

1 **Research Article**

2

3 **Slow fluctuation of Rac1 activity is associated with biological and transcriptional**  
4 **heterogeneity of glioma cells**

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## 1 **Summary**

2 Phenotypic heterogeneity of cancer cells is caused not only by genetic and epigenetic  
3 alterations, but also by stochastic variation of intracellular signaling molecules. Using cells  
4 that stably express Förster resonance energy transfer (FRET) biosensors, we here show  
5 correlation of Rac1 activity fluctuation with invasive property of C6 glioma cells. By  
6 long-term time-lapse imaging Rac1 activity in C6 glioma cells was found to fluctuate with a  
7 timescale significantly longer than the replication cycle. Because the level of Rac1 activity  
8 in each cell was robust to suspension-adhesion procedure, C6 glioma cells were sorted by  
9 Rac1 activity, yielding Rac1<sup>high</sup> and Rac1<sup>low</sup> cells. The Rac1<sup>high</sup> cells invaded more efficiently  
10 than did Rac1<sup>low</sup> cells in the Matrigel invasion assay. Among the top 14 membrane-related  
11 genes enriched in Rac1<sup>high</sup> cells, four genes were associated with glioma invasion and Rac1  
12 activity as examined by siRNA knockdown experiments. Among transcription factors  
13 enriched in Rac1<sup>high</sup> cells, Egr2 was found to positively regulate expression of the four  
14 membrane-related invasion-associated genes. The identified signaling network may cause  
15 the slow fluctuation of Rac1 activity and heterogeneity in invading capacity of glioma cells.

16

## 17 **Introduction**

18 Cancer cells originated from a single cell acquire phenotypic heterogeneity due to genomic  
19 instability or heritable epigenetic changes (Lengauer et al., 1998; Shackleton et al., 2009).  
20 This heterogeneity is of great advantage for the cancer progression and guarantees its  
21 insidious, highly invasive nature in tissues (Heppner, 1984; Rubin, 1990; Shackleton et al.,  
22 2009). Recently, however, it has also been reported that the fate and behavior of mammalian  
23 cells, including cancer cells, can be determined by stochastic gene expression variation  
24 (Brock et al., 2009). For example, patterns of signaling heterogeneity in monoclonal cancer  
25 cells can generate diverse phenotypes with different drug sensitivities (Singh et al., 2010).

26 A typical example of cancers that exhibit extensive heterogeneity is glioblastoma,  
27 which was previously termed glioblastoma “multiforme,” reflecting its histopathological  
28 divergence in size, shape, karyotype, etc. (Louis, 2006). Among the many experimental  
29 models of glioblastoma, the C6 glioma cell model has been frequently used to study  
30 invasiveness of glioblastoma cells (Grobbs et al., 2002). The C6 glioma cells implanted  
31 into syngeneic Wistar rats share many histological hallmarks with human glioblastoma and  
32 preferentially migrate along neuronal fibers and through the perivascular space, a pattern  
33 which resembles the spread of human glioblastoma.

34 Rho-family GTPases regulate cytoskeletal dynamics and thereby affect many cellular  
35 processes, including cell polarity, migration, vesicle trafficking and cytokinesis  
36 (Etienne-Manneville and Hall, 2002). In cancer cells, Rho-family GTPases play critical roles  
37 in manifesting the cancer cell-specific behavior (Sahai and Marshall, 2002). Rac1, for

1 example, accelerates tumorigenesis by regulating apoptosis, cell cycle progression, assembly  
2 and disassembly of tight junction and adherens junction, cell migration, and cell invasion  
3 (Mack et al., 2011). Importantly, these pleiotropic functions of Rho-family GTPases have  
4 been characterized by comparing cancer cells with non-cancer cells. Meanwhile, little is  
5 known about the heterogeneity and fluctuation of Rho-family GTPase activity within the  
6 cancer cell population.

7         Sorting cells with respect to a property of interest is essential to study the  
8 heterogeneity of cancer cells by genetic, epigenetic, biochemical, or cell biological analyses.  
9 Cell surface markers and cognitive antibodies have been routinely used for this purpose, but  
10 the methods used to sort cells depending on the intracellular activity of a signaling molecule  
11 are limited. Biosensors based on the principle of Förster resonance energy transfer (FRET)  
12 have been widely used to monitor the activity of the signaling molecules (Kiyokawa et al.,  
13 2006; Miyawaki, 2011); however, due to a lack of methods for the stable expression of the  
14 FRET biosensors, cell sorting with FRET biosensor-expressing cells has been a difficult  
15 task.

16         Recently, we developed methods to express FRET biosensors stably in cell lines and  
17 transgenic mice (Kamioka et al., 2012; Komatsu et al., 2011). With C6 rat glioma cells stably  
18 expressing a FRET biosensor for Rac1, we found that C6 glioma cells penetrating the brain  
19 parenchyma showed higher Rac1 and Cdc42 activities and lower RhoA activity than those  
20 advancing in the perivascular regions (Hirata et al., 2012). This observation urged us to  
21 investigate the mechanism by which the heterogeneity of Rho-family GTPase activity is  
22 generated, and the role of the heterogeneity in the invasion of glioma cells. For this purpose,  
23 we established a method to sort cells with respect to their levels of Rho-family GTPase  
24 activity. Then, by using next-generation sequencers, we identified genes whose expressions  
25 were correlated with Rac1 activity. Using this approach, we here show that slow fluctuation  
26 of Rac1 activity is associated with the heterogeneity of glioma invasion.

27

## 28 **Results**

### 29 **Distribution of Rac1 activity among C6 glioma cells**

30 We have shown that C6 glioma cells invading at the periphery of a tumor mass in the rat  
31 brain or a 3D spheroid exhibit higher Rac1 activity than those trailing such leader cells  
32 (Hirata et al., 2012). We speculated that such distribution of Rac1 activity among C6 glioma  
33 cells may be autonomously generated during cell growth and spreading. To test this idea, we  
34 prepared C6 glioma cells that stably express a FRET biosensor, Raichu-Rac1, and visualized  
35 Rac1 activity on glass-bottom dishes (Fig. 1A). We detected significant variation in Rac1

1 activity, which exhibited a typical normal distribution (Fig. 1B, C). Correlation between the  
2 Rac1 activity and the expression level of the biosensor was not observed (Fig. 1D)

3

#### 4 **Fluctuation and Robustness of Rac1 activity**

5 The normal distribution of Rac1 activity probably reflected the noise of the system (Brock  
6 et al., 2009). To study the mechanism underlying the generation of the noise, we time-lapse  
7 imaged Rac1 activity in C6 glioma cells for 5 days (Fig. 2A, Movie S1). C6 glioma cells  
8 expressing Raichu-Rac1 were seeded onto a glass-bottom dish having 282- $\mu$ m-diameter  
9 spot, which prevented cells from straying out from the visual field. To maintain cell density  
10 within the optimal range for cell growth, the Raichu-Rac1-expressing cells were  
11 co-cultured with parental C6 glioma cells. We chose spots having a single  
12 biosensor-expressing cell and one to several parental C6 cells for the tracking. Rac1  
13 activity was averaged over the entire cell area and plotted against time (Fig. 2B). Except for  
14 the rapid decline and increment during cell division, Rac1 activity exhibited fluctuation  
15 with timescales longer than the cell cycle (>40 hours). Consequently, after 5 days, when the  
16 single cells proliferated to 6 to 8 cells, Rac1 activity varied significantly among the  
17 daughter cells (Supplementary Fig. S1A). Analysis with power spectrum did not reveal any  
18 periodicity of Rac1 activity fluctuation (Fig. 2C). Of note, Rac1 activity did not  
19 significantly change before and after cell division, suggesting that the level of Rac1 activity  
20 was maintained by a mechanism that is robust to cell division (Fig. 2D). The range of Rac1  
21 activity after 5 days (Fig. 2B) was similar to the range of Rac1 activity observed for the cell  
22 population (Fig. 1C). Therefore, we concluded that the distribution of Rac1 activity was  
23 generated primarily by the slow fluctuation with timescales longer than the cell cycle. To  
24 understand the biological significance of the observed distribution of Rac1 activity, we  
25 examined the correlation between the cell area and Rac1 activity (Fig. 2E), and found a  
26 weak positive correlation. The positive, albeit weak, correlation between cell area and Rac1  
27 activity probably reflects the high level of Rac1 activity in lamellipodia (Itoh et al., 2002;  
28 Kraynov et al., 2000). We also examined the velocity of migration (Fig. 2E), but could not  
29 observe any clear correlation with Rac1 activity.

30 Next, to examine the robustness of the level of Rac1 activity, we detached the cells  
31 by trypsin, and then induced cell adhesion by trypsin inhibition in situ (Fig. 2F).  
32 Trypsinization induced cell rounding and decreased Rac1 activity. Following trypsin  
33 inhibition induced cell adhesion and restored the Rac1 activity. Notably, the relative Rac1  
34 activity of each cell did not change before, during, and after trypsinization (Fig. 2F). This  
35 observation agrees with the previous report that the suspension of adherent cells reduces  
36 Rac1 activity (del Pozo et al., 2000), and also suggests that the level of Rac1 activity in

1 each cell is maintained by a robust mechanism, which is not affected by the  
2 suspension-adhesion procedure.

3

#### 4 **FRET-based cell sorting to select Rac1<sup>high</sup> and Rac1<sup>low</sup> populations**

5 To understand the mechanisms underlying and roles played by the slow fluctuation and  
6 robustness of Rac1 activity, we attempted to examine the transcriptomes of C6 glioma cells  
7 with different levels of Rac1 activity. Encouraged by the observation that the  
8 suspension-adhesion procedure did not alter the relative Rac1 activity of each cell, we  
9 sorted C6 glioma cells depending on Rac1 activity with FACS. The FRET/CFP ratio was  
10 used as the index of FRET efficiency as in microscopy. The FRET/CFP ratio was  
11 independent of the expression level of the biosensor (Fig. 3A) as observed in 2D condition  
12 (Fig. 1D). C6 glioma cells in the highest and lowest decile with respect to the FRET/CFP  
13 ratio were named the Rac1<sup>high</sup> and Rac1<sup>low</sup> populations, respectively, and sorted (Fig. 3B).

14 There was a serious concern about whether the Rac1 activity monitored by the  
15 FRET/CFP ratio in FACS reflected the Rac1 activity of cells grown on the culture dishes,  
16 because Rac1 activity is closely associated with cell attachment (del Pozo et al., 2000). In  
17 Fig. 2F, we showed that suspension-adhesion procedure did not alter the relative Rac1  
18 activity of each cell among the cell population. Here, we quantified GTP bound to the  
19 endogenous Rac1 by pulldown assay. The amount of GTP-Rac1 in Rac1<sup>high</sup> cells was larger  
20 than that in Rac1<sup>low</sup> cells (Fig. 3C). Furthermore, we directly measured the GTP/GDP ratio  
21 by TLC after cell sorting. Both Rac1<sup>high</sup> and Rac1<sup>low</sup> cells were plated on the culture dishes  
22 and labeled with <sup>32</sup>P<sub>i</sub> for 2 hours, followed by TLC analysis to measure the GTP/GDP ratio  
23 on the biosensor (Fig. 3D). Although the difference in the GTP/GDP ratio between Rac1<sup>high</sup>  
24 and Rac1<sup>low</sup> was smaller than the difference between wild-type and GTPase-deficient  
25 mutant Rac1 proteins, the GTP/GDP ratio of Rac1<sup>high</sup> cells was constantly higher than that  
26 of Rac1<sup>low</sup> cells, providing a biochemical validation of the FRET-based cell sorting. In  
27 addition, we found that cells in G2/M phases were enriched in Rac1<sup>low</sup> cell (Supplementary  
28 Fig. S1B), which agrees with the observation that Rac1 activity transiently dropped during  
29 cell division (Fig. 2B).

30 We then examined the invasion of Rac1<sup>high</sup> and Rac1<sup>low</sup> cells into Matrigel by  
31 Boyden chamber assay. Cells that had reached the lower side of filter were counted after 22  
32 hours. Although the efficiency of invasion varied in each experiment, we constantly  
33 observed that Rac1<sup>high</sup> cells invaded into Matrigel significantly faster than did Rac1<sup>low</sup> cells  
34 (Fig. 3E). This observation agrees with the findings of our previous 3D spheroid assay that  
35 cells with higher Rac1 activity invaded into Matrigel at the front and guided cells with  
36 lower Rac1 activity (Fig. 1B) (Hirata et al., 2012).

1 Finally, to confirm our hypothesis that the distribution of Rac1 activity was caused  
2 by slow fluctuations, the Rac1<sup>high</sup> and Rac1<sup>low</sup> cells were cultured for up to nine days and  
3 re-analyzed by FACS. On the first day, the distribution of Rac1 activities within the sorted  
4 populations remained discrete, but after one week the distribution of Rac1 activity within  
5 each population was identical, supporting our hypothesis (Fig. 3F). We performed similar  
6 experiments with C6 glioma cells expressing a FRET biosensor for Cdc42. In agreement  
7 with the previous finding that the glioma cells invading at the front exhibit high Rac1  
8 activity and high Cdc42 activity (Hirata et al., 2012), a similar result was obtained with  
9 Cdc42<sup>high</sup> and Cdc42<sup>low</sup> cells (Fig. 3F). Furthermore, we retrogradely analyzed long-term  
10 time-lapse images (Supplementary Fig. S1C), and found that, in agreement with the FACS  
11 data, cells in the highest and lowest decile with respect to the FRET/CFP ratio were  
12 merging in fifty hours.

13

#### 14 **Transcriptional signatures of Rac1<sup>high</sup> and Rac1<sup>low</sup> cells**

15 To investigate the association of the Rac1 activity variation with transcriptional signatures,  
16 RNA-Seq analysis was performed with C6 glioma cells sorted by FACS. mRNA was  
17 isolated from the Rac1<sup>high</sup> and Rac1<sup>low</sup> populations of C6 glioma cells and sequenced. The  
18 expression difference of Rac1<sup>high</sup>/Rac1<sup>low</sup> was plotted against average expression (Fig. 4A).  
19 Similar analysis was performed for Cdc42 and RhoA to characterize the nature of the  
20 Rac1<sup>high</sup> and Rac1<sup>low</sup> populations (Fig. 4B). The difference in the expression of  
21 Rac1<sup>high</sup>/Rac1<sup>low</sup> was positively correlated with that of Cdc42<sup>high</sup>/Cdc42<sup>low</sup>. But there was  
22 no correlation between the expression differences of Rac1<sup>high</sup>/Rac1<sup>low</sup> and  
23 RhoA<sup>high</sup>/RhoA<sup>low</sup> or those of Cdc42<sup>high</sup>/Cdc42<sup>low</sup> and RhoA<sup>high</sup>/RhoA<sup>low</sup>. Again, this is in  
24 agreement with our previous observation that both Rac1 and Cdc42 activities were high in  
25 cells migrating at the front of glioma cells in rat brains and in 3D Matrigel (Hirata et al.,  
26 2012). For identification of differentially expressed genes, we used the weighted average  
27 difference method (WAD). The WAD method identified 713 differentially expressed genes  
28 using a cutoff of the top 5% of ranked genes. Gene ontology analysis based on biological  
29 process terms showed that the Rac1<sup>high</sup> phenotype is associated with the GPCR protein  
30 signaling pathway, cell-matrix adhesion, and electron transport chain in that order (Fig. 4C).  
31 The Rac1<sup>low</sup> phenotype is associated with cell division, cell cycle, and mitosis terms. In  
32 addition, analysis with cellular component terms showed that genes related to the  
33 respiratory chain, focal adhesion, and mitochondrial respiratory chain complex I, were  
34 enriched in the Rac1<sup>high</sup> population, and that genes related to the cytoplasm, nucleus, and  
35 integral to membrane were enriched in Rac1<sup>low</sup> population (Fig. 4D).

36

#### 37 **Identification of genes that regulate C6 glioma cell invasion**

1 Based on the RNA-Seq data, we attempted to identify genes that regulate glioma invasion.  
2 For this purpose, we focused on the top 23 up-regulated genes related to cell component  
3 term “membrane” (Table S1). Notably, 18 genes out of the 23 genes were up-regulated in  
4 Cdc42<sup>high</sup> cells in comparison to Cdc42<sup>low</sup> cells, strongly suggesting that a large part of  
5 Rac1<sup>high</sup> cells are overlapped with Cdc42<sup>high</sup> cells. Before starting detailed characterization  
6 of the membrane-related genes enriched in Rac1<sup>high</sup> cells, the difference in gene expression  
7 between Rac1<sup>high</sup> cells and Rac1<sup>low</sup> cells was confirmed by qPCR, except for Ecop, to  
8 which we failed to prepare specific primers. Next, we knocked-down top 10 genes with  
9 three different siRNAs, except for cathepsin L1 (ctsl1), against which we failed to prepare  
10 three effective siRNAs. From the remaining 12 genes, we arbitrarily chose and  
11 knocked-down Ntrk2, Freq, Il1rap, and Pstpip2. C6 glioma cells were then examined for  
12 their invasive potential by the Matrigel invasion assay. Among the 14 membrane-related  
13 genes, knockdown of MMP15, TSN17, Pstpip2, and Freq, which we call membrane-related  
14 invasion-associated genes, significantly inhibited C6 glioma cell invasion (Fig. 5A). Except  
15 for MMP15, knockdown of the membrane-related invasion-associated genes suppressed  
16 Rac1 activity, suggesting that TSN17, Pstpip2, and Freq promoted invasion via Rac1  
17 activation (Fig. 5B). Knockdown of membrane-related but invasion-irrelevant genes,  
18 Lgals3 and Rgs2, did not affect Rac1 activity. Notably, knockdown of TSN17 and Freq,  
19 but not Pstpip2, caused rounding of the cell shape (Fig. 5C). These two genes may be  
20 associated with Rac1-mediated membrane protrusion.

21 We next sought for transcription factors enriched in Rac1<sup>high</sup> cells (Table S2), and  
22 found that Egr2 was reproducibly enriched in Rac1<sup>high</sup> cells and Cdc42<sup>high</sup> cells. Similarly,  
23 we identified Elmo1 and PRex1 as Rac1 activators enriched in Rac1<sup>high</sup> cells (Table S3).  
24 Knockdown of Egr2 and Elmo1, but not PRex1, suppressed C6 cell invasion and decreased  
25 Rac1 activity (Fig. 5D, E). Unlike the knockdown of membrane-related genes, TSN17 and  
26 Freq, knockdown of Egr2 or Elmo1 decreased Rac1 activity without affecting the cell  
27 shape (Fig. 5F).

28 Thus, we identified genes of four membrane-related proteins, a transcriptional  
29 factor, and a Rac1 activator as invasion-associated genes enriched in Rac1<sup>high</sup> cells. Notably,  
30 knockdown of Egr2, Elmo1, Pstpip2, Freq, and TSN17 not only decreased the average  
31 Rac1 activity but also suppressed the fluctuation of Rac1 activity (Supplementary Fig. S2),  
32 implying that the fluctuation of Rac1 activity may be associated with the basal level of  
33 Rac1 activity.

34

### 35 **Hierarchy of the invasion-associated genes enriched in Rac1<sup>high</sup> cells**

36 To untangle the signaling network of the invasion-associated genes enriched in Rac1<sup>high</sup>  
37 cells, we first transiently activated Rac1 by rapamycin-induced Rac1 activation system

1 (Yagi et al., 2012). Upon rapamycin-induced membrane translocation of Tiam1, a GEF for  
2 Rac1, Rac1 activation was clearly detected by both FACS (Fig. 6A) and pulldown assay  
3 (Fig. 6B). Among the genes tested, only Egr2 was significantly induced rapidly and  
4 transiently (Supplementary Fig. S3). We next knocked down each gene and quantified the  
5 expression of the other invasion-associated genes by qPCR (Supplementary Fig. S4). The  
6 comprehensive knockdown experiments revealed intriguing features of the signaling  
7 network that regulates Rac1 activity (Fig. 6C). First, the membrane-related  
8 invasion-associated genes, Pstpip2, TSN17, Freq, and MMP15, could be clustered by the  
9 response to the knockdown of the other genes. Second and more importantly, we could  
10 infer the hierarchy of the genes by assuming that the knockdown of a gene decreases the  
11 expression of the downstream genes and increases the expression of the upstream gene(s)  
12 by a negative feedback loop. For example, Egr2 knockdown decreased expression of all  
13 four membrane-related invasion-associated genes, but not an invasion-irrelevant gene,  
14 Rgs2, or Elmo1. On the other hand, knockdown of the membrane-related  
15 invasion-associated genes increased Egr2 expression, suggesting the presence of negative  
16 feedback loops to Egr2. Because the effect of Egr2 knockdown on the expression of  
17 Pstpip2 was not significant, this gene may be placed in a different signaling pathway. From  
18 these data, we suggest a model of the gene network that regulates Rac1 activity and  
19 invasion of C6 glioma cells (Fig. 6D).

20

## 21 **Discussion**

22 The phenotypic heterogeneity of cancer cell populations is caused by genetic, epigenetic,  
23 and non-genetic mechanisms. The non-genetic mechanism that causes the variation of gene  
24 expression includes transcriptional and translational noises (Brock et al., 2009). Although  
25 the precise nature of such noise remains largely elusive, we can speculate that the gene  
26 expression variation would reflect the intracellular signaling activities. Here we established  
27 a technology to sort the cells depending on the activities of intracellular signaling  
28 molecules and to examine the effect of the activity variation of signaling molecules on the  
29 biological or transcriptional heterogeneity of cancer cells.

30

## 31 **FRET-based cell sorting**

32 The technology is based on two assumptions. First, the activity of the molecule of interest  
33 is maintained during FACS. Second, the transcriptome is not significantly perturbed during  
34 FACS. We had a serious concern as to whether the process of cell preparation, i.e.,  
35 trypsinization and suspension of adherent C6 glioma cells, might mask the intercellular  
36 variation in the activities of Rho-family GTPases that are observed both under 2D and 3D



1 conditions. In fact, it has been established that the suspension of adherent cells reduces  
2 their Rac1 activity (del Pozo et al., 2000). Contrary to our expectation, however, the  
3 intercellular variation in Rac1 activity was reproduced after the cell preparation. Reanalysis  
4 of Rac1 activity by FACS, TLC, or pulldown assay demonstrated that Rac1 activity was  
5 conserved in both the Rac1<sup>high</sup> and Rac1<sup>low</sup> cell populations (Fig. 3A, B). Time-lapse  
6 imaging confirmed that the relative Rac1 activity of each cell was maintained before and  
7 after cell division or suspension-adhesion procedure (Fig. 2D). These observations imply  
8 that the Rac1 activity in a single cell consists of basal and external cue-dependent Rac1  
9 activities. The external cue includes integrin, growth factors, etc., and rapidly changes Rac1  
10 activity upon input of the cues (Heasman and Ridley, 2008). Meanwhile, the basal Rac1  
11 activity is determined by intrinsic signaling status, which is robust to external cues and is  
12 subjected to fluctuation with longer timescales.

13 The second assumption that needs further consideration is the effect of cell sorting  
14 on the transcriptome. The ontology analysis of genes enriched in Rac1<sup>high</sup> cells showed  
15 close correlation to biological process terms that are linked to the function of Rac1 (Fig.  
16 4C). The first and second scores went to the GPCR pathway and cell-matrix adhesion. Both  
17 of these are related to cell migration, with which the functions of Rac1 are most often  
18 associated (Sahai and Marshall, 2002; Sander and Collard, 1999). Another major function  
19 of Rac1 is the regulation of NADH-mediated production of reactive oxygen species  
20 (Heasman and Ridley, 2008); therefore, it is not surprising that the electron transport chain  
21 was scored at the third position. Furthermore, in agreement with the finding that Rac1  
22 activity drops rapidly during cell division (Yoshizaki et al., 2003), the first to third scores  
23 of genes enriched in Rac1<sup>low</sup> cells went to the pathways of cell division, cell cycle, and  
24 mitosis (Fig. 4C). Furthermore, among the 23 genes up-regulated in the Rac1<sup>high</sup> population  
25 and classified under the cell component term “membrane”, 13 genes are known to be  
26 involved in cancer cell invasion (Table S1). These observations support our assumption  
27 that the transcriptional profiles are reasonably conserved during FACS.

28

### 29 **Genes associated with the Rac1<sup>high</sup> phenotype**

30 Among the 14 genes classified under the cell component term “membrane” and enriched in  
31 Rac1<sup>high</sup> cells, knockdown of 4 genes inhibited C6 glioma cell invasion in the Matrigel  
32 invasion assay. Previous reports have indicated or suggested that proteins encoded by the  
33 four genes are more or less associated with invasion of cancer cells. MMP15, matrix  
34 metaloprotease protein 15 of MT2-MMP, is expressed predominantly in glioblastoma  
35 (Lampert et al., 1998; Nakada et al., 1999), suggesting that MMP15 may play a major role  
36 in the degradation of extracellular matrix during glioma invasion. TSN17, tetraspanin 17,  
37 was recently shown to regulate ADAM10, which has been shown to be involved in cancer

1 progression (Dornier et al., 2012; Haining et al., 2012; Mochizuki and Okada, 2007).  
2 Pstpip2 regulates F-actin bundling and enhances filopodia (Chitu et al., 2005), which  
3 strongly argues for a role of this protein in glioma invasion. Freq is a calcium-binding  
4 protein expressed predominantly in neuronal cells (Dason et al., 2012; Nakamura et al.,  
5 2006). In primary cultured adult cortical neurons, overexpression of NCS1 induces neurite  
6 sprouting; however, the role of NCS-1 in glioma invasion has not been determined.

7 Genes responsive to the activation of Rac1 or Cdc42 have been identified by  
8 overexpressing constitutively active QL mutants of Rac1 or Cdc42 in NIH 3T3 cells  
9 (Teramoto et al., 2003). There are some similarities between this previous work and our  
10 present study. First, the expression profile of Rac1QL-expressing cells resembles that of  
11 Cdc42QL-expressing cells in the previous studies. We also found that the expression  
12 profile of Rac1<sup>high</sup> cells resembles that of Cdc42<sup>high</sup> cells (Fig. 4B, Table S1). Second, in  
13 cells expressing Rac1QL or Cdc42QL, genes related to the extracellular matrix and cell  
14 adhesion are enriched and genes related to the cell cycle are suppressed as in Rac1<sup>high</sup> cells  
15 (Fig. 4C). However, there are also some discrepancies. For example, collagen alpha 1 chain  
16 precursor was 3.1-fold enriched in Rac1QL-expressing cells, but was 0.56-fold suppressed  
17 in Rac1<sup>high</sup> cells. This difference may have been caused by the lack of GTPase activity in  
18 Rac1QL mutant, because the cycling between the GDP-bound and GTP-bound forms has  
19 been shown to play an important role in cell migration (Parrini et al., 2011). In another  
20 study, different levels of Rac1 were expressed in colorectal cancer cells to identify the  
21 target genes of Rac1 by microarray analysis (Gomez et al., 2007). However, we could not  
22 find any similarity to our data. In another study, C6 rat glioma cells were selected both in  
23 vitro and in vivo for high and low migratory phenotypes (Tatenhorst et al., 2005). By  
24 microarray analysis, thirty-one genes were found to be differentially expressed in  
25 association with migratory phenotypes. We could not detect a significant resemblance  
26 between the gene expression profiles of this study and our present findings. Thus, the  
27 constitutive activation (or suppression) and intrinsic fluctuation of Rac1 activity might  
28 cause different transcriptional phenotypes. Alternatively, the effect of Rac1 on  
29 transcriptional profiles might be cell-type specific. In any event, different approaches led to  
30 the identification of various genes related to glioma invasion. Further analyses will be  
31 required to find the cause of such divergence.

32

### 33 **Hierarchy of invasion-associated genes enriched in Rac1<sup>high</sup> population**

34 The comprehensive knockdown experiments strongly argued for the role of Egr2 as a  
35 master regulator of C6 glioma invasion. Knockdown of Egr2 suppressed the expression of  
36 the four membrane-related invasion-associated genes. In contrast, knockdown of the four  
37 membrane-related invasion-associated genes or Elmo1 increased the expression of Egr2,

1 implying negative feedback loops from the invasion phenotype to Egr2 expression.  
2 Microarray analyses have revealed enrichment of Egr2 in metastatic squamous cell  
3 carcinomas (Kim et al., 2006; Liu et al., 2008). Furthermore, in fibroblasts infected with  
4 Kaposi sarcoma-associated herpesvirus, Egr2 induces MMPs and Extracellular Matrix  
5 MetalloPRoteinase INducer (emmprin) (Dai et al., 2012). These observations strongly  
6 argue for the proposal that Egr2 is a key regulator of glioma invasion.

7

## 8 **Origin of the heterogeneity of Rac1 activity**

9 What causes the heterogeneity of Rac1 activity among C6 glioma cells? The five-day  
10 time-lapse image revealed that the distribution of Rac1 activity was caused by non-genetic  
11 slow fluctuation with time scales of more than 40 hours (Fig. 2A). This conclusion was also  
12 supported by the observation that the isolated Rac1<sup>high</sup> or Rac1<sup>low</sup> cell population restored  
13 the original distribution within one week (Fig. 3F, Supplementary Fig. S1C). Notably, our  
14 conclusion agrees with the variation of protein levels in human H1299 lung carcinoma cells  
15 (Sigal et al., 2006), in which the expression levels of proteins have been shown to fluctuate  
16 with a timescale of more than 40 hours. By the knockdown experiments against genes  
17 enriched in Rac1<sup>high</sup> population, we identified a gene network regulating Rac1 activity (Fig.  
18 6D). This network comprises both positive and negative feedback loops, which are  
19 sufficient to cause oscillation of a signaling network. Although we have not been able to  
20 confirm that the variation in Rac1 activity in vivo is also driven by the same mechanism,  
21 slow fluctuations of gene expression, and resulting fluctuation of Rac1 activity could  
22 generate glioma cells with different levels of invading capacity.

23

## 24 **Materials and Methods**

### 25 **Biosensors and cell lines**

26 C6 rat glioma cells were obtained from American Type Culture Collection and cultured in  
27 DMEM containing 10% FBS. The FRET biosensors for Rac1, Cdc42, and RhoA,  
28 Raichu-Rac1, Raichu-Cdc42, and Raichu-RhoA, respectively, were described previously  
29 (Itoh et al., 2002; Yoshizaki et al., 2003). For the establishment of stable cell lines  
30 expressing Raichu biosensors, we took two approaches. First, we replaced CFP with teal  
31 fluorescent protein (TFP) and delivered the expression cassettes by a retroviral vector into  
32 C6 glioma cells as described previously (Hirata et al., 2012). More recently, piggyBac  
33 transposon-mediated gene transfer was used to stably express Raichu biosensors with  
34 higher sensitivity (Komatsu et al., 2011; Yusa et al., 2009). The cells were single-cell  
35 cloned before further experiments unless described otherwise.

36

## 1 **Time-lapse FRET imaging**

2 FRET images were obtained and processed using essentially the same conditions and  
3 procedures as previously reported (Aoki and Matsuda, 2009). Cells were plated on 35  
4 mm-diameter glass-bottom dishes (AGC Techno Glass, Shizuoka, Japan) or  
5 micro-patterned glass-bottom dishes (CytoGraph; Dai Nippon Printing Co., Tokyo, Japan).  
6 Cells were imaged at 37°C in 5% CO<sub>2</sub> with an inverted microscope (IX81; Olympus,  
7 Tokyo, Japan) equipped with a x40 objective lens (UAPO/NA 1.35; Olympus), a x40  
8 objective lens (UPLSAPO/NA 0.95; Olympus), and a x60 objective lens (PlanApoPH/NA  
9 1.40; Olympus), a cooled CCD camera (Cool SNAP-HQ or Cool SNAP-K4; Roper  
10 Scientific, Tucson, AZ), an LED illumination system (CoolLED precisExcite; Molecular  
11 Devices, Sunnyvale, CA), an IX2-ZDC laser-based autofocus system (Olympus) and an  
12 MD-XY30100T-Meta automatically programmable XY stage (SIGMA KOKI, Tokyo,  
13 Japan). The following filters used for the dual emission imaging studies were obtained  
14 from Omega Optical (Brattleboro, VT): an XF1071 (440AF21) excitation filter, an XF2034  
15 (455DRLP) dichroic mirror, and two emission filters, XF3075 (480AF30) for CFP and  
16 XF3079 (535AF26) for yellow fluorescent protein (YFP). After background subtraction,  
17 FRET/CFP ratio images were created with MetaMorph software (Universal Imaging, West  
18 Chester, PA), and represented by intensity-modulated display mode. In the  
19 intensity-modulated display mode, eight colors from red to blue are used to represent the  
20 FRET/CFP ratio, with the intensity of each color indicating the mean intensity of FRET  
21 and CFP. For the quantification, the FRET and CFP intensities were averaged over the  
22 whole cell area, and the results were exported to Excel software (Microsoft Corporation,  
23 Redmond, WA).

## 24 **FRET-based Cell Sorting**

25 C6 glioma cells expressing Raichu-Rac1 were trypsinized, resuspended in PBS containing  
26 3% FBS, and analyzed and/or sorted with a FACSAria (Becton Dickinson, Franklin Lakes,  
27 NJ). We used the following combinations of lasers and emission filters for the detection of  
28 fluorescence from the biosensor: for the donor fluorescence of TFP and CFP, a 407 nm  
29 laser and a 480AF30 filter (Omega Optical); for the sensitized FRET from YFP, a 407 nm  
30 laser and a 535AF26 filter (Omega Optical); and for the acceptor fluorescence of YFP, a  
31 475 nm laser and a 535AF26 filter (Omega Optical). Cells were first gated for size and  
32 granularity to exclude cell debris and aggregates. For cell sorting, C6 glioma cells in the  
33 highest and lowest decile with respect to the FRET/CFP (or TFP) ratios were sorted as  
34 Rac1<sup>high</sup> and Rac1<sup>low</sup> populations, respectively, into DMEM containing 10% FBS. Small  
35 fractions of Rac1<sup>high</sup> and Rac1<sup>low</sup> were reanalyzed for validation and the remaining cells  
36 were snap-frozen and stored at -80°C until RNA extraction. Detailed data analysis was  
37 performed using FlowJo. 7.6 software (Tree Star Inc., Ashland, OR).

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## **TLC of guanine nucleotides bound to GTPases**

Guanine nucleotides bound to Raichu biosensors or GFP-tagged Rac1 proteins were quantified essentially as described previously (Gotoh et al., 1995). Briefly, cells were sorted by FACS and plated on 6-well dishes. After 3 hours, cells were metabolically labeled with  $^{32}\text{P}_i$  for 2 hours and lysed with lysis buffer. The cell lysates were clarified by centrifugation and used to immunoprecipitate Raichu biosensors or GFP-tagged Rac1 with an anti-GFP antiserum and Protein-A Sepharose. Guanine nucleotides bound to the immunoprecipitates were separated by TLC and quantitated with a BAS-1000 image analyzer.

## **Rac1 pulldown analysis**

Rac1 pulldown assay was performed according to the manufacturer's protocol (Cytoskelton, Inc, Denver, CO).

## **RNA extraction**

Total RNA was isolated with a Qiagen RNeasy Micro Kit (Qiagen, Hilden, Germany) or a Qiagen RNeasy Mini Kit, according to the manufacturer's protocol. RNA preparations were confirmed to be free of proteins using a NanoDrop ND-1000 instrument (Thermo Fisher Scientific Inc., Waltham, MA), and the integrity of these measurements was confirmed using a 2100 BioAnalyzer (Agilent Technologies, South Queensferry, UK). RNA that had an RNA integrity Number (RIN)  $\geq 8.6$  was used for RNA-Seq.

## **Library preparation and sequencing**

Total RNA was poly(A)-selected using poly(T) Dynabeads (Invitrogen, San Diego, CA). Sequencing libraries were prepared according to Illumina's mRNA-Seq protocol and sequenced at the Omics Science Center (OSC) RIKEN Yokohama Institute. Two independent libraries were analyzed for each data set. Sequence-read data have been submitted to the Sequence Read Archive at DDBJ (submission No. DRA000605).

## **Mapping and processing of RNA-Seq reads**

The reads of each dataset were aligned to the rat reference genome (rn4, Nov. 2004, version 3.4) using TopHat v1.3.0 (Trapnell et al., 2009). The resulting sequence alignment/map files in the BAM format were analyzed with Cufflinks version 0.8.0 (Trapnell et al., 2010) to compute fragments per kilobase of transcript per million mapped reads (FPKM). Genomic annotations were obtained from Ensembl in gene transfer format (GTF). We used only reads mapped to 20 or fewer sites on the genome. The WAD method (Kadota et al., 2008) was then performed on the data of pairs of cells to generate expression differences. Differentially expressed genes were filtered for a WAD ranking cutoff of the

1 top 5.0%. Gene Ontology (GO) annotations were used to assign biological functions to  
2 genes included in this study (Ashburner et al., 2000).

### 4 **3D Matrigel invasion assay**

5 3D Matrigel invasion assay was performed with a BD BioCoat Matrigel Invasion Chamber  
6 (Becton Drive, Franklin Lakes, NJ) according to the manufacturer's protocol. Briefly,  $2 \times$   
7  $10^4$  cells were seeded on the membrane with or without Matrigel precoating. After 22 hours,  
8 cells were fixed, stained for nuclei with propidium iodide, and imaged with an  
9 epifluorescence microscope. The number of nuclei was counted with MetaMorph software  
10 (Universal Imaging). Data is expressed as the percent invasion through the Matrigel Matrix  
11 and membrane relative to the migration through the control membrane.

### 13 **3D spheroid imaging**

14 Organotypic culture was prepared as described previously (Gaggioli et al., 2007). In a  
15 12-well plate coated with poly-(2-hydroxyethyl methacrylate) (Sigma, St. Louis, MO) and  
16 containing 1 ml serum-free CO<sub>2</sub>-independent medium (Invitrogen),  $10^6$  cells were cultured  
17 overnight with slow agitation to form small aggregates. The aggregates were embedded in  
18 6 mg/ml Matrigel, maintained in complete medium and observed under a two-photon  
19 microscope or a confocal microscope for up to 18 hours in an incubation chamber.

### 21 **Quantitative RT-PCR**

22 RNA was reverse-transcribed by a High Capacity cDNA Reverse Transcription kit  
23 (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Then, the  
24 expression levels of each gene and GAPDH used as a standard were analyzed by Power  
25 SYBR Green PCR Master Mix (Applied Biosystems) with ABI PRISM7300 Sequence  
26 Detection System (Applied Biosystems). The sequences of primers used for qPCR are  
27 shown in Table S4.

### 29 **siRNA-Knockdown experiments**

30 Stealth RNAi Negative Control Duplex and Stealth RNAi against MMP15 were purchased  
31 from Invitrogen. Mission siRNAs against the other genes were purchased from  
32 Sigma-Aldrich (St. Louis, MO). C6 cells stably expressing Raichu-Rac1 were transfected  
33 with  $20 \mu\text{M}$  siRNA by Lipofectamine 2000 (Invitrogen). Two days after transfection,  
34 cells were used for invasion assay, qPCR, or FRET imaging. The siRNA sequences are  
35 shown in Table S5.

### 37 **Rapamycin-induced Rac1 activation**

1 Rapamycin-induced Rac1 activation with FKBP-Tiam1 was reported previously (Yagi et  
2 al., 2012).

3

#### 4 **Supplementary Materials**

5 Figure S1. (A) Rac1 activity of a single C6 glioma cell and its daughter cells traced for five  
6 days. (B) Enrichment of cells in G2/M phases in Rac1<sup>low</sup> cell populations. (C) Time-lapse  
7 analyses of Rac1<sup>high</sup> and Rac1<sup>low</sup> cells.

8 Figure S2. Suppression of Rac1 activity fluctuation by the knockdown of genes enriched in  
9 the Rac1<sup>high</sup> cells.

10 Figure S3. Effect of Rac1 activation on the expression of invasion-associated genes.

11 Figure S4. Effect of knockdown of the invasion-associated genes on the expression of the  
12 other genes.

13 Table S1. Genes enriched in Rac1<sup>high</sup> population and related to "membrane".

14 Table S2. Transcription factors enriched in Rac1<sup>high</sup> population.

15 Table S3. Rac1 activators enriched in Rac1<sup>high</sup> population.

16 Table S4. Primer sequences used for qPCR.

17 Table S5. siRNA sequences.

18 Movie S1. Slow fluctuation of Rac1 activity during 5-day time-lapse imaging.

19

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31

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1 **Figure Legends**

2 **Fig. 1.** Activity variation of Rac1. (A) Schematic view of the Raichu-Rac1 FRET biosensor.  
3 (B) C6 glioma cells that stably expressed Raichu-Rac1 were grown on glass-bottom dishes  
4 and imaged to visualize the FRET/CFP ratio in the intensity-display mode with the  
5 FRET/CFP ratio ranges as indicated in the figures. Bar, 200  $\mu\text{m}$ . (C) The FRET/CFP ratio  
6 averaged for each cell in (B) is shown in the histogram, which could be normal distribution  
7 ( $p=0.92$ , Kolmogorov-Smirnov test and  $p=0.3$ , Shapiro-Wilk normality test). Analyses were  
8 performed by R(R Ver 2.12.1). (D) The FRET/CFP ratio and YFP intensity in each cell are  
9 plotted to show the independence of the Rac1 activity from the concentration of the  
10 biosensor.

11

12 **Fig. 2.** Fluctuation and Robustness of Rac1 activity. (A) C6 glioma cells expressing  
13 Raichu-Rac1 were plated on glass-bottom dishes with parent C6 glioma cells, which served  
14 as feeder cells at a low cell density. Cells were time-lapse imaged for 5 days  
15 (Supplementary Movie 1). Representative snap shots of FRET/CFP images and DIC  
16 images overlaid with FRET/CFP image are shown. (B) The time course of a single cell and  
17 its derivatives after smoothing by the Savitzky-Golay filter, except for the mitosis phase,  
18 during which period a surge of Rac1 activity was observed (asterisks and thin lines). The  
19 color of each arrow is used to depict each newborn cell. The data are also shown in  
20 Supplementary Fig. S1. (C) Power spectrum of Rac1 activity. Blue and red lines indicate  
21 normalized power spectra of analyzed cells ( $N = 58$ ) and the average, respectively. (D)  
22 Correlation of Rac1 activities before and after cell division. (E) Scatter plots show the  
23 relationship between Rac1 activity and the cell area and velocity of cells. (F) C6 glioma  
24 cells expressing Raichu-Rac1 in serum-free media were time-lapse-imaged. During the  
25 imaging, 1.25% Trypsin was added at 0.5 h. At 0.75 h, FBS was added to inactivate Trypsin.  
26 Bars, 20  $\mu\text{m}$ .

27

28 **Fig. 3.** Isolation of Rac1<sup>high</sup> and Rac1<sup>low</sup> cell populations by FACS. (A) C6 glioma cells  
29 expressing Raichu-Rac1 were analyzed by FACS. The Rac1 activity (FRET/CFP) did not  
30 correlate with the FRET biosensor concentration (YFP) in each cell. (B) The top and  
31 bottom 10% of cells were sorted to obtain Rac1<sup>high</sup> (red) and Rac1<sup>low</sup> (blue) populations.  
32 Small fractions of Rac1<sup>high</sup> and Rac1<sup>low</sup> cells were reanalyzed after sorting. (C) Rac1<sup>high</sup> and  
33 Rac1<sup>low</sup> cells were collected and analyzed by pulldown assay. Cells expressing  
34 CFP-Rac1V12 and CFP-Rac1N17 were used as positive and negative controls, respectively.  
35 (D) Rac1<sup>high</sup> and Rac1<sup>low</sup> cells were plated on dishes and cultured for 3 hours. Cells were  
36 labeled with <sup>32</sup>P<sub>i</sub> for 2 hours, lysed, and immunoprecipitated with an anti-GFP antibody,  
37 followed by TLC to quantify GTP and GDP bound to the FRET biosensor. . (E) Rac1<sup>high</sup>  
38 and Rac1<sup>low</sup> cells were used for the Matrigel invasion assay. (F) Rac1<sup>high</sup>, Rac1<sup>low</sup> cells,

1 Cdc42<sup>high</sup>, and Cdc42<sup>low</sup> cells were plated on dishes and cultured for the indicated periods  
2 and re-analyzed by FACS.

3

4 **Fig. 4.** RNA-Seq analysis of Rac1<sup>high</sup> and Rac1<sup>low</sup> cell populations. (A) poly(A)-selected  
5 RNA was isolated from Rac1<sup>high</sup> and Rac1<sup>low</sup> cell populations and used for RNA-Seq  
6 analysis. The relationship between average expression [ $\log_2(\text{Rac1}^{\text{high}} \times \text{Rac1}^{\text{low}}) / 2$ ] and  
7 expression difference of Rac1<sup>high</sup> vs Rac1<sup>low</sup> [ $\log_2(\text{Rac1}^{\text{high}}/\text{Rac1}^{\text{low}})$ ] is shown in the M-A  
8 plot. The WAD method identified 713 differentially expressed genes using cutoffs of the  
9 top 5% ranked genes. Cell populations enriched in Rac1<sup>high</sup> and Rac1<sup>low</sup> cells are depicted  
10 with pink and blue dots, respectively. The top 14 genes for the cellular component term  
11 “membrane” are marked in orange, except for Pstpip2, Freq/NCS-1, MMP15, and Tsn17,  
12 which are shown with red dots. Elmo1 and Egr2 are shown in green dots. (B) RNA-Seq  
13 analysis was similarly performed for the Cdc42<sup>high</sup>, Cdc42<sup>low</sup>, RhoA<sup>high</sup>, and RhoA<sup>low</sup> cell  
14 populations. Scatter plots of the expression differences are shown. (C and D) Gene ontology  
15 analysis with biological process terms (C) or cellular component terms (D) is shown. The  
16 p-value was calculated by Pearson product-moment correlation coefficients.

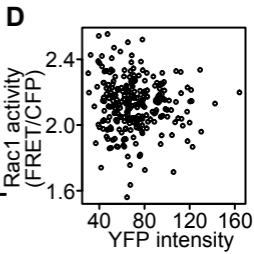
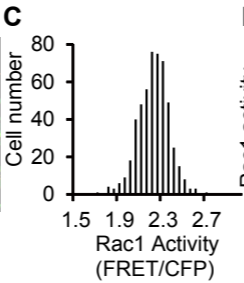
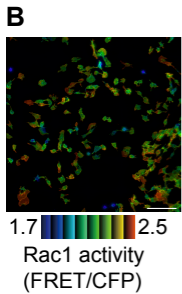
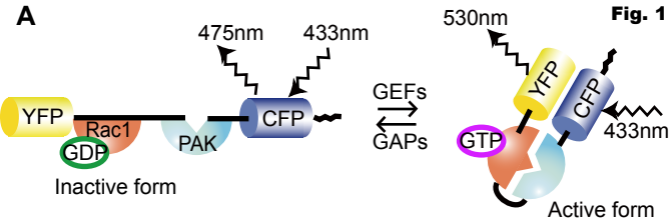
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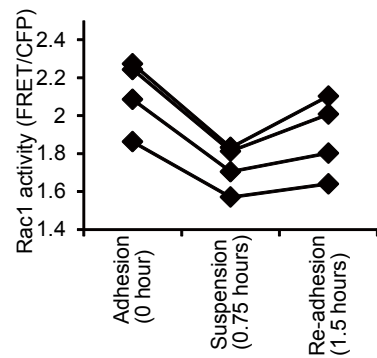
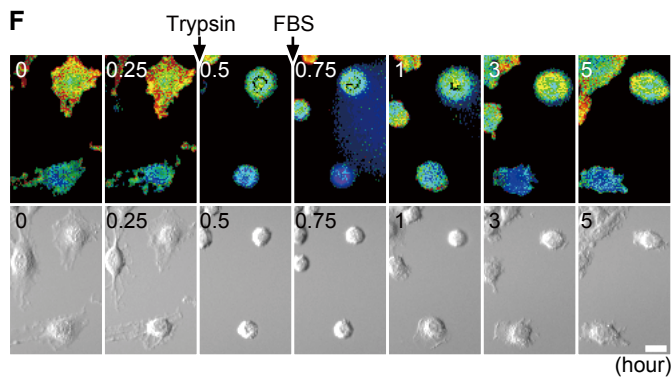
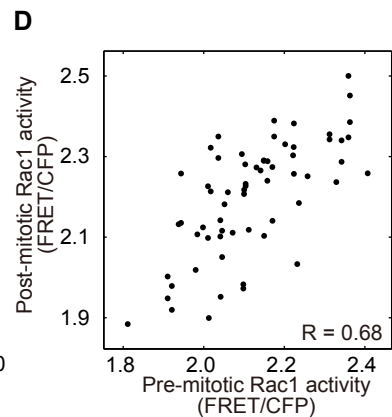
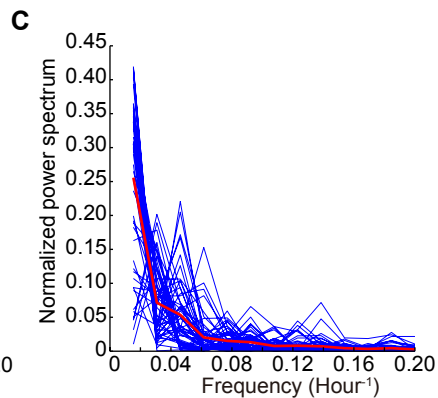
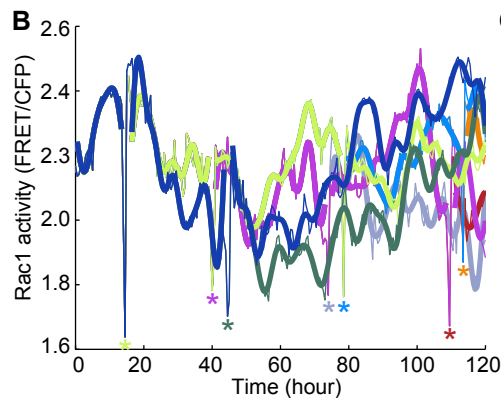
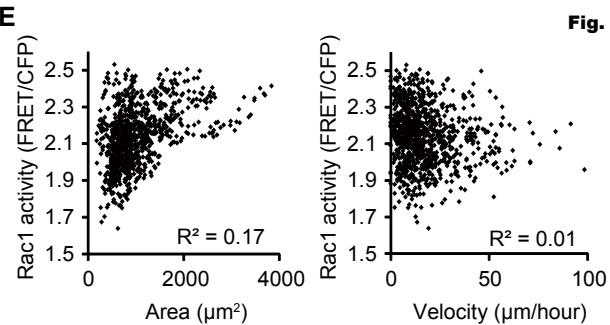
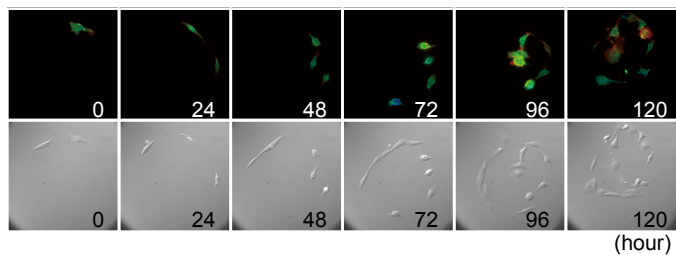
18 **Fig. 5.** Effect of knockdown of genes enriched in the Rac1<sup>high</sup> cell population on invasion  
19 and Rac1 activity. (A) For the top 14 genes enriched in the cellular component term  
20 “membrane”, three siRNAs for each gene were prepared and used to knockdown the target  
21 genes in C6 glioma cells. Cells were used for invasion analysis as described in the text. The  
22 results of two independent experiments are included. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001.  
23 P-value was calculated by two-tailed paired t-test. (B) Four genes associated with invasion  
24 phenotype in (A) and Lgals3 and Rgs2 used as controls were knocked down and  
25 FRET/CFP ratio values were quantified for each C6 glioma cells. Numbers of cells  
26 analyzed are shown at the bottom. (C) Representative snap shots of FRET/CFP images of  
27 C6 glioma cells transfected with the siRNAs. Bars, 100  $\mu\text{m}$ . (D, E, F) Effect of knockdown  
28 of Egr2, Elmo1, and PRex1 was also examined as in (A), (B), and (C). Bars, 100  $\mu\text{m}$ .

29

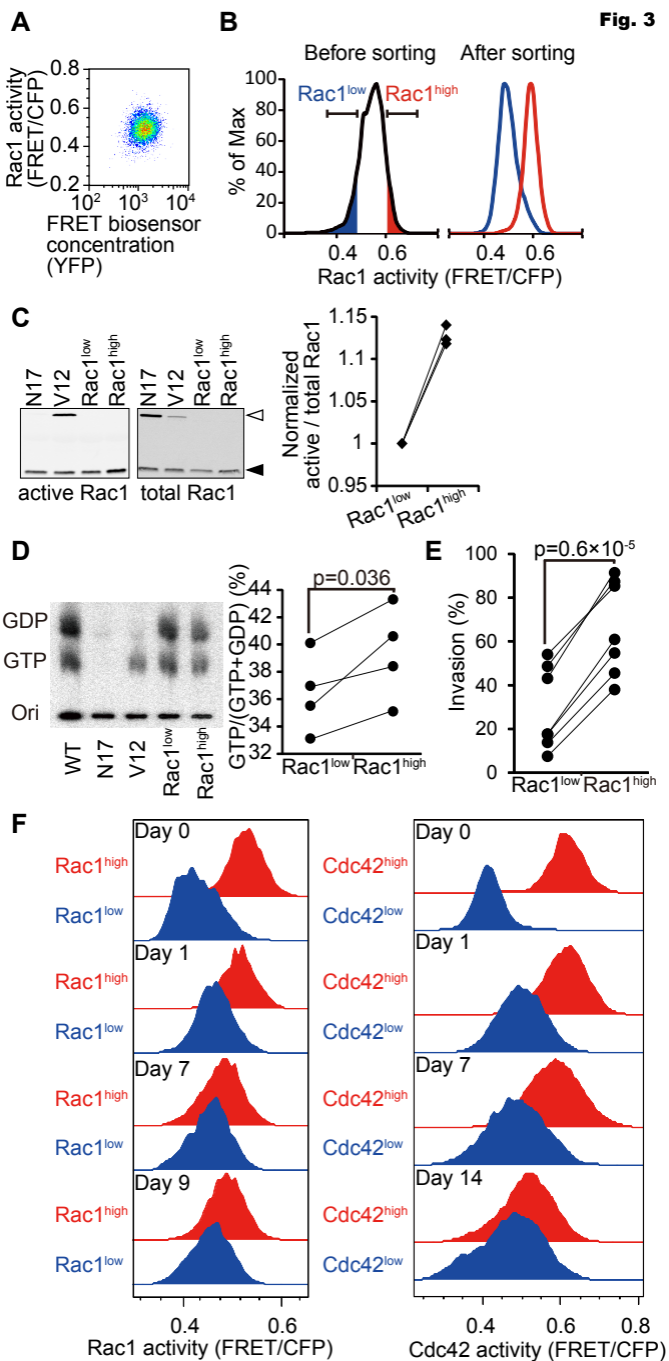
30 **Fig. 6.** Identification of a signaling network comprising the genes enriched in the Rac1<sup>high</sup>  
31 cell population and associated with invasion. (A, B) C6 glioma cells expressing  
32 Raichu-Rac1 alone or Raichu-Rac1, plasma membrane-targeted  
33 Lyn-FKBP12-rapamycin-binding domain (LDR) and FK506-binding protein (FKBP) fused  
34 with Tiam1 were stimulated with (solid line) 10  $\mu\text{M}$  rapamycin or the solvent DMSO  
35 (dashed line) for 30 min. Rac1 activity was examined with FACSaria (A) or by pulldown  
36 assay (B). C6 glioma cells expressing CFP-Rac1N17 and CFP-Rac1V12 were used as  
37 negative and positive controls, respectively. White and black arrows indicate CFP-Rac1 and  
38 endogenous Rac1, respectively. Densitometry for GTP-bound Rac1 was normalized to the

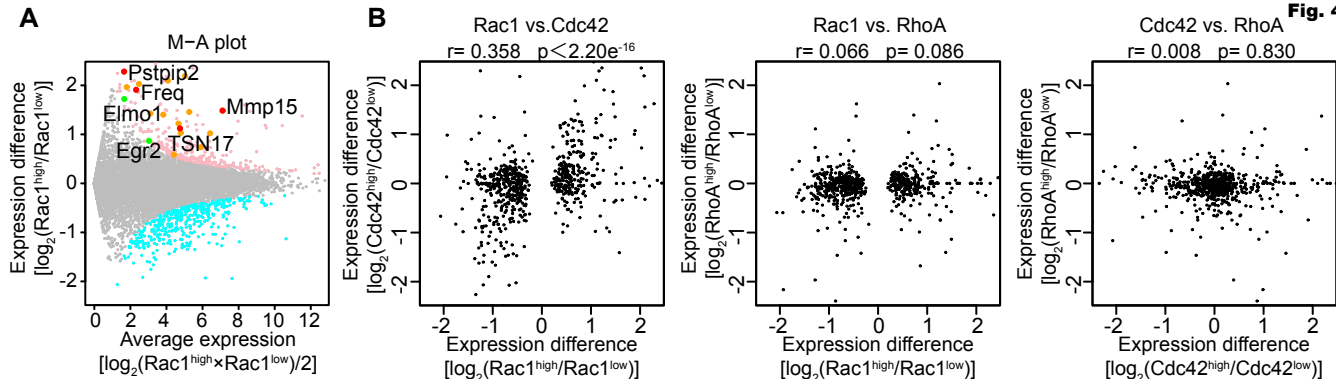
1 amount of the total Rac1.(C) Genes listed in the left column were knocked down as in Fig.  
2 5 or Rac1 was activated as in (A). mRNAs purified from the cells were used for qPCR  
3 analysis for the genes in the top row. Fold changes to the control siRNA-transfected cells  
4 are shown in the log(2) scale. The genes were clustered by nearest neighbor method. Data  
5 on invasion and Rac1 activity shown in Fig. 5 are also included. (D) A proposed model of  
6 Rac1 activity regulation in C6 glioma cells.  
7

**Fig. 1**







**C**Genes up-regulated in Rac1<sup>high</sup> population

Biological Process	Count	P-value
G-protein coupled receptor protein signaling pathway	4	7.36E-07
cell-matrix adhesion	6	3.94E-05
electron transport chain	6	7.55E-05
cellular response to mycophenolic acid	5	1.60E-04
Rac protein signal transduction	3	1.60E-04
positive regulation of endothelial cell proliferation	2	1.64E-04
negative regulation of MAP kinase activity	2	4.09E-04
bioluminescence	4	4.77E-04
glial cell migration	2	9.46E-04
ureteric bud development	4	1.47E-03

Genes up-regulated in Rac1<sup>low</sup> population

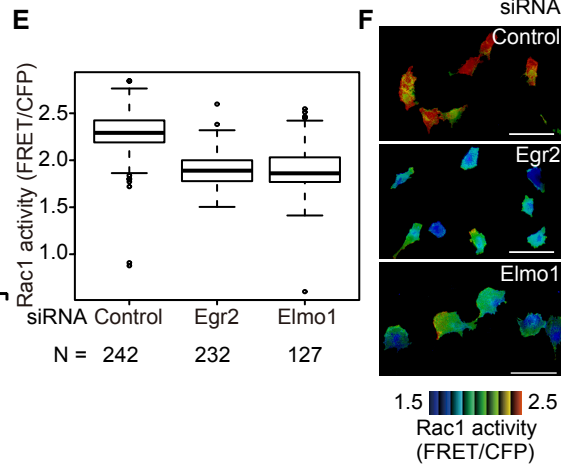
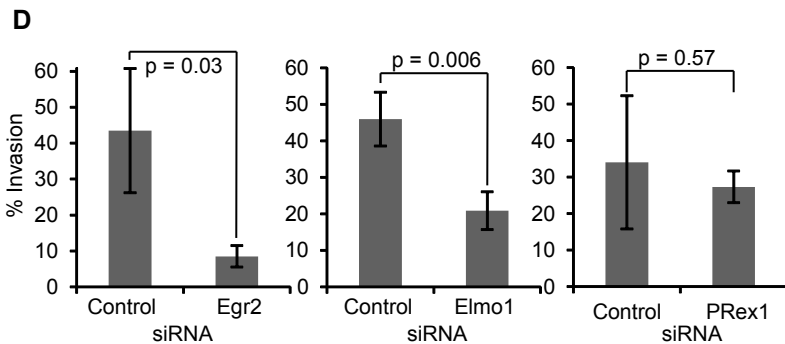
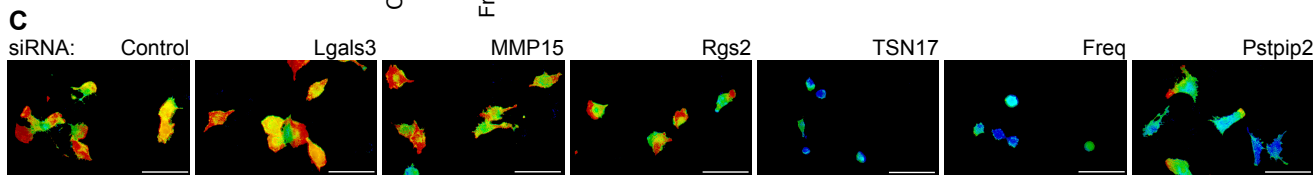
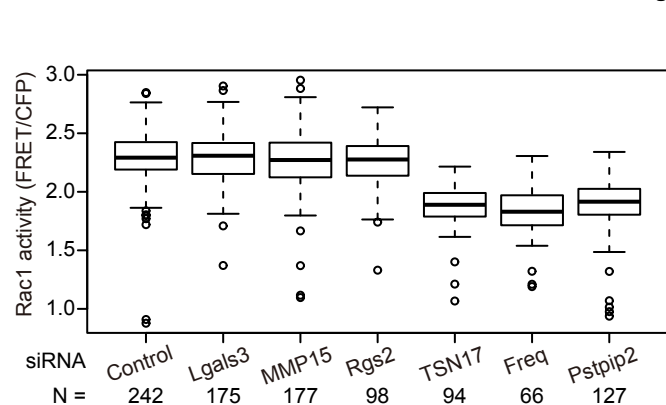
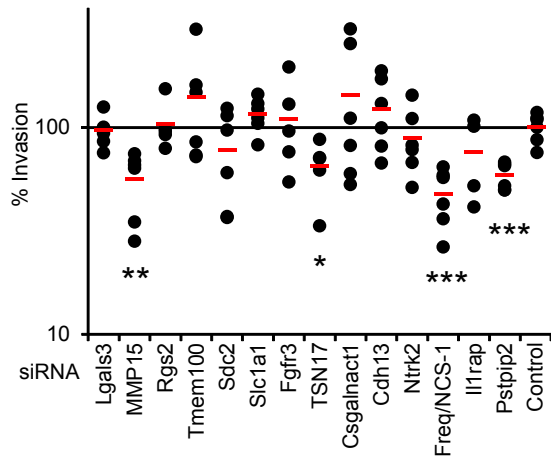
Biological Process	Count	P-value
cell division	24	3.81E-18
cell cycle	30	7.77E-18
mitosis	21	1.19E-16
G-protein coupled receptor protein signaling pathway	5	2.43E-10
G1/S transition of mitotic cell cycle	8	2.37E-07
regulation of cell cycle	9	5.35E-06
organ regeneration	9	6.03E-06
positive regulation of cell cycle cytokinesis	4	7.46E-06
mitotic cell cycle	4	9.77E-05
signal transduction	28	1.54E-04

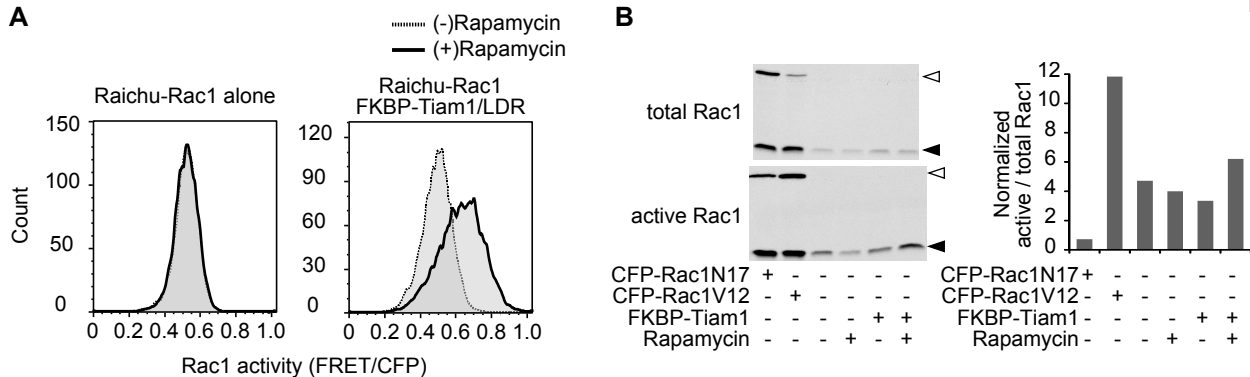
**D**Genes up-regulated in Rac1<sup>high</sup> population

Cellular Component	Count	P-value
respiratory chain	5	2.94E-05
focal adhesion	7	1.21E-04
mitochondrial respiratory chain complex I	4	4.09E-04
cytoplasmic vesicle membrane	5	4.63E-04
Golgi membrane	9	8.99E-04
mitochondrial membrane	5	9.81E-04
membrane	57	1.45E-03
endoplasmic reticulum	18	2.50E-03
Golgi apparatus	17	2.60E-03
integrin complex	3	3.78E-03

Genes up-regulated in Rac1<sup>low</sup> population

Cellular Component	Count	P-value
cytoplasm	114	1.79E-09
nucleus	110	9.47E-08
integral to membrane	40	1.90E-07
spindle pole	9	1.06E-06
extracellular matrix	15	1.72E-06
centrosome	17	2.63E-06
spindle	9	2.85E-06
chromosome	13	1.90E-05
cytoskeleton	19	1.74E-04
microtubule	11	4.57E-04

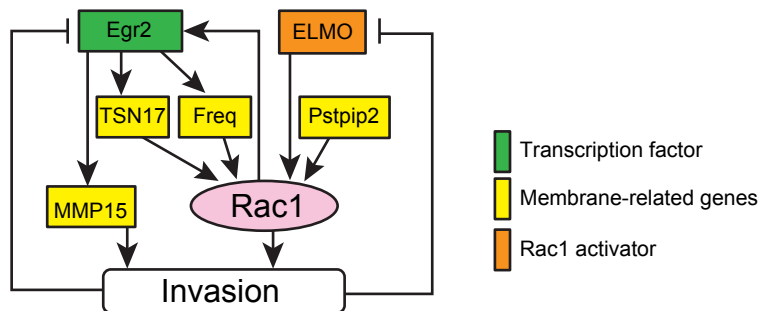




C

		mRNA								Invasion	Rac1 activity
		Rgs2	Lgals3	Egr2	Elmo1	Pstpip2	TSN17	Freq	MMP15		
Activation of Rac1		0.40	-0.34	1.86	0.14	-0.40	0.17	0.25	0.07		0.34
siRNA	Rgs2	-3.60	1.19	0.77	0.15	0.16	-0.94	-0.06	0.99	0.05	-0.01
	Lgals3	-0.33	-3.52	-0.13	0.61	-0.52	0.06	0.04	-0.83	-0.05	0.01
	Egr2	1.06	-0.07	-2.23	0.06	-0.12	-1.55	-1.44	-1.28	-2.32	-0.28
	Elmo1	-1.07	0.63	0.79	-1.99	-1.13	1.20	-0.03	-0.64	-1.25	-0.30
	Pstpip2	-1.61	0.82	0.09	0.62	-3.84	1.68	-0.24	0.87	-0.77	-0.26
	TSN17	1.90	0.65	1.16	0.92	0.30	-5.76	-0.14	-2.48	-0.62	-0.28
	Freq	2.22	1.23	2.91	2.19	1.30	0.97	-4.39	-0.28	-1.07	-0.32
	MMP15	1.06	0.72	3.10	1.97	0.57	0.28	0.14	-4.82	-0.84	-0.01

D



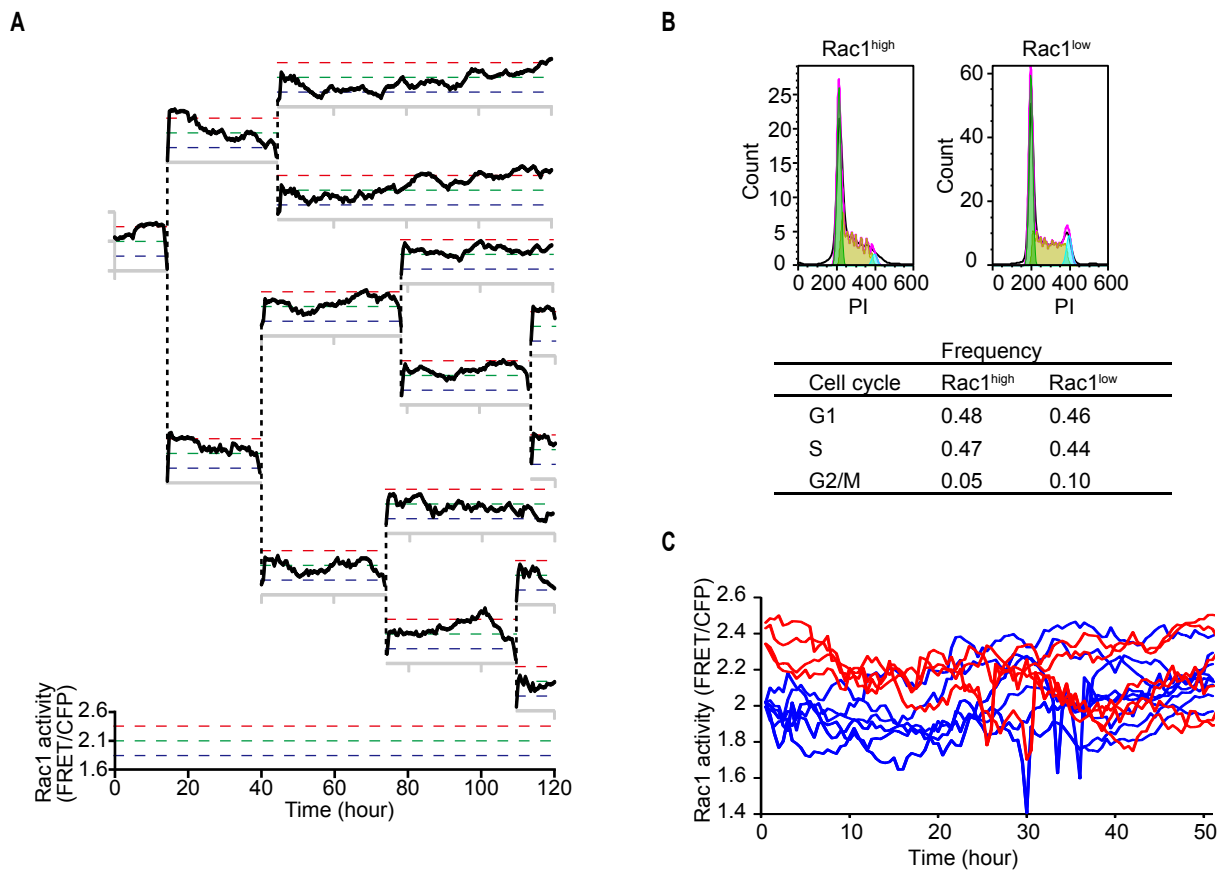


Figure S1. (A) Rac1 activity of a single C6 glioma cell and its daughter cells traced for five days. This figure corresponds to Fig. 2B in the text. The red, green, and blue dotted lines indicate FRET/CFP ratios of 2.35, 2.1, and 1.85, respectively. (B) Enrichment of cells in G2/M phases in Rac1<sup>low</sup> cell populations. Rac1<sup>high</sup> and Rac1<sup>low</sup> cells were fixed, stained with propidium iodide (PI), and analyzed by FACS. The pink lines represent fitting by the Watson-Pragmatic cell cycle model. Green, yellow and blue area indicate G1, S, and G2/M phases, respectively. (C) Time-lapse analyses of Rac1<sup>high</sup> and Rac1<sup>low</sup> cells. Figure S1. C6 glioma cells expressing Raichu-Rac1 were plated on glass-bottom dishes and time-lapse imaged for 5 days. Cells in the highest (red) and lowest (blue) decile with respect to the FRET/CFP ratio were selected and followed up to 50 hours.

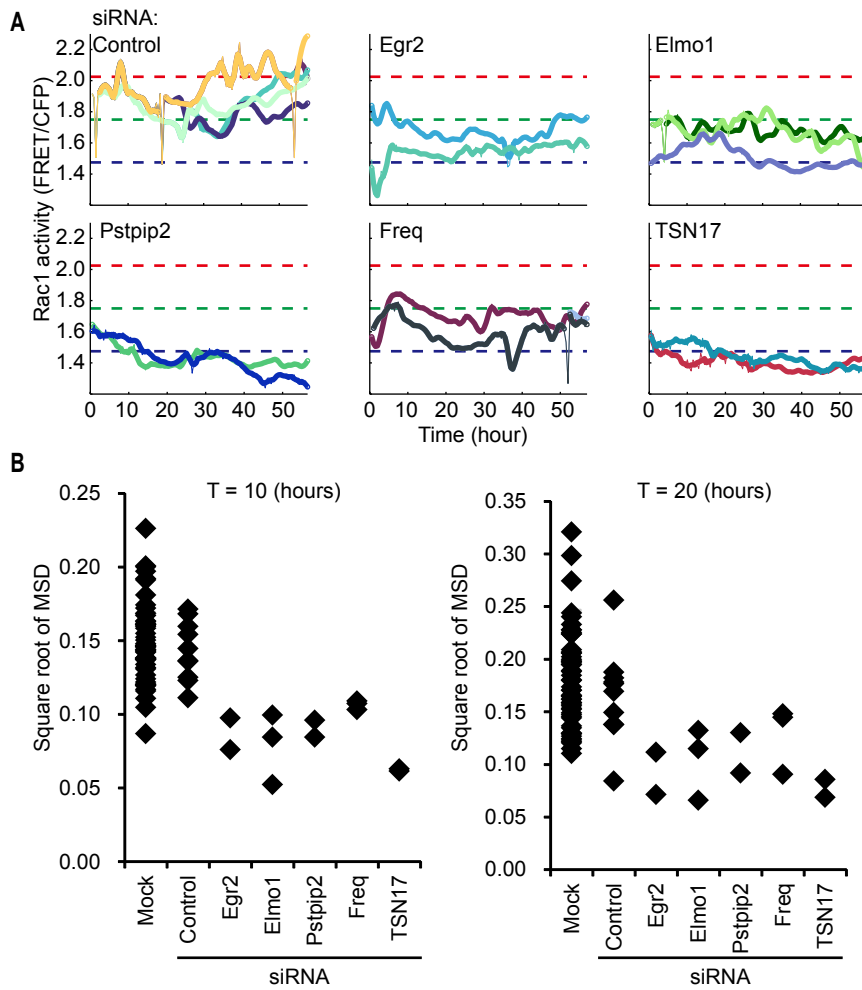


Figure S2. Suppression of Rac1 activity fluctuation by the knockdown of genes enriched in the Rac1<sup>high</sup> cells. (A) Egr2, Elmo1, Pstpip2, Freq, or TSN17 were knocked down as described in the legend to Fig. 5, followed by time-lapse imaging for at least 50 hours. (B) The time courses are smoothed by the Savitzky-Golay filter, except for the mitosis phase, during which a surge of Rac1 activity was observed. The effect of siRNA-knockdown was evaluated by square root of mean-square displacement (MSD) of the Rac1 activity during 10 or 20 hours window.

$$MSD = \frac{\sum_{t=1}^{T_{end}-T} \{x_{t+T} - x_t\}^2}{T_{end} - T},$$

where  $x_{t+T}$  and  $x_t$  indicate Rac1 activity at time  $t+T$  and  $t$ , respectively,  $T_{end}$  indicates total duration of imaging, and  $T$  is an arbitrary number of duration.

$$X = \sqrt{MSD},$$

where  $X$  indicates square root of MSD. These analyses were implemented in Matlab software (The Mathworks).

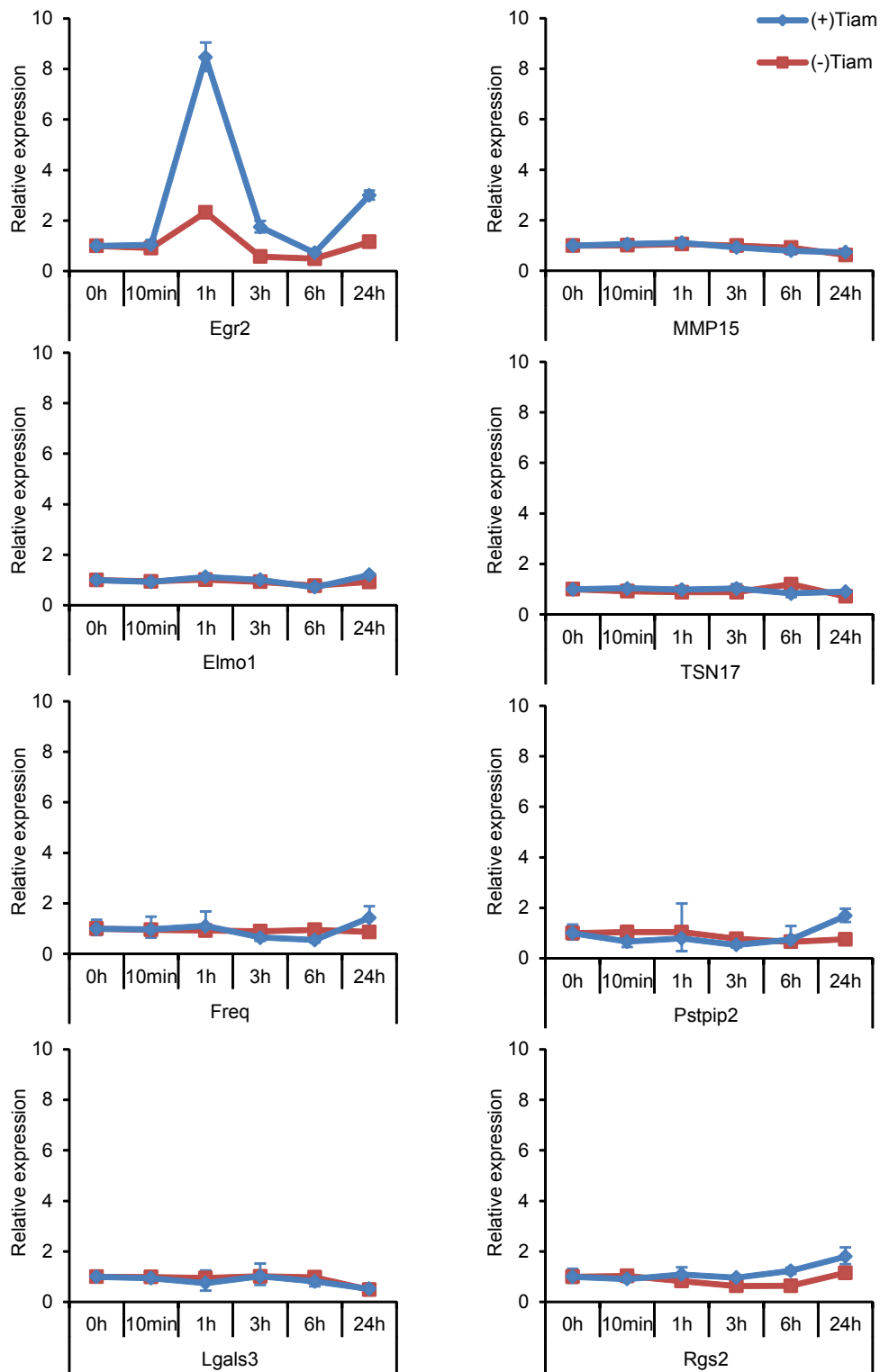


Figure S3. Effect of Rac1 activation on the expression of invasion-associated genes. C6 glioma cells expressing plasma membrane-targeted FKBP12-rapamycin-binding domain, and FK506-binding protein (FKBP) alone or FKBP fused with Tiam1 (blue) or without Tiam1 (red) were stimulated with 10  $\mu$ M Rapamycin. mRNAs depicted at the bottom were quantified by real-time PCR.

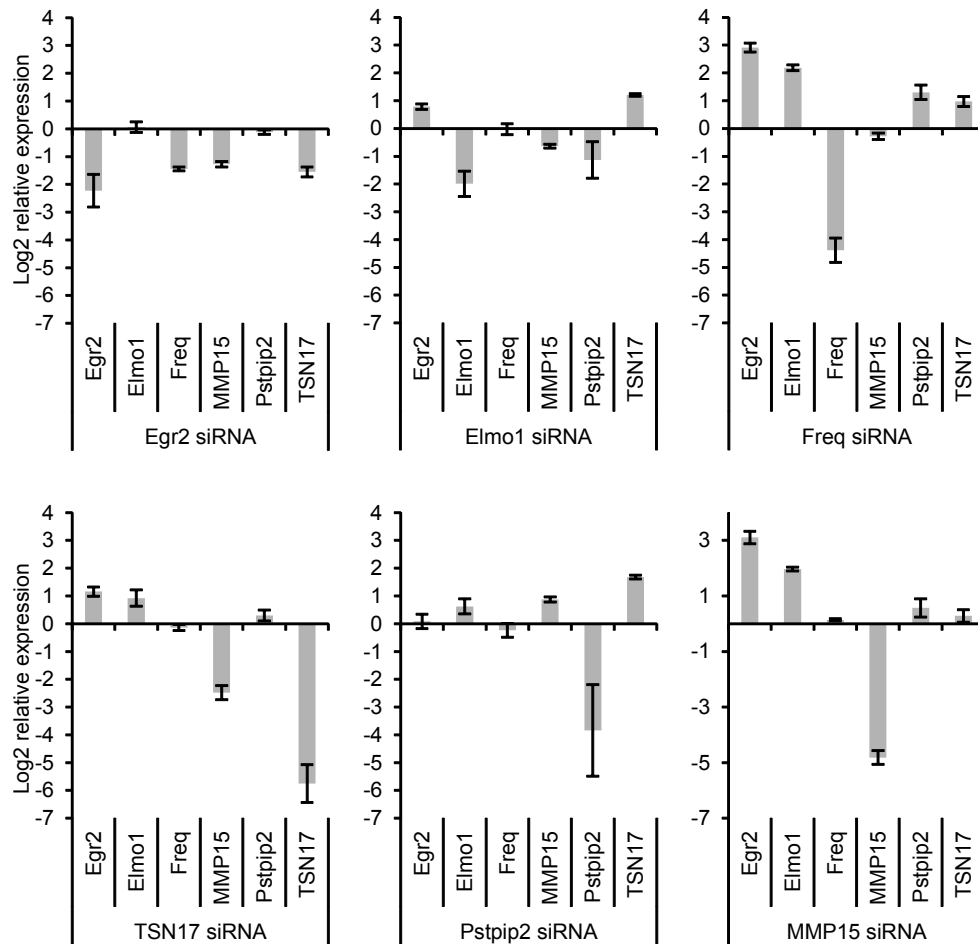


Figure S4. Effect of knockdown of the invasion-associated genes on the expression of the other genes. Genes listed at the bottom were knocked down by siRNAs. mRNAs purified from the cells were analyzed by qPCR for the listed genes. Fold changes to the control siRNA-transfected cells are shown in the log(2) scale. Bars, S.D.



**Table S1. Genes enriched in Rac1<sup>high</sup> population and related to "membrane".**

Ensembl gene ID	Symbol	Rac1 experiments				Cdc42	References related to invasion	
		Effect of knockdown on invasion*	WAD rank	RNA-Seq**		qPCR		RNA-Seq
				Exp.1	Exp.2			
ENSRNOG00000010645	Lgals3	→	10	3.06	6.83	2.07	0.96	1-9
ENSRNOG00000012622	Mmp15	↓	12	2.01	3.89	3.41	6.78	10-30
ENSRNOG00000003687	Rgs2	→	17	3.14	5.83	2.96	3.00	31-33
ENSRNOG00000018566	Ctsl1	N.A.	21	1.70	2.40	1.63	1.40	34-36
ENSRNOG00000002434	Tmem100	→	26	2.03	3.72	2.61	3.11	
ENSRNOG00000004936	Sdc2	→	38	1.82	2.26	2.02	0.97	37-42
ENSRNOG00000014816	Slc1a1	→	65	2.00	2.71	2.56	5.01	
ENSRNOG00000016818	Fgfr3	→	76	2.08	3.34	2.94	1.83	43-48
ENSRNOG00000018122	TSN17	↓	78	1.75	2.71	2.55	1.21	
ENSRNOG00000013024	Csgalnact1	→	87	2.82	5.91	5.92	1.65	
ENSRNOG00000014371	Cdh13	→	93	1.57	2.64	1.55	1.53	49-57
ENSRNOG00000006756	Maged1	N.A.	105	1.54	1.82	1.31	0.97	58-62
ENSRNOG00000018646	Hbegf	N.A.	112	1.60	1.84	1.42	1.55	63, 64
ENSRNOG00000018839	Ntrk2	→	128	2.86	2.52	2.35	2.08	65-70
ENSRNOG00000008761	Freq	↓	129	3.21	4.40	4.48	0.83	
ENSRNOG00000001928	Il1rap	→	137	1.58	1.76	1.80	2.06	
ENSRNOG00000006646	Ecop	N.A.	144	3.79	4.78	N.D.	1.05	
ENSRNOG00000004322	Sh3kbp1	N.A.	150	1.50	1.90	1.42	1.82	71-74
ENSRNOG00000019536	Nid67	N.A.	152	1.79	1.74	1.74	1.46	
ENSRNOG00000007060	Adfp	N.A.	179	1.31	1.80	1.72	0.98	
ENSRNOG00000009922	Prhr	N.A.	192	1.30	3.56	4.36	4.16	
ENSRNOG00000007638	Loxl3	N.A.	197	1.18	1.57	1.68	1.44	75, 76
ENSRNOG00000016987	Pstpip2	↓	211	3.80	6.23	5.51	3.07	77

\*Effect of knock-down on glioma invasion: See legend to Figure 5 in the main text; ↓, suppressed; →, not affected; N.A., not analyzed

\*\*RNA-Seq was performed twice. Fold increase of Rac1<sup>high</sup> vs Rac1<sup>low</sup>, or Cdc42<sup>high</sup> vs Cdc42<sup>low</sup> cell populations is shown.

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**Table S2. Genes enriched in Rac1<sup>high</sup> population and encoding transcription factors.**

Ensembl gene ID	Symbol	Rac1 experiments				Cdc42 experiments	
		RNA-Seq*		qPCR	average FPKM	RNA-Seq*	qPCR
		Exp.1	Exp.2				
ENSRNOG00000012262	Depdc7	1.51	1.88	1.18	33.57	1.15	N.A.**
ENSRNOG00000001314	Fam20c	1.35	1.84	1.19	16.25	0.88	N.A.
ENSRNOG000000019965	Tgfb1i1	1.61	2.04	1.03	12.96	0.85	N.A.
ENSRNOG00000000640	Egr2	1.35	2.41	3.28	6.00	3.98	5.35
ENSRNOG000000018841	Sox8	1.54	5.20	N.A.	1.52	1.61	N.A.
ENSRNOG00000004109	Zfpm2	3.63	4.99	N.A.	1.24	1.14	N.A.
ENSRNOG000000028648	Olig1	2.77	7.76	N.A.	1.07	N.A.	N.A.
ENSRNOG00000008826	Pax9	2.02	2.35	N.A.	0.95	1.42	N.A.

\*RNA-Seq was performed twice. Fold increase of Rac1<sup>high</sup> vs Rac1<sup>low</sup>, or Cdc42<sup>high</sup> vs Cdc42<sup>low</sup> cell populations is shown.

\*\*N.A.; not analyzed.

**Table S3. Genes enriched in Rac1<sup>high</sup> population and encoding Rac1 activators**

Ensembl gene ID	Symbol	Rac1 experiments				Cdc42 experiments		
		WAD	Exp.1	Exp.2	qPCR	WAD	Exp.1	qPCR
		rank				rank		
ENSRNOG00000018726	Elmo1	435	2.95	3.60	3.89	766	2.57	3.84
ENSRNOG00000016479	Plekhg4	504	0.71	0.55	0.60	9716	1.03	
ENSRNOG00000006952	Prex1	541	1.43	1.69	2.05	481	1.84	
ENSRNOG00000016728	LOC100362710	1103	0.69	0.52		6064	0.84	
ENSRNOG00000015026	ARHGB_RAT	1199	0.71	0.71		11806	1.00	
ENSRNOG00000023313	Arhgef19	1428	1.50	1.25		5272	1.12	
ENSRNOG00000001818	FGD4_RAT	1573	1.60	1.94		1197	2.47	
ENSRNOG00000020130	Arhgef1	1633	1.12	1.33		2366	1.16	
ENSRNOG00000004823	F1LUN1_RAT	1717	0.73	0.50		805	0.49	
ENSRNOG000000038970	Fgd1	1810	0.79	0.74		10120	0.98	
ENSRNOG00000017765	Net1	2280	0.72	0.79		6736	1.13	
ENSRNOG00000030266	Plekhg2	2391	1.38	1.43		4992	1.29	
ENSRNOG00000001304	Bcr	3026	0.85	0.76		10983	0.99	
ENSRNOG00000007733	Arhgef9	3154	3.67	6.11		260	2.98	
ENSRNOG00000014576	F1M4N6_RAT	3308	0.82	0.42		20750	0.93	
ENSRNOG00000000869	Arhgef6	3356	0.87	0.86		5928	0.90	
ENSRNOG00000001706	Kalrn	3384	0.87	0.73		385	0.42	
ENSRNOG00000020027	Arhgef2	3517	0.95	0.83		8618	0.97	
ENSRNOG00000022216	Abr	4824	0.95	0.83		2853	0.85	
ENSRNOG00000018683	Dock1	4854	0.94	0.89		1667	1.21	
ENSRNOG00000009910	Swap70	4941	1.21	1.02		10349	1.01	
ENSRNOG00000011203	Farp1	5098	1.00	1.17		6749	1.05	
ENSRNOG00000020485	Vav3	5141	1.57	1.09		3525	1.48	
ENSRNOG000000021569	D3ZTB8_RAT	5544	0.71	0.97		9806	0.97	
ENSRNOG00000012934	Arhgef7	6377	1.01	0.83		6127	1.09	
ENSRNOG00000007422	Vav2	6877	1.09	1.10		8559	0.95	
ENSRNOG000000024703	F1MA88_RAT	6881	1.03	0.83		5190	0.91	
ENSRNOG00000010964	Akap13	7218	1.09	1.04		2941	1.14	
ENSRNOG00000015894	Dock8	7232	1.01	0.81		1872	0.65	
ENSRNOG00000028426	Mcf2l	8326	1.00	0.75		2647	0.61	
ENSRNOG00000006570	Plekhg3	8421	0.92	0.95		8574	0.95	
ENSRNOG00000013321	Dock11	8933	1.89	2.78		20750	0.00	
ENSRNOG00000006701	Fgd6	9043	0.91	0.69		11143	1.08	
ENSRNOG00000005506	Arhgef5	9777	1.13	0.71		1085	0.48	
ENSRNOG00000011969	Dock9	9856	0.99	0.91		6614	0.90	
ENSRNOG00000004826	Sos2	9910	1.05	1.02		4542	1.15	
ENSRNOG00000023280	Als2	9935	0.97	1.10		5808	1.11	
ENSRNOG00000028090	Arhgef18	10122	1.02	0.92		5425	0.89	
ENSRNOG00000008924	Arhgef12	10923	1.13	0.92		6075	1.07	
ENSRNOG00000016011	Plekhg1	11624	1.03	1.01		11069	1.02	
ENSRNOG00000002001	Itsn1	11680	0.96	1.02		10061	0.98	
ENSRNOG00000018051	Farp2	11899	1.13	0.92		8672	0.93	
ENSRNOG00000016544	Rggef	12021	0.95	1.02		5957	0.71	
ENSRNOG00000013707	Spata13	12168	1.01	0.97		961	2.32	
ENSRNOG00000010652	Dock6	12180	0.70	0.59		11040	0.88	
ENSRNOG00000014549	Sgef	12550	1.17	0.86		20750	1.68	
ENSRNOG00000000502	Def6	15330	0.00	0.00		20750	0.00	
ENSRNOG00000000528	Fgd2	15330	0.00	0.00		20750	0.00	
ENSRNOG00000003435	Mcf2	15330	0.00	1.83		20750	0.00	
ENSRNOG00000004566	Arhgef15	15330	0.00	2.75		20750	0.00	
ENSRNOG00000010213	F1LTE6_RAT	15330	0.00	0.00		20750	0.00	
ENSRNOG00000014025	Rasgrf1	15330	0.00	0.92		20750	1.35	
ENSRNOG00000014035	Arhgef4	15330	0.82	0.31		20750	0.00	
ENSRNOG00000014363	Arhgef3	15330	1.12	0.99		20750	0.13	
ENSRNOG00000016225	Fgd3	15330	0.17	1.53		20750	3.77	
ENSRNOG00000016653	Ngef	15330	1.03	0.92		20750	0.38	

\*RNA-Seq of Rac1 was performed twice. Fold increase of Rac1<sup>high</sup> vs Rac1<sup>low</sup>, or Cdc42<sup>high</sup> vs Cdc42<sup>low</sup> cell populations is shown.

**Table S4. Primer sequences used for qPCR.**

Adfp-Forward	tatgcctgcaaggggcta	Adfp-Reverse	gggcattggcaacaatct
Cdh13-Forward	caaccacagaccaacgag	Cdh13-Reverse	caggggtgaaaggcagag
Csgalnact1-Forward	ccggtcagacttcatcaaca	Csgalnact1-Reverse	ggagatattccggtacaggtg
Ctst1-Forward	ttgtgtgactcctggaagaatc	Ctst1-Reverse	cctctaggaacccgatg
Depdc7-Forward	ctcccctcacgtctctacca	Depdc7-Reverse	gtccaatcgctcctcttgc
Ecop-Forward	gccgttctatgaagactgc	Ecop-Reverse	gccgttctatgaagactgc
Egr2-Forward	ctaccgggtggaagacctc	Egr2-Reverse	tctcccggctatgcaatg
Elmo1-Forward	cactattctcgattaaccacgctc	Elmo1-Reverse	ttgatgactgtattcgttcatgg
Fam20c-Forward	gaggcacaatgcgagatag	Fam20c-Reverse	gaggcactctgcgaaatc
Fgfr3-Forward	ctcaggagatgacgaagatgg	Fgfr3-Reverse	cggtcagtcaggtaaggag
Freq-Forward	cctggatgagaagttgaggtg	Freq-Reverse	ccactatgtccagcatctcg
Hbegf-Forward	tgaccacactaccgtcttgg	Hbegf-Reverse	cataacctcctcgctatgg
Il1rap-Forward	aagcagccaaggtgaaacag	Il1rap-Reverse	ctccagccagtaaacgtggt
Lgals3-Forward	aagcccaacgcaaaccagtat	Lgals3-Reverse	tcattgaagcgggggta
Loxl3-Forward	ccggttctcagactccaac	Loxl3-Reverse	ctggtcggagtcgactt
Maged1-Forward	caagagctatggctcagaaacc	Maged1-Reverse	agcaaggcgctgtcttctac
Mmp15-Forward	gaagacgccgaagtatacgc	Mmp15-Reverse	gctgggtaggtagccataga
Nid67-Forward	tcgctgaggatcccttg	Nid67-Reverse	gctgatagcatcattgttg
Ntrk2-Forward	accaatcgggagcatctct	Ntrk2-Reverse	gccaaactgagcagaagca
Prex1-Forward	ccatcaggaccctgtagac	Prex1-Reverse	gcagctggttcttccatc
Prhr-Forward	ggcgcatctcactgaagc	Prhr-Reverse	cgccagcactgcagatag
Pstpip2-Forward	gctgcagcggaaaaagac	Pstpip2-Reverse	tgcgagttcctctgttttg
Rgs2-Forward	aactttatcaagccttctctga	Rgs2-Reverse	acgctctgaatgcagcaag
Sdc2-Forward	ttgatggcctgtgtgtcg	Sdc2-Reverse	ggagctgctgtcaaggta
Sh3kbp1-Forward	gaggaacacatttcgcttg	Sh3kbp1-Reverse	gggaagcctgttatcagaca
Slc1a1-Forward	ttcctgcggaatcactgg	Slc1a1-Reverse	accaagactcctaccagatg
Tgfb1i1-Forward	aacctattgctggcaagtg	Tgfb1i1-Reverse	aacctctgcaaggaagtg
Tmem100-Forward	ggctcttctctccaagtca	Tmem100-Reverse	aggttcagaaagcctgacca
Tsn17-Forward	gcccttctcctgtgtgta	Tsn17-Reverse	ttgggtgtagatggagcctt



**Table S5. siRNA Sequences**

Ap1s2-1	GAAUGAAAGUUUAUUGAAATT	Itgb3-1	GCUUUGACGCCAUCUAGCATT
Ap1s2-2	CUGAUUACCCUGGAAUAATT	Itgb3-2	CAAGCAAUGUCCUUCAGCUTT
Ap1s2-3	GUGAAACCUUGUUUAUUGATT	Itgb3-3	CCAUGUUUGGCUACAAACATT
Cdh13-1	CUAUCAGGUACUCUGUUUATT	Lgals3-1	CACAGUGAAGCCCAACGCATT
Cdh13-2	CUUAUCAACUGUUUGUGGATT	Lgals3-2	CGGUCAAUGAUGUUCUUCUTT
Cdh13-3	CUAUGAGGUCUCAAGCCCATT	Lgals3-3	CCAACUGGCCCUAGUGCUUTT
Csgalnac-1	CCAUAAGCAUGAAUCCAATT	MMP15-1	UCUCCAGCACUGACCUUGCAUGGAAU
Csgalnac-2	GAUUUGACCUUGGACAUAATT	MMP15-2	GGACACCCAUUUUCGACGCACAUGAA
Csgalnac-3	CAUAGCAACCUCUAGUGATT	MMP15-3	ACAGAGAAGCUGGGCUGGUACAACU
Egr2-1	GUGACUAUUGUGGCCGUAATT	Ntrk2-1	GACAUCAUGUGGCUCAAGATT
Egr2-2	GUUUGACUAUGGUCUGCGATT	Ntrk2-2	CAAUGAAGAUGAUGUCGAATT
Egr2-3	GAAAGGAAGCGCCACACCATT	Ntrk2-3	CAAACAACGAGGUGAUAGATT
Elmo1-1	GUUUUAUGACUGUAACUGAATT	Pstpip2-1	GAAAGAAAAGGCAUCAAUUTT
Elmo1-2	GCAUUUCACUCCUCACUCATT	Pstpip2-2	CCAUCAUGUAUGAGAAUUUTT
Elmo1-3	GGAUGAACCAGGAAGAUUUTT	Pstpip2-3	GUGAAACUGGCCACUUCGATT
Fbxo23-1	CGCCAUUGCCUCCUCAAATT	Rgs2-1	GGUUGGCUUGCGAAGACUUTT
Fbxo23-2	GGCUAUACAUCGCGUCAAAATT	Rgs2-2	GAGAUAAACAUAAGACUUUCTT
Fbxo23-3	CCCAAUGACUGGAACCUCATT	Rgs2-3	CUUGCUGCAUUCAGAGCGUTT
Fgfr3-1	CACAUGACCUGUACAUGAUTT	Sdc2-1	CUUUAGCAUAGAAUAAUGATT
Fgfr3-2	GAGUCUAAUUCUCUUAUGATT	Sdc2-2	CAGUGUUCUGUGAAUAGCATT
Fgfr3-3	GCAUUAAAGCUCCGGCACCAT	Sdc2-3	CUGACAACAUCCCAACUGATT
Freq-1	CACCAAGUUCGCCACGUUUTT	Slc1a1-1	CAGAUUCUGGUGGAUUUCUTT
Freq-2	GAACGCUGAUGGGAAGCUATT	Slc1a1-2	CCACAAUCCUGGUAUAAUGATT
Freq-3	CGAAUUCAUCCAGGCUCUATT	Slc1a1-3	GGUAUUGUGUUAGUUGUGATT
Il1rap-1	GACUUACUGCAGCAAAGUUTT	Tmem100-1	GAUAUGCACAGCAUAAAUTT
Il1rap-2	GGUUGUACUGAAAUAGUGATT	Tmem100-2	CAAUAAUUGGACAGGAUGATT
Il1rap-3	CUCAUAUCUACUCGCCAAATT	Tmem100-3	GGGAUAACUCAUCUUCUUTT