Abstract

Title of Document: CELLULAR PATHWAYS INVOLVED IN EPITHELIAL-TO-MESENCHYMAL TRANSITIONS IN NEURAL CREST CELLS

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Abstract:

Neural crest cells are a population of multi-potent progenitor cells in the developing vertebrate embryo that undergo an epithelial-to-mesenchymal transition (EMT) and migrate extensively to generate diverse derivatives. As such, abnormal development of neural crest cells can lead to human congenital and hereditary malformations, diseases and cancers. Both internal molecular signals and external mechanical factors play essential roles in facilitating neural crest cell EMT. How cells modulate their adhesion machinery and dynamically reorganize their actin cytoskeleton to respond to the mechanical features of their external environment during EMT is not well understood. To evaluate the role of the actomyosin cytoskeleton during neural crest cell EMT and migration, midbrain neural folds that contain premigratory neural crest cells were dissected out from chick embryos, explanted into chamber slides, and incubated to allow for the formation of migratory neural crest cells. Time-lapse imaging technique was used to record cell behaviors. To elucidate cellular pathways controlling EMT and migration, chemical inhibitors (blebbistatin, Y-27632, latrunculin-A, and nocodazole) that perturb molecular cascades regulating cellular structures were employed. Effects of these perturbations on neural crest cell EMT and migration were quantified in terms of the spreading rate of the explants, and vorticity of collectively moving cell groups. We observed that blebbistatin treatment reduced the overall velocity of migratory neural crest cells to negligible levels. Moreover, migratory neural cells developed rounder cell bodies, and lamellipodia were transformed into filopodia at the periphery of the extract. Y-27632 treatment led to more neural crest cells coming out from these explants within a shorter time period compared to control. Nocodazole treatment blocked neural crest cell EMT and the resumption was dose-dependent. Latrunculin-A caused cell death at a very low concentration. These results implicate roles for non-muscle myosin II, the target of blebbistatin, and ROCK, the target of Y-27632, as well as microtubules and actin filaments, in chick midbrain neural crest cell EMT and migration. Actin crosslinkers such as α-actinin and actin-associated proteins like palladin also participate in pathways affected by these cytoskeletal inhibitors through their regulation of focal adhesion formation and cytoskeletal organization, thereby modulating cell stiffness and migration. We are also documented the distribution of α-actinin and palladin in migratory neural crest cells *in vivo*. Collectively, our studies have provided insight into specific cellular pathways regulating neural crest cell EMT and migration and the impact on various biophysical parameters upon perturbation of these pathways.

Cellular pathways involved in epithelial-to-mesenchymal transitions in neural crest cells

By

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Dedication

This thesis is dedicated to my grandfather who I never got to meet but miss a lot.

Acknowledgements

First and foremost I would like to thank my advisor, Dr. Lisa Taneyhill, for her guidance, extraordinary patience and support throughout my graduate studies. She has been an excellent mentor both personally and professionally. As her first international student, I truly appreciate her patience in correcting and shaping my academic altitude and techniques and her extra efforts in helping me improve my writing skills.

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I would also like to thank our collaborator, Christina Ketchum in Dr. Upadhyaya's lab, for her help in making those amazing movies and collecting quantitative data in my project.

Lastly I would like to extend thanks to my friends and family, especially my parents, Chuanfeng Li and Yanbin Li, my husband, Hao Cheng for providing love and support in my life. I would also like to thank my friends Yangyang Wang, Summer Hu, Meng Li, Christina Ketchum and Qiong Hu for providing support during my graduate study. I would also like to thank my sweet kitty, Oktoberfest (Oktty), for her company during my thesis writing.

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Chapter 1: Literature Review

1.1 Introduction

Early embryonic development is a crucial time period in which organisms form a highly structured, functioning body from a single, fertilized egg. Any abnormal development in this highly regulated process can lead to severe defects or be lethal. Different cell types with specific functions are generated and directed to their destinations under strict spatio-temporal control. During this process, the neural crest plays a critical role due to its multi-potent, transient, and migratory properties, and is the focus of my thesis research.

Neural crest cells undergo an epithelial-to-mesenchymal transition (EMT) to acquire motility so that they can migrate extensively to their target areas. Aggressive tumor cells share many characteristics with neural crest cells, contributing to the paradigm of tumor cell plasticity (Kasemeier-Kulesa, Teddy et al. 2008). Although we know that cell-cell and cell-extracellular matrix interactions must be altered to facilitate EMT and cell migration, the specific cellular pathways that are involved, and how these molecular signals function in this dynamic process, are poorly understood. In my thesis project, I explored the role of non-muscle myosin II (NM myosin II), Rho kinase (ROCK), actin and microtubules during neural crest cell EMT and migration. This project not only will give insight into neural crest cell EMT but will shed light on the potential pathways involved in tumor generation and progression.

In this study, we incorporate time-lapse imaging techniques to accomplish our goal of dynamically recording neural crest cell behaviors, under normal conditions and those in which the above pathways have been perturbed. We use chick embryos for our research model because they develop externally, allow easy access for neural crest cell dissection, and neural crest cells have been well characterized in them. Because of the

non-transparent 3D environment of the chick embryo, it is hard to track dynamic cell phase changes and movements associated with EMT *in vivo*. *In vitro* time-lapse imaging, however, allows us to easily record neural crest cell behaviors, providing us with a tractable experimental model with results translatable to neural crest cell EMT and migration *in vivo*.

1.2 An overview of the neural crest

The neural crest is a transient structure derived from the ectoderm. The development of the neural crest is strictly regulated by a series of events. The specification of the neural crest at the neural plate-ectoderm boundary occurs during gastrulation. In this process, a set of transcription factors, including Distalless-5 and Pax3/7, cooperatively keeps cells in the border region from becoming the neural plate or epidermis and induces a second set of transcription factors (FoxD3, Sox9, Id, Twist, and Snail) to direct the formation of the neural crest (Nikitina, Sauka-Spengler et al. 2009). Neural crest cells are multi-potent and thus give rise to a diverse array of derivatives, including sensory neurons and glia, skin pigment cells, portions of the heart, and the craniofacial skeleton (Nicole Le Douarin, Chaya Kalcheim 1999). Abnormal development of neural crest cells may lead to neurocristopathies or even cancers (Ramgolam, Lauriol et al. 2011)

1.3 EMT

Initially contained within the dorsal neural tube, neural crest cells undergo EMT and depart from their site of origin, migrating extensively throughout the embryo (Fig. 1). Premigatory neural crest cells need to lose intercellular tight and adherens junctions and reorganize their cytoskeleton in order to acquire motility, which is crucial for these cells to exit the dorsal neural tube. Neural crest cells then migrate towards their precise peripheral targets, allowing for the formation of a wide variety of derivatives and, eventually, a functioning vertebrate embryo.

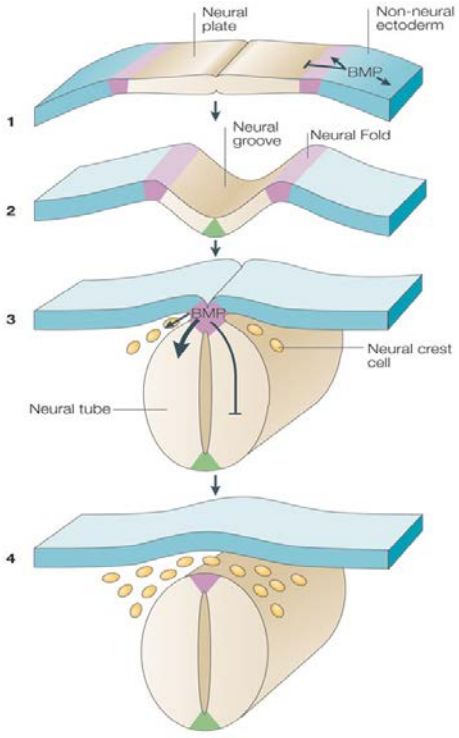

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Figure 1. Schematic illustrating the formation of the neural crest during chick neurulation. Adapted from (Liu 2005).

EMT is an orchestrated series of events in which cell-cell and cell-extracellular matrix (ECM) interactions are changed to release epithelial cells from the surrounding tissue. The cytoskeleton is reorganized to confer cells with the ability to move through a three-dimensional ECM, and a new transcriptional program is induced to maintain the mesenchymal phenotype (Fig. 2; (Kalluri and Weinberg 2009)).

The structural integrity of epithelial tissues is essential for the organism to maintain the protective barrier against environmental hazards externally and to create specific and physically controlled subdomains internally. Cell-cell interactions, including tight junctions, cadherin-based adherens junctions, gap junctions, and desmosomes connected to the intermediate filament cytoskeleton, collectively maintain epithelial structures. Integrins involved in cell-ECM interactions also help to maintain the epithelial state of the cell. Tissue polarity like apical-basal differences is also defined by cell-cell and cell-ECM contacts (Yeaman, Grindstaff et al. 1999). Many mesenchymal cells, however, do not have cell-cell contacts and defined cell polarity. Instead, mesenchymal cells have distinct cell-ECM interactions and cytoskeleton structures. At the same time,

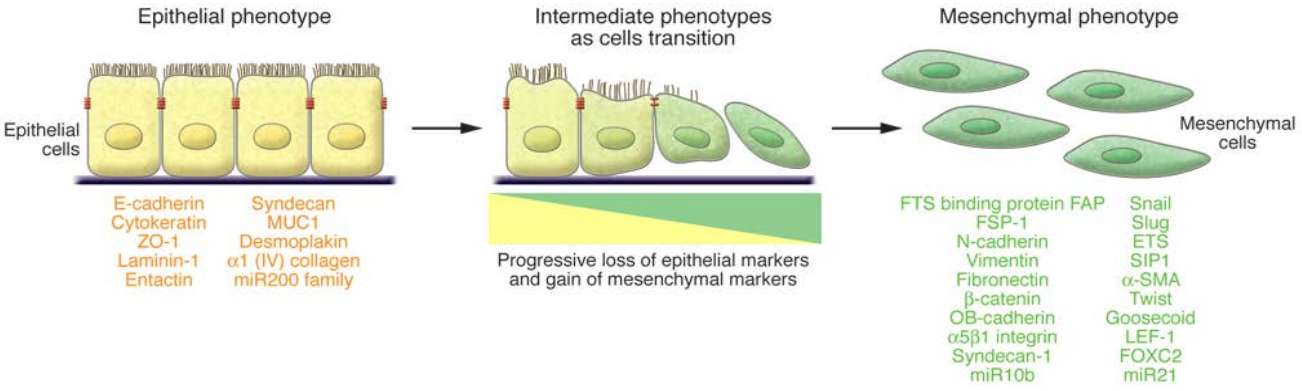

Figure 2. Schematic illustrating the morphology changes of cells and involved signal pathways during EMT. Through EMT, polarized epithelial cells transition into motile and ECM component–secreting mesenchymal cells. The figure above shows commonly used epithelial and mesenchymal cell markers. Adapted from (Kalluri & Weinberg, 2009).

mesenchymal cells produce signaling proteins to organize and remodel ECM, which, in turn, affects epithelial cells. These signaling proteins include but are not limited to growth factors of the epidermal (EGF), hepatocyte (HGF) and fibroblast (FGF) families, and transforming growth factor β (TGFβ) (Derek Radisky, 2005).

According to their different functions, EMTs are classified as three subtypes (Kalluri and Weinberg 2009) (Fig. 3). Type 1 EMTs play a role in many stages of development, including implantation and embryo formation (Zeisberg and Neilson 2009). This type of EMT neither causes fibrosis nor induces invasive phenotypes. Mesenchymal cells generated through type 1 EMTs have the potential to undergo a mesenchymal-to-epithelial transition (MET) to form secondary epithelia. For example, during delamination of the neural crest, a population of highly motile cells migrates to and incorporates into many different tissues (Radisky 2005). After arrival to their destinations, the cells may revert to their original epithelial phenotype through MET. Type 2 EMTs are associated with wound healing, tissue regeneration, and organ fibrosis (Zeisberg and Neilson 2009). Their initiation and continued occurrence depend on inflammation-inducing injuries. Once the provoking injuries or infections are removed, these EMTs cease. Type 2 EMTs can continue to respond to ongoing inflammation and eventually lead to organ destruction (Ivanova, Butt et al. 2008). Type 3 EMTs are related to cancer progression and metastasis. During carcinoma progression, epithelial cells lose their polarity and detach from the basement membrane (Zeisberg, Shah et al. 2005). Cell-ECM interactions and signaling networks are altered, while the composition of the basement membrane changes. EMT then occurs to facilitate the malignant phase of tumor growth. Type 3 EMTs are also involved in enabling cancer cells to enter the circulation and exit the blood stream at a remote site, where they may form micro- and macro-metastases, which may involve METs and thus a reversion to an epithelial phenotype (Zeisberg and Neilson 2009). It should be noted that the specific signals that determine EMTs to form in these three settings are not clearly defined. A common set of

genetic and biochemical elements, however, appears to underlie these diverse systems. Neural crest cells, as a highly invasive embryonic progenitor cell population, share many characteristics with cancer cells, making them a good model of tumor cell plasticity and invasiveness. (Kasemeier-Kulesa, Teddy et al. 2008).

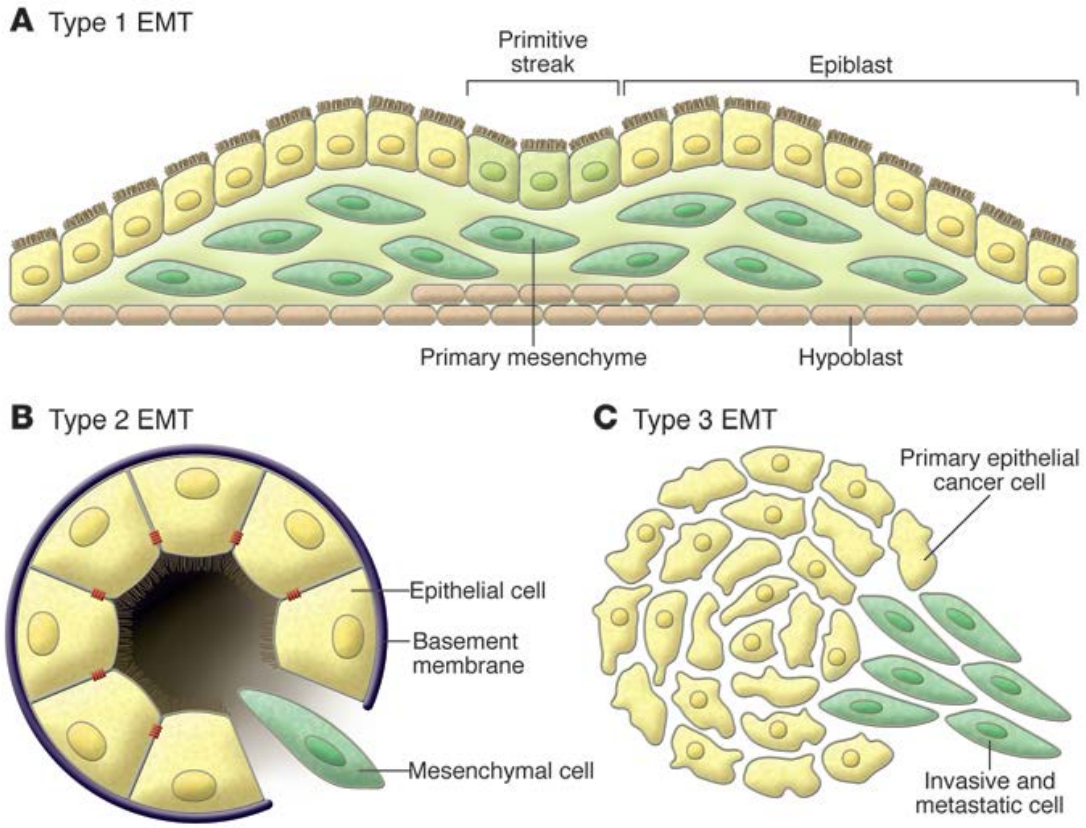

Figure 3. Classification of EMTs according to their biological functional differences. (A) Type 1 EMTs associate with implantation, embryogenesis, and organ development. (B) Type 2 EMTs are associated with tissue regeneration and fibrosis. (C) Type 3 EMTs occur in the context of tumor growth and cancer progression. Adapted from (Kalluri & Weinberg, 2009).

1.4 Neural crest cell migration

Negative and positive regulators collectively facilitate neural crest cell migration. Two main classes of these negative signaling molecules are ephrins and their Eph receptors and class 3 semaphorins and their neuropilin/plexin receptors (Baker and Antin 2003) (Mellott and Burke 2008). These signaling pathways induce the collapse of cell protrusions to prevent neural crest cells from entering specific zones. Neuropilins 1 and 2 (Nrp1/2) (Schwarz, Vieira et al. 2008) (Yu and Moens 2005) are expressed by cephalic neural crest cells and form a receptor specific for the class 3 semaphorins, allowing for Nrp1/2 binding. Some physical barriers like the otic placode/vesicle also prevent the entry of neural crest cells into specific regions in the head. Positive regulators are considered to fall into two categories: permissive factors, which promote general motility, and chemoattractants, which lead neural crest cells to specific regions. Molecular components of the ECM that support migration or increase motility are the examples of this first category. Putative chemoattractants must meet three criteria. First, the candidate should be expressed in neural crest cells; second, its loss should lead to cell dispersion, so that cells expressing the receptor for this factor develop a broader distribution than the control; and third, when assessed by time-lapse imaging, a localized source of the candidate should lead to directional movement (Theveneau and Mayor 2012). So far, the VEGF, PDGF and FGF family of signaling molecules ((McLennan, Teddy et al. 2010); (Trokovic, Jukkola et al. 2005); (Richarte, Mead et al. 2007)), the chemokine Stromal cell-derived factor 1 (Sdf1 or CXCL12) ((Fukui, Goto et al. 2007); (Olesnicky Killian, Birkholz et al. 2009)) and Cadherin-11, expressed in *Xenopus* neural crest cells (Kashef, Köhler et al. 2009), are potential neural crest cell chemoattractants. Interestingly, though, none of the candidates identified to date meets all three criteria described above.

1.5 Neural crest cell derivatives

Neural crest cells are an evolutionarily conserved cell population that gives rise to various cell types including pigment cells, craniofacial cartilage and the peripheral nervous system (Nikitina, Sauka-Spengler et al. 2009). In general, the type of derivative depends upon the axial level from which the neural crest cells originate and the time of their emigration from the neuroepithelium. For example, cephalic neural crest cells generated from the midbrain and rhombomere 1 and 2 will contribute to cranial ganglia and stop in a relatively dorsal position, while the subpopulations forming cartilage and bones of the face and neck will continue further migrating ventrally and invade the branchial arches. The cardiac neural crest cells, arising posterior to the otic placode from the anterior limit of rhombomere 4 and caudalward, migrate to the developing heart (Kirby and Hutson 2010), and the enteric crest, arising from somites 1 to 7, colonize the gut. The time of migration also influences the types of derivatives that neural crest cells form. Early migrating cranial neural crest cells populate the branchial arches to generate bone, cartilage, and connective tissue (skeletal structures), whereas the later wave of neural crest cells stays close to the central nervous system and generates the neurons and glia of the cranial ganglia (Graham, Begbie et al. 2004).

1.6 Role of intracellular molecules during cellular movement

Many cellular pathways have been suspected to participate in neural crest cell EMT and migration due to their primary roles in general cytoskeletal organization or cell motility. Fig. 4 shows interactions among four cytoskeletal components upon which we focused in our project. In addition, we performed immunohistochemistry for the actin crosslinking proteins α-actinin and palladin to further explore potential cytoskeletal changes after inhibitor treatment.

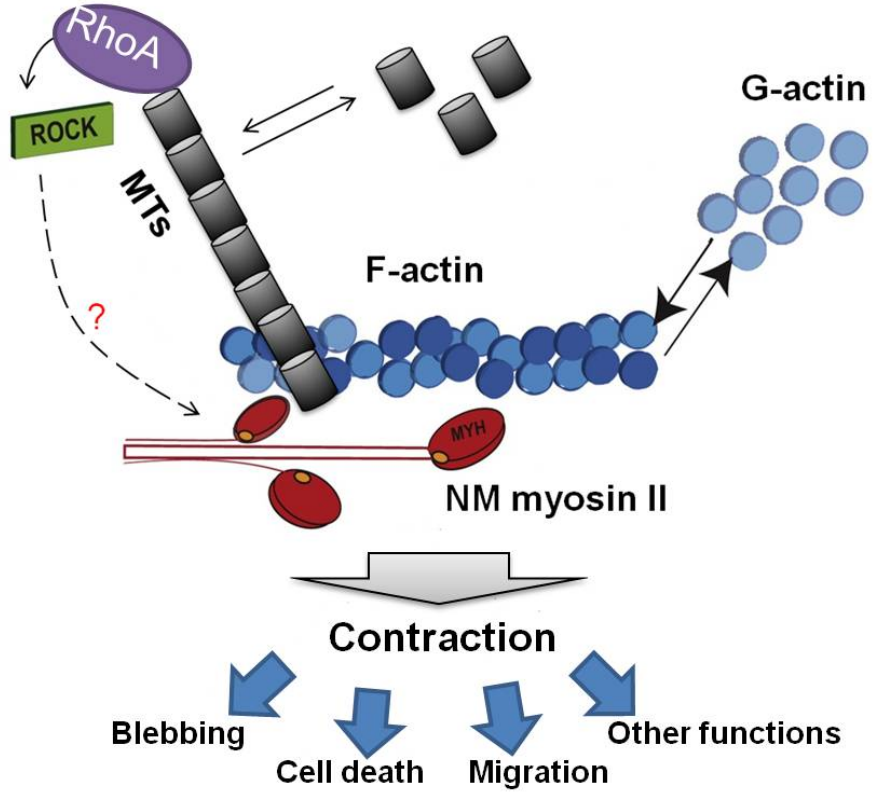

Figure 4. Cytoskeletal components interact with each other and facilitate cellular activities. The figure shows known interactions among myosin, actin, microtubule and ROCK.

***NM myosin II*:** NM myosin II is a myosin family member that binds to actin and has actin-linking and contractile properties. By phosphorylation of its light or heavy chains, NM myosin II controls cell adhesion, cell migration, and tissue architecture due to its position downstream of convergent signaling pathways (Vicente-Manzanares, Ma et al. 2009). Recent evidence implicates NM myosin II in neural crest cells. Berndt and Halloran (2008) showed that neural crest cells require blebbing or protrusive activity to undergo EMT in zebrafish (Berndt, Clay et al. 2008). They found that inhibition of myosin II or ROCK disrupted both blebbing and neural crest cell EMT. Myosin II contractility is also required for other forces generated in migratory cells, such as those exerted on focal adhesions (Mitchison and Cramer, 1996). Although the authors did not see an obvious effect of the myosin inhibitor blebbistatin on production of filopodia or lamellipodia, it remains possible that a significant effect of blebbistatin is instead on focal adhesion-related forces. In addition, the disassembly of apical junction complexes in calcium-depleted intestinal cells, which occurs through contractile F-actin ring intermediates, is blocked by blebbistatin treatment, indicating a requirement for NM myosin II activity in disassembly (Ivanov et al., 2004).

Rho: Rho proteins constitute a family of small signaling GTPases that reside within the Ras superfamily. Rho kinase (ROCK) is involved in many basic cellular processes that influence cell proliferation, differentiation, motility, adhesion, survival or secretion (Boureux, Vignal et al. 2007). ROCK leads to stabilization of actin filaments via LIM kinase and cofilin, and also leads to phosphorylation and activation of NM myosin II (Bresnick 1999) in some cell types. Liu and Jessell (1998) showed that inhibition of Rho by C3 exotoxin in chick neural tube explants caused a decrease in the number of migratory neural crest cells without affecting neural crest cell induction (Liu and Jessell 1998). Moreover, Rho inhibition did not affect neural crest cells that had already initiated migration, suggesting that Rho signaling may specifically alter EMT and not the ability of

neural crest cells to migrate. In contrast, a recent *in vivo* study showed that RhoA inhibition caused a decrease in filopodial retractions and cell velocity in migrating post-otic neural crest cells, suggesting that Rho signaling plays a significant role in later adhesion-based migration (Rupp and Kulesa 2007), at least in this subpopulation of neural crest cells. Shoval et al. (2012) reported that while Rho disappears from neural crest cell membranes upon delamination, active Rac1 becomes apparent in lamellipodia of mesenchymal cells (Shoval and Kalcheim 2012). Loss of Rac1 function at chick trunk levels inhibited neural crest cell migration but did not prevent emigration that is associated with N-cadherin down-regulation and the G1/S transition. Furthermore, inhibition of Rho stimulated premature Rac1 activity and consequent formation of lamellipodia, leading to neural crest cell migration (Groysman, Shoval et al. 2008). Recently, Clay and Halloran (2013) showed that Rho is activated in a discrete apical region of premigratory neural crest cells during EMT, and Rho-ROCK signaling is essential for apical detachment and generation of motility within the neuroepithelium (Clay and Halloran 2013). Previous conflicting results regarding Rho functions may be due to different species and/or axial levels examined. The role of ROCK pathway in chick cranial neural crest cell EMT and migration, however, remains unknown.

***Actin*:** Actin is the most abundant protein in many eukaryotic cells and is highly conserved among different organisms (Schmidt and Hall 1998). The globular monomeric form, G-actin, can reversibly assemble to form filamentous actin (F-actin) via a process controlled by a large number of actin-binding proteins. The functions of actin have been well studied in various cellular processes such as cell motility, cell division, muscle contraction, and cytokinesis (Schmidt and Hall 1998). Deletion of Integrin-linked kinase (ILK), a major regulator that links integrins to the actin cytoskeleton, from mouse neural crest cells results in embryonic lethality during late gestation (Arnold, Zang et al. 2013). This indicates actin, as the building block of cytoskeleton, plays an essential role during

the development of neural crest cells.

***Microtubules*:** Microtubules are filamentous structures created by self-assembly of α/β tubulin heterodimers (Nogales 2001; Amos 2004). Similar to F-actin microfilaments, microtubules are polarized by having fast-growing "+" and slow- growing "-" ends. In columnar epithelial cells, prominent bundles of microtubules align along the lateral plasma membrane. This population of microtubules orient their minus ends toward the cell apex and plus ends toward the cell base (Ivanov, McCall et al. 2006). The perijunctional space of differentiated epithelial cells is rich in microtubules (Ivanov, McCall et al. 2006). Microtubules appear to be involved in neural crest cell migration indirectly, since directional cell migration requires polarized alignment of the cytoskeleton with the microtubule organizing center. Francis et al. (2011) reported that knock-out mice deficient in the gap junction gene *connexin43* exhibit developmental anomalies associated with abnormal neural crest and proepicardial cell migration. These migration defects are due to a loss of directional cell movement and are associated with abnormal actin stress fiber organization and a loss of polarized cell morphology (Francis, Xu et al. 2011). Microtubules also interact with cadherins, core transmembrane proteins in adherens junctions, which play a critical role during neural crest cell EMT (Coles, Taneyhill et al. 2007; Brieher and Yap 2013).

Palladin: Palladin is an actin-associated protein that plays an essential role in maintaining cell morphology and cytoskeletal organization in different cell types (Goicoechea, Arneman et al. 2008). Palladin binds directly to actin-regulating proteins (e.g., Vasodilator-Stimulated Phosphoprotein (VASP), profilin, CLP36, lipoma preferred partner (LPP), and Eps8) and actin-crosslinking proteins (e.g., α-actinin, Lasp-1, and ezrin). The level of palladin expression appears to be tightly regulated by cells and is dramatically altered during EMT and cellular differentiation (Goicoechea, Arneman et al.

2008). Palladin and α-actinin co-localize in focal adhesions and stress fibers (Parast and Otey 2000), which consist of bundles of crosslinked actin and myosin. Palladin functions in maintaining adherens junctions, facilitates the formation of podosomes in migrating cells (Goicoechea, Bednarski et al. 2008), and, along with α-actinin in the mouse, is expressed in the proper spatio-temporal pattern to play a role in neural crest cell ontogeny (Luo, Liu et al. 2005; Tomita, Matsumura et al. 2005). The *palladin* knock-out mouse is embryonic lethal due to severe defects in cranial neural tube closure and herniation of the liver and intestine (Luo, Liu et al. 2005). These studies in both cultured cells and knock-out mice suggest that palladin's actin-organizing activity plays a central role in promoting cell motility. Whether palladin is an integral part of neural crest cell EMT, however, remains unclear.

***α-Actinin*:** α-Actinin is an actin-crosslinking protein that functions as a platform for the assembly of multi-protein complexes, and is uniquely positioned as an anchor between the actin cytoskeleton and the cytoplasmic domains of several cell surface adhesion proteins. α-Actinin is a ubiquitous protein, belonging to a highly conserved family of actin-binding proteins, the spectrin superfamily, which also contains the spectrins and dystrophin (Blanchard A, Ohanian V, et al 1989). α-Actinin isoforms have been found and characterized genetically and/or biochemically from a large variety of taxa (Sjöblom, Salmazo et al. 2008). Through alternative splicing and gene duplication, mammalian cells produce at least six different protein products from four *α-actinin* encoding genes, each found within a specific tissue type and expression profile (Sjöblom, Salmazo et al. 2008). Non-muscle actinin-1 is believed to play an essential role in regulating cell shape and cell motility (Otey et al., 1990, 1993; Glück et al., 1993; Glück and Ben-Ze'ev, 1994; Knudsen et al., 1995), while actinin-4 is associated with cell motility and cancer cell invasion (Honda, Yamada et al. 1998). Aside from its interactions with actin filaments, α-actinin has emerged as a major platform for a number of protein-protein interactions with many

cytoskeletal and regulatory proteins. There is no direct evidence showing how α-actinin functions during neural crest cell EMT.

In the following chapters, I detail my experiments aimed at examining the role of known molecular cascades regulating cell migration and/or cytoskeletal elements during neural crest cell EMT and migration. I have also examined the distribution of palladin and α-actinin pre- and post-pathway perturbation. My results have provided insight into specific cellular pathways regulating neural crest cell EMT and migration and the impact on various biophysical parameters upon pathway perturbation.

Chapter 2: Materials and Methods

Chicken Embryos

Fertilized chicken eggs (*Gallus gallus)* were obtained from B&E and Hy-Line North America, L.L.C., and incubated at 37° C in humidified incubators. The embryos were staged according to the number of somite pairs (somite stage, ss) and removed from the yolk sac, using forceps and scissors. The embryos were kept in Ringer's solution on ice before dissection.

Neural Crest Cell Explants

Neural fold tissue, containing premigratory neural crest cells, from 2 to 5 ss embryos was dissected out using tungsten needles in PB-1 standard medium and explanted in 8-well chamber slides coated with a 1:100 dilution of poly-L-lysine (Sigma) in cell culture water, followed by fibronectin (BD Bioscience) (Coles, Taneyhill et al. 2007)(Fig. 5). The collected tissues were incubated in cell culture media (DMEM with N2 supplement (GIBCO-BRL), penicillin, and streptomycin) at 37° C in humidified incubators. Depending upon the research aims; incubation time was shortened or extended. Cell culture media were gradually changed to L15 (Invitrogen), a CO_2 independent media, before imaging.

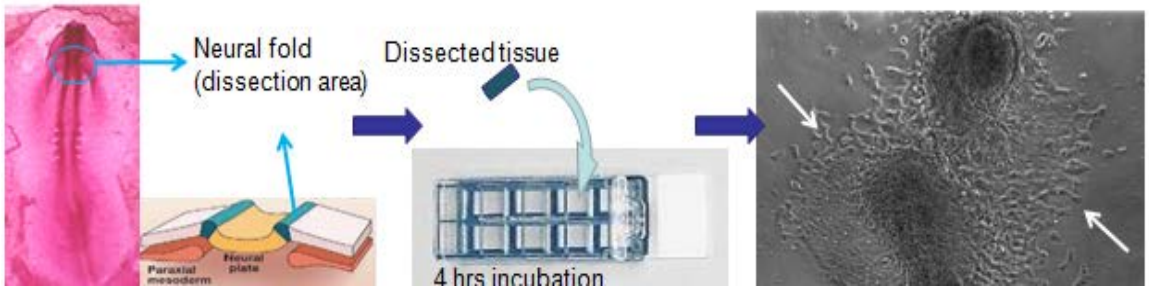

Figure 5. Neural fold explant assay. Neural fold tissues were dissected out and incubated in a chamber slide to allow for the formation of migratory neural crest cells for fixed and live imaging studies.

Applications of inhibitors and time-lapse Imaging

To examine effects on neural crest cell EMT, chemical inhibitors ((blebbistatin (Sigma-Aldrich), Y-27632 (Selleckchem), latrunculin A (Sigma-Aldrich) and nocodazole (Sigma)) were applied after an hour of explant incubation to first allow for the adherence of tissues to the bottom of slides (pre-incubation) (Fig. 6). Inhibitors were then added before imaging. The inhibitors were removed by washout if any cell behavioral changes were observed in their presence. Imaging was continued for another 6 hours or until cell death was noted. Pre-incubation time was extended to 2 to 4 hours to permit EMT when effects of inhibitors on neural crest cell migration were examined (Fig. 7). Inhibitors were added after 2 to 3 hours of imaging to make sure that cell migration had already commenced. If any cell behavior changes were observed, a washout was performed and cells continued to be imaged. Phase-contrast images were collected using an inverted microscope (TE2000 PFS, Nikon, Melville, NY) with a cooled CCD camera (Coolsnap HQ2, Photometrics, Tucson, AZ) and a 10x 0.30 NA objective lens. Images were collected at a frame interval of 30 seconds.

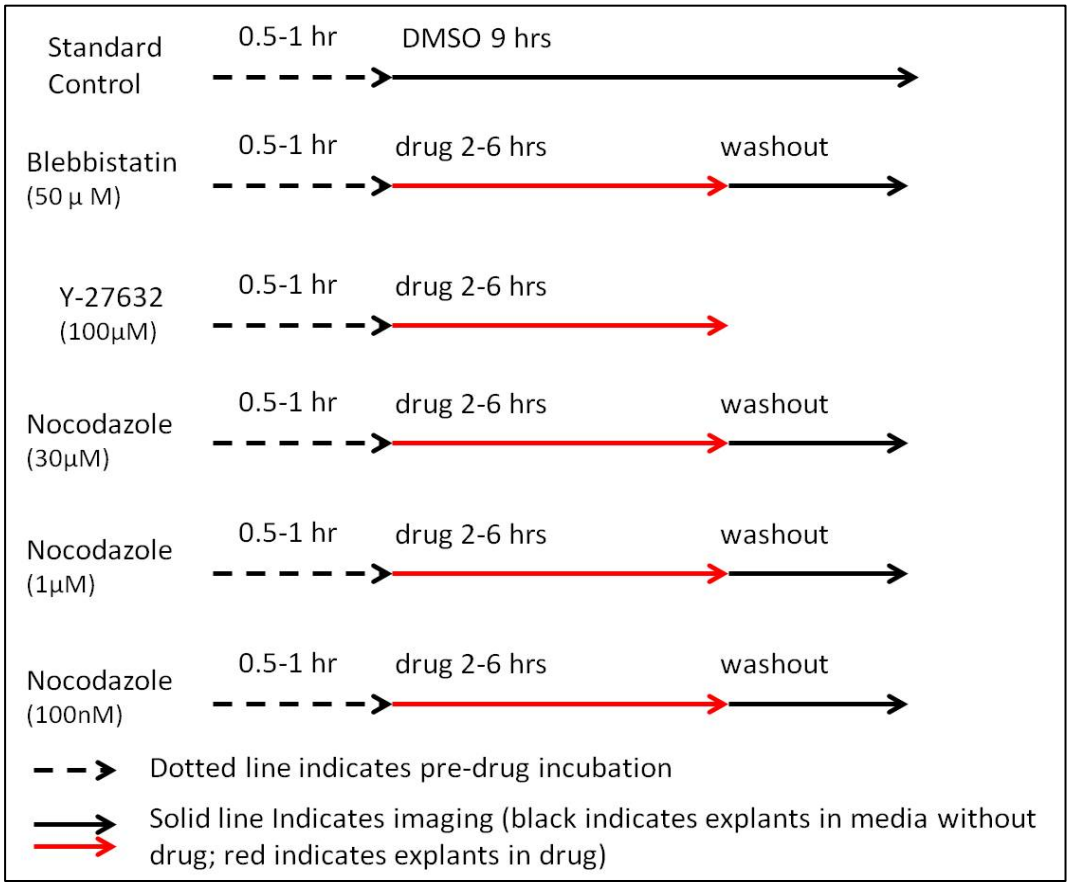

Figure 6. Experimental set-up for neural crest cell EMT assay. To examine perturbation effects on NCC EMT, inhibitors were added within an hour of incubation and removed if cell behaviors changed. 2mM blebbistatin and 5mM Y-27632 stocks were diluted to working concentrations in L15. Nocodazole stock was first diluted to 1mM using DMSO and further diluted to working concentrations.

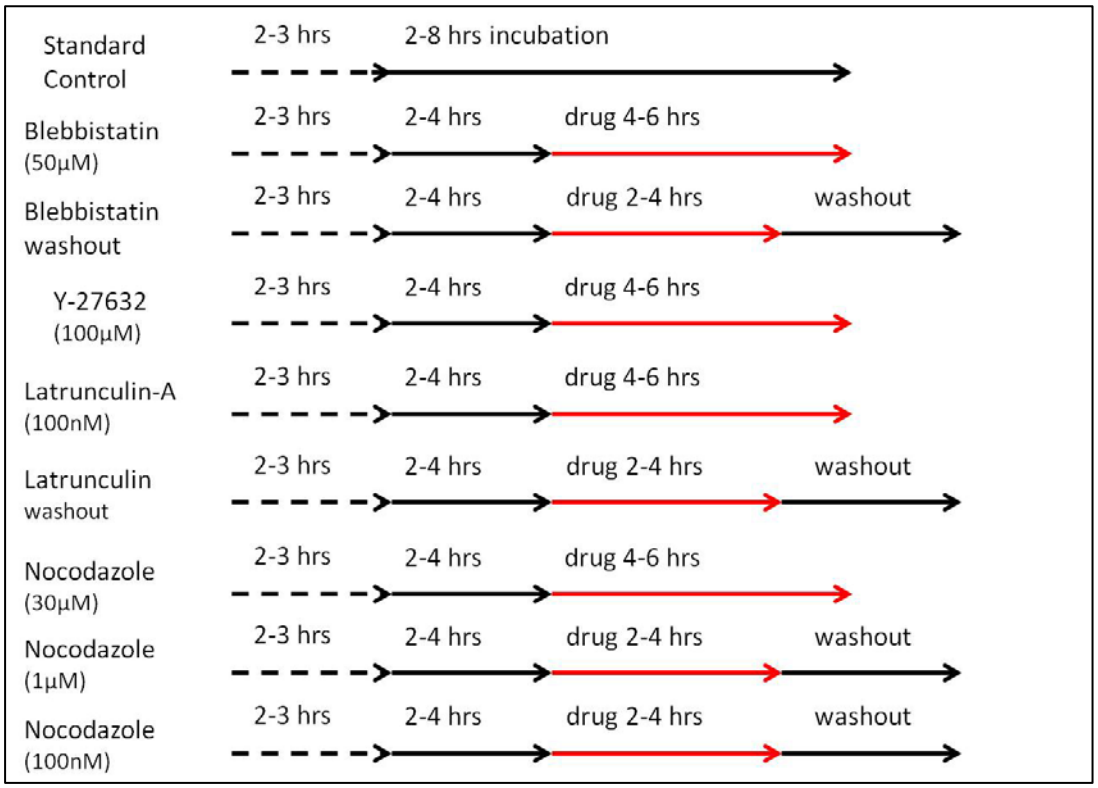

Figure 7. Experimental set-up for neural crest cell migration assay. Inhibitive effects on NCC migration were tested by recording cell behaviors using time-lapse imaging after adding inhibitors. A washout was performed if cell behaviors changed. 2mM blebbistatin and 5mM Y-27632 stocks were diluted to working concentrations in L15. Nocodazole stock was first diluted to 1mM using DMSO and further diluted to working concentrations. 10mM latrunculin-A was diluted to 100nM in L15.

Immunohistochemistry

Neural crest cell explants were fixed with cold 4% paraformaldehyde overnight and washed twice with 0.03% Triton X-100 in 1x PBS, 20 minutes each. To fix cells after blebbistatin treatment, the same concentration of blebbistatin used in the experiment was maintained with the fixative so that the cell morphology phenotype observed in the presence of blebbistatin could be captured. Antigen retrieval was then performed by incubating cells in 10mM sodium citrate at 96 ° C for 20 minutes. After cooling to room

temperature, the cells were rinsed with 1x PBS, blocked for 1 hour in 1x PBS containing 2% BSA and then incubated with primary antibodies (anti-palladin rabbit polyclonal antibody (1:800), Proteintech; anti-α-actinin mouse monoclonal antibody (1:500), Sigma) diluted in blocking solution overnight at 4 ° C. Cells were washed four times for 20 minutes with 0.03% Triton X-100 in 1x PBS. Secondary antibodies (Alexafluor 488 goat anti-rabbit IgG (1:1000) and Alexafluor 594 goat anti-mouse IgM (1:500), both from Invitrogen) were diluted in blocking solution and applied. Cells were then washed and stained with 4',6-diamidino-2-phenylindole (DAPI) to mark cell nuclei before being mounted with Aqua Poly/Mount. Samples were analyzed under the fluorescence microscope (Zeiss) or confocal microscope (Leica) at either 594 nm or 488 nm. Adobe Photoshop 9.0 was used to process images.

Video Analysis

Andor (South Windsor, CT) imaging software was used to make kymographs to calculate explant spreading. Kymographs made along a radial line across the advancing explant. Speed calculated from the slope of the kymograph edge as indicated in Figure 8. The velocity is measuring the speed of cell migration at the edge of the explants. PIV analysis was done using MATLAB (Mathworks, Natick MA) image analysis package PIVLab (http://pivlab.blogspot.com/). PIV analysis was used to calculate cell density, vorticity, and coherence of motion. Vorticity is an indicator of how much curl a field of vector displays (Fig 9). The live cell imaging and quantitative analysis was performed in Dr. Arpita Upadhyaya's lab in collaboration with graduate student Christina Ketchum.

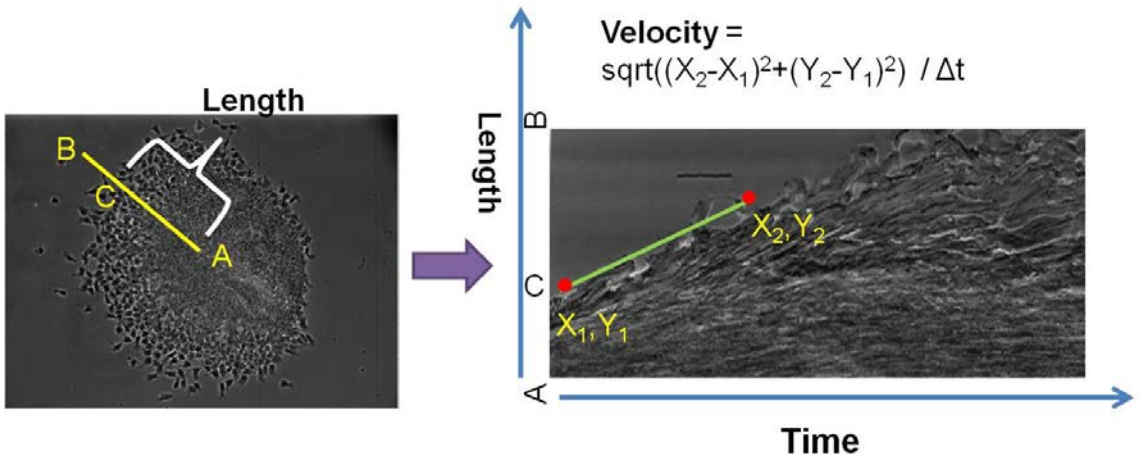

Figure 8. Measurement of velocity. Kymographs made along a radial line across the advancing explant. Speed calculated from the slope of the kymograph edge

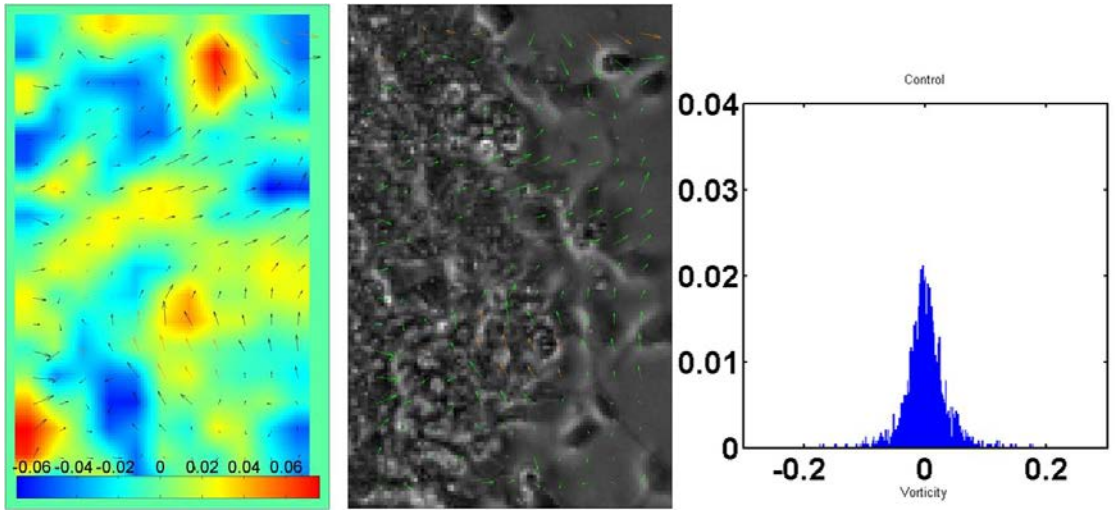

Figure 9. Measurement of vorticity. Heat Map plot of vorticity of a field of cells. Negative values correspond to counterclockwise curl. Positive values correspond to clockwise curl. Figure on the right shows a histogram of vorticity. The closer a distribution is to 0, the less curling and more linear the movement of the cells

Chapter 3: Cellular pathways involved in neural crest cell EMT

3.1 Inhibition of non-muscle myosin II by blebbistatin irreversibly blocks EMT

To first elucidate standard cell behaviors characterizing neural crest cells undergoing EMT, we performed time-lapse imaging on control-treated (DMSO) neural folds. Dissected tissue was preincubated in cell culture media for 0.5-1 hour at 37˚C to allow adherence to the fibronectin-coated chamber slide. Cell culture media was gradually changed to L15 (colorless CO_2-independent media) before imaging. Our observations indicate that it took approximately 2 hours for the first few cells to break cell-cell adhesions and become mesenchymal, as seen by the emergence of individual cells from the neural folds at a velocity of 0.3743μm/min (Fig.14), which reflects the speed of cell migration on the edge of the explants. As time passed, more cells proceeded through EMT and became individual mesenchymal cells (Fig. 10 and Movie 1 in supplemental data). We define the neural fold tissue and migratory neural crest cells emerged from the neural folds together as explants.

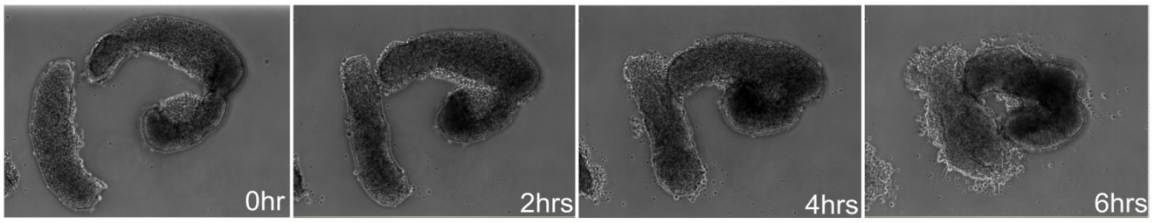

Figure 10. Cells undergo EMT and migrate out from control-treated (DMSO) neural folds. Successive panels show cells gradually emerging from neural fold tissue and undergoing migration.

To determine if NM myosin II is involved in neural crest cell EMT, we added blebbistatin (50μM) after attachment of neural folds. Blebbistatin binds to the ATPase intermediate with ADP and phosphate bound at the active site, and it slows down phosphate release (Kovács, Tóth et al. 2004). Time-lapse imaging showed that neural crest cell EMT was suspended (movie 2 in supplemental data). During the 2.5 hours of incubation in blebbistatin, no cells emerged from the neural fold (Fig. 11A), and the velocity of cells on the edge of the neural fold decreased to 0.0182μm/min (Fig. 14), which is a 20-fold decrease compared to controls and is statistically significant ($P < 0.05$). Cells appeared to be moving around within the neural fold, however, indicating that cells retained their motility at a certain level without properly functioning NM myosin II.

We then removed this block by washing out blebbistatin to examine its effect on EMT (Fig. 11B). Intriguingly, the overall motility of cells was increased to 0.3917μm/min, which is comparable to the controls (Fig. 14), as evidenced by an increasingly changed neural fold shape. Even 5 hours post-washout, not a single cell showed signs of mesenchymal morphology, indicating neural crest cell EMT failed to resume after the removal of blebbistatin. It is important to note, however, that the failure of EMT may be due to effects of residual blebbistatin not removed upon washout. Although the same washout successfully allowed for cell motility to resume in blebbistatin treatment of migrating neural crest cells (detailed in Chapter 4), the epithelial cells in the neural folds may be more sensitive to blebbistatin compared to migrating neural crest cells. Furthermore, washout of blebbistatin in the migration assay caused migratory crest cells to move towards the neural folds (see Chapter 4). It is possible that the same mechanism also is directing cell behavior during EMT because no neural crest cells emerged from the neural folds. Such explant contraction after washout was not as easily observed in our experimental set-up.

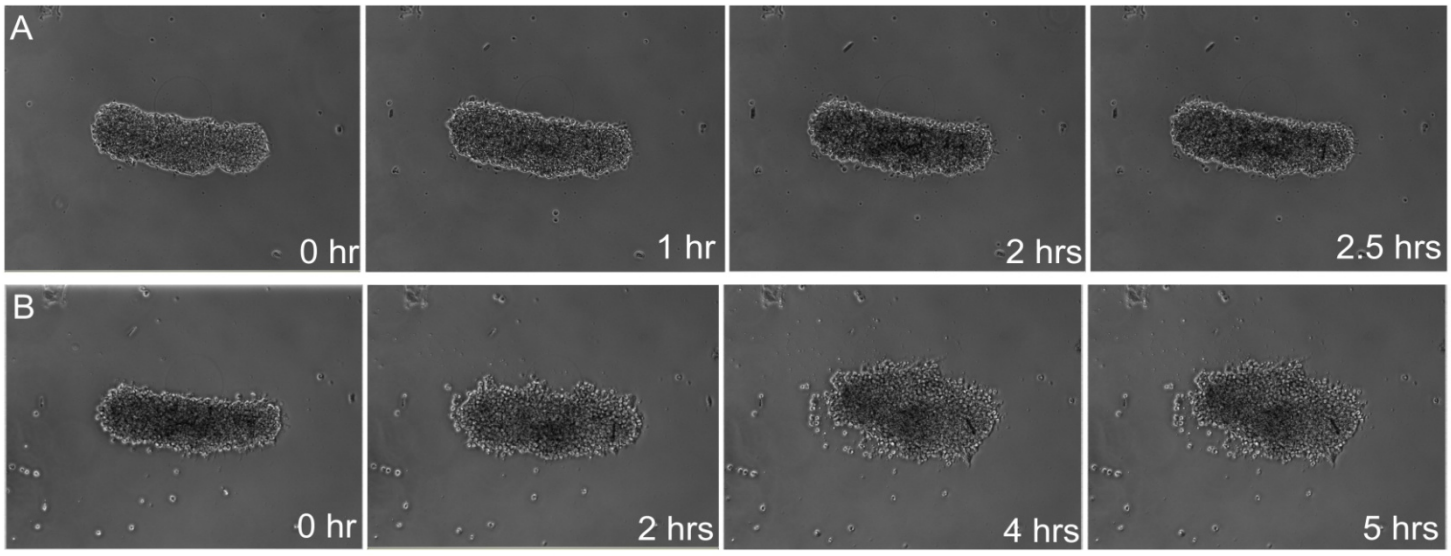

Figure 11. Blebbistatin treatment irreversibly blocks neural crest cell EMT. Panel A shows snapshots from time-lapse imaging recording cell behavior after adding blebbistatin. During 2.5 hours, no cells had come out of the explant. Panel B shows cell behaviors after removal of blebbistatin (washout).

In zebrafish, blebbistatin treatment in the hindbrain significantly decreased the number of cells undergoing EMT but did not give an obvious effect on production of filopodia or lamellipodia (Berndt, Clay et al. 2008). Our observations also show that blebbistatin treatment of midbrain neural crest cells decreases or even blocks EMT. This confirms that neural crest cells need NM myosin II to facilitate EMT.

3.2 Inhibition of ROCK by Y-27632 treatment promotes neural crest cell EMT

We tested the role of ROCK in neural crest cell EMT by employing the ROCK pathway inhibitor Y-27632. Y-27632 inhibits the ROCK by binding to the catalytic site (Ishizaki, Uehata et al. 2000). ROCK, as a downstream effector of Rho GTPases that regulates cytoskeletal dynamics in many aspects of cell motility (Raftopoulou and Hall 2004; Jaffe and Hall 2005), leads to stabilization of actin filaments via LIM kinase and cofilin, and also leads to phosphorylation and activation of NM myosin II (Bresnick 1999) in some cell types. Thus inhibition of ROCK not only allows us to test the function of this signaling pathway in controlling cell behaviors during EMT, but also may provide a means to inhibit NM myosin II activity and potentially corroborate our blebbistatin results. Our findings, however, indicate that at least in chick cranial neural crest cells, the ROCK pathway is unlikely to be functioning through NM myosin II because drastically different cell behaviors were observed in blebbistatin-treated versus Y-27632-treated neural folds.

Using the same experimental set-up, we noted that more neural crest cells migrated out in Y-27632-treated neural folds than in the controls (Fig. 12B, 4 and 6 hours). Within the first 2 hours, there is an apparent difference between Y-27632 treatment and control treatment (Fig. 12A) in that very few cells emerged from control-treated neural folds while a large number of cells migrated out from neural folds immersed in Y-27632. In the following 4 hours, many neural crest cells completed EMT and became migratory with a velocity of 0.4007μm/min, which is drastically greater than the velocity of cells immersed in blebbistatin (0.0182μm/min) and slightly higher than in controls (0.3743μm/min). Although there is no significant difference in the velocities between Y-27632-treated cells and the controls, more mesenchymal neural crest cells appeared to emerge from Y-27632-treated neural folds qualitatively (Fig. 12B, 4 and 6 hours, movie 3 in supplemental data). This suggests that Y-27632 treatment may promote neural crest cell EMT.

Interestingly, our observations in midbrain neural crest cells are not in agreement

with findings reported for later stage (10ss-18ss) zebrafish hindbrain neural crest cells, probably because hindbrain neural crest cells migrate through a different external environment (through the rhombomeres), which possess a different molecular milieu than the midbrain. This study showed that treatment with a ROCK inhibitor led to a reduction of neural crest cell EMT (Berndt, Clay et al. 2008) as assessed by *in situ* hybridization using *dlx2*, a marker of migratory neural crest cells. This lab also showed that inhibition of ROCK upon C3 exotransferase disrupted neural crest cell EMT in zebrafish (Clay and Halloran 2013). In agreement with a more recent finding in chick trunk neural crest cells that disrupting RhoA signaling induces migration of post-otic neural crest cells (Rupp and Kulesa 2007), we showed that in midbrain neural crest cells, inhibition of the ROCK pathway also induced more cells to undergo EMT.

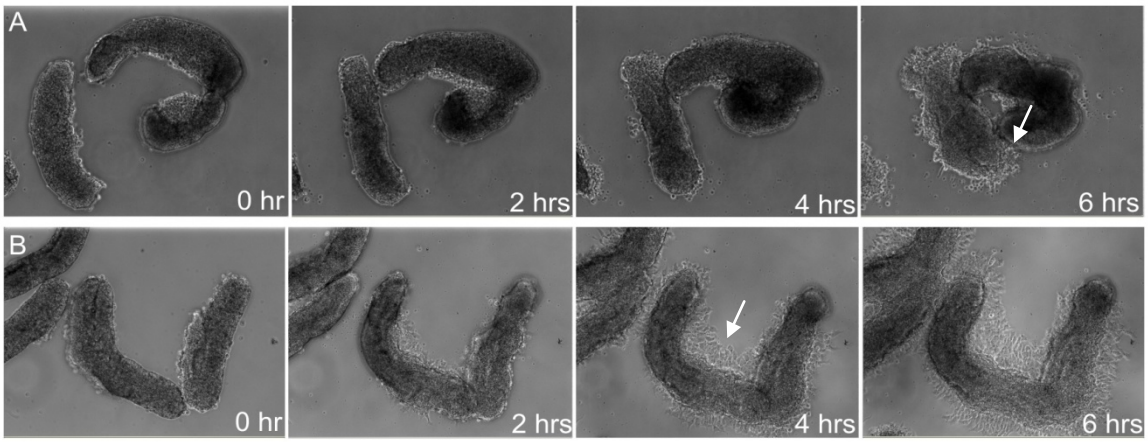

Figure 12. Y-27632 treatment promotes neural crest cell EMT. Panel A shows neural crest cells undergo EMT and migrating over 6 hours. Panel B shows more cells migrating out after Y-27632 treatment within the same period of time. Arrows show cells that migrated out.

3.3 Microtubule function is important for proper neural crest cell EMT

It has been reported that microtubule dynamics, along with RhoA, play an important role in EMT during chick gastrulation. In this example, embryos treated with nocodazole, a specific inhibitor of microtubules, showed a premature breakdown of the cell basement membrane, a crucial step in EMT (Nakaya, Sukowati et al. 2008). Microtubules also actively interact with cadherins, core transmembrane proteins in adhesion junctions, which play a critical role during EMT in neural crest cells (Coles, Taneyhill et al. 2007; Brieher and Yap 2013). Whether microtubules are involved in controlling neural crest cell EMT, however, is not known. To address this question, we employed nocodazole to perturb the polymerization of microtubules. Nocodazole binds to microtubule subunits and prevents heterodimers from repolymerizing (Saunders and Limbird 1997). A high concentration of nocodazole (30μM) results in the depolymerization of the whole microtubule lattice and affects cell junctions. Significantly lower concentrations (100nM) inhibit the dynamic activity of microtubules' plus ends (Nakaya, Sukowati et al. 2008) and can cause a decreased rate of locomotion in NRK fibroblasts cells by more than 60% but does not show a qualitative change in MT distribution by immunofluorescence (Liao, Nagasaki et al. 1995). As such, employing a range of concentrations of nocodazole in our study may give us different results through its differential effects. To test our hypothesis, we chose nocodazole concentrations of 30μM, 1μM, 100nM.

Using the same experimental set-up, we noted that no/very few neural crest cells emerged from neural folds treated with high concentrations of nocodazole (Fig. 13A). Moreover, the velocity of the cells was 0.0208μm/min (Fig. 14), 18-fold lower than that observed for cells in controls and thus significantly different. Neural folds became flat, with cell movements occurring within them (movie 4 in supplemental data). It was hard to determine if the cells were still alive by the end of the treatment because cells stayed within the neural folds. Nevertheless, we performed a washout experiment to assess effects after removal of nocodazole. After washout of 30μM nocodazole (Fig. 13B), we

observed a neglible number of cells emerging from the neural folds. Many cells appeared to be rounding up, probably dying (based upon their morphology) and spread out with a velocity of 0.1713μm/min (Fig. 14). This indicates that high concentrations of nocodazole may irreversibly block neural crest cell EMT. It is also possible that such high concentration of nocodazole inhibited many cellular processes, including EMT, which caused most cells to die and led to the failure of neural crest cell EMT in the presence of nocodazole and after washout.

In the1μM nocodazole treatment, the neural fold cells spread with a velocity of 0.3748μm/min (Fig. 14), comparable to the controls. The boundary of the neural folds became blurry, indicating cells were emerging from the neural folds (movie 5 in supplemental data). A few cells were able to come out, but they could not maintain their mesenchymal morphology as they rounded up shortly after emerging from the neural fold (Fig. 13C and movie 5), indicating that perhaps nocodazole affected EMT by interrupting the maintainence of the mesenchymal cell shape. It is also possible that cells were rounding up because they were dying. Washout experiments showed a decreased cell velocity (0.1227μm/min; Fig. 14); however, some cells were able to come out as individual mesenchymal cells and kept migrating. Most cells rounded up and died after 4 hours (Fig. 13D). This suggests that the block to neural crest cell EMT from 1μM nocodazole can be partially released, but this concentration may still be toxic to the cells and eventually cause cell death. The velocity of cells was greater in the presence of 1μM nocodazole than after washout. This suggests that lower concentrations of nocodazole may facilitate neural crest cell migration during EMT via some pathway that compensates for the perturbation of microtubules. Similar results were observed in migration assays with more significant data (detailed in Chapter 4).

100nM nocodazole treatment resulted in only a few cells emerging from the neural folds (Fig. 13E), but the effect is reversible after removal of nocodazole (movie 6 in supplemental data). The velocity of cells was 0.4560μm/min (Fig. 14) in the presence of

nocodazole, higher than the cells in controls (0.3743μm/min) and the cells in 1μM nocodazole (0.3748μm/min), but only a few cells came out from the neural folds (Fig. 13E). After washout, the velocity was unchanged (0.4534μm/min; Fig. 14) and still higher than the controls, but it appeared that more individual cells came out (Fig. 13F). These results imply that the partial block of EMT is removed after washout. Given that this low concentration of nocodazole has been observed to disrupt the plus end of microtubules, and that we observe a higher velocity than the control explants, this result indicates that the plus end of microtubules may be involved in neural crest cell EMT and that microtubules may play a significant role in regulating neural crest cell EMT.

In summary, 30μM nocodazole irreversibly blocks neural crest cell EMT, and may do so by causing cell death; 1μM nocodazole reversibly blocks neural crest cell EMT but causes cell death eventually; and 100nM nocodazole partially blocks neural crest cell EMT but the block can be removed by washout. These results indicate that microtubules are involved in neural crest cell EMT and that the resumption (or lack thereof) of neural crest cell EMT after nocodazole treatment is dose-dependent and/or could be absent due to lethal effects of nocodazole at higher concentrations.

We also examined the effects of the inhibition of actin polymerization by latrunculin-A treatment on neural crest cell EMT. Latrunculin A sequester monomeric actin in living cells (Yarmola, Somasundaram et al. 2000). Results looking at neural crest cell migration, however, showed that even very low concentrations of latrunculin A (100nM) irreversibly disrupted cell migration and led to cell death. Based on this result, we did not treat with latrunculin-A and document effects on EMT, since the effects of latrunculin A were very harsh to neural crest cells.

As summarized in Table 1, blebbistatin treatment irreversibly blocks EMT, Y-27632 treatment promotes EMT, the effect of nocodazole on EMT is dose-dependent, and latrunculin A severely impairs EMT and migration. This suggests NM myosin II, ROCK and microtubules are involved in neural crest cell EMT.

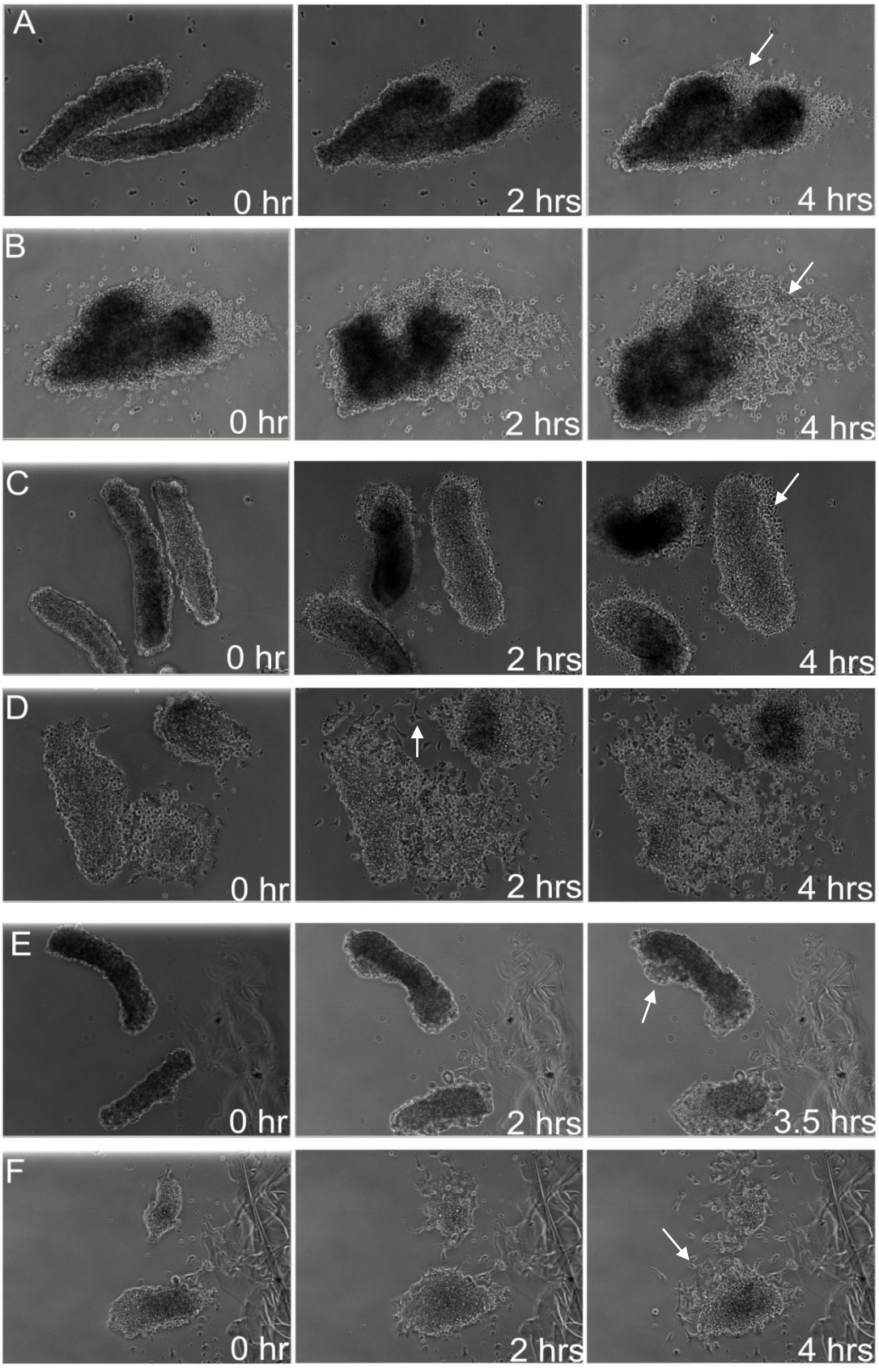
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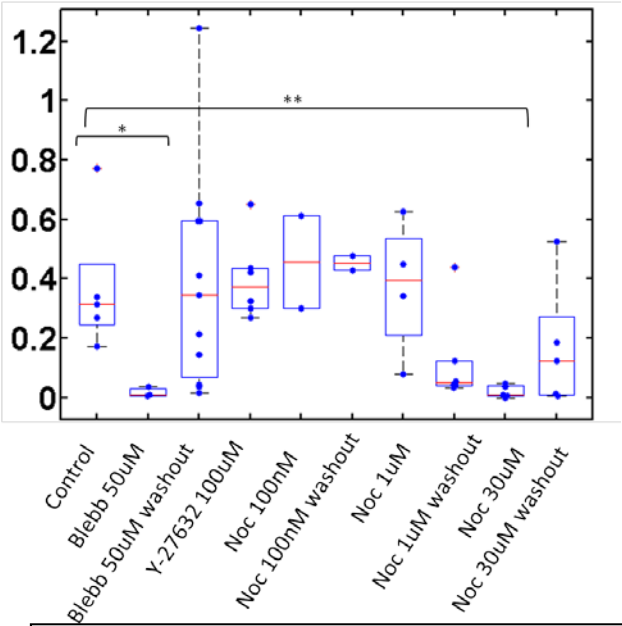
Figure 13. Different concentrations of nocodazole can block neural crest cell EMT but resumption of EMT is dose-dependent. Panels A, C, E show that neural crest cell EMT is blocked or partially blocked under 30μM, 1μM, 100nM nocodazole conditions (arrows indicate no/few cells could come out as mesenchymal cells). Panel B shows that no cells resume EMT and most cells appeared to be dead after the washout of 30μM nocodazole (arrow). Panel D shows that several cells resumed EMT (arrow), but cell death happened after 4 hours post-washout of 1μM nocodazole. Panel F shows the resumption of neural crest cell EMT post-100nM nocodazole washout (arrow).



MEANS in μm/min
Control = 0.3743
Bleb 50μM = 0.0182*
Bleb washout= 0.3917
Y27632 = 0.4007
Noc 100nM =0.4560
100nM washout =0.4534
Noc 1μM =0.3748
1μM washout =0.1227
Noc 30μM =0.0208**
30μM washout = 0.1713

Figure 14. Velocities of cells measured on the edge of the explants in EMT assay. Figure on the left shows the velocity comparisons among controls and each condition in EMT assay. Each bar represents mean±SD. N.S; *$P < 0.05$, **$P < 0.01$. Table on the left gives means of the velocities on each experimental condition.

Table 1. Summary of results for neural crest cell EMT assay

Treatment	Phenotype	Velocity (compared to control)
DSMO-treated (control)	Normal EMT	
50μM blebbistatin	Blocks EMT	Statistically decreases
Bleb washout	Resumes motility but not EMT	Comparable
Y-27632	Promotes EMT	Comparable
100nM nocodazole	Partially blocks EMT	Comparable
100nM noco washout	Resumes EMT	Comparable
1μM nocodazole	Blocks EMT	Comparable
1μM noco washout	Partially resumes EMT but cells later die	Comparable
30μM nocodazole	Causes cell death	Statistically decreases
30μM noco washout	Cells die	Comparable

Chapter 4: Cellular pathways involved in neural crest cell migration

4.1 Inhibition of NM myosin II by blebbistatin leads to cessation of cell migration, with neural crest cells migrating back towards the neural folds after washout of blebbistatin

To elucidate cell behaviors characterizing neural crest cell migration after EMT, we performed time-lapse imaging on inhibitor treated and control (DMSO-treated) neural crest cell explants. Dissected tissue was preincubated in cell culture media for 2 to 3 hours at 37˚C to allow adherence to the fibronectin-coated chamber slide and to permit EMT. Cell culture media was gradually changed to L15 before imaging. By the time we started imaging, there were several cells that had undergone EMT and became mesenchymal. In the following 2 to 10 hours of imaging, migratory mesenchymal cells spread out from the explants with a velocity of 0.5249μm/min (Fig. 22). We also observed extensive dynamics of cell-cell contacts during cell spreading and migration. At the same time, more cells from the interior of the explants underwent EMT and became mesenchymal cells (Fig. 15 and movie 7 in supplemental data).

We then examined the effect of blebbistatin on neural crest cell migration. We allowed the cells to undergo EMT and migrate out for 2 to 4 hours before adding blebbistatin (50μM) into media. Cell behaviors were recorded to make sure that cells developed to a similar stage among experiments with different inhibitor treatments. Cell morphology drastically changed in blebbistatin-treated explants. Cell migration was suspended, as the velocity of cell migration decreased to a negligible level (0.1133μm/min, Fig. 22). Cells exhibited blebbing behavior (Berndt, Clay et al. 2008) and developed rounder cell bodies, with lamellipodia disappearing and filopodia appearing (Fig. 16B). Within half an hour, cells accomplished morphological changes described above (movie 8 in supplemental data). Cells stayed alive while maintaining this

morphology with blebbistatin in the media (Fig. 16B). We expected to see resumption of normal motility after removal of blebbistatin. Intriguingly, cells fully resumed motility with a comparable migrating velocity (0.5943μm/min) to controls (Fig. 22), but they changed their direction and moved toward the neural folds, leading to the contraction of the explant (Fig. 16C). Neural crest cells quickly adopted a normal morphology, with flat cell bodies and lamellipodia and a reduction in filopodia. Within 5 hours after washout, cells appeared to be alive and had active cell-cell contacts. We also observed that a few cells that were relatively far away from the neural fold were unable to move back to the neural folds and ended up moving in random directions (in other movies recording blebbiatatin washout experiments, data not shown here).

We next examined the spatial dynamics of cell migration by measuring the vorticity for the movement of cells migrating out from the explants. Vorticity is an indicator of how much curl a field of cells displays (Vedula, Leong et al. 2012). The closer a distribution is to 0, the less curling and more linear the movement of the cells is. The control explants (Fig. 23) demonstrated a typical distribution of vorticity with the peak at zero on the x-axis. After washout of blebbistatin, the distribution of vorticity was much narrower, corresponding to lower vorticities and indicating that cells moved in a more linear manner. This suggests that cell migration may be directed by some yet unknown factors after the removal of blebbistatin.

The movement of neural crest cells back towards the original explanted tissue after removal of blebbistatin was first observed and documented in our experiments. Two possibilities could explain these observations. One is chemotaxis, which is the phenomenon whereby cells direct their movements according to certain chemicals in their environment. This would require a chemoattractant from the explant center or a chemorepellent from outside of the explant acting on the migratory neural crest cells. The latter one is unlikely happening because we did not include any other factor other than blebbistatin, which was removed by washout. It may be the case that some molecules

were induced and/or secreted from the explants after removal of blebbistatin. The suspected factor, however, remains unknown. The other explanation could be a change of cell polarity. Migratory neural crest cells changed their direction during explant contraction, which is one hallmark of directional migration. In order to initiate directional migration, cells need to break symmetry and polarize the migratory machinery. There is strong evidence that NM II contractility initiates symmetry-breaking by forming the prospective rear of the cell (Yam, Wilson et al. 2007), which allows for protrusion to occur at the opposite end. This might be an explanation of explant contraction. After washout of blebbistatin, NM myosin II recovered its function and reinitiated symmetry-breaking; however, the reason why cells changed the prospective rear after pausing by blebbistatin needs further exploration.

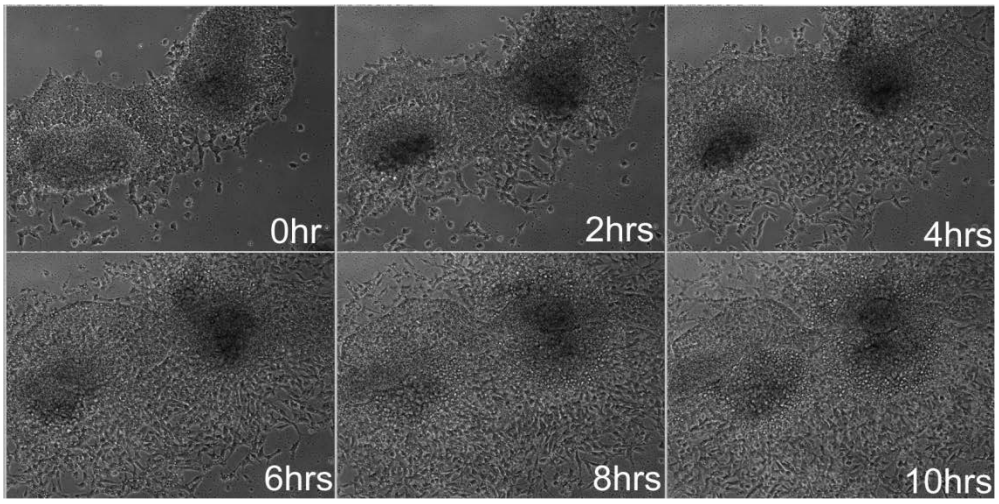


Figure 15. Cell migration in control-treated (DMSO) neural crest cell explants. Cells that had undergone EMT migrate out of neural folds and spread out as time passed. Cell-cell contacts were maintained while cells were migrating. More cells from neural folds undergo EMT and became migratory mesenchymal cells.

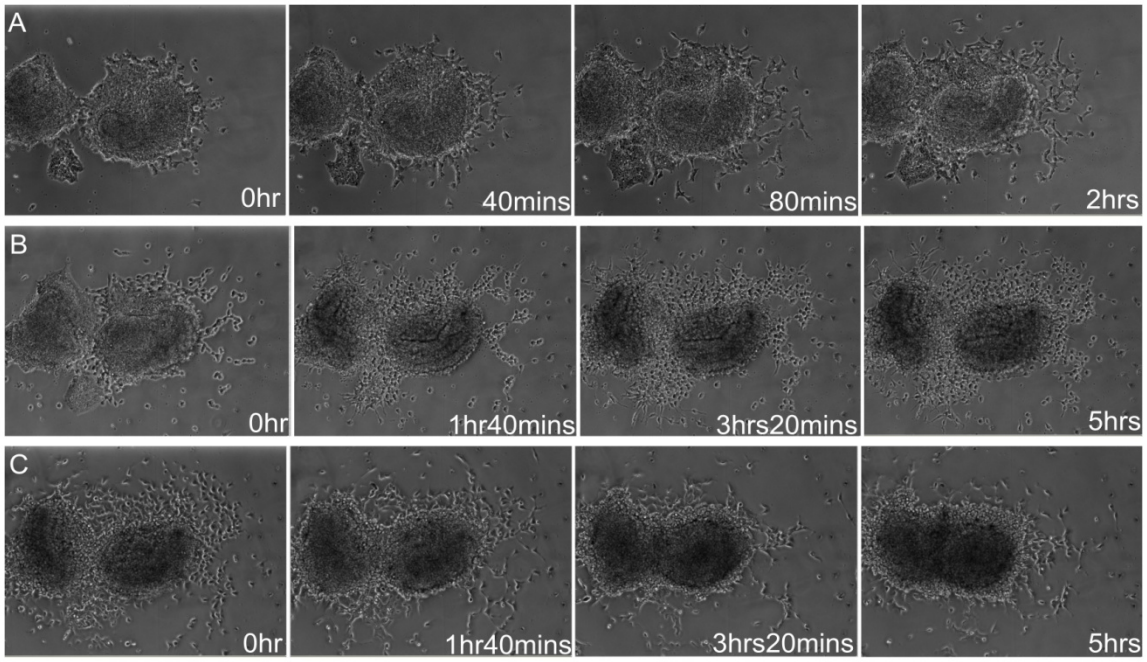


Figure 16. Inhibition of non-muscle myosin II by blebbistatin leads to cessation of cell migration, and neural crest cells migrate towards neural folds after washout of blebbistatin. Panel A shows pre-drug cell movement: cells behaved normally like controls. Panel B shows cells migration was suspended and cells developed rounder cell bodies after blebbistatin treatment. Panel C shows cells resuming motility upon washout of blebbistatin and having a normal morphology but changing their migratory direction back towards the neural folds.

4.2 Inhibition of Rho-kinase by Y-27632 affects neural crest cell migration

Using the same set-up, we examined the inhibition of ROCK by Y-27632 in migratory neural crest cells. After adding Y-27632, we did not observe any apparent cell morphology changes (Fig. 17 and movie 9 in supplemental data). The quantitative data, however, showed that the velocity of cell migration (0.7082μm/min) was actually increased compared to the controls (0.5249μm/min) (Fig. 22). This result indicates Y-27632 treatment accelerates neural crest cell migration and is consistent with its function in promoting neural crest cell EMT.

Vorticity of cells upon Y-27632 treatment had a broader distribution, which means cells in Y-27632-containing media moved in a curved or non-linear path. This indicates that ROCK may be involved in directional migration of neural crest cells. Previous studies have shown in *Xenopus* and zebrafish that Rho regulating directional migration of neural crest cells through inactivation of Rac (Cascone, Audero et al. 2003). Our findings have now established that a similar mechanism may be occurring in chick midbrain neural crest cells.

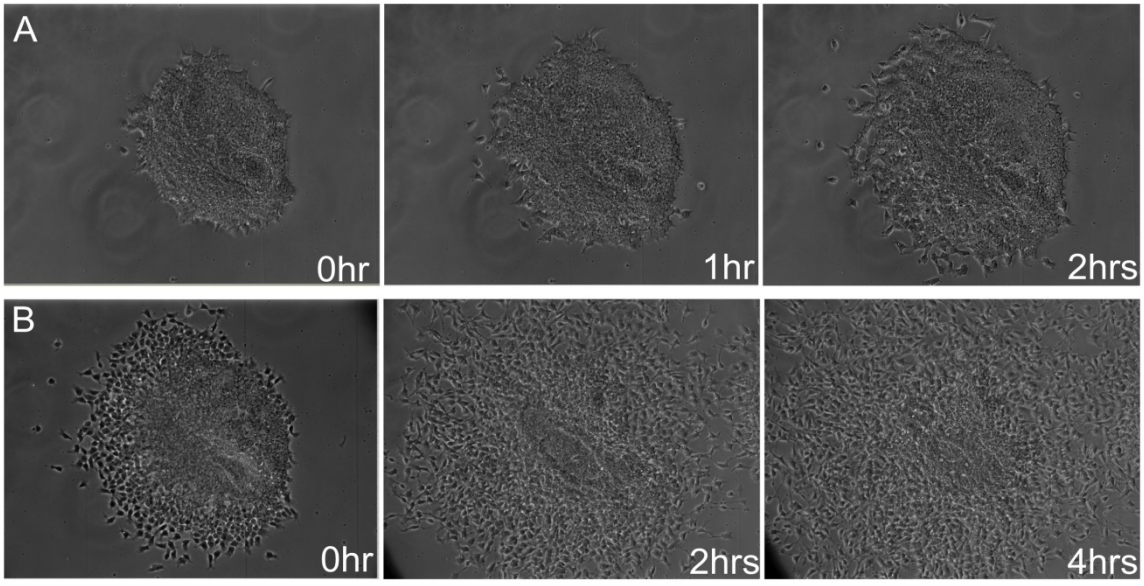


Figure 17. Inhibition of ROCK by Y-27632 accelerates neural crest cell migration. Panel A shows pre-drug cell movement: cells behaved normally like the controls. Panel B shows that the overall cell morphology did not change upon treatment with 100μM Y-27632.

4.3 Microtubule function is important for proper neural crest cell migration

Knowing that different concentrations of nocodazole have varying effects on microtubule polymerization, which affected neural crest cell EMT, we also tested the inhibitory effects of nocodazole in different concentrations on cell migration.

30μM nocodazole appeared to be toxic to cells (Fig. 18). Cell migration was suspended, with velocity decreasing to 0.0208μm/min (Fig. 22). Most cells also rounded up and died after 4 hours of drug treatment. Explant size decreased as a result of cell death, although a few cells appeared to survive (movie 10 in supplemental data).

We then tested 1μM nocodazole treatment on cells. Cell morphology did not change, but the velocity (0.4163μm/min) decreased a little (not significantly) compared to the controls (Fig. 22). Intriguingly, the velocity further decreased to 0.0534μm/min after removal of nocodazole by washout, which is significantly different from the controls. Eight hours after washout, cells failed to resume migratory capacity, and instead cell death was observed, although more cells appeared to survive compared to 30μM nocodazole treatment (Fig. 19B and movie 11 in supplemental data). This suggests that 1μM nocodazole is also toxic to neural crest cells. This result is similar to what we observed after 1μM nocodazole treatment of neural folds in the EMT assay. Cells were able to retain motility in the presence of 1μM nocodazole, but further lost their motility after removal of nocodazole. This implies that nocodazole may facilitate cell motility in some other way, which compensates for the loss, or partial loss of microtubule function, upon microtubule disruption. After removal of nocodazole, both disrupted microtubules and the unknown pathway(s) that nocodazole supports disappeared, leading to cessation of cell migration and eventually cell death.

Because a low concentration of nocodazole (100nM) had a reversible effect on neural crest cell EMT, we wanted to test if it would affect cell migration to see if the plus end of microtubules is involved in controlling migration. Interestingly, although no change in cell morphology was observed (Fig. 20A and movie 12 in supplemental), quantitative

data indicate that 100nM nocodazole accelerated the velocity to 0.7544μm/min, even faster than that observed in controls (0.5249μm/min) (Fig. 22). Even more surprising, the velocity significantly decreased to 0.2147μm/min compared to controls (P<0.01) after washout. This finding reveals that a relatively low concentration of nocodazole may promote migration. It also indicates that the polymerization of microtubules at the plus end may modulate neural crest cell migration. These findings are consistent with those observed in EMT assay.

Cell vorticity in 100nM nocodazole (Fig. 23) showed a broader distribution than controls, indicating cells moved more randomly. This result supports the notion that the factor(s) that maintains cell migratory direction in controls was weakened in the presence of nocodazole. Also, this finding suggests that the plus end of microtubules may play a role in directing cell migration in neural crest cells. In some cell types like HeLa and 293T cells, the function of the plus end of microtubule in facilitating directional cell migration has been reported (Zhang, Shao et al. 2013).

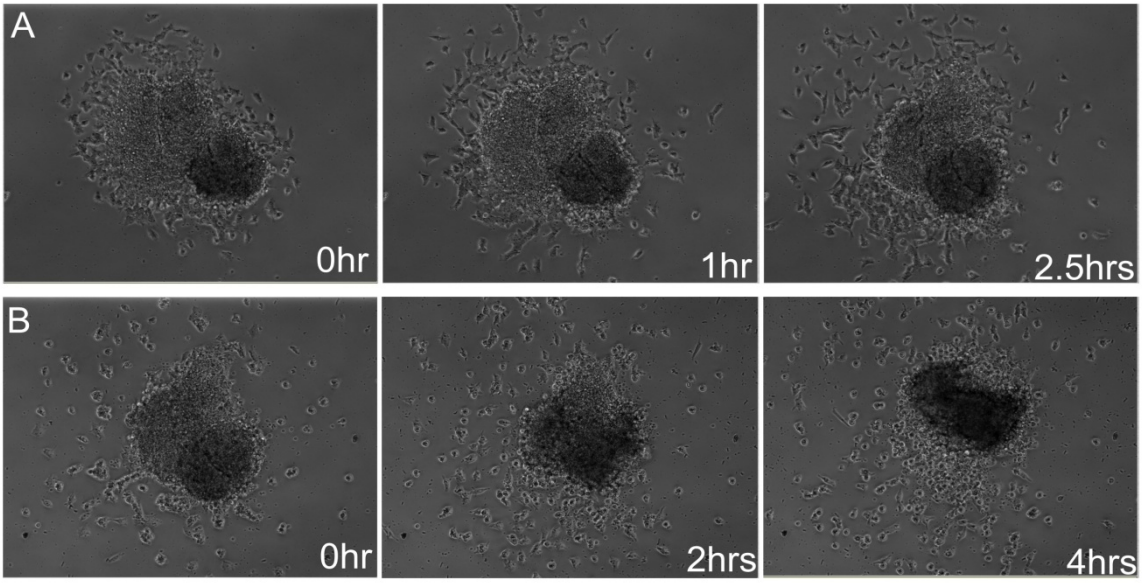


Figure 18. 30μM nocodazole suspended cell migration and caused cell death. Panel A shows pre-drug cell movement: cells behaved normally like controls. Panel B shows cells rounded up and gradually died after 30μM nocodazole treatment.

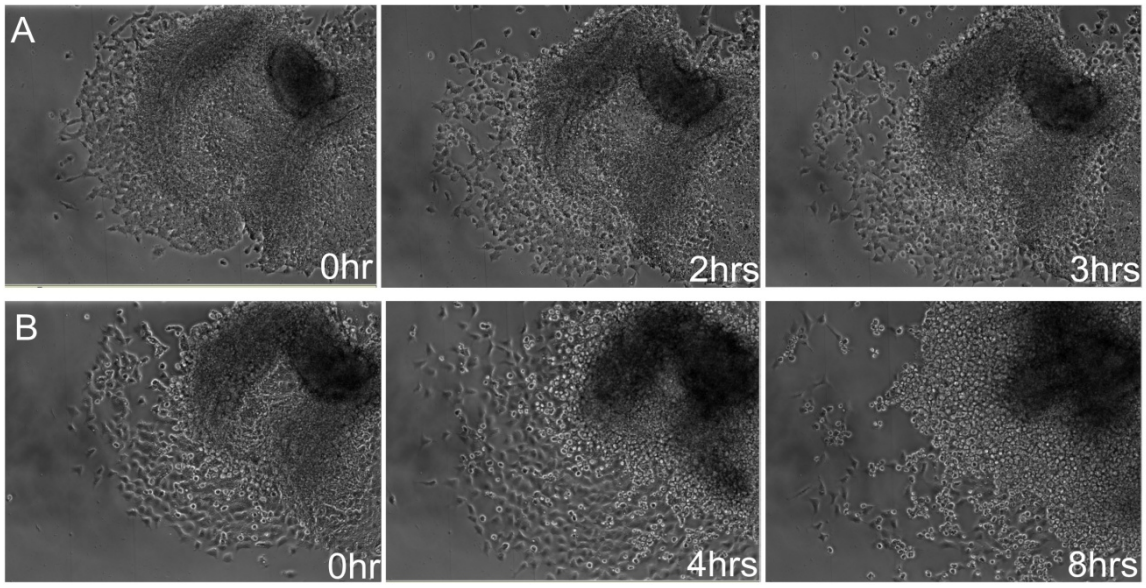


Figure 20. 1μM nocodazole slowed cell migration and caused cell death after washout. Panel A shows cells movement after adding 10μM nocodazole. Cell migration slowed down a little. Panel B shows that cells migration was further slowed after removal of nocodazole, with many cells dying after 8 hours.

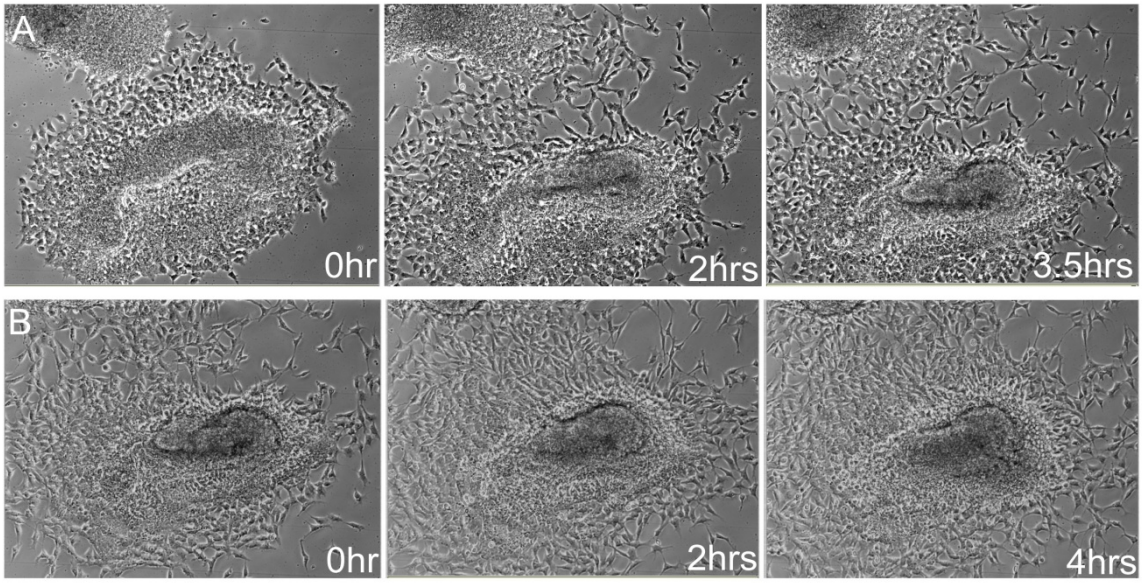


Figure 19. 100nM nocodazole did not have an apparent effect on neural crest cell migration. Panel A shows cells migrating normally after adding 100nM nocodazole. Panel B shows cells continued normal migration after washout.

4.4 Inhibition of actin polymerization by latrunculin A irreversibly suspends cell migration

Actin filaments are building blocks for the cytoskeleton involved in many basic cellular processes (Schmidt and Hall 1998). Knowing that inhibition of actin polymerization is detrimental in different cell types, we wanted to test what effect it would have on neural crest cells. Using the same experimental set-up, we noted that 100nM latrunculin A suspended cell migration to a significantly low level, with the velocity decreased to 0.1313μm/min (Fig. 22). We also observed explant contraction in some cases, with a velocity of 0.4352μm/min, likely due to cell death (data now shown here). Cell shapes were distorted, as cells rounded up within 4 hours post-drug treatment and appeared to be dying (Fig. 21 and movie 13 in supplemental data). After washout to remove latrunculin A, the cells in the explants became loose probably because of the inhibition of actin and the removal of media during by washout. This was not observed after washout of other inhibitors, indicating the importance of actin filaments in the cytoskeleton and the maintenance of explant contact with the fibronectin-coated chamber slide. Interestingly, blebbistatin and Y-27632 treatment can also interrupt actin, but in an indirect way through the interaction among NM myosin II, ROCK and actin filaments (as shown in Fig 4). Our observation indicates that proper actin activity is crucial to neural crest cell migration. Unlike many cell types, neural crest cells are very sensitive to latrunculin A.

As summarized in Table 2, blebbistatin reversibly blocks cell migration but cell migratory direction changes after removal of blebbistatin; Y-27632 accelerates cell migration; nocodazole has differential effects on neural crest cell migration based upon the concentration used; and latrunculin A causes cell death even at a very low concentration. This suggests NM myosin II, ROCK, microtubules and actin filaments are all involved in neural crest cell migration.

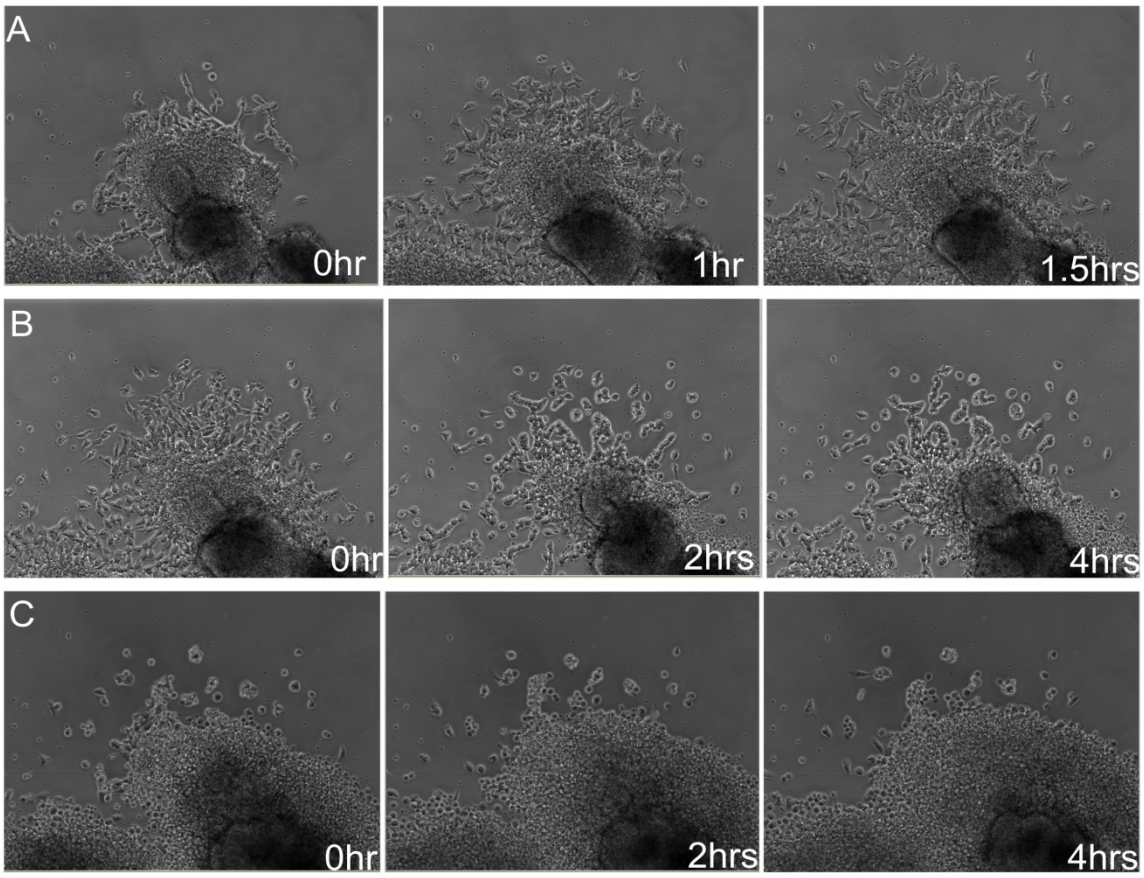
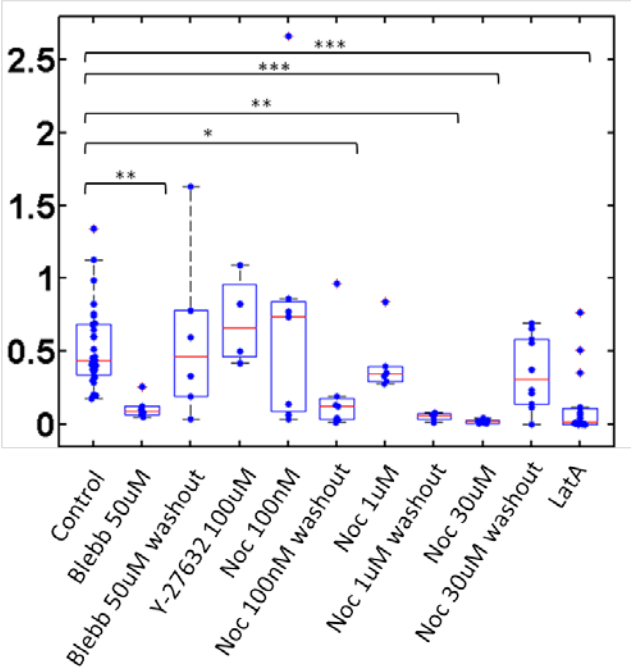


Figure 21. Inhibition of actin polymerization by latrunculin A irreversibly blocks cell migration. Panel A shows pre-drug cell movement: cells behaved normal like the controls. Panel B shows that 100nM latrunculin A suspended cell migration and caused cell death. Panel C shows this effect could not be released by washout and cells eventually died.



MEANS in µm/min
Control = 0.5249
Blebbistatin = 0.1133 **
Bleb washout= 0.5943
Y27632 = 0.7082
Noc 100nM =0.7544
100nM washout = 0.2147 *
Noc 1µM = 0.4163
1µM washout = 0.0534 **
Noc 30µM =0.0208 ***
30µM washout=0.3577
LatA = 0.1313 ***

Figure 22. Velocities of cells measured at the edge of the explants in the migration assay. Figure on the left shows the velocity comparisons among controls and each condition in migration assay. Each bar represents mean±SD. N.S; *P < 0.05, **P < 0.01, ***P < 0.001). Table on the left gives means of the velocities on each experimental condition.

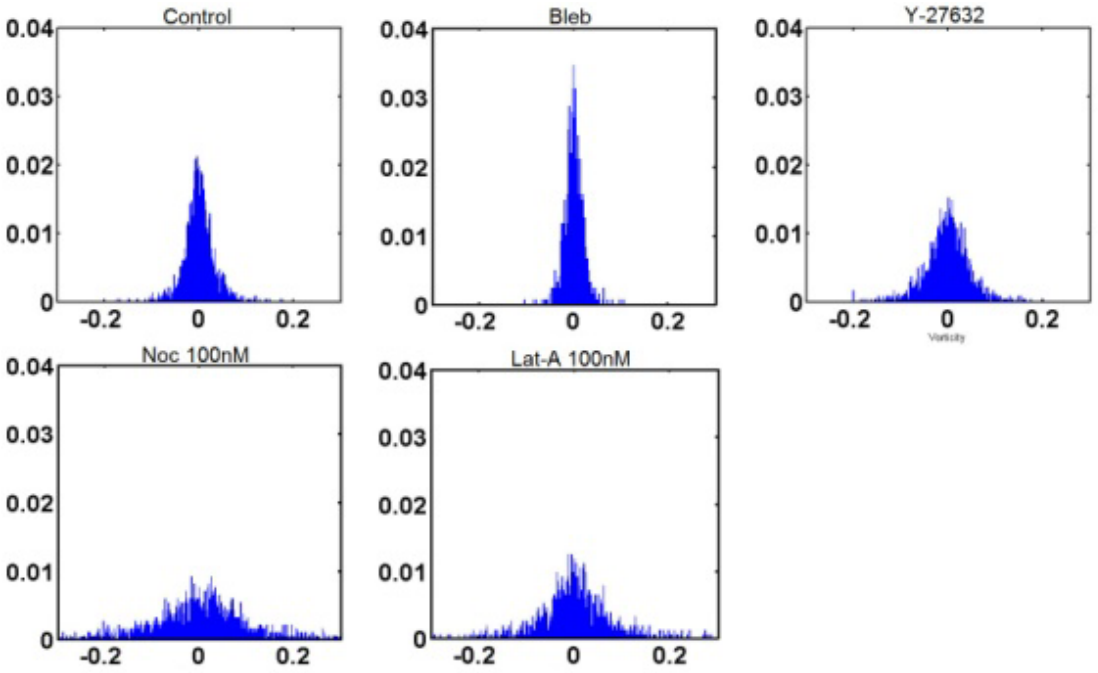


Figure 23. Vorticity of cells on explants treated in different inhibitors. The first row shows the distribution of vorticity in controls, after washout in blebbistatin treatment and in Y-27632 treatment; the second row shows the distribution of vorticity upon treatment with 100nM nocodazole and latrunculin.

Table 2. Summary of results for neural crest cell migration assay

Treatment	Phenotype	Velocity (compared to control)	Vorticity
DSMO-treated (control)	Normal migration		
50μM blebbistatin	Blocks migration	Statistically decreases	N/A
Bleb washout	Resumes migration but direction changes	Comparable	More directional
Y-27632	Normal migration	Increases	Less directional
100nM nocodazole	Normal migration	Comparable	Less directional
100nM noco washout	Normal migration	Statistically decreases	N/A
1μM nocodazole	Normal migration	Comparable	N/A
1μM noco washout	Normal migration but cells die later	Statistically decreases	N/A
30μM nocodazole	Causes cell death	Statistically decreases	N/A
100nM latrunculin A	Causes cell death	Statistically decreases	Less directional

Chapter 5: Distribution of α-actinin and palladin in neural crest cells

To examine the effects of chemical inhibitors on the distribution of α-actinin and palladin in neural crest cell explants, we performed immunohistochemistry on cells after inhibitor treatment. By looking at the distribution changes of these two actin-binding and/or actin-associating proteins compared to control explants treated with DMSO, we can get additional insight into the effects these inhibitor treatments had on the actin cytoskeleton.

5.1 Distribution of α-actinin and palladin in DMSO-treated (control) neural crest cell explants

We first examined α-actinin and palladin distribution DMSO-treated explants. α-actinin was found in filopodial and lamellipodial structures in the cytosol, while palladin co-localized with α-actinin but was also expressed in nuclei. This is the first time the distribution of α-actinin and palladin has been reported in cranial neural crest cells (Fig. 24 and Fig. 25).

α-actinin, as a focal adhesion protein, functions as a linker between the cytoplasmic domain of β-integrins and actin filaments (Critchley DR 1999). In avian trunk neural crest cells, α-actinin was shown to be localized to focal contacts and along actin microfilaments (Delannet, Martin et al. 1994) (Testaz, Delannet et al. 1999). It was also reported that in chick trunk neural crest cells (from the level of mid-otocyst to the third somite pair), α-actinin was localized around the nucleus, in focal adhesions, and in filopodia (Anthony R. Mwakikunga 2011), indicative of its function to bundle actin filaments into stress fibers. Our findings of the distribution of α-actinin in cranial crest cells are in agreement with what has been reported in trunk neural crest cells.

Palladin, an actin associated protein identified in 2000 (Parast and Otey 2000), directly binds to α-actinin and localizes to stress fibers, focal adhesions, and other

actin-based structures (Jin 2011). Although palladin is thought to be of critical importance in regulating cell motility (Chin and Toker 2010), there are no reports showing its location or function in neural crest cells. Our localization results suggest that palladin, along with α-actinin, may serve as a cytoskeletal scaffold and to cross-link actin. Moreover, palladin localization to neural crest cell nuclei indicates that it may have additional functions other than as an actin-crosslinker, such as nuclei-cytosol communication.

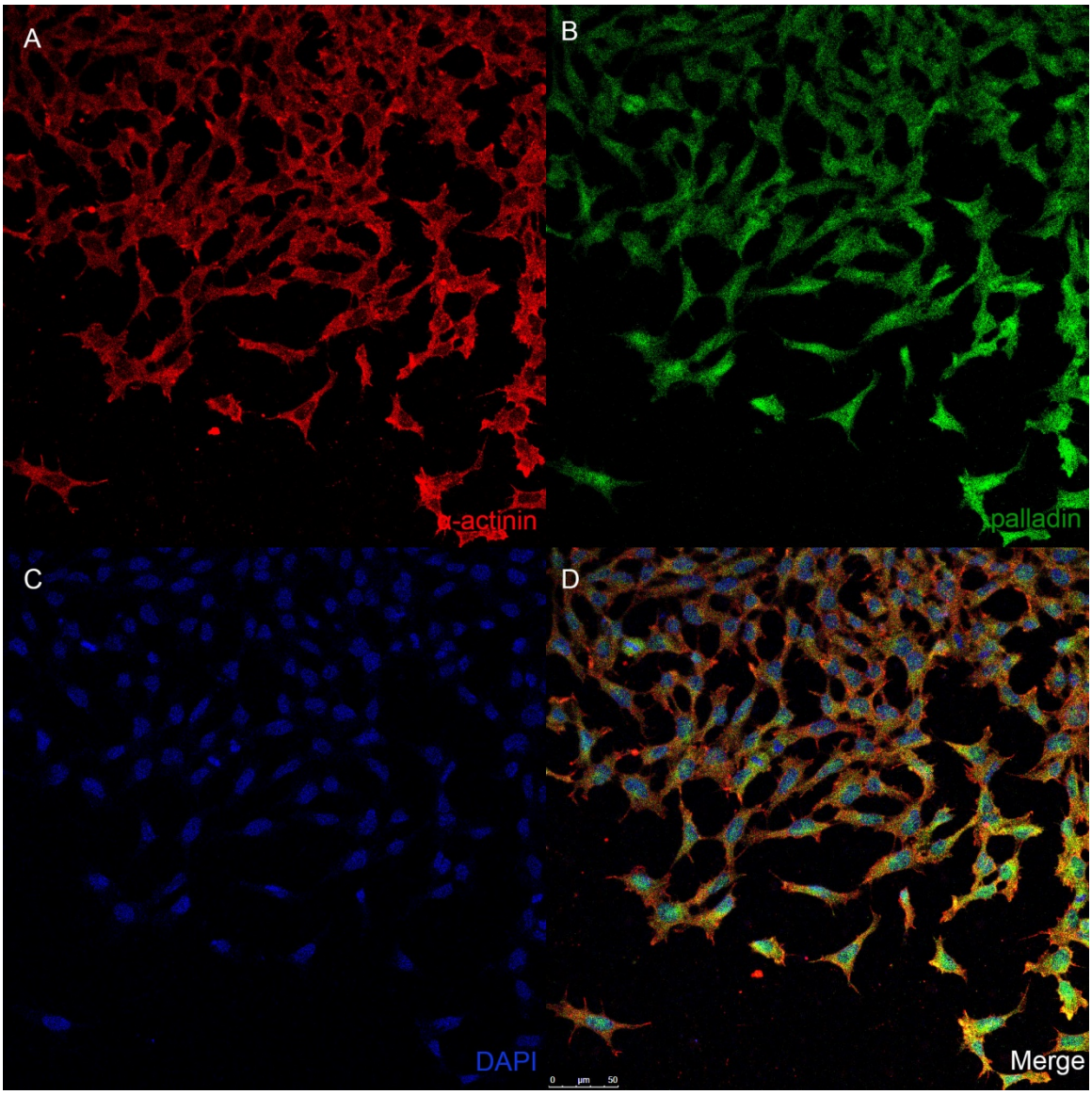


Figure 24. Distribution of α-actinin and palladin in control (DMSO-treated) explants. α-actinin (A, red) and palladin (B, green) co-localize to the cytosol, lamellipodia and filopodia; palladin also localizes to the nucleus. DAPI (C, blue) stains the nucleus. Rectangular area is magnified in Fig. 25. Bar = 50µM.

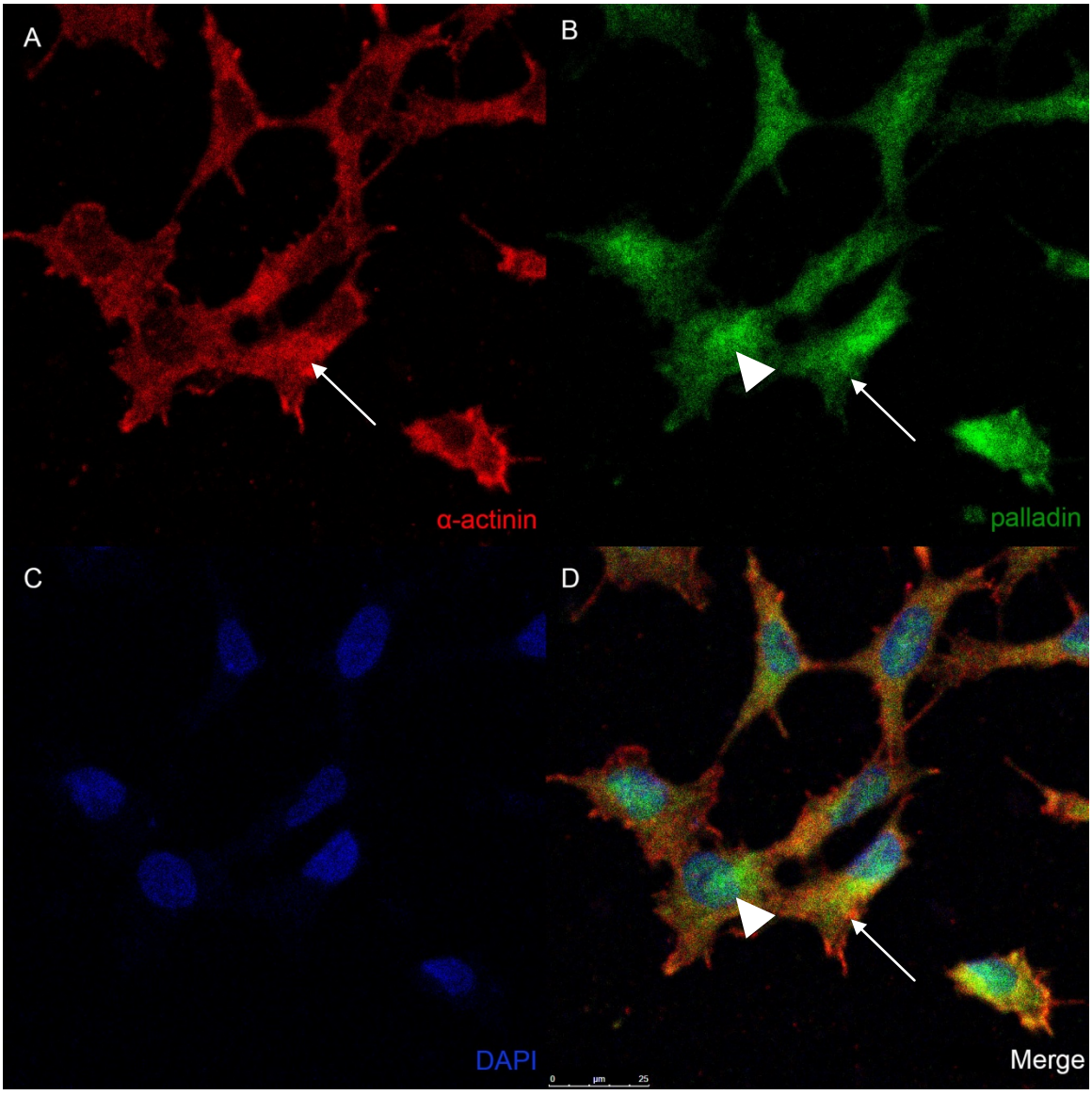


Figure 25. Distribution of α-actinin and palladin in control (DMSO-treated) explants (magnified image of Fig. 24 in rectangular area). α-actinin (A, red) and palladin (B, green) co-localize to the cytosol, lamellipodia and filopodia (arrows); palladin localizes to the nucleus (arrowhead). DAPI (C, blue) stains the nucleus. Bar = 25µM.

5.2 Distribution of α-actinin and palladin in blebbistatin-treated neural crest cell explants

To examine potential changes in the distribution of α-actinin and palladin in neural crest cell after inhibition of NM myosin II by blebbistatin, we fixed cells after blebbistatin treatment and performed immunohistochemistry. To maintain the blebbistatin treatment-induced change in cell morphology (as seen in Fig. 16B), blebbistatin (50μM) was added along with the fixative. Blebbistatin-treated neural crest cells developed rounder and smaller cell bodies (Fig. 26), lamellipodia disappeared, and filopodia appeared instead (Fig. 27, arrows). α-Actinin was observed in the cytosol and enriched in filopodia, and palladin co-localized with α-actinin in the cytosol and filopodia. Palladin was also noted in the nucleus and appeared to be more abundant there compared to controls.

In fibroblasts, NM myosin II localizes to actin bundles contacting growing adhesions, forming a striated pattern with α-actinin. In other cells, such as in neuronal growth cones, NM myosin II may have a more direct role controlling retrograde flow in the peripheral zone (Medeiros, Burnette et al. 2006) When NM myosin II is knocked out, knocked down by small interfering RNA or inhibited with blebbistatin, large actin bundles are not observed in the lamellum, whereas the lamellipodium remains intact (Ponti, Machacek et al. 2004). In many cells, inhibition of NM II with blebbistatin, or genetic deletion of NM II, increases protrusiveness (Vicente-Manzanares, Ma et al. 2009); however, we did not observe obvious protrusions in neural crest cells.

In order to examine whether α-actinin and palladin are involved in the contraction of explants after washout of blebbistatin and to identify potential reasons of explant contraction, we performed immunostaining after washout of blebbistatin (Figs. 28, 29). Cell morphology was slightly different from control treated cells with slimmer cell bodies and directional protrusions. Interestingly, α-actinin and palladin were not equally distributed in cells. Most cells had a stronger distribution of α-actinin and palladin in

regions of the cell facing the explants (Figs. 26, 27 arrows pointing at the explant). This asymmetric distribution of α-actinin and palladin may be related to the changed polarity of the neural crest cells. At the same time, palladin nuclear distribution strongly decreased after washout of blebbistatin compared to controls and blebbistatin treatment. Moreover, vorticity data shows cells have a more directional movement than controls after washout of blebbistatin. The shift in localization of palladin from the nucleus to the cytosol, and α-actinin to the edge of the cell facing the explant, correlates nicely with the accompanying vorticity change. This was the first time that the shift in localization of palladin from the nucleus to the cytosol was observed.

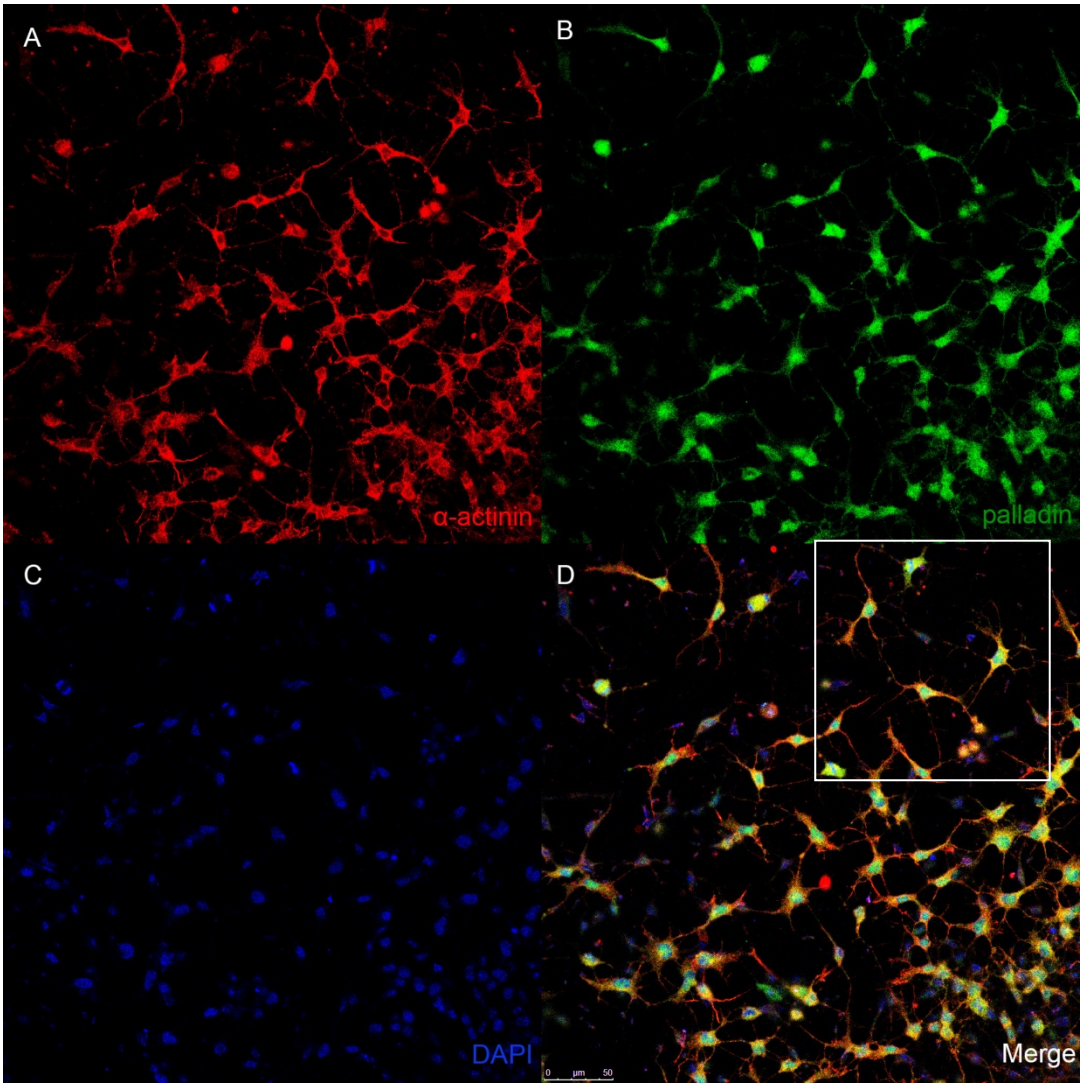


Figure 26. Distribution of α-actinin and palladin in blebbistatin-treated explants. α-actinin (A, red) and palladin (B, green) co-localize to the cytosol and filopodia; palladin localizes to the nucleus. DAPI (C, blue) stains the nucleus. Rectangular area is magnified in Fig. 27. Bar = 50µM

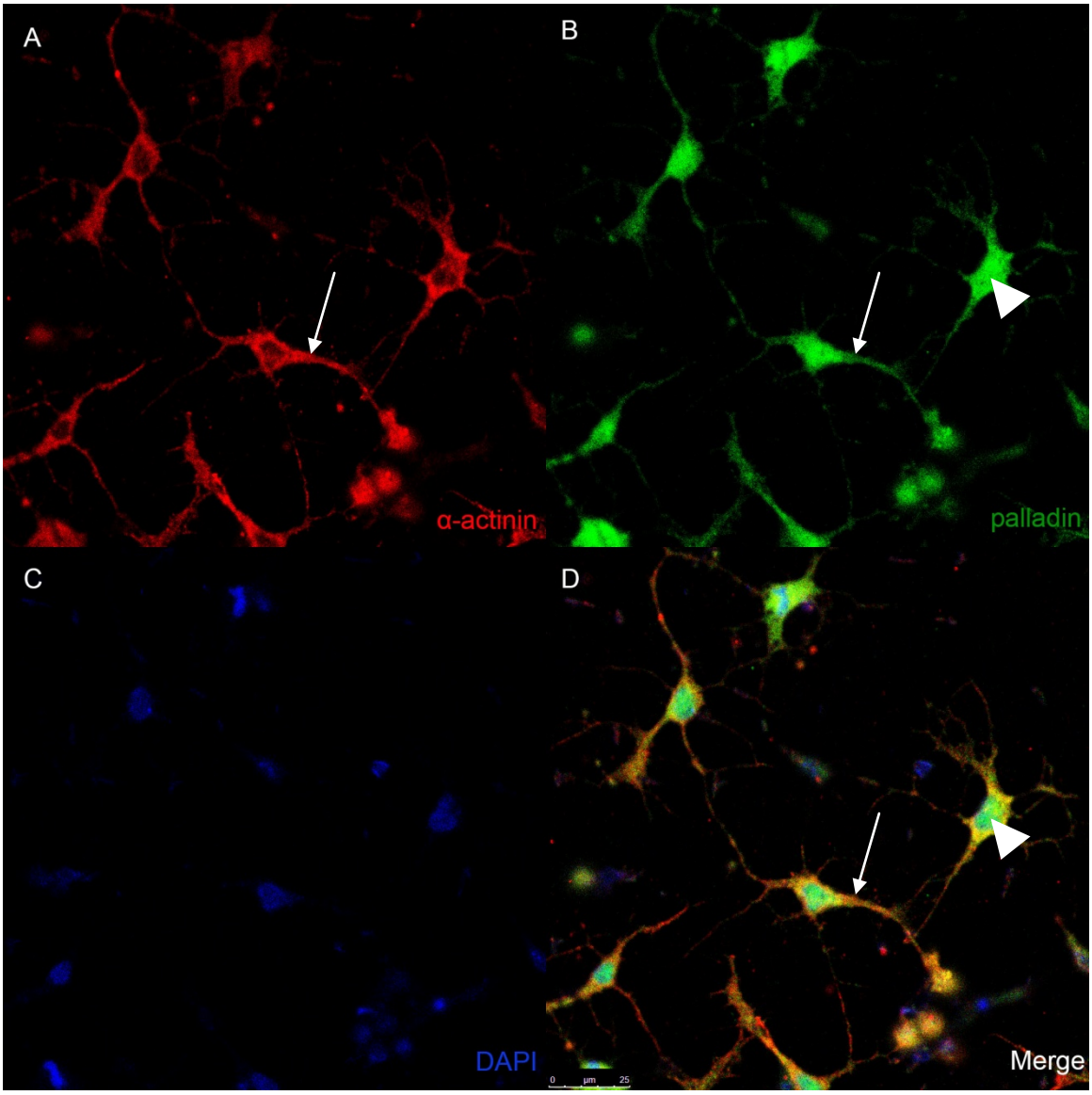


Figure 27. Distribution of α-actinin and palladin in blebbistatin-treated explants (magnified image of Fig. 26 in rectangular area). α-actinin (A, red) and palladin (B, green) co-localize to the cytosol and filopodia (arrows); palladin localizes to the nucleus (arrowhead). DAPI (C, blue) stains the nucleus. Bar = 25µM.

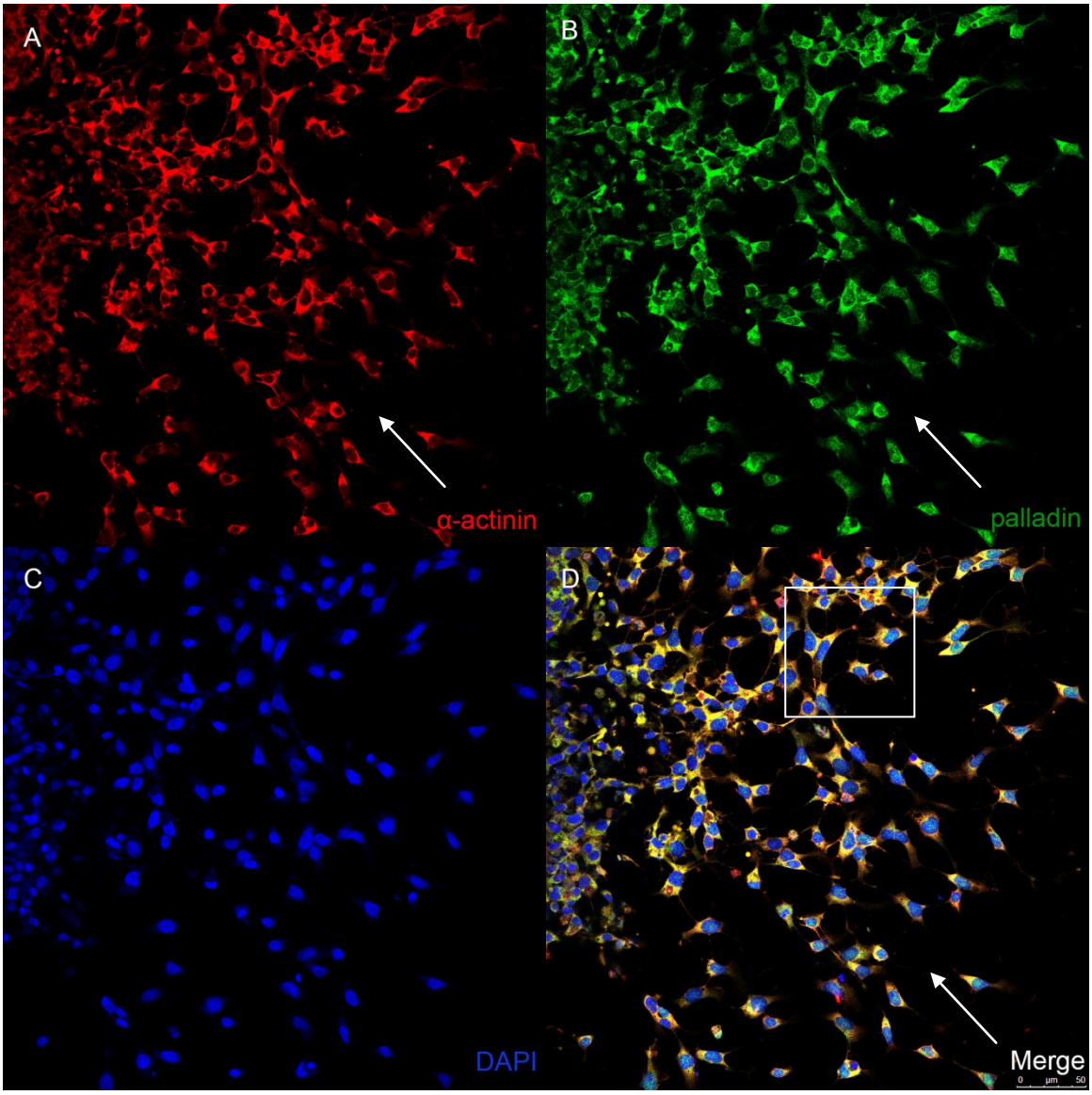


Figure 28. Distribution of α-actinin and palladin in blebbistatin-treated explants after washout to remove blebbistatin. α-Actinin (A, red) and palladin (B, green) co-localize to the cytosol but show an asymmetric distribution in cells. Expression of palladin in nuclei is much reduced compared to blebbistatin-treated explants. Cells also developed apparent polarized cell bodies. DAPI (C, blue) stains the nucleus. Rectangular area is magnified in Fig. 29. Bar = 50μM.

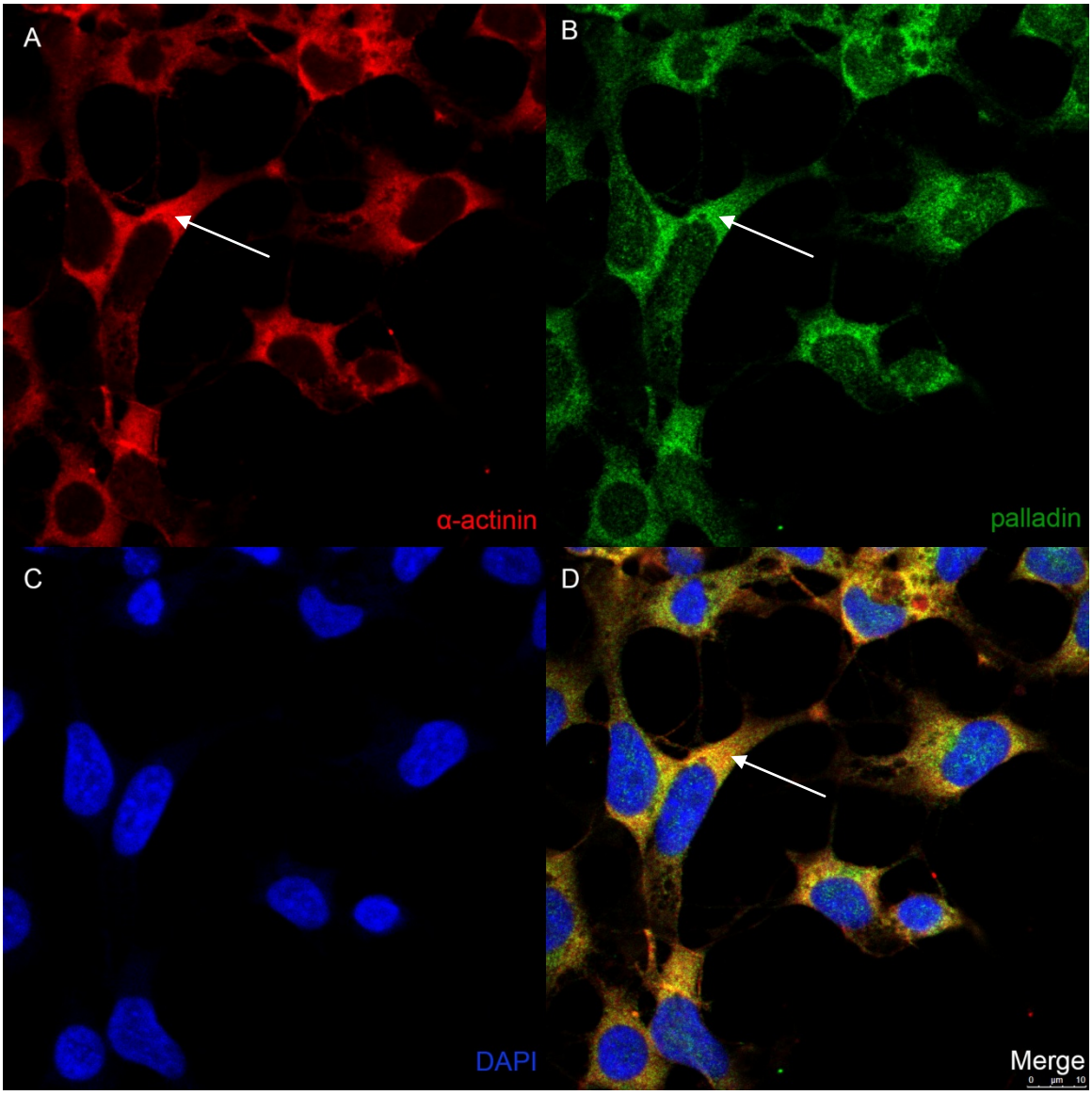


Figure 29. Distribution of α-actinin and palladin in blebbistatin-treated explants after washout to remove blebbistatin (magnified image of Fig. 28 in rectangular area). α-Actinin (A, red) and palladin (B, green) co-localize to the cytosol but show an asymmetric distribution in cells (arrows); palladin localization to the nucleus is reduced. Cells developed polarized cell bodies. DAPI (blue) stains the nucleus. Bar = 10μM.

5.3 Distribution of α-actinin and palladin in Y-27632-treated neural crest cell explants

To examine the change in the distribution of α-actinin and palladin in neural crest cell explants after inhibition of ROCK, and subsequently the Rho pathway, by Y-27632, we fixed cells after Y-27632 inhibition and performed immunohistochemistry. Compared to control-treated explants, Y-27632 treated cells did not have as flat of a cell shape and had more cell-cell contacts (Fig. 30) by filopodia and lamellipodia. α-actinin and palladin are also uniformly co-localized in the cytosol, and palladin showed reduced distribution in nuclei, less than controls (Fig. 31).

Rho GTPases are important regulators of both actin dynamics and cell-substratum adhesions in migratory cells, and thus are critical proteins involved in neural crest cells. The two subgroups of the Rho GTPases, Rho and Rac, with their effects on actin and adhesion dynamics, have been well studied (Bishop and Hall 2000; Wennerberg and Der 2004). Rho induces the formation of stress fibers and focal adhesions, while Rac induces lamellipodial extensions and the focal complexes at the leading edge of a migratory cell (McHardy, Warabi et al. 2005). In Swiss 3T3 fibroblasts, injection of Y-27632 induced rapid disassembly of the focal adhesions and active formation of lamellipodia and associated focal complexes, which were due to the activation of Rac (Rottner et al., 1999). It has been reported that Y-27632 treatment alters neural crest morphology by reducing stress fibers and focal adhesions (Groysman, Shoval et al. 2008). Also, in neurons, Y-27632 (10 μM) can trigger neuronal polarity formation at very early time points (Bito, Furuyashiki et al. 2000).

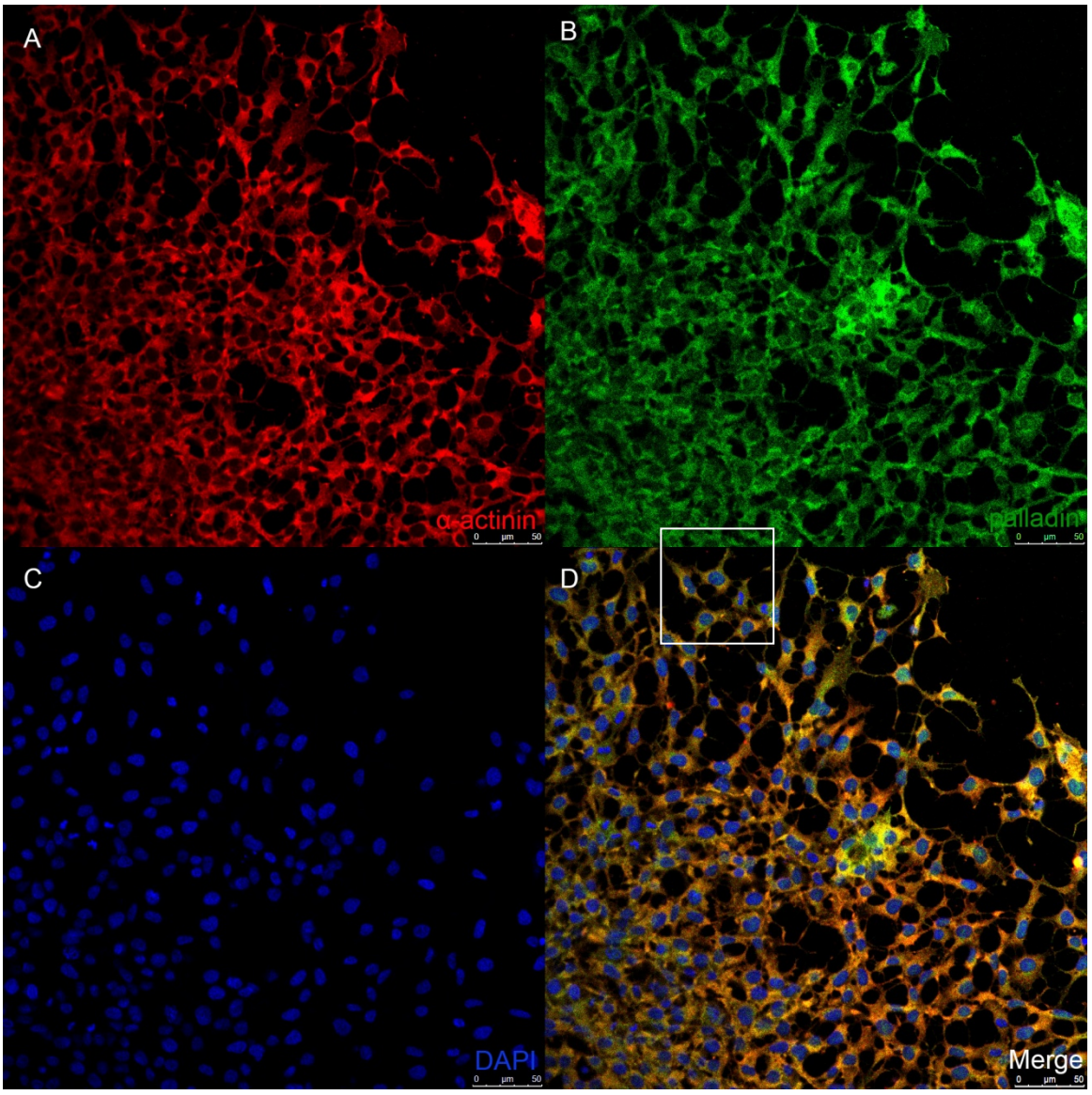


Figure 30. Distribution of α-actinin and palladin in Y-27632-treated explants. α-actinin (A, red) and palladin (B, green) co-localize to the cytosol; DAPI (C, blue) stains the nucleus. Rectangular area is magnified in Fig. 31. Bar = 50μM.

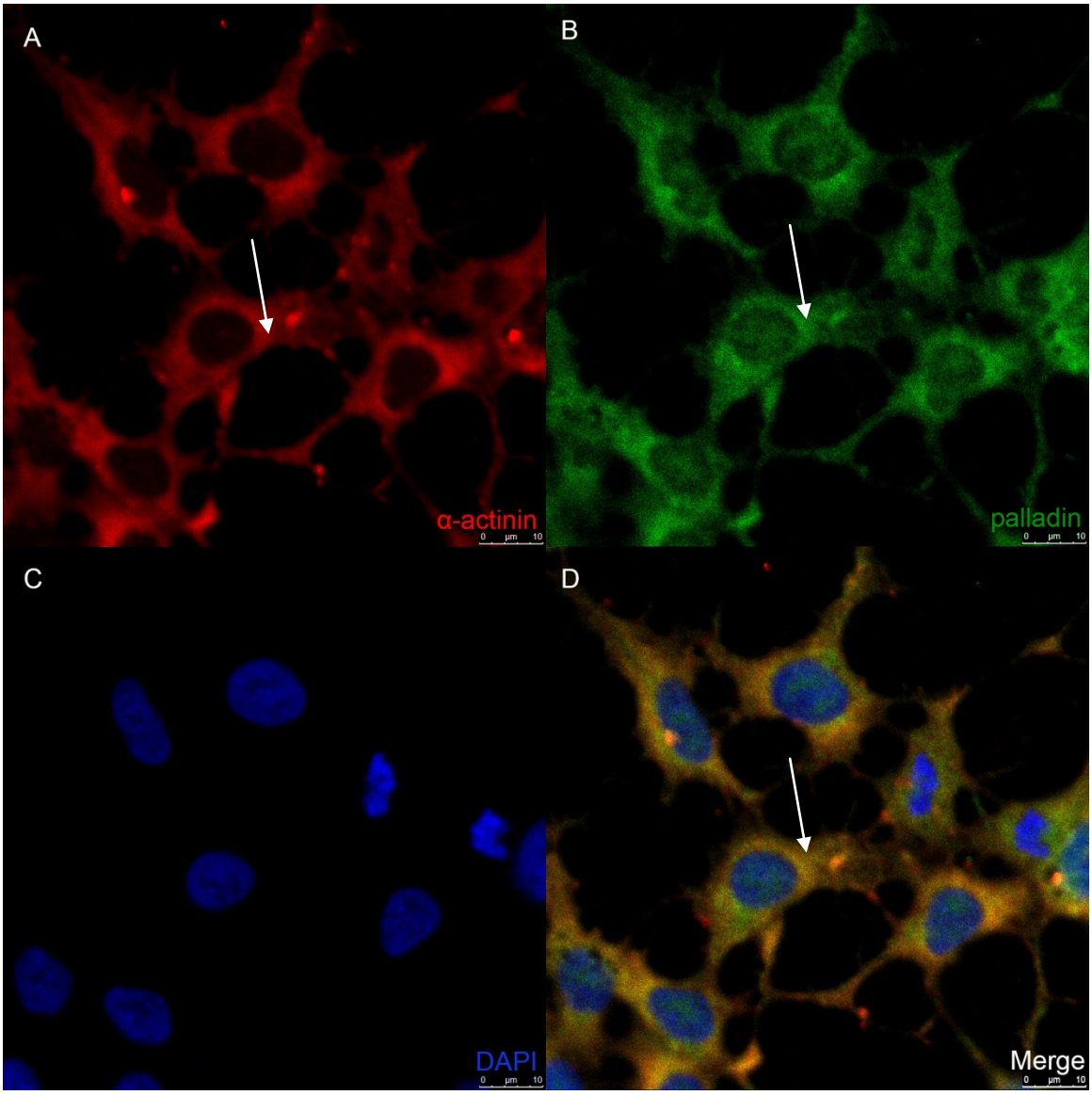


Figure 31. Distribution of α-actinin and palladin in Y-27632-treated explants (magnified image of Fig. 30 in rectangular area). α-actinin (A, red) and palladin (B, green) co-localize to the cytosol. DAPI (C, blue) stains the nucleus. Bar = 10μM.

5.4 Distribution of α-actinin and palladin in nocodazole-treated neural crest cell explants

To examine the changes in the distribution of α-actinin and palladin in neural crest cell after inhibition of the polymerization of microtubules by nocodazole in different concentrations, we fixed cells after nocodazole treatment and performed immunohistochemistry. Compared to control treated explants, different concentrations of nocodazole had different effects on cell morphology as well as the distribution of α-actinin and palladin (Figs. 32-38).

With 30μM nocodazole treatment, cells developed two drastically different morphologies (Fig. 32). As shown in Fig. 33, cells that appeared to survive in 30μM nocodazole developed very flat cell shapes, with α-actinin enriched in protrusions. From the time-lapse imaging data, we find that some neural crest cells that directly attach to the fibronectin bottom can survive this treatment. Most cells, like those shown in Fig. 34, however, have retracted cell bodies, with α-actinin and palladin condensed in several dot-like areas. These cells were dead shortly after 30μM nocodazole treatment.

With 1μM nocodazole treatment, neural crest cells adopted a flatter morphology (Figs. 35, 36) and seemed to have more filopodia compared to controls. A- αctinin and palladin co-localized in the cytosol, and α-actinin was enriched in filopodia. Palladin also appeared to localize more abundantly in nuclei compared to control cells.

100nM nocodazole treatment increased cell motilities in time-lapse imaging experiments, whereas the cell morphology and distribution of α-actinin and palladin was quite similar to the controls. Alpha-actinin is slightly more enriched in protrusions in the experimental cells than in controls (Figs. 37 and 38). The large red dots in the α-actinin staining are precipitates.

It has been shown that perturbation of microtubule depolymerization by nocodazole slows the movement of undifferentiated neural crest cells and causes retraction of processes led by the immature neurons, implicating microtubules in the

morphogenesis of neural crest cells into neurons (Haendel, Bollinger et al. 1996). Other than this finding, however, nothing is known about the function of microtubules in neural crest cells. Here, we report that nocodazole can alter cell morphology as well as the distribution of α-actinin and palladin.

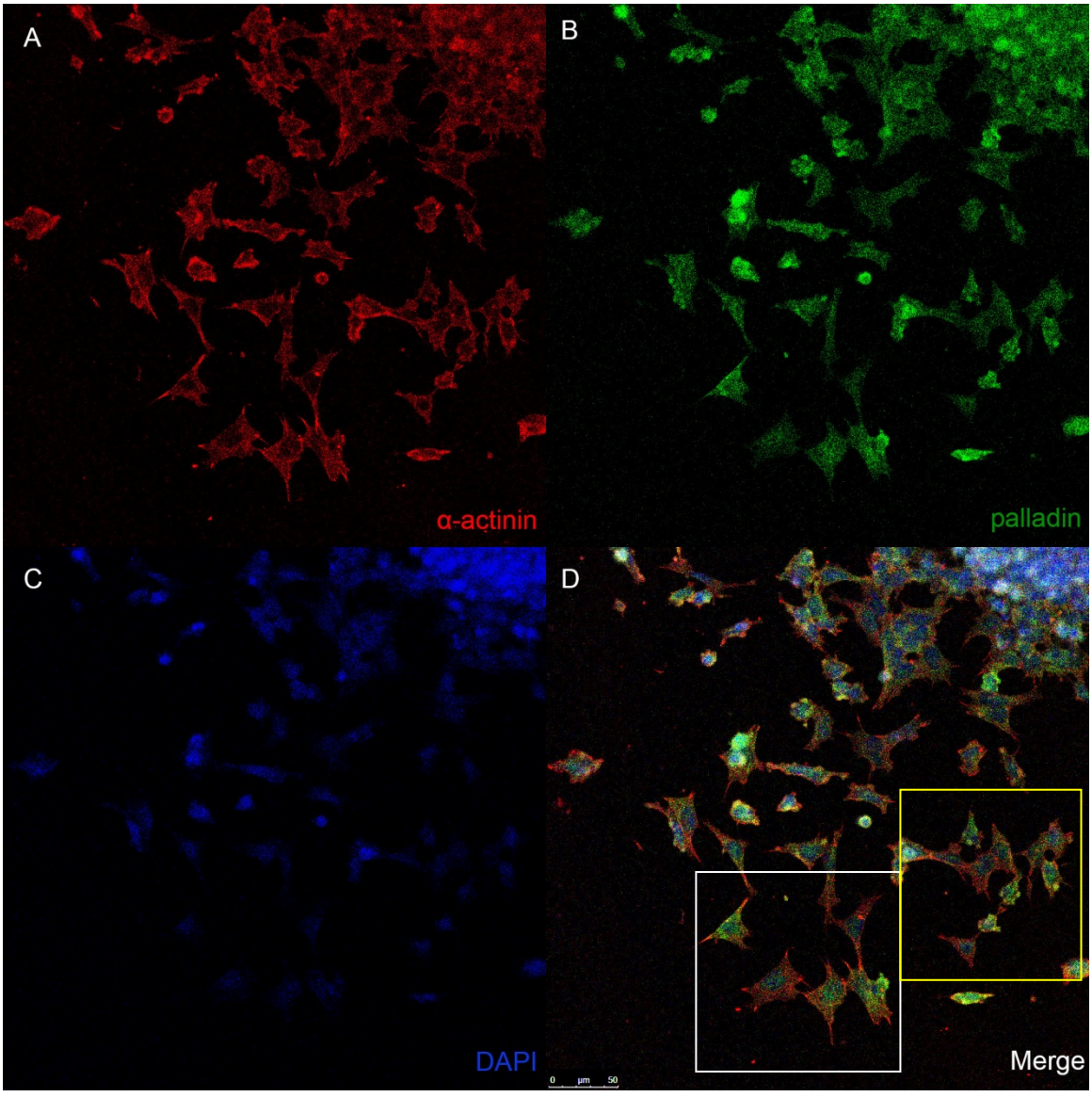


Figure 32. Distribution of α-actinin and palladin in 30uM nocodazole-treated explants. α-Actinin (A, red) and palladin (B, green) co-localize to the cytosol, α-actinin is more enriched in filopoida, and palladin localizes to the nucleus. DAPI (C, blue) stains the nucleus. Rectangular areas are magnified in Fig. 33 and Fig. 34. Bar = 50μM.

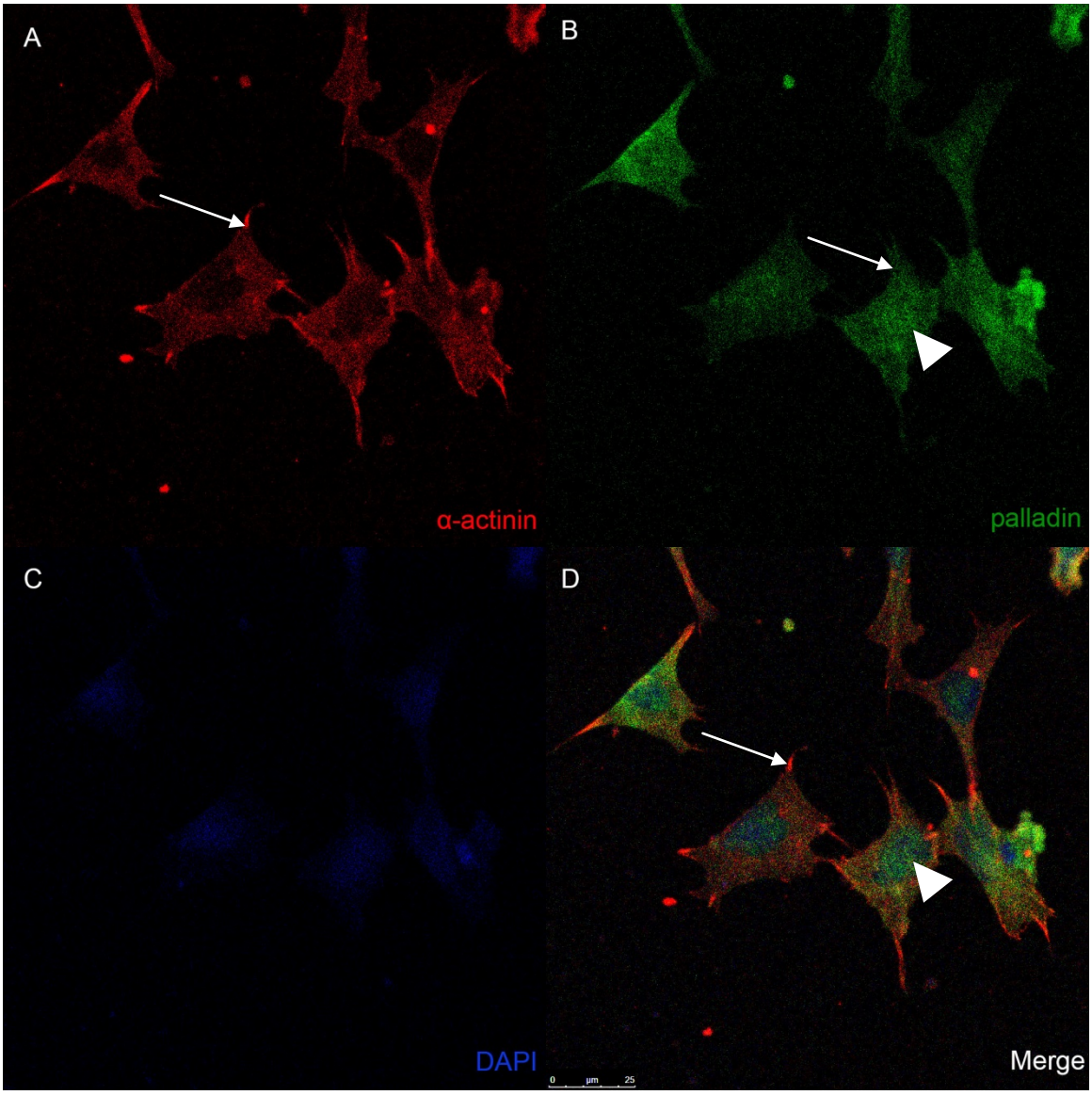


Figure 33. Distribution of α-actinin and palladin in 30uM nocodazole-treated explants (magnified image of Fig. 32 in white rectangular area). Cells surviving in 30uM nocodazole developed a flatter morphology. α-Actinin (A, red) and palladin (B, green) co-localize to the cytosol, α-actinin is enriched in filopodia (arrows), and palladin localizes to the nucleus (arrowheads). DAPI (C, blue) stains the nucleus. Bar = 25µM.

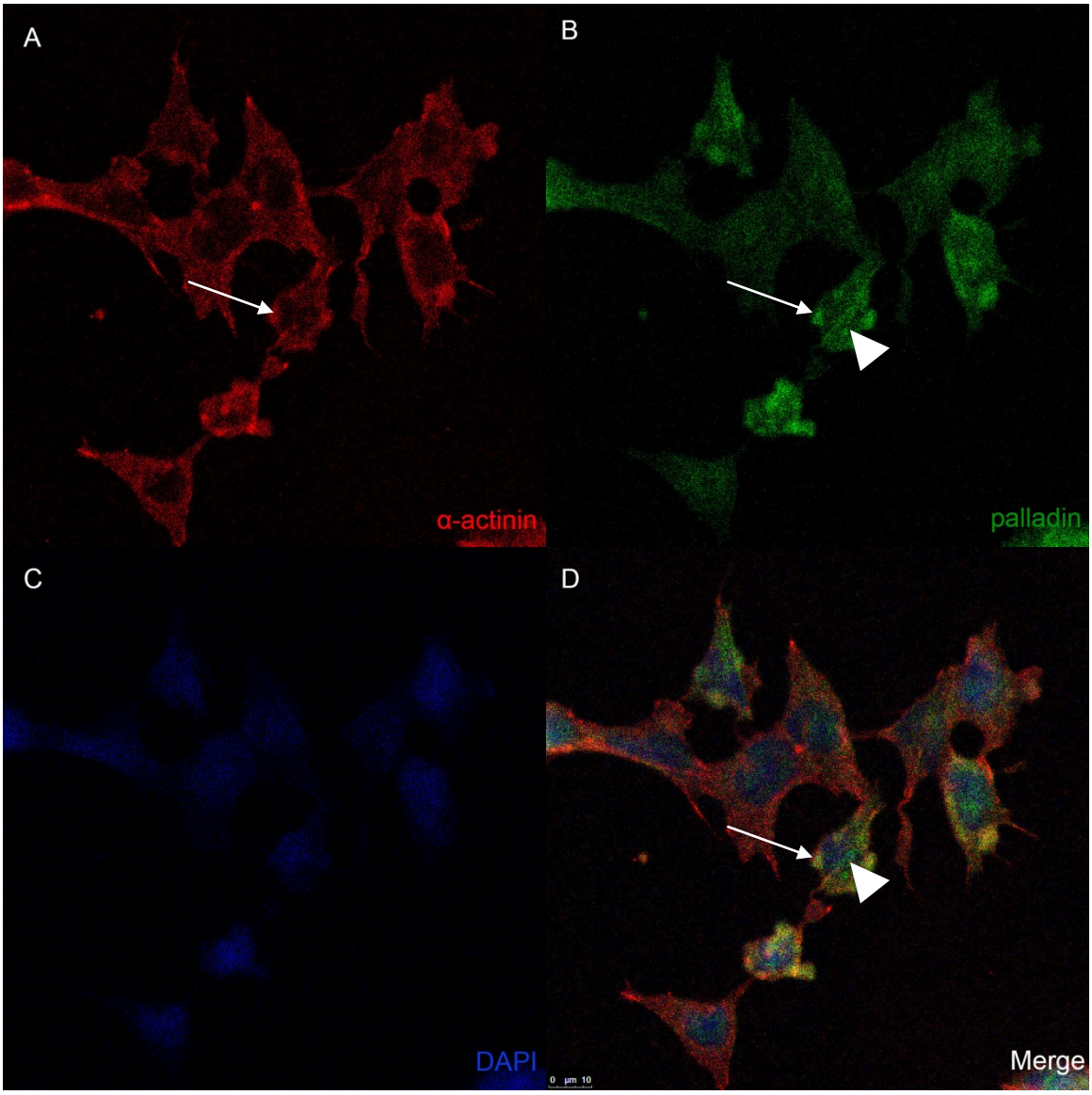


Figure 34. Distribution of α-actinin and palladin in 30uM nocodazole-treated explants (magnified image of Fig. 32 in yellow rectangular area). Dying cells are smaller, with α-actinin (A, red) and palladin (B, green) co-localizing and condensing into several dot-shape area in the cytosol (arrows). Palladin localizes to the nucleus in these cells (arrowheads). DAPI (C, blue) stains the nucleus. Bar = 10µM.

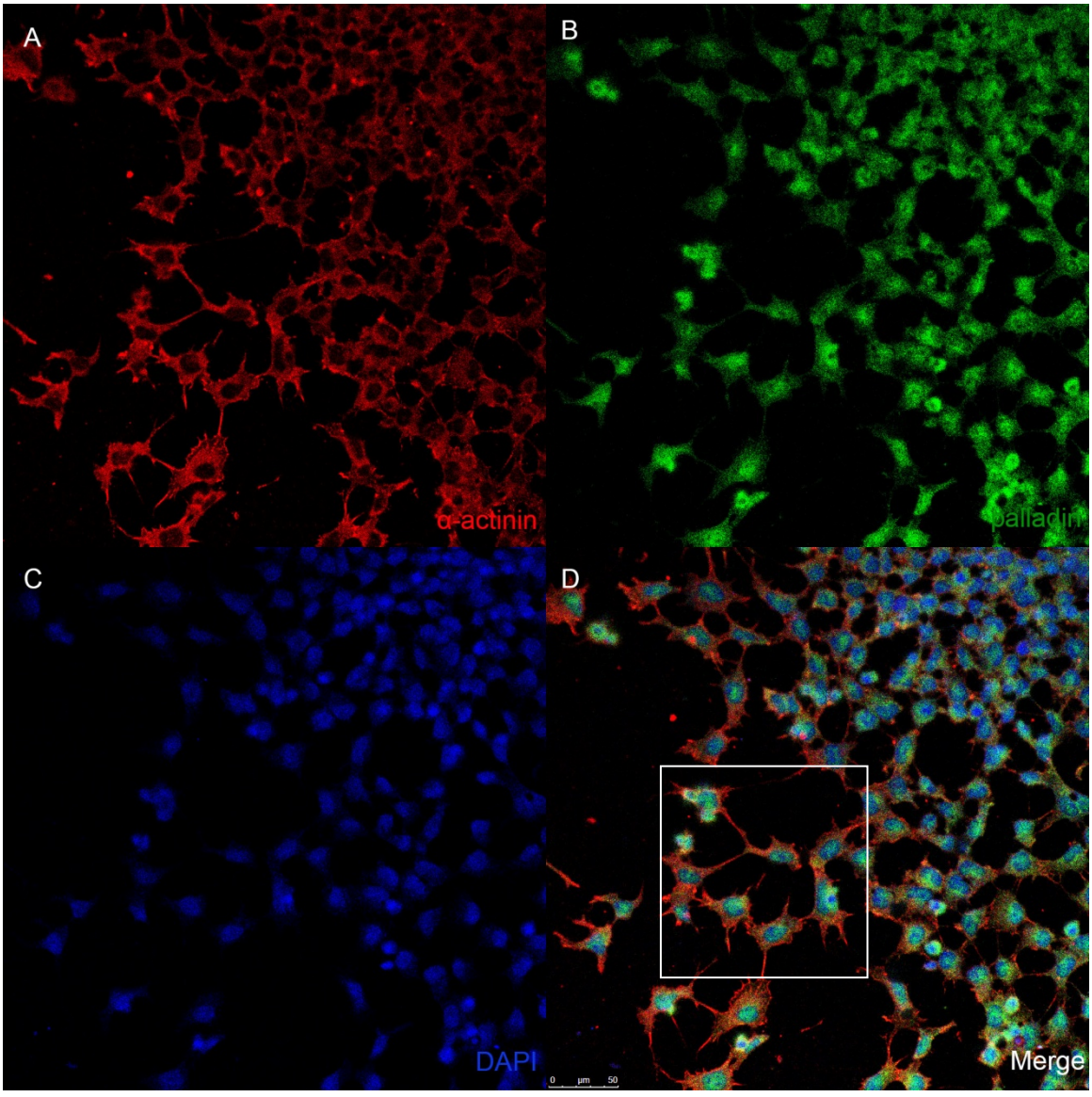


Figure 35. Distribution of α-actinin and palladin in 1uM nocodazole-treated explants. α-Actinin (A, red) and palladin (B, green) co-localize to the cytosol and filopodia, and palladin localizes to the nucleus. DAPI (C, blue) stains the nucleus. Rectangular area is magnified in Fig. 36. Bar = 50μM.

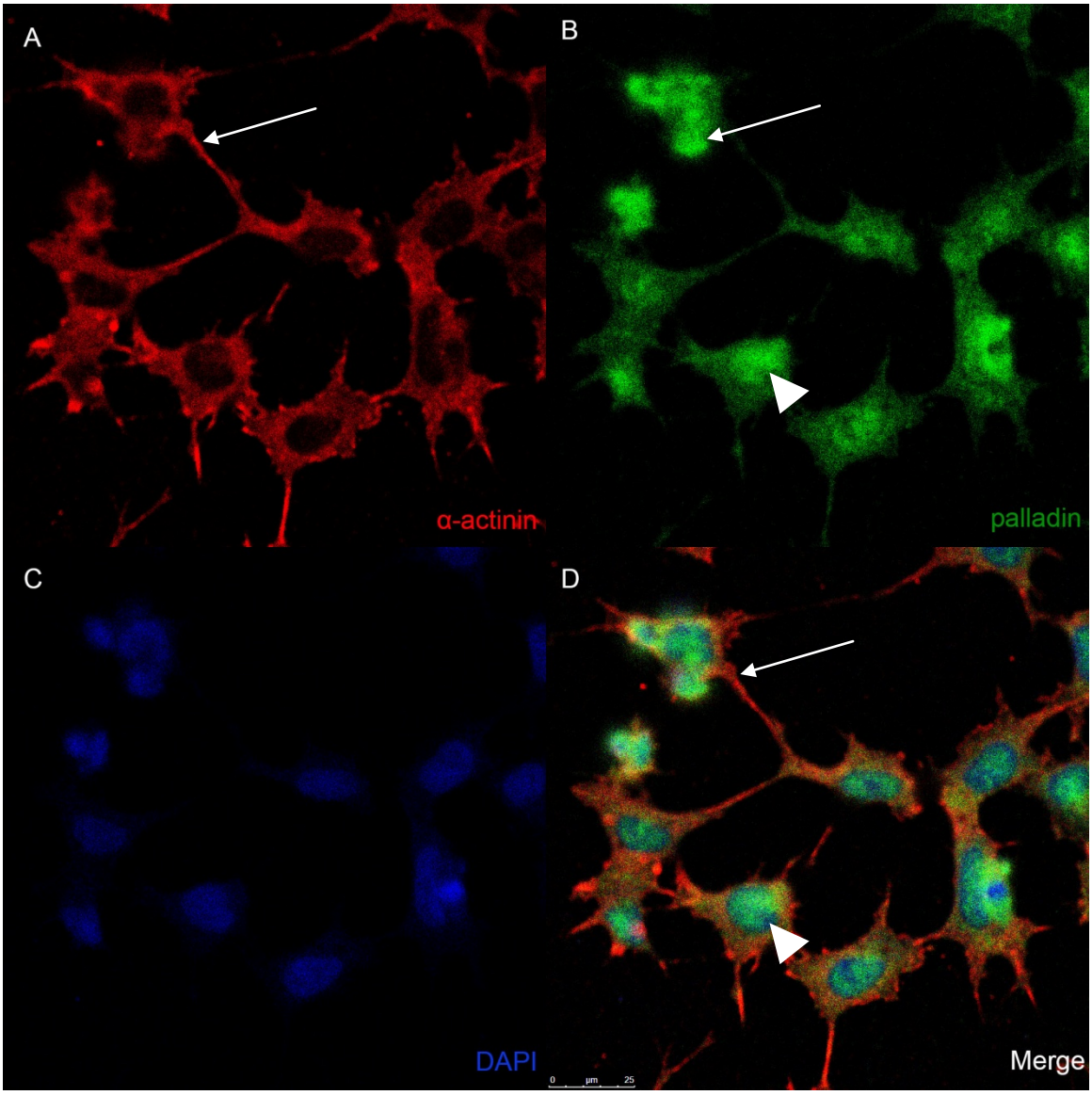


Figure 36. Distribution of α-actinin and palladin in 1uM nocodazole-treated explants (magnified image of Fig. 35 in rectangular area). α-actinin (A, red) and palladin (B, green) co-localize to the cytosol and filopodia, α-actinin is enriched in filopodia (arrows), and palladin localizes to the nucleus (arrowheads). DAPI (C, blue) stains the nucleus. Bar = 25µM.

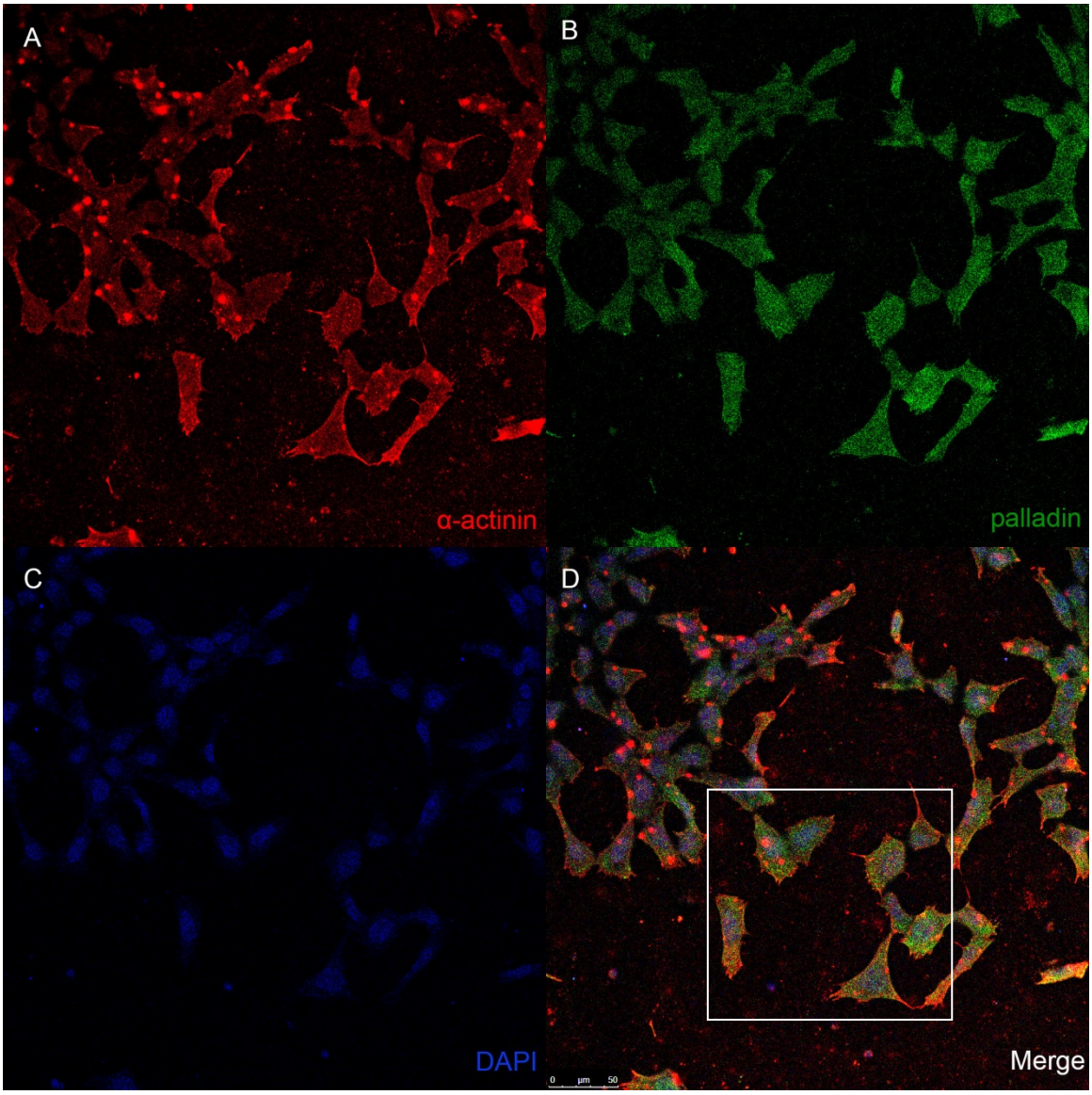


Figure 37. Distribution of α-actinin and palladin in 100nM nocodazole-treated explants. α-Actinin (A, red) and palladin (B, green) co-localize to the cytosol, and palladin localizes to the nucleus. DAPI (C, blue) stains the nucleus. Rectangular area is magnified in Fig. 38. Bar = 50µM.

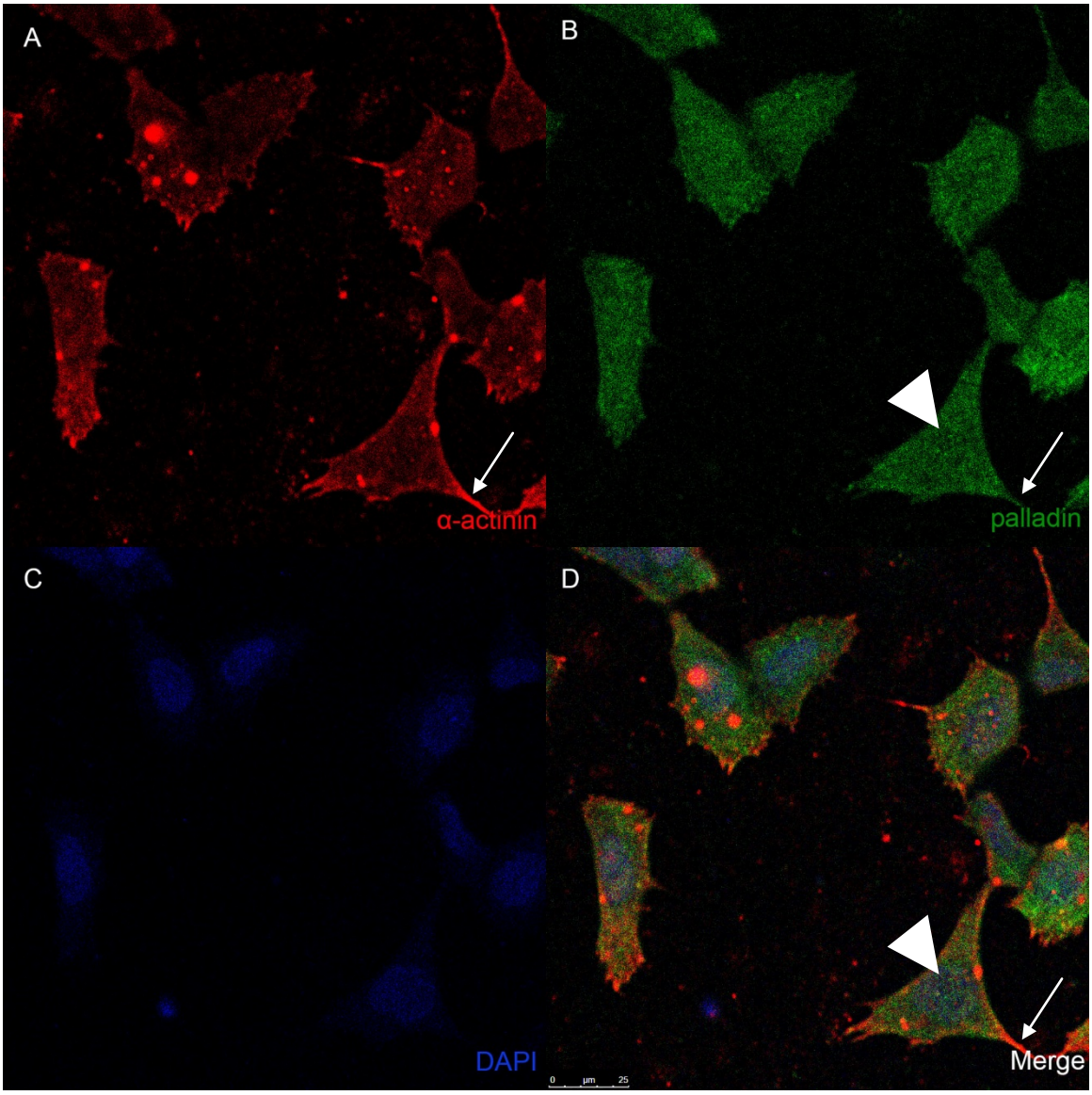


Figure 38. Distribution of α-actinin and palladin in 100nM nocodazole-treated explants (magnified image of Fig. 37 in rectangular area). α-Actinin (A, red) and palladin (B, green) co-localize to the cytosol, α-actinin is enriched in protrusions (arrows), and palladin localizes to the nucleus (arrowheads), but is reduced compared to the controls. DAPI (C, blue) stains the nucleus. The large red dots in the α-actinin staining are precipitates. Bar = 25µM.

5.5 Distribution of α-actinin and palladin in latrunculin A-treated neural crest cell explants

To examine the changes in the distribution of in neural crest cell after inhibition of the polymerization of actin filaments by latrunculin A, we fixed cells after latrunculin A inhibition and performed immunohistochemistry (Fig. 39). Because latrunculin A had a severe effect on cells and caused cell death, the cytoskeleton was likely partially distorted due to a reduction and/or loss of polymerization of actin. Immunostaining showed that cells looked smaller than controls due to retraction of the cell body. α-Actinin and palladin were localized to the cytosol. Palladin was also found in nucleus but appeared reduced in experimental cells compared to controls.

As the building blocks of cytoskeleton, disruption of actin filaments immediately leads to the arrest of neural crest cell movement and causes cell death (Haendel, Bollinger et al. 1996). We observed the same results with very low concentration of latrunculin A. We also first report the morphological changes of cranial neural crest cells treated with latrunculin A by analyzing the distribution of α-actinin and palladin.

As summarized in Table 3, α-actinin and palladin co-localize in the cytosol and in filopodia, while palladin localizes to nucleus. The nuclear localization of palladin shifted from the nucleus to the cytosol after washout of blebbistatin. At the same time, cells appeared to have asymmetric distribution of α-actinin and palladin in the cytosol after washout of blebbistatin. Y-27632 treatment resulted in uniform distribution of α-actinin and palladin in the cytosol.

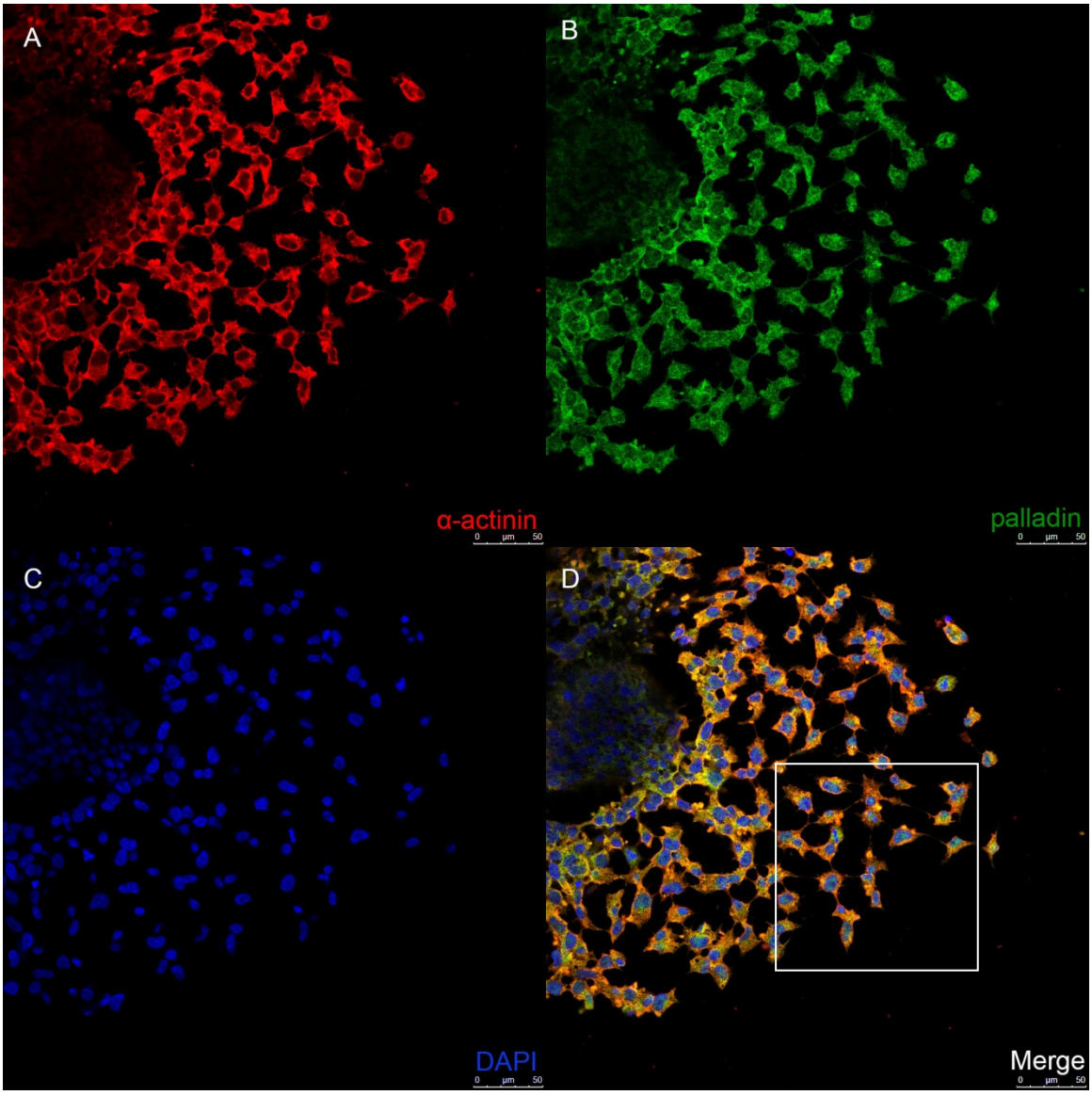


Figure 39. Distribution of α-actinin and palladin in 100nM latrunculin A-treated explants. Latrunculin A caused substantial cell death. α-Actinin (A, red) and palladin (B, green) are still co-localized to the cytosol. DAPI (C, blue) stains the nucleus. Rectangular area is magnified in Fig. 40. Bar = 50µM.

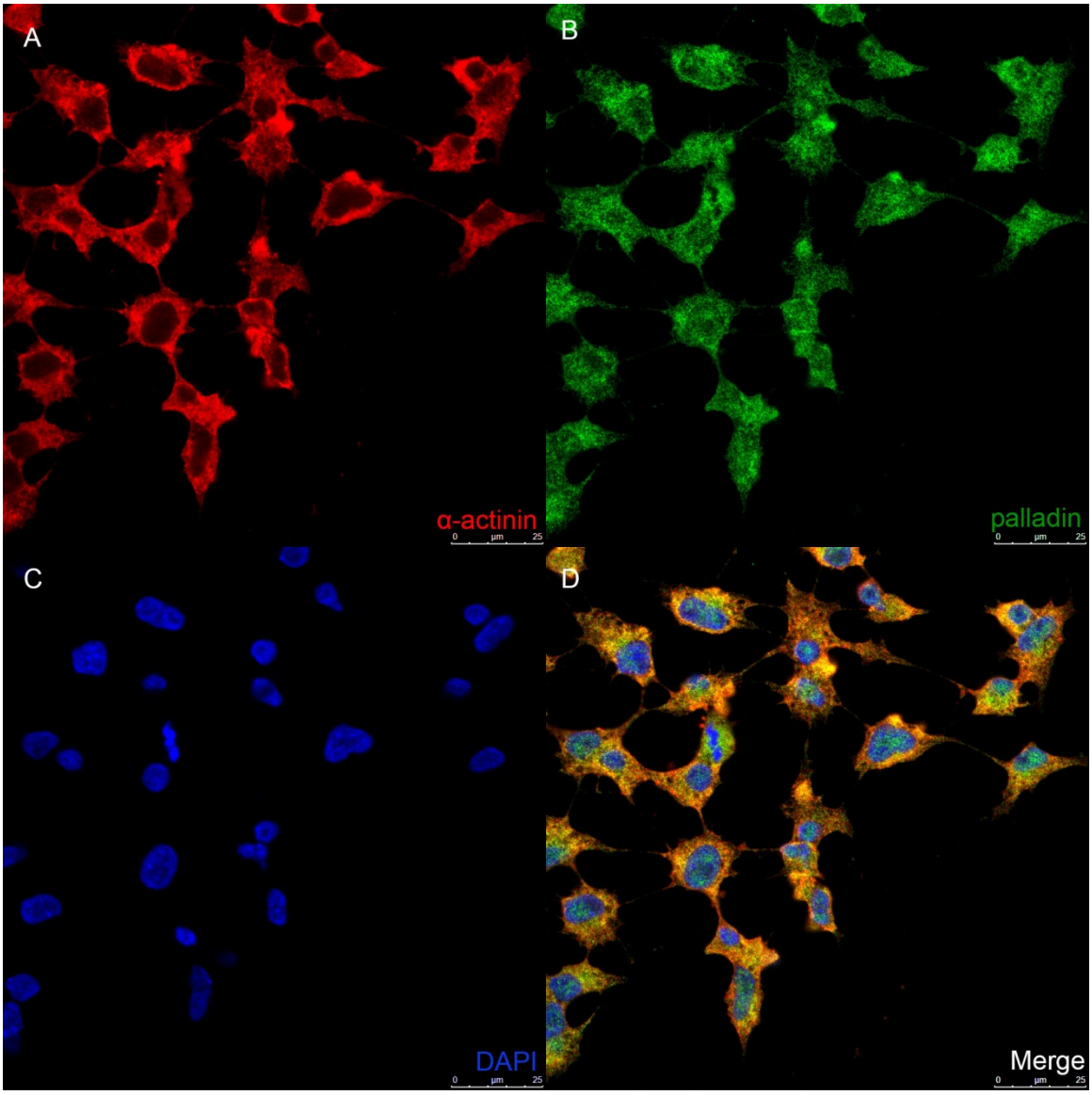


Figure 40. Distribution of α-actinin and palladin in 100nM latrunculin A-treated explants (magnified image of Fig. 39 in rectangular area). Latrunculin caused cell retraction and eventual death. α-actinin (A, red) and palladin (B, green) are still co-localized to the cytosol. DAPI (C, blue) stains the nucleus. Bar = 25μM.

Table 3. Immunohistochemistry results and relationship with vorticity

Treatment	Phenotype	Distribution of α-actinin	Distribution of palladin	Vorticity
DSMO-treated (control)	Normal migration	Cytosol, lamellipodia and filopodia	Nucleus, cytosol, lamellipodia and filopodia	
50μM blebbistatin	Blocks migration	Cytosol, filopodia	Nuclear localization much higher than controls, cytosol and filopodia	N/A
Bleb washout	Resumes migration but direction changes	Asymmetric in cytosol with more localization on the side toward neural fold	Very little nuclear localization, co-localization with α-actinin	More directional
Y-27632	Normal migration	Uniform distribution in cytosol	Less nuclear localization than controls, uniform distribution in cytosol	Less directional
100nM nocodazole	Normal migration	Cytosol, enriched in filopodia	Uniform distribution in cells	Less directional
1μM nocodazole	Normal migration	Cytosol, enriched in filopodia	More nuclear localization than controls, cytosol	N/A
30μM nocodazole	Causes cell death	Cytosol , enriched in filopodia (prior to death)	Nucleus and Cytosol	N/A
100nM latrunculin-A	Causes cell death	Cytosol (prior to death)	Nucleus and cytosol	Less directional

Chapter 6: Discussion and future plans

The neural crest is referred as the fourth germ layer because neural crest cells are able to give rise to variety of cell types, tissues and organs; it has fascinated developmental biologists for over a hundred years. Proper development of vertebrate embryos requires neural crest cells to undergo EMT and migrate to targets in a strictly regulated spatio-temporal manner. The specific cellular pathways regulating the processes, however, are poorly understood. Neural crest cells also share many similar properties with cancer cells as they are both migratory and plastic. Thus, deciphering the mechanisms controlling neural crest cell EMT and migration will shed light on the potential pathways that could be targeted for cancer therapies. Through time-lapse imaging, we were able to dynamically record neural crest cell behaviors. Here we show NM myosin II, ROCK, actin and microtubules to be critical factors involved in neural crest cell EMT and migration. We also performed immunohistochemistry of α-actinin and palladin, two actin-linkers, on the explants treated with inhibitors of above cytoskeleton components to test our hypothesis of the potential roles of these factors on neural crest cell migration.

Previous research in zebrafish embryos showed inhibition of NM myosin II by blebbistatin disrupted cell blebbing, which is required for hindbrain neural crest cells to undergo EMT in vivo (Berndt, Clay et al. 2008). Here we show in chick cranial neural crest cell explants, blebbistatin irreversibly blocks neural crest cell EMT in vitro. A previous study showed that the ROCK pathway regulates NM myosin II by phosphorylation and activation of NM myosin II (Bresnick 1999) in some cell types. Our findings, however, indicate that ROCK and NM myosin II likely are two distinct pathways in chick neural crest cells. We measured the velocity of cells on the edge of the explants to examine the spreading speed of the explants in order to characterize cell motility. In the

EMT assay, velocity drastically decreased to a negligible level (0.0182μm/min), 20-fold lower than the controls (0.3743μm/min). While cells were able to resume their motility after washout to a comparable level to the controls, they failed to resume EMT with no cells managing to come out from the neural fold after 5 hours. This finding is in agreement with what was found in hindbrain neural crest cell in zebrafish. In the neural crest cell migration assay, we showed that blebbistatin also blocks cell migration with a velocity decreased to 0.1133μm/min, 4.6-fold lower than the controls (0.5249μm/min); however, cell migration can be fully resumed to similar velocity (0.5943μm/min) as the controls after removal of blebbistatin. Intriguingly, cells changed their original direction to the neural folds causing the contraction of the explants. We noted that cells developed rounder cell bodies and increased number of filopodia in the presence of blebbistatin and resumed their normal morphology after removal of the drug. In the EMT assay, after removal of blebbistatin, epithelial cells became motile again but lost their ability to break the boundary of the neural folds and develop to individual mesenchymal cells. This indicates that NM myosin II may facilitate the loss of cell-cell adhesions at the boundary of the neural folds to promote neural crest cell EMT. In the migration assay, as mentioned in Chapter 4, chemotaxis and/or changes in cell polarity could be potential reasons for explant contraction after removal of blebbistatin. Our immunohistochemistry results, provide evidence for the theory of polarity changes through asymmetric distribution of α-actinin and palladin. The vorticity data showed that during explant contraction, cells appeared to be more linear or non-curled than cells in controls, which suggests alteration of NM myosin II by blebbistatin treatment and washout caused a change in the signals that direct neural crest cell migration. Collectively, NM myosin II has different functions during neural crest cell EMT and migration.

Functions of the Rho pathway during neural crest cell EMT and migration have been long discussed in both chick and zebrafish embryos. Although Rho has been implicated in neural crest cell EMT and migration, different research models have yielded conflicting

conclusions. Liu and Jessell first reported RhoB was expressed in the chick dorsal neural tube and migrating neural crest cells. This group also showed that inhibition of Rho by C3 exotoxin prevented the delamination of neural crest cells from trunk neural tube explants but had little effect on later migration of neural crest cells (Liu and Jessell 1998). Research on later stage zebrafish hindbrain neural crest cells also showed that the use of a ROCK inhibitor led to a reduction of EMT (Berndt, Clay et al. 2008). This group also recently showed inhibition of ROCK upon C3 exotransferase disrupted neural crest cell EMT in zebrafish (Clay and Halloran 2013). We found that the Rho inhibitor, Y-27632, promotes EMT, with more mesenchymal cells emerging from neural folds compared to the controls within the same time period. Consistent with our findings in the EMT assay, Y-27632 treatment also led to an increase the velocity of cells migrating out from the neural folds. This is the first time that an inhibitory effect of the Rho pathway has been documented in cranial neural crest cells. Our findings are in agreement with what has been observed in chick hindbrain neural crest cells. This study showed that alterations of RhoA *in vivo* induced more cells to become migratory(Rupp and Kulesa 2007). Another *in vitro* research on late stage trunk neural crest cells also reported C3 and Y-27632 both enhanced cell emigration without affecting cell proliferation (Groysman, Shoval et al. 2008). The conflicting findings may be caused by different species and/or axial levels. In addition, we also found in the vorticity test that Y-27632 treatment slightly altered cell behaviors toward a more curled direction. It has been reported in *Xenopus* and zebrafish that, through inactivation of Rac, RhoA is involved in regulation of neural crest cell directional migration (Matthews, Marchant et al. 2008). Our findings provided more data regarding the function of RhoGtpase in regulating cell directional migration.

Only one other report in chick trunk neural crest explants has examined microtubule function in neural crest cells. This study revealed that perturbation of microtubule depolymerization by nocodazole slowed the movement of undifferentiated neural crest cells and caused retraction of processes extended by the immature neurons, implicating

microtubules in the morphogenesis of neural crest cells into neurons (Haendel, Bollinger et al. 1996). In our EMT and migration assay using different concentrations of nocodazole, we found that the effect of nocodozole on both neural crest cell EMT and migration was dose-dependent. Similar dose-dependent effects were observed with endothelial cells. In this study, micromolar concentrations of nocodazole irreversibly changed the barrier function and caused cell death, whereas nocodazole in 100 to 200 nM concentrations reversibly increased the permeability of the endothelial monolayer (Smurova, Birukova et al. 2008). 30μM nocodazole caused irreversible blocks on neural crest cell EMT and migration leading to most cell death. This block may due to the toxicity of high concentration of nocodazole on cells that perturbs many cellular process including EMT and migration. 1μM nocodazole blocks neural crest cell EMT by affecting the cell capacity to maintain mesenchymal cell shape, but cells were able to move with a comparable velocity as the controls. Washout in the EMT assay led to a decrease in velocity, but the mesenchymal cell type was resumed, indicating a partial resumption of EMT even though cells eventually died after a few hours. Interestingly, the velocity decrease after washout was also observed in the migration assay and was significant. Although no apparent change in cell morphology was observed in presence of 1μM nocodozaole, cells eventually died 8 hours after washout. This suggests nocodazole potentially has an unknown function facilitating cell motility other than through microtubule depolymerization. This function compensates for the lost of microtubule function inhibited by nocodazole, leading to mild changes in cell behavior compared to after washout, in which the compensated function and microtubule function both disappear, causing a cessation of cell migration. 100nM also affects neural crest cell EMT, with few cells emerging from the explants that had an accelerated velocity compared to control and 1μM nocodazole treatment. This implicates the plus end of microtubule in neural crest cell EMT. The velocity did not change after washout in the EMT assay but significantly decreased in the migration assay, which is consistent with the findings in the

1μM nocodazole treatment on migration assay. Note that the velocity of cells in the migration assay in the presence of 100nM nocodazole is faster than the control and significantly decreased after washout, which further supports our hypothesis with regards to an additional function in facilitating cell motility provided by nocodazole. Vorticity data on 100nM nocodazole treatment showed a broader distribution, suggesting that the plus end of microtubules plays a role in cell migration potentially in modulating the directionality of cell movement.

A previous study has shown that microfilament disruption by cytochalasin immediately arrests the movement of neural crest cells and causes them to round-up (Haendel, Bollinger et al. 1996). An indirect study of actin filaments on ILK, a linker between integrins and actin cytoskeleton, showed that knock out of ILK in mouse neural crest cells led to embryonic lethality (Arnold, Zang et al. 2013). In agreement with previous findings, our experiment with 100nM latrunculin A caused drastic changes in cell morphology and cell death in migratory neural crest cells. This harsh effect could not be removed after washout of latrunculin-A. This suggests that actin filaments, as the building blocks of cytoskeleton, are involved in neural crest cell migration.

Although the *in vitro* experiment attempts to mimic the *in vivo* events during EMT and migration, this might not be the case *in vivo*. It might be different or contradictory to what we observed since cells receive cues from external environment including ephrins and semaphorins (that help define their migratory pathways) while they are migrating. The intrinsic molecular factors in charge of cell behaviors during EMT and migration may be different, since they may be regulated by external signals *in vivo*. With that, our findings still demonstrate, for the first time, the changes of cell behaviors, velocity, and vorticity after different inhibitor treatments during EMT and migration in chick cranial neural crest cells.

Further exploration could focus on validating these *in vitro* findings *in vivo* by employing knock-down techniques to eliminate specific pathway components. Several

intriguing findings also require further attention. Deciphering the underlying mechanism of cell movement after washout of blebbistatin will help understand pathways controlling directional cell migration. At the same time, the unknown pathway that nocodazole regulates with respect to facilitating cell motility also is worth exploring.

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