Cytoneme-mediated intercellular signaling in keratinocytes essential for epidermal remodeling

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Summary
The skin, the body's largest organ, functions as a primary defense mechanism. Epidermal stem cells supply undifferentiated keratinocytes that differentiate as they migrate toward the outermost skin layer. Although such a replenishment process is disrupted in various human skin diseases, its underlying mechanisms remain elusive. With high-resolution live imaging and in vivo manipulations, we revealed that Notch signaling between keratinocytes is mediated by signaling filopodia called cytonemes and is essential for proper keratinocyte differentiation and proliferation. Inhibiting keratinocyte cytonemes reduces Notch within undifferentiated keratinocytes, leading to abnormal differentiation and hyperproliferation, resembling human skin disease phenotypes. Overproduction of Interleukin (IL)-17 signal, associated with skin diseases like *psoriasis*, induces psoriatic phenotypes via cytonemes in zebrafish. Clathrin interactor 1 (*clint1*), crucial for epidermal homeostasis in zebrafish and humans, regulates cytoneme extension. Our study suggests that intercellular signaling between keratinocytes via cytonemes is critical for epidermal maintenance, and its misregulation could be an origin of human skin diseases.

Introduction
The vertebrate skin is a multilayered structure comprised of three main layers: the epidermis, the dermis, and the hypodermis (Akat et al., 2022). Each layer has distinct anatomical characteristics and serves specific functions. Of these layers, the epidermis, as the outermost layer, plays a pivotal role as the body's first line of defense against environmental threats such as pathogens. It also plays a crucial role in regulating body temperature and preventing water loss into the surrounding environment. The epidermis consists of various layers, including the stratum basale, which houses a single layer of stem cells, and several layers of differentiated cells, including the stratum spinosum, stratum granulosum, and stratum lucidum. The outermost layer, the stratum corneum, primarily comprises dead keratinocytes that are continuously shed and replenished by underlying cells differentiated from epidermal stem cells in mammals (Segre, 2006).
In zebrafish, the epidermis shares a similar composition to that of mammals. However, unlike mammals, aquatic animals lack a cornified layer and instead rely on mucous layer to protect the epidermis in the aquatic environment. The basal layer of zebrafish, equivalent to mammalian stratum basale, consists of stem cells that provide keratinocytes for replenishment during epidermal remodeling or wound healing. The

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periderm, comparable to the mammalian stratum granulosum and lucidum, is where fully differentiated keratinocytes are found. Similar to mammalian stratum granulosum, an intermediate layer is present between the basal layer and the periderm, housing undifferentiated keratinocytes (Chang and Hwang, 2011). This intermediate layer is formed during metamorphosis and persists throughout adulthood in zebrafish (Lee et al., 2014).

The maintenance of a healthy epidermal barrier requires on the intricately controlled proliferation and differentiation of keratinocytes, which are supplied from underlying basal stem cells (Blanpain and Fuchs, 2009). As keratinocytes migrate apically from the stem cell layer, they undergo differentiation while concurrently exhibiting proliferation within the intermediate layer. Ultimately, fully differentiated keratinocytes replenish the periderm. This replenishment mechanism is well-conserved in both zebrafish and humans. Its mis-regulation constitutes a pivotal factor in the etiology of various human skin diseases, including *psoriasis*, *atopic dermatitis*, and others (Chang and Hwang, 2011; Lowes et al., 2014; Nowell and Radtke, 2013; Schempp et al., 2009; Zhou et al., 2022). Studies have also revealed that maintaining a healthy epidermis requires complex intercellular communications between keratinocytes and the immune system, adding complexity to our efforts to comprehensively understand the mechanism (Blanpain and Fuchs, 2009; Park et al., 2021).

One of the major signaling pathways critical for keratinocyte differentiation and proliferation is the Notch pathway. Studies have demonstrated that Notch receptors and ligands exhibit spatially restricted expression patterns in layers in epidermis (Nowell and Radtke, 2013). Notch 1, 2, and 3 are notably prevalent in the undifferentiated keratinocytes in mice (Blanpain et al., 2006; Nickoloff et al., 2002; Thelu et al., 2002). Dysregulated Notch signaling has been linked to abnormal keratinocyte differentiation and hyperproliferation, which are one of the hallmarks of various skin diseases including *psoriasis* and *atopic dermatitis* (Gratton et al., 2020), highlighting the importance of proper Notch signaling in epidermal maintenance to retain the balance between keratinocyte differentiation and proliferation (Ota et al., 2014; Rangarajan et al., 2001). Nonetheless, the precise mechanisms underlying the initiation of Notch signaling in undifferentiated keratinocytes and its dynamic regulations are not yet understood.

Furthermore, various combinations of cytokines produced by different immune cells under inflammatory conditions can significantly contribute to the onset or progression of these skin diseases. Among these, interleukin-17 (IL-17) is a well-characterized cytokine closely associated with the symptoms of these conditions. The overproduction of IL-17 by T helper (Th)17 cells and other immune cells is a primary driver of certain skin diseases, such as *psoriasis*. Biologic treatments that inhibit IL-17 have proven to effectively alleviating the symptoms of these conditions (Mosca et al., 2021). Unfortunately, discontinuing these treatments often lead to symptom recurrence. Also known side effects associated with these inhibitors include headaches, nasopharyngitis, and infections (Campa et al., 2016). Consequently, a definitive cure remains elusive currently (Fragoulis et al., 2016; Lowes et al., 2014).

Thus, IL-17 is one of the key molecules that contribute to the development of *psoriasis* and other skin diseases. The IL-17 pathway exerts its effects on keratinocytes, endothelial cells, and immune cells resulting in the production of additional cytokines

that promote positive feedback loops, sustaining the inflammatory state (Mosca et al., 2021). However, the underlying cellular and molecular mechanisms of this process are not yet fully understood. Therefore, gaining a comprehensive understanding of the interplay between keratinocytes, the Notch pathway, IL-17, and other players is essential for advancing the treatment of these diseases.

Intercellular signaling plays a critical role in skin development and maintenance. In recent years, a growing body of evidence has demonstrated that cells can establish communication through long, thin cellular protrusions extended by cells involved in either sending or receiving signals. These protrusions can be categorized based on their cytoskeletal composition, mode of signal delivery, morphology, and other characteristics. Examples include cytonemes, airinemes, tunneling nanotubes, intercellular bridges, migrasomes, exophers, and more (Eom, 2020; Kornberg and Roy, 2014b; Ma et al., 2015; Melentijevic et al., 2017; Zhang and Scholpp, 2019). These specialized cellular protrusions have been observed in diverse cell types and species, ranging from fruit flies and sea urchins to zebrafish and mice, with their signaling roles experimentally confirmed *in vivo* (Daly et al., 2022; Eom, 2020; Hall et al., 2023; Kornberg and Roy, 2014a; Zhang and Scholpp, 2019).
These specialized protrusions are significantly longer than typical filopodia and establish direct contact with target cells and serve as highways for transporting signaling molecules for major signaling pathways such as Hedgehog, Wnt, TGFβ, FGF, Notch in many *in vivo* and *in vitro* contexts (Daly et al., 2022). These signaling cellular protrusions temporally exist and have thin actin or actin/tubulin-based filaments. Consequently, live-imaging is considered one of the most effective methods for observing these structure. For that reason, zebrafish has emerged as an excellent model system for studying these cellular protrusions. The transparent nature of their early developing embryos makes zebrafish particularly well-suited for this purpose. Moreover, zebrafish are vertebrates that share common mechanisms with mammals in terms of epidermal remodeling and maintenance (Eisenhoffer et al., 2017; Li et al., 2011; Martinez-Navarro et al., 2019).

In this study, we show evidence that cytonemes extended by fully differentiated keratinocytes play a crucial role in activating Notch signaling in undifferentiated keratinocytes, thus contributing to the maintenance of epidermal homeostasis and remodeling in zebrafish. Furthermore, we demonstrate that IL-17, a key player in the pathogenesis of human skin diseases, and *clint1*, a gene required for epidermal homeostasis, regulate keratinocyte cytonemes (Dodd et al., 2009; Sahlen et al., 2021). Collectively, our findings shed light on the mechanisms that govern the regulation of skin replenishment through keratinocyte cytonemes, providing a novel perspective on understanding how skin diseases can originate from keratinocytes autonomously through the mediation of cellular protrusions.

Results

Differentiated keratinocytes extend cytoneme-like cellular protrusions

In previous studies, we have reported a unique type of signaling cellular protrusions known as ‘airinemes’ and have investigated their signaling roles in pigment cells of zebrafish (Bowman et al., 2023; Eom, 2020; Eom et al., 2015; Eom and Parichy, 2017; Park et al., 2022). To extend our understanding of airinemes in other cell types, we employed random cell labelling and identified several cell types that extend airineme-like protrusions featured by highly curved filaments and large vesicles at their tips (Eom, 2020; Eom et al., 2015; Lee et al., 2014). Fully differentiated keratinocytes, marked by *krt4*, were among the cell types displaying these airineme-like protrusions (Lee et al., 2014) (Figure 1A, arrowhead). These keratinocytes intriguingly also extend cytoneme-like protrusions, characterized by relatively straight filaments and a lack of vesicle at the tips (Eom, 2020; Kornberg and Roy, 2014b; Zhang and Scholpp, 2019) (Figure 1A, B, arrowheads). We observed that while airineme-like protrusions are rare, cytoneme-like are more frequently observed in these keratinocytes (Figure 1C). Consequently, we conducted a more in-depth characterization of these cytoneme-like protrusions. It is noted that cytonemes primarily consist of actin filaments, whereas airinemes are both actin and tubulin-based structures (Eom, 2020). To affirm their nature, we utilized an actin marker, *LifeAct-mRuby*, which clearly labeled these cytoneme-like protrusions while showing no co-localization with *tubulin-mCherry* (Figure 1D, E). Additionally, cytoneme-like protrusions were significantly reduced with actin inhibitor treatment (Figure S1). Therefore, based on their cytoskeletal composition and morphology, we classified them as cytonemes.

Keratinocytes marked by *krt4* expression extend cytonemes with the highest frequency during post-embryonic stages, specifically between 6.5 to 7.5 SSL (Standardized Standard Length (Parichy et al., 2009)) (Figure 1C). It is worth noting that embryonic keratinocytes are replaced by post-embryonic cells around these stages, suggesting the potential role of cytonemes in epidermal remodeling and maintenance (Lee et al., 2014; Parichy et al., 2009). These keratinocyte cytonemes exhibit an approximate speed of 2.98µm/min during extension and retract at 1.9µm/min, with an average length of 18.21µm (Figure 1F, G).

Keratinocyte cytonemes establish physical contact with underlying undifferentiated keratinocytes

Similar to mammals, the zebrafish epidermis consists of three layers. We specifically visualized the periderm, which is the outermost layer and consists of *krt4*+ fully differentiated keratinocytes. The intermediate layer, where undifferentiated keratinocytes reside, was marked with *krtt1c19e* promoter driving tdTomato expression, pseudo-colored in magenta (Chang and Hwang, 2011; Lee et al., 2014). The basal layer was also labelled with *krtt1c19e* but exhibited a typical polygonal morphology (Figure 2A-A”). This allowed us to distinguish all three epidermal layers using the *Tg(krt4:lyn-EGFP; krtt1c19e:lyn-tdTomato)*.

In addition to *krt4*+ keratinocytes, we explored whether undifferentiated keratinocytes (*krtt1c19e*+) also extend cytonemes or other types of cellular protrusions during metamorphic stages. However, we observed that cytoneme-like protrusions from undifferentiated keratinocytes were significantly fewer in numbers compared to those

from fully differentiated keratinocytes, and we did not observe cytoneme-like protrusions from basal stem cells (Figure 2B). In this study, our focus was primarily on cytonemes from *krt4+* differentiated keratinocytes.
To investigate the role of keratinocyte cytonemes, we first asked what the target cells of those cytonemes are. High resolution confocal imaging revealed that cytonemes originating from peridermal keratinocytes (*krt4+*) establish physical contact with underlying undifferentiated keratinocytes (*krtt1c19e+*) in the intermediate layer. Cross-sectional views clearly showed that the tips of these cytonemes stop at the intermediate layer, with no observed instances reaching the basal layer (Figure 2C, red dashed circles).

Cytoneme-mediated signaling regulates keratinocyte differentiation and proliferation

The observations that cytonemes most often observed during epidermal remodeling and their target cells are undifferentiated keratinocytes led us to hypothesize that cytoneme-mediated signaling play a pivotal role in regulating keratinocyte differentiation and proliferation.
To test, we generated a transgenic line capable of temporally expressing a dominant negative form of cdc42 (cdc42DN) in peridermal keratinocytes, a strategy frequently used to inhibit cytoneme extension in various contexts (Daly et al., 2022; Zhang and Scholpp, 2019). We implemented a dually inducible TetON system in the transgene, denoted as *Tg(krt4:TetGBDTRE-v2a-cdc42DN),* and immersed the transgenic and control fish in water containing inducer drugs, doxycycline and dexamethasone, for temporal cdc42DN transgene induction (Eom et al., 2015; Knopf et al., 2010). To minimize potential off-target effects of cdc42 manipulation, we determined the drug concentrations at which normal filopodial or lamellipodial activities were maintained while inhibiting cytoneme extensions (Video S1). We employed two control groups in this experiment: one consisting of a transgenic line with no drugs and the other comprising non-transgenic fish treated with the same drug concentrations as the experimental group. Under these conditions, we observed a significant reduction in the frequency of cytoneme extension in the cdc42DN-expressing transgenic line within the drug treated group (Figure 3A). In contrast to the two control groups, the peridermal layer in the cytoneme-inhibited group displayed severe disorganization, with areas devoid of *krt4+* keratinocytes (Figure 3B”, arrowhead). Furthermore, the expression of the undifferentiated keratinocyte marker, *krtt1c19e* (magenta), was dramatically increased in the periderm when cytoneme extension was inhibited (Figure 3B-B”, C). Additionally, we found a substantial increase in the number of keratinocytes within the peridermal layer in the cytoneme-inhibited group (Figure 3D). However, it is important to note that cytoneme inhibition did not affect the cell death rate or the number of goblet cells (Figure 3E, 2A’, Figure S2A). These findings strongly suggest that cytoneme-mediated signaling controls keratinocyte differentiation and proliferation.

Cytonemes activate Notch in undifferentiated keratinocytes

Previous studies have established the role of Notch signaling in controlling keratinocyte differentiation and proliferation. However, the mechanisms triggering Notch signaling in keratinocytes have remained obscure (Moriyama et al., 2008; Nguyen et al., 2006;

Rangarajan et al., 2001). To investigate whether the target cells of cytonemes, specifically undifferentiated keratinocytes in the intermediate layer, are responsive to Notch signaling, we crossed Notch signal reporter line, *Tg(Tp1:H2B-mCherry),* with *Tg(krt4:lyn-EGFP; krtt1c19e:lyn-Tdtomato)* to label all three epidermal layers (Parsons et al., 2009). We observed that only undifferentiated keratinocytes in the intermediate layer exhibit Notch responsiveness, whereas keratinocytes in the periderm or basal layer do not (Figure 4A, arrowheads, Figure S3).

Subsequently, we tested whether Notch activation in undifferentiated keratinocytes is dependent on cytonemes. First, we validated the *Tp1* Notch reporter line, *Tg(Tp1:EGFP)* by measuring cytoplasmic EGFP signal before and after Notch inhibitor, LY411575 treatment, which demonstrated a significant decrease in Notch signaling, as expected (Figure S4A, B). Next, we measured the difference in *Tp1* intensity before and after cytoneme inhibition. Notably, we observed a significant reduction in Notch signal under conditions where cytonemes were compromised (Figure 4B, Figure S4C). We also found more abundant Notch activated keratinocytes during metamorphic stages (Figure S4D, Figure 1C). These suggest cytonemes are responsible for Notch activation in undifferentiated keratinocytes.

We then asked why Notch activation is dependent on cytonemes since peridermal keratinocytes and underlying intermediate keratinocytes are in constant contact with each other. We revealed that intermediate keratinocytes exclusively express *lfng* (lunatic fringe), which potentially act as a default inhibitor of Notch activation in these cells (Dale et al., 2003; Panin et al., 1997) (Figure S5A, S3). However, this needs further studies to understand how cytonemes overcome this Notch signaling barrier or potentiate the signal.

Next, we hypothesized that if Notch activation by cytonemes is indeed essential for keratinocyte differentiation and proliferation, then direct inhibition of Notch signal in undifferentiated keratinocytes should yield phenotypes similar to those observed when cytonemes are inhibited (Gratton et al., 2020). To investigate this, we generated a transgenic line capable of temporally expressing dominant negative form of Suppressor of Hairless (SuHDN) in the undifferentiated keratinocytes, *Tg(krtt1c19e:TetGBDTRE-v2a-SuHDN)* (Eom et al., 2015). We observed that Notch inhibition in the undifferentiated keratinocytes resulted in a disorganized periderm, abnormally increased expression of undifferentiated keratinocyte marker, and hyperproliferation of keratinocytes in the periderm (Figure 4C-C”, D, E, F, Figure S4C). These results closely mirrored the phenotypes observed when we inhibited cytoneme extension in peridermal keratinocytes (Figure 3B-B”, C, D, Figure S4C). These observations suggest that Notch signaling, activated by cytonemes in undifferentiated keratinocytes, plays a critical role in epidermal remodeling and maintenance.

It has been documented that both hypo- or hyper-activation of Notch signaling can lead to the onset of skin diseases (Abdou et al., 2012; Gratton et al., 2020). Thus, we further investigated whether gain-of-function experiments for Notch also disrupt epidermal homeostasis. We overexpressed the Notch IntraCellular Domain (NICD) in undifferentiated keratinocytes, using the *Tg(krtt1c19e:nVenus-v2a-NICD)*. Consistent with previous studies in mammals and the cytoneme or Notch loss-of-function experiments, we observed disorganized periderm, abnormal keratinocyte differentiation and hyper-proliferation (Figure S5B-B’, C, D). These results imply that uncontrolled

Notch signaling in keratinocytes could lead to disruption of epidermal remodeling and maintenance in both zebrafish and mammals.

Interleukin-17 regulates keratinocyte cytonemes

The aberrant differentiation and hyperproliferation of keratinocytes observed when cytoneme extension was inhibited in peridermal keratinocytes (*krt4*+) or Notch signaling was inhibited in undifferentiated keratinocytes (*krtt1c19e*+) are hallmark features of many human skin diseases such as *psoriasis*, *atopic dermatitis* and more (Armstrong and Read, 2020; Gratton et al., 2020) (Figure 3, 4). Also, it has been well established that impaired Notch signaling is associated with some of these human skin disorders (Gratton et al., 2020; Nowell and Radtke, 2013). Thus, we asked which key molecules are critical for the onset or progression of these skin diseases and whether they have an impact on keratinocyte cytoneme signaling.

One of them is interleukin-17 (IL-17) and we hypothesized IL-17 signaling could influence cytoneme extension in peridermal keratinocytes. Given that IL-17 is a cytokine released into the extracellular space, and to test its role in peridermal keratinocytes, we decided to overexpress the IL-17 receptors. It is noted that interleukin-17 receptors and ligands are evolutionarily conserved across chordates (Gonzalez-Fernandez et al., 2020; Wu et al., 2011).

To test, we generated transgenic lines that cell-autonomously overexpressed il17 receptor D (*il17rd*) or il17 receptor A1a (*il17ra1a*) in fully differentiated peridermal keratinocytes (*krt4*+), *Tg(krt4:nVenus-v2a-il17rd)* and *Tg(krt4:il17ra1a-v2a-mCherry)*. Both of these IL-17 receptor-overexpressing transgenic lines exhibited a significant reduction in cytoneme extension, resulting in a decreased Notch activation in undifferentiated keratinocytes (Figure 5A, B). Moreover, similar to the two previous manipulations, we observed a severely disorganized periderm, an upregulation of the undifferentiated keratinocyte marker expression, and hyperproliferation of keratinocytes within the periderm (Figure 5C-C', D, E, Figure S3).

To assess whether IL-17 signaling regulates keratinocyte differentiation and proliferation through cytonemes, we crossed two transgenic lines: *Tg(krt4:TetGBDTRE-v2a-cdc42DN)* and either *Tg(krt4:nVenus-v2a-il17rd)* or *Tg(krt4:il17ra1a-v2a-mCherry)*. If cytoneme extension and IL-17 signaling are part of the same pathway, we would expect to observe a more severe inhibition of cytoneme extension when these manipulations are combined compared to individual manipulations. Indeed, when inhibited cytoneme extension with the cdc42DN along with *il17rd* or *il17ra1a* overexpression, we observed a more significant inhibition of cytoneme extension than when manipulated individually (Figure 5F).

Next, we sought to understand how the IL-17 signal impacts cytoneme extension. Studies have shown that IL-17 signaling can regulate actin cytoskeleton in keratinocytes (Borowczyk et al., 2020; Das et al., 2019). To evaluate whether *il17rd* overexpression alters the actin cytoskeleton, thus cytonemes, we compared keratinocyte microridges, which are actin cytoskeleton-rich structures, between control and il17rd-overexpressed peridermal keratinocytes (Pinto et al., 2019; van Loon et al., 2020). Our observation revealed severe disorganization of microridges in the *il17rd* overexpressed keratinocytes, coupled with a significant up-regulation of *RhoA* GTPase expression in these cells (Figure 5G, H). These data suggest that IL-17 signaling negatively regulates

cytoneme extension by unbalancing small GTPases that are essential in control of actin cytoskeleton (Daly et al., 2022; Hall, 1998; Jaffe and Hall, 2005; Stanganello et al., 2015).

Our data collectively support the idea that cytoneme-mediated intercellular signaling between peridermal and underlying undifferentiated keratinocytes is essential for epidermal remodeling and maintenance. IL-17 functions as a regulator capable of affecting cytoneme extension by modulating the actin cytoskeleton in keratinocytes. Consequently, our data suggest that the overproduction of IL-17 ligands by immune cells in conditions such as psoriatic or other skin diseases leads to epidermal imbalance through altered cytoneme signaling in keratinocytes.
In addition, recent human Genome-Wide Association Studies (GWAS) have identified a close association between *clint1* and individuals with *psoriasis* and *atopic dermatitis* (Sahlen et al., 2021). It has also been reported that *clint1* is important for epidermal homeostasis and development in zebrafish (Dodd et al., 2009). Given these reported findings, we investigated to determine whether *clint1* plays a role in epidermal maintenance through its influence on keratinocyte cytonemes. Indeed, we found that *clint1* mutants exhibited a significant decrease in cytoneme extension in peridermal keratinocytes, accompanied by reduced Notch signaling in undifferentiated keratinocytes. As a result, these mutant fish displayed abnormal keratinocyte differentiation and hyperproliferation of keratinocytes (Figure 6). These findings suggest that *clint1* is yet another regulator of cytoneme-mediated intercellular signaling in keratinocytes.

In conclusion, the intercellular signaling facilitated by cytonemes between fully differentiated and undifferentiated keratinocytes is crucial for the maintenance and remodeling of the epidermis. Our study implies that the patients with human skin diseases may have defective cytoneme signaling between keratinocytes.

Discussion

It is intriguing to observe that keratinocytes have the ability to extend both cytoneme and airineme-like protrusions. While these protrusions differ in their extension frequency and developmental context, this observation implies that a single cell type can employ two distinct types of cellular protrusions for signaling, each capable of transferring unique signaling molecules to potentially different targets.
Previous research in *Drosophila* has demonstrated that the air sac primordium (ASP) can extend multiple cytonemes that appear morphologically identical but convey different signaling molecules (Roy et al., 2011). However, it remains unknown whether the same cell type can extend two morphologically (and functionally) distinct protrusions that might also serve distinct functions in separate contexts. Consequently, future studies aimed at unraveling the regulation of these two types of protrusions could provide insights into how cells choose their signaling modalities based on their specific signaling requirements and environmental cues.
We have asked why cytoneme-mediated signaling is necessary between fully differentiated- and undifferentiated keratinocytes, even though they are separated by

opposing cell membranes. Notably, we have identified the Notch signaling pathway as being involved, which operates through membrane receptor and ligand interactions between adjacent cells. This led us to hypothesize that Notch signaling between cells in these layers is suppressed by Fringe proteins, which modulate Notch signaling. In our research, we have discovered that *lunatic fringe (lfng)* is exclusively expressed in the intermediate layer in zebrafish ((Panin et al., 1997), Figure S3). Early studies in mice have also reported that three fringe genes exhibit differential expression in different epidermal layers (Thelu et al., 1998). Hence, it appears that Notch signaling is tightly regulated or inhibited by default between epidermal layers in both zebrafish and mice. While it is well documented that Notch signaling is essential for keratinocyte differentiation and proliferation, the mechanism by which Notch signaling is initiated remains unclear. Our study suggests that cytonemes from fully differentiated keratinocytes in the periderm can activate Notch signaling in undifferentiated keratinocytes in intermediate layer. However, further investigations are necessary to fully comprehend how cytoneme-mediated signaling coordinate with Lunatic fringe or other Fringes to trigger the Notch signal.

It is widely believed that the overproduction of IL-17 is a major contributing factor to *psoriasis*, and many studies have demonstrated that IL-17 signaling triggers inflammation (Liu et al., 2020). IL-17 affects various cellular targets, such as keratinocytes, neutrophils, endothelial cells, promoting tissue inflammation (Blauvelt and Chiricozzi, 2018). Thus, further studies are essential to determine whether uncontrolled cytoneme-mediated signaling in keratinocytes correlates with the onset or progression of psoriatic or other skin diseases. Nevertheless, our data strongly indicate that cell-autonomous overexpression of *il17rd* in peridermal keratinocytes (*krt4*+) can induce psoriasis-like phenotypes by inhibiting cytoneme extensions, which subsequently leads to a reduction in Notch signaling in undifferentiated keratinocytes. A diminished Notch signal has been observed in psoriatic lesions (Ota et al., 2014). Our study suggests that cytoneme-mediated intercellular communication between keratinocytes plays a crucial role in epidermal maintenance, and its dysregulation may contribute to the development of human skin diseases. This idea has yet to be fully appreciated, and it could open up new avenues for understanding the onset or progression of human skin diseases.

We have demonstrated that IL-17 can influence cytoneme extension by regulating RhoA and Cdc42 GTPases, ultimately affecting actin polymerization. Consequently, it would be intriguing to investigate whether genes associated with *psoriasis* or other human skin diseases also function as regulators of cytonemes. For instance, the epidermal-specific deletion of inhibitor of NF-kB kinase 2 (IKK2) or the double knock out of the c-Jun and JunB genes in mice resulted in psoriasis-like phenotypes in a T cell-independent manner (Pasparakis et al., 2002; Zenz et al., 2005). Among the various roles of IKK and JunB, they are also known to regulate the actin cytoskeleton (Dubin-Bar et al., 2008; Lennikov et al., 2018; Otani et al., 2016). Thus, it is conceivable that dysregulated cytoneme-mediated signaling may contribute to psoriatic conditions in these contexts.

Similarly, we have shown that *clint1* mutants exhibited compromised cytoneme signaling and keratinocyte hyperproliferation (Figure 6) (Dodd et al., 2009). Clint1(Clathrin Interactor 1) plays an important role in vesicle trafficking, and it is well established that endocytic pathways are critical for multiple steps in cytoneme-mediated

morphogen delivery (Kalthoff et al., 2002). Therefore, it is interesting to investigate whether clint1 functions as a cytoneme regulator could provide valuable insights. Another important question requires an answer: the proper remodeling and maintenance of the epidermis seemingly depend on a well-controlled supply of keratinocytes from basal stem cells. Our study demonstrated the relay of cytoneme-mediated signals from the periderm to the underlying undifferentiated keratinocytes, leading to their differentiation and subsequent replacement of peridermal keratinocytes. However, the mechanism through which basal stem cells detect the need for an increased population of keratinocytes to maintain the intermediate layer remains unclear. This process doesn't necessarily involve cellular protrusion-mediated signaling. However, we discovered cytoneme-like protrusions originating from undifferentiated keratinocytes (Figure 2B). Our preliminary data, not presented in this manuscript, suggests that these cellular protrusions project downward, indicating their interaction with basal stem cells. Although further investigation is needed, it is conceivable that a replenishment signal from external sources or the periderm can be transmitted to stem cells through a multi-step cytoneme-mediated intercellular signaling pathways.

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Competing interests
The authors declare no competing interests.

Figure legends

Figure 1. Differentiated keratinocytes extend cytoneme-like cellular protrusions
(A) Airineme-like protrusion (arrowhead), and (B) cytoneme-like protrusions (arrowheads) labelled in *krt4:palmEGFP* injected keratinocytes. (C) Keratinocytes expressing *krt4* extend cytoneme-like protrusions most frequently during metamorphic stages [6.5-7.5 SSL [34]; N=457 cells, 19 larvae total]. A cytoneme-like protrusion (arrowhead) labeled for (D) F-actin, revealed by *LifeAct-mRuby* but did not colocalize (E) with tubulin, revealed by *Tuba-mCherry*. (F) Keratinocyte cytonemes extend faster than they retract (N=125 cells, 4 larvae total), with an average length (G) of 18.21µm (N=190 cells, 4 larvae total). Scale bars: 20µm (A, B, D, E). Error bars indicate mean ± SEM.

Figure 2. Keratinocyte cytonemes establish physical contact with underlying undifferentiated keratinocytes

(A-A”) Postembryonic zebrafish epidermis of *Tg(krt4:lyn-EGFP;krtt1c19e:lyn-tdTomato)* comprises three distinct layers. (A) Expression of *lyn-EGFP* in *krt4+* cells in the periderm layer. (A’-A”) Expression of *lyn-tdTomato* (pseudo-colored magenta) in *krtt1c19e+* cells in (A’) the intermediate layer and (A”) the basal layer. Note that the green circular labelling in (A’, arrowheads) represents mucous-secreting goblet cells. (Chang and Hwang, 2011) (B) Undifferentiated keratinocytes (KC) extend significantly fewer cytoneme-like protrusions (P<0.0001, N=49, 3 larvae; N=91 cells, 6 larvae total). (C) Cytonemes from fully differentiated keratinocytes make physical contact with undifferentiated keratinocytes in the intermediate layer (red dotted circles). Note that dotted lines indicate the layers in the cross-sectional views. Scale bars: 20µm (A-A”, C). Error bars indicate mean ± SEM.

Figure 3. Cytoneme-mediated signaling regulates keratinocyte differentiation and proliferation

(A) Expression of *cdc42DN* effectively inhibits cytoneme extension ($F_{2, 231}$ = 25.38, P<0.0001, N=234 cells, 10 larvae total). (B) Periderm layer of *Tg(krt4:TetGBDTRE-v2a-Cdc42DN;krt4:lyn-EGFP;krtt1c19e:lyn-tdTomato)* without drug treatment. (B’) Periderm layer of *Tg(krt4:lyn-EGFP;krtt1c19e:lyn-tdTomato)* with drug treatment. (B’’) Periderm layer of *Tg(krt4:TetGBDTRE-v2a-cdc42DN;krt4:lyn-EGFP;krtt1c19e:lyn-tdTomato)* with drug treatment exhibits disorganization and regions lacking *krt4+* keratinocytes (arrowhead). Cytoneme inhibition increases *krtt1c19e* expression in the periderm (C) ($F_{2, 387}$ = 226.7, P<0.0001, N=39 larvae total) and leads to an increased number of keratinocytes within the periderm (D) ($F_{2, 18}$ = 27.36, P<0.0001, N=21 larvae total). (E) The cell death rate in the periderm is not affected by cytoneme inhibition. Tested with acridine orange incorporation ($F_{2, 17}$ = 3.192, P=0.0666, N=20 larvae total). Statistical significances were assessed by One-way ANOVA followed by Tukey’s HSD post hoc test. Scale bars: 20µm (B-B”). Error bars indicate mean ± SEM.

Figure 4. Cytonemes activate Notch in undifferentiated keratinocytes

(A) Postembryonic zebrafish epidermis of *Tg(krt4:lyn-EGFP;krtt1c19e:lyn-tdTomato;TP1:H2BmCherry)* shows Notch responsiveness in undifferentiated keratinocytes within the intermediate layer but not in the periderm or basal layer. (B) Inhibition of cytonemes reduces Notch responsiveness in undifferentiated keratinocytes. ($F_{2, 33}$ = 48.27, P < 0.0001, N=36 larvae total). (C-C”) Notch inhibition by expressing SuHDN in undifferentiated keratinocytes results in the disorganization of periderm (C”). (C) Properly organized periderm layer of *Tg(krtt1c19e:tetGBDTRE-v2a-SuHDN;krt4:lyn-EGFP;krtt1c19e:lyn-tdTomato)* without drug treatment and (C’) *Tg(krt4:lyn-EGFP;krtt1c19e:lyn-tdTomato)* with drug treatment. (D-F) Notch inhibition (D) increases *krtt1c19e* signal in the periderm ($F_{2, 397}$ = 89.47, P < 0.0001, N=40 larvae total), (E) reduces Notch responsiveness in undifferentiated keratinocytes ($F_{2, 32}$ = 13.18, P < 0.0001, N=35 larvae total), and leads to (F) an increased number of keratinocytes in the periderm ($F_{2, 19}$ = 6.620, P = 0.0066, N=22 larvae total). Statistical significances were assessed by One-way ANOVA followed by Tukey’s HSD post hoc test. Scale bars, 20µm (C-C”). Error bars indicate mean ± SEM.

Figure 5. Interleukin-17 regulates keratinocyte cytonemes

Overexpression of *il17rd* or *il17ra1a* in *krt4+* keratinocytes results in (A) a significant reduction in cytoneme extension ($F_{2, 325}$ = 38.48, P < 0.0001, N=328 cells, 10 larvae total), and (B) decreased Notch responsiveness ($F_{2, 34}$ = 23.16, P < 0.0001, N=37 larvae total). (C-C') The periderm is disrupted in (C') *Tg(krt4:nVenus-v2a-il17rd;krt4:lyn-EGFP;krtt1c19e:lyn-tdTomato)* compared to the (C) properly arranged periderm of *Tg(krt4:lyn-EGFP;krtt1c19e:lyn-tdTomato)*, resulting in (D) an increased *krtt1c19e* signal in the periderm (D) ($F_{2, 287}$ = 109.7, P < 0.0001, N=29 larvae total) and (E) an increased number of keratinocytes in the periderm ($F_{2, 15}$ = 27.79, P < 0.0001, N=18 larvae total). (F) Simultaneous overexpression of *cdc42DN* and *il17rd* or *il17ra1a* in *krt4+* keratinocytes further reduces cytoneme extension (*il17rd*: P < 0.0001, N=164 cells, 7 larvae total, *il17ra1a*: P < 0.0001, N=115 cells, 6 larvae total). (G) *il17rd*-overexpressing *krt4+* keratinocytes exhibit disorganization of microridges (arrowheads). (H) FACS sorted *krt4+* peridermal cells show significantly elevated *RhoA* expression (P< 0.0047, N=5 independent experiments). Statistical significances were assessed by One-way ANOVA followed by Tukey's HSD post hoc test and Student t test. Scale bars: 20µm (C, G). Error bars indicate mean ± SEM.

Figure 6. *clint1* regulates epidermal maintenance via keratinocyte cytonemes
(A-A') *clint1* mutants exhibit a disorganized periderm. (B-E) *clint1* mutants show (B) a lower frequency of cytoneme extension as compared to controls (WT, *cx41.8*$^{-/-}$) ($F_{2, 186}$ = 14.55, P<0.0001, N=10 larvae total), (C) reduced Notch responsiveness (P<0.0001, N=12 mutants, N=10 controls), (D) increased *krtt1c19e* expression in the periderm (P<0.0001, N=13 mutants, N=6 controls), and (E) a greater number of keratinocytes within the periderm (P<0.001, N=8 mutants, N=6 controls). Statistical significances were assessed by One-way ANOVA followed by Tukey's HSD post hoc test and Student t test. Scale bars, 20µm (A-A'). Error bars indicate mean ± SEM.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish

Adult zebrafish were maintained at a constant temperature of 28.5°C under a 16:8 (L:D) cycle. The zebrafish used in this study were wild-type AB^{wp} or its derivative WT(ABb). Additionally, transgenic lines used include *Tg(krt4:lyn-EGFP)*^{sq18}*, Tg(krtt1c19e:lyn-tdTomato)*^{sq16} provided by T. Carney; *Tg(Tp1:EGFP)*^{um13}*, Tg(Tp1:H2BmCherry)*^{jh11} provided by M. Parsons*; clint1a*$^{hi1520Tg/+}$*(AB),* obtained from the Zebrafish International Resource Center (ZIRC). The sexes of individual fish could not be distinguished since experiments were performed before the development of secondary sexual characteristics. All zebrafish stocks used in this study were known to produce balanced sex ratios, ensuring that experiments sampled similar numbers of males and females. All animal work conducted in this study received approval from the University of California Irvine Institutional Animal Care and Use Committee (protocol #AUP-22-023) and adhere to institutional and federal guidelines for the ethical use of animals.

METHOD DETAILS

Transgenesis and transgenic line production

Transgenic fish lines with cell-type specific and temporally inducible expression of human *cdc42DN* and *SuHDN* were generated following the protocol described by Eom et al., (2015). These constructs were driven by a 2.2kb *krt4* promoter obtained from T. Carney for the expression in fully differentiated keratinocytes and a 3.9kb *krtt1c19e* promoter, also from T. Carney, for the expression in undifferentiated keratinocytes. The efficiency of gene expression in both *Tg(krt4:TetGBDTRE-v2a-cdc42DN)*$^{ir.rt3}$ and *Tg(krtt1c19e:TetGBDTRE-v2a-SuHDN)*$^{ir.rt18}$, induced by doxycycline (Dox; Sigma-Aldrich) and dexamethasone (Dex; Sigma-Aldrich), was performed in both F0 mosaic fish and non-mosaic stable lines, with consistent results. The coding sequence of *il17rd* (ZDB-GENE-020320-5) and *il17ra1a* (ZDB-GENE-070705-242) were isolated from WT(ABb) cDNA and used to generate constructs driven by the *krt4* promoter, which were assembled using Gateway assembly into the pDestTol2CG2 destination vector to produce *Tg(krt4:nVenus-v2a-il17rd)*$^{ir.rt16}$ and *Tg(krt4:il17ra1a-v2a-mCherry)*$^{ir.rt17}$. Constitutive Notch activation used the intracellular domain of notch1a (NICD) linked by 2a sequence to nVenus and driven by the *krtt1c19e* promoter to produce *Tg(krtt1c19e:nVenus-v2a-NICD)*$^{ir.rt19}$. The lunatic fringe reporter line, *Tg(lfng:mCherry)*$^{ir.rt20}$, was generated by first amplifying the *lfng* promoter sequence (ZDB-GENE-980605-16) using WT gDNA and then used Gateway assembly into the pDestTol2-exorh:EGFP destination vector [Addgene #195983]. These constructs were then injected into WT(ABb) fish to generate mosaic F0 fish, which were then bred in pairs to find germline carriers to produce non-mosaic transgenic lines. Actin was examined with *LifeAct-mRuby* [Addgene #166977] (Barros-Becker et al., 2017) driven by *krt4* promoter. Microtubules in *krt4+* keratinocytes and cytonemes were labelled using subclones from Tuba1-mCherry [(Addgene #49149] (Friedman et al., 2010).

Drug treatments

Zebrafish were kept in E3 medium with or without drugs (Dox/Dex) during both the light and the dark cycles for long-term drug treatment experiments. For overnight time-lapse images, fish subjected to drug-treatment were transferred to fresh E3 medium 30min before explant preparation to minimize background fluorescence from the drugs. Zebrafish with *krt4:palmEGFP* injected TetGBD-containing transgenics and non-transgenic siblings were treated with 37.5µM Dox and 50µM Dex one day prior to time-lapse imaging, while fish for long-term drug treatment received 27.5 µM Dox and 50µM Dex starting from SSL7.0. Notch inhibitor, LY411575 (10mM; Thomas Scientific) was prepared in DMSO (Sigma-Aldrich), and fish were treated with drug [LY411575 (3µM)] or vehicle from SSL7.0. For acute drug administration, Cdc42 inhibitor ML141 (Sigma-Aldrich), prepared in DMSO, was added to fish medium [ML141 (2µM)] 30min prior to explant preparation for time-lapse imaging.

Acridine Orange Cell Death Assay

Acridine Orange (5mg/mL; Sigma-Aldrich) was prepared in Invitrogen UltraPure™ Distilled Water. Fish were washed in 1x E3 medium 30min before being transferred to 1x E3 medium containing AO (2µg/mL). The fish were incubated in AO for 30min and then rinsed in regular 1x E3 medium three times for 10min each to minimize background fluorescence during imaging. Dead cells, pseudo-colored in magenta, were counted for all groups.

Time-lapse and still imaging

Ex vivo time-lapse imaging of zebrafish epidermal cells within their native tissue environment was acquired at 3-minute intervals for 10 hours. This was achieved using a 40x water-immersion objective on a Leica TCS SP8 confocal microscope equipped with a resonant scanner and two HyD detectors. Larvae were imaged at SSL7.5, unless otherwise specified. For still images, the region directly underneath the dorsal fin of the zebrafish was captured, with a total of 10 tiles per fish.

RT-PCR, qRT-PCR and FACS

Zebrafish at SSL7.5 were skinned (N=15 per group), and the dissected tissues were placed in cold 1x PBS. Tissues were washed by pipetting up and down and then centrifuged to remove the supernatant. Next, 1mL Gibco Stem® Pro® Accutase Cell Dissociation Reagent was added to resuspend tissues, and the tube was incubated for at least 10 min at 37°C to dissociate the cells. After incubation, the cells were passed through 40µm cell strainers to remove large tissue chunks before subjecting them to FACS (Fluorescence-activated cell sorting). FACS enabled the separation and collection of desired cell populations based on different cell membrane markers. EGFP+ and tdtomato+ cells were collected for downstream non-quantitative RT-PCR and quantitative RT-PCR. For cDNA synthesis, the Invitrogen Cells Direct™ One-Step qRT-PCR Kit was employed. Quantitative PCR was conducted on an Applied Biosystems QuantStudio™ 3 Real-Time PCR Instrument using custom TaqMan probes for *rhoab* (Dr03439241_mH) and *actin* (Dr03432610_m1). Quantitative PCRs were run with at least three triplicates for each sample. Non-quantitative RT-PCR amplifications were performed with 40 cycles (*actb1, dlc, lfng, krt4, krt19, il17rd, notch2, notch3*, *il17ra1a*) using Takara PrimeStar GXL DNA Polymerase. *actb1*: 5'- CATCCGTAAGGACCTGTATGCCAAC- 3', 5'- AGGTTGGTCGTTCGTTTGAATCTC- 3' (Hu et al., 2016); *krt4*: 5'- TCAACCAGGTCTATCTCTTACTCC- 3', 5'- AACAGGCTCTGGTTGACAGTTAC- 3'; *krt19*: 5'- GCTACCACCTTCTCCAGCGGAAG- 3', 5'- TGCAGCACCAAATCCTCCACCAG- 3'; *lfng*: 5'- CATATCGCGGAAAAGTCGTGGTC- 3', 5'- GCTTATCCGCTTCCCTCCTGCTG- 3'; *dlc*: 5'- CGGGAATCGTCTCTTTGATAAT- 3', 5'- CTCACCGATAGCGAGTCTTCTT- 3'; *il17rd*: 5'- CTGGGTCACGGAGACTTGCGC- 3', 5'- TGGCGTGTTTCCCCAGCGGAC-3'; *il17ra1a*: 5'- TCACCTTTATACCTGTAGTGCTG- 3'; 5'- CATGTTTTCTCTGACATCAAAAC-3'; *notch2*: 5'- GAGTGTGTGGACCCGTTAGTATG- 3', 5'- GCAGGCATCATCAATGTGACAC- 3'; *notch3*: 5'- TCAGGATTGTTCTCTCGTTGATG- 3', 5'- GTGTTAAAGCATGTACCACCATTG- 3'.

Cell Counts

For all experiments, 6 out of 10 tiles of still images, which represent the central trunk area directly beneath the dorsal fin, were selected for cell counts. To ensure consistency across all samples and minimize discrepancies resulting from variations in fish trunk widths, a region of interest (ROI), 290µm x 200µm per tile, was created using the rectangle selection tool in ImageJ. Z-stack imaging was confined to the superficial/periderm layer based on the cross-sectional view, and the final count included both *krt4*+ and *krtt1c19e*+ cells at the periderm layer.

Intensity analyses

To analyze Notch expression level, tile images were merged separately for each fish. Merged images were then sectioned in ImageJ using the polygon selection tool to encompass only the fish trunk, and maximal projection was utilized to obtain the mean gray value. For *krtt1c19e*+ expression in the periderm layer, the z-stack was set as previously described, and the mean gray value of *krtt1c19e*+ expression was obtained.

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism software version 9.0.0 for Windows (GraphPad Software, San Diego, CA, USA) was used to perform statistical analyses. Continuous data were evaluated by Student t-test and Post hoc means were compared by Tukey-Kramer HSD.

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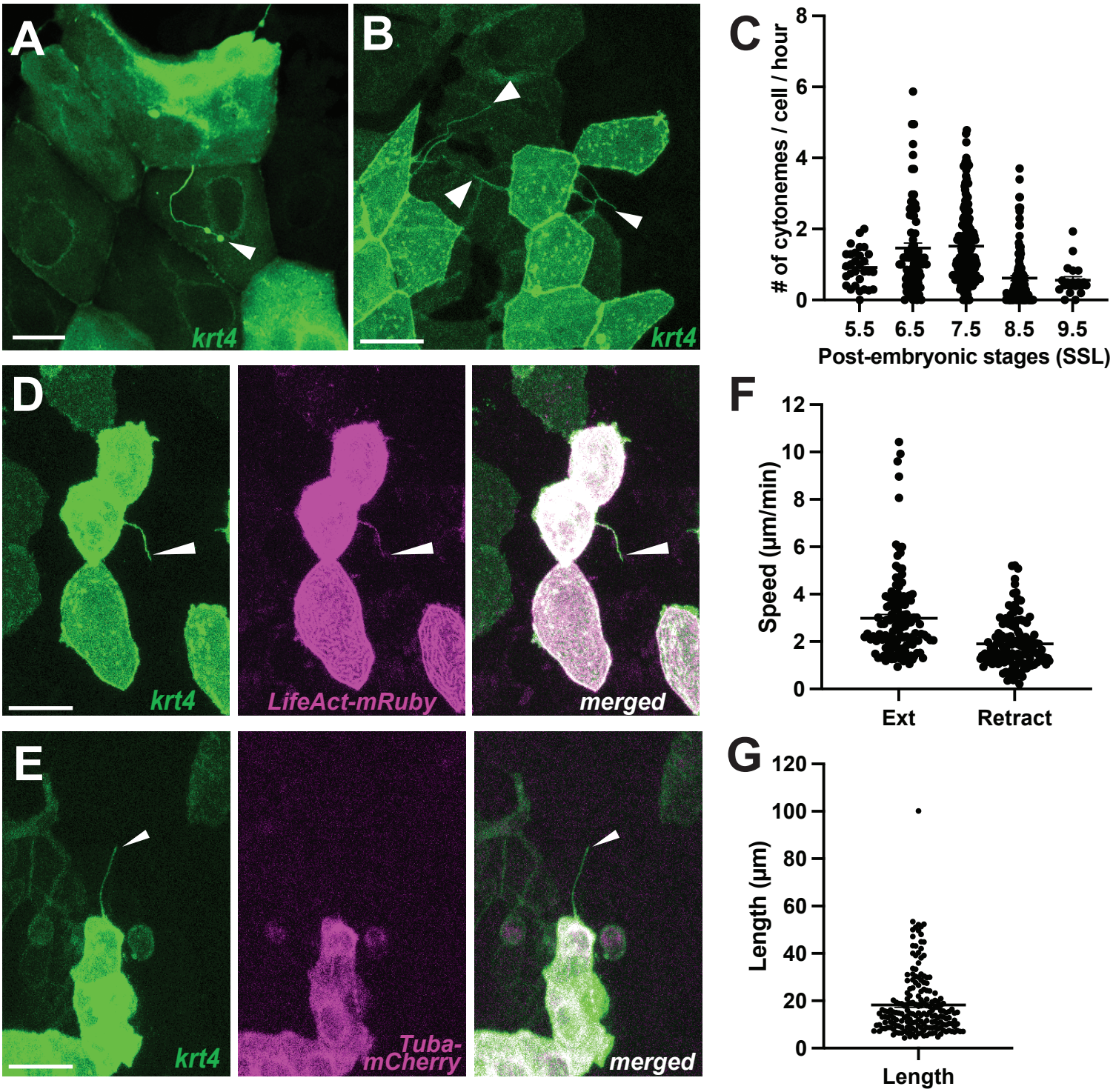

A
krt4
B
krt4
C
# of cytonemes / cell / hour
0
2
4
6
8
5.5
6.5
7.5
8.5
9.5
Post-embryonic stages (SSL)
D
krt4
LifeAct-mRuby
merged
E
krt4
Tuba-
mCherry
merged
F
Speed (µm/min)
0
2
4
6
8
10
12
Ext
Retract
G
Length (µm)
0
20
40
60
80
100
120
Length

Figure 1

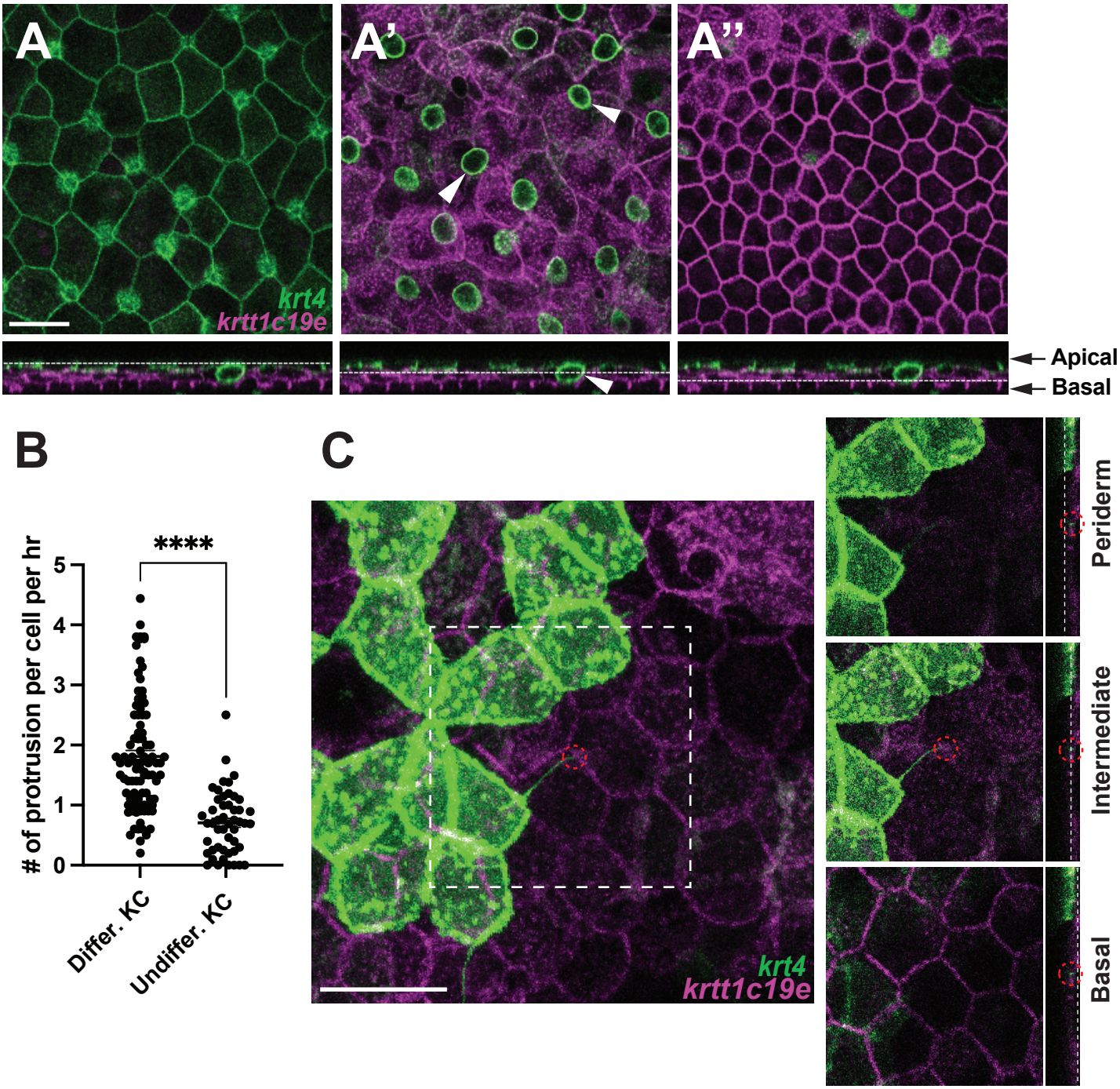

A
A'
A''
krt4
krtt1c19e
Apical
Basal
B
C
****
# of protrusion per cell per hr
0
1
2
3
4
5
Differ. KC
Undiffer. KC
krt4
krtt1c19e
Periderm
Intermediate
Basal

Figure 2

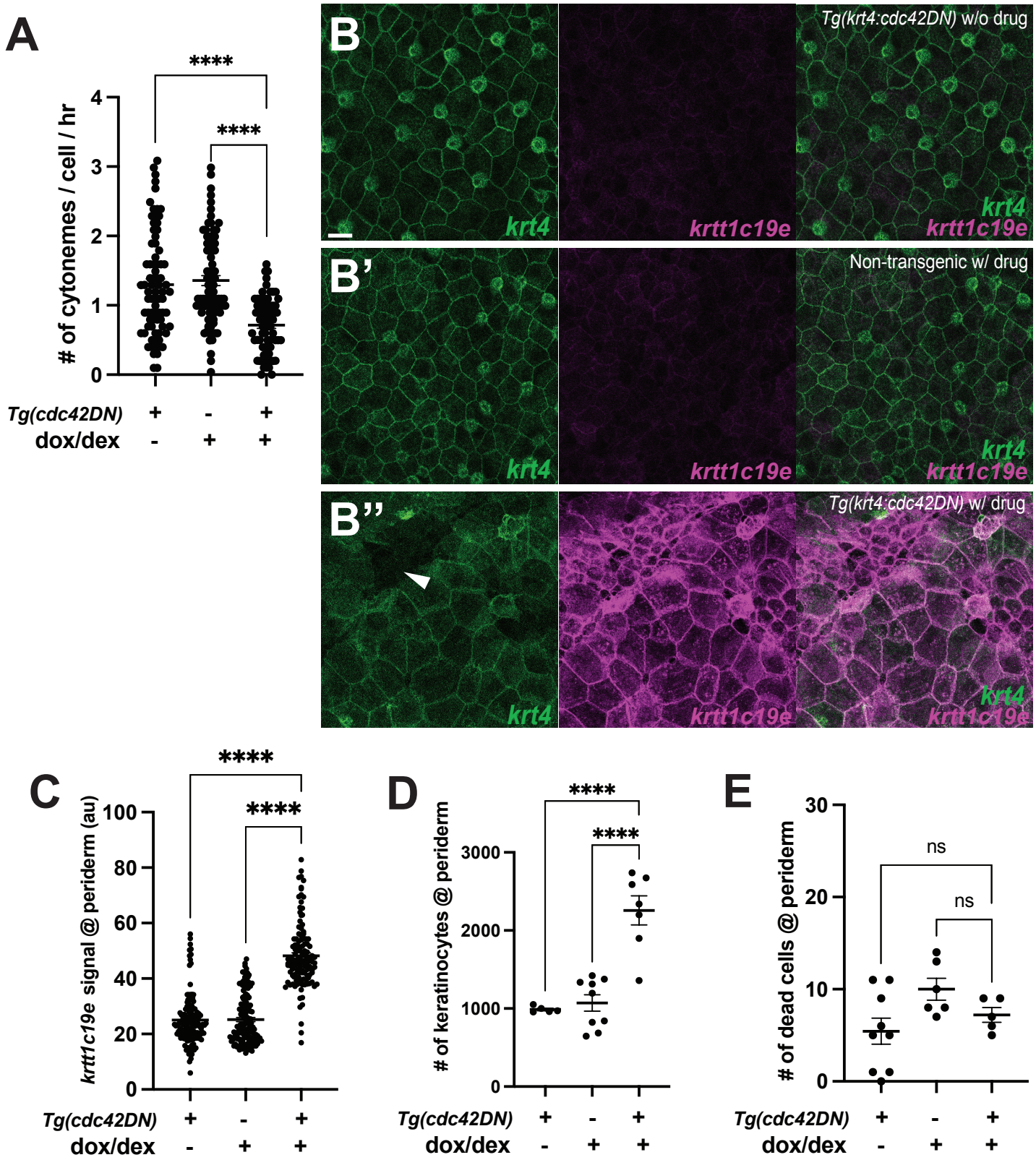
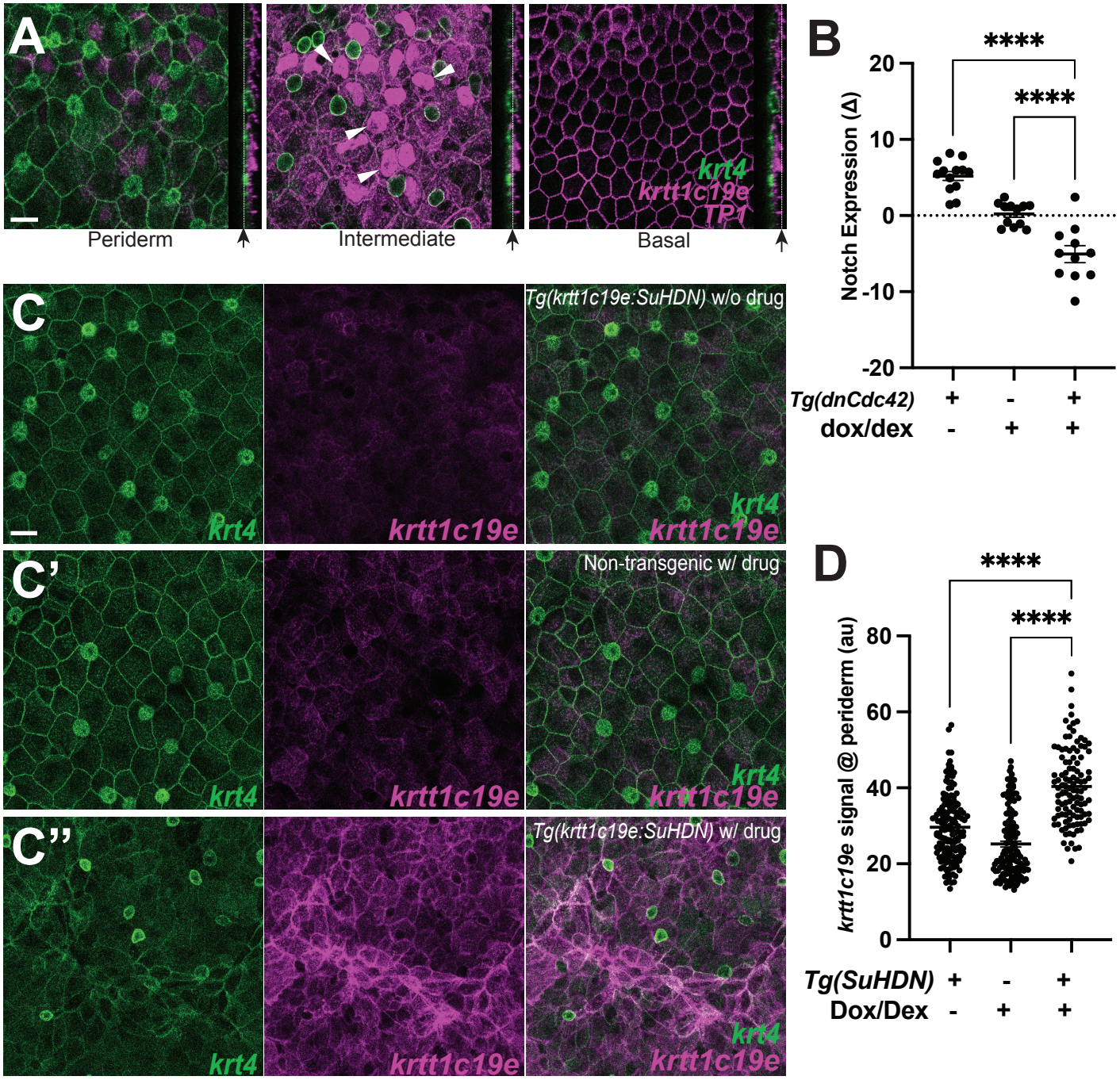

A
****
****
# of cytonemes / cell / hr
0
1
2
3
4
Tg(cdc42DN) + - +
dox/dex - + +
B
Tg(krt4:cdc42DN) w/o drug
krt4
krtt1c19e
krt4
krtt1c19e
B'
Non-transgenic w/ drug
krt4
krtt1c19e
krt4
krtt1c19e
B''
Tg(krt4:cdc42DN) w/ drug
krt4
krtt1c19e
krt4
krtt1c19e
C
****
****
krtt1c19e signal @ periderm (au)
0
20
40
60
80
100
Tg(cdc42DN) + - +
dox/dex - + +
D
****
****
# of keratinocytes @ periderm
0
1000
2000
3000
Tg(cdc42DN) + - +
dox/dex - + +
E
ns
ns
# of dead cells @ periderm
0
10
20
30
Tg(cdc42DN) + - +
dox/dex - + +

Figure 3


A
Periderm
Intermediate
Basal
krt4
krtt1c19e
TP1
B
****
****
Notch Expression (Δ)
20
10
0
-10
-20
Tg(dnCdc42) + - +
dox/dex - + +
C
Tg(krtt1c19e:SuHDN) w/o drug
krt4
krtt1c19e
krt4
krtt1c19e
C'
Non-transgenic w/ drug
krt4
krtt1c19e
krt4
krtt1c19e
C''
Tg(krtt1c19e:SuHDN) w/ drug
krt4
krtt1c19e
krt4
krtt1c19e
D
****
****
krtt1c19e signal @ periderm (au)
80
60
40
20
0
Tg(SuHDN) + - +
Dox/Dex - + +

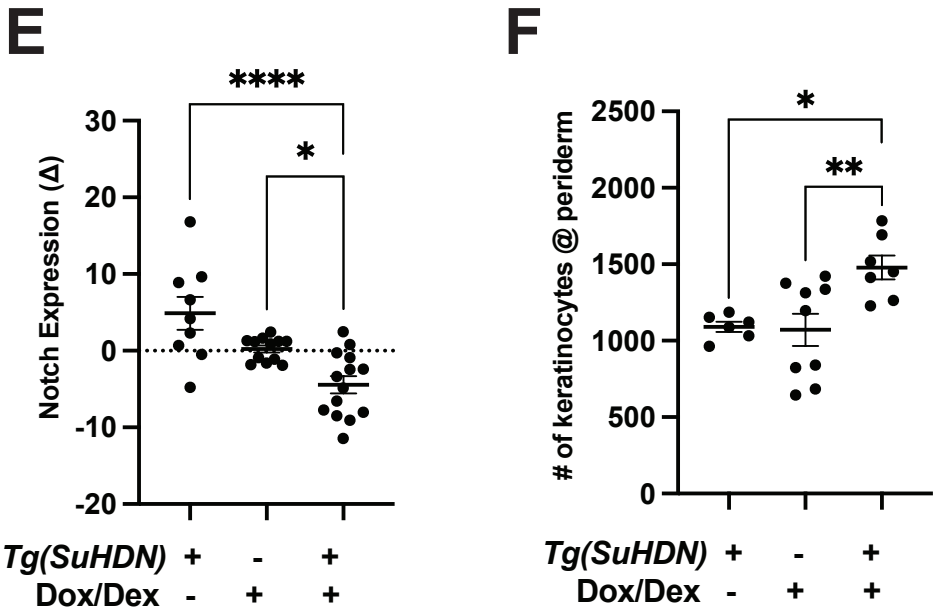

E
****
*
Notch Expression (Δ)
30
20
10
0
-10
-20
Tg(SuHDN) + - +
Dox/Dex - + +
F
*
**
# of keratinocytes @ periderm
2500
2000
1500
1000
500
0
Tg(SuHDN) + - +
Dox/Dex - + +

Figure 4

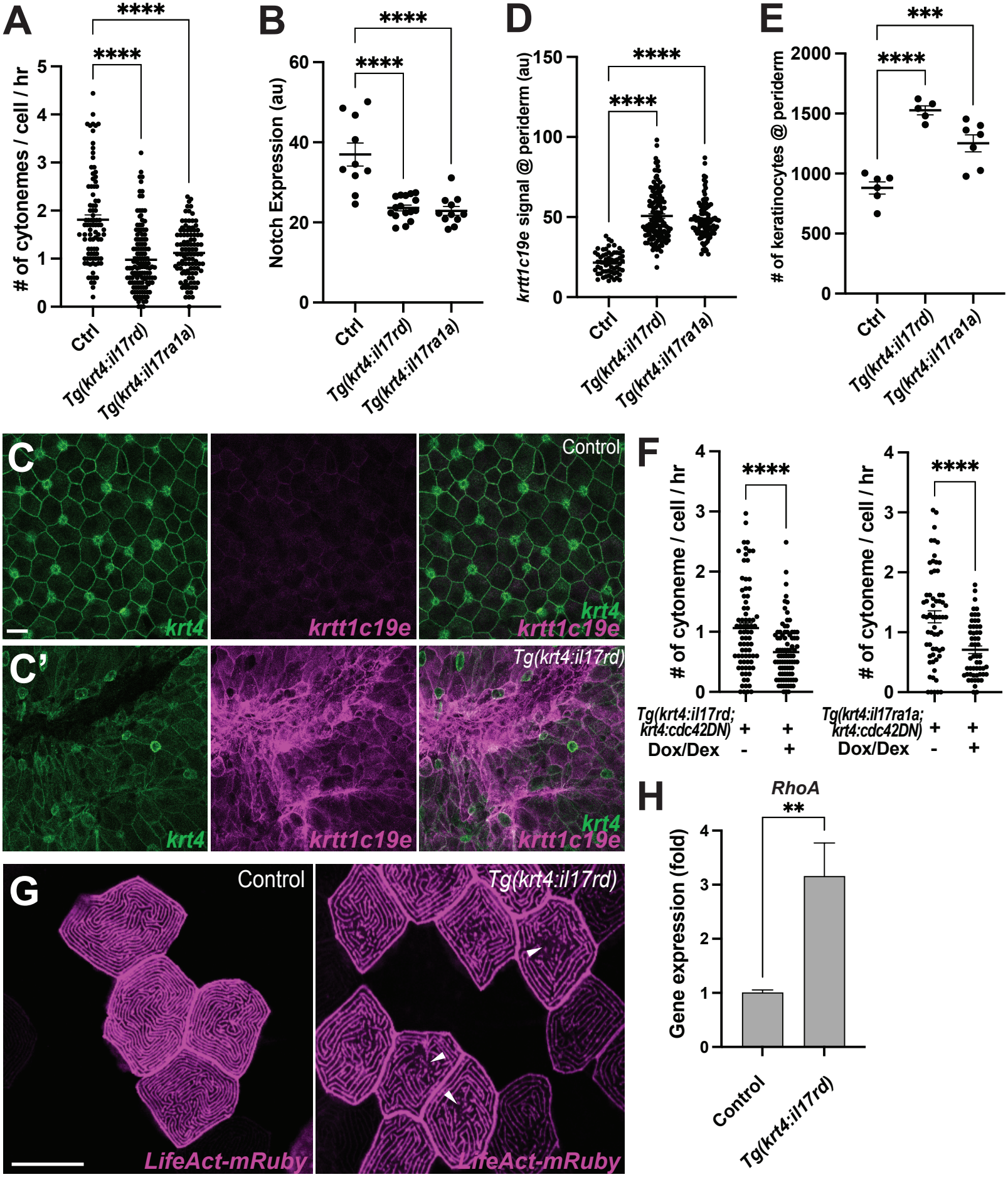

A
# of cytonemes / cell / hr
****
****
Ctrl
Tg(krt4:il17rd)
Tg(krt4:il17ra1a)
B
Notch Expression (au)
****
****
D
krtt1c19e signal @ periderm (au)
****
****
E
# of keratinocytes @ periderm
***
****
C
krt4
krtt1c19e
Control
krt4
krtt1c19e
C'
Tg(krt4:il17rd)
F
# of cytoneme / cell / hr
****
****
Tg(krt4:il17rd; krt4:cdc42DN)
Tg(krt4:il17ra1a; krt4:cdc42DN)
Dox/Dex
G
Control
Tg(krt4:il17rd)
LifeAct-mRuby
H
RhoA
**
Gene expression (fold)

Figure 5

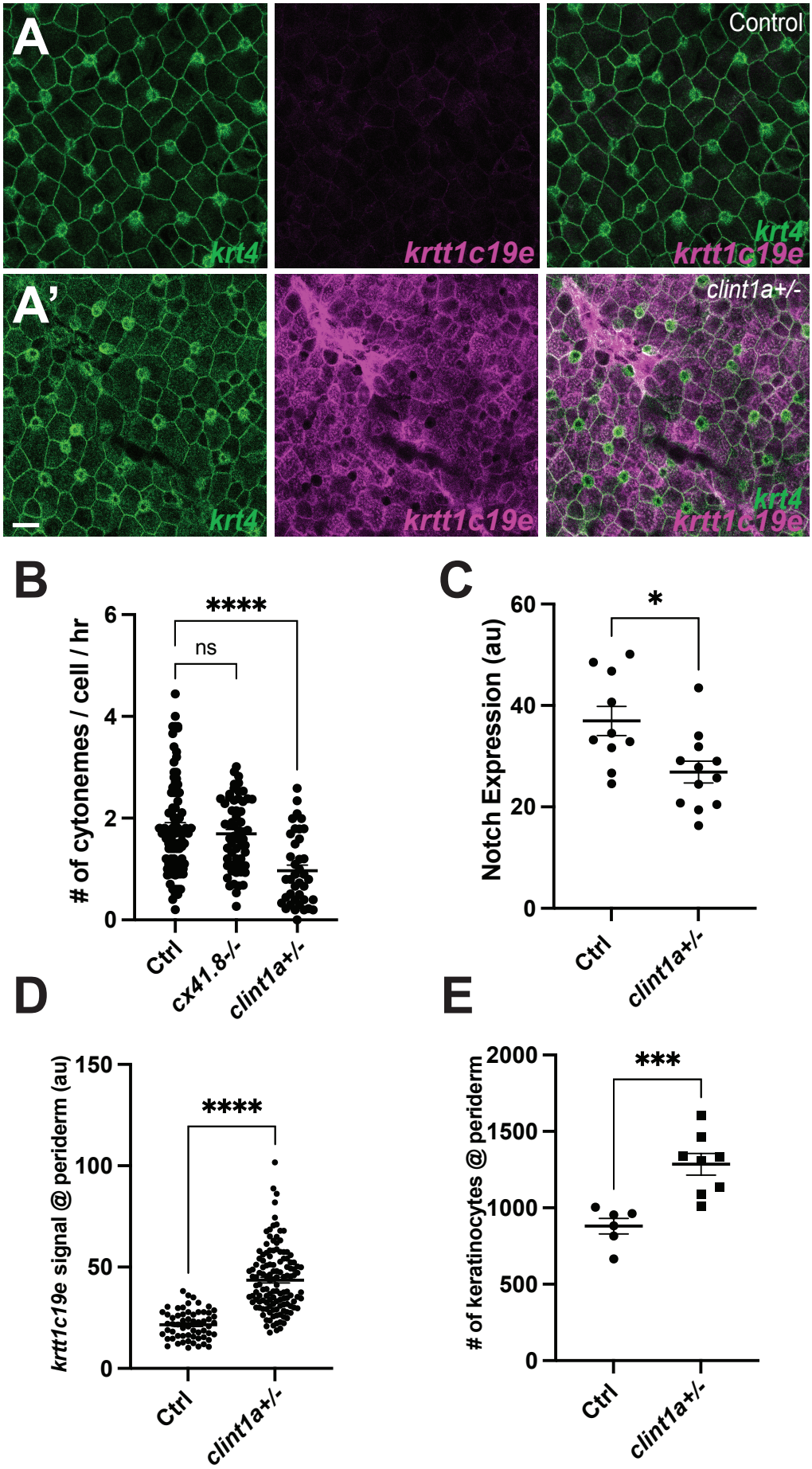

A
krt4
krtt1c19e
Control
krt4
krtt1c19e
A'
krt4
krtt1c19e
clint1a+/-
krt4
krtt1c19e
B
# of cytonemes / cell / hr
****
ns
Ctrl
cx41.8-/-
clint1a+/-
C
Notch Expression (au)
*
Ctrl
clint1a+/-
D
krtt1c19e signal @ periderm (au)
****
Ctrl
clint1a+/-
E
# of keratinocytes @ periderm
***
Ctrl
clint1a+/-

Figure 6