Cytoplasmic RAP1 mediates cisplatin resistance of non-small cell lung cancer

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Cytotoxic chemotherapy agents (e.g., cisplatin) are the first-line drugs to treat non-small cell lung cancer (NSCLC) but NSCLC develops resistance to the agent, limiting therapeutic efficacy. Despite many approaches to identifying the underlying mechanism for cisplatin resistance, there remains a lack of effective targets in the population that resist cisplatin treatment. In this study, we sought to investigate the role of cytoplasmic RAP1, a previously identified positive regulator of NF-κB signaling, in the development of cisplatin resistance in NSCLC cells. We found that the expression of cytoplasmic RAP1 was significantly higher in high-grade NSCLC tissues than in low-grade NSCLC; compared with a normal pulmonary epithelial cell line, the A549 NSCLC cells exhibited more cytoplasmic RAP1 expression as well as increased NF-κB activity; cisplatin treatment resulted in a further increase of cytoplasmic RAP1 in A549 cells; overexpression of RAP1 desensitized the A549 cells to cisplatin, and conversely, RAP1 depletion in the NSCLC cells reduced their proliferation and increased their sensitivity to cisplatin, indicating that RAP1 is required for cell growth and has a key role in the development of cisplatin resistance in NSCLC cells. The RAP1-mediated cisplatin resistance was associated with the activation of NF-κB signaling and the upregulation of the antiapoptosis factor BCL-2. Intriguingly, in the small portion of RAP1-depleted cells that survived cisplatin treatment, no induction of NF-κB activity and BCL-2 expression was observed. Furthermore, in established cisplatin-resistant A549 cells, RAP1 depletion caused BCL2 depletion, caspase activation and dramatic lethality to the cells. Hence, our results demonstrate that the cytoplasmic RAP1 axis represents a key pathway to cisplatin resistance in NSCLC cells, identifying RAP1 as a marker and a potential therapeutic target for cisplatin resistance of NSCLC.

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Lung cancer is a leading cause of tumor-related mortality around the world. Non-small-cell lung cancer (NSCLC) represents a heterogeneous group of tumors that account for >85% of the newly diagnosed lung cancer incidents. Multiple approaches have been applied for the treatment of NSCLC, among which are the apoptosis-induction chemotherapy agents such as cisplatin (CP). But unfortunately, considerably high rate of relapse occurs following CP treatment despite the approaches aiming to overcome resistance. It is thus necessary to develop strategies that act on the CP-resistant subpopulation of NSCLC and reverse the resistance in these tumor cells.

During the past decade, great effort has been made to identify the extratelomeric functions of telomere-associated proteins. One of these factors is RAP1 (repressor/activator protein-1), also known as TRF2IP (telomeric repeat binding factor-2 interacting protein-1), which was originally identified as part of the telomere-binding complex. Considered as a shelterin protein component, RAP1 has been found to associate with multiple types of human cancer, including NSCLC. However, genetic modification studies have revealed that RAP1 is not required in the maintenance of human telomere length and telomere heterochromatic structure, indicating that other mechanisms must be involved regarding the function of RAP1 in oncogenesis. Indeed, RAP1 was identified to bind with extratelomeric sites through a consensus motif, helping with gene transcriptional regulation. Meanwhile, cytoplasmic RAP1 has been revealed for its essential role in modulating NF-κB signaling. Specifically, the RAP1–NF-κB axis was demonstrated to promote invasion of breast cancer cells. Given the broad activation of NF-κB signaling in human cancer, one would infer that RAP1 might contribute to the progression of other types of tumors via a similar mechanism.

NF-κB activity has been detected in both lung cancer and preneoplastic pulmonary lesions and associated with the resistance to chemotherapy. In lung cancer cells, independent studies have shown that cytotoxic chemotherapy using gemcitabine or TNF-α could induce NF-κB activity and combining with NF-κB inhibitor has been considered to overcome resistance. Further, NF-κB signaling has been shown to positively regulate the expression of the apoptosis inhibitor BCL-2 and the cancer stem-like cells, a population associated with therapy resistance, were
discovered to have both high BCL-2 expression and NF-κB hyperactivation in multiple types of human cancer.\textsuperscript{29,30} These results suggest that BCL-2 overexpression might be, at least part of, the consequence of NF-κB activation that accounts for chemotherapy resistance. More specifically, NF-κB signaling was recently assessed in cervical cancer and epidermoid carcinoma cells to inhibit apoptosis and mediate CP resistance.\textsuperscript{31,32} However, the mediator between chemotherapy agent and NF-κB activity remains poorly understood, making it difficult to identify the target that could re-sensitize those resistant tumor cells to cytotoxic agents.

In this report, we evaluated a mediating role of cytoplasmic RAP1 in tumorigenesis of NSCLC through NF-κB signaling. RAP1 is highly expressed in high-grade NSCLC samples, facilitates NF-κB activation which is required for the proliferation of NSCLC cells, upregulates BCL-2 expression and mediates the resistance of NSCLC cells to CP. Meanwhile, we observed a synergistic effect between CP treatment and RAP1 deletion in tumor suppression. Our results demonstrate an extratelomeric function for RAP1 in tumor progression and suggest that RAP1 is a potential therapeutic target for CP-resistant NSCLCs.

**Results**

Expression of cytoplasmic RAP1 is associated with malignancy in NSCLC patients. It was previously reported that cytoplasmic RAP1 was detected in breast cancer tissue,
and its pathology scores were positively correlated with the tumor grades. To test the hypothesis that cytoplasmic RAP1 is a biomarker for higher-grade NSCLC, we immunostained for RAP1 and quantified its cytoplasmic and nuclear expression in 93 lung adenocarcinoma and 75 lung squamous cell carcinoma tissues. The peri-tumoral normal tissue was used to reflect RAP1 expression in non-malignant cells (Supplementary Tables S1 and S2). Both cytoplasmic and nuclear RAP1 were increased in NSCLC tumors compared with normal tissues, but the difference in cytoplasmic RAP1 appears to be greater (Figures 1a, b, f, Supplementary Figures S1a and b). A higher level of cytoplasmic RAP1 expression was associated with a higher grade of NSCLCs (Figures 1c, d, Supplementary Figures S1c and d). Moreover, higher cytoplasmic RAP1 expression was associated with poorer prognosis of adenocarcinoma patients (Figure 1e; information of squamous cell carcinoma patients’ survival is unfortunately not available). These analyses identify cytoplasmic RAP1 as an indicator of high-grade NSCLC, suggesting that it may have a critical role in cancer progression.

**RAP1 is required for the growth of NSCLC cells.** Although cytoplasmic RAP1 protein levels are higher in high-grade NSCLC tissues than in low-grade NSCLC tissues, it is also detected in most examined low-grade NSCLCs, prompting us to speculate that cytoplasmic RAP1 could be a signature to distinguish NSCLC cells from the normal compartment. Therefore, we compared the expression of RAP1 between three NSCLC cell lines and two lung epithelial cell lines. The NSCLC cells tested here include adenocarcinoma A549 and PC9, together with squamous cell carcinoma HCC827, while the normal cells include bronchial epithelial cells 16HBE and small airway epithelial cells HSAEC1-KT. Intriguingly, in NSCLC cells, we detected an overall higher mRNA and protein expression of RAP1, particularly in the cytoplasmic fraction compared with normal lung epithelial cells (Figures 2a and b). The increased cytoplasmic expression of RAP1 in NSCLC cells was further confirmed by immunofluorescence (Figure 2c and Supplementary Figure S2), leading to a hypothesis that RAP1 facilitates malignancy by working in the cytoplasmic compartment.

Owing to the challenge to specifically inhibit cytoplasmic RAP1, we tested the alternative hypothesis that RAP1 mediates NSCLC cell proliferation. Therefore, lentiviruses encoding either one of the two short hairpin RNA (shRNA) against RAP1 (shRAP1-1 and shRAP1-2) or a scramble shRNA were applied to three different NSCLC cell lines. RAP1 knockdown by shRAP1-1 or shRAP1-2 was evident both at the mRNA (Figure 2d) and protein levels in both cytoplasmic and nuclear fractions (Supplementary Figure S3b). RAP1-deleted NSCLC cells exhibited defect in cell viability and growth over culture (Figure 2e). Consistently, when plated at the same number, RAP1-deleted NSCLC cells formed fewer colonies after a 3-week culture (Figure 2f and Supplementary Figure S4). These results demonstrate that RAP1 is required for the growth of A549 cells.

**RAP1 induces NF-κB signaling in NSCLC cells.** Having proven the requirement of RAP1 in lung cancer cell proliferation, we next studied the mechanism by which RAP1 mediates cell growth. The assays described above do not attribute the function of RAP1 exclusively to the cytoplasmic compartment. However, RAP1 per se is insufficient for telomere protection, suggesting that nuclear RAP1 has very minor role in maintaining genomic stability in hyperproliferative tumor cells. Thus we speculated that cytoplasmic RAP1 is the main contributor to induce cell proliferation. To test this hypothesis, we detected the effect of RAP1 deletion on the activity of NF-κB signaling, a known downstream effector of cytoplasmic RAP1. As was proven by previous studies, cytoplasmic RAP1 acts as IKK adaptor proteins which mediate the phosphorylation of p65. Indeed, when RAP1 was deleted in A549 cells, we observed a decrease of phosphorylated p65 in the nuclear fraction (Figures 3a and b). Phosphorylation of IκBα, which promotes NF-κB signaling, was also decreased with the deletion of RAP1 (Figure 3a and Supplementary Figure S5a). Intriguingly, transcription of IκBα was significantly suppressed but the total IκBα protein was only moderately decreased after RAP1 deletion (Figures 3a and b), probably due to a positive feedback of NF-κB signaling. Both IκBα and phospho-IκBα are mainly expressed in the cytoplasmic fractions. No significant changes in IKK protein levels or in its phosphorylation were observed upon RAP1 deletion (Supplementary Figures S5b and c). To further demonstrate the inhibition of NF-κB signaling, we evaluated the transcription of IL-1, MCP-1, and CD44, which are known to be positively regulated by activated NF-κB. Clearly, RAP1 deletion led to the downregulation of these factors (Figure 3c). Together with the observation that RAP1 mediates NSCLC cell growth, these results suggest that the expansion of NSCLC cells is positively regulated by RAP1-mediated NF-κB activation.

**RAP1 mediates CP resistance in NSCLC cells.** Activated NF-κB is known to render the cancer cells resistant to chemotherapy. Moreover, CD44, whose transcription is negatively regulated by RAP1-mediated NF-κB signaling, was discovered to be enriched in a CP-resistant population. Therefore, we tested the hypothesis that RAP1 suppresses the sensitivity of NSCLC cells to CP. First, the RAP1 shRNA or scramble shRNA-transduced A549 cells were treated with different doses of CP. As depicted in Figure 4a, a relative high dose of CP (>2 μM) eliminated almost all cells in both groups. Nevertheless, when less CP was applied, significantly fewer RAP1-deleted cells survived the treatment (Figure 4a). Next, we investigated the effect of overexpressed RAP1 on CP sensitivity. Unexpectedly, when A549 cells were transduced to overexpress RAP1, they displayed only modest increase of growth (Figure 4b); however, a larger amount of CP was required to eliminate RAP1-overexpressed cells to an extent similar to that achievable with the control cells (Figure 4c). To get a further understanding of RAP1 in mediating CP resistance, we performed a competition assay in which normal A549 cells (GFP+) were mixed with RAP1-overexpressing or -deleted cells (GFP−) at a 1:1 ratio before applied to CP treatment (Figure 4d). When treated with CP over time, the cells harboring overexpressed RAP1 became the dominant population while RAP1-deficient cells got
rapidly eliminated (Figure 4d). These results indicate that RAP1 renders lung cancer cells resistant to CP treatment. CP acts through generating DNA damage in the proliferative cancer cells, which ultimately leads to their apoptosis. However, RAP1 does not seem to have significant impact on CP induction of DNA damage per se (Supplementary Figures S6a and b). Hence, we questioned whether RAP1 acts on regulating the apoptotic status of CP-treated cells. Not surprisingly, an induction of cleaved caspase-3 was detected in A549 cells following CP treatment (Figure 4e). In the cells
harboring overexpressed RAP1, despite a similar baseline extent of apoptosis, CP did not trigger an obvious upregulation of cleaved caspase-3; in contrast, CP facilitated the RAP1-deleted cells to express a high level of cleaved caspase-3 at an early time point during the treatment (Figure 4e). Similarly, the proportion of Annexin5+ apoptotic cells after CP treatment was decreased in RAP1-overexpressing cells and, conversely, increased in RAP1-deleted cells (Figure 4f). When examining the antiapoptosis factor BCL-2, we discovered a positive correlation between RAP1 and BCL-2 expression even without CP treatment (Figure 4e, comparing columns 1, 3 and 5). CP treatment slightly induced BCL-2 expression in cells transduced with control and RAP1-overexpression vectors, which might be a negative feedback of facilitated apoptosis (Figure 4e). However, little increase of BCL-2 was observed in CP-treated, RAP1-deleted cells (Figure 4e), suggesting that RAP1 is necessary for BCL-2 induction in response to CP. Thus we would conclude that RAP1 inhibits CP-induced apoptosis to mediate CP resistance.

**CP resistance is associated with RAP1-dependent NF-κB activation.** To further investigate the correlation between RAP1 expression and CP sensitivity, we treated A549 cells with increasing doses of CP to generate the cells bearing different extents of resistance (Figure 5a). Surviving cells were harvested at multiple time points to evaluate the RAP1 expression. Shown in Figure 5a, in the viable cells that sustain the escalating dosage of CP, cytoplasmic but not nuclear RAP1 expression was gradually induced, supporting our hypothesis that cytoplasmic RAP1 marks CP resistance. Moreover, similar induction was also observed when examining NF-κB activity, as shown by changes in pp65 and p-IκBα (Figure 5b). Notably, the increase of pp65 and p-IκBα showed a delay when compared with RAP1 expression, suggesting their roles as the responders to RAP1 when encountering CP in the environment. Transcription of IL-1, MCP-1 and CD44 was also facilitated along the treatment process, which further demonstrated the activation of NF-κB signaling (Figure 5c).

To clarify the requirement of RAP1 in activating NF-κB signaling in response to CP, we further applied the sequential CP treatment to RAP1-overexpressing and -deleted cells. Consistent with the results depicted above, RAP1-overexpressing cells displayed an enhanced baseline NF-κB activity, which was further strengthened by CP to a larger extent compared with the untransduced cells (Figure 5d). At the latest time point analyzed in this assay, however, there was no significant difference of NF-κB activity between the RAP1-overexpressing and the untransduced cells (Figure 5d, columns 7 and 8), a result probably owing to the effect of continuous CP treatment in activating and saturating NF-κB signaling. In contrast, 0.5 μM of CP showed considerable...
toxicity to RAP1-deleted cells, and 1 μM of CP eliminated almost all the cells (Figures 4a and 5e). More importantly, treatment using 0.1 and 0.5 μM of CP did not induce NF-κB signaling in the surviving RAP1-deleted cells, although considerable amount of cells still survived under these conditions (Figures 5f and g). Therefore, RAP1 is an intermediate mediator for CP-induced NF-κB activation. Together, these results support the conclusion that RAP1 is necessary for the induction of NF-κB in the cells surviving CP treatment. Further, given the observation by previous studies...
that NF-κB signaling regulates BCL-2 transcription,\textsuperscript{27} the RAP1–NF-κB–BCL2 axis is inferred in the lung cancer cells to induce CP resistance.

**RAP1 deletion is lethal to CP-resistant NSCLC cells.** Having demonstrated the role of RAP1 in inhibiting CP-induced apoptosis, we then aimed to harness RAP1 as a target to overcome CP resistance. As described above, RAP1 deletion increased the sensitivity of NSCLC cells to CP (Figure 5e), so we next evaluated the capability to target RAP1 in cells that have already established resistance. We generated CP-resistant A549 cells (A549R) using protocols described previously.\textsuperscript{34,35} These A549R cells displayed upregulated cytoplasmic RAP1 and induced NF-κB signaling.
The tolerance of these cells to CP was well characterized by treating with high dose of CP, which eliminated 60–90% of normal A549 cells within 24 h (Supplementary Figure S7a). The BCL-2 expression was much higher in A549R cells compared with parental A549 cells, probably contributed by the continuous CP treatment in maintaining resistance (Figure 6d and Supplementary Figure S7c). Inspired by the effect described above that RAP1 deletion sensitizes cells to CP treatment, we sought to attenuate CP resistance in A549R cells through deleting RAP1. Surprisingly, lethality was observed almost immediately after applying lentiviruses encoding sh-RAP1 to A549R cells (Figure 6c). To rule out the possibility that the CP used to maintain resistance might induce mortality, we cultured CP-resistant cells in CP-free media for 24 h before inducing RAP1 deletion. Such CP clearance did not affect the lethality. The IF assay of cleaved caspase-3 (red) after RAP1 deletion was analyzed (Figure 6e). Flow cytometric analysis for Annexin-V staining after RAP1 deletion was analyzed (Figure 6f). Figure 6d. RAP1-deleted A549 cells and scramble shRNA-transduced A549R cells were cultured in media containing 1 μM CP (+) or CP-free media (−) for 24 h before lysis to harvest protein. Lentiviral shRAP1 was introduced to A549R cells pre-cultured in CP-free media for 24 h and protein harvested 12 h after transduction. Cleaved caspase-3 and BCL-2 were detected by western blotting. Figure 6f. IF assay of cleaved caspase-3 (red) in untreated A549 and A549R cells transduced with scramble shRNA or shRAP1. Flow cytometric analysis for Annexin-V staining in A549 and A549R cells after RAP1 deletion. A549 cells were maintained in CP-free media and A549R cells were preconditioned in CP-free media for 24 h before analysis. Statistics were generated from three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test; error bar: ± S.D.
tolerance of the cells to CP, but RAP1-depleted cells still could not survive for 7 days (Figure 6c and Supplementary Figure S4d). Consistently, although scramble shRNA-transduced A549R cells had minor induction of apoptosis in response to CP treatment, an upregulation of cleaved caspase-3 and the almost complete loss of BCL-2 were observed in the RAP1-deleted A549R cells even though these cells had undergone CP-free culture prior to transduction (Figure 6d). Expression of cleaved caspase-3 in RAP1-deleted A549R cells was significantly higher than untreated RAP1-deleted A549 cells and similar to CP-treated RAP1-deleted A549 cells (Figures 6d and e). Therefore, RAP1 deletion alone is sufficient to cause an overwhelming lethality in the A549R cells through hyperactivation of apoptosis, for which further CP treatment is no longer required.

Discussion

Here we have identified an extratelomeric function of RAP1 acting through the NF-κB signaling to promote lung cancer cell growth and mediate CP resistance (Figure 7). Our results show that cytoplasmic RAP1 is more enriched in human high-grade NSCLCs, which is agreed by previous observations in breast cancer.17 Several independent studies have confirmed that NSCLC cells acquired malignancy-associated phenotypes after repetitive exposure to the cytotoxic agent CP,33,36 we demonstrate here that similar treatment also leads to the induction of RAP1 expression. The facilitating role of RAP1 in NSCLC progression is supported by our findings that shRNA-mediated RAP1 knockdown severely impairs the growth of lung adenocarcinoma and squamous carcinoma cell lines. One might argue that RAP1 deletion targets not only the cytoplasmic fraction but also the nuclear fraction. However, nuclear RAP1 has been demonstrated to be dispensable for maintaining chromosome integrity.13 Also, the p-IκBα level was always associated with the RAP1 expression in this study, indicating that the main function of RAP1 in NSCLC cells comes from its cytoplasmic fraction.

Notably, while overexpressing RAP1 resulted in only a modest increase of cellular proliferation, it rendered the cells more resistant to CP treatment. Many studies have showed synergistic effects between CP and NF-κB inhibitor but the induction of NF-κB by CP seemed indirect.31,32 CP acts directly on the double helix DNA to generate damage. As we observed only minor or no effect of RAP1 on CP induction of DNA damage in NSCLC cells (Supplementary Figure S6), we infer that RAP1 mediates CP-resistance through reducing cytotoxic response to the DNA damage induced by CP. We speculate that CP-induced DNA damage triggers the synthesis of shelterin-forming genes, including RAP1; RAP1 is not required for the chromatin protection but, when transported to the cytoplasm, acts as a mediator of CP resistance through activating NF-κB signaling and subsequently inducing BCL-2 expression (Figure 7). Notably, we did observe a small fraction of RAP1-depleted cells that survived after CP treatment, pointing out the existence of redundant or additional mechanism that mediates CP resistance even in the absence of RAP1. However, those remaining cells failed to activate NF-κB signaling even after CP exposure, demonstrating that NF-κB induction is RAP1 dependent.

Interestingly, after RAP1 deletion, the transcription of IκBα, which was both an inhibitor and a downstream target of NF-κB,17,29 was remarkably decreased. The total IκBα protein, however, was only moderately decreased as a reduction of IκBα phosphorylation will cause less IκBα protein to be degraded. The synergistic effect we found between RAP1 deletion and TNF-α treatment (Supplementary Figure S8) could further prove that targeting RAP1 is able to inhibit NF-κB activity, as TNF-α resistance is also correlated with NF-κB activity.37–39 It is elucidated here that BCL-2 could be, at least one of, the downstream effectors of activated NF-κB signaling contributing to CP resistance. Given the activity of NF-κB on the promoter region of BCL-2 demonstrated by previous research,26,40 RAP1 is most likely to mediate CP resistance through a transcriptional facilitation of the negative apoptosis regulator via NF-κB. The RAP1–NF-κB–BCL2 regulatory axis partially explains why cytoplasmic RAP1 is highly expressed in tissues of advanced NSCLCs and associated with poorer clinical outcome, although further analysis is needed to examine RAP1 expression in NSCLCs pre- and post-CP therapy. Moreover, as BCL-2 was demonstrated to induce NF-κB signaling,41,42 it is thus possible that the mutual regulation between NF-κB and BCL-2 could act as a positive feedback loop, providing an intriguing direction to investigate whether an intermediate increase in RAP1 is able to cause CP resistance similar to highly overexpressed RAP1.

We observed a very intriguing and potentially highly significant effect in the established CP-resistant NSCLC cells, where RAP1 deletion led to an almost complete elimination of the cells. It was hypothesized that the hyperactivated NF-κB in these cells will generate large amount of TNF-α, leading to the induction of apoptosis when the NF-κB signaling is disrupted by loss of RAP1. However, mortality still occurred when fresh media containing negligible amount of TNF-α was applied immediately after RAP1 deletion, suggesting that TNF-α is not the main reason for apoptosis induction. One possible
Cell viability and colony formation analysis. Cell viability was analyzed by Cell Counting Kit 8 (Dojin, Kumamoto, Japan) according to the manufacturer’s instructions and calculated as the percentages of control plated under the same conditions, based on the procedure of previous studies.44,45 Cell proliferation was calculated as fold expansion in comparison to the viable cells plated. Colony formation was performed as described previously.46

Western blotting analysis. Whole-cell lysate was generated with RIPA lysis and extraction buffer supplemented with protease inhibitors and phosphatase inhibitors (Thermo). Electrophoresis and transfer were based on standard procedures as previously reported.47 Protein blots were probed with primary antibodies against Actin (Cell Signaling Technology #4970), Histone H3 (Cell Signaling Technology #4499), RAP1 (Cell Signaling Technology #5433), p65 (Cell Signaling Technology #8242), pSer536 (Cell Signaling Technology #3033), IxBr (Cell Signaling Technology #4814), IxBr-pSer32 (Cell Signaling Technology #2859), H2AX (Abcam, Cambridge, UK #11174), BCL-2 (Cell Signaling Technology #2870) and cleaved caspase-3 (Cell Signaling Technology #1638) followed by HRP-conjugated secondary antibodies (Cell Signaling Technology #7074 and #7076). Immunoblots were detected using ECL reagents (Thermo).

Immunofluorescence. Cells were digested with trypsin and then plated for 24 h to adhere to chamber slides (Millipore, Billerica, MA, USA). Then the cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton-X and blocked with 2% of bovine serum albumin. Staining was performed using primary antibodies against IκκBα, p65, pSer536, IxBr, p-IxBr, cleaved caspase-3 (Cell Signaling Technology) same as above and H2AX (Abcam #11174) for 12–18 h followed by secondary staining using Alexa fluoro594 goat-anti-rabbit or goat-anti-mouse IgG (Proteintech, Chicago, IL, USA) for 1 h and DAPI for 10 min. Fluorescence was detected via a TCS SP8 confocal microscope (Leica) and processed by Image J (NIH, Bethesda, MD, USA).

Flow cytometry. Trypsinized cells were washed and resuspended in PBS supplemented with 2% FBS. For GFP detection, cells were directly proceeded to analysis with Sidak–Bonferroni correction (three or more groups).

Conflict of Interest
The authors declare no conflict of interest.

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