MicroRNA-223 Suppresses the Canonical NF-κB Pathway in Basal Keratinocytes to Dampen Neutrophilic Inflammation

Graphical Abstract

Highlights
- miR-223-deficient zebrafish display augmented neutrophilic inflammation
- Elevated NF-κB activation increases inflammation in miR-223−/− embryos
- miR-223 suppresses canonical NF-κB pathway in basal epithelial cells
- Both epithelial and phagocytic miR-223 regulate inflammation

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In Brief
microRNA-223 is dysregulated in many inflammatory conditions such as cancer and asthma, yet its physiological role is not clear. Zhou et al. demonstrate that microRNA-223 in both phagocytes and epithelial cells cooperate to suppress the canonical NF-κB pathway in epithelial cells to restrict the magnitude of inflammation.

Data and Software Availability
GSE94996

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MicroRNA-223 Suppresses the Canonical NF-κB Pathway in Basal Keratinocytes to Dampen Neutrophilic Inflammation

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SUMMARY

MicroRNA-223 is known as a myeloid-enriched anti-inflammatory microRNA that is dysregulated in numerous inflammatory conditions. Here, we report that neutrophilic inflammation (wound response) is augmented in miR-223-deficient zebrafish, due primarily to elevated activation of the canonical nuclear factor κB (NF-κB) pathway. NF-κB over-activation is restricted to the basal layer of the surface epithelium, although miR-223 is detected throughout the epithelium and in phagocytes. Not only phagocytes but also epithelial cells are involved in miR-223-mediated regulation of neutrophils' wound response and NF-κB activation. Cull1a/b, Traf6, and Tab1 are identified as direct targets of miR-223, and their levels rise in injured epithelium lacking miR-223. In addition, miR-223 is expressed in cultured human bronchial epithelial cells, where it also downregulates NF-κB signaling. Together, this direct connection between miR-223 and the canonical NF-κB pathway provides a mechanistic understanding of the multifaceted role of miR-223 and highlights the relevance of epithelial cells in dampening neutrophil activation.

INTRODUCTION

Neutrophilic inflammation is critical for host defense and tissue repair. Neutrophils are recruited by multiple “intermediate” and “end-target” chemoattractants released by tissue-resident sentinel cells upon activation by pathogen or damage-associated molecular pattern molecules (Kim and Luster, 2015; Kołaczkowska and Kubés, 2013). Activated neutrophils release oxidants, proteases, and antimicrobial proteins to eliminate pathogens or damaged cells, which also causes collateral tissue damage (Nathan, 2006). As a result, chronic or rampant neutrophilic inflammation drives the immunopathology involved in numerous human diseases, including those directly involving an immune component such as rheumatic arthritis and those that are not obviously linked such as diabetes, neurodegenerative disease, and cancer (Borregaard, 2010; Nathan, 2006). Therefore, fine-tuning the magnitude and resolution of neutrophilic inflammation is critical for the host to restore homeostasis.

It is estimated that 30%–80% of genes in humans are regulated by microRNAs (miRNAs) (Lu and Clark, 2012). miRNAs are small (20–22 nucleotides) non-coding RNA molecules that typically suppress the translation and the stability of transcripts through partial complementarity (Ha and Kim, 2014; Jonas and Izaurralde, 2015). miRNAs are fine-tuners that suppress target gene expression at modest levels, yet master regulators that suppress multiple genes in the same pathways (Gurol et al., 2016; Orellana and Kasinski, 2015). These features suggest that miRNAs may be suitable modulators for the duration and the magnitude of inflammation. Several miRNAs, including miR-223, miR-155, miR-146, and miR-125b, have a role in the differentiation and function of the innate immune system (Johnnids et al., 2009; Lindsay, 2008; Lu and Liston, 2009). However, as a result of the complex and dynamic interactions between multi-tissues that coordinate an inflammatory response, the functions of these miRNAs in immune response are not fully understood.

miR-223 has previously been observed primarily in the myeloid lineage, especially neutrophils (Chen et al., 2004; Johnnids et al., 2008), and is implicated in many inflammatory disorders, infections, and cancers (Haneklaus et al., 2013). Global miRNA expression profiling revealed dysregulation of miR-223 in numerous conditions, however, no consensus between the level of miR-223 and the type or progress of the diseases can be concluded (reviewed in Haneklaus et al., 2013). Opposing roles of miR-223 have been reported, suggesting that miR-223 plays parts of a complex regulatory network with profound impact from the surrounding tissues.

The first in vivo characterization of miR-223 was performed in a miR-223 loss-of-function mouse model (miR-223−/−), where an increase in granulopoiesis, as well as hyper-mature and hyper-responsive neutrophils were observed (Johnnids et al., 2008). This strain displayed increased susceptibility to Mycobacterial infection, which is due to increased neutrophil accumulation in the lung (Dorhoi et al., 2013), where a cell-autonomous role of miR-223 in neutrophil recruitment was suggested. NLR family
pyrin domain containing 3 (NLRP3) inflammasome activity was also regulated by miR-223 in primary murine neutrophils (Bauernfeind et al., 2012). In addition to the immune system, Harraz et al. (2012) revealed that miR-223, delivered by adenovirus to the brain, protected mice from an ischemic reperfusion brain injury.

In addition, there is a body of literature related to the function of miR-223 in myeloid cells in vitro, with yet to be verified in vivo relevance. As one of the most abundant miRNAs in macrophages, miR-223 responds to stimuli to control the production of interleukin (IL)-6 and IL-1β (Chen et al., 2012) or promotes alternative macrophage activation to inhibit diet-induced adipose tissue inflammation and insulin resistance by targeting Pknox1, Rasa1, and Nfat5 in murine macrophages (Ying et al., 2015; Zhuang et al., 2012). MiR-223 is also associated with macrophage differentiation through targeting IKKβ (Li et al., 2010). Furthermore, macrophages and monocytes secrete miR-223 in microvesicles or associated high-density lipoproteins, which are delivered into non-immune cells such as endothelial cells (Ismail et al., 2013; Tabet et al., 2014). Those transferred miRNAs are functionally active, indicating the potential of miR-223 as a central mediator for intercellular cross-talk.

Aside from the immune cells, miR-223 is required for stem cell differentiation, such as the differentiation of mesenchymal stem cells into adipocytes and osteoblasts (Guan et al., 2015). In cancers, the role of miR-223 is conflicting. For example, miR-223 is upregulated in acute lymphoblastic leukemia and bladder cancers, but downregulated in chronic lymphoid leukemia and hepatocellular carcinoma (Chiaretti et al., 2010; Gottardo et al., 2007; Stamatopoulos et al., 2009; Wong et al., 2008). Elevated miR-223 supports migration and invasion in gastric cancer cells but has opposite effect in esophageal cancer cells and human cervical cancer (Li et al., 2011a, 2011b; Tang et al., 2015).

In this study, we took advantage of the zebrafish system, which offers a unique genetic and imaging platform to dissect the interplay of various tissues during inflammation. Moreover, zebrafish are a suitable model for studying innate immunity as they possess conserved innate immune cells and signaling molecules as human (Renshaw and Trede, 2012). We discovered an unexpected importance of nuclear factor-κB (NF-κB) activation in epithelial cells at the center of miR-223 regulated neutrophilic inflammation, which provides insights into the multifaceted role of miR-223 in various inflammatory conditions.

RESULTS

miR-223 Deficiency Delays the Resolution of Neutrophilic Inflammation

The gene structure and mature sequence of miR-223 are conserved within vertebrates (Roberto et al., 2015). Similar to mice and human (Johnnidis et al., 2008), miR-223 expression was significantly higher in neutrophils in zebrafish (Figures S1A and S1B), supporting zebrafish as a suitable model for miR-223 research. We then generated a miR-223-deficient (miR-223−/−) zebrafish line using the CRISPR/Cas9 system. An allele containing an 18-bp mismatch to the wild-type (WT), which is predicted to disrupt the hairpin structure of pre-miR-223 was selected (Figures 1A and 1B). A complete loss of miR-223, with no alterations of two other miRNAs expressed in neutrophils (miR-142-3p and miR-92a), was detected in the miR-223−/− fish (Figure 1C). No apparent abnormalities in development, viability, or life-span were noted in the miR-223−/− fish, similarly to the miR-223−/− mice (Johnnidis et al., 2008) and a recently generated miR-223−/− zebrafish line (Kasper et al., 2017). In addition, no differences in total neutrophil numbers, spontaneous inflammation, or cell death in 3-day post fertilization (dpf) embryos were noted (Figures 1D, 1E, and S1C–S1F).

We next investigated the function of miR-223 in a self-resolving inflammation model. Specifically, a tailfin transection injury in 3 dpf embryos results in a rapid recruitment of neutrophils that peaks at 1 hr post wounding (1 hpw), which is spontaneously resolved by 6 hpw. Significant increases in neutrophils at the wound region in miR-223−/− embryos were detected at both time points (Figures 1F and 1G). The miR-223−/− embryos has a phenotype comparable to miR-223−/−, possibly due to the self-reinforced expression of miR-223 (Fazi et al., 2005). As a result, adult fish (miR-223+/+ and miR-223−/−) from the same parents (miR-223+/−) were used to produce embryos for experiments. To better identify the source of neutrophils at the wound vicinity at 6 hpw, photoconversion-enabled neutrophil fate-mapping was performed (Deng et al., 2011) (Figure S2A). Green neutrophils present at the wound at 1 hpw were photo-converted into red neutrophils. Five hours later, the percentage of red neutrophils remaining at the wound in miR-223−/− was modestly higher than that in WT, indicating a defect in neutrophil reverse migration (migration away from the wound) (Figures S2B and S2C). Furthermore, the number of green neutrophils at the wound in 6 hpw miR-223−/− was significantly higher than that in WT, suggesting a continuous recruitment of neutrophils in the miR-223−/− background (Figure 2D). Altogether, the excessive neutrophilic inflammation in miR-223−/− embryos was primarily a result of continuous neutrophil recruitment, with a minor defects in reverse migration.

Neutrophil-Intrinsic miR-223 Is Required to Control Neutrophilic Inflammation

Because miR-223 has been known as a myeloid-specific miRNA, we next determined whether miR-223 regulates neutrophilic response cell-autonomously. To specifically knock down miR-223 in neutrophils, a miR-223 sponge, containing six bulged binding sites of miR-223, was expressed under the lysozyme C (lyzC) promoter (Hall et al., 2007) (Figure 2A). Indeed, embryos expressing the miR-223 sponge accumulated more neutrophils at the wound at 6 hpw (Figures 2B and 2C; Movie S1). To further confirm the neutrophil-intrinsic role of miR-223, a neutrophil-specific miR-223-overexpression line, Tg(lyzC:miR-223-RFP) pno9 was generated and crossed into the miR-223−/− background (Figure 2D). The neutrophil-specific rescue partially restored the miR-223 expression level and partially rescued the over-inflammation phenotype (Figures 2E and 2F), suggesting that miR-223 regulates inflammation, at least partially, inside neutrophils. A control rescue with RFP alone did not yield any phenotype (Figures 2D, 2E, and 2G).

NF-κB Pathway Is Activated in miR-223−/− Embryos

To fully understand how miR-223 regulates neutrophilic inflammation, microarray analysis of both WT and miR-223−/−
Figure 1. miR-223-Deficient Embryos Display Augmented Neutrophil Response to Tissue Injury

(A) Sequences of pre-mir-223 in WT and miR-223 mutant embryos. Mutated nucleotides are labeled in red.

(B) The hairpin structures of miR-223 in WT and mutant embryos predicted by Centroidfold.

(C) Expression of miR-223, miR-142-3p, and miR-92a in WT and miR-223/C0/C0 embryos determined by qRT-PCR.

(D and E) Representative images (D) and quantification (E) of total neutrophil numbers in WT and miR-223/C0/C0 embryos. The number of neutrophils in the boxed region were quantified. Scale bars, 100 μm.

(F and G) Representative images (F) and quantification (G) of neutrophil recruitment to tail transection sites in WT and miR-223/C0/C0 embryos at 1 hpw and 6 hpw. Data are representative of three independent experiments (E and G) or are pooled from three independent experiments (C). Mean ± SD. ***p < 0.001 and ****p < 0.0001, unpaired Student’s t test. See also Figures S1 and S2.
Figure 2. Neutrophil-Intrinsic miR-223 Regulates Neutrophilic Inflammation

(A) Schematics of Tol2-lyzC-Gal4-crys-CFP construct, injected into WT embryos to generate the transgenic line Tg(lyzC: Gal4-Vp16, crys:CFP)pu8; Tol2-UAS-miR-223 sponge that contains 6 bulged miR-223 binding sites after the UAS element and the Dendra2 control plasmid. (B and C) miR-223 sponge or Dendra2 control plasmids in (A) were injected into embryos from Tg(lyzC: Gal4-Vp16, crys:CFP)pu8 and Tg(lyzC: mCherry-H2B) cross. Tailfins were transected at 3 dpf. Representative images at indicating time points are shown in (B), and neutrophil recruitment at 6 hpw is shown in (C).

(D) Schematics of Tol2-lyzC-miR-223/RFP constructs, injected into WT embryos to generate the transgenic line Tg(lyzC: RFP-miR-223)pu9 and the control line Tg(lyzC: RFP)pu10. (E) The transgenic lines illustrated in (D) were crossed into the miR-223−/− background. The siblings without RFP were used as negative control. miR-223 expression in indicated groups was quantified by qRT-PCR (mean ± SD).

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embryos, unwounded or at 1 and 6 hpw, was performed. Transcriptome analysis revealed that 166, 115, and 114 genes (that have known human orthologs) were upregulated over 1.5-fold in miR-223−/− at indicated time points (Figure S3A). This cutoff was selected according to a previous report that miR-223 modulates gene expression at modest levels (Baek et al., 2008). The top four pathways altered are inflammatory response, cancer, organismal injury and abnormalities, and reproductive system disease. Among the genes involved in inflammatory response, those in the interferon pathways and NF-κB pathways were enriched.

To identify the biological relevant pathway, a CRISPR screen was performed, aiming to identify genes whose suppression would rescue the over-inflammation phenotype in miR-223−/− embryos. The genes initially screened were: Ifr3 and Ifr7 (interferon regulatory transcription factors), Isg15 and Isg16 (type 2 interferons), Csf3r and Csf3rb (type 1 or type 2 interferon receptor subunits (Aggad et al., 2010), St2a/b and Jak2a (signaling molecules), Myd88 (the adaptor of Toll-like receptor [TLR] and IL1R), Caspa (Caspase1 ortholog), and Ifit1 (interferon-stimulated genes 1) were enriched. One representative experiment of three independent repeats is shown. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001, unpaired Student’s t test (C, F, and G) or one-way ANOVA (E). See also Movie S1.

Loss of miR-223 Elevates NF-κB Activation in Basal Epithelial Cells

To determine the dynamics of the NF-κB activation, we utilized a NF-κB reporter line Tg(NFκB:GFP), where GFP transcription is controlled by NF-κB recognition sequences (Kanter et al., 2011). miR-223−/− embryos displayed elevated GFP signal in the tail fin at steady state, as well as at the wound margin at 6 hpw (Figures 4A and 4B; Movie S2), which is consistent with our microarray results (Figure S6). As expected, the NF-κB inhibitor BAY, significantly downregulated GFP expression at wound (Figure 4C). Interestingly, the GFP+ cells in the fin were immobile with an epithelium-like morphology (Figure 4D). The tailfin epithelium in zebrafish larva are composed of one apical layer and one basal layer. With immunofluorescence, we discovered that the GFP signal was restricted to the Tp63-positive (Lee and Kimelman, 2002) basal layer (Figure 4E). Collectively, NF-κB pathway was elevated in basal epithelial cells in miR-223−/− embryos.

miR-223 Directly Suppresses Cul1a/b, Traf6, and Tab1

Despite the established importance of the canonical NF-κB pathway in inflammation, a direct link of this pathway with miR-223 has not been reported. We used three algorithms (Target scan, PicTar, and miRanda) (Grimson et al., 2007; John et al., 2004; Krek et al., 2005) and identified four genes in the NF-κB pathway: Cul1a, Cul1b, Traf6, and Tab1 as potential miR-223 targets (Figure 3C). Cul1 is an essential component of the SCF (complex of SKP1, CUL1, and F-box protein) E3 ubiquitin ligase. The SCF E3 ligase complex (SKP1-Cul1-β-TrCP1) ubiquitinates IκB and activates the canonical NF-κB pathway (Vileneuve et al., 2010). Cul1a and Cul1b are duplicated orthologs in zebrafish. TRAF6 (tumor necrosis factor [TNF] receptor-associated factor protein 6) and TAB1 (transforming growth factor (TGF)-β activated kinase 1 binding protein 1) are signaling components in the canonical NF-κB pathway. The mRNA levels of Cul1a and Traf6 were significantly elevated in miR-223−/− at 1 hpw, suggesting that they are regulated by miR-223 (Figure 3D). However, there is a lack of reduction in Cul1b and Tab1 transcripts, which may reflect the fact that miRNAs do not always degrade the target miRNAs (Cipolla, 2014). To verify the direct suppression of Cul1a, Cul1b, Traf6, and Tab1 by miR-223, dual-luciferase reporter assays were performed. As expected, miR-223 significantly inhibited the expression of luciferase reporters fused with the 3′ untranslated regions (3′UTRs) of Cul1a, Cul1b, Traf6, or Tab1, but not with a control 3′UTR (Figure 3E).

miR-223 Suppresses NF-κB Activation in Basal Epithelial Cells in a Phagocyte-Dependent and -Independent Fashion

Because miR-223 is highly expressed in phagocytes, we then sought to determine whether the elevated wound signal is a result of hyperactive phagocytes. Morpholinos that disrupt macrophages development (Pu.1) (Rhodes et al., 2005) and inhibit neutrophil and macrophage motility (Rac2) (Deng et al., 2011; Rosowski et al., 2016) were used to remove all phagocytes at the wound margin (Figure 4F). The elevated NF-κB signal in wounded epithelial cells was partially inhibited in the phagocyte-deficient miR-223−/− embryos (Figure 4G), suggesting both intra- and extra-phagocyte regulation.
Furthermore, the elevated NF-κB signal in unwounded fin was not affected by phagocytes (Figure S6), suggesting a surprising possibility that miR-223 directly regulates NF-κB pathway in epithelial cells, whereas no, or very low, expression of miR-223 in epithelial cells was reported. Thus, whether miR-223 is expressed in the epithelium was determined. Neutrophils,
Figure 4. NF-κB Pathway Is Elevated in Basal Epithelial Cells in miR-223−/− Embryos

The NF-κB reporter line Tg(NF-κB:GFP) was crossed into the miR-223−/− and matched WT background. 

(A and B) Representative images (A) and quantification (B) of GFP signal. Mean fluorescence intensity (MFI) in the yellow square (A) in unwounded embryos or at the wound edge at 6 hpw was quantified. White arrows, neuromast cells constitutively expressing NF-κB signal.

(C) Quantification of GFP signal at the wound edge at 6 hpw in miR-223−/− embryos treated with DMSO or BAY.

(D) Representative confocal image of GFP+ cells in miR-223−/− embryos.

(E) Immunofluorescence of GFP and Tp63 (basal cell marker) in Tg(miR-223−/−, NF-κB:GFP) embryos. Nucleus were stained with DAPI. Representative confocal images of vertical view are shown in (E).

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macrophages, and basal epithelial cells were sorted from the 3 dpf embryos. The quality of cell sorting was validated by RT-PCR using lineage-specific markers (Figure 5A). Mature miR-223 was enriched for 2-fold in basal epithelial cells compared with the whole embryo (Figure 5B).

To demonstrate that basal epithelium-intrinsic miR-223 regulates the NF-κB pathway, an RFP-miR-223 sponge mRNA was injected into embryos at the 4-cell stage. It was previously reported that injecting mRNAs at 4- to 8-cell stage led to mosaic expression predominantly in basal epithelial cells (Gault et al., 2014), and the same observation was confirmed using TdTomato-CAAX mRNA (Figure 5C). When the RFP-miR-223 sponge mRNA was delivered this way, its expression was also restricted to the close proximity to the apical layer (Figure 5D). Embryos expressing RFP-miR-223 sponge had elevated NF-κB signals at the wound edge and enhanced neutrophil wound response at 6 hwp, compared to embryos receiving the RFP control (Figures 5E–5G). To further validate that miR-223 downregulates the target gene expression in basal epithelial cells, transcripts associated with the ribosomes in the basal epithelium were isolated. We have optimized the published protocol (Heiman et al., 2014) that no contamination from the phagocytes were detected (Figure 5H). The translation of Cull1a/b, Traf6, and Tab1, but not Cxcl8, was indeed enhanced in the miR-223-deficient larvae after wounding (Figure 5I). Therefore, miR-223 in basal epithelial cells regulates neutrophilic inflammation and NF-κB pathway in a cell autonomous manner.

To be noted, apical epithelial cells also contain modest, yet detectable levels of miR-223 (Figures 6A and 6B). Therefore, a transgenic line that expresses the miR-223 sponge specifically in apical epithelial cells, Tg(krt4: RFP-miR-223 sponge)pu12,” was generated, in which a significantly enhanced recruitment of neutrophils was also observed (Figure 6C). The translation of Cull1a/b, Traf6, and Tab1, but not Cxcl8, was indeed enhanced in the miR-223-deficient larvae after wounding (Figure 5I). Therefore, miR-223 in basal epithelial cells regulates neutrophilic inflammation and NF-κB pathway in a cell autonomous manner.

Furthermore, an accelerated tailfin regeneration was observed in miR-223-deficient larvae, especially at 72 hwp and 96 hwp (Figures 6G and 6H), possibly due to the enhanced NF-κB activation that is known to promote cell proliferation (Brantley et al., 2001). This result further supports that miR-223 regulates physiological processes in epithelial cells.

miR-223 Regulates NF-κB Pathway in Human Bronchial Epithelial Cells

Zebrafish skin is a suitable model for human mucosal epithelium (Enyedi et al., 2016). To extend our findings to humans, the expression of miR-223 was evaluated in human cells. Indeed, we detected miR-223 in the immortalized human bronchial epithelial cells (HBECs), but not in the human embryonic kidney 293 cells (HEK293T). MiR-223 expression was further elevated in the H441 human lung cancer cell line (Figure 7A). Moreover, human CUL1 and TAB2 were bioinformatically identified as potential targets of miR-223, which was further confirmed in luciferase reporter assays (Figure 7B). Additionally, overexpression of miR-223 in HEK293T cells suppressed NF-κB activation with or without stimulation using heat-killed P. aeruginosa. In HBECs, miR-223 overexpression suppressed, whereas its inhibition enhanced the activation of NF-κB after P. aeruginosa stimulation (Figure 7C). Together, our results indicated that miR-223 regulates NF-κB pathway in human cells.

**DISCUSSION**

Here, we have reported that miR-223 regulates distinct signaling pathways in multiple tissues that coordinate the resolution of neutrophilic inflammation. In basal epithelial cells, miR-223 suppresses the canonical NF-κB signaling by directly targeting multiple components in the signaling cascades. Phagocytes also contribute to suppress basal cell NF-κB activation during inflammation. In the apical epithelium, miR-223 promotes the resolution of neutrophilic inflammation in a separate mechanism. Our observation is in line with the seminal work performed by McDonald et al. (2010) that complex overlapping chemokines and lipid mediators, produced by the phagocytes and injured tissue coordinate to recruit neutrophils to a sterile injury.

The most surprising observation is that miR-223 modulates the inflammatory signaling, visualized by the NF-κB reporter, primarily in epithelial cells in a cell-intrinsic manner. This phenomenon is in line with the report that the reactive oxygen species, which are essential for neutrophil wound response, are primarily restricted to the injured epithelium (Niethammer et al., 2009), whereas the phagocytes were not the primary source as previously speculated. Neutrophils are recruited to tissue injury sites by a hierarchical system of chemoattractive signals. Other signaling molecules possibly produced by the injured epithelium are the metalloproteases, such as mmp9 (LeBert et al., 2015) and mmp13 (Lisse et al., 2016), as well as ATP and proinflammatory lipid mediators, released as a consequence of osmotic swelling of the epithelial cells and their nucleus (Enyedi et al., 2016; Gault et al., 2014). In addition, the formyl-peptide signals produced by injured hepatocytes recruits neutrophils to the necrotic core in mice (McDonald et al., 2010). It remains to be determined how these signals are modulated by miR-223 and the related NF-κB pathway. It remains to be determined whether neutrophil maturation is also regulated by the function of miR-223 outside the phagocytes. Neutrophils in miR-223 knockout mice are hypermature and hyperactive (Johnnidis et al., 2008), however, the mechanism is not clear.

Indeed, the importance of the epithelial cells in inflammation is increasingly recognized. A good example is that epidermal cells utilize fatty acid β-oxidation, which again was previously reported.
Figure 5. miR-223 Suppresses NF-κB Activation in Basal Epithelial Cells in a Cell-Intrinsic Manner

(A and B) Macrophages, neutrophils, and basal epithelial cells were sorted from 3 dpf embryos. (A) RT-PCR of lineage-specific markers. *mpeg*, macrophage marker; *mpx*, neutrophil marker; *tp63*, basal epithelial cell marker; *myoD*, muscle cell marker; *ef1a*, loading control; WE, whole embryo; NTC, non-template control. (B) qRT-PCR of miR-223.

(C) Tdtomato-CAAX mRNA was injected into Tg(krt4:GFP)pu11 embryos at 4-cell stage. Representative confocal images from the lateral view of 3 dpf larvae are shown.

(D) RFP-miR-223 sponge mRNA was injected into Tg(krt4:GFP)pu11 embryos at 4-cell stage. A representative confocal image from the vertical view of 2 dpf larvae is shown.

(E and F) Tg(NF-κB:GFP) were injected with miR-223 sponge or the RFP control mRNA at 4-cell stage. Representative images and quantification of GFP signal at the wound edge at 6 hpw are shown.

(G) Quantification of neutrophil recruitment to the wound in embryos injected with miR-223 sponge or RFP mRNA at 4-cell stage.

(H and I) Tol2-tp63-GFP-L10a was injected into WT or miR-223/C0/C0 embryos. At 3 dpf, cell-specific mRNA-ribosome complexes were isolated by anti-GFP antibodies at 1 hpw.

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investigated in the immune cells, to fuel the production of matrix metalloproteinase and coordinated the immune response during cutaneous inflammation (Hall et al., 2014). Because the expression level of miR-223 changes under many disease conditions, further animal or human work is still needed to elucidate the physiological significance of miR-223-NF-kB in various disease conditions. It is speculated that zebrafish skin is a good model for human mucosal epithelium (Enyedi et al., 2016). Here, we detected the presence of miR-223 in human bronchial epithelial cells, cells in the lower respiratory track that are critical for asthma pathogenesis (Erie and Sheppard, 2014). In line with our observation, miR-223 is upregulated in both bronchial and alveolar epithelial cells upon lipopolysaccharide (LPS) challenge in mice (Sugatani and Hruska, 2007). In addition, a recent study (Maes et al., 2016) reported a significant upregulation of miR-223 in sputum of patients with severe neutrophilic asthma, supporting a physiological role of miR-223 in mucosal epithelial cells.

Our result also indicates a neutrophil-intrinsic role of miR-223 in regulating neutrophil recruitment, which is consistent with the previous observation that miR-223 regulates neutrophil recruitment in a blood cell-intrinsic manner (Dorhoi et al., 2013). Neutrophils isolated from miR-223-deficient mice produce more Cxcl2 and Ccl3 upon Mycobacterial infection. Cxcl2 is a neutrophil chemoattractant. Ccl3 stimulates neutrophils to produce proinflammatory mediates such as platelet-activation factor and lipid leukotriene B4 (LTB4) to recruit other neutrophils (Reichel et al., 2009). In an elegant study, Lämmermann et al. (2013) have shown that neutrophils secret LTB4 in exosomes to amplify the range of neutrophil recruitment during neutrophil swarming (Majumdar et al., 2016).

Here, we visualized that miR-223-deficient phagocytes also have a direct impact on the injured epithelium. Many molecules produced by the phagocytes, including reactive oxygen species, granule enzymes, inflammatory mediates, and neutrophil extracellular traps can lead to further tissue damage and amplifies inflammation. Many of these molecules are regulated by the NF-κB transcription factor or are direct miR-223 targets in human neutrophils such as the cysteine proteases cathepsin L and Z (Baek et al., 2008). It is also possible that activated phagocytes deliver additional miR-223 into the epithelium via exosomes or microvesicles, as demonstrated recently in mice (Neudecker et al., 2017). Inhibiting the Cxcl8 receptor Cxcr2 or the high-affinity LTB4 receptor Ltb4r1 did not reduce neutrophil recruitment in the miR-223 knockout (data not shown). It is likely that miR-223 regulates redundant or other inflammatory signals to coordinate neutrophil recruitment. Further work is required to fully dissect the mechanism.

The adaptor protein Myd88 was detected as a suppressor to the over-inflammation induced by miR-223 deficiency. Both the TLRs and the IL-1/18 receptor can activate the canonical NF-κB signaling through Myd88. Due to the poor characterization of the zebrafish IL-1/18 pathway and the high functional redundancy of the TLRs (Li et al., 2017; Sepulcre et al., 2009), it is difficult at present to pinpoint the receptor(s) that activates the NF-κB pathway during tissue injury. The nuclear factor NF-κB plays a complex role in inflammation and cancer (Hoesel and Schmid, 2013; Lawrence, 2009). On one hand, it promotes inflammation by producing pro-inflammatory cytokines and chemokines. On the other hand, it resolves inflammation by promoting leukocytes apoptosis, the production of anti-inflammatory cytokines and survival of somatic tissue under stress. Despite the vast number of predicted and validated miR-223 targets, here, we report that miR-223 directly targets four different components in the canonical NF-κB pathway in zebrafish and human, providing a significant advance in miR-223 biology. In human cells, besides CUL1 and TAB2 (Figure 6), TRAF6 and TAB1 also harbor miR-223 binding sites in their longest splice variants. Furthermore, miR-223 can suppress IKKα, downregulating the alternative NF-κB pathway (Li et al., 2010). Together, miR-223 possibly regulates the NF-κB activation differentially in various tissues, as disease progress, as a result of alternative splicing, or balancing the canonical and the alternative pathways. In light of the complexed biological function of the NF-κB pathway, our work provides a mechanistic understanding of the multifaceted and multilayered role of miR-223 in inflammatory diseases and cancer.

**EXPERIMENTAL PROCEDURES**

**Animals**

The zebrafish experiment was conducted in accordance to the internationally accepted standards. The Animal Care and Use Protocol was approved by The Purdue Animal Care and Use Committee (PACUC), adhering to the Guidelines for Use of Zebrafish in the NIH Intramural Research Program (protocol number: 1401001018). Embryos at 3 days post fertilization, when the sex is not determined, were used for experiments if not otherwise noted.

The miR-223-deficient line and other transgenic lines were generated as described in the Supplemental Information.

**Cell Culture**

HEK293T, HBEC, and H441 were obtained from American Type Culture Collection (ATCC) and maintained as described in the Supplemental Information.

**Zebrafish Handling**

Microinjection, tailfin wounding, Sudan black staining, fluorescence-activated cell sorting, and immunofluorescent staining were carried out as described in the Supplemental Information.

**Live Imaging**

Time-lapse fluorescence images, NF-κB:GFP reporter imaging, confocal imaging, and photoconversion assay were performed as described in the Supplemental Information.

(H) RT-PCR of lineage-specific markers as described in (A).

(i) Real-time qPCR of indicated genes. Data are pooled from three independent experiments (mean ± SD).

Scale bars, 20 μm (C and D), 100 μm (E). Data are representative of three independent experiments (F and G) or of two independent experiments (A and B).

*p < 0.05, **p < 0.01, and ***p < 0.0001, unpaired Student’s t test.
Figure 6. Fin Regeneration Is Accelerated in miR-223-Deficient Embryos

(A) Apical epithelial cell were sorted from the Tg(krt4:GFP)pu11 and RT-PCR of lineage-specific markers were performed as described in Figure 5A. krt4, apical epithelial cell marker.

(B) Real-time qPCR of miR-223.

(C) Quantification of neutrophil recruitment at 6 hpw in embryos from Tg(krt4: RFP-miR-223 sponge)pu12 and Tg(krt4: RFP)pu13.

(legend continued on next page)
Microarray
Microarray analysis was performed following MIAME guidelines as described in the Supplemental Information.

CRISPR Screening
Transient gene knockout were performed as described in the Supplemental Information.

NF-κB Reporter Assay
A NF-κB reporter plasmid were generated and dual luciferase reporter assay was performed as described in the Supplemental Information.

Translating Ribosome Affinity Purification
WT or miR-223−/− embryos were injected with Tol2-tp63-GFP-L10a plasmid and translating ribosome affinity purification (TRAP) was performed as described in the Supplemental Information.

Statistical Analysis
Statistical analysis was performed with Prism 6 (GraphPad). The statistical significance of differences between groups was compared with an unpaired two-tailed Student’s t test, paired Student’s t test, or ANOVA. For dual luciferase reporter assays, each psiCHECK2 reporter was normalized to the control pCDNA vector and evaluated with paired Student’s t test. A p value of <0.05 was accepted as statistically significant.

DATA AND SOFTWARE AVAILABILITY
The accession number for the microarray results reported in this paper is GEO (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94996): GSE94996.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two movies and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.01.058.

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(F) Representative confocal images of embryos from Tg(NF-κB:GFP/krt4: RFP-miR-223 sponge)pu12 at 6 hwp. Tailfin regeneration in WT and miR-223−/− embryos. Tailfin transection was performed at 3 dpf. The length of regenerated tailfin was measured as the distance between the blue and yellow dash lines.

(G) Representative images of tailfins at indicated time points.

(H) Quantification of the length of tailfin at different time points (mean ± SD; n > 20 in each group). Scale bars, 100 μm (D and G), 20 μm (F). Data are representative of three independent experiments (C, E, and H) or of two independent experiments (A and B). *p < 0.05, **p < 0.01, and ***p < 0.0001, unpaired Student’s t test.
AUTHOR CONTRIBUTIONS

Q.D. and W.Z. designed research and wrote the manuscript. W.Z., A.Y.H.-H., T.G., and X.Z. performed experiments and analyzed data. S.E.W.-H. and J.L.F. performed and analyzed microarray. A.S.P. and A.L.K. performed experiments with human cells.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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Supplemental Information

MicroRNA-223 Suppresses the Canonical NF-κB Pathway in Basal Keratinocytes to Dampen Neutrophilic Inflammation

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Figure S1. miR-223 deficiency does not lead to cell death in zebrafish, related to Figure 1. Neutrophils were isolated by fluorescence-activated cell sorting from 3 dpf zebrafish embryos. (A) RT-PCR of indicated cell markers. *mpeg*: macrophage marker; *mpx*: neutrophil marker; *myoD*: muscle cell marker; *ef1a*: loading control. WE: whole embryo. NTC: non-template control. (B) RT-qPCR of miR-223 expression in neutrophils, presented as the relative expression to whole embryos normalized to U6. Two individual experiments are shown. (C) Representative
images and (D) Quantification of the neutrophil numbers in the fin in WT and $miR-223^{-/-}$ embryos. One representative experiment of three independent repeats is shown. NS, $P > 0.05$, unpaired student $t$-test. (E, F) Representative images of TUNEL staining and quantification of TUNEL$^+$ cells in the fin in WT and $miR-223^{-/-}$ embryos. WT embryos treated with DNase I were used as the positive control. Yellow arrows indicate TUNEL$^+$ cells. Scale bars, 100 µm. NS: $P > 0.05$, unpaired student $t$-test.
Figure S2. Enhanced neutrophil recruitment in miR-223⁻/⁻ embryos results from both continuous neutrophil recruitment and a defect in neutrophil reverse migration, related to Figure 1. (A) Schematics of photoconversion-enabled neutrophil fate-mapping assay. Embryos from Tg(miR-223⁻/⁻, mpx: Dendra2) or Tg(mpx: Dendra2) were used. Photolabled neutrophils remaining at the wound indicate a defect in reverse migration. (B) Representative confocal images of embryos at three time points: before, immediately after, and 5 hours after photoconversion. Red neutrophils are photoconverted neutrophils. Red star indicates the injury site. (C) Percentage of red neutrophils remaining at the wound at 6 hpw. (D) Quantification of the number of green neutrophils at the wound at 6 hpw. Scale bars, 20 µm. * P < 0.05 and **** P < 0.0001, unpaired student t-test.
Figure S3. Genes in the NF-κB and interferon pathways are upregulated in miR-223⁻/⁻ embryos, related to Figure 3. (A) Microarray was performed for both WT and miR-223⁻/⁻ embryos unwounded and at 1h and 6 h post wounding. Upregulated genes in miR-223⁻/⁻ embryos...
were grouped into five classes at each time point. Inflammatory response related genes were selected for further pathway analysis. (B) Schematics of the genes in three signaling pathways that were selected for CRISPR/Cas screening: interferon pathway, NF-κB pathway, and related inflammasome pathway.
Figure S4. Myd88 knock out rescues over-inflammation in miR-223−/− embryos, related to Figure 3. (A, B) Representative images and quantification of efficiency of transient knock out of GFP using the CRISPR/Cas system. Single guide RNAs targeting GFP were injected into embryos from $Tg(lyzC: GFP)$ together with Cas9 proteins, or Cas9 protein alone. The number of green neutrophils at 3 dpf was counted. (C) CRISPR screening. Two separate sgRNAs were designed for each gene. Pooled sgRNAs targeting one gene or two genes were injected with Cas9 protein into miR-223−/− embryos. Neutrophil recruitment to the wound at 6 hpw was quantified using gfp sgRNA as a control. Genes in the three pathways were color coded separately.
Figure S5. Mutation efficiency of transient knock out of *Myd88*, *Cul1a*, and *Cul1b* with the CRISPR/Cas system, related to Figure 3. The gene locus targeted by sgRNAs was amplified by PCR and sequenced by next-generation sequencing from embryos injected with sgRNAs and Cas9 protein. (A-C) Representative images showing the alignment of individual reads to the reference sequence of indicating gene by Integrative Genomics Viewer. Red arrow represents one locus of the gene targeted by sgRNAs. Black arrow presents a read with wild-type sequence of the gene.
Figure S6. Phagocytes do not regulate NF-κB activation in uninjured zebrafish tail fin, related to Figure 4. Quantification of NF-κB signal in the tail in unwounded WT and miR-223⁻/⁻ embryos. Data are representative of three independent experiments. NS, $P > 0.05$, unpaired student $t$-test.
Figure S7. NF-κB related genes are upregulated in unwounded embryos and at 1 hpw, related to Figure 4. (A, B) Relative expression of indicated genes was measured by RT-qPCR before wounding and at 1 hpw in WT and miR-223−/− embryos. Data are pooled from three independent experiments (mean and s.d.). * P < 0.05 and ** P < 0.01, unpaired student t-test.
**Movie S1. Neutrophil recruitment to tailfin transection site, related to Figure 2.**

Lateral view of neutrophils expressing miR-223 sponge or Dendra2 control in the background where all the other wild-type neutrophils are red, responding to a tailfin injury. Massive recruitment of neutrophils were observed in the miR-223 sponge expressing embryos. Scale bar, 100 µm.

**Movie S2. Dynamics of NF-κB signaling after tailfin transection, related to Figure 4.**

Lateral view of the wound response in embryos from *Tg(miR-223\textsuperscript{−/−}, NF-κB:GFP, lyzC: RFP)* or *Tg(NF-κB:GFP, lyzC: RFP)*. Note enhanced GFP intensity in *miR-223\textsuperscript{−/−}* embryos. Scale Bar, 100 µm.
Supplemental Experimental Procedures

**Animals.** To generate a miR-223 deficient fish line, two individual single guide RNAs (sgRNAs) targeting *pre-mir-223* were designed: sgRNA1 binding site: 5’-TTAGAGTATTTTGACAGACTG-3’, and sgRNA2 binding site: 5’-TTTGTCAAATACCCCAAGAG-3’. The T7 promoter and the scaffold were added using overlapping PCR with the following primers listed from 5’ to 3’:

sgRNA1-P1:
GCGGCCTCTCTAATACGACTCACTATAGGGTTAGAGTATTGTGACAGACTGGTTTTAGAGCTA, sgRNA1-P2:
TGACAGACTGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAGGCTAGTCCGTTATCAAC, sgRNA1-P3:
GATCCGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTA
ACT; sgRNA2-P1:
GCGGCCTCTCTAATACGACTCACTATAGGGTTTTGTCATAATACCCCAAGAGGTTTTAGAGCTA, sgRNA2-P2:
ACCCTGAAGAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC, sgRNA2-P3:
GATCCGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTA
ACT. Single guide RNAs were *in vitro* transcribed using MEGAscript T7 kit (Invitrogen) with the purified PCR products as templates. To make Cas9 mRNA, the plasmid pT3TS-nCas9n (Addgene Plasmid #46757) (Jao et al., 2013) was linearized by XbaI and purified. Cas9 mRNA was generated using mMESSAGE mMACHINE T3 kit (Invitrogen). A mix of sgRNA1 (100 ng/µl), sgRNA2 (100 ng/µl) and Cas9 mRNA (150 ng/µl) was injected into one-cell stage
embryos. The embryos were raised to sexual maturity and the F1s were screened by sequencing the \textit{mir-223} locus. The homozygous mutant were obtained after several generations of backcross and incross.

To generate transgenic zebrafish lines, plasmids containing the tol2 backbone were coinjected with transposase mRNA into embryos at one-cell stage as described (Deng et al., 2011). The construct tol2-\textit{lyzC-Gal4-Vp16-crys}-CFP was generated with Gateway Cloning (Thermo Fisher) using the tol2 kit (Kwan et al., 2007) to make \textit{Tg(lyzc: Gal4-Vp16; crys: CFP)}\textsuperscript{pu8}. Founders were screened with green eyes.

To generate a miR-223 over expression line, \textit{Tg(lyzC: RFP-miR-223)}\textsuperscript{pu9}, a 317 bp genomic DNA sequence containing miR-223 was PCR amplified using the following primers listed from 5’ to 3’: forward CTGGCAGTACGGGCTCAGGAGGAAGAGGGAGGAGTAAAATTGAAT, reverse TAACAGCAGTTGGCTAATGAATGTTGTCATCCTCCACATTTTGCA, and cloned into the BbsI site in the intron of the vector (De Rienzo et al., 2012). GFP was replaced by RFP and then inserted into a tol2 backbone with \textit{lyzC} promoter. A transgenic line expressing the empty backbone, \textit{Tg(lyzC: RFP)}\textsuperscript{pu10} was generated as the control.

To generate miR223 sponge line, \textit{Tg(krt4: RFP-miR-223 sponge)}\textsuperscript{pu12}, a sequence containing six copies of bulged miR-223 binding sites was synthesized as a gBlocks Gene Fragment (Integrated DNA Technologies) and inserted downstream of RFP in a pME construct. The final construct was generated by Gateway Cloning. Founders were selected with red apical epithelial cells. A line with RFP \textit{Tg(krt4: RFP)}\textsuperscript{pu13} was made as the control. The line \textit{Tg(krt4: GFP)}\textsuperscript{pu11} was also generated for cell sorting. Multiple founders were obtained for each line and their offspring display similar phenotypes.
Cell Culture. HEK-293T cells, HBEC and H441 were obtained from American Type Culture Collection (ATCC), and maintained in their specific media in 37°C, 5% CO2 incubator. HEK-293T cells were cultured in 10%FBS 4.5g/glucose DMEM with sodium bicarbonate. HBEC cells were cultured in Keratinocyte Serum Free Media (KSFM), supplemented with 50µg/ml Bovine pituitary extract (BPE), 5ng/µl Epidermal growth factor (EGF) and 1X Penicillin-Streptomycin (PS). H441 cells were cultured in RPMI 1640 Medium supplemented with 10%FBS and 1XPS. Cells were checked monthly for mycoplasma using the e-Myco plus Mycoplasma PCR Detection Kit (Bulldog Bio 25234).

Microinjection. For constructs, 1 nl of mixture containing 25 ng/ul DNA plasmid and 35 ng/µl Transposase mRNA was injected into the cytoplasm of one-cell stage embryos. For morpholinos, 3 nl of solution containing 200 µM $Pu.1$ MO (5′-GATATACTGATACTCCATTGGTGGT-3′) and 100 uM $Rac2$ MO (5′-CCACCACACACTTTATTGCCTGAT-3′) were injected into the yolk of one-cell stage embryos. For mRNAs, 500-600 pg $RFP$ mRNAs, $TdT$omato-$CAAX$ mRNAs or $RFP$-$miR$-223 sponge mRNAs were injected into the yolk of 4-cell stage embryos.

Tailfin wounding, Sudan black staining, Immunofluorescent staining, and TUNEL staining. Tailfin wounding, Sudan black staining, and immunofluorescent staining were carried out with 3 dpf embryos as described (Deng et al., 2011). Briefly, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline overnight at 4°C and stained with Sudan black, TUNEL label Mix (Roche), or double-immunostained for anti-GFP (abcam, ab13970) and anti-Tp63 (GeneTex, GTX124660) primary antibodies. Goat anti-Rabbit IgG (H+L), Alexa Fluor 568 (cat# A-11011) and Goat anti-Chicken IgY (H+L), Alexa Fluor 488 (cat# A-11039) (ThermoFisher) were used as the secondary antibodies, and DAPI (Sigma) stained nuclei. Neutrophils within 200 µm to the cut sites were quantified.
**Isolation of RNA and quantitative PCR.** Total RNA was purified with a mirVana miRNA purification Kit (ThermoFisher). For miRNA assays, miRNAs were reverse-transcribed using Universal cDNA Synthesis Kit II (Exiqon). miRNA qRT-PCR was performed with ExiLENT SYBR Green master mix (Exiqon). A LightCycler 96 Real-Time RCP system (Roche Life Science) was used for the assay. Results were normalized to U6 or miR-92a. Primers used in the assays are: miR-223-3p (205986), miR-142-3p (204291), miR-92a (204258), dre-U6 (206999), and hsa-U6 (203907). For messenger RNAs, cDNAs were synthesized with Transcriptor First Strand cDNA Synthesis Kit (Roche). qRT-PCR was performed using FastStart Essential DNA Green Master (Roche). Results were normalized to *Rpl13a* or *Acta1b*. The efficiency of the primers were calculated using Real-time PCR Miner and corrected for the relative expression (Zhao and Fernald, 2005). Primers used for qRT-PCR are listed from 5’ to 3’:

*Cul1a*: forward CAGTTCAAGAAGCACCTCACA, reverse GCTCAACACCTGAATGCTGA;

*Cul1b*: forward TCCTCCAACAAAAACCCTGT, reverse TGCTCCTCGCAATCGT;

*Traf6*: forward GCACCTTTTGGATGCTGTA, reverse CATGTGCATCTGTGTAACCTCCT;

*Tab1*: forward CTAACGTAGGGTGGTCAGAATCAGG, reverse AGTCTGGTCAGCTCCTTTCA;

*Cxcl8*: forward GTCGCTGCACTGAACAGAA, reverse CTTAACCCATGGAGCAGG;

*Rpl13a*: forward CTGAAACCCACACGGTGGT, reverse CAGCTTGGCCTTTTCTCTT;

*Aimp1*: forward ACGAGGGCAAAAAGATGAAA, reverse CACCTTCGCATCTCCTTT;

*Cp*: forward GCTCCGAGAACTCAAGAAACA, reverse GTGTCGGGACGAAACCATT;

*Acta1b*: forward GCCCTGGCAGTTTGTCGAC, reverse GTCGTCACACATCCTTGATATTCT.
Live imaging. Time-lapse fluorescence images and NF-κB-GFP reporter images were acquired with AXIO Zoom V16 microscope (Zeiss). Embryos at 3 dpf were put on a glass-bottom dish. Images were analyzed using ImageJ. For GFP fluorescence intensity measurements, images within an experiment were collected using identical camera settings and background was subtracted with the rolling ball radius as 50. Mean fluorescence intensity of interested areas with identical size in one experiment was valued by Measurement in ImageJ and plotted in Prism (GraphPad) software. For confocal imaging, images were obtained using a laser-scanning confocal microscope (LSM 710, Zeiss) with a 1.0/20 x objective or a 2.0/40x water immersion objective lens.

Photoconversion assay. Photoconversion assays were performed as described (Yoo and Huttenlocher, 2011). Briefly, Tg(miR-223-/-, mpx-Dendra2) embryos were wounded with a needle around the tailfin region at 3 dpf. At 1 hpw, neutrophils expressing Dendra2 at the wound were photoconverted into red by 405 nm laser using a laser-scanning confocal microscope (LSM 710, Zeiss) with a 1.0/20 x objective, and the number of photoconverted neutrophils was recorded. After 5 hours, the number of red neutrophils at the wound was counted. The percentage of neutrophils remaining at wound was calculated as the ratio of the number of red neutrophils at 6 hpw to the number of red neutrophils at 1 hpw.

Microarray. Embryos were collected before wounding and at 1 h and 6 h post wounding. Total RNAs were extracted with Trizol (Invitrogen) and further cleaned up with RNeasy MinElute kit (Qiagen). The quality and integrity of RNAs were confirmed using Agiloent Nano RNA QC chip (Sequencing Center of Purdue University). Transcriptomic microarray analysis was conducted using the one-color hybridization strategy to compare gene expression profiles among the different time points with a zebrafish V3 array (Agilent Technologies). Following hybridization,
arrays were washed and scanned on an Agilent Technologies SureScan Microarray Scanner. Array image data was extracted using Agilent Feature Extraction Software and data was uploaded onto GeneSpring for statistical analysis. Microarray analysis was performed following MIAME guidelines. The data was deposited into Gene Expression Omnibus (GSE94996).

**CRISPR screening.** The high efficiency guides without off targeting were selected using CRISPRScan (Moreno-Mateos et al., 2015). Two individual sgRNAs were synthesized for each gene. 1 nl of solution containing 400 ng/µl sgRNAs and 400 ng/µl Cas9 protein (PNA, CP01) was injected into the cytoplasm of miR-223⁻/⁻ embryos at one-cell stage. When knock out two genes simultaneously, 200 ng/ul of sgRNAs for each gene were used. Tailfins were transected at 3 dpf, and embryos were fixed at 6 hpw. Neutrophil recruitment was measured and compared with embryos injected with gfp sgRNA. To determine the mutation efficiency of sgRNAs for *Myd88*, *Cul1a*, and *Cul1b*, injected embryos were collected at 3 dpf. The gene locus around the sgRNA binding sites of each gene was PCR amplified and followed by library construction using Nextera and sequencing using an Illumina MiSeq 300 at the sequencing center of Purdue University.

Primers used to synthesize templates of sgRNAs are listed from 5’ to 3’:

**gfp sgRNA forward:**

TAATACGACTCACTATAGGGCGAGGGCGATGCCACCTAGTTTTAGAGCTAGAAATAGCAAG;

**Myd88 sgRNA1 forward:**

TAATACGACTCACTATAGGGCGAGGGCGATGCCACCTAGTTTTAGAGCTAGAAATAGCAAG;

**Myd88 sgRNA2 forward:**

TAATACGACTCACTATAGGGCGAGGGCGATGCCACCTAGTTTTAGAGCTAGAAATAGCAAG;

**Myd88 sgRNA3 forward:**

TAATACGACTCACTATAGGGCGAGGGCGATGCCACCTAGTTTTAGAGCTAGAAATAGCAAG;
AAG; Caspa sgRNA1 forward:
TAATACGACTCACTATAAGGGTACAGGTGGCTCCGGCTGTTTTAGAGCTAGAAATAGC
AAG; Caspa sgRNA2 forward:
TAATACGACTCACTATAAGGGCCTGTTTGGCCAGTGAGTTTTAGAGCTAGAAATAGC
AAG; Il1b sgRNA1 forward:
TAATACGACTCACTATAAGGGATGGGAGCGGAGCCTGTTTTAGAGCTAGAAATAGC
AAG;
Il1b sgRNA2 forward:
TAATACGACTCACTATAAGGGCTGTCAGGGCCAGGTACGTTTTAGAGCTAGAAATAGC
AAG; Crfb5 sgRNA1 forward:
TAATACGACTCACTATAAGGGCTGTCAGGGCCAGGTACGTTTTAGAGCTAGAAATAGC
AAG; Crfb5 sgRNA2 forward:
TAATACGACTCACTATAAGGGCTGTCAGGGCCAGGTACGTTTTAGAGCTAGAAATAGC
AAG; Crfb17 sgRNA1 forward:
TAATACGACTCACTATAAGGGCTGTCAGGGCCAGGTACGTTTTAGAGCTAGAAATAGC
AAG; Crfb17 sgRNA2 forward:
TAATACGACTCACTATAAGGGCTGTCAGGGCCAGGTACGTTTTAGAGCTAGAAATAGC
AAG; Stat1a sgRNA1 forward:
TAATACGACTCACTATAAGGGCTGTCAGGGCCAGGTACGTTTTAGAGCTAGAAATAGC
AAG; Stat1a sgRNA2 forward:
TAATACGACTCACTATAAGGGCTGTCAGGGCCAGGTACGTTTTAGAGCTAGAAATAGC
AAG; Stat1b sgRNA1 forward:
TAATACGACTCACTATAAGGGCTGTCAGGGCCAGGTACGTTTTAGAGCTAGAAATAGC
AAG; Stat1b sgRNA2 forward:
TAATACGACTCACTATAAGGGCTGTCAGGGCCAGGTACGTTTTAGAGCTAGAAATAGC
AAG; *Stat1b* sgRNA2 forward:
TAATACGACTCACTATAGGAGGATGTGTTGGCATAACAGTTTTTAGAGCTAGAAATAGC
AAG; *Jak2a* sgRNA1 forward:
TAATACGACTCACTATAGGGATGGAGTTCTCCCTCCGTTTTATAGAGCTAGAAATAGC
AAG; *Jak2a* sgRNA2 forward:
TAATACGACTCACTATAGGGCCGCGCGCCGGAGCCAGTTTTTAGAGCTAGAAATAGCAAG; *Ifng1* sgRNA1 forward:
TAATACGACTCACTATAGGGCCGACGCTTGCAAAAGGATTGTAGCTAGAAATAGC
AAG; *Ifng1* sgRNA2 forward:
TAATACGACTCACTATAGGGATGAAGCTACAAAGGAGGTTTTAGAGCTAGAAATAGCAAG; *Ifng2* sgRNA1 forward:
TAATACGACTCACTATAGGGATGAAGCTACAAAGGAGGTTTTAGAGCTAGAAATAGCAAG; *Irf3* sgRNA1 forward:
TAATACGACTCACTATAGGGATGAAGCTACAAAGGAGGTTTTAGAGCTAGAAATAGCAAG; *Irf3* sgRNA2 forward:
TAATACGACTCACTATAGGGATGAAGCTACAAAGGAGGTTTTAGAGCTAGAAATAGCAAG; *Irf7* sgRNA1 forward:
TAATACGACTCACTATAGGGATGAAGCTACAAAGGAGGTTTTAGAGCTAGAAATAGCAAG; *Irf7* sgRNA2 forward:
TAATACGACTCACTATAGGGATGAAGCTACAAAGGAGGTTTTAGAGCTAGAAATAGC
AAG; *Cul1a* sgRNA1 forward:

TAATACGACTCACTATAGGGGTGACGAGGGCCGCAAGTTTTAGAGCTAGAAATAG

AAG; *Cul1a* sgRNA2 forward:

TAATACGACTCACTATAGGGAGGAGCTCAGTTTGTGGTTTTTAGAGCTAGAAATAGC

AAG; *Cul1b* sgRNA1 forward:

TAATACGACTCACTATAGGGGAGGAGCTCAGTTTGTGGTTTTTAGAGCTAGAAATAGC

AAG; *Cul1b* sgRNA2 forward:

TAATACGACTCACTATAGGGCGTCGGGAGTGTGATGAGTTTTAGAGCTAGAAATAGC

CAAG. The reverse primer for all sgRNA templates is 5’-

AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAA

CTTGCTATTTTCTAGCTCTAAAC-3’.

**Dual luciferase reporter assays.** The dual luciferase reporter assay was performed as described (Gonzalez-Martin et al., 2016). Briefly, *miR-223* was cloned into human expression vector pCDNA. The 3’UTR fragments of zebrafish *Cul1a, Cul1b, Traf6, Tab1*, and human *CUL1, TAB2* were cloned into the psiCHECK-2 vector (Promega). The pCDNA and psiCHECK-2 constructs were co-transfected into HEK293T cells using Lipofectamine 3000 (Invitrogen). After 48h, cells were lysed and luciferase activity was measured using the Dual Glo-Luciferase assay system (Promega, PF-E2920) on Synergy 2(Biotek). The renilla luciferase activity was normalized to the firefly luciferase activity. Three independent repeats were performed for each 3’UTR. The ratio of renilla luciferase activity to firefly luciferase activity of each 3’ UTR for the control pCDNA vector was set as 1.

Primers used for amplifying 3’UTR are listed from 5’ to 3’:
**NF-κB reporter assay.** To generate a NF-κB reporter plasmid, five copies of NF-κB binding sites were cloned into psiCHECK2 (Promega) to control the expression of firefly luciferase (hluc+). HEK293T cells were seeded in 12 well plates and transfected with 1 µg of the reporter plasmids and 250 ng of miR-223 over-expression plasmid or the vector control using Lipofectamine 3000 (Invitrogen). HBEC cells were seeded in 12 well plates and transfected with 500 ng of the NF-κB reporter, together with 250 ng of miR-223 over-expressing construct, 500 ng of the miR-223 sponge or corresponding control plasmids using FuGENE HD (Promega). After 48h, cells were stimulated with PBS or heat-killed *P. aeruginosa* for 6 hours. The cells were then lysed and luciferase activity was measured using the Dual Glo-Luciferase assay system (Promega, PF-E2920) on Synergy 2 (Biotek). The firefly luciferase activity was
normalized to the renilla luciferase activity and further normalized with the PBS-treated vector control. Three independent repeats were performed.

**Fluorescence activated cell sorting.** Trypsin dissociated cells of Tg(lyzC: GFP)(Vincent et al., 2016), Tg(mpeg: Dendra2)(Vincent et al., 2016), Tg(krt4: GFP)pu11 and embryos injected with Tol2-tp63-AcGFP plasmid (a generous gift from Dr. Sandra Rieger, MDI Biological Laboratory) were sorted by FACS as described(Deng et al., 2011). The quality of sorting was demonstrated by RT-PCR amplifying different cell type markers using QIAGEN OneStep Ahead RT-PCR kit (Qiagen).

Primers are listed from 5’ to 3’:

- *mpeg*: forward CTTTAATTCAGAGCCACGGAGGAGC, reverse GTAGACAACCCTAAGAAACCACAGG;
- *mpx*: forward ACCAGTGAGCCTGAGACACGCA, reverse TGCAGACACCGCTGGCAGTT;
- *tp63*: forward GGGATAAAAGGAAGAAAACTAGGG, reverse GCCCCAGGCTTTGTATACTGA;
- *krt4*: forward CTATGGAAGTGGTCTTGGTGGAGG, reverse CCTGAAGAGCATCAACCTTGGC;
- *myoD*: forward CCTTGCTTCAACACCAACGACATG, reverse GTCATAGCTGTTCCGTCTTCTCGTC;
- *ef1a*: forward TACGCCTGGGTGTTGGACAAA, reverse TCTTCTTGATGTATCCGCTGA.

**Translating ribosome affinity purification (TRAP).** Tissue-specific TRAP was performed as described (Heiman et al., 2014). Briefly, 25 µl/sample Dynabeads Protein G (Invitrogen; 100-07D) were collected on a magnetic rack and incubated with 10 µl anti-GFP antibody (Invitrogen A11122) for 2 hours at room temperature and then washed. More than 100 WT or miR-223−/−
embryos injected with Tol2-tp63-GFP-L10a plasmid were collected at 1 hpw and homogenized by a prechilled Dounce homogenizer containing cell-lysis buffer on ice. Cell lysate supernatant was incubated with coated beads at 4°C for 16-18h with end-over-end mixing on a tube rotator. After incubation, the beads were collected with a magnet and washed for four times with high-salt buffer. RNAs were eluted from beads and further purified by RNeasy MinElute kit (Qiagen). The quality of tissue-specific TRAP was identified by RT-PCR amplifying different cell type markers using OneStep Ahead RT-PCR kit (Qiagen). The quantification of the target genes was performed by one-step quantitative RT-PCR with SuperScript III Platinum one-step qRT-PCR kit (Invitrogen).
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