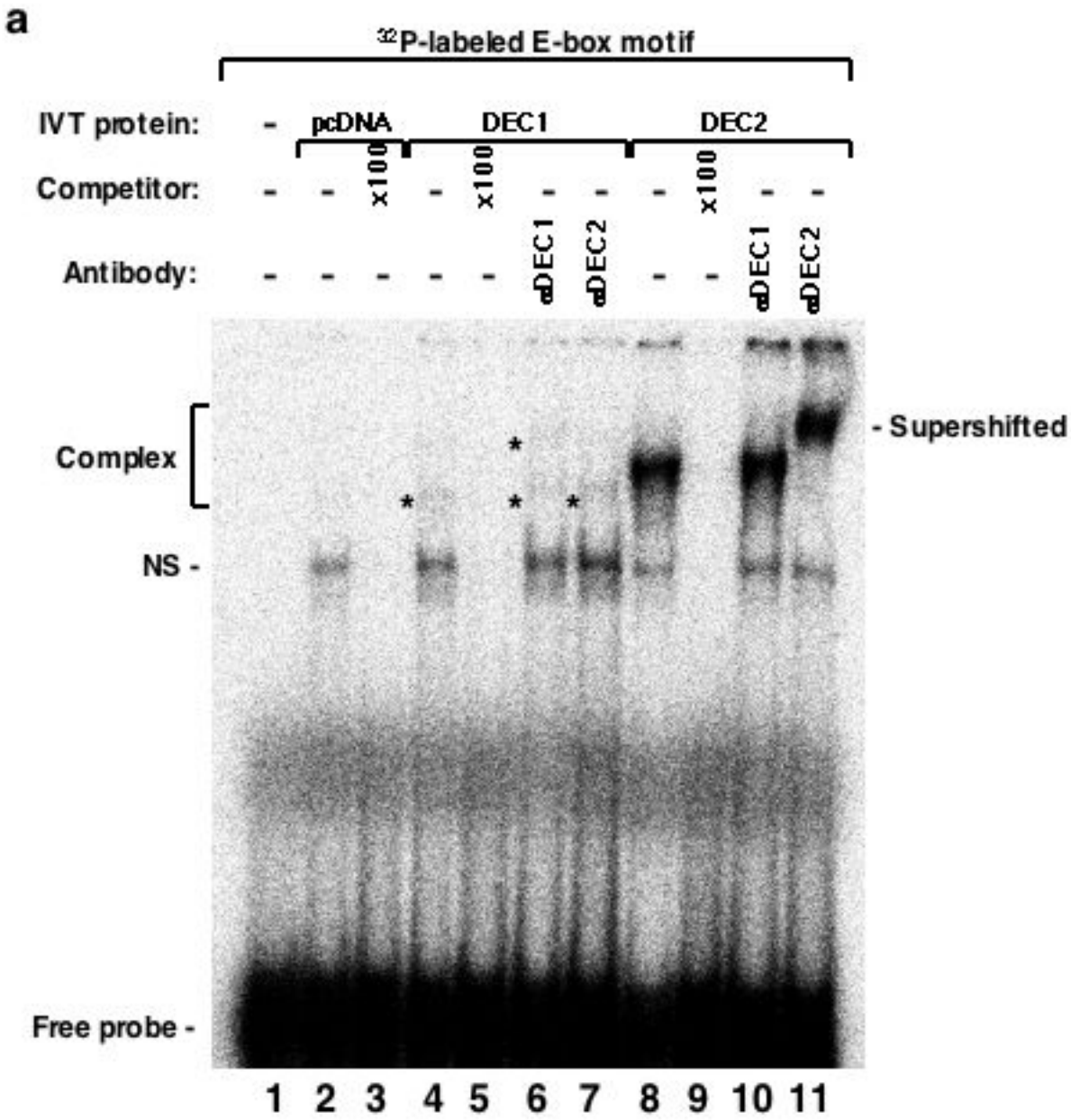

a
32P-labeled E-box motif
IVT protein:
-
pcDNA
DEC1
DEC2
Competitor:
-
-
x100
-
x100
-
-
-
x100
-
-
Antibody:
-
-
-
-
-
αDEC1
αDEC2
-
-
αDEC1
αDEC2
Complex
NS -
Free probe -
- Supershifted
1 2 3 4 5 6 7 8 9 10 11

Figure 4a

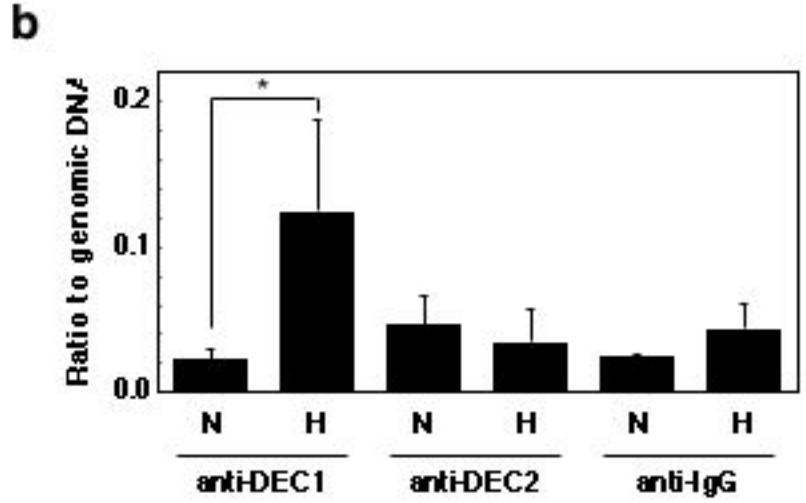

b
Ratio to genomic DNA
0.2
0.1
0.0
*
N
H
N
H
N
H
anti-DEC1
anti-DEC2
anti-IgG

Figure 4b

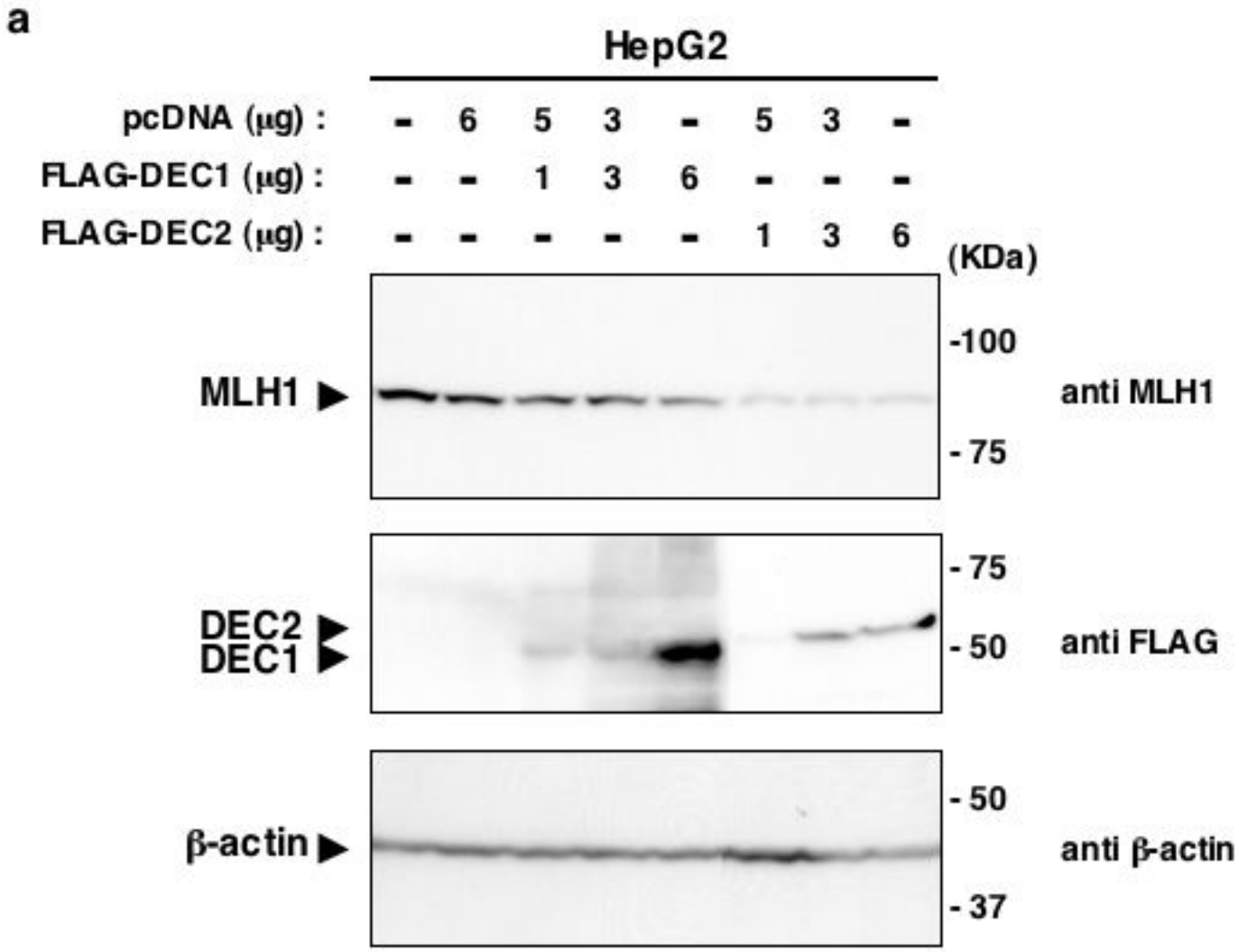

a
HepG2
pcDNA (μg) : - 6 5 3 - 5 3 -
FLAG-DEC1 (μg) : - - 1 3 6 - - -
FLAG-DEC2 (μg) : - - - - - 1 3 6
(KDa)
MLH1
-100
- 75
anti MLH1
DEC2
DEC1
- 75
- 50
anti FLAG
β-actin
- 50
- 37
anti β-actin

Figure 5a

b

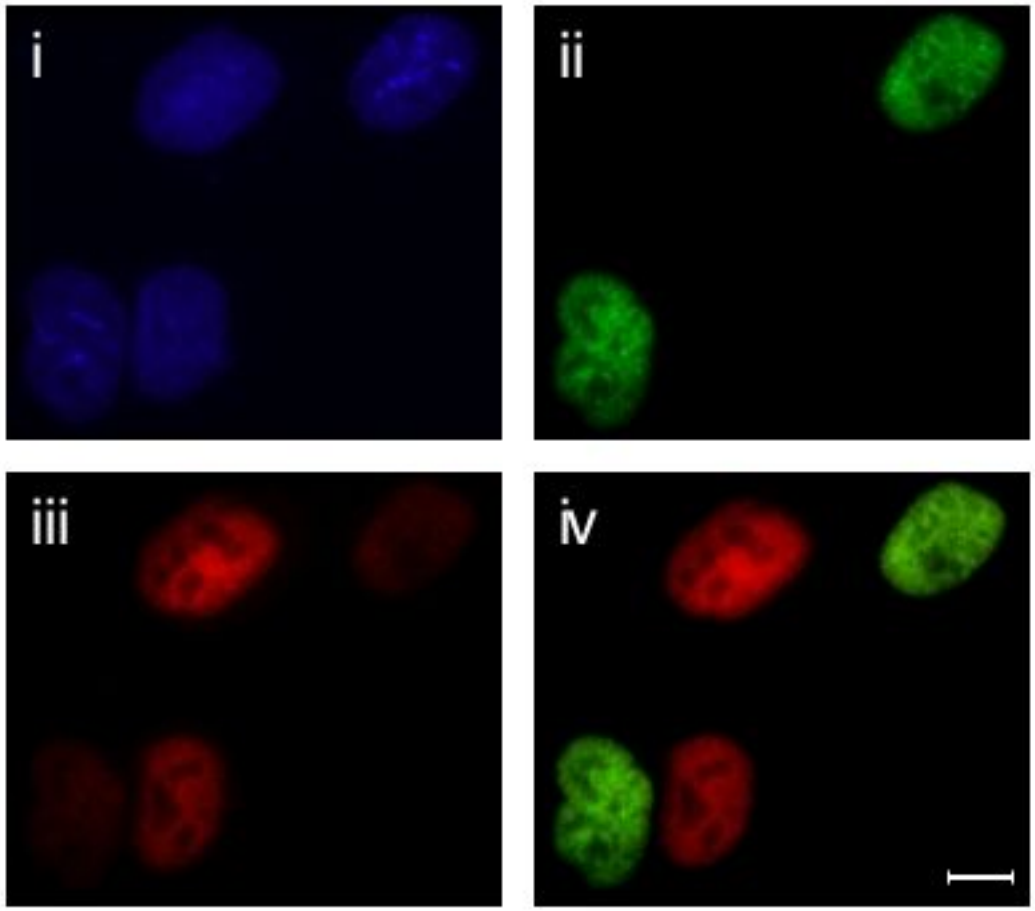


Figure 5b

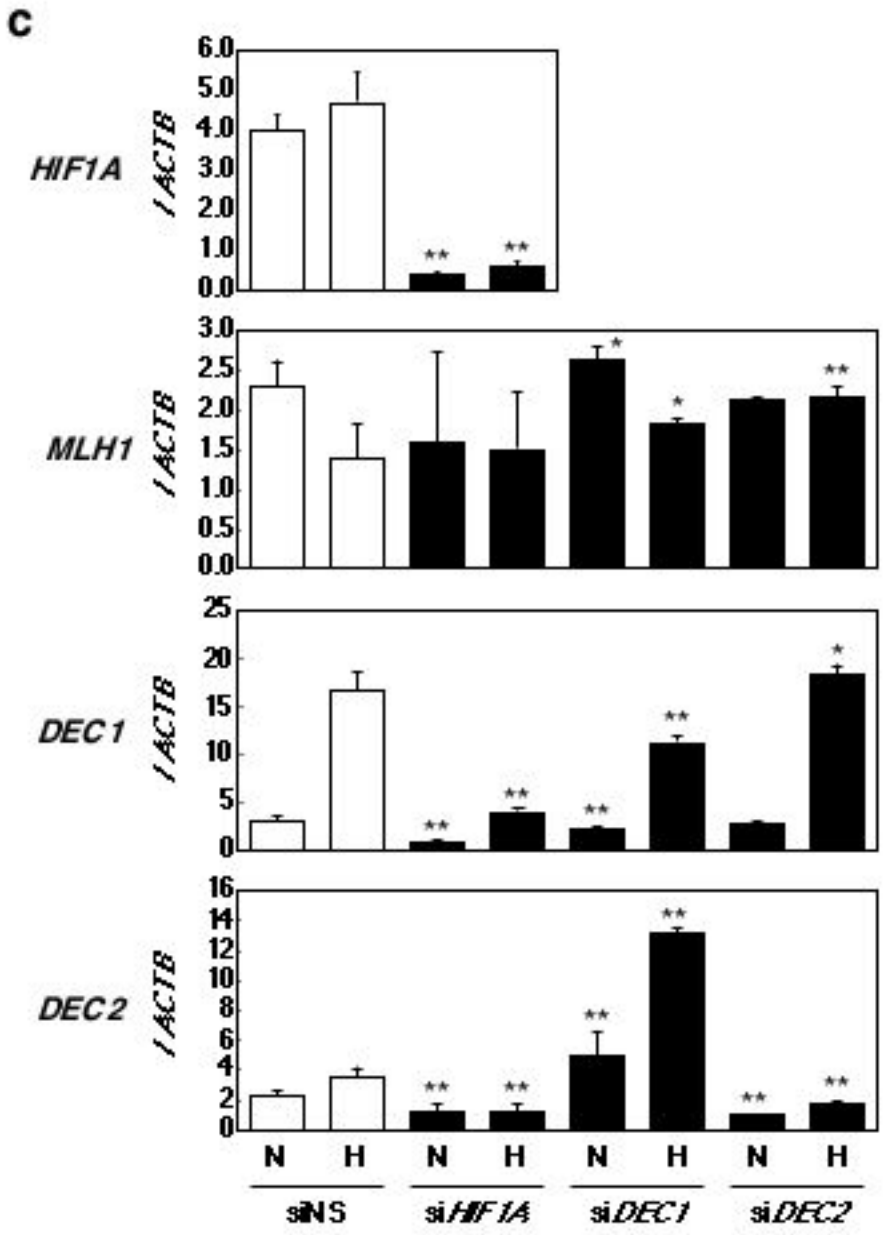

c
HIF1A
MLH1
DEC1
DEC2
/ACTB
6.0
5.0
4.0
3.0
2.0
1.0
0.0
3.0
2.5
2.0
1.5
1.0
0.5
0.0
25
20
15
10
5
0
16
14
12
10
8
6
4
2
0
**
*
N
H
siNS
siHIF1A
siDEC1
siDEC2

Figure 5c

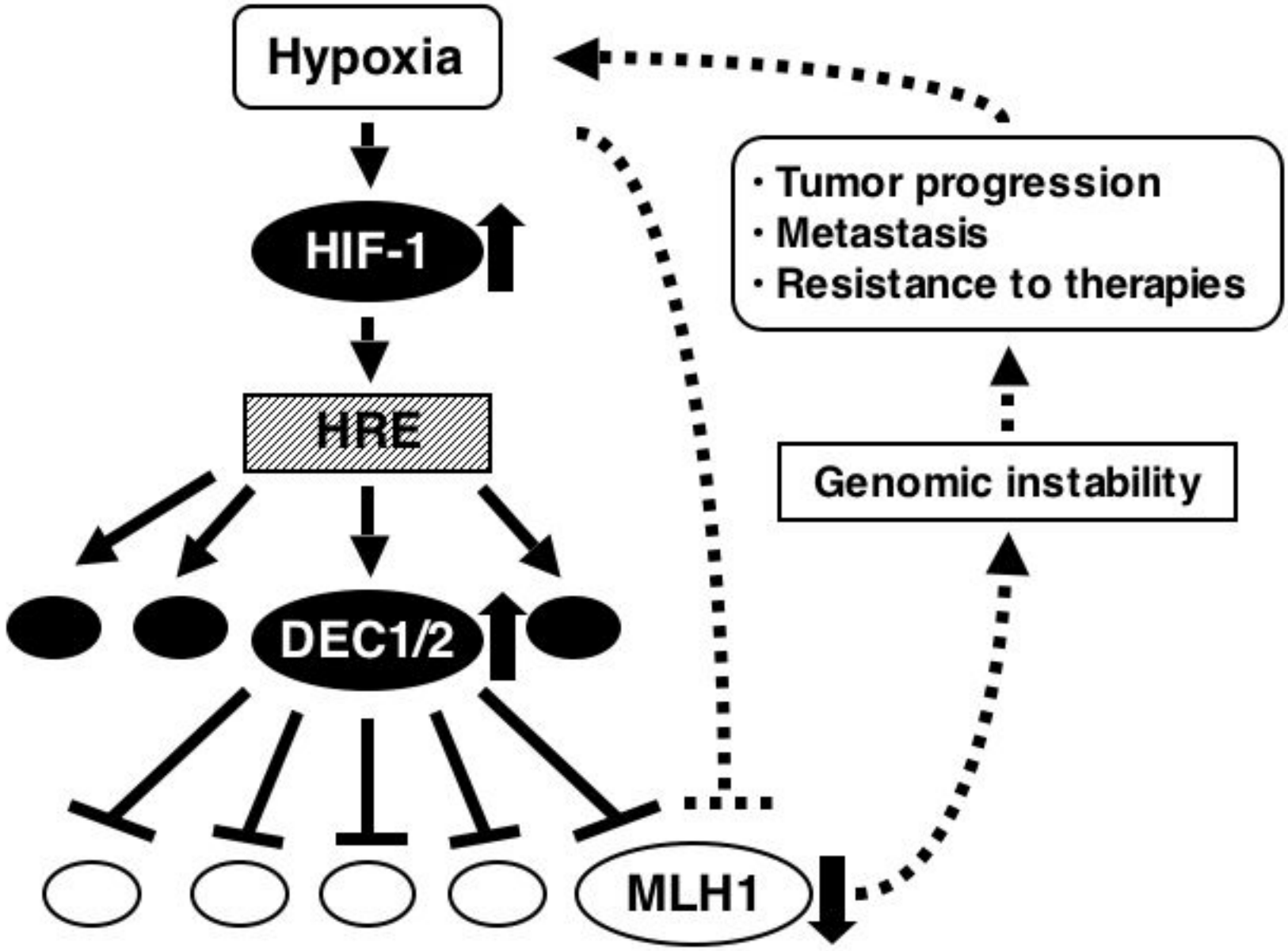

Hypoxia
HIF-1
HRE
DEC1/2
MLH1
· Tumor progression
· Metastasis
· Resistance to therapies
Genomic instability

Figure 5d