Gαi2-Mediated Regulation of Microtubules Dynamics and Rac1 Activity Orchestrates Cranial Neural Crest Cell Migration in *Xenopus*

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Abstract

During cell migration, a complex process intricately influenced by chemical and mechanical cues, the actin and tubulin cytoskeletons serve as central players, dictating cell morphology, polarity, focal adhesion dynamics, and overall motility. Despite the well-established involvement of heterotrimeric G proteins in cell migration, the precise underlying mechanism remains elusive. This study delves into the intricacies of Gαi2 role in cranial neural crest cell migration, revealing its interaction with tubulin and microtubule-associated proteins like EB1 and EB3, suggesting a regulatory function in microtubule dynamics modulation. Gαi2 knockdown led to stabilized microtubules, altered cell morphology, disrupted polarity, increased Rac1-GTP concentration at the leading edge and cell-cell contacts, distorted Par3 and ζPKC localization and impaired cortical actin localization and focal adhesion disassembly. Significantly, treatment with nocodazole, a microtubule-depolymerizing agent, effectively decreased Rac1 activity and rescued cranial neural crest cell morphology, actin distribution, and overall cell migration. Our findings shed light on the intricate molecular mechanisms underlying cranial neural crest cell migration and highlight the pivotal role of Gαi2 in orchestrating microtubule dynamics through EB1 and EB3, modulating Rac1 activity during this crucial developmental process.

Introduction

Cell migration plays a crucial role throughout an organism's lifespan. During embryogenesis, it drives various processes such as germ layer formation and organogenesis. In adults, proper wound healing, immune response and tissue homeostasis rely on accurate cell migration, while abnormal cell migration is associated with severe pathologies, including cancer metastasis (Chang et al., 2013; Mayor and Etienne-Manneville, 2016; Ridley et al., 2003; Vedula et al., 2013; Trainor P.A., 2010). The process of cell migration is orchestrated by a cascade of molecular and morphological events. These events encompass cell polarization, protrusion generation, substrate adhesion, cell body translocation through contraction and release of adhesions, ultimately leading to cell detachment (Ridley et al., 2003; Abercrombie et al., 1971; Heath and Dunn, 1978; Huttenlocher et al., 1995; Lauffenburger and Horwitz, 1996). Actin microfilaments and microtubules are crucial for cell shape, cell adhesion and motility, and their remodeling is vital during these events (Akhshi et al., 2014; Garcin and Straube, 2019). The crosstalk between actin microfilaments and microtubules involves small GTPases from the Rho family and other components of the cytoskeletal networks. These factors govern cell polarity, actin polymerization and actomyosin contractility (Seetharaman and Etienne-Manneville, 2020; Garcin and Straube, 2019; Bouchet and Akhmanova, 2017; Schaks et al., 2019). Additionally, microtubules participate in trafficking and deliver molecules to the leading edge of the migrating cell, influencing mechanosensing and regulating focal adhesion (FA) dynamics, migration velocity, persistence and directionality (Etienne-Manneville, 2013; Wang et al., 2018).

The neural crest (NC) has long served as a valuable model for studying cell migration during development and has contributed to our understanding of the chemical and mechanical cues that govern this process (Barriga et al., 2018; Kelleher et al., 2006; Mayor and Theveneau, 2013; Toro-Tapia et al., 2018). The NC, originating at the neural plate border, represents a transient embryonic cell population that undergoes extensive migration to differentiate into various tissues (Szabó et al., 2018). During the pre-migratory stage, cranial NC cells establish an epithelium with distinct apico-basal polarity. Following specification, these cells undergo an epithelial-mesenchymal transition (EMT), enabling

them to acquire motility, lose their epithelial polarity and undergo a switch from the highly adhesive E-cadherin to the less adhesive N-cadherin. This transition facilitates their migration and colonization of adjacent tissues (Bronner-Fraser and Sauka-Spengler, 2008; Kuriyama and Mayor, 2008; Steventon and Mayor, 2012; Theveneau and Mayor, 2012a). In polarized cranial NC cells, Rac1 and Cdc42 exhibit activity at the leading edge, facilitating the formation of cell protrusions (Matthews et al., 2008; Ridley et al., 2011; Liu et al., 2013). Conversely, RhoA activity at the rear of the cell regulates cell contraction (Theveneau et al., 2010; Shoval and Kalcheim, 2012). Furthermore, in *Xenopus*, Par3 plays a negative regulatory role in Rac1 activity at cell-cell contacts by inhibiting Rac-GEF Trio, thereby inducing microtubules catastrophe during cranial NC cell migration (Moore et al., 2013). As previously mentioned, the interaction between actin and microtubules is mediated by small GTPases from the Rho family (Rodriguez et al., 2003).

Numerous studies have demonstrated that heterotrimeric G-protein-mediated signals are involved in cell migration, including in NC cell (Cotton and Claing, 2009; Nobes and Hall, 1995; Kjoller and Hall, 1999; Sah et al., 2000; Rohde and Heisenberg, 2007; Fuentealba et al., 2013; Toro-Tapia et al., 2018; Leal et al., 2018). Recent studies have demonstrated that Ric-8A, a Guanine Nucleotide Exchange Factor (GEF) for Gα subunits of heterotrimeric G proteins, plays a regulatory role in NC cell migration by influencing polarity properties (Leal et al., 2018) and facilitating focal adhesion formation through Gα13 subunit (Fuentealba et al., 2013; Toro-Tapia et al., 2018). Nevertheless, all the Gα family subunits from heterotrimeric G proteins have been implicated in cell migration (Cotton and Claing, 2009), and their expression has been observed in the NC region of *Xenopus* embryos (Fuentealba et al., 2016). Specifically, the Gαi/o family has been strongly associated with cell migration through chemotactic signaling in other tissues (Han et al., 2005; Hwang et al., 2007; Pero et al., 2007; Zarbock et al., 2007). Treatment with pertussis toxin (PTX), an inhibitor of Gαi/o family protein function, results in impaired chemotaxis in macrophages (Wiege et al., 2013). Gαi2 is expressed in NC cells (Fuentealba et al., 2016) and it has been implicated in the regulation of cell migration in other tissues through a GPCR-dependent mechanism (Hwang et al., 2007; Hwang et al., 2015). Additionally, studies conducted on Gαi2 knockout mice demonstrate a significant reduction in the macrophages and neutrophils recruitment to inflammatory sites (Han et al., 2005; Zarbock

et al., 2007; Wiege et al., 2012). Notably, Gαi subunit has been reported to interact with microtubules motor components such as dynein and polarity molecules, thereby influencing asymmetric cell division controlling microtubules pulling forces in a GPCR independent mechanism (Kiyomitsu T., 2019; Schaefer et al., 2001; Yu et al., 2000; Villaseca et al., 2022). Despite the established role of Gαi2 in chemotaxis through GPCR activation, limited information is available regarding its specific mechanism by which it controls cell migration (Villaseca et al., 2022). Hence the primary aim of this study was to unravel the mechanism by which Gαi2 controls cranial NC cell migration using *Xenopus* as an *ex vivo* model.

Our findings highlight the essential role of Gαi2 in microtubules dynamic to modulate the establishment of accurate polarity proteins and focal adhesion disassembly, thereby facilitating NC cell migration. Under Gαi2 knockdown conditions, we observed a decreased in microtubules dynamics and an increase in Rac1 activity. In this sense, our findings showed a functional interplay between Gαi2 with tubulin and other microtubules dynamic components, such as EB1 and EB3, possible controlling downstream effector like Rac1. Therefore, regulating actin cytoskeleton, protrusion formation and focal adhesion disassembly. Consistently, we showed that low concentrations of nocodazole reduce Rac1 activity in Gαi2 depleted cells and restore cranial NC cell morphology, actin distribution and consequently cell migration. This study offers new insights into the previously unrecognized function of Gαi2 in regulating microtubule dynamics to ensure proper polarization, cell morphology and focal adhesion dynamics during cell migration.

Results

Gαi2 is required for cranial neural crest cell migration

Since cranial NC cells express Gαi2 during migration stages (Fuentealba et al., 2016), we analyzed whether it plays any role on NC migration. To achieve this, we performed loss-of-function studies by injecting a translational inhibitory antisense morpholino oligonucleotide against to Gαi2 (Gαi2MO), in *Xenopus* embryos, followed by WISH against the NC marker *snail2*. The injection of Gαi2MO or ControlMO into one dorsal and one ventral blastomere of eight-cells stage embryo did not interfere with NC induction, as the distribution of *snail-2* expressing cells remained unaffected at stage 16 (**Fig. 1A**). To evaluate cranial NC cell migration, we performed WISH against *snail-2* and quantified the phenotype by measuring the total length of the three migratory routes (mandibular, hyoid and branchial) at stage 22-23, as depicted in (**Fig. 1F)**. The results showed severe alterations in migration, with the localization of *snail-2* positive cells affected at stage 22-23 (**Fig. 1B,C,E,G**) in 60-70% (n=49) of injected embryos, showing impaired NC cell migration. Importantly, we demonstrated that migration defects can be rescued in Gαi2MO embryos (n=31) upon co-injection with Gαi2 mRNA that does not bind the Gαi2MO, indicative that the morphant phenotype observed is specific (**Fig. 1D,G)**. Western blot analysis of Gαi2 shows that the Gαi2MO significantly reduced the levels of the protein (**Fig. 1H,I**). In addition, the injection of Gαi2MO had no effect on Epithelial-Mesenchymal Transition (EMT), as recognized by the expression of E-cadherin and N-Cadherin, similar to control NC cells (**supplementary material Fig. S1A-F**). Together, these observations show that Gαi2 is required for cranial NC cell migration *in vivo*.

To further characterize the effect of Gαi2MO, NC explants from stage 17 embryos were cultured on fibronectin, and their migratory behavior was then observed and recorded using time-lapse imaging. To quantify cell dispersion, we employed Delaunay triangulation (Carmona-Fontaine et al., 2011), revealing a severe impairment in Gαi2 morphant cells migration compared to control cells (**Fig. 1J-M**, **supplementary material movie S1**). As it is showed in (**Fig. 1J,K**), the injection of Gαi2MO resulted in a significant reduction in the

area covered by cranial NC cells after 4 hours. Taken together these results support the notion that Gαi2 controls cranial NC cell migration.

Gαi2 is required to regulate cell morphology during migration

To further explore the influence of Gαi2 during cranial NC cell migration, we conducted an analysis of the impact of Gαi2 knockdown on cell morphology. To achieve this, we used NC explants, which were injected with mRNAs encoding H2B-Cherry and membrane-GFP to label nuclei and cell membrane, respectively (**Fig. 1N,O**), and lifeactin-GFP to label actin cytoskeleton (**supplementary material Fig. S1G,H**). Cell morphology was evaluated under both control and morphant conditions, focusing on cell shape (**Fig. 1N-Q**) and protrusion dynamics (**supplementary material Fig. S1G,H)**. We found that Gαi2 depleted NC cells exhibited larger and more stable protrusions when compared with control cells, accompanied by a notable reduction in cell circularity (**Fig. 1N-R**). The quantification of the protruding and retraction area showed that cranial NC cells with reduced Gαi2 exhibited a significant increase of approximately 70% in the protruding area whereas control cells only displayed a protruding area of 30%. Conversely, the retractile area in the morphant cells decreased in comparison to the control cells, with values of 30% and 60%, respectively (**Fig. 1R,T**). The speed of protrusion growth versus the speed of retractions was also analyzed using ADAPT software as is explain in the methodology section. It was found that both the control cells and the morphant cells did not show significant differences in the protrusion growth rate. However, the morphant cells exhibited a clear tendency to be slower than the control cells, presenting a rate of 0.89 μm/min ± 0.88 for morphant cells compared to 2.06 μm/min ± 0.51 for control cells (**Fig. 1S**). Moreover, morphant cells exhibited a significant decrease in retraction speed (0.26 μm/min ± 0.13) compared with control cells (2.04 μm/min ± 0.72) (**Fig. 1U**), suggesting that Gαi2 could be influencing the cellular machinery involved in cell body retraction during migration. Therefore, these results collectively, indicate that Gαi2 is required for NC migration *in vivo* and *in vitro*, and its loss of function generates longer cell with bigger protrusions.

Gαi2 regulates microtubules dynamic during migration

To further explore the potential molecular mechanism by which Gαi2 regulates the morphology and migration of cranial NC cells, we proceeded with the analysis of microtubules cytoskeletal components. With the Gαi/o antecedents about its control on microtubules array during asymmetric cell division (Wang et al., 2005), we first analyzed microtubules cytoskeletal organization through immunofluorescence against α-tubulin. Our findings reveal that microtubules from control cells exhibit a radial arrangement extending from the centrosome towards the leading edge (**Fig. 2A,C).** Conversely, besides an elongated shape, Gαi2 morphant cells exhibit an aberrant cytoskeleton characterized by concentrated distribution of microtubules towards the leading edge **(Fig. 2B,D).** To enhance visualization, we conducted a 3D deconvolution (**Fig, 2C,D, supplementary material movie S2).** This observation is also further supported by the fluorescent intensity quantification graphs, which depict that Gαi2 morphant cells display a clear increase in the tubulin fluorescens signal toward the leading edge (**Fig. 2E,F**).

To further characterize the effect of Gαi2 on microtubules organization we injected mRNA encoding EB3-GFP, a GFP-tagged protein that binds to the plus-end of microtubules. High-resolution imaging of growing microtubules was performed using confocal microscope live-cell imaging. We conducted time-lapse tracking of EB3-GFP comets at both 3-second and 1.5-second intervals and quantified various parameters, including growth speed, growth length, comet lifetime, and pauses, using automated analysis with the PlusTipTracker software for Matlab (Applegate et al., 2011; Moore et al., 2013). Comets were classified as either slow or fast and short-lived or long-lived, determined by the mean growth velocity (11 μm/minute) and comet lifetime (23 seconds). Inhibition of Gαi2 expression reduced the proportion of fast, short-lived microtubules in favor of slow microtubules of both short and long-lived (**Fig. 2G,H,I, supplementary material movie S3**). Consequently, microtubules in Gαi2 morphant cells grow more slowly (8.52 μm/min) than those in control cells (10.53 μm/min) (**Fig. 2J**). These results indicate that microtubules originating from Gαi2 morphant cells are less dynamic. Importantly, no significant differences were observed between control cells and Gαi2 morphant cells in the pauses rate experienced by EB3-GFP labeled comets (**Fig. 2K**). On the other hand, the

percentage of microtubules catastrophes was calculated using the MTrackJ plugin in ImageJ. For this purpose, we tracked EB3-GFP comets and compared the number of microtubules undergoing catastrophes (disappearing comets) with the total number of microtubules (comets) at the leading edge and cell-cell contact. In control cells, microtubules at the cell-cell contact experienced a higher percentage of catastrophes compared to microtubules at the leading edge (**Fig. 2L**). However, upon silencing Gαi2, the percentage of catastrophes, although similar between leading edge and cell-cell contact, was significantly lower at the cell-cell contact compared to control cells (**Fig. 2L**). This suggests that Gαi2 is required to promote microtubules catastrophe during the migration of *Xenopus* cranial NC cells, which correlates with a decrease in microtubules dynamics. Therefore, suppression of Gαi2 expression leads to increased microtubules stability, resulting in reduced dynamic behavior and a decreased incidence of catastrophes when compared to the control cell population.

To continue elucidating the role of Gαi2 in regulating cranial NC cells migration through the control of microtubules distribution and dynamics, an analysis was conducted to determine which population of microtubules is concentrated in the cell. Our results revealed a significant increase in acetylated tubulin levels, indicative of more stable microtubules in Gαi2 morphant cells, whereas control cells exhibited reduced acetylated tubulin towards the protrusions at the leading edge (**Fig. 3A,B**). This observation was further validated by western blot analysis, which quantification graph display a gray value of approximately 1.5, in morphant cells compare with 0.5 in control cells (**Fig. 3C,D**). In addition, the subcellular localization of Gαi2 in relation to α-tubulin was subjected to analysis, demonstrating colocalization of both proteins within the same subcellular compartment (**Fig. 3E**). The quantification, using the Pearson coefficient, demonstrated a strong correlation with a value of 0.743, while the overlap coefficient was 0.779 (**Fig. 3F**). These results are supported through co-immunoprecipitation assays, which show that Gαi2 and tubulin are part of a common interaction complex (**Fig. 3G**) along with microtubules plus-end proteins, such as EB1 **(Fig. 3H**) and EB3 (**Fig. 3I**), which are associated with tubulin to regulate microtubule dynamics (Pérez et al., 1999; Bieling et al., 2007). Therefore, these findings suggest a functional relationship between Gαi2 and plus-end binding proteins that regulate microtubule dynamics. The significant alterations in tubulin

organization observed under Gαi2 morphant conditions and the increased presence of stable tubulin at the leading edge further support this notion.

For a more comprehensive insight into the role of Gαi2 in regulating cranial NC cells migration through the control of microtubules dynamics, we conducted a rescue experiment using a low concentration of nocodazole (65 nM) and cell dispersion *in vitro* was analyzed by Delaunay triangulation. Nocodazole is known to interfere with microtubules polymerization. Our results indicated that the treatment with this concentration of nocodazole had no significant impact on the dispersion of control cells (**Fig. 3M,N, supplementary material movie S4**). However, in the context of Gαi2 morphant cells, where we observed that Gαi2MO blocked cranial NC cells dispersion (**Fig. 3J,K,N**), treatment with 65 nM of nocodazole efficiently rescued cell dispersion (**Fig. 3L,N, supplementary material movie S4**) and additionally cell morphology (**supplementary material movie S5)**, suggesting that the loss of function of Gαi2 increases microtubules stability, phenotype that can be rescued by microtubule depolymerization using nocodazole.

All together, these findings further support the notion that Gαi2 control cranial NC cell morphology and migration regulating microtubules dynamics, probably by interacting with microtubules plusTip proteins such as EB1 and EB3.

Gαi2 controls cell polarity during cranial neural crest cell migration via microtubule dynamics

Microtubules and actin filaments interact via Rho family of GTPases (Rac1, Cdc42, RhoA), controlling cell polarity, actin polymerization, and contractility (Rodriguez et al., 2003). Microtubules play a crucial role in cell migration, affecting cellular trafficking, morphology, and polarity (Etienne-Manneville, 2013). Altered microtubule polymerization inhibits Rac1 activity, impacting lamellipodium formation (Laan et al., 2008; Waterman-Storer et al., 1999). Several studies have proposed that Gαi is involved in regulating mitotic spindle dynamics, thereby influencing asymmetric cell division and cellular polarity (Siller et al., 2009; Williams et al., 2014; Villaseca et al., 2022), which is turn is known to be regulated by the Rho family GTPases (Gotta et al., 2001; Cowan and Hyman, 2007). In this

study we found that treating Gαi2 morphant cranial NC cells with nocodazole resulted in the rescue of cell dispersion and morphology, both behaviors related to actin and microtubules cytoskeleton organization that are finely tuning by the Rho GTPases family proteins. Therefore, to delve deeper into the functional mechanism of Gαi2 in cranial NC cells migration, we aimed to determine whether Gαi2 plays a crucial role in establishing proper cellular polarization during cell migration. To achieve this, we explored whether Gαi2 regulates the subcellular distribution of active Rac1 in cranial NC explants under Gαi2 loss-of-function conditions, considering Rac1 pivotal roles in cranial NC migration and contact inhibition of locomotion (CIL) (Carmona-Fontaine et al., 2011; Moore et al., 2013; Leal et al., 2018). Hence, we employed mRNA encoding Rac1 small GTPase-based probe, enabling specific visualization of the GTP-bound states of this protein. Cranial NC explants were cultured on fibronectin, and the dynamic localization of the active GTPase was monitored using live-cell confocal microscopy. Consistent with earlier observations by Carmona-Fontaine et al. (2011), in control cranial NC cells, active Rac1 displayed prominent localization at the leading edge of migrating cells, whereas its presence was reduced at cell-cell contacts (white arrows in **Fig. 4A**). On the contrary, in comparison to the control cells, Gαi2 morphants exhibit a pronounced accumulation of active Rac1 protein in the protrusions at cell-cell contacts (white arrow in **Fig. 4B, supplementary material movie S6**). As microtubules polymerization locally activates Rac1, resulting in an increase in Rac1 activity towards the protrusions (Waterman-Storer et al., 1999), we aimed to explore whether the microtubules depolymerizing drug nocodazole could reduce the levels of active Rac1 in Gαi2 morphant cells. As demonstrated by the results in (**Fig. 4C-F)**, the administration of nocodazole (65 nM) diminished the levels of active Rac1 in Gαi2 morphant cells. Additionally, immunofluorescence assays showed a change at the localization, accompanied by reduced fluorescence intensity, of both polarity markers, Par3 and ζPKC (**Fig. 4G-J**), suggesting a severe impact on cellular polarity under diminished Gαi2 expression.

The extension of the plasma membrane to form lamellipodia is primarily driven by actin polymerization, a process mainly regulated by Rac1 (Ridley A., 2015). The concurrent inhibition of active Rac1 at the leading edge significantly impacts cellular polarity and migration (Moore et al., 2013), making any changes in the levels of these

GTPases critically influential. To further comprehend the impact of Gαi2 knockdown on Rac1 activity as we found in this work, we proceeded to investigate the potential rescue of cell migration behavior by employing a pharmacological inhibitor (NSC23766) to modulate Rac1 activity. Remarkably, our results show partial restoration in cranial NC cells dispersion following a 5-minute treatment with a low concentration of the Rac1 inhibitor (20 nM of NSC23766) (**Fig. 4K-O, supplementary material movie S7)**. The Rac1 inhibitor was administered by adding the molecule to the solution, leading to its inhibitory effect being observed both at the leading edge and the cell-cell contacts, significantly disrupting cell migration. This effect was evident in the control cells treated with the Rac1 inhibitor, as they exhibited a complete loss of migratory capacity due to reduced cell dispersion (**Fig. 4N,O, supplementary material movie S7**). This finding suggests that the observed partial rescue of migration may be attributed to the global inhibition of Rac1 throughout the cell, resulting from the method of treatment. All these findings collectively indicate that Gαi2 plays a pivotal role in the regulation of cell polarization during cranial NC cell migration, possible controlling Rac1 activity via microtubules dynamics. A decrease in Gαi2 levels leads to distinct changes in the subcellular localization of all the analyzed markers. Therefore, these alterations could be potentially be associated to disruptions in the microtubule network which, in turn, may impact the proper transportation of cell polarity proteins.

As we mention above, Rac1 activity controls protrusion formation, consequently we next analyzed actin cytoskeleton organization by immunofluorescence against F-actin. We found that in control cells cortical actin is typically localized at the leading edge establishing normal protrusions (**Fig. 4P**). On the contrary, Gαi2 morphant cells display not only enlarged protrusions, as demonstrated above, but also an aberrant cytoskeleton marked by disorganized cortical actin cables, nearly disappearing at the leading edge (**Fig. 4Q**). Therefore, considering that Gαi2 knockdown conditions showed microtubules highly concentrated towards the protrusions at the leading edge (**Fig. 2B**), and nocodazole treatment was able to rescue cell dispersion (**Fig. 3J-N**), we proceeded to evaluate whether cortical actin organization can also be rescued with nocodazole. Consistently, the treatment of Gαi2 morphant cells with 10 μM of nocodazole resulted in a partial restoration of the normal organization of cortical actin (**Fig. 4R**), supported by the fluorescence intensity

quantification graph (**Fig. 4S,T**). All together these findings suggest that Gαi2 inhibit Rac1 activity in the protrusions and probably actin turnover, via microtubules dynamic.

Gαi2 regulates focal adhesion dynamics by controlling their disassembly during cell migration via microtubules dynamic

Our ongoing investigation reveals that Gαi2 morphant cells exhibit noticeable migration defects and substantial alterations in cellular morphology, particularly manifesting a significant increase in protrusion area. Moreover, these cells display distinct changes in the organization of the actin and microtubules cytoskeletons, characterized by a concentrated distribution of microtubules towards the leading edge. Considering the well-established role of microtubules in facilitating the transport of proteins involved in diverse dynamic processes, including focal adhesion dynamics, we are prompted to explore the potential association of these abnormal morphological and cytoskeletal phenotypes with defects in focal adhesion assembly or disassembly. To assess the contribution of Gαi2 in the focal adhesion morphology and dynamic, an immunohistochemical analysis was conducted against β-integrin and phospho-paxillin (p-Pax), both key proteins involved in focal adhesion assembly. It has been reported that integrins, once in their active conformation, recruit several adapter proteins with kinase activity, including FAK, Src, and Paxillin (Parsons et al., 2010). Cells with Gαi2 loss of function exhibited an increase in the area of focal adhesions compared to control cells (**Fig. 5A-D**). Additionally, the dynamics of focal adhesions was evaluated by conducting time-lapse imaging of a GFP-tagged form of FAK expressed in explanted NC cells. Our results revealed a significantly higher stability of focal adhesion in Gαi2 morphant cells compared to control cells (**Fig. 5E-H**, **supplementary material movie S8**). These findings suggest that integrins and the adapter protein FAK comprising the focal adhesions remain localized at the cell edge for an extended duration. These results suggest that Gαi2 morphant cells exhibit a slower disassembly dynamic of focal adhesions, in contrast to control cells, which may be related to the regulatory role of Gαi2 on microtubules organization.

Therefore, to evaluate the dynamics of microtubules-dependent focal adhesion assembly and disassembly, both control and Gαi2 morphant conditions, were synchronized

using 65 nM of nocodazole to deplete the microtubules network, thereby immobilizing the adhesions on the cell membrane. Subsequently, the cells were washed to restore microtubules polymerization, and facilitate cargo transport, and fixed at various time points to establish a temporal profile of focal adhesions assembly and disassembly dynamics. Immunofluorescence staining was performed against p-Pax and α-tubulin, and the area of focal adhesions was analyzed over time. In control cells, a rapid decrease in focal adhesion area is observed within 30 seconds after the nocodazole washes (**Fig. 5I,K**). Furthermore, a drastic increase in focal adhesion area occurs at one minute (**Fig. 5K,** green bars), indicating a 30-second period for both assembly and disassembly. In contrast, Gαi2 morphant cells exhibit a reduction in focal adhesion area only after 5 minutes, which persists until 10 minutes (**Fig. 5J,K,** red bars), revealing a time frame of approximately 5 minutes for the disassembly process, significantly different from the control cells, which exhibited a brief 30-second between each process. These findings demonstrate that the focal adhesion disassembly process is significantly slower in Gαi2 morphant cells compared to control cells (**Fig. 5K**). Taken together, these data strongly support the conclusion that Gαi2 exerts a regulatory role in the disassembly dynamics of focal adhesions, by controlling microtubules dynamic, resulting in a significant impact on *in vitro* migration.

Collectively, our results provide compelling evidence that Gαi2 exerts significant control over cell morphology and cell-matrix adhesion by regulating microtubule dynamics through the involvement of the small GTPase, Rac1. This regulatory mechanism enables the coordination and crosstalk between the actin and tubulin cytoskeletons, ultimately facilitating the proper collective migration of cranial NC cells.

Discussion

For effective migration, dynamic microtubules are crucial. They act as transport routes for proteins, facilitating the delivery and recycle of membrane components and signaling molecules to the leading edge of migrating cells (Bershadsky et al., 1996; Bouchet and Akhmanova, 2017; Etienne-Manneville, 2013; Garcin and Straube, 2019). Gα subunits of heterotrimeric G proteins are key regulators of migration in various cell types

(Cotton and Claing, 2009), with Gαi subunits playing pivotal roles in immune cell migration (Hwang et al., 2007; Wiege et al., 2012; Zhong et al., 2012; Hwang et al., 2015). In this study, we demonstrate that the functional deficiency of Gαi2, a protein we previously identified as having significant expression in *Xenopus* cranial NC cells and cranial NC-derived cells (Fuentealba et al., 2016), does not impair NC induction and the transition from E-cadherin to N-cadherin expression. As a result, the primary role of Gαi2 become evident during the dynamic process of active NC migration. We made a significant finding that the Gαi2 subunit of heterotrimeric G proteins plays a pivotal role in regulating microtubule dynamics by their regulatory proteins, such as EB1 and EB3. This regulation, in turn, influences cellular polarity components, including Rac1 GTPase, and has a direct impact on actin polymerization and, consequently, the dynamics of focal adhesions (**Fig. 6**). These cellular processes are crucial in governing cell migration.

Our initial findings reveal that the functional loss of Gαi2 significantly inhibits both *in vivo* and *in vitro* migration. Interestingly, during our *in vitro* dispersion assays on fibronectin, control cells displayed radial dispersion, facilitated by the initial contact inhibition force, which allowed them to separate from the group and migrate. In contrast, Gαi2 morphant cells exhibited reduced dispersion efficiency, showing a tendency to remain within the cellular cluster. This cellular behavior in the absence of Gαi2 is consistent with the observations made by Wiege et al. in 2012, where they reported that Gαi2-/- mouse-derived macrophages lost their migratory capacity towards inflamed regions in alveolitis and peritonitis cases, indicating a compromised cellular motility. Therefore, in order to gain deeper insights into the mechanism underlying Gαi2-mediated regulation of cellular migration, we conducted an analysis of cellular morphology, with a specific focus on actin dynamics, in both the control and Gαi2 morphant conditions. As observed in our results, the Gαi2 morphant condition exhibited a statistically significant increase in protrusion area compared to control cells, whereas the retraction area was considerably reduced in the Gαi2 morphant condition. Additionally, our assessment of retraction velocity demonstrated a significant decrease in Gαi2 morphant cells, suggesting a prolonged stability of protrusions over time compared to control cells, which exhibited minimal retraction and tended to display larger protrusion areas. These findings implicate Gαi2 in potential signaling

pathways involved in the regulation of protrusion formation thereby impacting the dynamic of cell migration.

This notion is consistent with our examination of tubulin distribution that unveiled a distinctive pattern in Gαi2 morphant cells, with microtubules concentrated towards the leading edge in parallel bundles, in contrast to control cells exhibiting radial tubulin distribution from a defined centrosome. This suggests that Gαi2 loss provoke changes in the microtubule dynamics towards the leading edge, potentially interfering with cortical actin formation in this region. Gαi proteins regulate astral microtubule organization during asymmetric cell division (Yu et al., 2000; Parmentier et al., 2000; Gotta and Ahringer, 2001; Schaefer et al., 2001; Villaseca et al., 2022) probably by modulating microtubule dynamics. In their absence, microtubule catastrophes decrease *in vitro*, as Gαi inhibits assembly and activates tubulin intrinsic GTPase, enhancing microtubule dynamics by removing the GTP cap (Roychowdhury et al., 1999; Roychowdhury and Rasenick, 2008). Our specific analysis of microtubule dynamics in Gαi2 morphant cells, using the GFP-tagged microtubule plus-end tracking protein EB3, revealed a remarkable increase in microtubule stability over time. The growth rate of microtubule in these cells was slower compared to control cells, and the frequency of microtubule catastrophes was reduced. Interestingly, in addition to microtubules prominent concentration at the leading edge, they exhibit a compelling phenotype wherein EB3 appears to remain persistently associated with them, resulting in a distinctive long-bundle morphology as opposed to the characteristic comet-like appearance. Furthermore, our investigation revealed that Gαi2 forms an interaction complex with α-tubulin and plus-end-binding proteins of microtubules, such as EB1 and EB3, known to regulate microtubule dynamics (Mimori-Kiyosue et al., 2000; Akhmanova et al., 2015; Nehlig et al., 2017; Serre et al., 2019). Consistent with these results, when examining the population of microtubules primarily concentrated at the leading edge, we observed a higher percentage of acetylated microtubules in Gαi2 morphant cells compared to control cells. Acetylated microtubules are known to exhibit greater stability (Eshun-Wilson et al., 2019). Earlier investigations have demonstrated that cranial NC cells can be prompted to migrate by reducing microtubules acetylation, even in soft matrices (Marchant et al., 2022). Additionally, research has indicated that modulations in microtubule stability are essential for establishing a polarized microtubule cytoskeleton

in motile cells (Garcin and Straube, 2019). Supporting these, a recent study has proposed that the acetyltransferase MEC-17 directly promotes α-tubulin acetylation, leading to the inhibition of cell motility by increasing cell spreading area (Lee et al., 2018). Our findings align with this notion, as we observed that Gαi2 morphant cells exhibit a migration-inhibited phenotype displaying and elongated protrusion area in the presence of highly acetylated and concentrated microtubules at the leading edge. These findings strongly suggest that Gαi2 may also play a role in modulating microtubule dynamics as part of its interaction with regulatory proteins, such as EB1 and EB3.

During cell migration, microtubules extend to the periphery, and their dynamics are influenced by local factors, thereby impacting actin polymerization pathways (Akhshi et al., 2013). Microtubules and actin microfilaments work together to meet cellular demands (Etienne-Manneville et al., 2013; Dogterom et al., 2019; Garcin et al., 2019; Seetharaman et al., 2020). Microtubules participate in protein trafficking that regulates actin polymerization, including EB1 and mDia1-2, which control actin filament assembly from microtubules plus-ends (Henty-Ridilla et al., 2016). Some studies show that as microtubules grow toward the migratory edge, plus-end-binding proteins like CLIP170 enhance their stabilization (Galjart et al., 2010) and closely associate with mDia1, promoting actin polymerization from microtubules plus-ends, contributing to lamellipodia and filopodia formation (Henty-Ridilla et al., 2016). Our findings support this idea, as we observed a complete restoration of the migration *in vitro* and partial restoration of cortical actin and cell morphology in Gαi2 morphants using the microtubule-depolymerizing drug nocodazole. Disassembling the concentrated microtubules at the leading edge indicated that reduced Gαi2 expression induces significant changes in microtubule dynamics, leading to their occupation into the cortical actin domains. This, in turn, disrupts actin polymerization, which can be partially rescued with nocodazole.

GTPase Rac1 plays a vital role in actin cytoskeleton remodeling, particularly at the leading edge during cell migration (Nobes et al., 1995; Ridley et al., 2015). Therefore, the absence of Gαi2 is expected to increase active Rac1 at the leading edge, a behavior confirmed in this study and consistent with our findings of increased protrusion areas upon Gαi2 loss. Previous studies, had linked Gαi2 to CXCR4 and Elmo1/Dock180 complex suggesting a potential molecular interplay with Rac1, promoting its activation (Li et al.,

2013). Additionally, the known association of Gαi signaling with Src kinase in ovarian cancer cells activating Rac1 (Ward et al., 2015), provides insights into observed cellular morphology changes in our study. Interestingly, our analysis of the cell cortex revealed that Gαi2 loss of function results in a paradoxical phenomenon: cortical actin is diminished, despite the presence of more stable protrusions. This contradicts the expected increase in active Rac1 and consequent actin polymerization. Other studies have reported that microtubule assembly promotes Rac1 signaling at the leading edge, while microtubule depolymerization stimulates RhoA signaling through guanine nucleotide exchange factors associated with microtubule-binding proteins at the cell rear (Best et al., 1996; Garcin and Straube, 2019; Waterman-Storer et al., 1999; Bershadsky et al., 1996; Moore et al., 2013). This mechanism would contribute to establishing the antero-posterior polarity of cells, crucial for maintaining migration directionality, underscoring the significance of regulating microtubule dynamics in directed cell migration. These findings closely align with the results obtained in this investigation, demonstrating that Gαi2 loss of function reduces microtubule catastrophes and promotes tubulin stabilization, resulting in increased localization of active Rac1 at the leading edge and cell-cell contacts. This strikingly contrasts with the normal cranial NC migration phenotype, where Rac1 is suppressed at cell-cell contacts during CIL, leading to a shift in polarity towards the cell-free edge to sustain directed migration (Theveneau et al., 2010; Shoval and Kalcheim, 2012; Leal et al., 2018). In addition, our findings here, where we observed a partial rescue at the Gαi2 morphant migratory behavior using a low concentration of Rac1 inhibitor (NSC23766) strongly support this possible mechanism. Some studies in epithelial cells have associated Par3 and ζPKC with cell-cell adhesion complexes, crucial for apical-basal cell polarity, and like Gαi in asymmetric cell division (Hao et al., 2010). Par3 is also implicated in regulating microtubule dynamics and Rac1 activation through interaction with Rac-GEF Tiam1 (Chen et al., 2005). Additionally, Par3 has been found to regulate CIL in *Xenopus* cranial NC cells, promoting microtubule catastrophe and inhibiting Rac1/Trio signaling during cell collision (Moore et al., 2013). These results support our findings as Gαi2 morphant cells showed altered subcellular localization of Par3 and ζPKC, possible due to impaired microtubule dynamics, leading to a delayed delivery of these proteins to their required sites.

Collectively, all these findings suggest that Gαi2 inhibit Rac1 activity in the protrusions and probably actin turnover, via microtubules dynamic.

Cell migration relies on the detection of various signaling cues, including chemical gradients (chemotaxis), adhesion gradients (haptotaxis), and extracellular matrix (ECM) stiffness gradients (durotaxis) (Piacentino et al., 2020). Microtubules stabilize ECM receptors, called integrins, which form focal adhesions for cell to anchor to the substrate and generate traction forces (Shacks et al., 2019; Rottner et al., 2019; Dogterom et al., 2019; Kaverina et al., 1999). Microtubules also facilitate trafficking of integrins, chemokine receptors, and essential adaptor proteins to regulate focal adhesion dynamics, including Calpain-2 (Etienne-Manneville et al., 2013; Garcin et al., 2019; Dogterom et al., 2019; Seetharaman et al., 2020). It has been reported that plus-end microtubule-binding proteins, such as EB1, interact with focal adhesion adaptor proteins, including talin, focal adhesion kinase (FAK), Paxillin, and Src (Bouchet et al., 2016; Dubois et al., 2017). Interestingly, our findings demonstrate interactions between Gαi2 and EB1 revealing that the Gαi2 subunit positively regulates microtubule dynamics, a regulation likely contributing to the maintenance of focal adhesion dynamics during cell migration. FAK, activated by Src-induced phosphorylation, regulates focal adhesion size by recruiting growth factor receptor-binding protein 2 (Grb2) and dynamin for integrin internalization (Mitra et al., 2005; Ezratty et al., 2005, 2009; Wang et al., 2011). Interestingly, Gαi may impact this process, as it is known to interact with PI3K and Grb2 in response to growth factors like EGF (Cao et al., 2009; Zhang et al., 2015; Li et al., 2016; Liu et al., 2018; Nusková et al., 2021; Villaseca et al., 2022). This suggests that in the absence of Gαi, Grb2 might be unable to recruit dynamin, potentially hindering integrin internalization and reducing focal adhesion size. Therefore, investigating dynamin localization and integrin trafficking in Gαi2-depleted conditions could provide valuable insights. In our investigation, Gαi2 knockdown cells exhibited significantly reduced focal adhesion disassembly dynamics a process that dependent of microtubules dynamics. This is consistent with previous findings in human fibroblasts and endothelial cells treated with pertussis toxin (PTX), an uncoupler of Gαi subunit from its receptor (Sugiura et al., 1996; Orr et al., 2002; Orr et al., 2003). Additionally, treatment with membrane-permeable peptides blocking Gαi2 subunit function, effectively hindered thrombospondin (TSP)-mediated focal adhesion disassembly.

TSP, a matricellular protein that activates PI3K to promote the disassembly of stress fibers and focal adhesions (Orr et al., 2002), has also been studied in chicken embryo NC cells, where it was found to enhance migration speed (Tucker et al., 1999).

Finally, in our model, Gαi2 emerges as a key player in microtubule dynamics and cellular polarity. Diminished Gαi2 levels hinder efficient direction changes of new protrusions, as primary protrusions exhibit higher stability and active Rac1 levels compared to controls. Additionally, increased protrusion area, slower retraction velocity and enhanced cell-matrix adhesion further underscore Gαi2 role. Notably, treating Gαi2 knockdown cells with nocodazole, a microtubule-depolymerizing agent, and NSC73266, a Rac1 inhibitor, rescues the effects observed and in turns migration. This response is analogous to findings with Par3, a regulator of microtubules catastrophe during CIL (Moore et al., 2013), offering insights into the delicate balance between microtubule dynamics and Rac1-GTP levels for proper cell polarity during collective migration. These results position Gαi2 as a crucial master regulator, orchestrating polarity, morphology and cell-matrix adhesion dynamics by modulating microtubules dynamics through EB1 and EB3 proteins interaction and crosstalk between actin and tubulin cytoskeletons, thus ensuring effective collective migration of cranial NC cells (**Fig. 6).**

Materials and Methods

Embryo manipulating and microinjections

Xenopus tropicalis and *Xenopus laevis* embryos were obtained by *in vitro* fertilization as previously described (Maldonado-Agurto et al., 2011; Toro-Tapia et al., 2018) and staged as described in Newport and Kirscher, 1982 and Nieuwkoop and Faber, 1994. Embryos were injected at the 8-cell stage as previously described (Aybar et al., 2003). MO against *Xenopus* Gαi2 was designed by GeneTools to target the 5' UTR site of *X. tropicalis (X.t)* and *X. laevis (X.l)* transcripts (Gαi2MO: 5'-CGACACAGCCCCAGATAGTGCGT-3'). *X.t* and *X.l* embryos were injected with 20 and 35 ng of Gαi2MO, respectively. Equimolar concentrations of standard control MO (Control MO: 5'-

CCTCTTACCTCAGTTACAATTTATA-3') were used as previously described (Toro-Tapia et al., 2018). Fluorescein-dextran (Invitrogen, D1821; 3µg) or Rhodamine-dextran (Invitrogen, D1824; 5µg) were used as linage tracers. Plasmids were linearized and mRNA transcribed as described before (Harland and Weintraub, 1985). The following mRNAs were co-injected: H2B-Cherry (100 pg *X.t.*, 200 pg *X.l.*), H2B-GFP(100 pg *X.t.*, 200 pg *X.l.*), membrane-GFP (100 pg *X.t.*, 200 pg *X.l.*), EB3-GFP (100 pg *X.t.*, 200 pg *X.l.*), V5Gαi2 (100 pg *X.t.*, 200 pg *X.l.*) and Lifeact-GFP (150 pg *X.t.*, 300 pg *X.l.*). In the case of mRNA from the GTPase-based probes pGBD-GFP or rGBD-mCherry, injections were carried out the same way as described before (Leal et al., 2018).

Whole mount *in situ* hybridization

Constructs with pSP72/snail2 and pBluescript/Twist from Labonne and Bronner-Fraser, 2000 and Mayor et al., 2000 were linearized with the restriction enzymes BglII and SmaI, respectively. For antisense RNA probe synthesis, we used Sp6 RNA Polymerase (NEB) with dig-UTP as previously described (Toro-Tapia et al., 2018). Stage 16 (for induction) or stage 23-24 (for migration) fixed embryos were processed as previously described in Maldonado-Agurto et al., 2011. Probe detection was performed with alkaline phosphatase conjugated anti-DIG antibodies (Roche) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as described before (Toro-Tapia et al., 2018). Images were collected using an Olympus SZ61 stereomicroscope and a Leica DFC450 camera from the University of Concepcion.

Explants and microdissection

NC cells dissection was conducted following the protocol by Alfandari et al., 2003, and fibronectin dishes were prepared as previously described (Theveneau et al., 2010; Toro-Tapia et al., 2018) using 10 µg/ml of fibronectin (Sigma) for plastic dishes and 50 µg/ml for glass dishes. When required, nocodazole (Sigma, M1404) or NSC23766 (Rac inhibitor, Sigma, SML0952) were added in the DFA medium (53mM NaCl, 5mM Na_2CO_3, 4.5mM K-Gluconate, 32mM Na-Gluconate, 1mM $MgSO_4$, 1mM $CaCl_2$, 0.1% BSA and 50µg/ml

streptomycin) to provide final concentrations of 10 μM-65 nM and 20 nM, respectively. NC explants were imaged using high magnification time-lapse on a Leica SP8 confocal microscope from University College London in UK and Centro de Microscopía Avanzada (CMA-BioBio) from University of Concepción, Chile. For low-magnification experiments, a Leica DM5500 compound microscope was utilized.

Cell lysates and co-immunoprecipitation

Embryos from stages 23-24 were collected in lysis buffer as previously described (Toro-Tapia et al., 2018). Total protein was obtained and quantified by Bradford Method, using 25 μg for Western Blot and 0.5 mg/1 mg for co-immunoprecipitation assays. For co-immunoprecipitations, lysates were precleared with protein A/G beads (Thermo Fisher Scientific) to clean it properly. Then, they were incubated in protein A/G beads conjugated with V5 and Gαi2 antibodies, respectively. We used A/G proteins without antibody and A/G proteins with normal mouse IgG antibody as control. The immunoprecipitations were finally eluted at 37 degrees Celsius in 1% SDS for 1 hour. Proteins were separated by SDS-PAGE gels and transferred to nitrocellulose membranes. The proteins were detected using several antibodies (mentioned in the next section).

Western blotting and immunostaining

Western blots were performed as described previously (Kuriyama and Mayor, 2009) using the following antibodies: anti Gαi2 (Santa Cruz, L-5; 1:1000); anti GAPDH (Novus Biologicals, 1D4; 1:1000); anti V5-probe (Santa Cruz, sv5-pk; 1:5000); anti acetylated tubulin (Sigma, 611B1; 1:1000); anti α-tubulin (Sigma, T9026; 1:500); anti GFP (ThermoFisher, C163; 1:500); anti EB1 (Sigma, E3406; 1:100); mouse IgG HRP (Jackson ImmunoResearch Laboratories, 1:10000); rabbit IgG HRP (Jackson ImmunoResearch Laboratories, 1:10000). Blots were revealed using a Western Lightening Plus-ECL kit (PerkinElmer) and Ultracruz Autoradiography Film Blue (Santa Cruz Biotechnology). Re-blots were performed using low pH stripping solution (1% p/v SDS, 25 mM glycine-HCl). Band intensity was measured using ImageJ (NIH). Immunostaining was performed

previously described (Toro-Tapia et al., 2018) using the following antibodies: α-tubulin (Sigma, T9026; 1:100), acetylated tubulin (Sigma, 611B1; 1:100), Par3 (Santa Cruz, sc-5598; 1:20), ζ-PKC (Santa Cruz, sc-8393; 1:20), anti-phosphopaxilin (Invitrogen, pY118; 1:20), anti β-integrin (DSHB, 8C8; 1:10), anti N-cadherin (DSHB, 5D3; 1:10), rabbit IgG Alexa 488 (Invitrogen, A11034; 1:100) and mouse IgG Alexa 488 (Invitrogen, A11017; 1:200) antibodies. Hoescht (Thermo Fischer Scientific, 1:1000), Phalloidin 546 and Phalloidin 633 (Life Technologies, 1:500) were applied with the secondary antibody. The imaging was performed in a Zeiss LSM780 Confocal Microscope using 40X and 63X oil objectives (CMA-BioBio), University of Concepción.

Cell dispersion and morphology

Cell dispersion was studied by time-lapse using DMX6000 Zeiss microscope from University of Concepción equipped with a motorized stage and a Leica camera (DFL420) with a N Plan ApoChromat 20x/0.25 NA objective controlled by LAS EZ software. Frames were obtained every 3 minutes for 4 hours in *X.t* and every 5 minutes for 10 hours in *X.l*. The dispersion rate was quantified by Delaunay triangulation using a plugin for ImageJ as previously described (Carmona-Fontaine et al., 2011), comparing the final and initial average area for each condition. For cell morphology time-lapse, the images were acquired using a 40x objective in a SP8 Confocal Microscope (University College London), tracking cells injected with membrane-GFP and lifeactin-GFP. For actin dynamics, the images were acquired using a 63X objective in an inverted SP8 Confocal Microscope (CMA-BioBio from University of Concepción). The frames were collected every 20s for 30 minutes in total and processed subtracting frame per frame to measure extension/retraction area and using ADAPT plugin from ImageJ to calculate extension/retraction perimeter and extension/retraction speed (Barry et al., 2015).

Microtubule dynamics analysis

EB3-GFP images were acquired at intervals of 1.5 or 3 seconds per frame for a total duration of 5 minutes, utilizing the SP8 Confocal Microscope from CMA-BioBio at the

University of Concepción. Temperature conditions were regulated using an incubator chamber, set to 28 degrees Celsius for *X.t.* and 23 degrees Celsius for *X.l.* The recorded movies were analyzed automatically through plusTipTracker (Applegate et al., 2011), as previously described (Moore et al., 2013). The following parameters were applied: 8 frames as the maximum gap length, 3 frames as the minimum track length, 5-25 pixels as the search radius range, 35 as the maximum forward angle, 10 as the maximum backward angle, 1.5 as the maximum shrinkage factor and a 2-pixel fluctuation radius. For MtrackJ analysis, 5x5 µm squares were selected at the front and back of a cell. The total number of EB3-GFP +tips that disappeared within the square during a 2 minute period was compared with the number of EB3-GFP +tips entered to the square. Microtubules present within the square in the final frame were excluded from the analysis.

Statistical analysis

The normality of data sets was assessed using Kolmogorov-Smirnov's test, D'Agostino-Pearson test, and Shapiro-Wilks tests in Prism6 (GraphPad). For data sets that exhibited a normal distribution, we employed the T-student test (two-tail) or one-way ANOVA for comparison. In cases where the data sets did not follow a normal distribution, such as in cell dispersion assays, we utilized Mann-Whitney's test or nonparametric ANOVA (Kruskal-Wallis) in Prism6. Cross-comparisons were conducted only if the overall P-value of the ANOVA was less than 0.1.

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Figure Legends

Figure 1. Gαi2 is required for NC migration *in vivo* and *in vitro*, and knockdown generates longer cells with bigger protrusions.

A-D. *In situ* hybridization against *snail2* on *Xenopus* embryos at stage 16 to analyze *in vivo* NC induction n = 16 (A), and at stages 22-23 to study *in vivo* NC migration (B-D). n control = 49, n morphant = 49, n rescue = 31. nis: non-injected side, is: injected side.

E. Percentage of migratory/non-migratory phenotype showing a dramatic impairment of NC cell migration in Gαi2 knockdown condition.

F. Schematic representation of *Xenopus* embryos showing the cranial NC streams in purple, illustrating that the migration length was measured at the midpoint to the full width of the three cranial NC streams.

G. Quantification of the migration length in B, C and D. Each point is the mean value of a total of 6 independent experiments (n= 3 embryos from each condition in each experiment). Error bars are ± s.e.m. **P≤0.01, ****P≤0.0001 (two-tailed Student's t-test)

H-I. Western blot of lysates from *Xenopus* embryos injected with Gαi2 morpholino shows efficiently Gαi2 knockdown.

J-K. Explants of cranial NC cells were extracted from control embryos and embryos injected with Gαi2 morpholino, and migration was evaluated under the different conditions during 4 hours by time-lapse.

L-M. Delaunay triangulation was performed to quantify cell dispersion. The average area from total dispersion after 4 hours was plotted. Error bar: s.e.m., ****P≤0.0001, n = 13 explants (Kruskal-Wallis non-parametric test). 10X magnification.

N-O. Embryos were injected with membrane-GFP to analyze cell morphology.

P-Q. Circularity and protruding area parameters were measured in 3 random cells for each experiment (n = 3 experiments per condition). Error bars correspond to ± s.e.m. **P≤0.01; ***P≤0.001 (T independent test).

R-U. The percentage of protruding area, the percentage of retractable area, the protrusion growth rate and retraction rate were quantified using the ADAPT software from ImageJ. Error bars correspond to ± s.e.m. **P≤0.086; ***P≤0.001; ns: non-significant. T independent test.

Figure 2. Gαi2 knockdown increases microtubules stability.

A-B. Immunofluorescence against α-tubulin. Pseudocolor scale shows that Gαi2MO increases microtubules distribution towards the leading edge. Magnification: 63X.

C-D. Tubulin 3D reconstruction in control and Gαi2 knockdown conditions.

E. Quantification of tubulin fluorescence intensity. Gαi2 dramatically increased tubulin signal towards the leading edge. Error bars: s.e.m. *** $P \leq 0.001$. n= 3 cells were analyzed per condition for each of three independent experiments.

F. Schematic representation of the tubulin distribution measurements from the edge of the nucleus to the cell cortex. T: tubulin.

G-H. Images were obtained every 1.5 seconds for a total of 5 minutes using Leica SP8 confocal microscopy. Videos of EB3GFP-labeled cells were cropped to a region of interest, and they were analyzed using plusTipTracker software to detect microtubule growing tips via +TIP tracking proteins (Applegate et al., 2011). Magnification: 63X.

I. Cells were separated into four groups based on growth rate (slow < 11 µm/min < fast) and duration (short < 23 sec < long). Cells injected with Gαi2MO present a higher percentage of slow microtubules, and a lower percentage of fast microtubules compared to control cells.

J. The average growth rate of Gαi2 morphant microtubules is 8.52 µm/min ± 0.301 and is significantly lower compared to control cells, whose growth rate is on average 10.53 µm/min ± 0.297. ANOVA statistical analysis for multiple conditions and Student's T-Test (two-tailed) were performed. N = 3 experiments per condition.

K. The quantification of the arrests (pauses) of the comets did not show significant differences between control cells and morphant cells for Gαi2.

L. Using TrackMate plugin of ImageJ, the trajectory of comets was tracked in time, specifically those that enter or leave a pre-established region of interest at the cell-cell contact and at the leading edge. At the end of the 2.5 minutes, a relationship was made between the comets that remain in the area versus the total comets that entered the area. Control cells show a 53.8% of catastrophes at the leading edge and 75.1% at cell-cell contact. On the other hand, Gαi2 morphant cells show 42.9% of catastrophes at the leading edge versus only 47.3% at the cell-cell contact.

Figure 3. Gαi2 knockdown increases microtubules stability, phenotype rescued with nocodazole.

A-B. Immunofluorescence against acetylated tubulin (green) and actin (red) in control and Gαi2 morphant explants to localize stable microtubules. Under Gαi2 knockdown conditions acetylated tubulin increases towards the leading edge.

C-D. Western blot performed to measure the quantity of acetylated tubulin under control and Gαi2 knockdown conditions, showing an increase in the acetylated tubulin amount in morphant conditions.

E. Immunofluorescence against endogenous Gαi2 and α-tubulin proteins to study subcellular localization.

F. Protein colocalization was analyzed using Image's JACoP software whose Pearson coefficient was 0.743, while the overlap coefficient was 0.779.

G. Coimmunoprecipitation assays show that Gαi2 is interacting with α-tubulin. normal IgG: non-related IgG; IPP V5: immunoprecipitation conjugated to V5 antibody; input: total lysate. n total = 3 lysates per condition.

H-I. Coimmunoprecipitation assays show that Gαi2 interacts with EB1 and EB3, plusTip proteins that regulate microtubules dynamic. W/A: Without antibody; normal IgG: non-related IgG; IPP V5: immunoprecipitation conjugated to V5 antibody; input: total lysate; elu: eluted. n total = 3 lysates per condition.

J-M. Cranial NC explants were extracted from stage 17 *Xenopus* embryos, previously injected with Gαi2MO. The cell dispersion in each condition was evaluated during 10 hours through timelapse. All embryos were co-injected with H2B-Cherry and membrane GFP for individual cell monitoring and quantification of migration. Four conditions were evaluated: Control, Gαi2MO, Gαi2MO + 65 nM Nocodazole and Control + 65 nM Nocodazole. Under each representative image, Delaunay triangulation is shown in each condition at the final time (10 hours).

N. The rate between the average areas corresponding to the final time were plotted at 10 hours. Nocodazole can rescue Gαi2 morphant phenotype and does not affect control migration. Significance was assessed with a Mann-Whitney test for non-parametric data (****, $p<0.0001$). Error bars: s.e.m. 10X magnification. Control n = 21 explants; Gαi2MO

n = 24 explants; Gαi2MO + 65 nM Nocodazole n = 36 explants; Control + 65 nM Nocodazole n = 30 explants; 3 experiments.

Figure 4. Gαi2 inhibits Rac1 activity in the protrusions and actin turnover via microtubules dynamic.

A-B. Pseudocolor scale for visualization of changes in the active Rac1 localization via pGBD-GFP probe. This probe contains the Rac1 binding domain of the effector protein PAK1 fused to GFP, which makes it possible to observe the localization of active Rac1 over time. In control cells active Rac1 is located towards the leading edge and its presence is reduced at the cell-cell contact (white arrow). In Gαi2 morphant cells active Rac1 is localized throughout the cell, and highly concentrated in the cell protrusion at the cell-cell contact (white arrow).

C-E. Gαi2 morphant cells treated with nocodazole show a decrease in the levels of active Rac1.

F. Quantification of active Rac1 fluorescence intensity in time. The significance was evaluated with a Mann-Whitney test for non-parametric data (***, $p<0.001$; *, $p<0.1$). Error bars: s.e.m. Magnification: 40X. (experimental N = 3).

G-H. Immunofluorescence against Par3 (green). Red: phalloidin, blue: Hoechst. Gαi2MO delocalizes Par3 from cell-cell contact.

I-J. Immunofluorescence against ζ-PKC (green). Red: phalloidin, blue: Hoechst. Gαi2MO delocalizes ζ-PKC from cell cortex.

K-N. Cranial NC explants were cultured into fibronectin and cell dispersion was evaluated during 4 hours through timelapse. All embryos were co-injected with H2B-Cherry and membrane GFP to monitor individual cells and quantify cell dispersion. Four conditions were evaluated: Control, Gαi2MO, Gαi2MO + 20 nM NSC23766 and Control + 20 nM NSC23766. Delaunay triangulation of a representative explant of each condition at the final time (4 hours) is show below every explant.

O. The radius between the average areas corresponding to the final time were plotted at 4 hours to evaluate cell dispersion. Significance was assessed with a Mann-Whitney test for non-parametric data (****, $p<0.0001$). Error bars: s.e.m. 10X magnification. Control n = 15

explants; Gαi2MO n = 18 explants; Gαi2 Mo + 20 nM NSC23766 = 14 explants; Control + 20 nM NSC23766 = 12 explants; 3 trials.

P-R. Actin was stained with phalloidin to visualize cortical actin in Control, Gαi2 morphant condition and Gαi2 knockdown condition under 65 nM nocodazole treatment.

S. Quantification of actin fluorescence intensity showing that Gαi2 MO severely affects cortical actin distribution. Error Bars: s.e.m. *** P≤0.001, * P≤0.1. n= 3 cells were analyzed per condition for 3 each independent experiments.

T. Schematic representation of the measurements for actin distribution specifically from the edge of the nucleus to the cell cortex. A: actin.

Figure 5. Gαi2 morpholino inhibits focal adhesion turnover via microtubules stability.

A-B. Immunostaining against β1-integrin in Control and Gαi2 morphant conditions to detect focal adhesions.

C-D. Quantification of the area and number of focal adhesions. Statistical analysis using t-student (**, p<0.01; ns: non-significative; n explants = 43).

E-F. Images obtained by SP8 confocal microscopy timelapse following FAK-GFP localization in control and Gαi2 morphant conditions. Magnification: 63X.

G-H. Quantifications of changes in the focal adhesion area during time and focal adhesion stability. Focal adhesion from Gαi2 morphant cells are more stable over time, compared to control cells. Statistical analysis using t-student (**, p<0.01; ****, p<0.0001; n explants = 11).

I-J. Immunofluorescence performed to detect endogenous p-Pax (red) and endogenous α-tubulin (green) in control and Gαi2 knockdown conditions, under nocodazole treatment (10 µM).

K. Normalized graph from Gαi2 knockdown condition shows an increase in the focal adhesion area, decreasing the disassembly dynamic of focal adhesion after nocodazole treatment. We can observe that control focal adhesions disassembly starts at 0.5 minutes, however Gαi2 morphant explants show a delay in focal adhesion disassembly, which start 5 minutes after nocodazole wash out. Error bars: s.e.m., * p < 0.1; ** p> 0.01; **** p < 0.0001. n explants = 27, 3 experimental replicates. Magnification: 63X

Figure 6. Gαi2 controls cranial NC migration by regulating microtubules dynamics.
A. In the control condition, Gαi2-expressing cells maintain dynamic microtubules by the Gαi2 binding to tubulin and EB proteins. These dynamic microtubule networks promote cortical actin formation in the leading cells, facilitating rapid focal adhesion disassembly and enabling a change in migration direction controlling Rac1 activity at the leading edge.
B. Conversely, in Gαi2 knockdown conditions, cells have reduced microtubules dynamics, a decreased catastrophe rate, stable and larger focal adhesions, and slower and more stable microtubules wherein EB3 appears to remain persistently associated with them. Stable microtubules lead to enhanced Rac1 activation at the leading edge, interfering with cortical actin formation, and inhibiting collective migration.

Supplementary material

Supplementary Figure S1. Gαi2MO does not affect cadherin switching during EMT.
A-F. Immunofluorescence assays were performed in cranial NC cell explants using anti-E-cadherin (E-cadh: green, A-C) and anti-N-cadherin (N-cadh: green, D-F) to detect the cadherin switching. E-cadherin is localized at cell-cell contact on pre-migratory cell explants (A) and N-cadherin is localized at cell-cell contact on migratory explants from Control MO (E) and Gαi2 knockdown conditions (F). Phalloidin is showed in red and nuclei were stained with Hoechst (blue).
G-H. Example of protrusion and retraction dynamics measurements using ADAPT plugin for ImageJ. The area of interest was segmented. Protrusion dynamics are showed in green and retraction dynamics are showed in red.

Supplementary Movie S1. Gαi2 is required for cranial NC cell migration *in vitro* in *Xenopus.*

Supplementary Movie S2. 3D reconstruction of microtubules cytoskeleton in control and Gαi2 knockdown conditions.

Supplementary Movie S3. Gαi2 morpholino disrupt microtubule dynamics.

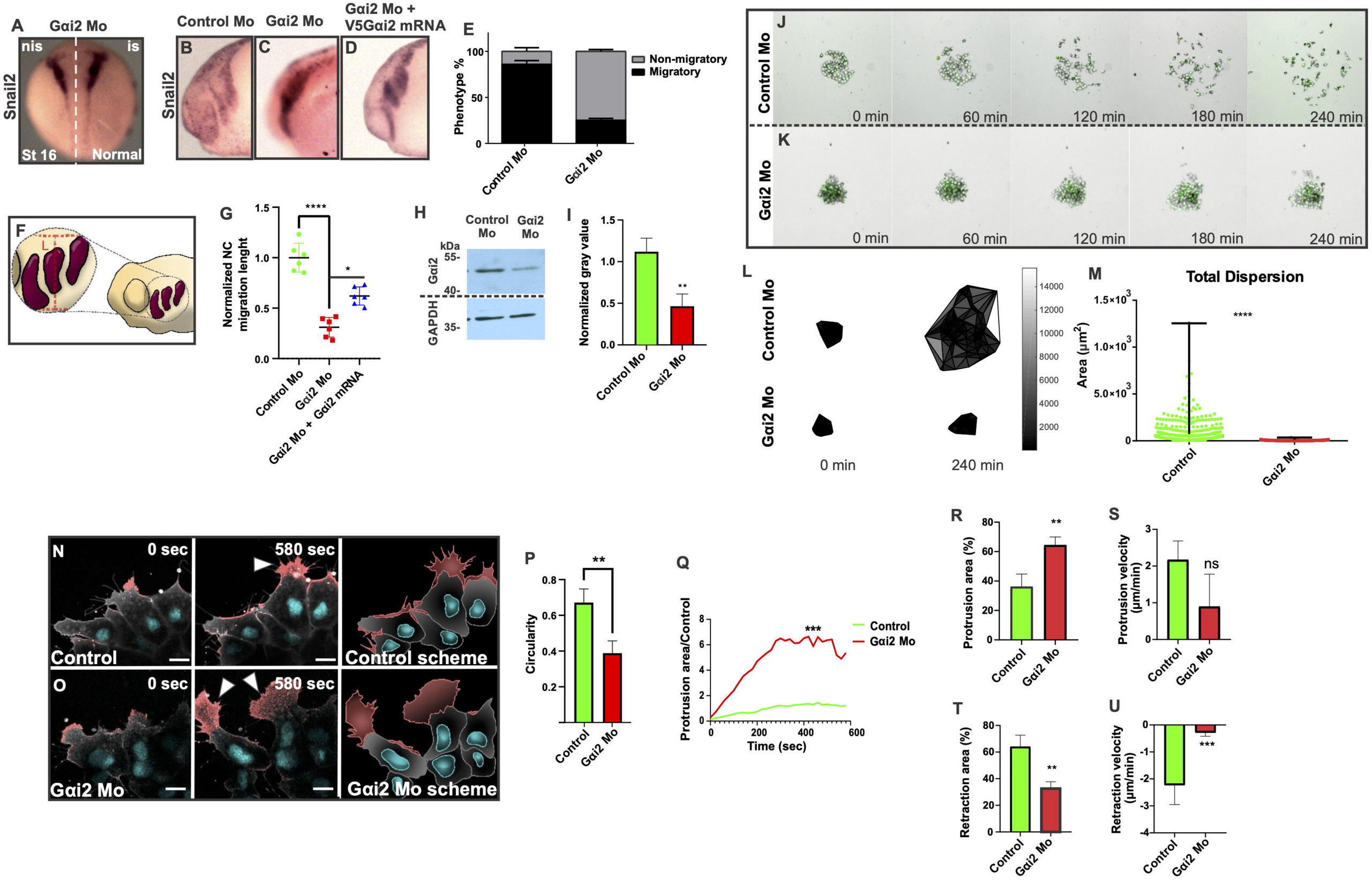
Supplementary Movie S4. Nocodazole restore *in vitro* cranial NC cell migration in Gαi2 knockdown conditions.

Supplementary Movie S5. Gαi2MO affects cell morphology and nocodazole can restore cell morphology under morphant conditions.

Supplementary Movie S6. Gαi2 knockdown condition affects active Rac1 localization during CIL in cranial NC cells.

Supplementary Movie S7. Rac1 inhibitor partially restore cranial NC cell migration in Gαi2 knockdown conditions.

Supplementary Movie S8. Gαi2 morphant cells show an increase in the focal adhesion stability.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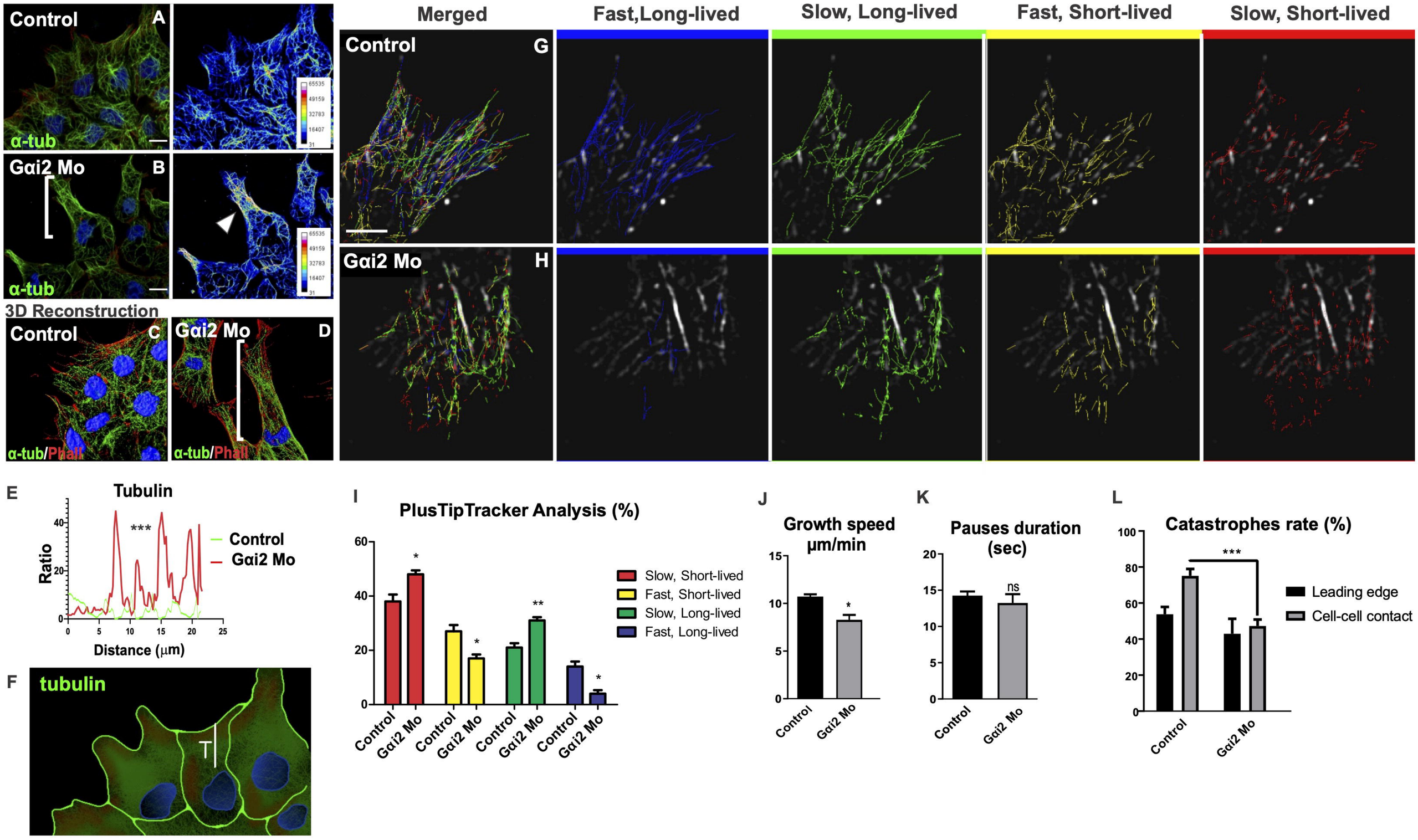
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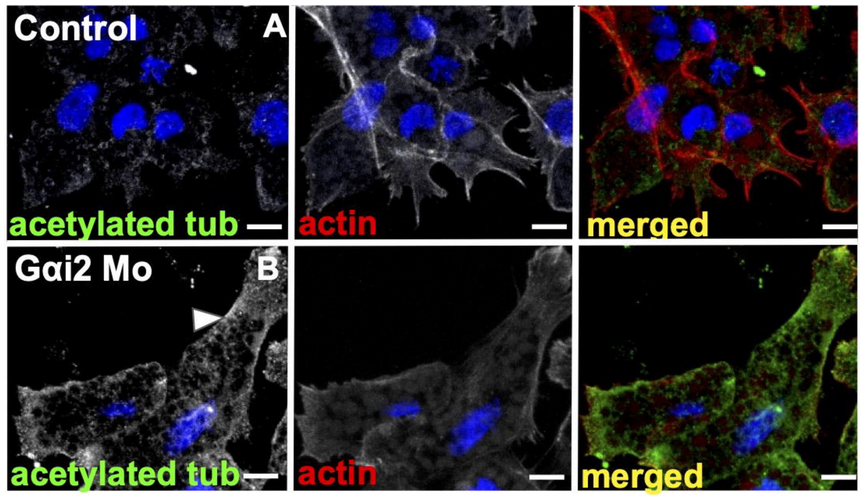
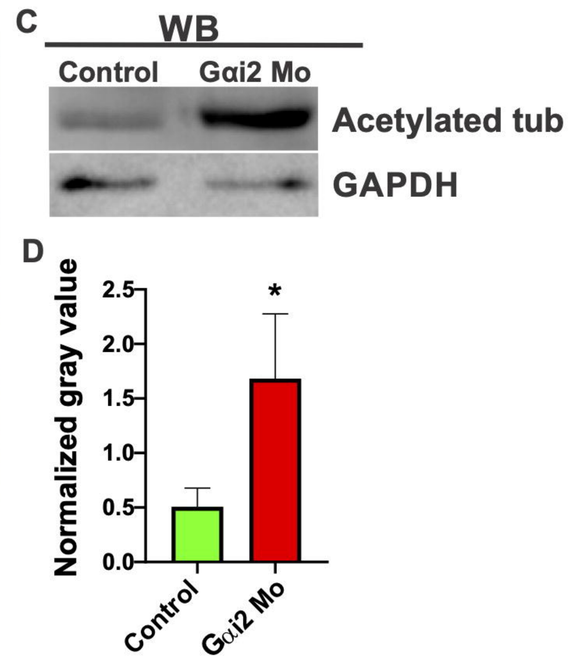
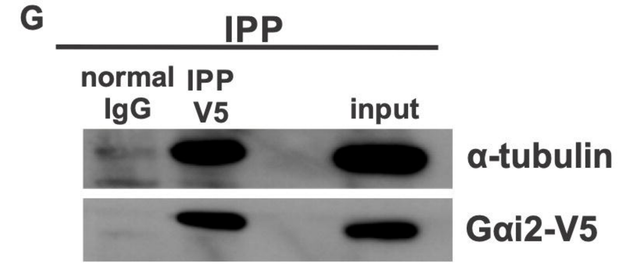
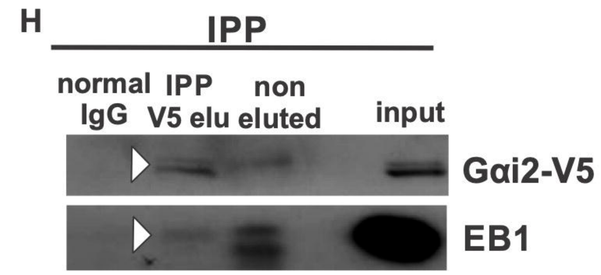
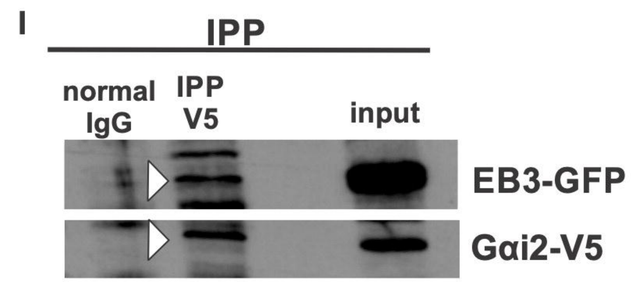
Leading edge
Cell-cell contact

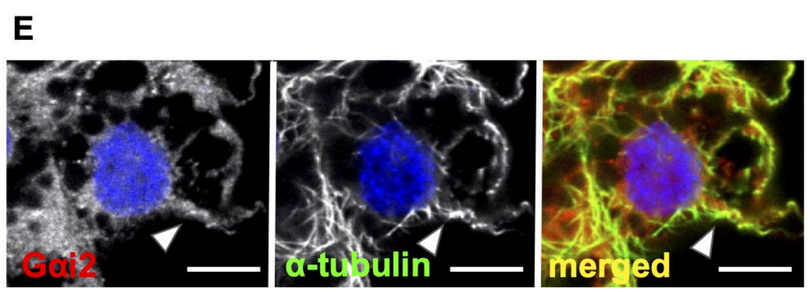




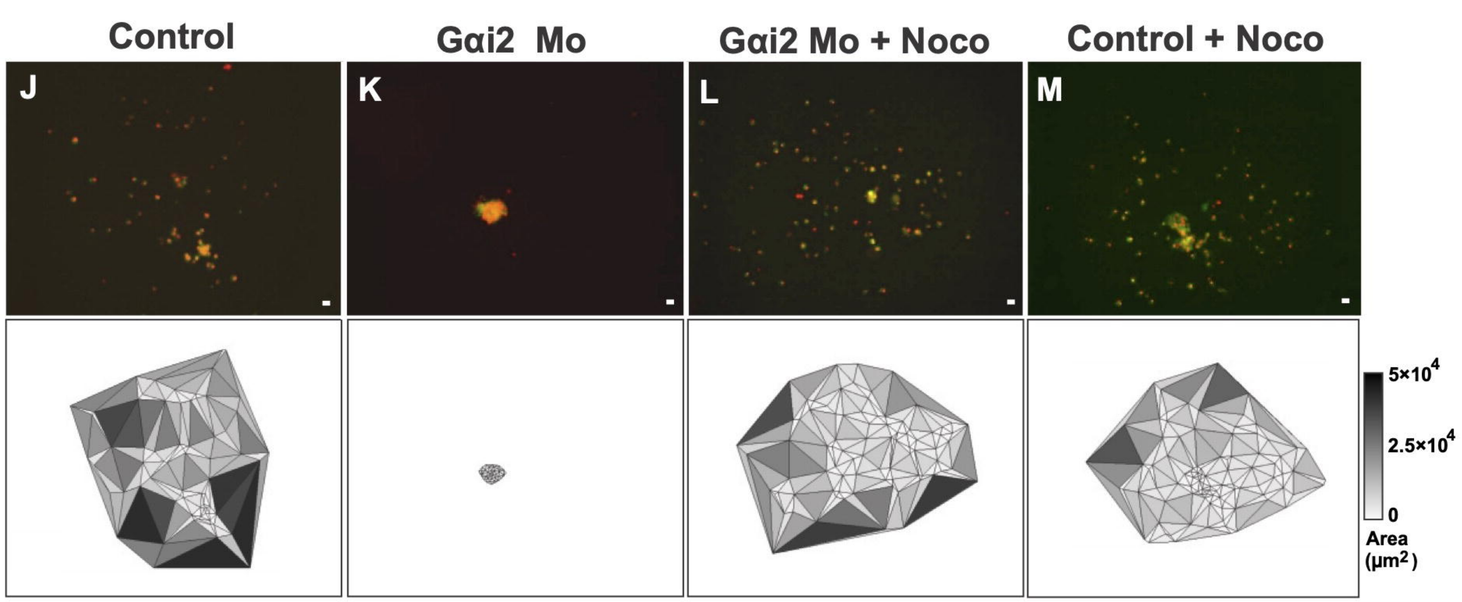


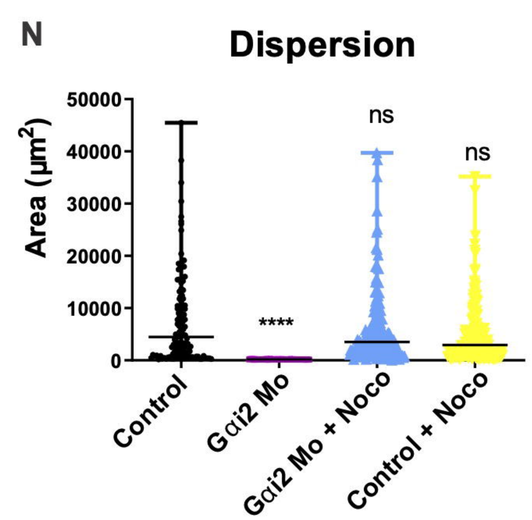


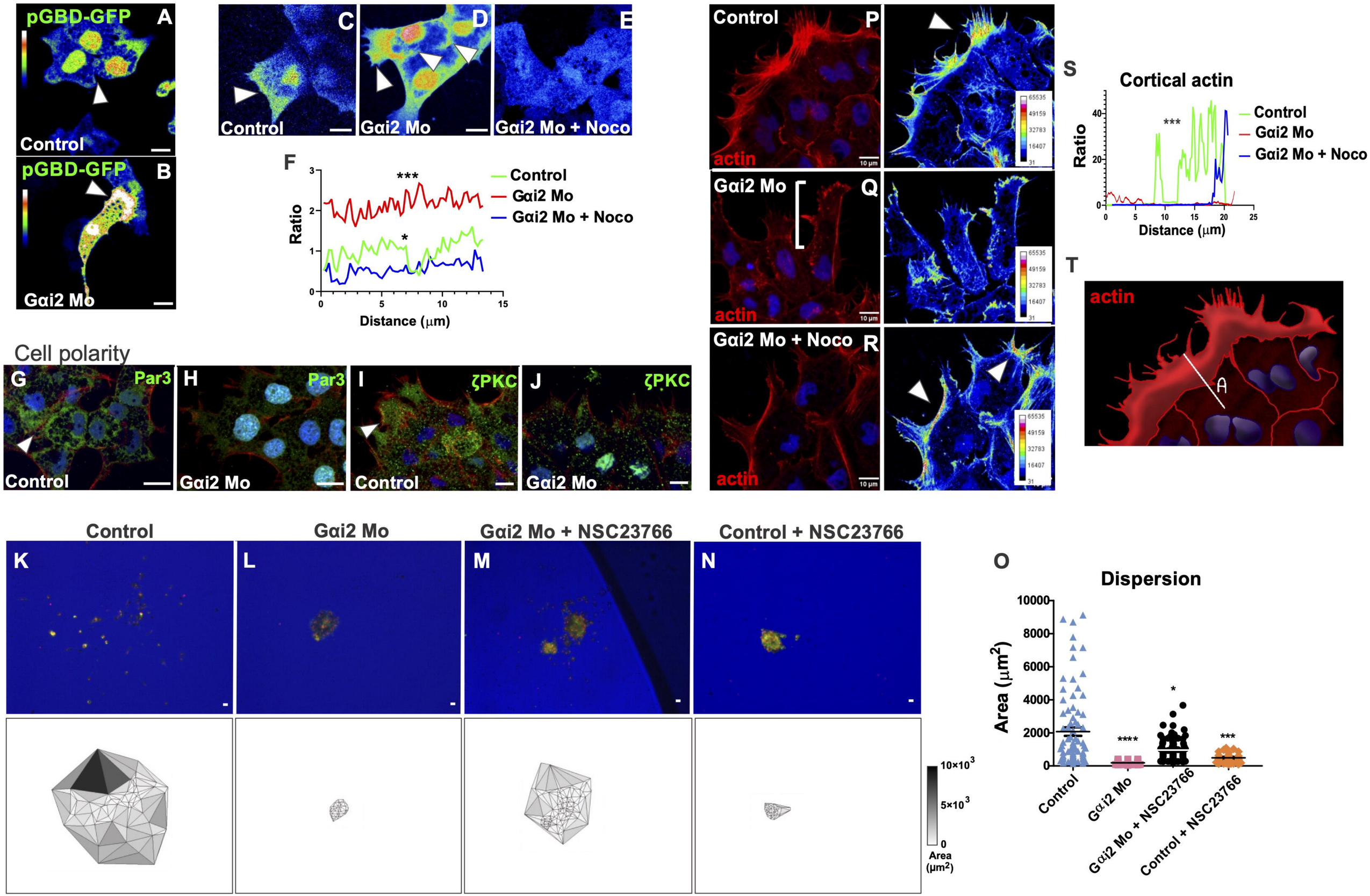


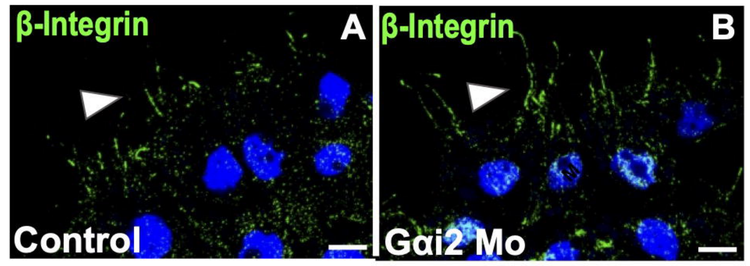


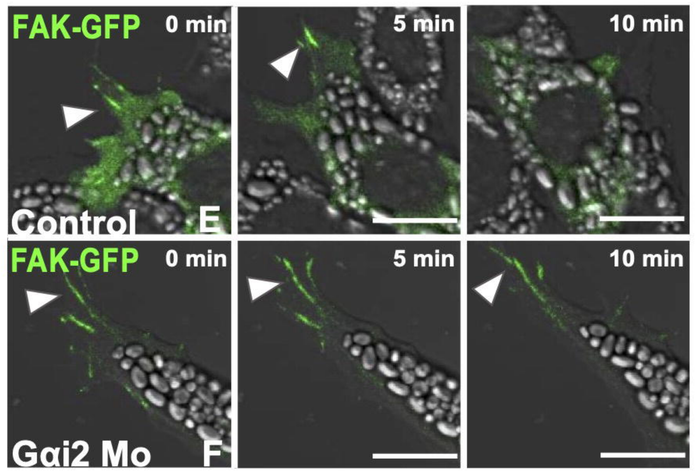
F	Cell cortex
Pearson	0.743
Overlap	0.779

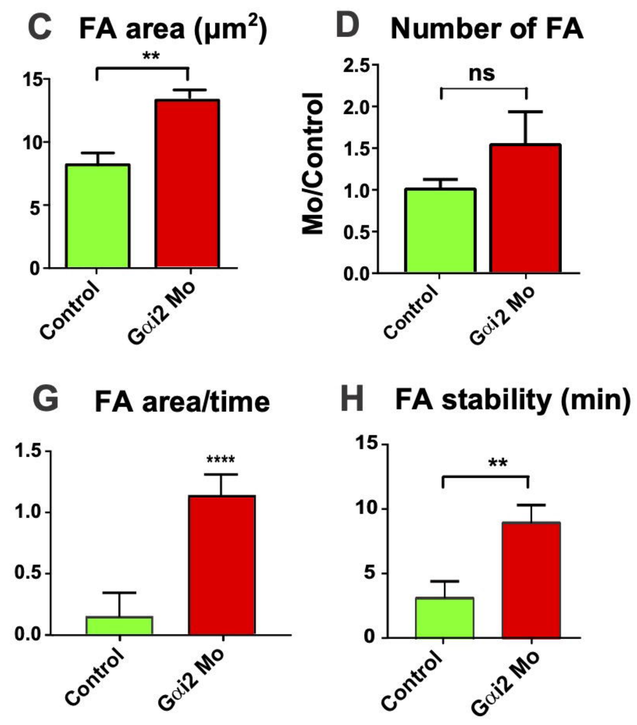


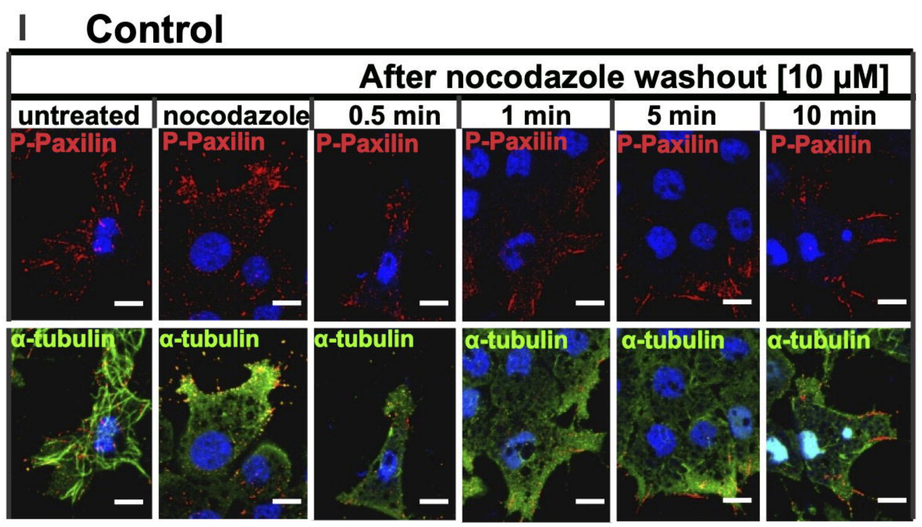


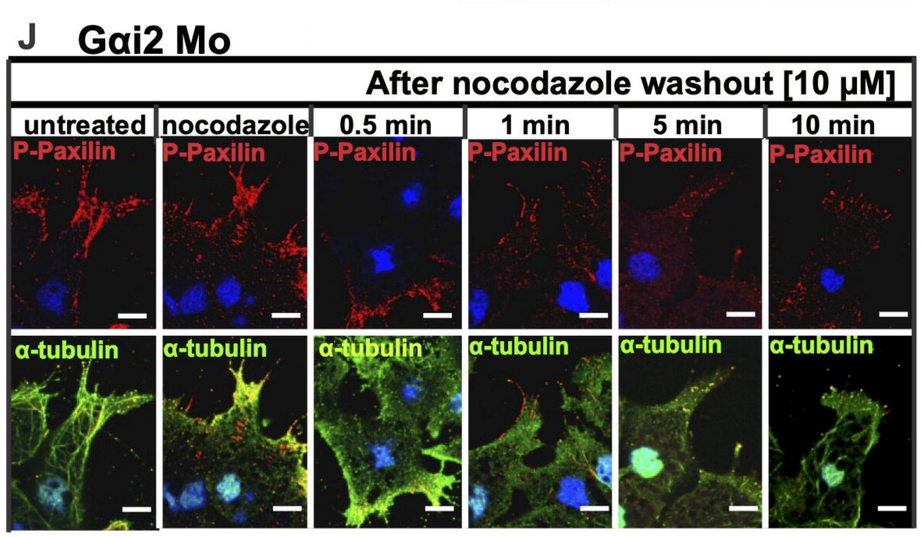

pGBD-GFP
A
Control
pGBD-GFP
B
Gαi2 Mo
C
Control
D
Gαi2 Mo
E
Gαi2 Mo + Noco
F
Ratio
Distance (μm)
Control
Gαi2 Mo
Gαi2 Mo + Noco
Cell polarity
G
Par3
Control
H
Par3
Gαi2 Mo
I
ζPKC
Control
J
ζPKC
Gαi2 Mo
Control
Gαi2 Mo
Gαi2 Mo + NSC23766
Control + NSC23766
K
L
M
N
Area (μm²)
O
Dispersion
Area (μm²)
Control
Gαi2 Mo
Gαi2 Mo + NSC23766
Control + NSC23766
P
actin
Q
R
S
Cortical actin
Ratio
Distance (μm)
T
actin
A

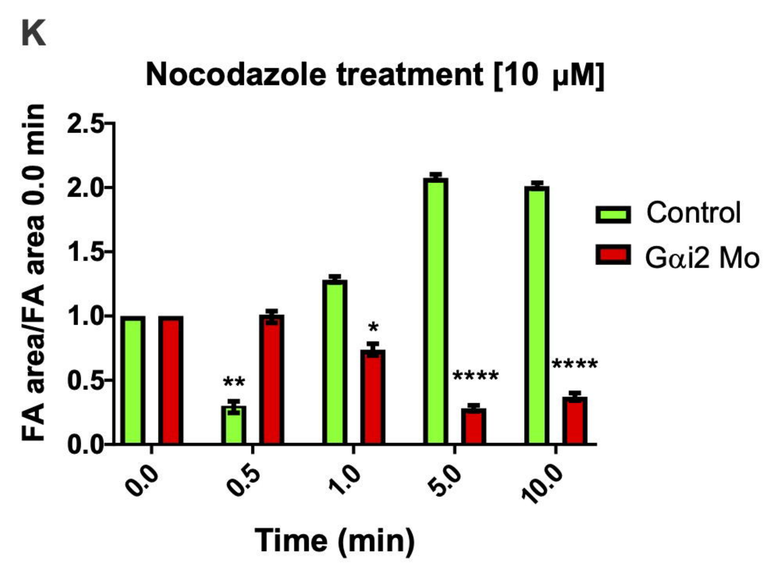

β-Integrin
A
Control
β-Integrin
B
Gαi2 Mo


FAK-GFP
0 min
5 min
10 min
Control
E
FAK-GFP
0 min
5 min
10 min
Gαi2 Mo
F

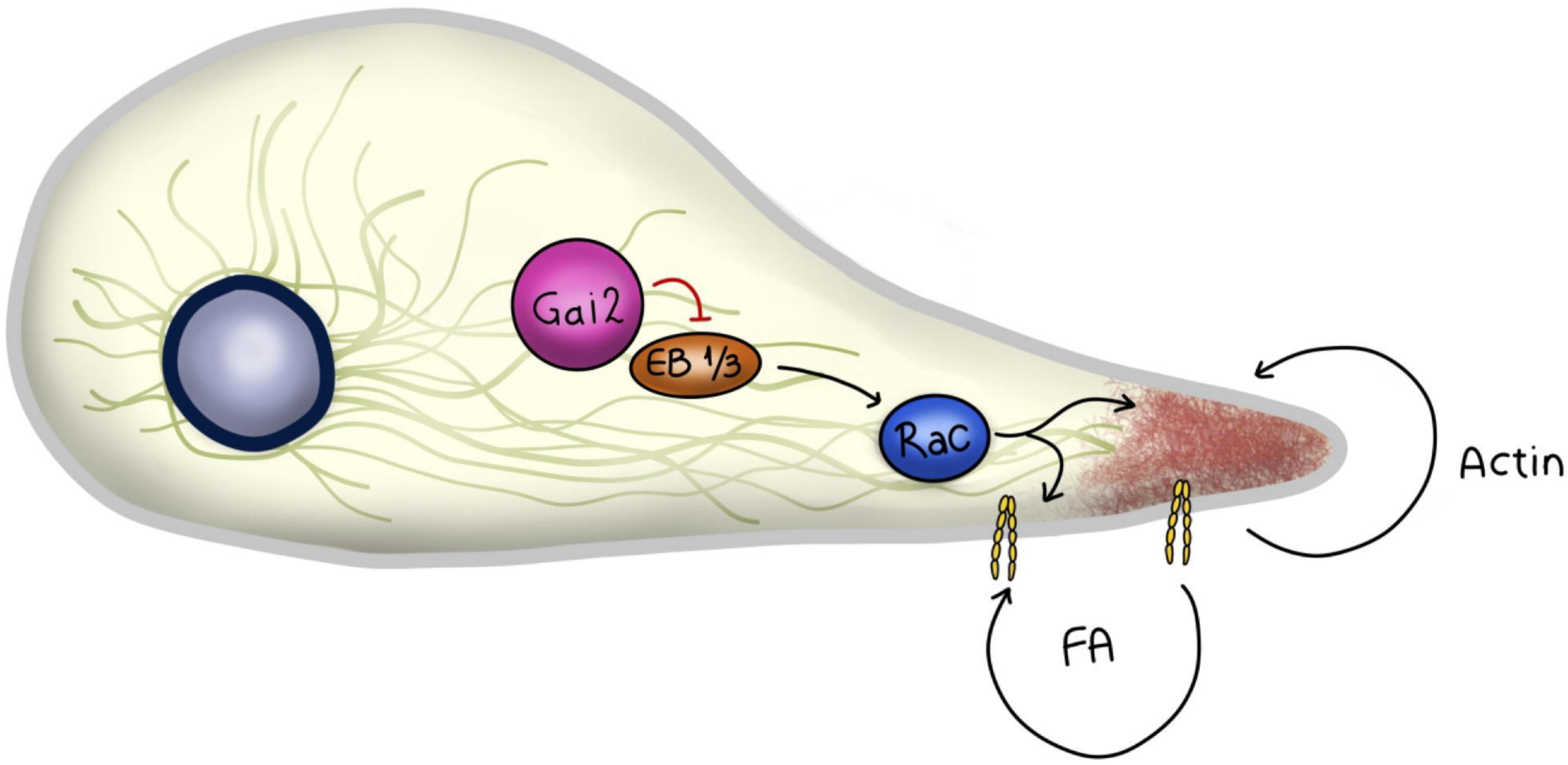

C
FA area (μm²)
**
15
10
5
0
Control
Gαi2 Mo
D
Number of FA
ns
Mo/Control
2.5
2.0
1.5
1.0
0.5
0.0
Control
Gαi2 Mo
G
FA area/time
****
1.5
1.0
0.5
0.0
Control
Gαi2 Mo
H
FA stability (min)
**
15
10
5
0
Control
Gαi2 Mo


I
Control
After nocodazole washout [10 μM]
untreated
nocodazole
0.5 min
1 min
5 min
10 min
P-Paxilin
α-tubulin

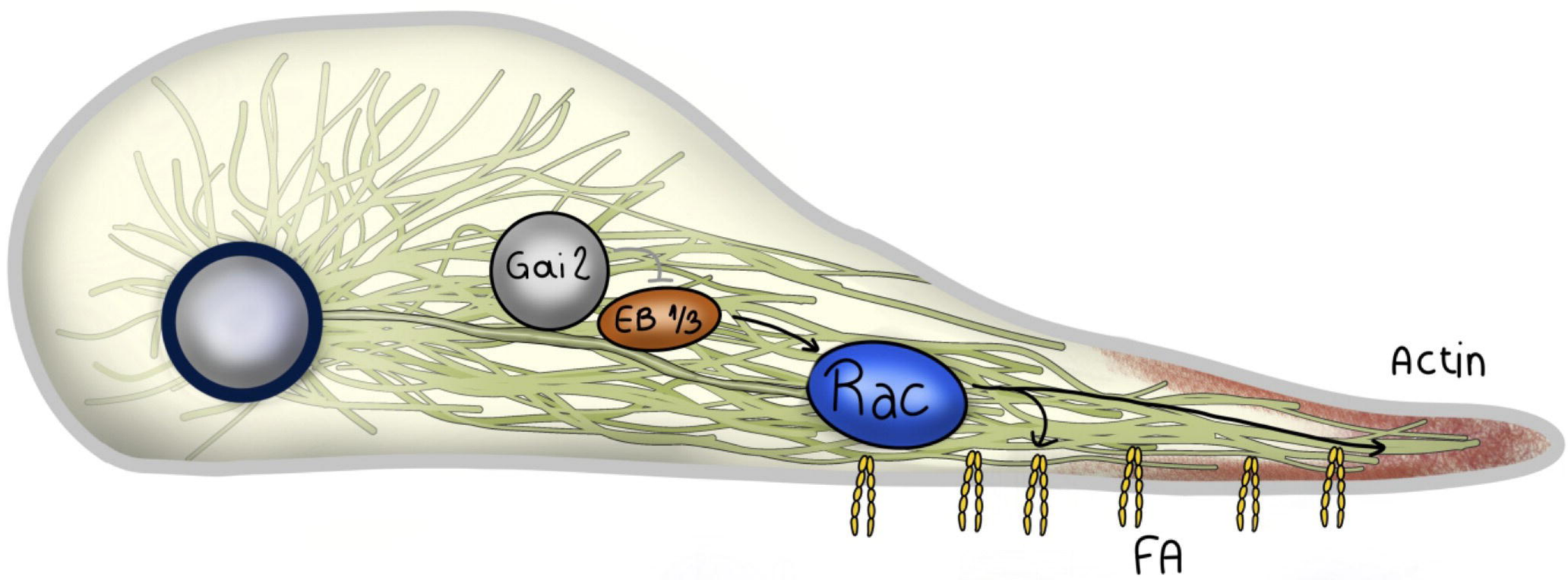

J
Gαi2 Mo
After nocodazole washout [10 μM]
untreated
nocodazole
0.5 min
1 min
5 min
10 min
P-Paxilin
α-tubulin

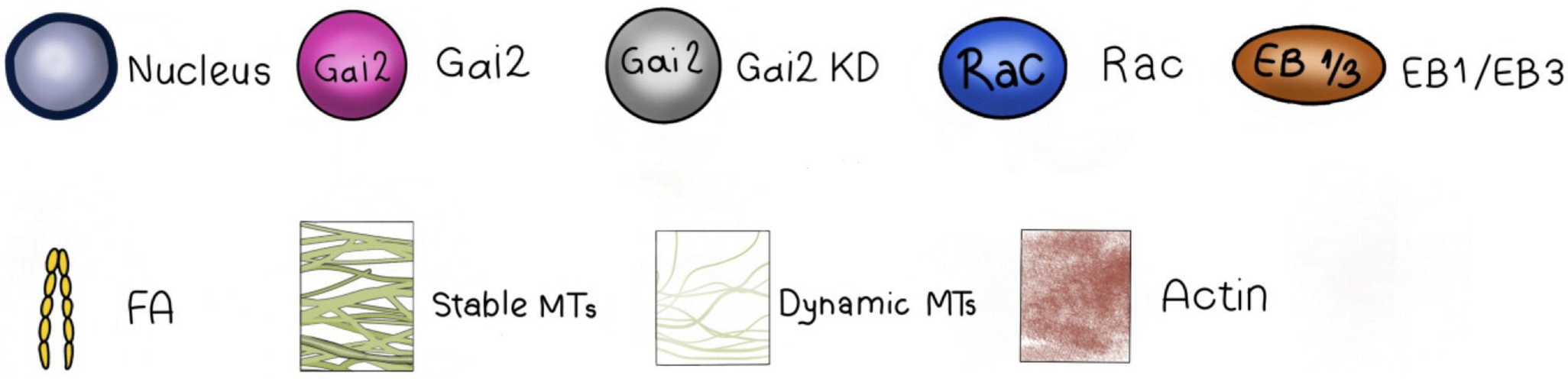

K
Nocodazole treatment [10 μM]
FA area/FA area 0.0 min
2.5
2.0
1.5
1.0
0.5
0.0
**
*
****
****
0.0
0.5
1.0
5.0
10.0
Time (min)
Control
Gαi2 Mo

A


Gai2
EB 1/3
RaC
Actin
FA

B


Gai2
EB 1/3
Rac
Actin
FA


Nucleus
Gai2
Gai2
Gai2
Gai2 KD
RaC
Rac
EB 1/3
EB1/EB3
FA
Stable MTs
Dynamic MTs
Actin