C3G-MEDIATED SUPPRESSION OF ONCOGENE-INDUCED FOCUS FORMATION IN FIBROBLASTS INVOLVES INHIBITION OF ERK ACTIVATION, CYCLIN A EXPRESSION AND ALTERATIONS OF ANCHORAGE-INDEPENDENT GROWTH.

Carmen Guerrero, Susana Martín-Encabo, Alberto Fernández-Medarde, and Eugenio Santos.

Centro de Investigación del Cáncer, IBMCC, Universidad de Salamanca-CSIC, 37007-Salamanca, Spain.

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Correspondence:

E. Santos
Centro de Investigación del Cáncer, IBMCC
Universidad de Salamanca-CSIC
Campus Miguel de Unamuno
37007 Salamanca, Spain
tel: 34-923-294720
fax: 34-923-294743
esantos@usal.es

SUMMARY

We showed previously that exogenous overexpression of C3G, a guanine nucleotide releasing factor (GEF) for Rap1 and R-Ras proteins, blocks the focus-forming activity of cotransfected, activated, *sis*, *ras* and v-*raf* oncogenes in NIH 3T3 cells. In this report, we show that C3G also interferes with *dbl* and R-*Ras* focus forming activity and demonstrate that the transformation suppressor ability of C3G maps to its Crk-binding region (SH3-b domain). Using full-length C3G and C3GΔCat mutant, lacking catalytic domain, we showed here that overexpression of cotransfected C3G or C3GΔCat inhibited oncogenic $Hras^{lys12}$-mediated phosphorylation of ERK, without altering Ras and Raf-1 kinase activation. We also showed that, overexpressed C3G and C3GΔCat inhibited viability of oncogenic Ras-induced colonies in soft agar, indicating that C3G interferes with the anchorage-independent growth of Ras-transformed cells in a Rap1-independent manner. Consistent with both observations, overexpression of exogenous C3G and C3GΔCat also caused downregulation of Ras-induced cyclin A expression. All together, our results indicate that C3G interferes with at least two separate aspects of oncogenic transformation -cell cycle progression and loss of contact inhibition-, and that these inhibitory effects probably account for its transformation suppressor activity.

INTRODUCTION

The C3G gene product is a modular protein acting as an exchange factor for the Rap and R-Ras proteins in fibroblasts (Gotoh et al., 1995; Gotoh et al., 1997; Ohba et al., 2000). C3G binds to the N-terminal SH3 domain of the adaptor protein Crk through four proline-rich stretches encompassing the central region of the molecule (residues 282-616 of the human protein) (Knudsen et al., 1994; Tanaka et al., 1994). The C3G molecule also encompasses CDC25-H and REM domains characteristic of the GEFs for the Ras family of proteins (Quilliam et al., 2002).

C3G is activated by multiple signals depending on the cell type (McLeod et al., 1998; Reedquist and Bos, 1998; Sakkab et al., 2000; Yokote et al., 1998; York et al., 1998; Zhu et al., 1998) and also can act as a mediator in the activation of several pathways, including the JNK, ERK and Rac signaling pathways (Mochizuki et al., 2000; York et al., 1998) (Abassi and Vuori, 2002; Ishimaru et al., 1999).

C3G signaling also plays a crucial role in integrin-mediated cell adhesion and migration (Arai et al., 2001; Arai et al., 1999; de Jong et al., 1998; Uemura and Griffin, 1999), assembly and stabilisation of focal adhesion (Li et al., 2002; Voss et al., 2003) and vascular myogenesis (Voss et al., 2003), a function that probably involves activation of Rap1 or R-Ras (Arai et al., 2001; Arai et al., 1999; de Jong et al., 1998; Uemura and Griffin, 1999). In fact, disruption of C3G expression in mice affects adhesion and spreading of embryonic fibroblasts (Ohba et al., 2001). Cell adhesion plays an essential role in regulating proliferation during the G1 phase of the cell cycle, and loss of substrate adhesion is a requirement for oncogenic transformation (Guadagno et al., 1993). Oncogenic Ras induces anchorage-independent growth in rodent fibroblast cell lines, measured as the ability to form colonies in semisolid medium. It has been described that Ras drives cyclin A expression, a most critical step for induction of anchorage-independent growth by this oncogene (Yang et al., 1998).

We have demonstrated previously that exogenously overexpressed C3G suppresses Ras-mediated transformation, an effect that appears to be independent of its CDC25-H, catalytic domain (Guerrero et al.,

1998). The aim of this study was to map the specific region of C3G required for its transformation suppression activity and to determine the mechanisms by which C3G exerts such an effect. Here we show that the Crk-binding domain is responsible for the transformation suppression ability of C3G, and that this is not exclusive of Ras-mediated pathways but seems to be a more general mechanism affecting other oncogenes like *dbl* or R-*ras*. Our data suggest that the mechanism of suppression involves inhibition of Ras-dependent ERK activation, and cyclin A expression. Furthermore, such an inhibitory effect is accompanied by a decrease in viability of cells in non-adherent conditions. Therefore, at least two aspects of oncogenic transformation, cell cycle regulation and anchorage-independent growth, are altered by overexpression of C3G. These alterations may account, at least in part, for the inhibitory effect of C3G on oncogene-mediated transformation of NIH 3T3 fibroblasts.

RESULTS

Transformation suppressor activity of C3G maps to its SH3-binding domain.

We have previously shown that the transformation suppressor activity of C3G on *sis*, *ras* and *raf* oncogenes was independent of its catalytic domain (Guerrero et al., 1998). To further characterize the C3G region responsible for the suppression effect, we constructed additional deletion mutants including ΔNC3G (lacking the first 636 nucleotides of the gene) and C3GSH3-b (containing only the Crk-binding region; n625-n1982) which were cloned into mammalian expression vector pLTR2 (DiFiore et al., 1987) (Figure 1a). These constructs were transfected into NIH 3T3 cells alone or in combination with a Neo-selectable construct expressing oncogenic H-*ras* (pMEXneo Hras^{lys12}, (Benito et al., 1991)). Transfectants were selected with G418 and/or HAT/*gpt* according with plasmid combinations. Protein extracts from NIH3T3 cells cotransfected with Ras and these new C3G constructs and selected for double resistance (neo+gpt), showed a high level of expression of both C3G clones and Hras^{lys12} peptides (Figure 1b). We observed in several independent experiments that both the ΔNC3G and the C3GSH3-b constructs were able to decrease about 40-75% the number of Ras-induced foci (Figure 1c,d), a reduction similar to that observed with pLTR2C3G and pLTR2C3GΔCat constructs (Figure 1c) (Guerrero et al., 1998). These results confirmed that the SH3-b region is necessary for C3G to exert its transformation suppressor activity.

Fig. 1

C3G suppresses the transforming ability of dbl and R-ras oncogenes.

To determine whether the transformation suppressor effect of C3G is limited to components of Ras-dependent pathways or it involves more general mechanisms, we studied the effect of our C3G mutant constructs on *dbl* and R-*ras* cotransfected oncogenes. Figures 2 a,b show that C3G was also able to inhibit foci formation by these two oncogenes and that, as for *ras*, *raf* and *sis*, this effect was mediated by the SH3-b domain. Likewise, as observed with Ras (Guerrero et al., 1998), constructs in which the catalytic domain had been eliminated (C3GΔCat) showed an even higher inhibitory effect than the complete C3G

Fig. 2

over R-*ras* and *dbl* induced transformation (Figure 2a, b). Expression of both proteins was monitored by Western blotting (data not shown). These results indicate that the suppressor activity of C3G is not restricted to Ras pathways but it seems to involve a more general mechanism.

C3G does not affect Ras, Raf-1 and MEK-1 activation.

Results from our previous work (Guerrero et al., 1998) indicated that C3G blocked malignant transformation triggered by components of the Ras-Raf pathway. Thus, a first question is whether C3G directly affects Ras and/or Raf-1 activities. We performed Ras activity assays on cells permanently transfected with C3G, alone or in combination with oncogenic *ras*. Figure 3a showed that the presence of C3G did not affect the amount of Ras-GTP complex present in cells treated with FBS for various periods of time. Furthermore, regarding Raf-1, we also showed in transient tranfections that C3G did not affect either the mobility shift due to Raf-1 phosphorylation (Figure 3b). Since Raf-1 can be phosphorylated either for activation or for inhibition (Dhillon et al., 2002; Marais et al., 1995; Sozeri et al., 1992), to ensure that C3G is not affecting Raf-1 activity we studied, in permanently transfected clones, the phosphorylation status of MEK1, a known Raf-1 substrate, using an anti phosphoMEK1 antibody that specifically recognizes the serine residues (ser217 and ser221) phosphorylated by Raf-1. Figure 3c showed that C3G did not affect Raf-1-dependent MEK1 phosphorylation, suggesting that, as with Ras, Raf-1 activity is not altered by C3G.

Fig. 3

C3G expression inhibits Ras-induced ERK phosphorylation.

To gain further insight into the mechanism of suppression, and given that both Ras and Raf are components of the ERK/MAPK pathway, we studied whether C3G affects Ras-mediated ERK activation, measured as p44/42 ERK phosphorylation. Permanently transfected clones expressing C3G and $Hras^{lys12}$, alone or in combination, were starved for 20 h and stimulated for 5 minutes with 10% FBS or 25 ng/ml PDGF before determination of the levels of phosphorylated ERK. Figure 4a showed that oncogenic H-Ras-mediated phosphorylation of ERK is inhibited in clones expressing C3G. We confirmed this result in

transiently transfected cells with the same DNAs. Figure 4b showed that the levels of Ras-mediated ERK phosphorylation decreases both in starved and exponentially growing cells that transiently overexpress C3G. This inhibitory effect was not Rap1 or R-Ras-mediated since C3GΔCat mutant, lacking the catalytic domain, also exhibited the same effect on ERK activation (Figure 4c).

Fig. 4

C3G alters anchorage-dependent properties of fibroblast cells

We have previously shown that C3G does not affect NIH 3T3 cells viability in adherent conditions, and that C3G expressing cells exhibit a similar growth rate to cells transfected with vector alone (Guerrero et al., 1998). Besides faster growth, another indicator of tumorigenicity is the ability of transformed cells to form colonies in semisolid medium. Thus, we studied whether C3G may affect anchorage-independent growth of oncogenic Ras-expressing cells. NIH 3T3 clones expressing C3G, C3GΔCat or $Hras^{lys12}$ alone or in combination, were seeded in soft agar as described in Materials and Methods, and the number and size of resulting, growing colonies was determined. Figure 5 showed that expression of C3G and C3GΔCat reduced dramatically the growth (measured as the size of the colonies and the number of colonies larger than ~3000 μm^3) of Ras-induced proliferating colonies in soft agar which is in concordance with its suppression role. This result clearly indicate that C3G exerts a strong inhibitory effect over the ability of Ras transformed cells to grow in soft agar independently of its exchange activity.

Fig. 5

The above results suggest that C3G expressing cells may display higher substrate dependence, and that C3G may confer augmented adhesive properties to the cells. To study this hypothesis, and based on recent reports showing that C3G plays an essential role for adhesion to laminin and gelatin (Voss et al., 2003) we studied the adhesiveness of C3G and C3GΔCat expressing cells using laminin-1 coated wells. Figure 6 showed that ectopic expression of C3G increases ~40% to 60% the adhesion to laminin-1 of wild type NIH 3T3 cells or cells cotransfected with $Hras^{lys12}$. However, expression of C3GΔCat mutant did not increase the adhesion to laminin. These results indicate that the higher adhesiveness conferred by overexpression of C3G is Rap1-dependent and does not account for the transformacion suppression effect on oncogenic transformation.

Fig. 6

Effects of C3G on cell cyclin A expression

Mitogenic signals promote G1 phase entry and cell cycle progression (Peeper et al., 1997), which is regulated by cyclin-CDKs complexes (Takuwa and Takuwa, 2001). The levels of most CDKs are relatively constant during the cell cycle (Yam et al., 2002); thus, the regulation of CDKs activities must be dependent of the corresponding cyclins. Since cyclin A is essential for anchorage independent growth and its expression is regulated by Ras (Kang and Krauss, 1996; Takuwa and Takuwa, 2001; Yang et al., 1998), we studied whether C3G affects cyclin A expression. NIH 3T3 clones expressing C3G and/or $Hras^{lys12}$ were grown exponentially and the levels of cyclin A determined by immunoblotting. Figure 7 showed that Ras-induced cyclin A expression was clearly inhibited by both C3G and C3GΔCat overexpression, which is consistent with the inhibitory effect of C3G or C3GΔCat on $Hras^{lys12}$-mediated growth in suspension. Other cell cycle components studied like cyclin D and cyclin E were not altered by C3G (data not showed).

Fig. 7

DISCUSSION

Previously, we have shown that overexpression of transfected C3G suppresses oncogenic transformation mediated by cotransfected Ras oncogenes in NIH 3T3 fibroblasts. We also observed that such suppression was independent of the presence of the CDC25-H, catalytic, domain in the transfected C3G constructs (Guerrero et al., 1998). In the present work we have demonstrated that the C3G region responsible for this effect maps to its amino-terminal, SH3-binding domain. Such an observation suggests that Rap1 (a small GTPase activatable by C3G), in spite of its previous depiction as a Ras antagonist (Sakoda et al., 1992), may not be the intermediary of the process of C3G-dependent suppression of transformation.

We have also shown here that C3G inhibits focus formation induced not only by *ras* but also by the *dbl* and R-*ras* oncogenes. This observation suggests that the suppressor function of C3G may involve more general mechanisms, not limited only to Ras oncogenes or pathways. The inhibitory effect on R-Ras-mediated transformation is somewhat paradoxical, as R-Ras has also been described to be activated by C3G, although the GEF that exhibits highest exchange activity on R-Ras is Grf1 (Gotoh et al., 1997). It has to be mentioned that these published results refer to work using COS7 cells and thus the differences in cell type may account for some of these discrepancies.

To get an insight into the suppression mechanism, and considering that malignant transformation by Ras is the result of the cooperation between various downstream pathways (Hamad et al., 2002; Rodriguez-Viciana et al., 1997), we studied the effect of C3G on some of the Ras effectors. Our results showed that whereas C3G does not affect activation of either Ras, Raf-1 or MEK1, it clearly inhibits Ras-induced ERK1/2 phosphorylation. The effect of C3G on Ras-mediated ERK activation was not due to the particular characteristics of the permanently transfected clones, as i) the clones used were, in fact, mass cultures of the corresponding permanent transfections and ii) we were able to confirm the results in transiently transfected cell cultures. Obviously, the observed effect was much lower under these conditions due to the limited transfection efficiency of NIH 3T3 cells (around 10%). In this case, we could

only observe the inhibitory effect of C3G on ERK activation in non-stimulated cells (both starved and serum-growing non-synchronic cultures), but not in growth factor-stimulated cells, probably due to the masking effect of the PDGF- or serum-dependent ERK activation on the 90% non-transfected cells. C3GΔCat mutant, lacking the catalytic domain, also inhibited Ras-mediated ERK activation which is in agreement with its transformation suppression effect (Guerrero et al., 1998) and confirms that ERK participates in the suppression mechanism. It should be mentioned here that C3G is only affecting the serum-mediated short term activation of ERK, since we have previously shown that sustained ERK activity is not altered (Guerrero et al., 1998).

We have also observed that C3G but not C3GΔCat exert an inhibitory effect on the PI3K-Akt pathway, which suggest that this pathway seems not to be involve in the transformation suppression mechanism (data not shown).

Ras induces anchorage-independent growth *via* multiple pathways, by down-regulating multiple adhesion-regulated cell cycle events, such as cyclin A expression (Guadagno et al., 1993; Kang and Krauss, 1996; Yang et al., 1998). Cyclin A is required for initiation and progression through S phase (Takuwa and Takuwa, 2001), and its expression is dependent on cell adhesion (Guadagno et al., 1993). In fact, only cells overexpressing cyclin A are able to cycle in suspension (Guadagno et al., 1993; Kang and Krauss, 1996; Schulze et al., 1996). This is in concordance with our results where cells expressing C3G and C3GΔCat showed lower levels of cyclin A and reduced ability to grow in suspension (Figs. 5,6,7). Thus, according to our results, C3G antagonizes cyclin A expression (probably through inhibition of ERK) an adhesion-regulated cell cycle event. Increased cyclin A expression has been detected in many tumors (Yam et al., 2002); thus, our results showing reduced expression of cyclin A in C3G and C3GΔCat expressing cells support the notion of C3G as a transformation suppressor gene by a mechanism that does not involve Rap1 activation.

Our results are in agreement with previous work showing that the activation of ERK and some cell cycle events like cyclin A expression, are the key components involved in the ability of cells to growth in non-adherent conditions (Howe et al., 1998; Renshaw et al., 1997). These author showed that the

activation of ERK by MEK1, but not the assembly of focal adhesions or the phosphorylation of focal adhesion proteins is the critical step that is altered in cells growing in suspension. This is in concordance with our results where not only C3G, which may activate focal adhesion proteins *via* Rap1, but also C3GΔCat inhibit growth in soft agar.

In conclusion, the results presented here indicate that C3G inhibits Ras-mediated oncogenic transformation, independently of its guanine nucleotide exchange activity, by interfering with the activation of the ERK pathway, which then results in inhibition of cyclin A expression and anchorage independent growth. In addition, our results also show an effect of C3G on other oncogenes, like *dbl* and R-*ras*, suggesting that the inhibitory effect of C3G on malignant transformation may involve more general mechanisms, probably affecting components of the cell adhesion machinery. Further studies are necessary to clarify the function and the mechanisms by which C3G exerts its inhibitory effect on oncogene-mediated transformation. The fact that C3G inhibits ERK activity without affecting other components of the Ras-ERK pathway suggests the existence of putative effector molecule(s) able to interact with the Crk binding domain of C3G and thus inhibiting ERK activation by still unknown mechanism(s). An interesting possibility, consistent with the results reported here, would be the activation of a potential phosphatase(s) acting downstream of C3G, that might be responsible for the observed inhibition of ERK phosphorylation. Further studies will be necessary to identify such a hypothetical phosphatase, or any other potential effector(s) mediating the inhibitory role of C3G on oncogenic suppression.

MATERIALS AND METHODS

Construction of expression vectors for the ΔNC3G and C3GSH3-b fragments.

Construct ΔNC3G was made by deletion of the first 212 aa. of C3G. A *Psh*AI-*Bam*HI fragment containing nucleotides from n637 to n3248 (considering n1 the A in the first ATG codon) was subcloned into pSP72/*Eco*RV-*Bam*HI, and subcloned again as a *Bgl*II-*Bam*HI fragment into pCEVKZHA (Miki et al., 1989). From the new construct, the ΔNC3G fragment was transferred by *Xho*I-*Bgl*II digestion into pLTR2 *Xho*I-*Bam*HI (DiFiore et al., 1987).

C3GSH3-b domain was cloned into pLTR2 by PCR amplification of a fragment enclosing from amino acid 209 to 660, comprising all the polyproline sequences important for SH3-binding, using oligos AF33 (GCTC**CTCGAG**ATGAAGCAGGGACGTCCGTC) and AF34 (CTTC**GGATCC**CCGACTGAGCCGACTCC), containing respectively a *Xho*I and a *Bam*HI site. After checking for the absence of mutations by sequencing, the resulting fragment was cloned into pLTR2 by *Xho*I-*Bam*HI digestion.

Permanent transfections and quantitation of focus-forming activity.

NIH3T3 cells were transfected by the calcium phosphate method (Chen and Okayama, 1987) and individual colonies isolated as described previously (Guerrero et al., 1998).

Focus forming activity was quantified after the transfection experiments by growing transfected NIH3T3 cells in the absence of selection for two weeks. The plates were then stained with a Giemsa solution and the number of morphologically transformed foci was counted.

Transient transfections

NIH 3T3 cells were transiently transfected using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals). FuGene 6 was diluted in 300 µl serum-free medium and DNA added at a ratio 3:1 (18 µl

FuGENE: 6 μg DNA). After 15 min incubation, the mixture was added dropwise to 60-70% confluent cells in 100 mm plates. After 24 hour of incubation at 37ºC and 5% CO_2, cells were serum-deprived for additional 24 hours and then treated with the corresponding agonists.

DNAs used in the transfection experiments included the constructs described above (ΔNC3G and C3GSH3-b fragments cloned in the pLTR2 vector for *gpt* selection), as well as clones described in a previous work (Guerrero et al., 1998). Mammalian expression constructs of the different oncogenes were obtained from several sources: $Hras^{lys12}$ in pMEXneo (Benito et al., 1991), *dbl* in pZIPneoSV (Eva and Aaronson, 1985), R-*ras* in pMEXneo (Saéz et al., 1994). Plasmids pLTR2 (DiFiore et al., 1987) and pMEXneo (Benito et al., 1991) were used as control vectors in every transfection experiment.

In all experiments (both in transient and permanent transfectants) cells were stimulated with 20% FBS (fetal bovine serum) for 5 min or 25 ng/ml PDGF (platelet derived growth factor) for 2 min. PD98059 (50 μM, 2h before agonists) was used as negative controls of ERK activation.

Antibodies and immunological studies

In this study we have used the following antibodies: anti C3G #1008 (Guerrero et al., 1998), anti C3G (C-19) and C3G (H-300), anti H-Ras (C-20), anti R-Ras (C-20), anti Dbl (3), anti Raf-1 (C-12), anti ERK-1 (K-23) and anti cyclin A (C-19) from Santa Cruz Biotechnologies (Santa Cruz, CA); anti Ras M90 (Lacal and Aaronson, 1986); anti phospho-p44/42MAPK (thr202/tyr204) and anti phosphoMEK-1/2 (Ser217/221) from Cell Signaling Technology, Inc. (Beverly, CA); anti MEK1 from PharMingen (San Diego, CA).

First antibodies were used at 1 μg/ml for #1008 antibody, 2 μg/ml for M90 antibody or 0.2 μg/ml for the rest. Anti-mouse or anti-rabbit Horseradish Peroxydase conjugated antibodies (Amersham Biosciences, Uppsala, Sweden) were used as secondary antibodies. The authenticity of the signal was checked in parallel experiments in which the antibody was incubated for 30 minutes at room temperature with an excess of a specific blocking peptide (20-40 μg). Detection was performed using the ECL-western blot detection system (Amersham Biosciences).

Ras activity assays

Cells were grown to confluence in 100-mm dishes and serum starved for 20-24 h followed by incubation with 10% fetal bovine serum (FBS) for the indicated times. After treatment, cells were washed twice with cold PBS and the levels of Ras-GTP determined using a "Ras activation assay kit" (Upstate Biotechnology, Lake Placid, NY) as described previously (Guerrero et al., 2002).

Raf-mobility shift assay

Activated Raf-1 was detected by mobility shift assay on NIH 3T3 cells transiently transfected with C3G and/or $Hras^{lys12}$, since phosphorylated Raf-1 shows a reduced electrophoretic mobility. 40μg protein were loaded onto a 8% gel and the amount of Raf-1 determined by inmunoblotting with anti Raf-1 (C-12) from Santa Cruz Biotechnologies (Paasinen-Sohns and Holtta, 1997).

Soft agar assay

Serial cell dilutions at 10^6, 10^5 and 10^4 cells/ml were mixed with 0.5% Noble Agar in 1X DMEM + 10% CS (calf serum) supplemented with G418, Killer HAT or both (100 μl of cell suspension in 4 ml of the above mixture) and added to 60 mm plates containing 4 ml 1% Agar + 1X DMEM + 10% CS. Plates were incubated a 37ºC in a humidified incubator and fed once a week for 14 days with 200 μl 1 x DMEM + 10% CS. Then, plates were stained with 1 ml of 0.005% Crystal Violet for more than 1 hour and colonies counted using a dissecting microscope. Size of colonies was measured as volume applying the equation $4/3\ \pi r^3$, where r is the radius of foci.

Cell adhesion assay

Cell attachment was assayed in microtiter plates as described (Matter and Laurie, 1994), http://iprotocol.mit.edu/protocol/292.htm. Briefly, cells were tripsinized, diluted to 2.0 x 10^5/ml in serum-

free medium, added to the wells (previously coated overnight at 4ºC with 40 µg/ml laminin-1) and placed in incubator for 30-60 min at 37º C and 5% CO_2. BSA (1%) was used as negative control. Cells were fixed by incubation with freshly diluted 1% glutaraldehyde in PBS for 10 min at room temperature and stained with 100 ul/well of freshly filtered 0.1% crystal violet for 25 min. After washing the cells, the dye was eluted with 0.5% Triton X-100 and the absorbance measured at 595 nm.

Statistical analysis

Data are represented as mean ± SEM. As comparisons were made between two experimental groups an unpaired Student's t test was used. Results were considered significant when $p<0.05$.

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LEGENDS TO FIGURES

Figure 1. Transformation suppressor activity of C3G maps to its SH3-b domain (**a**) Schematic representation of human C3G structure and deletion mutants of functional domains utilized in this work. REM: Ras, exchange motif; SH3-b: SH3-binding or Crk-binding domain; CDC25-H: catalytic or CDC25-homology domain. (**b**) Expression of $Hras^{lys12}$, C3G and mutants ΔNC3G and C3GSH3-b in stable, double contransfectant NIH 3T3 clones. (**c, d**) Effect of the different C3G mutants on Ras-mediated focus forming activity in NIH 3T3 cells. 4 micrograms of each C3G mutant, cloned in pLTR2, were contransfected with 0.1 micrograms of $pMEXneoHras^{lys12}$. (**d**) Representative Giemsa-stained plates, showing the effect of ΔNC3G and C3GSH3-b mutants on the number of Ras-induced foci, and (**c**) histogram representing average number of $Hras^{lys12}$-induced foci and SD corresponding to four independent experiments. Data are normalized to the average number of $pMEXneoHras^{lys12}$-induced foci (~20000 foci /pmol DNA) represented here as 100. *$p<0.05$; **$p< 0.01$ vs pLTR2 + $pMEXneoHras^{lys12}$.

Figure 2. Effect of the C3G mutants on R-*ras*- and *dbl*-mediated transformation. (**a**). Effect of the different C3G mutants on R-*ras*-mediated transformation. 4 micrograms of each C3G mutant, cloned in pLTR2 were cotransfected with 5 micrograms of pMEXneoR-Ras. Representative Giemsa-stained plates showing the effect of the different mutants on the number of R-Ras-induced foci (upper panel) and histogram representing average number of R-*ras*-induced foci and SD corresponding to three independent experiments (lower panel). Data are normalized to the average number of pMEXneoR-Ras-induced foci (~300 foci /pmol DNA) represented here as 100. *$p<0.05$; **$p< 0.01$ vs pLTR2 + pMEXneoR-Ras.

(**b**). Effect of C3G mutants on *dbl*-induced focus forming activity. 4 micrograms of each C3G mutant, cloned in pLTR2 were contransfected with 0.1 micrograms of pZIPNeoSV-Dbl. Representative Giemsa-stained plates showing the effect of the different mutants on the number of *dbl*-induced foci (upper panel) and histogram representing average number of *dbl*-induced foci and SD corresponding to three independent experiments (lower panel). Data are normalized to the average number of pZipNeoSV-Dbl-

induced foci (~5000 foci /pmol DNA) represented here as 100. *p<0.05; **p< 0.01 vs pLTR2 + pZipNeoSV-Dbl.

Figure 3. C3G does not inhibit Ras, Raf-1 or MEK-1 activation. (**a**) Starved NIH 3T3 cells overexpressing C3G and/or $Hras^{lys12}$, were stimulated with 20% FBS (fetal bovine serum) at the indicated times and the levels of Ras-GTP determined. The panels are representative Western blots showing activated Ras (Ras-GTP) and total Ras proteins. (**b**) C3G does not change Raf-1 electrophoretical mobility. NIH 3T3 cells were transiently transfected with pLTR2 or pLTR2C3G (5 μg) in the presence or absence of $pMEXneoHras^{lys12}$ (1 μg), serum-starved for 24 hours and then stimulated with PDGF 25 ng/ml for 2 min. Representative Western blot showing the levels and shift mobility of Raf-1 detected with an anti Raf-1 polyclonal antibody (see Materials and Methods). (**c**) C3G does not interfere with MEK1 phosphorylation. MEK-1 phosphorylation was detected by inmunoblotting with anti phosphoMEK1 antibodies in serum-starved permanent transfected clones treated with PDGF in the presence or absence of PD98059 (50 μg/ml 2h, before PDGF). PhosphoMEK1 blots were stripped and reblotted against total MEK1 protein. Upper and lower panels are representative Western blots showing phosphoMEK1 (p-MEK1) and total MEK1 proteins respectively.

Figure 4. C3G inhibits ERK phosphorylation. (**a, c**) NIH 3T3 clones permanently transfected with the indicated DNAs (pLTR2, pLTR2C3G, pLTR2C3GΔCat $pMEXneoHras^{lys12}$), were serum-starved and treated with FBS or PDGF as indicated and the amount of phosphoERK (p-ERK) determined by inmunoblotting. PhosphoERK blots were stripped and reblotted for total ERK protein. Western blots showing levels of C3G, C3GΔCat and H-Ras proteins are also indicated. C3G levels were detected with antibody antiC3G (C-19) against the last 19 amino acids of C3G protein and C3GΔCat was detected with anti C3G (H-300) that recongnize the first 300 amino acids of the protein. C: serum-starved control. S: serum-growing cells. (**b**) Transient transfections of NIH 3T3 cells with the indicated DNAs were serum-

starved (C) or left with serum (S) and the amount of phosphoERK, H-Ras and C3G determined by inmunoblotting. All panels are representative Westerns of three independent experiments.

Figure 5. C3G effect on Ras-induced anchorage-independent growth. C3G and C3GΔCat inhibits oncogenic $Hras^{lys12}$-induced growth in soft agar. **(a)** Representative pictures of cell cultures transfected with the indicated DNAs growing in soft agar. **(b)** Mean ± SEM of two independent experiments indicating number of colonies which volume is ≥ 3000 μm^3. * $p<0.001$ vs pLTR2 + $Hras^{lys12}$ cells. **(c)** Volume of foci growing in soft agar. Values represent the mean ± SEM from two separate experiments in which at least 40 foci were measured. * $p<0.001$ vs pLTR2 + $Hras^{lys12}$.

Figure 6. C3G increases cell adhesion to laminin. (a) Representative pictures of the corresponding permanent transfected clones attached to laminin-1. **(b)** Mean ± SEM of three independent experiments performed in quadruplicate, ** $p <0.0001$ vs pLTR2, # $p < 0.001$ vs pLTR2 + $Hras^{lys12}$.

Figure 7. C3G effect on cyclin A expression. C3G overexpression affects cyclin A expression in permanent clones transfected with $Hras^{lys12}$, alone or in combination with C3G or C3GΔCat. The Western blot in this figure is representative of two separate independent experiments. S: cells growing in steady-sate conditions.

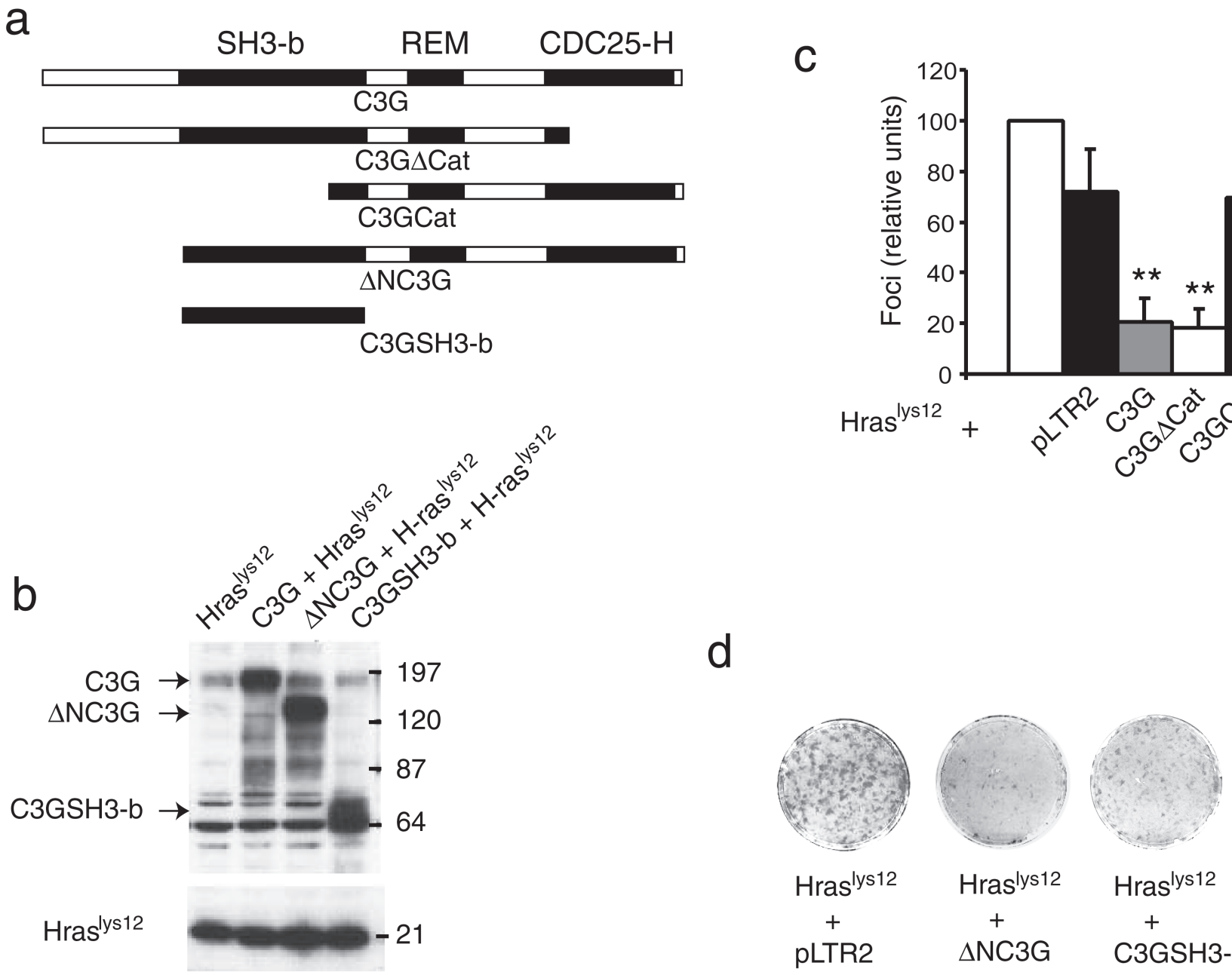

a
SH3-b
REM
CDC25-H
C3G
C3GΔCat
C3GCat
ΔNC3G
C3GSH3-b
b
Hras^lys12
C3G + Hras^lys12
ΔNC3G + H-ras^lys12
C3GSH3-b + H-ras^lys12
C3G
ΔNC3G
C3GSH3-b
Hras^lys12
197
120
87
64
21
c
Foci (relative units)
120
100
80
60
40
20
0
**
**
*
Hras^lys12 +
pLTR2
C3G
C3GΔCat
C3GCat
ΔNC3G
C3GSH3-b
d
Hras^lys12 + pLTR2
Hras^lys12 + ΔNC3G
Hras^lys12 + C3GSH3-b

Figure 1

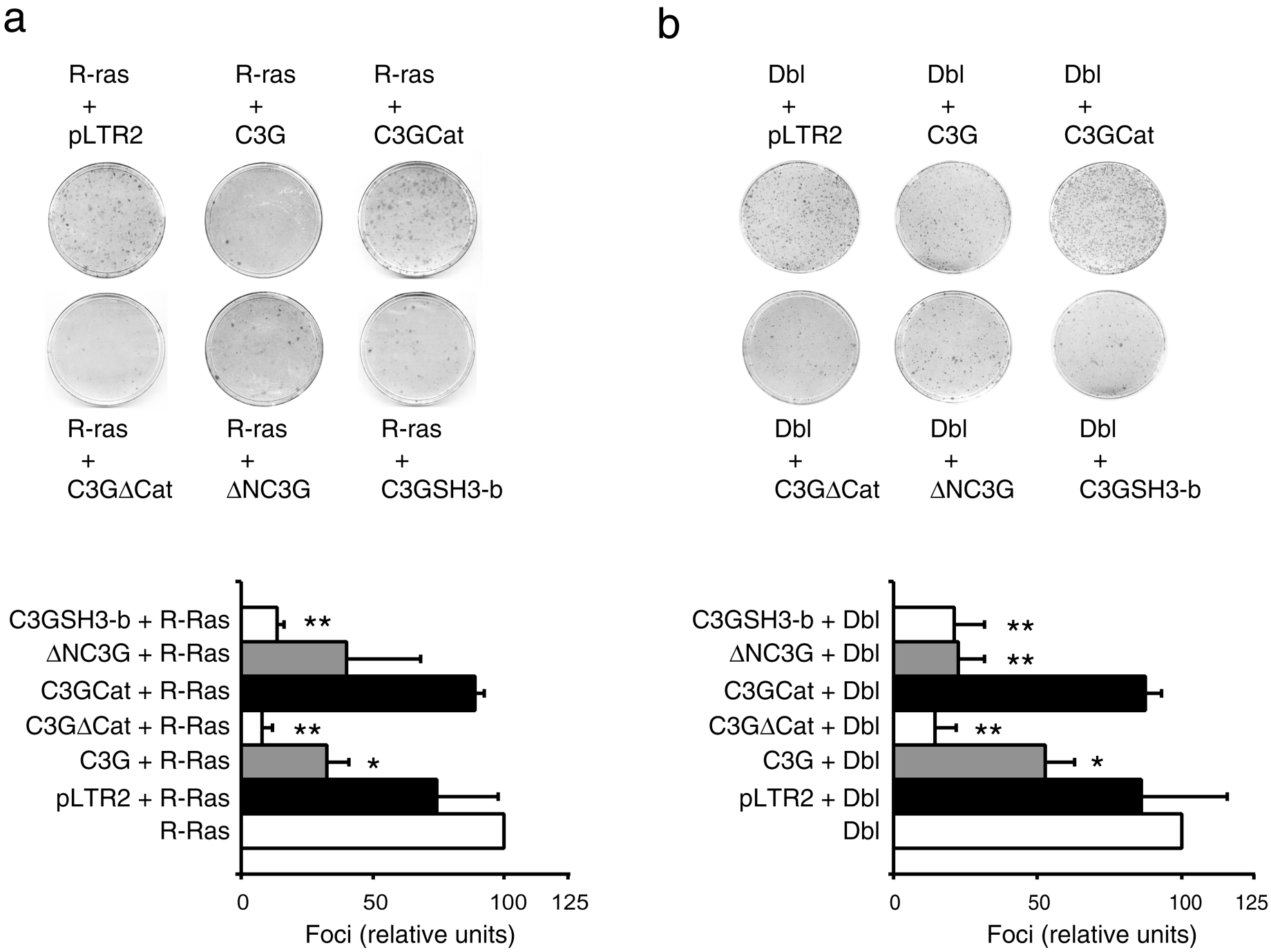

a
R-ras + pLTR2
R-ras + C3G
R-ras + C3GCat
R-ras + C3GΔCat
R-ras + ΔNC3G
R-ras + C3GSH3-b
b
Dbl + pLTR2
Dbl + C3G
Dbl + C3GCat
Dbl + C3GΔCat
Dbl + ΔNC3G
Dbl + C3GSH3-b
C3GSH3-b + R-Ras
ΔNC3G + R-Ras
C3GCat + R-Ras
C3GΔCat + R-Ras
C3G + R-Ras
pLTR2 + R-Ras
R-Ras
**
**
*
0
50
100
125
Foci (relative units)
C3GSH3-b + Dbl
ΔNC3G + Dbl
C3GCat + Dbl
C3GΔCat + Dbl
C3G + Dbl
pLTR2 + Dbl
Dbl
**
**
*
0
50
100
125
Foci (relative units)

Figure 2

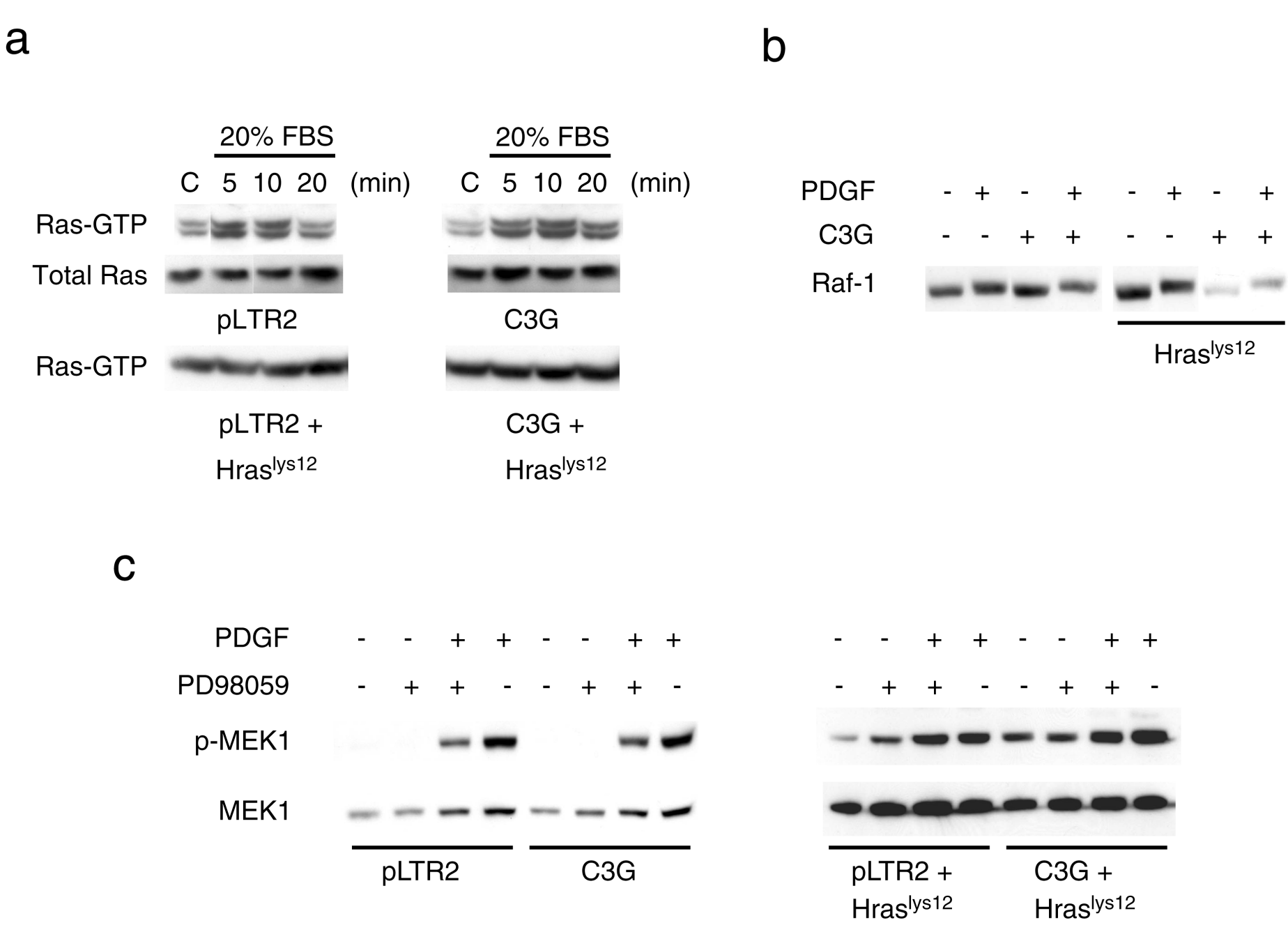

a
20% FBS
20% FBS
C 5 10 20 (min)
C 5 10 20 (min)
Ras-GTP
Total Ras
pLTR2
C3G
Ras-GTP
pLTR2 +
Hras^lys12
C3G +
Hras^lys12
b
PDGF - + - + - + - +
C3G - - + + - - + +
Raf-1
Hras^lys12
c
PDGF - - + + - - + +
PD98059 - + + - - + + -
p-MEK1
MEK1
pLTR2
C3G
- - + + - - + +
- + + - - + + -
pLTR2 +
Hras^lys12
C3G +
Hras^lys12

Figure 3

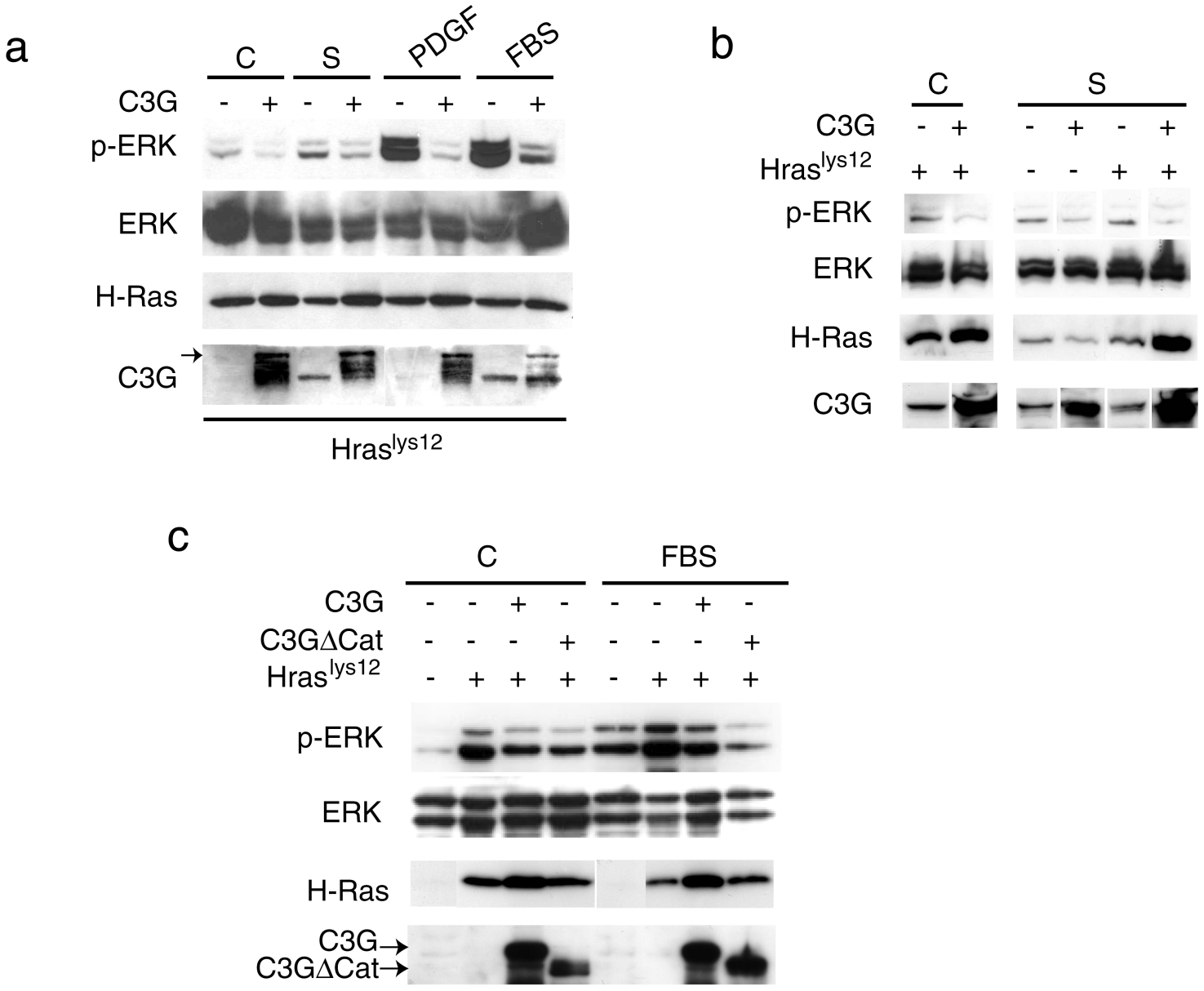

a
C
S
PDGF
FBS
C3G
- + - + - + - +
p-ERK
ERK
H-Ras
C3G
Hras^lys12
b
C
S
C3G
- + - + - +
Hras^lys12
+ + - - + +
p-ERK
ERK
H-Ras
C3G
c
C
FBS
C3G
- - + - - - + -
C3GΔCat
- - - + - - - +
Hras^lys12
- + + + - + + +
p-ERK
ERK
H-Ras
C3G→
C3GΔCat→

Figure 4

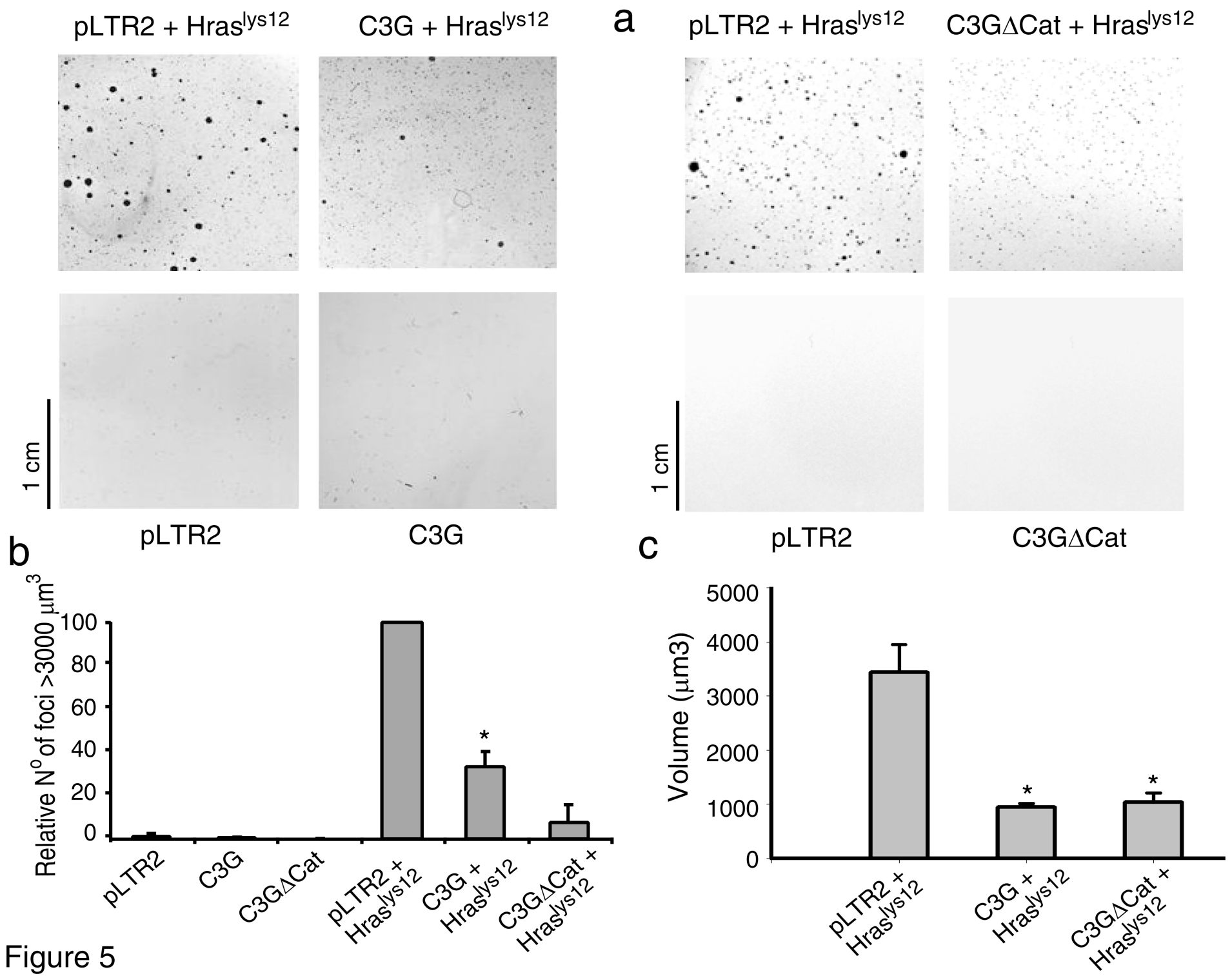
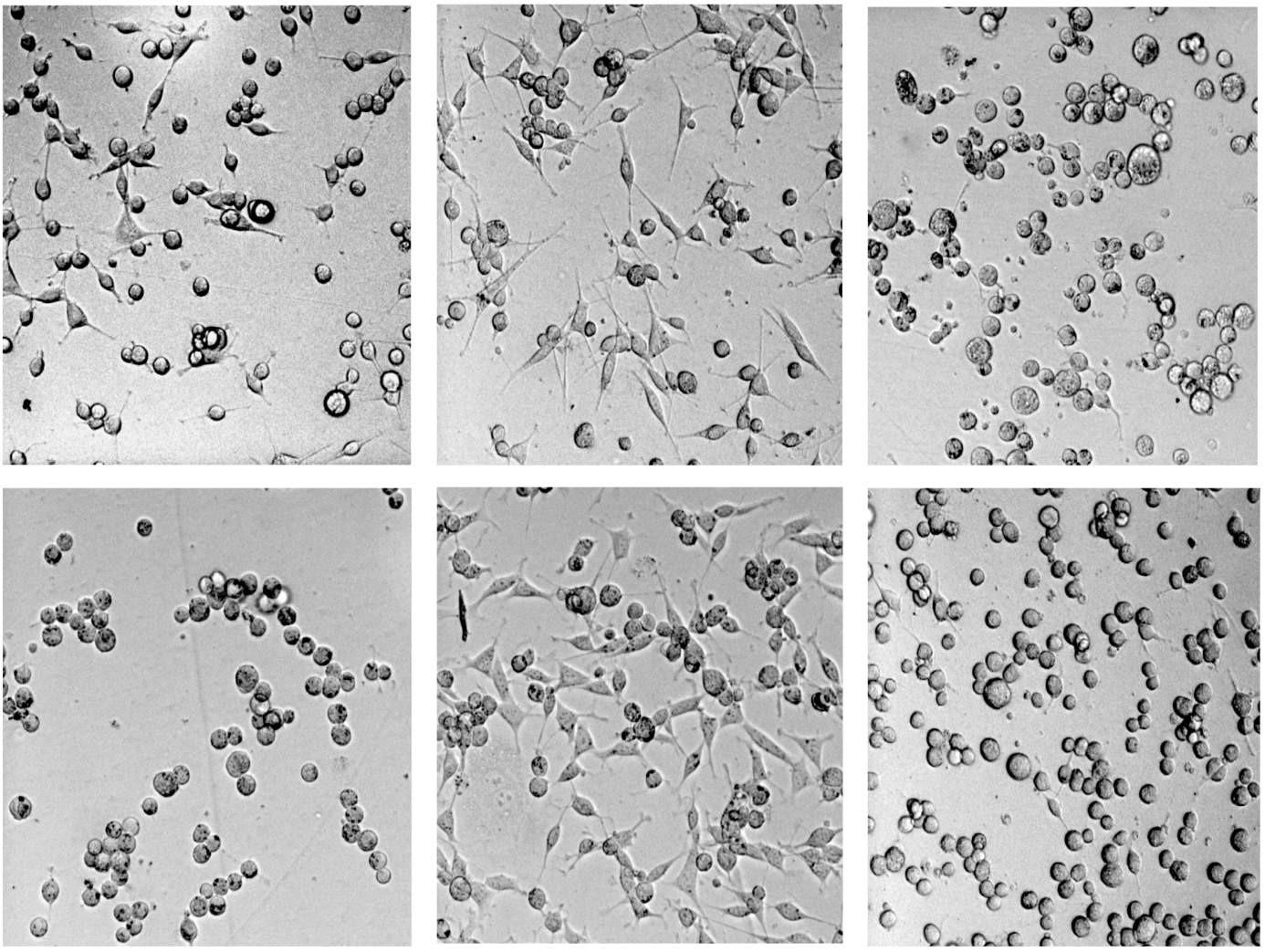

a
pLTR2 + Hras^lys12
C3G + Hras^lys12
pLTR2 + Hras^lys12
C3GΔCat + Hras^lys12
1 cm
1 cm
pLTR2
C3G
pLTR2
C3GΔCat
b
Relative N° of foci >3000 µm3
100
80
60
40
20
0
*
pLTR2
C3G
C3GΔCat
pLTR2 + Hras^lys12
C3G + Hras^lys12
C3GΔCat + Hras^lys12
c
Volume (µm3)
5000
4000
3000
2000
1000
0
*
*
pLTR2 + Hras^lys12
C3G + Hras^lys12
C3GΔCat + Hras^lys12

Figure 5

a


pLTR2 + Hras^lys12
C3G + Hras^lys12
C3GΔCat + Hras^lys12
pLTR2
C3G
C3GΔCat

b

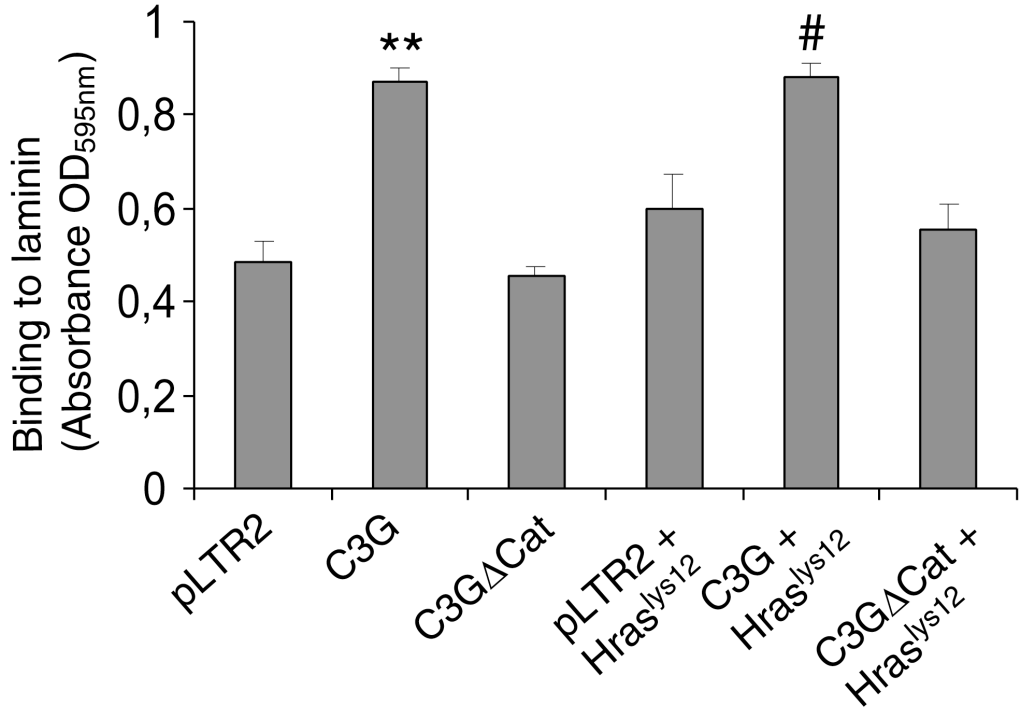

Binding to laminin
(Absorbance OD595nm)
1
0,8
0,6
0,4
0,2
0
**
#
pLTR2
C3G
C3GΔCat
pLTR2 + Hras^lys12
C3G + Hras^lys12
C3GΔCat + Hras^lys12

Figure 6

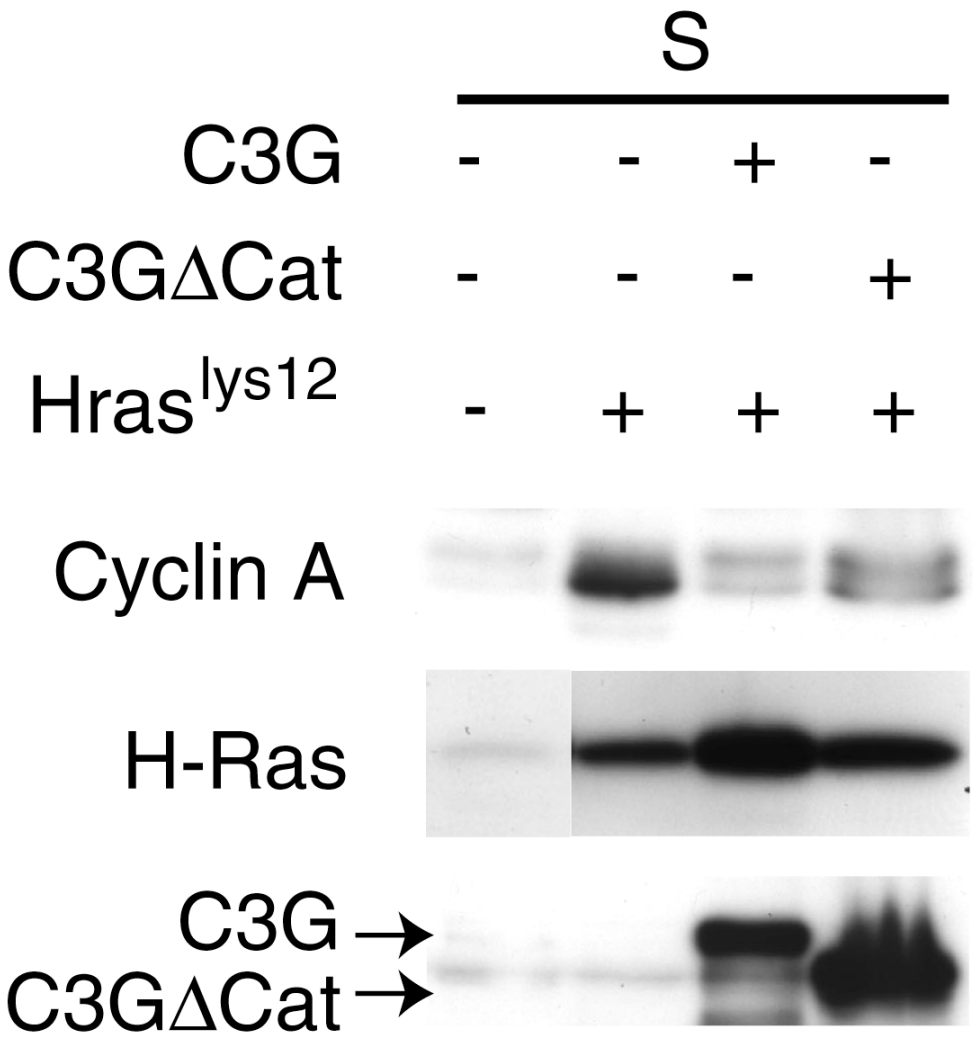


Figure 7