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Up-regulation of *ras-Gap* genes is reversed by a MEK inhibitor and doxorubicin in ν -Ki-*ras* transformed NIH/3T3 fibroblasts

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Abstract

Ras-GTPase-activating proteins (Ras-GAPs) have been implicated both as suppressors of Ras and as effectors in regulating cellular activities. To study whether Ras-GAPs have roles in tumor cell survival or not, mRNA levels of *ras*-related genes were measured in *v*-Ki-*ras*-transformed (DT) and the parental NIH/3T3 cells, using real-time PCR. mRNA levels of *p120-Gap*, *Gap1^m*, and *PIK3CA* were increased in DT cells compared with NIH/3T3 cells. *p120-Gap* and *PIK3CA* genes were induced by addition of serum or epidermal growth factor to serum-starved DT cells. Three anticancer drugs, an ERK kinase (MEK) inhibitor PD98059, a topoisomerase II poison doxorubicin (adriamycin), and a histone deacetylase inhibitor trichostatin A, selectively blocked the overexpression of *p120-Gap* and *Gap1^m* genes in DT cells. These drugs also caused reversion of DT cells to the adherent shape associated with growth arrest. Our results suggest that *p120-Gap* and *Gap1^m* genes provide important biomarkers for cancer therapies.

Keywords: *ras*; *ras-Gap*; *Gap1^m*; expression; MEK; doxorubicin; trichostatin A.

Introduction

Ras proteins (Ki-, Ha-, and N-Ras) are three closely related members of the Ras family, which controls various cellular processes, such as growth and proliferation [1]. Oncogenic mutants of *ras* genes are found in about 30 % of human tumors. Especially, pancreatic adenocarcinoma is the tumor with the highest incidence of Ki-*ras* point mutations, occurring in 70 to 90% of all cases [2].

Ras cycles between a GTP-bound form (Ras-GTP) and a GDP-bound form (Ras-GDP). The active Ras-GTP is converted to the inactive Ras-GDP by intrinsic GTPase activity, which is accelerated by the GTPase-activating protein (Ras-GAP) [1]. Oncogenic Ras is associated with Ras-GAP [3], where it is insensitive to catalysis by GAP [4]. Therefore, oncogenic Ras always remains in the GTP-bound form, which cannot switch off the downstream signal.

Several types of GAP proteins for Ras have been identified in mammalian cells, including p120-GAP, neurofibromin (the product of *Nf1* tumor-suppressor gene) and GAP1 [5]. The classical Ras-GAP, p120-GAP, has two Src-homology (SH)2 and one SH3 domains. SH domains of p120-GAP function as effectors by associating with several tyrosine-phosphorylated proteins [5], which induce Ras-dependent gene expression [6] and repress adhesion [7]. In addition, p120-GAP is involved in Ras transformation [8, 9], cell survival [10, 11, 12] tumor cell invasion [13], and *v*-Src-induced cytoskeletal disruption [14]. Based on these findings, p120-GAP proteins show promise as a target for anticancer approaches [11]. However, it is still not clear that p120-GAP contributes to the tumor cell activity, since full Ras function requires multiple downstream pathways, i.e. cascades from Raf to extracellular signal-regulated kinase (ERK), from p120-GAP to p190 Rho-GAP, and from phosphatidylinositol 3-kinase (PI3K) to AKT [1].

The 100 kDa Ras-GAP1 family contains two closely related proteins, GAP1^m [15]

and GAP1^{IP4BP} [16] / GAPIII (a mouse homologue of GAP1^{IP4BP}) [17]. Though roles of GAP1 are not completely clear, its distinct perinuclear localization [18] leads us to expect that GAP1^m might be involved in the transcriptional process. Physiologically, GAP1 has the G protein (G α 12)- [19] and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄)-binding sites [20], and activates inositol tetrakisphosphate (IP₄)-gated Ca²⁺ influx [21]. The same line of evidence is provided by us that bradykinin and Ins(1,3,4,5)P₄ induce continuous Ca²⁺ influx in oncogenic *ras*-transformed NIH/3T3 fibroblast DT cells [22], predicting the up-regulated GAP1 function [23, 24].

To know whether *Ras-Gap* gene expressions are up-regulated and linked to the tumor biological activity in *ras*-transformed cells, we compared mRNA expressions of four *ras-Gap* genes (*p120-Gap*, *Gap1^m*, *GapIII*, *Nf1*) between DT [25] and parental NIH/3T3 cells, and studied their sensitivity to anti-cancer drugs. We also measured the viral oncogene *v-Ki-ras*, *PIK3CA* which encodes the p110 α catalytic subunit of PI3K [1], *p53* that encodes a transcription factor which induces cellular senescence in response to oncogenic Ras signals [26], and *PTEN* tumor suppressor gene that encodes a phosphatase [27] which inhibits *ras* transformation in NIH/3T3 cells [28].

Materials and methods

Cell culture. *v-Ki-ras*-transformed NIH/3T3 fibroblasts (DT) [25] and parental mouse NIH/3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal calf serum (FCS). *v-Ha-ras*-transformed rat fibroblasts (tet-H-*ras*/CREF: tet-off) and parental cells (tet-H-*ras*/CREF: tet-on) [29] were grown as the above condition. 2 x 10⁵ cells (transformants) or 3-5 x 10⁵ cells (parental

cells) were seeded in 100-mm dishes and grown for 2 days to meet the exponentially proliferating phase.

RNA isolation and cDNA purification. Total RNA from cells at the exponential phase was isolated by RNA-zol, and further treated with RNase-free DNase I. Absence of contaminating genomic DNA in RNA samples was verified by QRT-PCR up to 40 cycles using β -actin primers. Reverse transcription reactions were performed by 5 μ g of total RNA in 20 μ l of DNase-, RNase-free water with murine reverse transcriptase and random hexadeoxynucleotides, using a first-strand cDNA synthesis kit.

Real-time quantitative reverse transcription-PCR (QRT-PCR). Primers were designed to amplify segments of <150 bp to maximize efficiency, which are summarized in Table 1. Expressions of the above genes were determined by QRT-PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). PCR reaction was performed with SYBR Green, the double-strand DNA-selective fluorescent dye. 25- μ l of reaction mixture was composed of 12.5 μ l of 2X SYBR Green PCR Master Mix, 500 nmol/l forward primer, 500 nmol/l reverse primer and 1.1 ng of first-strand cDNA. Data were analyzed using GeneAmp 7700 sequence detection system (SDS) software (version 1.6.3) and converted into threshold cycle (Ct) values. Expression of the housekeeping gene, β -actin, was used to normalize for variances in input cDNA. To adjust for variations in the amount of input cDNA, a Ct value for each gene was normalized against the Ct value for the housekeeping gene, i.e., $\Delta Ct = Ct$ (specific gene) – Ct (β -actin). A linear standard curve representing 4-fold dilutions of DT stock cDNA (1:2.5, 1:10, 1:40, 1:160) versus ΔCt was used for evaluating gene expression of unknown samples. All experiments were performed in triplicate and repeated 2 times. The PCR products were also examined using 2% agarose gel electrophoresis. After

ethidium bromide staining, bands were visible only at the expected molecular weights for each target mRNA product.

Western blot. Cells were treated with SDS buffer including 0.2% β -mercaptoethanol. 15 μ g of the samples for each lane was analyzed on a 10% SDS-PAGE followed by blotting on a nitrocellulose membrane. The membrane was incubated with antibodies, and subsequently visualized using the chemiluminescent solution. The visualized signals was analyzed by J-image software.

Cell count. Viable cell numbers were determined by trypan blue dye exclusion counted in a Neubauer-type hemocytometer. All experiments were performed in duplicate and repeated 3 times.

Sources of Drugs. EGF, doxorubicin and monoclonal anti- β -actin antibodies were purchased from Sigma (St. Louis, Mo., USA). PD98059 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA) or CALBIOCHEM (San Diego, CA, USA). TSA was obtained from Wako Pure Chemicals (Osaka, Japan). RNA-zol was obtained from BIOTECH LAB (Houston, TX, USA), and DNase I from Takara biomedical (Otsu, Japan). The first-strand cDNA synthesis kit was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). SYBR Green Master Mix was obtained from Applied Biosystems (Foster City, CA, USA). Primers were synthesized at Hokkaido System Science (Sapporo, Japan). Mouse monoclonal antibodies against human Ras-GAP was obtained from BD Biosciences Pharmingen (San Diego, CA, USA). Goat anti-mouse IgG antibodies and chemiluminescent solution were obtained from Pierce (Rockford, IL, USA). Molecular weight size marker was purchased from Invitrogen.

Results and Discussion

p120-Gap, Gap1^m and PIK3CA overexpression in DT cells

In this study we investigated the biological activity of oncogenic *v*-*Ki-ras* transformed clone (DT cells) by measuring mRNA levels of *ras*-related genes. Fig. 1A shows comparative mRNA expression analysis of DT cells *versus* parental NIH/3T3 cells. *p120-Gap* and *Gap1^m* mRNA expressions were higher in DT cells by 9.3-fold and 12.2-fold over the parental cells, respectively (mean value). *PIK3CA* expression was also higher by 6.2-fold. mRNA expressions of other target genes, *GapIII*, *Nf-1*, *PTEN* and *p53*, were at the similar levels between DT and NIH/3T3 cells. p120-GAP protein expressions were also increased in DT cells by 2.6-fold (Fig. 1B).

We also tested mRNA levels of *ras-Gap* genes in the different cells. In another *v*-*Ki-ras* transformant, 635 cells, *p120-Gap* and *Gap1^m* expressions were higher by 2.1-fold and 4.2-fold over the parental NIH/3T3 cells, respectively. Similarly, in *v*-*Ha-ras*-transformed fibroblasts with the regulatable *Ha-ras*(V12) gene (tet-H-*ras*/CREF) [29], *p120-Gap* and *Gap1^m* expressions were also higher over the parental (*ras* off) cells by 3.5-fold and 3.5-fold, respectively (data not shown). These results indicate that transformation of *ras* may cause increase in mRNA levels of *p120-Gap* and *Gap1^m*, though the extent is variable. The different expression levels might be related to the tumor biological activity, as the doubling time in DT cells (10.3 hrs in the present study) is shorter than that in 635 cells (20 hrs) or tet-H-*ras*/CREF cells (18.6 hrs).

Our results are in accord with the role of *PIK3CA* implicated as an oncogene in ovarian cancer [30]. Besides our results are novel in that *p120-Gap* and *Gap1^m* genes are involved in *ras*-transformation. Therefore, it could be predicted that the up-regulated *ras-Gap* genes as well as *PIK3CA* facilitate DT cell survival.

Next, we studied effects of growth factors on gene expressions. 2 hrs after stimulation of DT cells with 10 % FCS or EGF (25 ng/ml), *p120-Gap* expression was increased by 8.0- and 5.0-fold of the serum-starved level, respectively (Fig. 2A-a and -c). *PIK3CA* expression was also strongly induced after stimulation with growth factors. *Gap1^m* expression was slightly induced after stimulation with FCS and EGF by 3.3-fold and 2.9-fold, respectively. On the other hand, apparent changes of *GapIII* and *Nf-1* expressions were not detected either by FCS or EGF in this study (Fig. 2A-a). As shown in Fig. 2A-b and -d, neither FCS nor EGF affected the steady-state mRNA levels of all tested genes in parental NIH/3T3 cells. These results indicate that mRNA induction of *p120-Gap*, *Gap1^m* and *PIK3CA* genes is growth factors-dependent in *v*-*Ki-ras*-transformed DT cells. Similar results were observed in the *v*-*Ha-ras* transformants: 2 hrs of serum-deprivation resulted in decrement of *p120-Gap* expression by -8.2-fold, and subsequent addition of EGF caused increment by 3.8-fold (Fig. 2B-a).

PD98059 selectively reduces p120-Gap and Gap1^m mRNA expressions in DT cells

To determine whether these overexpressed genes are positively or negatively involved in DT cell survival, effects of several anti-cancer drugs on gene transcription were studied. Treatment of DT cells with the specific MEK inhibitor PD98059 (50 μ M) for 48 hrs selectively decreased the expression levels of *p120-Gap* and *Gap1^m* mRNA to -9.6-fold and -12.3-fold, respectively (Fig. 3A-a). 6 other genes stayed at almost the same level. PD98059 did not affect the steady-state mRNA levels of all target genes in NIH/3T3 cells (Fig. 3A-b). We also performed a time course analysis of mRNA levels of *p120-Gap* and *Gap1^m* genes in PD-treated DT cells. PD98059 caused rapid decreases during the initial 4 hrs in the expression of both *p120-Gap* and *Gap1^m*, followed by gradual decreases

during the next 5 - 24 hrs (data not shown). The result suggests that expressions of *p120-Gap* and *Gap1^m* are regulated by factor(s) downstream of MEK. A plausible one is the activator protein-1 (AP-1) transcription factor, such as cJun and Fra1 [31].

Blocking overexpression of ras-Gap genes by doxorubicin in DT cells

To determine whether common chemotherapeutic agents affect target gene transcription in DT cells, we focused on the topoisomerase II (topo II) poison, doxorubicin (adriamycin). Doxorubicin (10 μ M) treatment for 4 hrs caused selective reduction in *Gap1^m* expression to -5.1-fold among 8 genes tested in DT cells (Fig. 3B-a). Suppression of *Gap1^m* expression after 4 hrs of treatment was prior to apparent growth arrest or apoptosis (Fig. 4B). Doxorubicin did not affect the steady-state mRNA levels of all target genes in NIH/3T3 cells (Fig. 3B-b). Time course analysis shows that *p120-Gap* expression level was also reduced to -6.5-fold after 12 hrs of doxorubicin treatment (Fig. 4A-a). After 24 hrs of treatment, *p120-Gap* and *Gap1^m* expressions were equally reduced (-9.9 and -9.6-fold). In contrast to this, Expressions of 6 other genes were less modulated until after 12 hrs of treatment (Fig. 4A). Thus, doxorubicin treatment down-regulated overexpression of *p120-Gap* and *Gap1^m* genes in DT cells. These results implicate that the reduced *ras-Gap* mRNA levels contribute to the growth inhibitory properties of doxorubicin. For a mechanism of down-regulation of *ras-Gap* genes, the topo II poison doxorubicin might suppress the expression of immediate early genes. A plausible target is the AP-1, since AP-1 activity reflects the tumor sensitivity to doxorubicin [32].

Effect of trichostatin A on ras-Gap and p53 mRNA expressions in DT cells

Histone deacetylase (HDAC) inhibitors induce morphological reversion in various tumors [33], thus hold promise as anticancer agents. We studied whether trichostatin A (TSA), a specific HDAC inhibitor, regulates the transcription of *ras*-related genes in DT cells. TSA (100 ng/ml) treatment for 12 hrs reduced *p120-Gap*, *Gap1^m* and *p53* expressions of DT cells to -5.3, -4.6, and -12-fold of the pre-treated level, respectively (Fig. 3B-a). It did not affect the steady-state mRNA levels of all target genes in NIH/3T3 cells, with the exception of *p53* (-13-fold) (Fig. 3B-b). We found a rapid suppression with a partial recovery of *p120-Gap* expression, -9.3-fold for 4 hrs and -5.0-fold for 24 hrs of treatment (data not shown).

Effects of PD98059, doxorubicin and TSA on morphology and growth in DT cells.

Finally, in order to understand the biological effects of *ras-Gap* mRNA overexpression, we studied the modulation of cell morphology and growth by above agents (Fig. 3D). After 24 hrs of treatment with PD98059 (50 μ M), DT cells of round, thick and non-adherent shapes were altered to be long, flat and adherent shapes, more similar to those of NIH/3T3 cells. After 24 hrs of treatment with doxorubicin (10 μ M), survived cells were converted to a flat and adherent shape. TSA (100 ng/ml) treatment for 24 hrs also induced adherent shape in DT cells, as reported previously [33]. The number of DT cells treated with PD, doxorubicin, TSA for 24 hrs was reduced to 39 ± 13 %, 12 ± 3 %, 23 ± 12 % of untreated DT cells (n=6), respectively. Thus, treatment with these drugs caused growth arrest and morphological reversion of DT cells to adherent and flat shapes. As p120 Ras-GAP suppresses focal adhesion by its association with p190 Rho-GAP [7], our results suggest that the up-regulated *p120-Gap* causes repression of Rho signaling, leading to the decreased focal adhesion, thereby increases motility in DT cells.

We focused on two sets of information by profiling of mRNA expression in this study. One is the causal information for tumorigenesis, as discussed above. Another is to find biomarkers for the new molecular specific therapy. Our results indicate that *p120-Gap* and *Gap1^m* are included in important biomarkers for evaluating the biological activity, and for anti-cancer treatments in some of *ras*-mutated tumors.

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Figure Legends

Fig. 1. Monitoring of expression in *v*-Ki-*ras*-transformed and control mouse fibroblasts.

(A-a) The relative expression level of 7 genes was determined in RNA samples from *v*-Ki-*ras*-transformed DT and parental NIH/3T3 cells using real-time quantitative PCR. Housekeeping normalized units (Δ threshold cycle) for each gene obtained in the PCR analysis were used to determine the fold-change among samples. Bars represent fold-changes of the mRNA level of particular genes when comparing DT with parental NIH/3T3 cells. Positive values indicate that the transcript is more abundant in DT than NIH/3T3 cells (overexpressed), and negative values indicate the opposite (underexpressed). Data are expressed as the mean \pm S.D., performed in duplicate and repeated 2 times.

(A-b, c) Each plot represents the baseline-subtracted fluorescence intensity (ΔR_n) that reflects mRNA levels of *p120-Gap* (b), *Gap1^m* (c), or β -actin genes. Horizontal lines indicate threshold lines set in the exponentially increasing area calculated by SDS software.

(B) Comparison of Ras-GAP expression pattern by immunoblotting in DT cells and parental NIH/3T3 cells. The top bands marked by the arrow indicate full-length (115 kDa) p120 Ras-GAP proteins.

Fig. 2. Genes induced in response to fetal calf serum (FCS) or epidermal growth factor (EGF) in DT and NIH/3T3 cells.

(A-a, b) DT cells (a) and NIH/3T3 cells (b) were serum-starved for 2 hrs (pre), followed by treatment with 10% FCS (shaded bars) or EGF (25 ng/ml) (hatched bars) for 2 hrs

(post). Bars represent fold-changes of the mRNA level of 8 genes when comparing the stimulated cells (post) with serum-starved control cells (pre). Data are expressed as the mean \pm S.D. performed in duplicate and repeated 2 times. (A-c, d) Representative amplification plots for *p120-Gap* and β -actin. (B) *v*-Ha-*ras*-transformed (tet-H-*ras*/CREF: tet-off) and parental (tet-H-*ras*/CREF: tet-on) cells originally in 10 % FCS condition were serum-starved for 2 hrs, followed by addition of EGF (25 ng/ml) for 2 hrs.

Fig. 3. Effects of PD98059, doxorubicin and trichostatin A on mRNA levels of *ras*-related genes and the cell morphology in DT and parental cells

(A-C) Treatment with 50 μ M PD98059 for 48 hrs (A), 10 μ M doxorubicin for 4 hrs (B), 100 ng/ml trichostatin A for 12 hrs (C) in DT (a) and NIH/3T3 (b) cells. Bars represent fold-changes of the mRNA level of 8 genes when comparing drug-treated cells with untreated cells. Data are expressed as the mean \pm S.D. (D) Phase contrast images of DT, NIH/3T3, DT cells treated for 24 hrs with 50 μ M PD98059, 10 μ M doxorubicin, or 100 ng/ml trichostatin A.

Fig. 4. mRNA expression levels of *ras*-related genes in doxorubicin-treated DT cells.

(A) Each plot represents the fold-change of the mRNA level of 8 particular genes (a-c) when comparing DT cells treated with 10 μ M doxorubicin for indicated periods with untreated cells. Each symbol represents the mean \pm S.D. (B) Effects of doxorubicin on the DT cell growth. Each plot represents the fold-change of cell numbers when comparing DT cells treated with 10 μ M doxorubicin (●) with untreated cells (○). Each symbol represents the mean \pm S.D.

Table 1. Primer sequences for real-time QRT-PCR

m-p120- <i>Gap</i>	F: 5`-CGTGGTGTACAGCAGCATGTACT-3` R: 5`-AGGCTGCTACCTGATGTCA-3`
m- <i>Gap1m</i>	5`-AGTTCACCATTGAGGATTCT-3` 5`-CTCATGAGGTTTCATCGAGCTT-3`
m- <i>GapIII</i>	5`-CTGAGACTGAGTGAGGTCATT-3` 5`-GAGACCCTGGCACTCGAAGAT-3`
m- <i>Nf-1</i>	5`-GCAGGAGAACCTCTGCCTGACA-3` 5`-CATACACAGGACAGGAGCAAGT-3`
m- <i>PIK3CA</i>	5`-GGCTCTGGAATGCCAGAACTA-3` 5`-CCACCATGATGTGCATCATTTCAT-3`
m- <i>PTEN</i>	5`-GGCTAGCAGTTCAACTTCTGTGACT-3` 5`-GGATCAGAGTCAGTGGTGTGACA-3`
m- <i>p53</i>	5`-TCACCTCACTGCATGGACGATCT-3` 5`-TGTGCTGCAGGAGCTCCTGACA-3`
m-v-Ki- <i>ras</i>	5`-ACTTGTGGTAGTTGGAGCTA-3` 5`-GGAGTCCTCTATCGTAGGATCA-3`
m-c-Ki- <i>ras</i>	5`-CACTTCCAGCATGTCCTAGA-3` 5`-CTAGCAGTGGAAAGCTAGA-3`
m- β -actin	5`-CTCTAGGCACCAAGGTGTGAT-3` 5`-GGTACTTCAGGGTCAGGATA-3`
r-p120- <i>Gap</i>	5`-GTTGCACCACCAGAGCCAGTA-3` 5`-CAGAATGGCTCGTACACGTCTT-3`
r- <i>Gap1m</i>	5`-GTACGACTGTTGCTGCATCACA-3` 5`-GTCAGCCACCGCAGTGATAA-3`
r- β -actin	5`-TGGCTCCTAGCACCATGAAGATCA-3` 5`-GGACAGTGAGGCCAGGATAGA-3`

Fig. 1

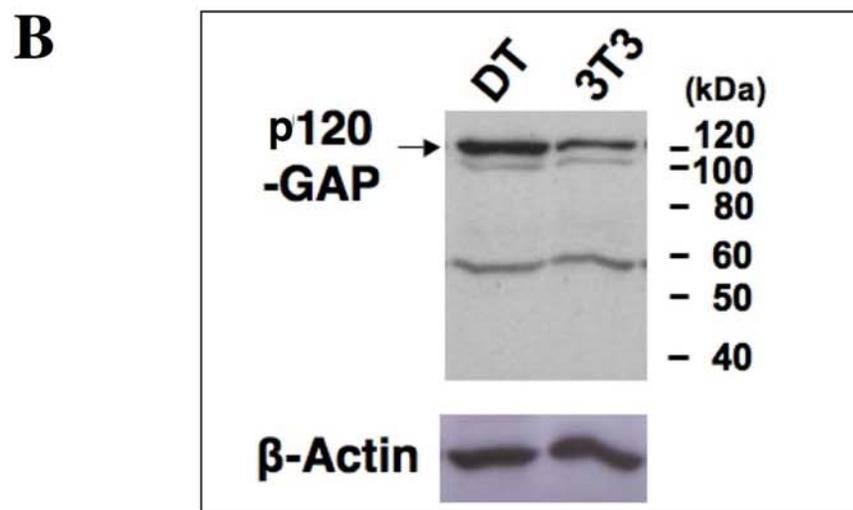
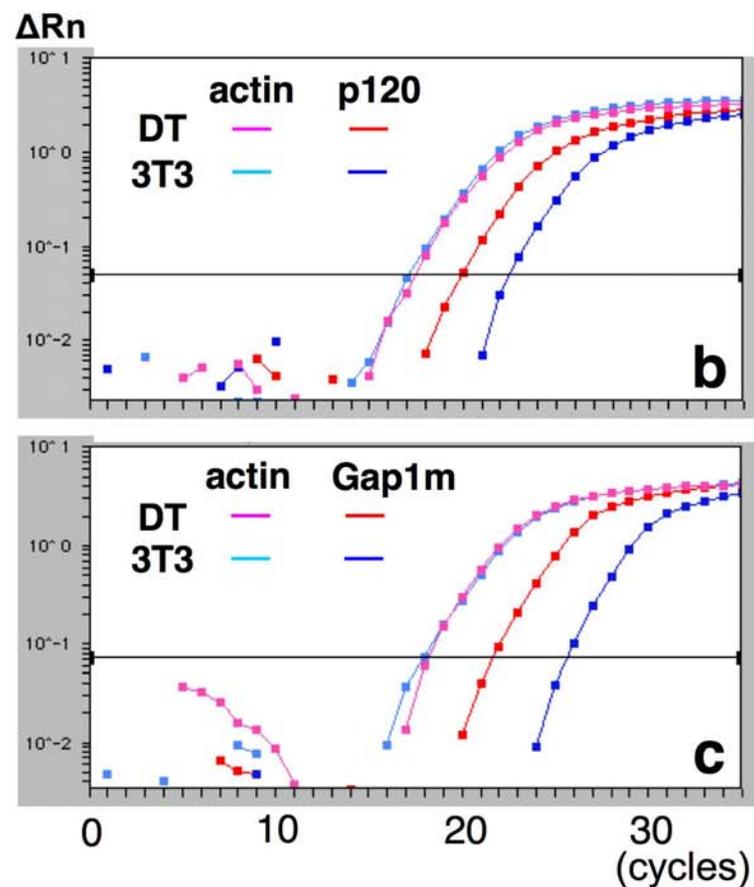
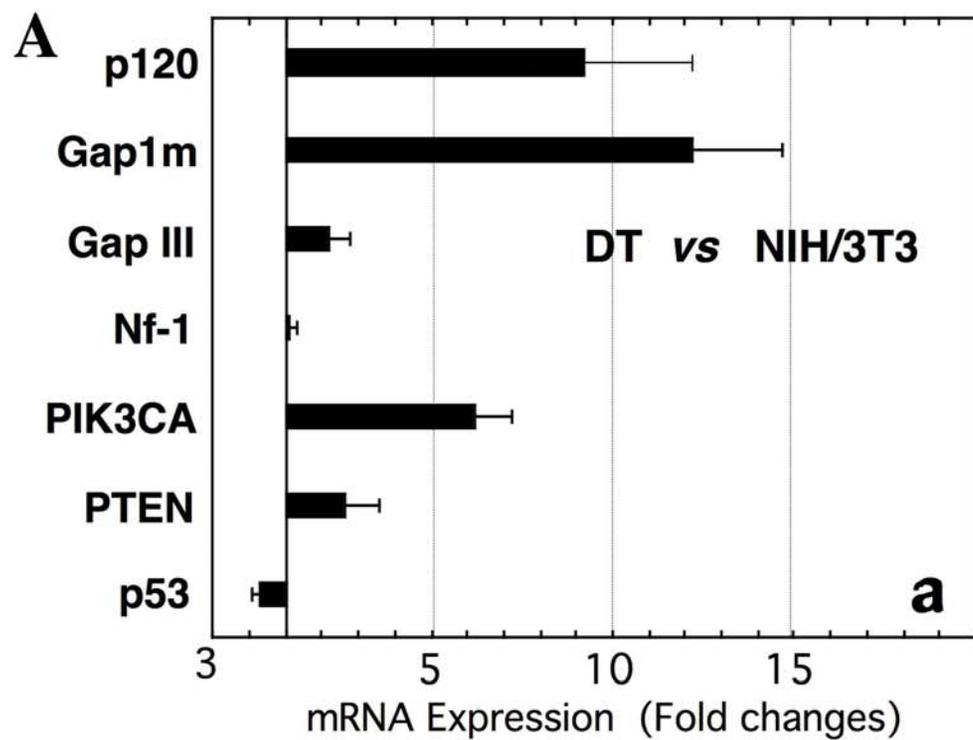
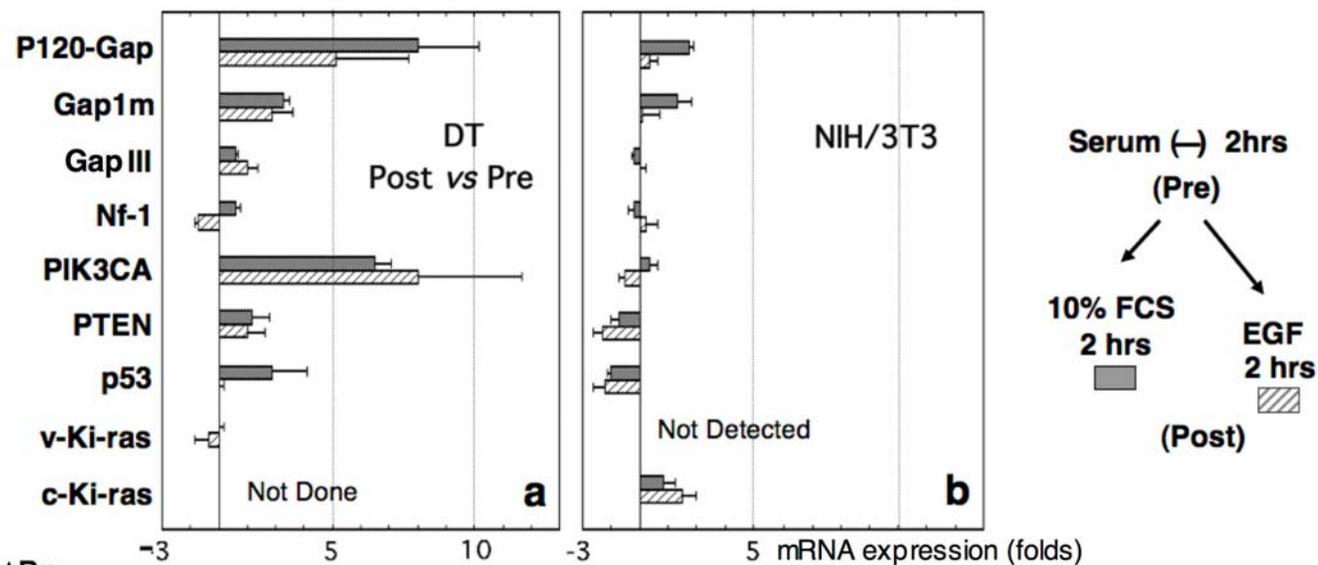
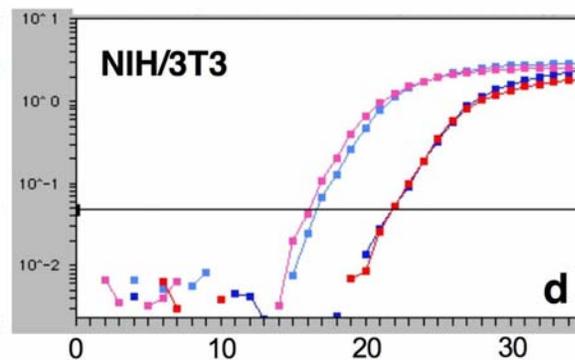
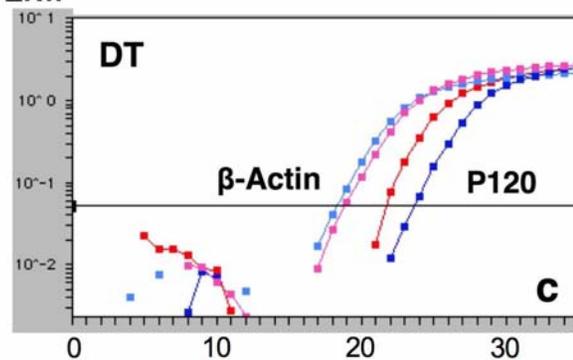


Fig. 2

A



ΔRn

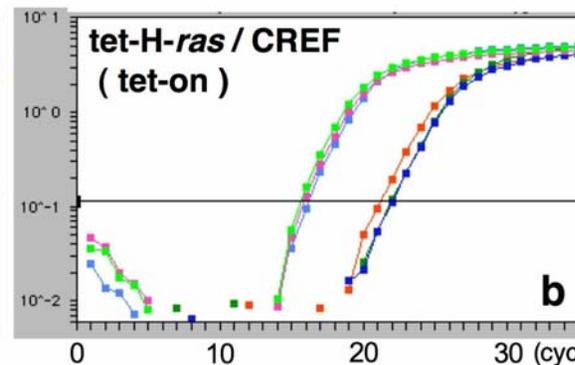
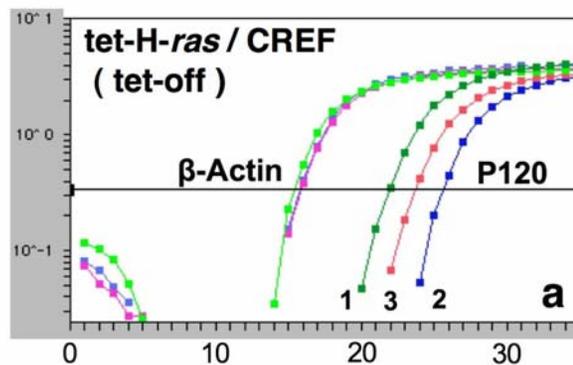


Serum (→) 2hrs

↓

EGF 2hrs

B



1 **10% FCS**

↓

2 **Serum (→) 2hrs**

↓

3 **EGF 2hrs**

(cycles)

Fig. 3

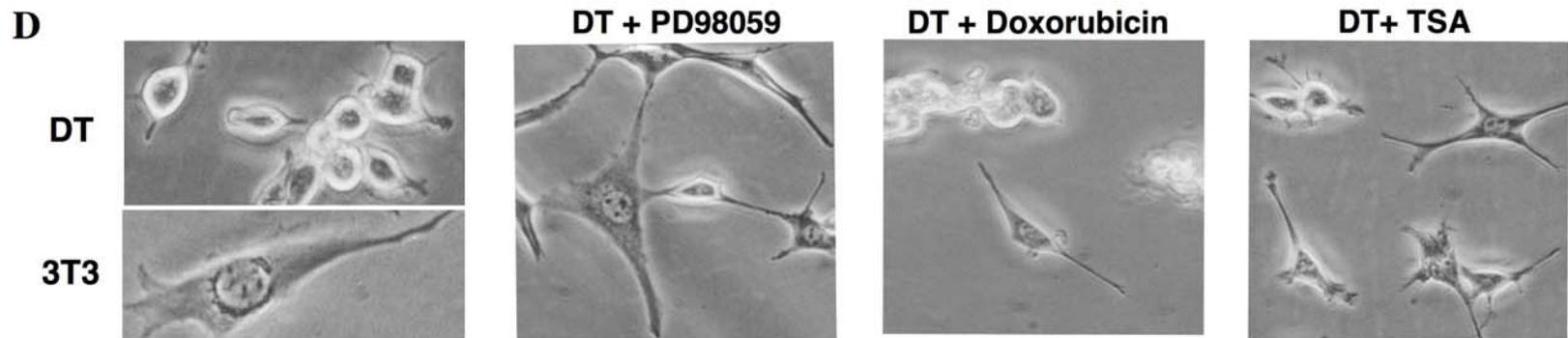
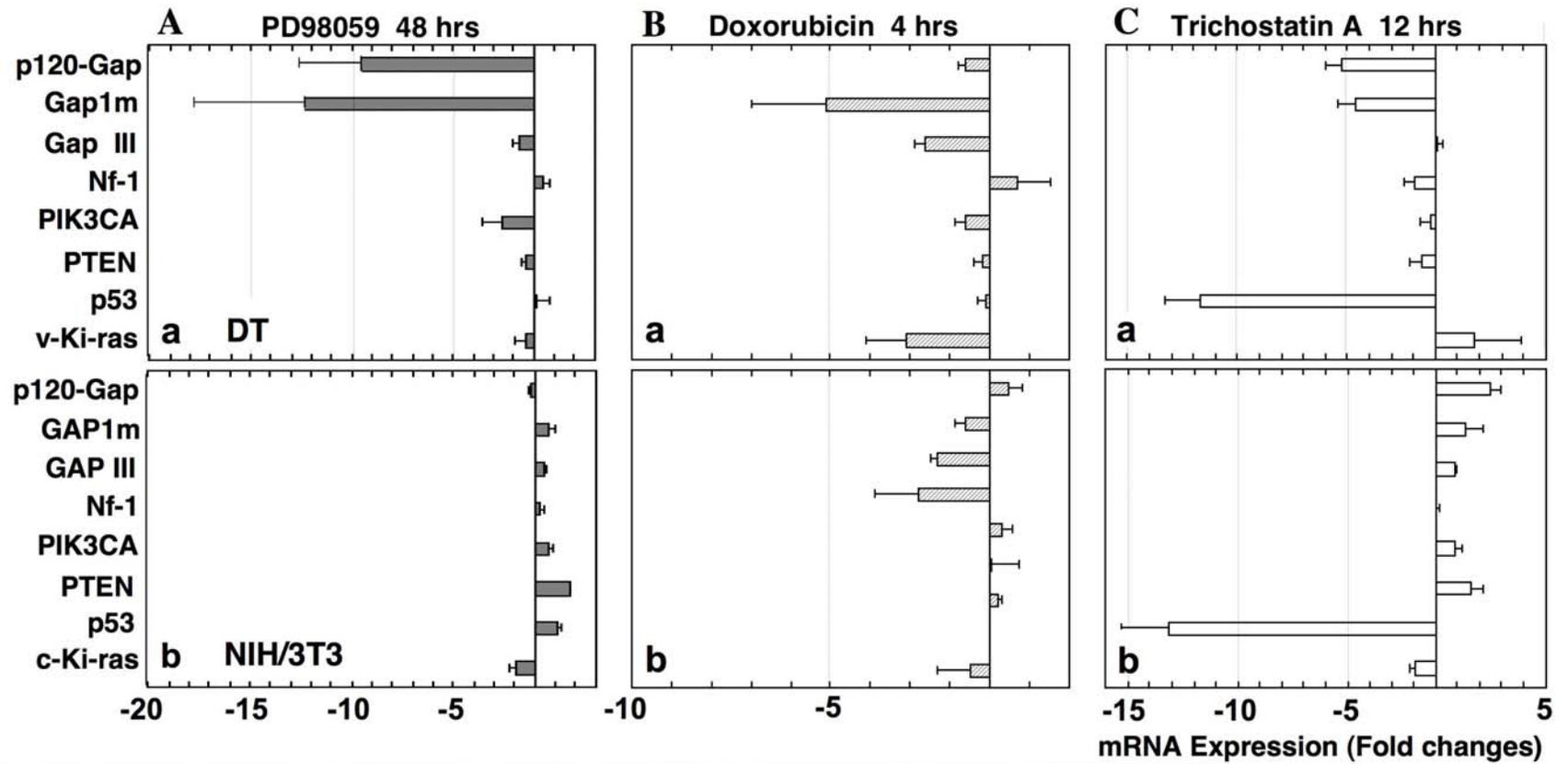


Fig. 4