Gain-of-function mutations in granulocyte colony–stimulating factor receptor (CSF3R) reveal distinct mechanisms of CSF3R activation

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Granulocyte colony–stimulating factor (G-CSF or CSF3) and its receptor CSF3R regulate granulopoiesis, neutrophil function, and hematopoietic stem cell mobilization. Recent studies have uncovered an oncogenic role of mutations in the *CSF3R* gene in many hematologic malignancies. To find additional *CSF3R* mutations that give rise to cell transformation, we performed a cellular transformation assay in which murine interleukin 3 (IL-3)–dependent Ba/F3 cells were transduced with WT CSF3R plasmid and screened for spontaneous growth in the absence of IL-3. Any outgrowth clones were sequenced to identify *CSF3R* mutations with transformation capacity. We identified several novel mutations and determined that they transform cells via four distinct mechanisms: 1) cysteine- and disulfide bond–mediated dimerization (S581C); 2) polar, non-charged amino acid substitution at the transmembrane helix dimer interface at residue Thr-640; 3) increased internalization by a Glu-524 substitution that mimics a low G-CSF dose; and 4) hydrophobic amino acid substitutions in the membrane-proximal residues Thr-612, Thr-615, and Thr-618. Furthermore, the change in signaling activation was related to an altered CSF3R localization. We also found that CSF3R-induced STAT3 and ERK activations require CSF3R internalization, whereas STAT5 activation occurred at the cell surface. Cumulatively, we have expanded the regions of the CSF3R extracellular and transmembrane domains in which missense mutations exhibit leukemogenic capacity and have further elucidated the mechanistic underpinnings that underlie altered CSF3R expression, dimerization, and signaling activation.

Granulocyte colony–stimulating factor (G-CSF;[4] also known as CSF3) and its receptor CSF3R have long been recognized to play important roles in the regulation of granulopoiesis, neutrophil function, and hematopoietic stem cell mobilization (1). Wildtype (WT) CSF3R consists of three structural domains: extracellular, transmembrane, and cytoplasmic, which regulate ligand binding, dimerization, and downstream signaling activation/termination of the receptor, respectively (2–4). Similar to other class I cytokine receptor family members, CSF3R lacks intrinsic kinase activity and is coupled with intercellular non-receptor tyrosine kinases, including JAK/STAT, MAPK/ERK, and PI3K3/AKT, via its cytoplasmic tyrosine residues (5). Activation of STAT5 has been linked to proliferation and survival signaling of hematopoietic progenitor cells (6–9). STAT3 activation is known to be critical for G-CSF–induced cell differentiation and negative regulation of CSF3R signaling by inducing SOCS3 activation (5, 8, 10, 11).

Recent studies have uncovered an oncogenic role of CSF3R mutations across all CSF3R domains in various hematologic malignancies. Briefly, CSF3R cytoplasmic truncation mutations, including CSF3R isoform IV, were shown to disrupt receptor internalization and/or degradation, leading to overexpression of the receptor on the cell surface, inducing sustained STAT5 and reduced STAT3 activation in a G-CSF–dependent manner (12–14). Clinically, these truncation mutations are associated with leukemia transformation of severe congenital neutropenia and a higher disease relapse rate of childhood acute myeloid leukemia, and coupled with CSF3R membrane-proximal and transmembrane mutations to induce chronic neutrophilic leukemia (15–17). In addition, recent studies have shown that CSF3R membrane-proximal and transmembrane mutations are prevalent and causative mutations in chronic neutrophilic leukemia (17, 18). Mechanistically, these mutations demonstrate increased dimerization of the receptor, leading to constitutive and enhanced G-CSF–independent JAK-STAT and ERK activation (19, 20). More recently, we have identified a gain-of-function CSF3R extracellular domain mutation, W341C, in an acute myeloid leukemia patient (21).

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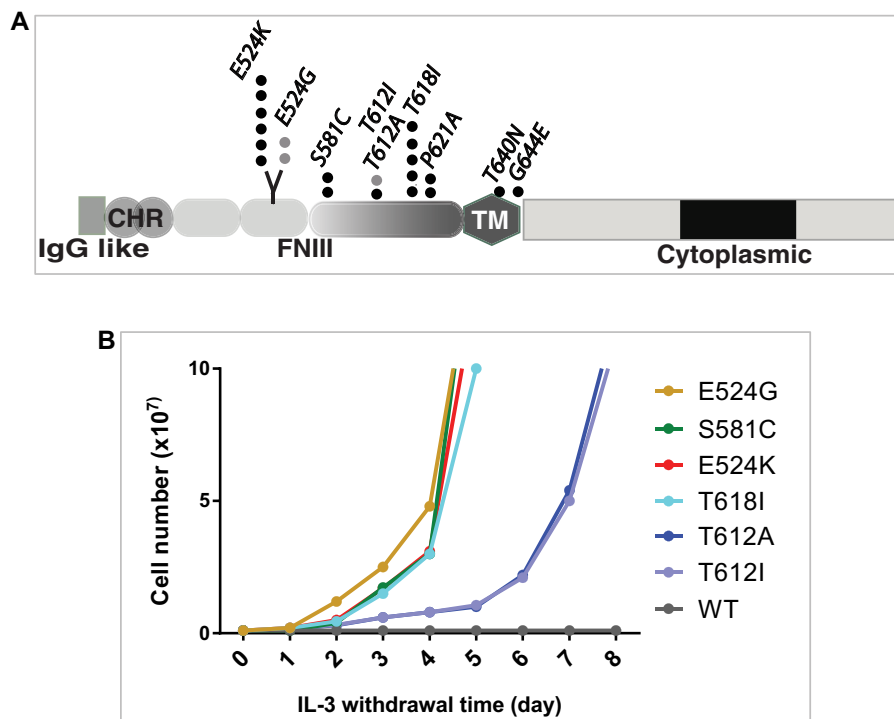J. Biol. Chem. (2018) 293(19) 7387–7396 **7387**

Figure 1. Identification of gain-of-function CSF3R mutations. *A*, schematic illustration of human CSF3R protein with domain architecture, position, and type of mutations that occurred during Ba/F3 transformation assays. *B*, representative transformation assay of Ba/F3 cells expressing CSF3R variants. *IgG like*, IgG-like domain; *CHR*, cytokine receptor homology region; *FNIII*, fibronectin-like type III domain; *TM*, transmembrane domain.

W341C was further shown to transform cells via cysteine- and disulfide bond–mediated dimer formation.

In the past, we have observed that Ba/F3 cells harboring CSF3R WT can sometimes spontaneously transform at later time points in a Ba/F3 IL-3 withdrawal assay due to the acquisition of *bona fide* oncogenic mutations in CSF3R, such as the T618I mutation (22). We therefore took advantage of this Ba/F3 spontaneous transformation model and performed sequencing of the outgrown clones to identify novel CSF3R activating mutations that would further inform us about the biology of this receptor.

Results

Identification of gain-of-function CSF3R mutations through sequencing of spontaneously transformed, CSF3R-expressing Ba/F3 cells

The CSF3R T618I mutation was previously found to induce constitutive receptor activation and transform Ba/F3 cells with fast kinetics (around 3–4 days) (17, 19, 23), whereas ectopic expression of CSF3R WT could sometimes lead to Ba/F3 transformation upon extended culture (>9 days) (22). We sequenced these transformed CSF3R WT Ba/F3 cells with primers covering most of the transgene. All of the autonomous CSF3R WT Ba/F3 clones showed an acquired single point mutation not present at detectable levels at the start of the experiment but likely selected during the growth factor withdrawal. Through this approach, we identified nine missense mutations (Fig. 1A and Fig. S1). These included two well-characterized activating mutations (T618I and T640N) (17–19, 24). Among the nine mutations identified, two (T640N and G644E) are located in the transmembrane domain. Interestingly,

T612A, T612I, and P621A are located in the same membrane-proximal region as the T618I mutation, whereas E524K, E524G, and S581C are located in the fourth and fifth fibronectin-like type III domains.

To confirm that these mutations induce cytokine-independent cell growth, we generated viral constructs expressing these mutations and performed an IL-3 withdrawal assay. E524K, E524G, S581C, T612A, T612I, and T640N transformed cells with fast kinetics. In contrast, P621A and G644E transformed cells with long latency, and further sequencing showed acquisition of E524K and T640N mutations in these transformed clones, respectively (Fig. 1B). We, therefore, focused on the molecular mechanisms of the mutations that conferred robust ligand-independent growth and did not acquire additional mutations. Having identified novel activating mutations in CSF3R, we next investigated the mechanisms by which these mutations lead to cellular transformation.

Cysteine- and disulfide bond–mediated dimerization

Previously, we have shown that mutations generating an additional cysteine in the extracellular domain of CSF3R form intramolecular disulfide bonds, leading to constitutive receptor dimerization (21). To test the possibility that the same mechanism was eliciting transforming capacity of CSF3R S581C, we first validated that S581C, but not S581R, could transform cells (Fig. 2A). Moreover, we observed markedly increased dimers of the mutant protein compared with WT under nonreducing conditions, which were abrogated on a reducing gel (Fig. 2B). In contrast, other mutations, T618I, T612A, and E524K, demonstrated only subtle dimerization under nonreducing conditions, which was similar to levels of WT dimerization in the
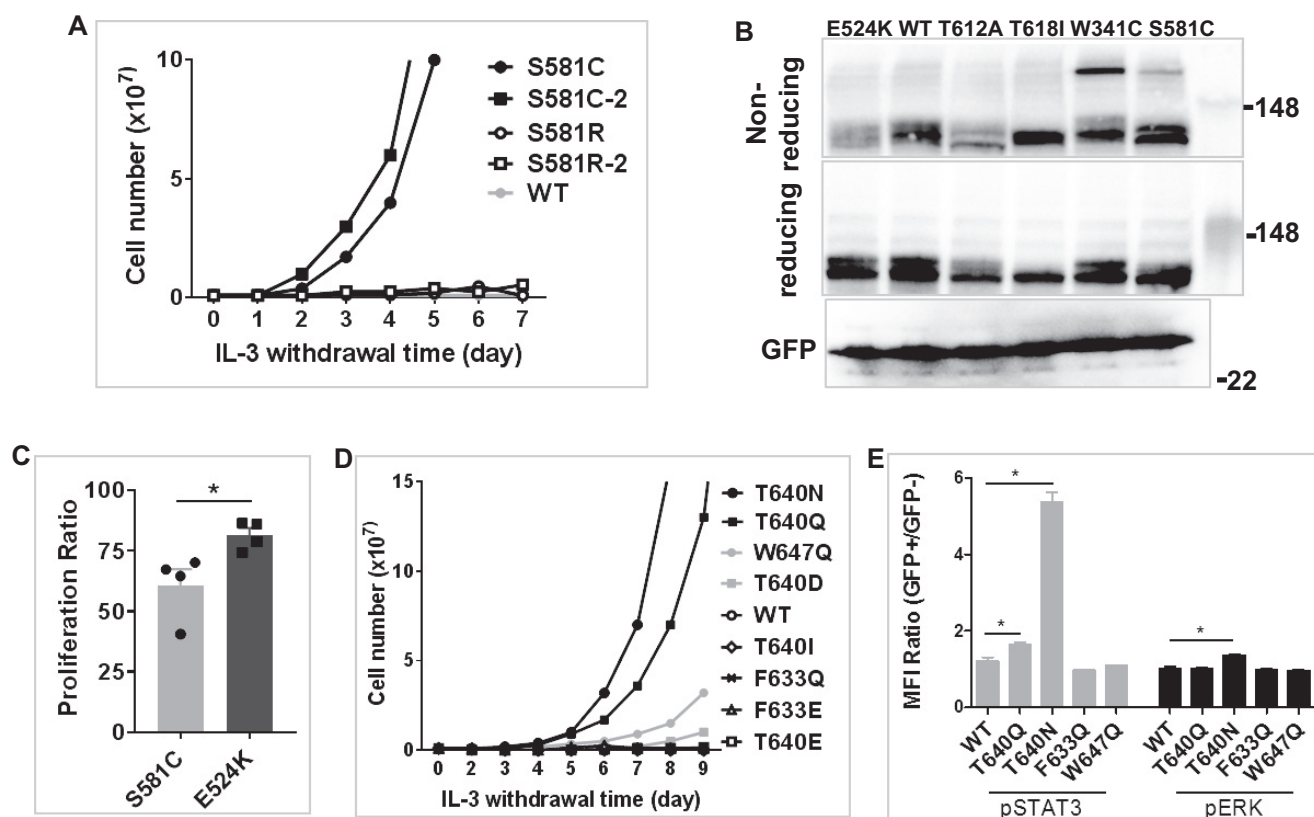
Figure 2. S581C transforms cells by cysteine- and disulfide bond–mediated dimerization. *A*, representative Ba/F3 cell transformation assay showing oncogenic potential of S581C but not S581R. *B*, CSF3R expression determined by nonreducing (*upper panel*) and reducing (*lower panel*) immunoblots in transfected HEK293T/17 cells expressing CSF3R variants. *C*, transformed Ba/F3 cells were cultured in medium with or without β-ME (0.2 mM) for 3 days. Cell number was counted. The graph depicts mean ± S.E. of proliferation ratio (cell number in medium with β-ME/cell number in medium without β-ME). *D*, polar, noncharged amino acid substitution at Thr-640 transforms cells. A representative Ba/F3 cell transformation assay showing oncogenic potential of T640N, but not T640I, F633E, and T640E, is shown. Images shown are representative of three independent experiments. *E*, the graph depicts pERK and pSTAT3 mean fluorescence intensity (*MFI*) ratios of GFP-expressing (*GFP+*) cells compared with GFP-negative (*GFP−*) cells. Statistical significance was assessed using a two-tailed nonparametric Student's *t* test (Mann-Whitney test) comparing each condition with the respective CSF3R WT and expressed as * ($p < 0.05$). *Error bars* represent S.E.

presence or absence of G-CSF stimulation (Fig. S2*A*). Consistent with these findings, we also observed enhanced growth inhibition of S581C-transformed cells *versus* E524K-transformed cells after exposure to a reducing agent, β-ME (Fig. 2*C*). Using coimmunoprecipitation, previous studies have demonstrated increased dimer formation of T618I and T640N relative to WT receptor (18, 19). These data suggest that T618I and G-CSF–stimulated CSF3R WT may form dynamic nonstable dimers, which are different from stable dimers formed by cysteine-mediated disulfide bonding.

Polar, noncharged amino acid substitution at Thr-640 transforms cells

T640N was proposed to promote dimer stabilization by forming polar hydrogen bonds between the transmembrane α helices in a pair of dimerized receptors (24). Similar mechanisms have been characterized in other receptors (thrombopoietin receptor MPL W505N (25) and CSF2RB V499E (26)). To confirm this hypothesis, we created a nonpolar substitution, isoleucine, at this position. T640I did not transform Ba/F3 cells (Fig. 2*D*), consistent with the prediction that isoleucine would not engage in intramolecular hydrogen bonding interactions with the opposing transmembrane helix.

Plo *et al.* (24) demonstrated by molecular modeling that the Thr-640 residue (annotated as Thr-617 in their study) is likely to be at this helix dimer interface. Based on their structural model, the G644E substitution that we identified in our Ba/F3 outgrowth experiments would not be predicted to be at the helix interface. In accordance, the G644E mutation did not confer transformation capacity. However, it is important to note that this glutamic acid substitution creates a negative charge, which could cause electrostatic repulsion between dimer pairs. To test whether a charged substitution at Thr-640 or hydrophilic amino acid substitution at other amino acid positions located in the dimer interface could activate the receptor, we generated additional artificial mutations at Thr-640, Phe-633, and Trp-647, which were predicted to be located at the dimer interface by the modeling study of Plo *et al.* (24). We observed that T640Q transformed Ba/F3 cells and induced ERK and STAT3 activation, whereas F633E/Q, T640D/E, and W647Q did not transform Ba/F3 cells or demonstrated robust ERK and STAT3 activation (Fig. 2*E* and Fig. S3). These data indicate that only polar, noncharged amino acid substitution at the most energetically favorable amino acid position, Thr-640, can confer cell transformation potential (Fig. S2*B*).
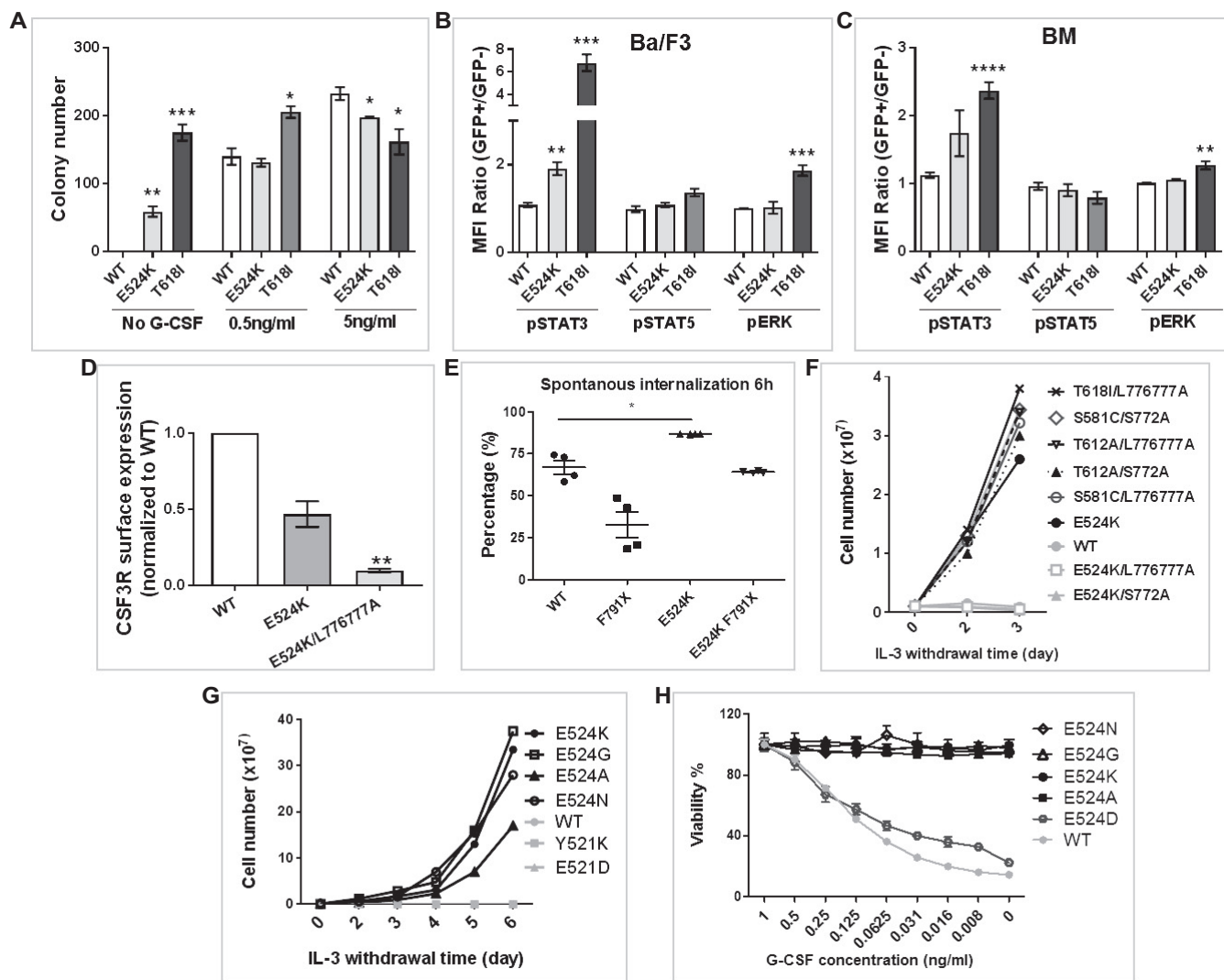
Figure 3. E524K transforms cells by increased internalization and STAT3 activation. *A*, representative images of the cfu assay demonstrate ligand-independent growth of E524K and T618I but not WT. The graphs depict ligand-independent signaling activation of E524K- and T618I-expressing Ba/F3 (*B*) and BM (*C*) cells. *D*, surface expression of CSF3R of Ba/F3 cells expressing CSF3R WT, E524K, and E524K/L776A/L777A was determined by FACS. To minimize the influence of CSF3R expression by transduction intensity, we normalized CSF3R expression by GFP expression. The graph shows -fold changes of CSF3R/GFP mean fluorescence intensity (*MFI*) ratio normalized to CSF3R WT. *E*, the graph depicts percentages of CSF3R internalization after 6-h cycloheximide treatment normalized to DMSO control. *F*, representative transformation assay of Ba/F3 cells expressing single and compound mutations. *G*, representative Ba/F3 cell transformation assay showing oncogenic potential of Glu-524 mutations. *H*, Ba/F3 cells expressing CSF3R WT and mutants were plated with a concentration gradient of G-CSF after removing IL-3, cultured for 72 h, and then subjected to 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay. A representative graph depicts percentage of cell viabilities (mean ± S.E.) normalized to the highest G-CSF concentration (1 ng/ml) for each cell line. Images shown are representative of at least three independent experiments. Data shown are mean ± S.E. of biological replicates from at least three independent experiments. Statistical significance was assessed using a two-tailed nonparametric Student's *t* test (Mann–Whitney test) comparing each condition with the respective CSF3R WT and expressed as * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). *Error bars* represent S.E.

Increased internalization and STAT3 activation transform cells

To understand the mechanism by which the E524K and E524G mutations transform cells, we first performed bone marrow (BM) cfu and signaling activation assays. Interestingly, we observed that E524K induced ligand-independent cell growth and increased STAT3 activation but did not increase STAT5 or ERK activation (Fig. 3, *A–C*, and Fig. S4*A*). In addition, E524K demonstrated reduced receptor surface expression and increased receptor internalization, although receptor degradation was not affected (Fig. 3, *D and E*, and Fig. S4*B*). To further elucidate the association of the altered receptor internalization and the cell transformation capacity, we mutated the

dileucine (Leu-776/777) and serine (Ser-772) in the internalization domain that are known to be important for receptor internalization (13). We observed that these internalization domain mutations (L776A/L777A or S772A) completely abrogated the surface expression and transformation capacity of E524K but did not influence the transformation potential of T618I, T612A, or S581C (Fig. 3, *D and F*). These data indicate that the internalization domain is important for the transforming potential of E524K. We further performed mutagenic analyses and found that E524K, E524A, E524G, and E524N, but not E524D, transformed Ba/F3 cells and induced G-CSF–independent cell growth (Fig. 3, *G and H*), indicating that amino
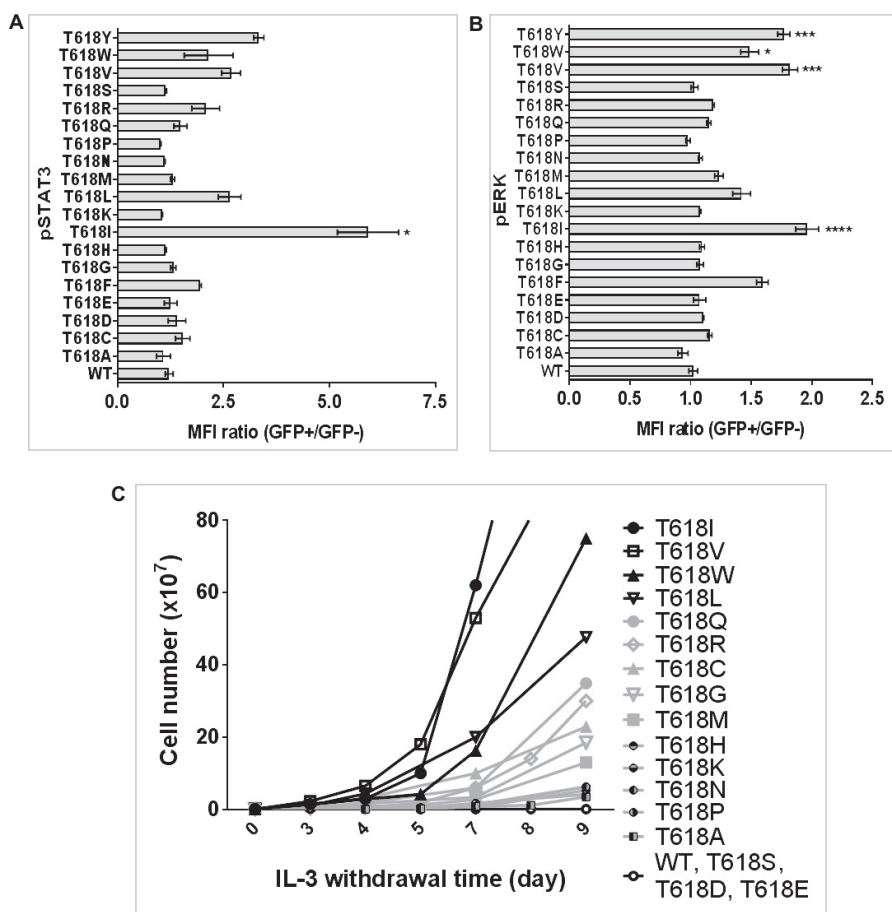
Figure 4. Increasing hydrophobicity in the membrane-proximal region confers oncogenic potential. Graphs depict pSTAT3 (*A*) and pERK (*B*) mean fluorescence intensity (*MFI*) ratios of GFP-expressing (*GFP*+) cells compared with GFP-negative (*GFP*−) control cells determined by phosphoflow. *C*, representative transformation assay of Ba/F3 cells expressing CSF3R Thr-618 mutants. Data shown are mean ± S.E. of biological replicates from at least three independent experiments. Images shown are representative of three independent experiments. Statistical significance was determined using one-way ANOVA and Kruskal–Wallis test comparing each condition with the respective CSF3R WT and expressed as *p* values (*, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$). *Error bars* represent S.E.

acids with negative charge may be important for the normal function of CSF3R surface transportation at amino acid 524. However, further structural analyses are needed to validate this hypothesis.

Increasing hydrophobicity in the membrane-proximal region confers cell oncogenic potential

The receptor activation by membrane-proximal mutations, T618I and T615A, was previously characterized to be related to altered receptor glycosylation patterns, leading to increased dimerization of the receptor (19). We identified T612A, T612I, T615A, and T618I mutations but not other amino acid substitutions at these residues in leukemia patients or spontaneously transformed Ba/F3 clones. To further understand the different amino acid preference of CSF3R membrane-proximal mutations that can confer transformation potential, we generated all amino acid substitutions of the Thr-618 residue. We observed that aliphatic and aromatic hydrophobic amino acid substitution including T618F, T618L, T618V, T618W, and T618Y cause similar increases of STAT3 and ERK phosphorylation and similar robust cell-transforming capacity as T618I (Fig. 4, *A–C*). In contrast, we observed that T615A and T612A/I/L transformed Ba/F3 with fast kinetics and induced ligand-inde-

pendent pSTAT3/pERK activation, but T612F and T615F/I/L did not (Fig. S4, *A–C*). Based on the amino acid features, we propose that increasing the hydrophobicity (especially to aliphatic and aromatic amino acids at Thr-618I and to aliphatic hydrophobic amino acids at Thr-612 and Thr-615) is required for cell-transforming capacity of CSF3R transforming membrane-proximal mutations. Further structural analysis will be helpful to understand the mechanisms associated with the amino acid preference.

Because STAT3 alone is able to transform cells, we performed mutagenesis and an inhibitor assay to investigate the significance of ERK activation. We observed that, similar to the JAK inhibitor ruxolitinib, the MEK inhibitor trametinib alone was able to inhibit the colony-forming ability of T618I, but not a control drug, imatinib (Fig. S6A). In addition, we mutated Tyr-727 and Tyr-767, which were characterized to be docking sites for STAT3 (11). We observed that T618I/Y727F/Y767F demonstrated reduced STAT3 activation, whereas the cytokine-independent growth was unchanged (Fig. S5, *B–D*), indicating that the ERK activation and the remaining STAT3 activation are sufficient for the oncogenesis of T618I. These data suggested that ERK activation is impor-
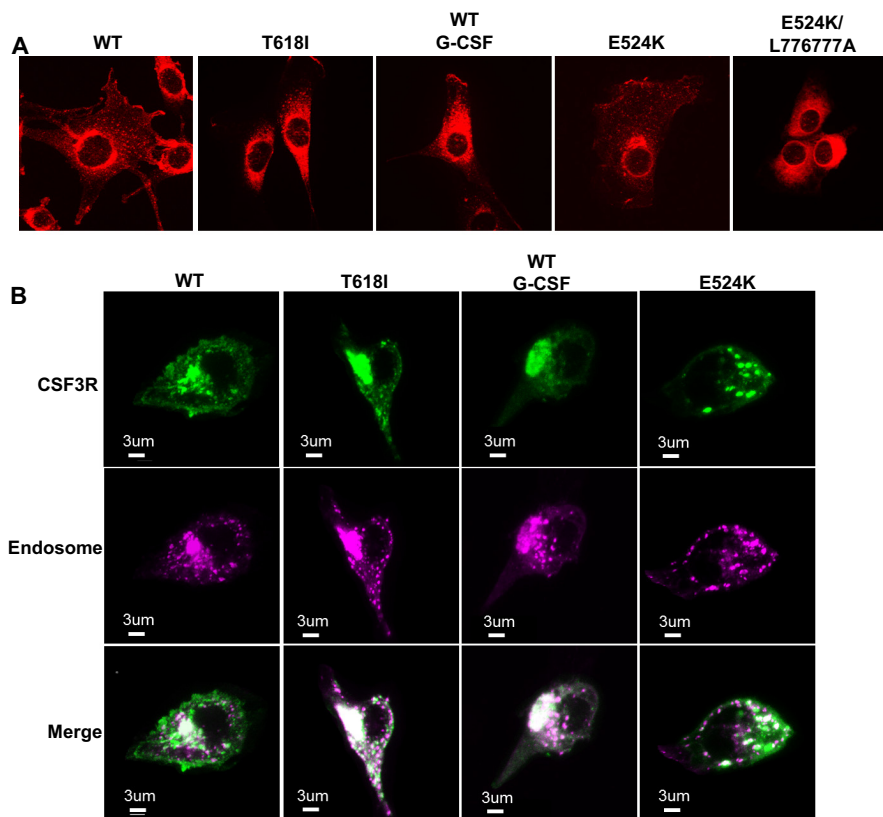
Figure 5. Differential localization of CSF3R activating mutations. *A*, images demonstrate receptor expression of CSF3R WT, T618I, E524K, and E524K/L776A/L777A and CSF3R WT stimulated with 50 ng/ml G-CSF for 30 min. *Red*, CSF3R. *B*, images demonstrate receptor expression and endosome colocalization of CSF3R WT, CSF3R T618I, CSF3R WT stimulated with 50 ng/ml G-CSF for 30 min, and CSF3R E524K. *Green* color indicates CSF3R; *magenta* indicates early endosome; *white* indicates colocalization.

tant for the oncogenesis of T618I and that adding ERK pathway inhibition might be a treatment option for patients with T618I mutation.

Differential subcellular localization of CSF3R activating mutations

We performed fluorescence immunostaining to better understand the mechanisms of differential signaling pathway activation elicited by different CSF3R mutations. In accordance with the FACS data (Fig. 3D), reduced or absent surface expression was observed with CSF3R E524K or E524K/L776A/L777A, respectively (Fig. 5A). Consistent with our previous studies, we observed significantly reduced surface expression of CSF3R T618I (Fig. 5A) (14). In addition, T618I demonstrated an altered receptor expression pattern similar to that observed with ligand-stimulated CSF3R WT. For both of these constructs, there is significant colocalization with a Rab5a-red fluorescent protein fusion construct that marks early endosomes, suggesting that the early endosome could be the location of these mutants when they are no longer surface-localized (Fig. 5B). Slightly reduced surface expression and receptor endosome colocalization were also observed with E524K (Fig. 5B). Interestingly, we observed predominantly endoplasmic reticulum accumulation of E524K/L776A/L777A (Fig. 5A and Fig. S6), which may contribute to the loss of surface expression and defective function of this mutant.

To further characterize the correlation of receptor endocytosis and signaling activation, we performed a time course G-CSF treatment of different CSF3R mutants. We observed significant reduction of STAT3 and ERK but sustained STAT5 activation with the L776A/L777A mutant, indicating that STAT3 and ERK activation require receptor endocytosis, whereas STAT5 activation is positively correlated with surface expression of the receptor (Fig. 6A). The full spectrum of the features and mechanisms of different CSF3R mutants are summarized in Fig. 6B and Table 1.

Discussion

Under normal conditions, CSF3R is activated by its specific ligand, G-CSF. Binding of G-CSF to CSF3R induces receptor dimerization, leading to transphosphorylation of the C-terminal tyrosines, which become available as docking sites for various downstream coupled mediators to form protein–protein interactions, resulting in a cascade of phosphorylation reactions including JAK/STAT, MAPK/ERK, and PI3K3/AKT (27, 28).

Different signaling pathways are mediated by different activating mutations and exert distinct functions. Under steady-state conditions, STAT3 is known to be essential for driving the G-CSF–mediated myeloid differentiation and to provide negative feedback by inducing SOCS3 activation, thereby attenuating JAK/STAT and MAPK/ERK activation (10, 27). Surpris-
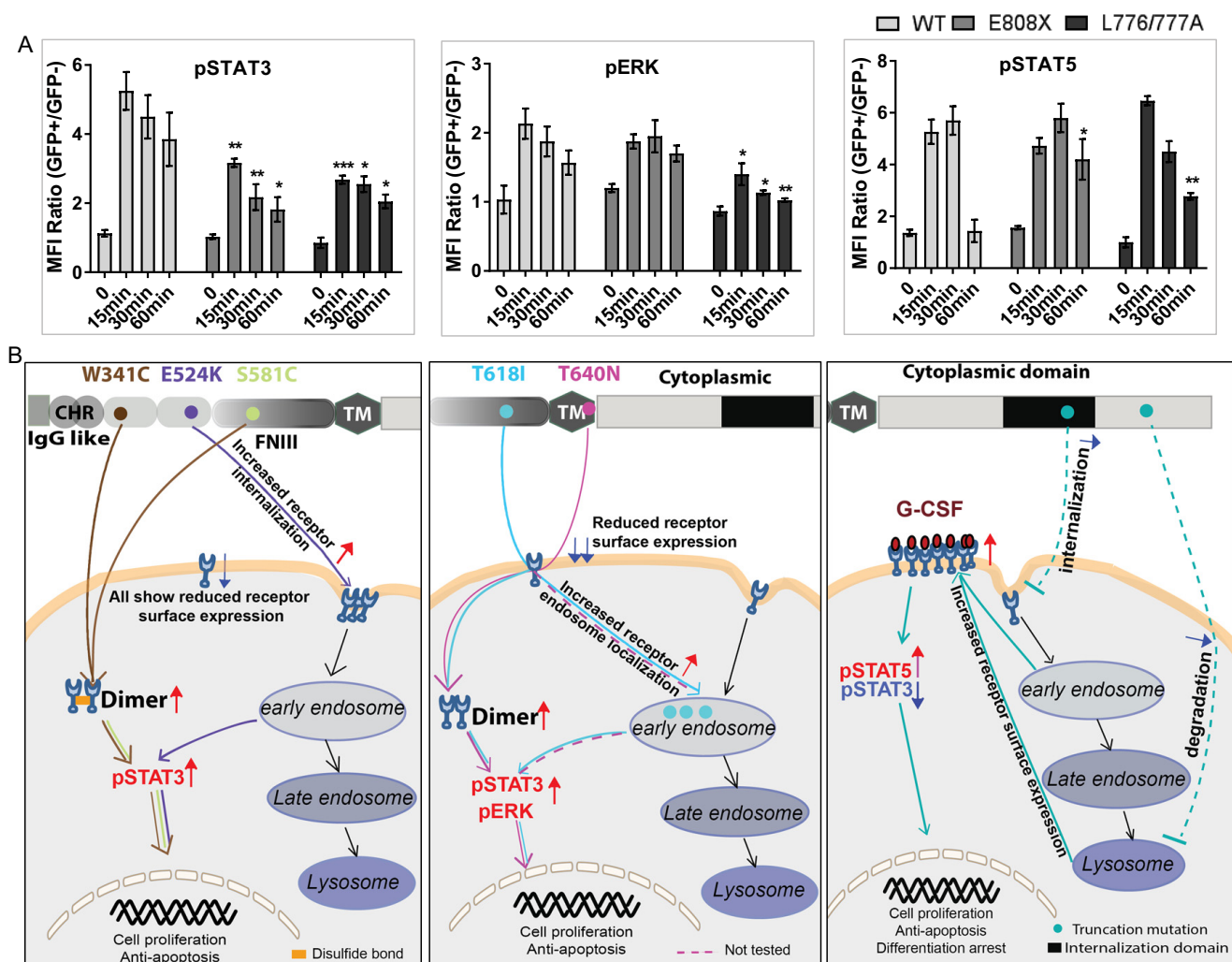
Figure 6. *A*, CSF3R-mediated STAT3 and ERK but not STAT5 activation is endocytosis-dependent. Graphs demonstrate STAT3, ERK, and STAT5 activation after a time-course G-CSF stimulation determined by phosphoflow. Data shown are mean $\pm$ S.E. of biological replicates from at least three independent experiments. Statistical significance was determined using one-way ANOVA and Kruskal–Wallis test comparing each condition with the respective CSF3R WT at the same time point and expressed as *p* values (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *Error bars* represent S.E. *MFI*, mean fluorescence intensity. *B*, distinct mechanisms of CSF3R gain-of-function mutations. The figure summarizes distinct mechanisms of CSF3R gain-of-function mutations from previous and the current studies. *Left panel*, W341C and S581C induce increased dimers via disulfide bond formation, leading to constitutive STAT3 activation and reduced receptor cell surface expression. E524K induces increased receptor internalization, resulting in reduced receptor surface expression and constitutive STAT3 activation. *Middle panel*, structural changes of T618I and T640N lead to increased receptor dimerization and endosome localization, resulting in constitutive STAT3 and ERK activation as well as significantly reduced receptor surface expression. Notably, T618I induces increased STAT5 activation in Ba/F3 cells but not BM cells. We use data from BM cells. *Right panel*, CSF3R cytoplasmic truncation mutations interrupt receptor internalization and/or degradation, leading to increased receptor surface expression and sustained G-CSF–induced STAT5 activation and reduced STAT3 activation. These gain-of-function CSF3R mutations ultimately lead to dysregulated cell proliferation/apoptosis and/or reduced cell differentiation. *IgG like*, IgG-like domain; *CHR*, cytokine receptor homology region; *TM*, transmembrane domain; *FNIII*, fibronectin-like type III domain.

Table 1

Different CSF3R gain-of-function mutations activate CSF3R through distinct mechanisms

$-$ indicates decreased. $+$ indicates increased. NK, not known; co-IP, coimmunoprecipitation.

	W341C and S581C	T618I, T615A, and T612A/I	T640N	E524K	Truncation mutation
Structure	Extracellular	Membrane proximal	Transmembrane	Extracellular	Cytoplasmic
Mechanism	Cysteine disulfide bond	Hydrophobicity, glycosylation	Increased hydrophilicity	NK	Lack of internalization or dephosphorylating domain
CSF3R expression	Decreased $-$	Decreased $-$ $-$	Decreased $-$ $-$	Decreased $-$	Increased $+$ $+$
Localization (major)	Surface/endosome[a]	Endosome $+$ $+$	Endosome $+$ $+$[a]	Surface/endosome $+$	Surface $+$ $+$
Internalization	No change[a]	No change	No change[a]	Increased	Decreased or no change
Degradation	No change	No change	No change	No change	Decreased
pSTAT3	Increased $+$	Increased $+$ $+$	Increased $+$ $+$	Increased $+$	Decreased
pERK	No change	Increased	Increased	No change	Decreased
pSTAT5	No change	No change	No change	No change	Increased
Dimer (detection method)	Disulfide bond dimer (nonreducing gel)	Increased (co-IP)	Increased (co-IP)	NK	NK

[a] No validation performed.

ingly, our data showed that STAT3 activation alone is sufficient to transform Ba/F3 cells and generate colonies in the absence of G-CSF, indicating a prosurvival role of CSF3R-mediated STAT3 activation. This is consistent with a previous study in which STAT3 was shown to be essential for the expansion of immature granulocytes in emergency granulopoiesis (29). STAT5 activation is known to promote cell survival and proliferation and contributes to the leukemogenic potential of CSF3R truncation mutations, which is G-CSF–dependent (7). Surprisingly, STAT5 is not activated in the context of T618I and E524K mutations, indicating that STAT5 activation might be G-CSF–dependent. CSF3R-mediated MAPK/ERK activation is less well-characterized compared with the JAK/STAT pathway. A previous study showed that STAT3 knockout mice demonstrated augmented cell proliferation and survival, which were attributed to increased ERK activation, indicating that MAPK/ERK activation has prosurvival functions in this context (29). In line with this finding and a recent study by Rohrabaugh *et al.* (20), our study showed that the MEK inhibitor trametinib significantly impaired T618I-driven cytokine-free colony-forming capability. Excitingly, combination of an ERK and a JAK inhibitor demonstrates a synergistic cytotoxic effect. Future clinical trials evaluating this combination in CSF3R T618I–driven disease are warranted.

Receptor localization and endocytosis are important for receptor signaling transduction. It is well-characterized that internalization of receptors, including CSF3R, from the cell surface to lysosomes results in degradation of the receptor, which can attenuate receptor signaling (10, 13). Defective receptor trafficking is attributed to the tumorigenesis of multiple oncogenic alterations such as altered ubiquitination (*e.g.* CBL mutations or cytokine receptor mutations disrupting CBL binding) (30, 31), rapid receptor recycling (*e.g.* HER2) (32), and disrupted internalization and/or degradation (*e.g.* EGFRvIII (33)). Similarly, previous studies have shown that CSF3R truncation mutations disrupt receptor internalization/degradation, leading to increased receptor surface expression and G-CSF hypersensitivity (13, 14). Surprisingly, in our study, we found that E524K induced increased receptor internalization and transformed cells. These data seem to be contradictory, however, as previous studies have shown that many receptors remain dimerized with sustained kinase activity in endosomes, including EGFR and TrkA (34–36). Increased levels of endocytic trafficking of hepatocyte growth factor receptor MET was shown to lead to cell transformation (37). In accordance, we observed in our study that mutation of the dileucine internalization domain of CSF3R, L776A/L777A, resulted in significantly reduced STAT3 and ERK activity but sustained STAT5 activation, suggesting that CSF3R-mediated STAT3 and ERK activation is internalization-dependent. In addition, T618I showed high endosome localization with concomitant constitutive STAT3 and ERK activation. Similarly, the E524K mutation demonstrated increased internalization and constitutive STAT3 activation, indicating that increased endocytosis leads to STAT3 and ERK activation.

However, how E524K enhances receptor internalization is unknown. We hypothesize that the amino acid change at 524 causes a conformation change of the receptor that mimics low levels of G-CSF stimulation and leads to increased internalization of the receptor. However, the effect is not strong enough to stimulate ERK and STAT5 activation. Further structural analyses are needed to unveil the full mechanism.

The detailed mechanisms underlying hydrophobic amino acid substitution at the membrane-proximal region of CSF3R that lead to endosome localization and cell transformation are unclear. There appear to be two possible mechanistic explanations: first that increased hydrophobicity in this region is structurally important for ligand independence and/or second that larger hydrophobic substitutions have a more disruptive effect on nearby glycosylation in this region. Unfortunately, a lack of structural information for this portion of the receptor makes accurate modeling of the structural effects of these large hydrophobic substitutions challenging. Understanding the precise relationship among these amino acid substitutions, receptor conformational changes leading to activation, and altered glycosylation will be the subject of future studies.

Distinct signaling activation of cytokine receptors has been demonstrated previously for FLT3 (38) and CSF2RB (39) gain-of-function mutations. However, a connection of the different active mutations with differential receptor localization has yet to be characterized. We identified four groups of CSF3R gain-of-function mutations, further revealing a correlation among distinct alteration of receptor structure, receptor endocytosis trafficking, and signaling activation. Collectively, these findings help shed new light on the normal biology of CSF3R as well as the mechanisms of pathogenesis of different CSF3R mutations. Similar mechanisms may also apply to the oncogenic potential of other mutated cell surface receptors, a topic that merits further evaluation.

Experimental procedures

Cell culture

Ba/F3 cells were grown in RPMI 1640 medium with 10% FBS and 15% WEHI conditioned medium. HEK293T/17 and NIH/3T3 cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS. L-Glutamine, penicillin/streptomycin, and fungizone were supplemented to all culture media. All cells were obtained from ATCC. *Mycoplasma* contamination was tested every other month to ensure that *Mycoplasma*-free cells were used in all experiments.

Retroviral vector production and transduction

CSF3R constructs were generated as described previously into a MSCV-IRES-GFP retroviral vector via the Gateway Cloning System (17) (Invitrogen) and confirmed by Sanger sequencing. Retrovirus was produced by HEK293T/17 cells and infected into Ba/F3 cells. Transduced Ba/F3 cells with equal intensity of GFP were sorted by flow cytometry (FACS Aria II, BD Biosciences).

Ba/F3 IL-3 withdrawal assay

Stably transduced Ba/F3 cells were washed three times and resuspended in cytokine-free media. The number of viable cells was determined on a Guava Personal Cell Analysis System (Millipore) every 1–2 days.

Transgene amplification and sequencing

Genomic DNA from outgrown cells was harvested using the DNeasy Blood and Tissue kit (Qiagen) and amplified using vector-specific primers (forward, 5′-CCCTTTGTACACCCTA-AGCCTCCGCC-3′; reverse, 5′-GGAAAGACCCCTAGAAT-GCTCGTCAA-3′). More information is provided in the supporting methods.

FACS analysis and phosphoflow

CSF3R surface expression was determined by staining with anti-CD114 antibody, and signaling activation was determined by phosphoflow as described previously (14, 21). More information is provided in the supporting methods.

Immunoblotting

Immunoblotting was performed as described previously (19). Detailed information is provided in the supporting methods.

cfu assay

BM cells were infected with retrovirus expressing CSF3R variants followed by FACS. Three to seven thousand cells per well were seeded into a 6-well plate with 1.1 ml of Methocult M3534 methylcellulose medium (StemCell Technologies) in the absence or presence of 0.5 and 5 ng/ml G-CSF or in the presence or absence of ruxolitinib, trametinib, and imatinib for 7–10 days. Images were taken, and colonies (>50 cells) were counted using STEMvision™ colony counting software (StemCell Technologies).

Immunofluorescence staining

Immunofluorescence staining was performed on NIH/3T3 and HEK293T/17 cells and imaged with an apotome microscope (ApoTome.2, Zeiss) and a Zeiss LSM780 confocal microscope. Detailed information is provided in the supporting methods.

Statistical analysis

GraphPad Prism 5 software was used to perform statistical analyses. The data are presented as the mean ± S.E. Statistical significance was determined using Student's two-tailed t tests or one-way ANOVA and expressed as a p value (*, $p < 0.05$).

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