

The role of microtubules in neutrophil polarity and migration in live zebrafish

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Summary

Microtubules control cell motility by positively regulating polarization in many cell types. However, how microtubules regulate leukocyte migration is not well understood, particularly in living organisms. Here we exploited the zebrafish system to study the role of microtubules in neutrophil migration *in vivo*. The localization of microtubules was visualized in motile neutrophils using various bioprobes, revealing that, in contrast to what has been seen in studies *in vitro*, the microtubule organizing center is positioned in front of the nucleus (relative to the direction of migration) in motile neutrophils. Microtubule disassembly impaired attraction of neutrophils to wounds but enhanced the polarity of F-actin dynamics as measured by the distribution of stable and dynamic F-actin. Microtubule depolymerization inhibited polarized phosphoinositol 3-kinase (PI(3)K) activation at the leading edge and induced rapid PI(3)K independent motility. Finally, we show that microtubules exert their effects on neutrophil polarity and motility at least in part by the negative regulation of both Rho and Rac activity. These results provide new insight into the role of microtubules in neutrophil migration in a living vertebrate and show that the motility of these professional migratory cells are subject to distinctly different rules from those established for other cell types.

Key words: Chemotaxis, Microtubule, Neutrophil, Zebrafish

Introduction

Directed cell migration is the result of a complex interplay between the actomyosin cytoskeleton, adhesion, and the microtubule network (Rodriguez et al., 2003; Small et al., 2002). More than 40 years ago, the pioneering work of Vasiliev on cultured mouse and human fibroblasts has shown that one of the major mechanisms by which microtubules contribute to cell migration is by the positive regulation of cell polarity (Vasiliev et al., 1970). Since Vasiliev's findings, it has become apparent that the microtubule cytoskeleton regulates cell migration in a cell type-dependent manner. Microtubule disassembly impairs cell migration in many types of cells such as fibroblasts, tumor cells and neurons (Etienne-Manneville, 2004; Kaverina and Straube, 2011; Mogilner and Keren, 2009; Siegrist and Doe, 2007; Small et al., 2002; Vinogradova et al., 2009; Watanabe et al., 2005; Wittmann and Waterman-Storer, 2001), whereas it promotes motility of neutrophils, but impairs their directionality (Niggli, 2003; Xu et al., 2005). In contrast, fish keratinocytes migrate directionally without microtubules (Euteneuer and Schliwa, 1984).

In addition to the context-dependent role of microtubules in cell migration, the location where microtubules nucleate and radiate during cell motility also depends on the cell type. In non-leukocyte cells, microtubules generally nucleate from the microtubule organizing center (MTOC) in front of the nucleus (relative to the direction of migration) and radiate towards the leading edge (Etienne-Manneville, 2004; Kaverina and Straube,

2011; Mogilner and Keren, 2009; Small et al., 2002; Vinogradova et al., 2009; Watanabe et al., 2005; Wittmann and Waterman-Storer, 2001). On the other hand, leukocytes are often described as having microtubules and the MTOC at the rear of the cell (uropod) (Friedl and Weigelin, 2008; Sánchez-Madrid and del Pozo, 1999; Sánchez-Madrid and Serrador, 2009), which is in sharp contrast with other cell types. This idea is supported by multiple primary research reports using lymphocytes *in vitro* (Lee et al., 2004; Ratner et al., 1997; Serrador et al., 1997; Takesono et al., 2010). However, where microtubules are localized in neutrophils migrating *in vitro* is more controversial: several studies suggested that the MTOC is localized in front of the nucleus (Malech et al., 1977; Schliwa et al., 1982) and others suggested that microtubules nucleate between nuclear lobes (Anderson et al., 1982) or behind the nucleus (Anderson et al., 1982; Xu et al., 2005). In contrast to non-leukocyte cells where the microtubules radiate towards the leading edge, the microtubule arrays extend towards the rear in neutrophils (Eddy et al., 2002; Xu et al., 2005). Little is known about how microtubules regulate neutrophil migration in three-dimensional (3D) tissue environments *in vivo*. Evidence that the biological context matters is revealed by studies showing that what is essential for migration *in vitro* can be dispensable *in vivo*, or vice versa (Lämmermann et al., 2008; Yoo et al., 2010).

To assess microtubule dynamics and function during neutrophil migration *in vivo*, we used zebrafish larvae, which

have been an emerging tool to study immune responses and leukocyte migration in physiological contexts (Deng et al., 2012; Deng et al., 2011; Mathias et al., 2006; Niethammer et al., 2009; Trede et al., 2004; Yoo et al., 2010; Yoo and Huttenlocher, 2011; Yoo et al., 2011). Previously we have shown that phosphoinositol 3-kinase (PI(3)K) regulates neutrophil motility through both the modulation of Rac-mediated protrusion and polarity of F-actin dynamics in zebrafish (Yoo et al., 2010). Here, we investigated the role of microtubules during neutrophil 3D motility. We demonstrate that microtubules nucleate in front of the nucleus, mainly radiating towards the uropod, and that microtubule disassembly induces neutrophil motility at least in part through the activation of both Rho and Rac in a PI(3)K-independent manner.

Results

To determine how microtubules regulate neutrophil motility *in vivo*, first we sought to visualize microtubule dynamics in neutrophils migrating rapidly in intact tissues in living zebrafish. Such imaging has not been demonstrated *in vivo*. Neutrophils migrate spontaneously and display apparent random motility in the mesenchyme of the head at 2–3 days post fertilization (dpf) by as-yet-undefined mechanisms (Yoo et al., 2010). We used this system to study neutrophil motility as previously reported (Yoo et al., 2010). We constructed multiple bioprobes and expressed them in neutrophils by using the neutrophil-specific *mpx* or *lyz* promoter to visualize microtubule dynamics in zebrafish larvae (Kitaguchi et al., 2009; Mathias et al., 2006). We first employed GFP-tubulin, which has traditionally been used to image microtubules in live cells (Small et al., 2002). GFP-tubulin revealed a relatively bright dot in

front of the nucleus (Fig. 1A; supplementary material Movie 1). This punctate dot is presumably the MTOC because the nucleation site of microtubule arrays appears as a point containing the strongest signals (Anderson et al., 1982; Eddy et al., 2002; see also below). In migrating neutrophils, the dot appeared to lead the nucleus (Fig. 1A), based on comparison of GFP-tubulin to soluble mCherry, which, unlike GFP-tubulin, is small enough to pass freely into and out of the nucleus.

In our imaging setting using rapidly migrating neutrophils in zebrafish, the GFP-tubulin failed to reveal structures likely to be individual microtubules due to high background fluorescence. Consequently, we next tried three tandem GFPs fused to the consensin microtubule-binding domain (EMTB-3xGFP) which was previously shown to provide higher contrast (relative to GFP-tubulin) microtubule imaging in live cultured cells as well as echinoderm embryos (Faire et al., 1999; von Dassow et al., 2009). EMTB-3xGFP yielded more signal than GFP-tubulin, and also showed a punctate dot in front of the nucleus and some linear structures (presumably microtubules) radiating from the putative MTOC (Fig. 1B; supplementary material Movie 2). However, the EMTB probe only rarely yielded structures that could clearly be interpreted as microtubules and therefore we tried an additional probe, EB3-GFP. EB3 is a plus end-binding protein (+TIP) (Hu et al., 2008; Stepanova et al., 2003). +TIPs specifically label plus ends at low expression (Stepanova et al., 2003; Stramer et al., 2010; Xu et al., 2005). Thus, +TIPs, such as CLIP170, have been used to label the whole microtubule arrays as well as plus ends (Stramer et al., 2010; Xu et al., 2005). Imaging of EB3-GFP revealed that

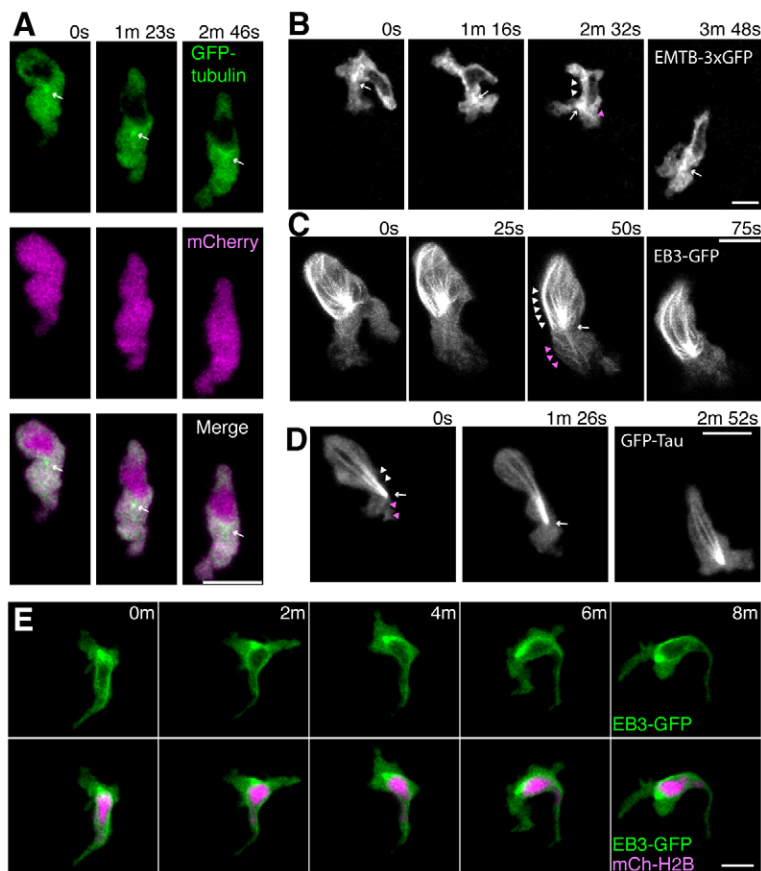


Fig. 1. Localization of microtubules during neutrophil motility in live zebrafish. (A) Time-lapse imaging of a neutrophil expressing GFP-tubulin and mCherry. Arrows indicate a punctate dot in front of the nucleus. Note that fluorescent signals of GFP-tubulin are excluded from the nucleus. (B) Time-lapse imaging of a neutrophil expressing EMTB-3xGFP. A punctate dot, presumably the MTOC, is indicated by arrows. Arrowheads indicate linear structures (presumably microtubules) extending to the leading edge (magenta) and to the uropod (white). (C) Time-lapse imaging of a neutrophil expressing EB3-GFP. An arrow indicates the MTOC and arrowheads indicate microtubules extending to the leading edge (magenta) and to the uropod (white). (D) Time-lapse imaging of a neutrophil expressing GFP-Tau. An arrow indicates the MTOC and arrowheads indicate microtubules extending to the leading edge (magenta) and to the uropod (white). (E) Time-lapse imaging of a neutrophil expressing EB3-GFP and a nucleus probe mCh-H2B. Data are representative of more than three separate time-lapse movies. Scale bars: 10 μ m.

microtubules nucleate from a MTOC positioned in front of the nucleus and mainly radiate towards the tail (Fig. 1C; supplementary material Movie 3). We also noticed that very faint microtubule filaments occasionally enter the pseudopods at the leading edge, but microtubule arrays radiating backwards were more dominant. Although the location of the nucleus was obvious due to exclusion of fluorescent signals from the nucleus (see also above), we confirmed that microtubules nucleate in front of the nucleus using a nucleus probe, mCherry-histone H2B (Fig. 1E; supplementary material Movie 4). The MTOC was mainly localized in front of the nucleus, but occasionally and transiently, it also localized on the side of the nucleus (supplementary material Fig. S1; Movie 4). We also used GFP-Tau, which is widely used to visualize microtubules in *Drosophila* and *Xenopus in vivo* (Brand, 1995; Kwan and Kirschner, 2005). This microtubule probe also revealed that microtubules nucleate in front of the nucleus and radiate towards the uropod (Fig. 1D; supplementary material Movie 5). Localization of the microtubule arrays towards the uropod is consistent with previous findings *in vitro* (Eddy et al., 2002; Xu et al., 2005), but the MTOC localization in front of the nucleus is different from the prevailing idea that leukocytes have the MTOC at the rear of the cell (Friedl and Weigelin, 2008; Sánchez-Madrid and del Pozo, 1999; Sánchez-Madrid and Serrador, 2009). Thus, we employed γ tubulin-GFP (Joshi, 1993), which labels the MTOC, to further confirm our findings and to exclude the possibility that localization of the MTOC is due to overexpression effects of the microtubule probes. Live imaging by γ tubulin-GFP unambiguously showed that the MTOC is localized in front of the nucleus (Fig. 2; supplementary material Movie 6). While none of the microtubule probes employed permitted us to assay the dynamics of individual microtubules, possibly because the microtubules in these rapidly migrating cells turnover very rapidly, they nonetheless collectively indicate that microtubules nucleate in front of the nucleus and radiate towards the uropod during neutrophil motility in zebrafish. Immunostaining of zebrafish microtubules using multiple fixation conditions was also not successful, presumably due to the rapid turnover of microtubules or proteolytic enzymes in neutrophils.

We next assessed how microtubules regulate neutrophil migration *in vivo*. Microtubule depolymerization using nocodazole or colchicine impairs neutrophil directional migration but enhances neutrophil motility *in vitro*, presumably through Rho activation (Niggli, 2003; Xu et al., 2005). To determine whether this knowledge could be also applied to neutrophil motility *in vivo*, first we investigated the effects of microtubule depolymerization on neutrophil wound attraction, which is suitable for studying directional migration of neutrophils *in vivo* (Mathias et al., 2006; Niethammer et al., 2009; Yoo et al., 2010; Yoo et al., 2011). We examined the effects of microtubule disassembly induced by nocodazole on neutrophil attraction to wounds at 30 minutes after wounding, a time point when neutrophil accumulation at wounds is not affected by reverse migration (Mathias et al., 2006; Niethammer et al., 2009; Yoo et al., 2010; Yoo and Huttenlocher, 2011; Yoo et al., 2011). Microtubule depolymerization impaired neutrophil directional attraction to wounds (Fig. 3A), consistent with findings in neutrophils *in vitro* (Xu et al., 2005) and macrophages in zebrafish (Redd et al., 2006). Next we focused on the effects of microtubule depolymerization on neutrophil random motility and F-actin polarity in the mesenchymal tissues of the

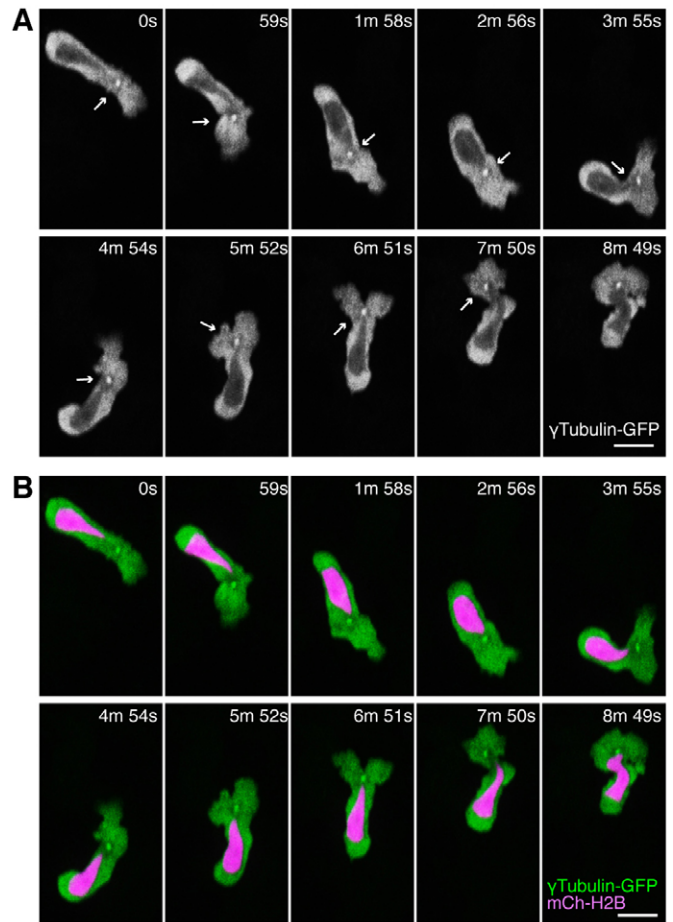


Fig. 2. The MTOC is localized in front of the nucleus during neutrophil motility in live zebrafish. (A) Time-lapse imaging of a neutrophil expressing tubulin-GFP. Arrows indicate the MTOC in front of the nucleus. (B) Simultaneous imaging of tubulin-GFP and a nucleus probe mCherry-histone H2B in the same cell with A. Data are representative of more than three separate time-lapse movies. Scale bars: 10 μ m.

head. Microtubule depolymerization with nocodazole enhanced motility and induced a more round, compact morphology (Fig. 3B–D; supplementary material Movie 7). Microtubule inhibition also enhanced polarity of F-actin dynamics (Fig. 3E), which was detected with Lifeact-Ruby (a probe for all F-actin) and GFP-UtrCH (a probe for stable F-actin) (Burkel et al., 2007; Riedl et al., 2008; Yoo et al., 2010). Nocodazole particularly emphasized tail localization of stable F-actin detected by GFP-UtrCH, presumably due to the well-established Rho-myosin activation by microtubule depolymerization (Niggli, 2003; Rodriguez et al., 2003; Wittmann and Waterman-Storer, 2001; Xu et al., 2005). Our findings are mainly consistent with findings reported for neutrophils *in vitro* (Xu et al., 2005), but one noticeable difference is that we did not observe decreased dynamic F-actin at the leading edge after microtubule depolymerization. This is in contrast to *in vitro* effects of microtubule depolymerization (Xu et al., 2005): nocodazole treatment disturbs F-actin at the leading edge of neutrophils *in vitro*. This implies that, in 3D tissues *in vivo*, microtubule disassembly activates ‘backness’ signals such as stable F-actin, presumably through Rho, at the tail, but that it may also maintain

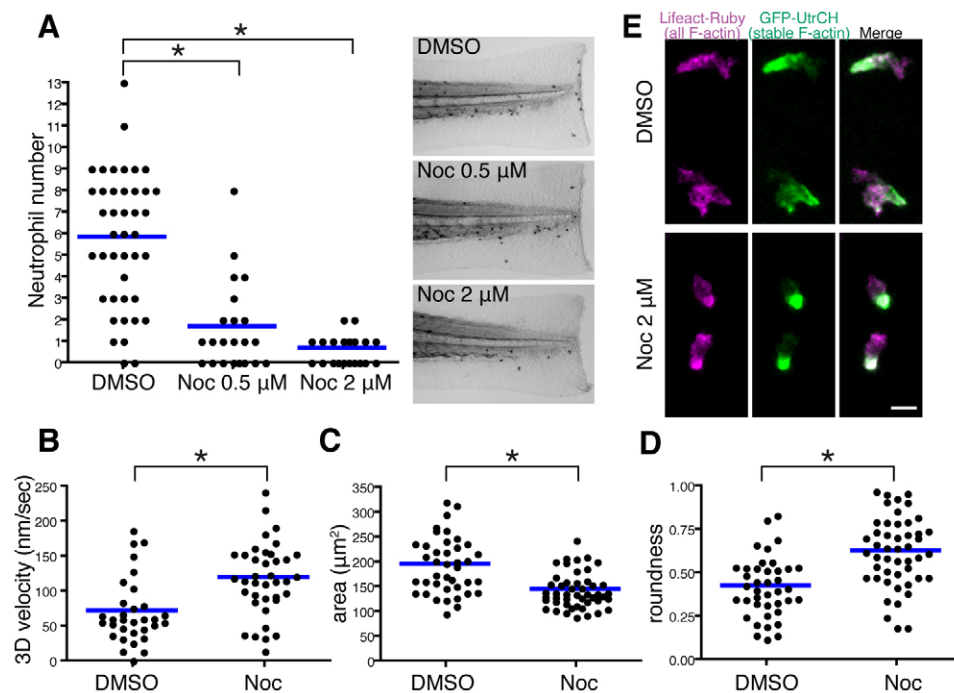


Fig. 3. Microtubule depolymerization impairs neutrophil wound attraction but enhances neutrophil motility and F-actin polarity. (A) Neutrophil recruitment to wounded fins at 30 minutes post wounding. Neutrophils were stained with Sudan Black. $n=42$ (DMSO), 22 (Noc 0.5 μM) and 22 (Noc 2 μM). (B–D) Microtubule depolymerization with nocodazole treatment enhances neutrophil 3D motility [(B), $n=32$ (DMSO), $n=38$ (Noc)], makes cells compact [(C), $n=40$ (DMSO), $n=48$ (Noc)] and induces a more round morphology [(D), $n=40$ (DMSO), $n=48$ (Noc)]. Tg(mpx:GFP-UtrCH) was used for live analysis. (E) Nocodazole treatment enhances polarity of F-actin dynamics in neutrophils. Rear localization of stable F-actin detected by GFP-UtrCH is particularly emphasized after microtubule depolymerization. Tg(mpx:GFP-UtrCH) was crossed with Tg(mpx:Lifeact-Ruby). $*P<0.05$, one-way ANOVA with Dunnett post-test (A) and two-tailed unpaired t -test (B–D). Data in A are representative of at least three separate experiments and three time-lapse movies were analyzed for data in B–D. Scale bar: 10 μm .

‘frontness’ signals (dynamic F-actin) during neutrophil migration.

To investigate the molecular mechanisms by which microtubules regulate neutrophil motility, we focused on PI(3)K signaling, which we recently found to regulate neutrophil motility in all contexts examined in zebrafish larvae, including wound attraction, reverse migration away from wounds, attraction to bacteria and interstitial motility in the head (Deng et al., 2012; Yoo et al., 2010). Interestingly, microtubule depolymerization-mediated neutrophil migration *in vitro* is independent of PI(3)K and is, in fact, inhibitory to PI(3)K signaling (Niggli, 2003; Xu et al., 2005). PHAKT-GFP (PH domain of AKT), a bioprobe for PI(3)K products PI(3,4,5)P₃–PI(3,4)P₂, and mCherry were expressed in neutrophils to detect PI(3)K signaling by ratiometric analysis (Yoo et al., 2010). In control, PI(3,4,5)P₃–PI(3,4)P₂ is localized at the leading edge as previously reported (Yoo et al., 2010), but microtubule depolymerization completely depleted PI(3,4,5)P₃–PI(3,4)P₂ signals at the leading edge (Fig. 4A,B; supplementary material Movie 8). This indicates that PI(3)K signaling is inhibited during nocodazole-induced neutrophil motility, suggesting that microtubule disassembly mediates neutrophil migration in a PI(3)K-independent manner. To test this hypothesis, we investigated whether microtubule depolymerization can rescue migration and F-actin polarity defects induced by PI(3)K inhibition. PI(3)K inhibition with LY294002 impairs neutrophil motility and F-actin polarity as previously reported (Fig. 5A,B) (Yoo et al., 2010). Nocodazole treatment not only reversed the effects of PI(3)K inhibition but further increased neutrophil migration and F-actin polarity compared to DMSO treatment (Fig. 5A,B; supplementary material Movie 9). Our findings indicate that microtubule disassembly induces neutrophil motility in a PI(3)K-independent manner, in agreement with *in vitro* studies (Niggli, 2003; Xu et al., 2005). Moreover, the findings

suggest that microtubule disruption is sufficient to rescue migration of PI(3)K inhibited cells *in vivo*, in contrast to localized Rac activation (Yoo et al., 2010).

Next we investigated how microtubule inhibition promotes neutrophil motility without PI(3)K activation. Rho was an obvious target because Rho, Rho kinase and myosin 2 are activated by microtubule disassembly in diverse cells including tumor cells, fibroblasts, *Xenopus* oocytes and leukocytes (Etienne-Manneville, 2004; Kaverina and Straube, 2011; Mogilner and Keren, 2009; Rodriguez et al., 2003; Small et al.,

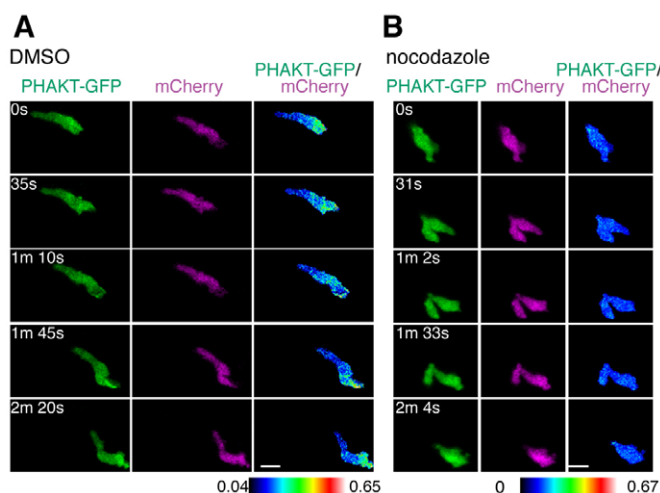


Fig. 4. Microtubule disassembly inhibits PI(3)K activation at the leading edge. (A) Time-lapse ratiometric imaging (PHAKT-GFP/mCherry) of PI(3,4,5)P₃–PI(3,4)P₂ dynamics during neutrophil random motility in the head. (B) Microtubule depolymerization inhibits localization of PI(3,4,5)P₃–PI(3,4)P₂ at the leading edge. Scale bars: 10 μm .

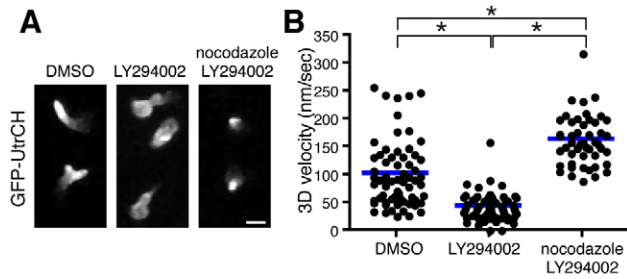


Fig. 5. Microtubule disassembly induces neutrophil polarity and motility in a PI(3)K-independent manner. (A) Microtubule depolymerization with nocodazole reverses F-actin polarity defects caused by PI(3)K inhibition. (B) Microtubule disassembly reverses PI(3)K inhibition-induced migration arrest and enhances neutrophil motility compared with control. $n=66$ (DMSO), 64 (LY294002) and 48 (nocodazole, LY294002). $*P<0.05$, one-way ANOVA with Tukey post-test. Three time-lapse movies were analyzed for data for (B). Scale bar: 10 μm .

2002; Takesono et al., 2010; Vinogradova et al., 2009; Watanabe et al., 2005; Wittmann and Waterman-Storer, 2001). Although how microtubule disassembly activates Rho pathways is not completely understood, Rho GEFs, particularly GEF-H1 and p190RhoGEF, which are activated upon release from microtubule arrays, are considered to be important for this process (Krendel et al., 2002; Ren et al., 1998; Small et al., 2002; van Horck et al., 2001). In addition, activation of Rho signaling mediates microtubule disassembly-induced neutrophil motility *in vitro* (Niggli, 2003; Xu et al., 2005). Thus, we examined whether Rho is also responsible for neutrophil motility induced by microtubule depolymerization *in vivo*. To specifically inhibit Rho in zebrafish neutrophils, we expressed a dominant negative RhoA T19N, which binds to GDP but not GTP, in neutrophils using the neutrophil-specific mpx promoter (Yoo et al., 2010). As previously reported, RhoA T19N inhibits neutrophil motility, inducing a rounded morphology (Fig. 6A,B) (Yoo et al., 2010). Microtubule disassembly using nocodazole slightly mobilized RhoA T19N expressing neutrophils but this effect was subtle, and generally neutrophils with Rho inhibition did not migrate after nocodazole treatment (Fig. 6A). This is consistent with the idea that microtubules regulate cell motility through Rho. However, we found that Rho-inhibited cells protruded the leading edge extensively after nocodazole treatment (Fig. 6B; supplementary material Movie 10). In combination with the data that dynamic F-actin at the leading edge is intact after microtubule

depolymerization (Fig. 3E), this suggested the possibility that microtubule disassembly may maintain or even promote frontness signals in addition to its well-known role in mediating Rho-mediated backness signals. To test this possibility, we focused on Rac2, a hematopoietic specific Rho family GTPase, which regulates neutrophil motility, promoting F-actin polymerization downstream of PI(3)K (Deng et al., 2011). As previously reported (Deng et al., 2011), Rac2 knockdown by morpholino antisense oligonucleotide inhibits neutrophil motility, making neutrophils more round and compacted than control neutrophils (Fig. 7A–C). We tested whether microtubule disassembly reverses motility arrest induced by Rac2 knockdown, as it did with PI(3)K-inhibited cells (Fig. 5B). If microtubule depolymerization induces cell motility only through enhancing Rho-mediated actomyosin tail contraction (Niggli, 2003; Xu et al., 2005), nocodazole should release the motility arrest caused by Rac2 knockdown. We found that nocodazole induced a further round, compact morphology of Rac2-deficient neutrophils, like nocodazole-treated control neutrophils, but that it did not induce neutrophil motility (Fig. 7A–C; supplementary material Movie 11). In addition, transgenic expression of dominant negative Rac2 D57N in neutrophils (Deng et al., 2011) also inhibited nocodazole-induced motility (Fig. 7D; supplementary material Movie 12). This indicates that Rac2 is necessary for microtubule depolymerization-induced motility in addition to Rho. To further test whether microtubule depolymerization directly activates Rac as well as Rho in mammalian neutrophils in a cell autonomous manner, we purified primary human neutrophils and performed affinity precipitation assays with GST-RBD and GST-PBD, to which only the active GTP-bound forms of Rho and Rac bind respectively (Benard et al., 1999; Nishita et al., 2002; Ren et al., 1999). As shown in Fig. 7E, microtubule disassembly directly activated Rac in addition to Rho in primary human neutrophils (Fig. 7E). Taken together, our data suggest that microtubule disassembly induces neutrophil 3D motility through both Rac and Rho-dependent pathways.

Discussion

Here we investigated microtubule dynamics and function during neutrophil migration *in vivo*. In accordance with reported *in vitro* findings, we found that microtubule disruption impairs neutrophil directed migration to wounds but induces cell motility and polarity in a PI(3)K-independent manner. We also found that Rho mediates nocodazole-induced neutrophil motility. These findings

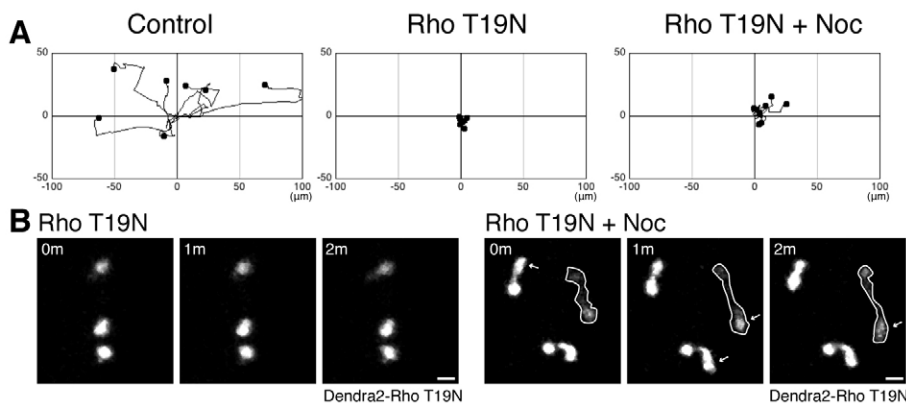


Fig. 6. Rho regulates microtubule disassembly-mediated neutrophil motility. (A) Expression of a dominant negative RhoA T19N prevents microtubule depolymerization-induced neutrophil motility. Dendra2-Rho T19N was expressed in Tg(mpx:Lifeact-Ruby) and neutrophil motility was tracked for 30 minutes. (B) Nocodazole induces protrusion in Rho-inhibited cells. Data in A are representative of at least three time-lapse movies. Scale bars: 10 μm .

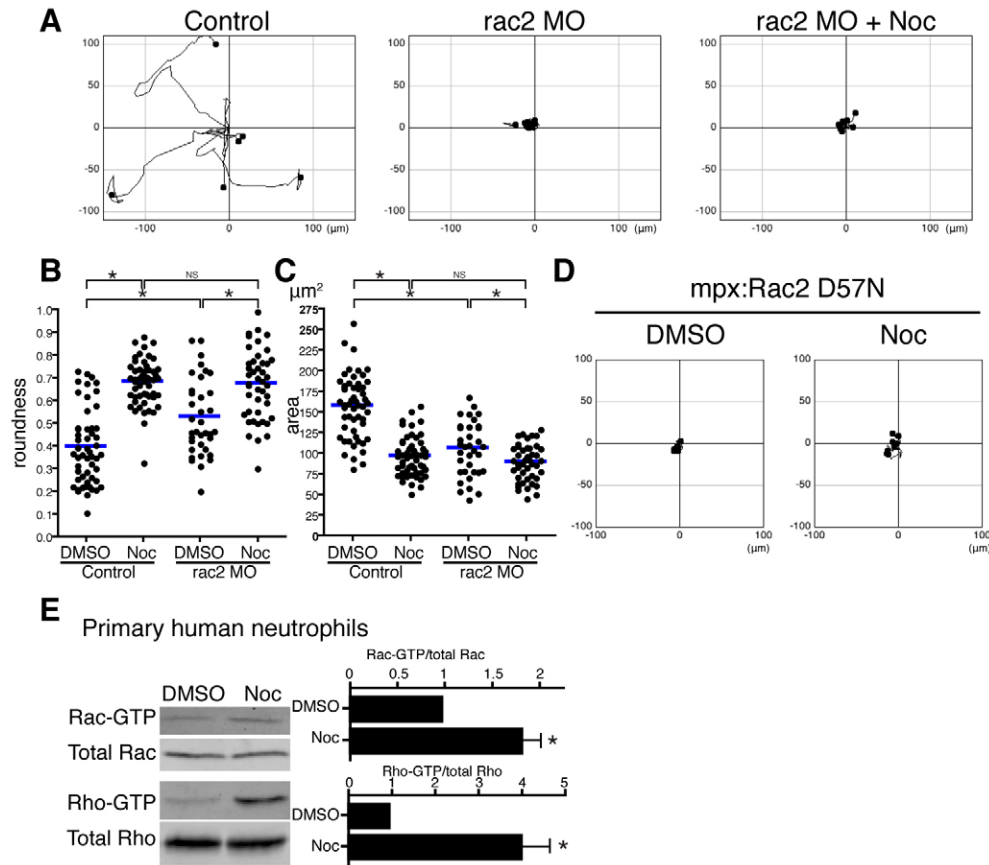


Fig. 7. Rac regulates microtubule disassembly-mediated neutrophil motility. (A) *rac2* MO inhibits microtubule depolymerization-induced neutrophil motility. Neutrophil motility was tracked for 30 minutes in Tg(*mpx*:GFP-UtrCH). (B) Rac2 knockdown makes neutrophils round, but nocodazole treatment makes Rac2-inhibited cells more round. $n=53$ (Control, DMSO), 52 (Control, Noc), 36 (*rac2* MO, DMSO) and 44 (*rac2* MO, Noc). (C) Rac2 knockdown makes neutrophils compact, but nocodazole treatment makes Rac2-inhibited cells more compact. $n=54$ (Control, DMSO), 52 (Control, Noc), 36 (*rac2* MO, DMSO) and 44 (*rac2* MO, Noc). (D) Transgenic neutrophil specific expression of dominant negative Rac2 D57N inhibits microtubule depolymerization-induced neutrophil motility. Neutrophil motility was tracked for 30 minutes in Tg(*mpx*:mCherry-2A-Rac2D57N). (E) Affinity precipitation assays with GST-RBD and GST-PBD to detect Rho-GTP and Rac-GTP, respectively ($n=3$). Microtubule disassembly directly activates Rac in addition to Rho in primary human neutrophils. Data in A and D are representative of at least three time-lapse movies. Three time-lapse movies were analyzed for data for B and C. Data in E are means \pm s.e.m. of three separate experiments. * $P<0.05$, one-way ANOVA with Bonferroni post-test (B,C) and two-tailed paired t -test (E).

are consistent with previous findings *in vitro*, but further provide a physiological context for neutrophil migration. In addition, we elucidated two previously unidentified roles of microtubules during neutrophil motility *in vivo*. First, microtubule arrays nucleate in front of the nucleus and mainly radiate towards the uropod. Second, Rac is activated by microtubule depolymerization in primary human neutrophils and is necessary for nocodazole-induced neutrophil motility *in vivo*.

Leukocytes are often described as having the microtubule cytoskeleton and MTOC behind the nucleus at the uropod (Friedl and Weigelin, 2008; Sánchez-Madrid and del Pozo, 1999; Sánchez-Madrid and Serrador, 2009). We found that microtubule arrays radiate towards the uropod, which is consistent with the idea that leukocytes have microtubules at the uropod. Microtubules visualized in zebrafish neutrophils have similar structures to those imaged in fixed human neutrophils *in vitro* (Eddy et al., 2002). However, our finding that microtubules nucleate from the MTOC in front of the nucleus is very different from what has been reported from *in vitro* studies. This suggests

that we need to revise the widespread model that leukocytes have the MTOC at the rear.

Our findings that microtubule depolymerization activates Rac in addition to Rho is also different from what was previously thought. Microtubule-mediated antagonistic regulation of Rho, presumably through Rho GEFs such as GEF-H1 and p190RhoGEF, has been observed in diverse systems including tumor cells, fibroblasts, *Xenopus* oocytes and leukocytes (Etienne-Manneville, 2004; Kaverina and Straube, 2011; Mogilner and Keren, 2009; Rodriguez et al., 2003; Small et al., 2002; Takesono et al., 2010; Vinogradova et al., 2009; Watanabe et al., 2005; Wittmann and Waterman-Storer, 2001). On the other hand, microtubule polymerization is known to activate Rac in fibroblasts (Waterman-Storer et al., 1999; Wittmann and Waterman-Storer, 2001), suggesting a concept that microtubules regulate Rho negatively and Rac positively. However, using zebrafish neutrophils *in vivo* and primary human neutrophils *in vitro*, we have shown that microtubule disruption activates Rac and induces neutrophil motility in a Rac-dependent manner. The

difference between our findings and previous findings are most likely due to difference in the cell types. How microtubules regulate Rac in neutrophils is not clear yet, but we speculate that two mechanisms are possible. The first possibility is that similar mechanisms to microtubule-mediated Rho regulation, such as regulation of Rac GEFs, might regulate Rac activity in neutrophils. The second possibility is that the well-known antagonistic effects of Rho on Rac activity (Small et al., 2002; Wittmann and Waterman-Storer, 2001) might occur locally in neutrophils due to the highly polarized sequestration of Rho at the tail, leading to relative activation of Rac at the leading edge and also at the whole cell level. To further elucidate the role of Rho GTPase signaling in zebrafish neutrophils would require tools that are not currently available in zebrafish including inducible or tissue specific knockdowns. Moreover, we tried to use biosensors for Rho GTPase activation in zebrafish neutrophils but the probes had toxic effects precluding further analysis of the polarity of Rho GTPase activation in zebrafish neutrophils *in vivo* at this time.

Our current findings *in vivo*, together with previous *in vitro* studies (Niggli, 2003; Xu et al., 2005), suggest that microtubules regulate neutrophil directional migration positively and motility negatively. Both Rac and Rho are involved in microtubule-mediated suppression of motility, but how microtubules control directional migration remains elusive. One interesting correlation with the directional migration defects induced by microtubule disassembly is that nocodazole inhibits PI(3)K activation at the leading edge. PI(3)K regulates neutrophil motility through the regulation of both Rho and Rac (Van Keymeulen et al., 2006; Yoo et al., 2010). This implies the possibility that microtubule disassembly renders neutrophils blind to attraction signals due to inactivation of PI(3)K, which is indispensable for orchestrating cell migration upstream of Rho and Rac. In the current study, we addressed how microtubules regulate cell motility through Rho and Rac, but it is noticeable that both Rho and Rac also regulate microtubules dynamics (Palazzo et al., 2001; Takesono et al., 2010; Wittmann et al., 2004), suggesting intricate feedback systems among Rho, Rac and microtubules.

Beyond the insights of our findings to the basic mechanisms that regulate leukocyte migration, it is intriguing to note that many microtubule depolymerizing agents are clinically used as anti-inflammatory drugs to treat chronic inflammatory diseases (Cocco et al., 2010). However, the mechanisms by which microtubule depolymerizing drugs alleviate inflammatory symptoms are not known. Neutrophils from patients with autoinflammatory diseases show migration defects (Huttenlocher et al., 1995; Lokuta et al., 2005). Further, resolution of neutrophilic inflammation is accomplished by neutrophil reverse migration away from wounded tissues in zebrafish (Mathias et al., 2006; Yoo and Huttenlocher, 2011). Neutrophil reverse migration away from injured tissues has also been observed in mice (Woodfin et al., 2011). Taken together with our current findings that microtubule disassembly induces neutrophil motility *in vivo*, this suggests an intriguing paradigm that inflammatory diseases might occur due to leukocyte migration defects or retention of leukocytes at inflammatory sites, which could be resolved by microtubule disassembly-mediated neutrophil motility.

Here, for the first time, we have visualized the dynamics of microtubules during neutrophil migration in intact tissues *in vivo*. We demonstrated that the microtubule cytoskeleton suppresses neutrophil polarity and motility through negative regulation of

both Rho and Rac activity in a PI(3)K-independent manner. Together with our previous findings that PI(3)K regulates neutrophil motility through both the modulation of Rac-mediated protrusion and polarity of F-actin dynamics, we have now begun to understand the complex mechanisms that regulate 3D neutrophil migration *in vivo*. Undoubtedly, by using the genetically tractable and optically transparent vertebrate, zebrafish, we will be able to further illuminate the molecular mechanisms that orchestrate cell migration within complex tissues *in vivo*.

Materials and Methods

Zebrafish maintenance and general procedures

Adult AB zebrafish and larvae were maintained as described previously (Yoo et al., 2010). For live imaging or wounding assay, larvae were anesthetized in E3 containing 0.2 mg/ml Tricaine (ethyl 3-aminobenzoate, Sigma-Aldrich). To prevent pigment formation, some larvae were maintained in E3 containing 0.2 mM N-phenylthiourea (PTU, Sigma-Aldrich). For all experiments, 2.5–3 dpf larvae were used. When drugs were used, larvae were pretreated at least for 20 minutes before experiments (0.5–2 μ M nocodazole, 65 μ M LY294002). Neutrophils were stained with Sudan Black as previously described (Yoo et al., 2010; Yoo and Huttenlocher, 2011).

Morpholino injection

Three nanoliters of Rac2 MO (Deng et al., 2011) at a final concentration of 100 μ M was injected into 1-cell-stage embryos. Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5.0 mM HEPES pH 7.1–7.3] was injected as a control.

Plasmid construction and injection

GFP- α tubulin was subcloned into the backbone vector with the mpx promoter (Mathias et al., 2006), minimal tol2 elements (Urasaki et al., 2006) and a SV40 polyadenylation sequence as previously described (Yoo et al., 2010). EMTB-3xGFP (Faire et al., 1999; von Dassow et al., 2009), EB3-GFP (Hu et al., 2008; Stepanova et al., 2003), GFP-Tau (human Tau: IMAGE:40007445), mCherry-histone H2B (von Dassow et al., 2009), γ tubulin-GFP or Dendra2-Rho T19N (Yoo et al., 2010) was subcloned into the backbone vector with the lyz promoter (Kitaguchi et al., 2009), minimal tol2 elements (Urasaki et al., 2006) and a SV40 polyadenylation sequence. Three nanoliters of solution containing 12.5 ng/ μ l DNA plasmid and 17.5 ng/ μ l transposase mRNA was injected into the cytoplasm of one-cell stage embryos.

Image acquisition and analysis

Except data of Fig. 2 and supplementary material Movie 6, time-lapse fluorescence images were acquired in 2–3 dpf larvae with a confocal microscope (FluoView FV1000, Olympus) using a NA 0.75/20 \times objective or a NA 1.10/60 \times water immersion objective lens as previously described (Yoo et al., 2010). For images in Fig. 2 and supplementary material Movie 6, we used a Spinning disk confocal microscope (Yokogawa CSU-X) with confocal scanhead on a Zeiss Observer Z.1 inverted microscope (NA1.3/60 \times water immersion objective). A Photometrics Evolve EMCCD camera was used to acquire the images. Z-series images were acquired using a 0.4 μ M step size and 300 EM gain. Maximum intensity projection images were made using the Zen 2011 (blue edition) software (Carl Zeiss). Neutrophils were tracked and analyzed by using plugins MTrackj (3D tracking), Manual tracking (2D tracking) and Chemotaxis and Migration tool (ibidi) for ImageJ (NIH, Bethesda, MD). Neutrophil area and roundness were measured using 'analyze' function of ImageJ. To measure roundness, $4 \times \text{area} / \pi \times \text{major_axis}^2$ was calculated. Data of time-lapse images represent at least three separate movies.

Purification of human neutrophils and affinity precipitation assays

Blood was obtained from healthy donors with informed consent. Neutrophils were purified using Polymorphprep (Nycomed Pharma AS) as previously reported (Yoo et al., 2011). Purified neutrophils were suspended in PBS at $1.5\text{--}2.5 \times 10^7$ cells/ml. 1×10^7 Cells were pretreated with DMSO or 10 μ M nocodazole in a 37°C/5% CO₂ incubator for 30 minutes. Affinity precipitation assays of Rho and Rac were performed as previously described (Nishita et al., 2002). Briefly, cell activation was stopped by addition of equal volume of 2 \times lysis/kinase buffer for 20 min on ice. After centrifugation, the supernatant was mixed with 60 μ g GST-RBD or 15 μ g GST-PBD bound to glutathione-Sepharose (Cytoskeleton) and incubated at 4°C for 1 h. The bead pellets were washed three times with 1 \times lysis/kinase buffer, suspended in 35 μ l of Laemmli sample buffer, and analyzed by immunoblotting using an antibody to RhoA (Cell Signaling, 67B9) or Rac (Cell Signaling, no. 2465).

Statistics

Assuming Gaussian distribution of overall population of values, P values were derived by the following analyses. Two-tailed unpaired *t*-test: Fig. 3B–D. Two-tailed paired *t*-test: Fig. 7E. One-way ANOVA with Dunnett post-test: Fig. 3A. One-way ANOVA with Tukey post-test: Fig. 5B. One-way ANOVA with Bonferroni post-test: Fig. 7B,C.

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