Dynamics of in vivo ASC speck formation

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Activated danger or pathogen sensors trigger assembly of the inflammasome adaptor ASC into specks, large signaling platforms considered hallmarks of inflammasome activation. Because a lack of in vivo tools has prevented the study of endogenous ASC dynamics, we generated a live ASC reporter through CRISPR/Cas9 tagging of the endogenous gene in zebrafish. We see strong ASC expression in the skin and other epithelia that act as barriers to insult. A toxic stimulus triggered speck formation and rapid pyroptosis in keratinocytes in vivo. Macrophages engulfed and digested that speck-containing, pyroptotic debris. A three-dimensional, ultrastructural reconstruction, based on correlative light and electron microscopy of the in vivo assembled specks revealed a compact network of highly intercrossed filaments, whereas pyrin domain (PYD) or caspase activation and recruitment domain alone formed filamentous aggregates. The effector caspase is recruited through PYD, whose overexpression induced pyroptosis but only after substantial delay. Therefore, formation of a single, compact speck and rapid cell-death induction in vivo requires a full-length ASC.

Introduction

Inflammasomes are large, supramolecular structures that signal the detection of danger or pathogenic stimuli by specific pattern-recognition receptors, including some NOD-like receptor (NLR) family members (Broz and Dixit, 2016; Sharma and Kanneganti, 2016). Inflammasome signaling ultimately leads to the activation of the effector caspase-1 through proximity-induced, auto-proteolytic cleavage (Hauenstein et al., 2015). Activated caspase-1 can proteolytically process cytokines as well as trigger pyroptosis, a proinflammatory form of regulated cell death, through cleavage of Gasdermin D (Man and Kanneganti, 2016). During pyroptosis, cells swell after the N-terminal domain of Gasdermin D assembles into pores in the plasma membrane, leading to its rupture and the release of intracellular contents and membrane vesicles (Vande Walle and Lamkanfi, 2016; Vincze and Silke, 2016). The adapter molecule apoptosis-associated speck-like protein containing a CARD (ASC) is central to the inflammasome assembly process (Hoss et al., 2017). ASC is composed of two protein–protein interaction domains of the death-domain superfamily, a pyrin domain (PYDα) and a caspase activation and recruitment domain (CARDα) joined by a flexible linker (de Alba, 2009). This enables ASC to interact with both PYD-containing receptors and the CARD-containing procaspase-1, thus bridging sensor and effector molecules (Broz and Dixit, 2016).

Upon activation, inflammasome-forming receptors oligomerize and nucleate the prion-like aggregation of ASC, enabling the subsequent clustering of caspase-1 (Cai et al., 2014; Lu et al., 2014). During this process, ASC is rapidly depleted from its steady-state, homogeneous cellular distribution and self-associates to form a single punctum inside the cell of ~1 µm in diameter, called a speck (Masumoto et al., 1999; Fernandes-Alnemri et al., 2007). The fast and irreversible assembly of ASC into specks maximizes the amount of activated caspase-1, ensuring high signal amplification (Kagan et al., 2014; Broz and Dixit, 2016).

Structural methods used to analyze specks in vitro showed that ASC assembles into filaments in which PYDα forms a rigid, cylindrical core, whereas CARDα is directed outward through a flexible attachment (Lu et al., 2014; Sborgi et al., 2015). The external orientation of CARDα, in addition to enabling the recruitment of downstream signaling elements, allows intra- and interfilament cross-linking through CARDα–CARDα interactions. Indeed, recent cell culture studies showed that preventing CARDα interactions by single-point mutagenesis (Dick et al., 2016) or use of an intracellular alpaca antibody fused to a fluorescent protein (Schmidt et al., 2016) abolishes speck formation, but not a PYDα filament assembly. However, whether in vivo, assembled specks also share this cross-linked filament arrangement has not been analyzed with structural methods.

Specks can be visualized using light microscopy by expressing ASC fused to a fluorescent protein from a transgene
(Fernandes-Alnemri et al., 2007; Cheng et al., 2010). The switch from a diffuse signal throughout the cell to one single bright point is considered a readout and a proxy for inflammasome activation (Stutz et al., 2013; Sester et al., 2015; Beilharz et al., 2016; Tzeng et al., 2016). However, experimentally expressed constructs increase the cellular concentration of ASC and, given the protein’s high tendency to aggregate if overexpressed (Hoss et al., 2017), the risk that speck formation occurs without an inflammatory stimulus also increases. The aforementioned study by Schmidt et al. (2016) represented the first time that endogenous ASC was visible using live cell imaging, rather than immunofluorescence. However, because speck formation is abolished by the use of the alpaca antibody, this tool cannot be used to assess speck formation in vivo.

Inflammasome function has mainly been studied in cells of the innate immune system, such as macrophages. However, many pathogens and toxic agents first enter the body through epithelia that form the interface between body and environment, which evidently requires innate immune-surveillance mechanisms (Yazdi et al., 2010). In spite of that, little is known about the role of the inflammasome and ASC in these or other tissues, such as endothelium or connective tissue, which are also composed of cells that contribute to a global inflammatory response (Yazdi et al., 2010; Peeters et al., 2015; Santana et al., 2016).

For example, although ASC is present in mammalian epidermis (Feldmeyer et al., 2010) and acts as a tumor suppressor in keratinocytes (Drexler et al., 2012), whether speck formation leads to pyroptosis in those cells is unknown. Studying the responses of native tissues in vivo using murine models, however, is challenging because of limited imaging accessibility.

The zebrafish (Danio rerio) is a genetically and optically accessible model organism for studying diseases and for drug screening (Renshaw and Trede, 2012; van der Vaart et al., 2012; Torracca et al., 2014; Lin et al., 2016), in which in vivo innate immune responses can be studied in the context of a whole organism (Renshaw and Trede, 2012; Angosto and Mulero, 2014; Kuri et al., 2016). The zebrafish genome contains more than 10 times as many NLR genes as the mouse or human genomes (Stein et al., 2007; Hansen et al., 2011; Howe et al., 2016). However, it has only one gene encoding ASC (named pycard, here referred to as asc) with a PYD–CARD domain structure; a zebrafish homologue of caspase-1 (named caspa) has also been identified. Although caspa has an N-terminal PYD domain, instead of a CARD domain, when cotransfected with zebrafish ASC in mammalian cells, the two proteins colocalize in a single speck (Masumoto et al., 2003).

We use zebrafish to study ASC function in tissues, such as skin, in which inflammasome signaling has not been addressed in vivo. The transparency of the zebrafish makes this model especially well-suited to study ASC-mediated inflammasome formation using speck formation as the readout. For this purpose, we generated a line in which the endogenous asc was tagged with GFP using CRISPR/Cas9 technology, allowing body-wide, in vivo analysis of speck formation.

This tool, together with an asc-inducible expression system with which we visualize the ultrastructure of specks formed in vivo, revealed that speck formation in keratinocytes can occur within the nucleus and that macrophages engulf pyroptotic cellular debris. Furthermore, the expression of the separate ASC domains shows both PYDα and CARDα cluster in filamentous aggregates. PYDα aggregates are sufficient to elicit cell death at a reduced rate, showing CARDα is required both for maximal speck clustering and cell death efficiency. Finally, by generating a Caspase-1 orthologue knockout, we conclude that speck formation unleashes Caspase-dependent pyroptosis in keratinocytes in vivo.

## Results

### Tissue-specific expression of ASC

ASC has been shown to be expressed in the skin, digestive tract, bone marrow, and peripheral blood leukocytes, among other tissues in humans (Masumoto et al., 2001), and most myeloid lineage cell lines also express asc constitutively (Hoss et al., 2017). However, no encompassing analysis addressing the spatial distribution of its expression sites within an organism has been made. To investigate the role of ASC in vivo, we first characterized gene and protein expression in zebrafish by RT-PCR in situ hybridization and immunofluorescence with a newly generated antibody against zebrafish ASC. The expression of asc is detectable from the morula stage onward, and adult hematopoietic tissues also express asc (Fig. S1 A). In 3-d-post-fertilization (dpf) larvae, asc RNA is present throughout the epidermis and in the area around the gills (Fig. 1 A and Fig. S1 B), where it has previously been reported to have a role in pharyngeal arch development (Masumoto et al., 2003). Sections showed expression in internal tissues, such as the intestinal epithelium and individual asc-expressing cells in the brain (Fig. 1, B and C; and Fig. S1, C–G’). The lateral line system and some internal tissues, such as the notochord and muscle, lacked ASC. Immunostainings showed ASC presence in the epidermis from 1 to at least 5 dpf (Fig. S1, I–O). Transgenic tissue-specific markers identified the ASC-expressing cells in the skin as both enveloping layer (EVL) and basal keratinocytes (Fig. 1, D and D’). In these cells, the protein is seen both in the cytoplasm and the nucleus (Fig. 1 E). All macrophages express ASC, as do most neutrophils (Fig. 1, F and G), but not all cells labeled by the myeloid lineage reporter spi1b express ASC (Fig. 1 H).

### Endogenous ASC and specks visualized in vivo in a knock-in transgenic line

To be able to study ASC in vivo, we generated a transgenic CRISPR knock-in line through homology-dependent repair in which the endogenous protein is fused with GFP, called Tg(asc:asc-EGFP) (Fig. S1, P–R). In agreement with the aforementioned results, transgenic embryos have ASC–GFP throughout the entire epidermis, in nuclear and cytoplasmic compartments as well as in the intestinal epithelium (Fig. 2, A–C’). ASC–GFP is also expressed in myeloid cells (Fig. 2 D). Microgliia, the tissue-resident macrophages of the brain (Peri and Nüsslein-Volhard, 2008) were ASC+, as were cells in the caudal hematopoietic tissue; many (but not all) of which were labeled by the spi1b reporter transgene. At all stages examined, muscle cells and other internal tissues were devoid of GFP.

We observed the sporadic appearance of GFP specks in the epidermis of Tg(asc:asc-EGFP) larvae (Video 1). Without exception, specks were contained in dead or dying cells, as shown in brightfield images in which these cells were rounded and dislodged from the rest of the epithelium (Fig. 3, A–C). The reason for spontaneous speck formation in these examples is unclear. To determine whether inflammatory stimuli could trigger speck formation in epidermal cells, we exposed Tg(asc: asc-EGFP) embryos to high concentrations of copper sulfate.
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(CuSO₄), a compound toxic to zebrafish larvae (Olivari et al., 2008; d’Alençon et al., 2010; Hernandez et al., 2011). The epidermis of these larvae showed signs of stress, with many deformed cells forming a rugged, instead of a smooth, epithelium and had significantly increased numbers of specks (Fig. 2 D). Cells containing a speck were rounded and dislodged from the rest of the epithelium, which is indicative of cell death. However, not all abnormal epidermal cells had specks (Fig. 2, E and E’), suggesting CuSO₄ exposure triggers a range of stress symptoms, and speck formation may occur as an indirect consequence of CuSO₄-induced toxicity to the skin. Because toxicity-induced speck formation in the skin resulted in undesired side effects, making this an inadequate system with which to address the dynamics and consequences of speck formation in vivo, we tested other, more-direct means of triggering speck formation.

Speck formation in vivo is induced by NLR or ASC overexpression

When ASC is present at endogenous concentrations, activated members of the NLR protein family, among other receptors, can trigger speck formation. Under overexpression conditions, however, the propensity of ASC to spontaneously aggregate in cultured cells is well documented (Masumoto et al., 1999; Stutz et al., 2013; Sester et al., 2015). We, therefore, tested whether these stimuli resulted in speck formation in live fish. Overexpressing a PYD-containing zebrafish NLR (ENSDARP00000137642.1) lacking the leucine-rich repeat domain led to ASC–GFP speck formation in epidermal cells of the Tg(asc:asc-EGFP) line, showing that the GFP-tagged, endogenous ASC responds appropriately to its direct stimulus (Fig. 4 A and Video 2). We also used an overexpression system, based on the construct

Figure 1. asc is expressed during zebrafish early development. asc whole-mount, in situ hybridization (wish) of 3-dpf zebrafish larvae (A) with cross (B) and longitudinal (C) sectioning of plastic embedded wish sample showing expression in epidermis, intestinal epithelium, and cells located in the brain. Bars: (full larvae) 300 µm; (sections) 100 µm. Immunostaining of ASC in 3-dpf Tg(krt4:GFP) larva (D). Optical cross section of lateral fin showing GFP expression in the EVL and ASC expression on both epidermal layers (D'). WT 3-dpf larva immunostained for ASC, together with nuclear envelope marker lamin and DAPI shows its nuclear and cytoplasmic localization (E). Immunostaining of 3-dpf Tg(mpeg1:EGFP) (F), Tg(lyz:DsRed2) (G), and Tg(spi1b:GAL4,UAS:TagRFP) (H) larvae showing expression of ASC in macrophages, neutrophils, and a single myeloid cell in the caudal hematopoietic tissue (H, white arrowhead). Bars: (full larvae) 300 µm; (all others) 30 µm.

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HSE:asc-mKate2, in which mKate2-tagged ASC was expressed under the control of a heat-shock promoter, which allowed us to induce ASC expression throughout the fish, including cells that do not express it endogenously. Transient expression of ASC–mKate2 from this construct led to the appearance of specks, whereas mKate2 alone had a cytoplasmic distribution (Fig. S2 A). Speck formation was not caused by the mKate2 fused to the ASC or by heat-shock–related stress because overexpressing ASC with other tags and using other expression systems also resulted in speck formation (Fig. S3, B–E). To simultaneously and stably induce ASC–mKate2 overexpression in all cells, we generated the transgenic line Tg(HSE:asc-mKate2) (Fig. 4 B). A quantification of speck formation over time in transgenic embryos showed that, from 2.5 h post–heat shock (hphs), the number of specks increases rapidly, and then, plateaus at around 17 hphs (Fig. 4 C and Video 3). Each cell formed only one speck, concomitant with the depletion of the cytoplasmic pool of ASC–mKate2 (Fig. 4 D and Video 3). Although muscle cells do not express asc endogenously, the heat-shock–induced ASC–mKate2 also assembled into a single speck in these cells. When we overexpressed ASC–mKate2 in Tg(asc:asc-EGFP) embryos, specks that formed in muscle cells were constituted exclusively by ASC–mKate2 (Fig. 4 E), whereas in epidermal cells, the endogenous ASC–GFP was recruited to the ASC–mKate2 speck (Fig. 4 F and Video 2). These results suggest that overexpression of ASC or its upstream receptors trigger speck formation and bypass the need for an inflammatory stimulus to activate inflammasome signaling.
Specks are formed by large, filamentous assemblies of ASC

Based on cryo-EM structures of in vitro–assembled PYD$_A$ filaments and EM data of ASC specks reconstituted in vitro (Lu et al., 2014), specks are thought to be composed of cross-linked filaments that aggregate into a sphere (Lu and Wu, 2015). To characterize the structure of in vivo–formed specks, we used correlative light and electron microscopy (CLEM; Fig. 5, A and B). We visualized ultrastructural details of specks formed in muscle cells after inducing ASC–mKate2 expression in the Tg(HSE:asc-mKate2) line. Specks in muscle cells form a cluster of 700 nm in diameter, consisting of highly intercrossed filaments (Fig. 5 B and Video 4). A three-dimensional model of the filaments reveals that the aggregated ASC filaments form a globular structure (Fig. 5 C and Video 4). These data are a strong indication that the filamentous organization observed from in vitro studies is also true of in vivo, assembled specks.

Mutating conserved, predicted phosphorylation sites abrogates speck formation

Activation of ASC, similar to other inflammasome components, is subjected to regulation by posttranslational modifications (Hoss et al., 2017). Thus, the speck formation we observed should depend on those modifications as well. We used the overexpression system to test whether ASC in zebrafish was regulated through phosphorylation by the JNK and spleen tyrosine kinase (Syk) signaling pathways, as reported for mammalian ASC (Hara et al., 2013; Lin et al., 2015). An in silico analysis, as used by Hara et al. (2013), predicted several potential JNK and Syk phosphorylation sites in zebrafish ASC (Table S1). Three sites corresponded to residues within the CARD$_A$ that are conserved in mouse and human ASC (Fig. 5 D and Fig. S2 F). We mutated those three sites (Y152F, T160F, and T170A) and one additional site in PYD$_A$, which was not conserved (T38A). Because muscle cells do not express asc endogenously, we were able to use these cells for an in vivo analysis of speck formation by mutant proteins and avoid interference from the WT ASC. Transiently expressed ASC–mKate2, containing the four mutations, formed a striated pattern or large, filamentous aggregates in muscle cells, rather than a compact speck (Fig. 5 E). By expressing constructs with single mutations, we found that the Y152F mutation was sufficient to disrupt the speck formation entirely (Fig. 5 F), similar to the corresponding mutations in mouse (Y144A) or human (Y146A) ASC, which also caused defective speck formation.

Figure 3. Live imaging of endogenous speck formation in the Tg(asc:asc-EGFP) line. Time-lapse imaging of a keratinocyte with a speck. Single plane merged with the brightfield is shown. (A) Yellow arrowheads highlight a second cell that appears to surround the speck-containing cell. Full time-lapse is included in Video 1. Live imaging of specks in the dorsal epidermis (B) and ventral fin (C) of 3-dpf Tg(asc:asc-EGFP) larvae. Merge with brightfield plane shows each speck is within a cell with altered morphology (dashed yellow line). (D) Tg(asc:asc-EGFP) 3-dpf larvae were treated with 25 µM CuSO$_4$ for 1 h. At 1 and 3 h after treatment, the number of specks per larva were quantified (one-way ANOVA; ****, P < 0.0001). Live imaging of untreated and treated larvae showing high damage of epidermis and increase in specks (E), examples in treated embryo of single cells displaying altered morphology with and without speck formation (E'). Bars: (full larvae) 300 µm; (all others) 30 µm.
These results support the notion that speck formation caused by the experimental conditions used here is under the control of conserved ASC post-translational regulatory mechanisms, and assembly, therefore, follows the physiologic signaling pathway.

Speck formation leads to keratinocyte pyroptosis

It is well established that speck formation can cause cell death by macrophage pyroptosis in culture. However, the first barrier a pathogen must overcome to establish infection is the epithelial...
surfaces that cover the body, which, as we have shown, express high levels of ASC. In spite of this, very little is known about the function and dynamics of ASC activation and speck formation in this important tissue. Because inducing asc expression in the Tg(HSE:asc-mKate2) line allowed us to study cell type–specific responses to speck formation, we compared responses of keratinocytes, which endogenously express asc, to muscle cells, which do not. We observed starkly different responses to speck formation. Keratinocytes round up within minutes after speck formation, whereas muscle cells show no visible change over ≥10 h; during which, the speck continuously increases in size (Fig. 6 A and Video 5). The response in epidermal cells was independent of the method used to overexpress ASC (Fig. S2 G). That the appearance of ASC–mKate2 specks is associated with the same morphological changes as those seen after the formation of endogenous ASC–GFP specks suggests that inflammasome signaling is being activated in these cells as a result of overexpression-induced speck formation.

We quantified cell death in the Tg(HSE:asc-mKate2) line with acridine orange (Fig. 6 B). Before specks assemble, Tg(HSE:asc-mKate2) and control larvae show similar levels of staining. However, after speck formation, cell death was significantly higher in heat-shocked, transgenic larvae (Fig. 6 C). Most of the acridine orange staining was located in the skin (Fig. S2 H); and keratinocytes, but not muscle cells, accumulated acridine orange in their surroundings after speck formation (Fig. S2 I). This, together with the observed changes in morphology, suggested that keratinocytes were undergoing cell death upon speck formation. To test that, we monitored the cellular changes in response to speck formation, specifically in EVL keratinocytes, using Tg(krt4:GFP, HSE:asc-mKate2) larvae (Fig. 6 D and Video 5). All GFP+ cells that formed a speck showed classic signs of pyroptosis (Vande Walle and Lamkanfi, 2016) <15 min after speck formation, including rounding up, detachment from the epithelia, and loss of plasma membrane integrity. We analyzed the process of cell extrusion by

Figure 5. ASC specks are highly intercrossed, filamentous structures whose clustering is altered by point mutations. (A–C) CLEM of high-pressure frozen 3 dpf Tg(HSE:asc-mKate2) larvae at 18 hphs. Low magnification electron micrograph (A, left) and overlay with red channel (A, right) imaged with light microscope. Black arrowhead shows location of speck. (B) Area of interest (red box) imaged with electron microscope. TEM tomography slice of the speck (B, black arrowhead) and overlay with 3D reconstruction of speck after manual tracking of individual filaments (B'). (C) Zoom in of three-dimensional reconstruction model. Entire TEM tomography stack and three-dimensional model are found in Video 4. Bars, 10 μm, unless otherwise indicated. (D) Results from phosphorylation-site analysis using the online tool GPS version 2.1.1, depicting Syk and JNK-specific, predicted phosphorylation sites in zebrafish ASC. Full results are found in Table S1. (E) Live imaging of larvae transiently expressing HSE:asc(4xmut)-mKate2, containing four missense mutations (T38A, Y152F, T160A, and T170A). (F) Single muscle cell in larva transiently expressing either HSE:asc-mKate2, or HSE:asc(4xmut)-mKate2, or HSE:asc(Y152F)-mKate2. Bars, 30 μm.
Figure 6. ASC speck formation in keratinocytes leads to cell death. (A) Time-lapse imaging of speck formation in keratinocyte (top) and muscle cell (bottom) in 3 dpf Tg(HSE:asc-mKate2) larva at 3 hphs. (B) Drastic, morphological changes occur only in keratinocytes. Tg(HSE:asc-mKate2) larvae, and negative siblings were stained with acridine orange and imaged at 2.5 and 15 hphs. 3D rendering of individual larvae manually segmented to exclude the head, heart and yolk regions. (B') Acidine orange spots in segmented region were quantified using 3D image analysis software (white spots); spots positive in the red channel were excluded (magenta spots). (C) Histogram of acridine orange spots in each group shows only transgenic larvae at 15 hphs have significantly higher cell death (one-way ANOVA; ****, P < 0.0001). (D) Time-lapse imaging of Tg[HSE:asc-mKate2, krt4:GFP] larvae at 3 hphs showing morphological changes in EVL keratinocyte upon speck formation (white arrowhead). Enlarged view of EVL keratinocyte (dashed white outline) of single plane with the brightfield (D'). (E) Time-lapse imaging of Tg(HSE:asc-mKate2) injected with lynGFP mRNA for membrane visualization at 8 hphs. Epidermal layer shows extrusion and gap closure after speck formation. (E') Single plane showing extruded keratinocyte. (F) Time-lapse imaging of 3-dpf Tg(krt1c19e:Tomato) larva transiently expressing HSE:asc-GFP, showing plasma membrane collapse and cell extrusion after speck formation in keratinocytes. All time-lapses are included in Video S. Bars, 30 µm.
labeled the plasma membrane with a membrane-targeted GFP (lynGFP) and observed that speck formation led to extrusion of the pyroptotic cell from the epithelial sheet, with surrounding cells sealing the gap (Fig. 6E and Video 5). This was also seen after transient overexpression of ASC–turbo-GFP (tGFP) in a reporter line labeling the membranes of keratinocytes (Fig. 6F and Video 5). These results show that keratinocytes undergo pyroptosis within 15 min of speck formation.

Effect of speck formation by nuclear ASC
Both when detected by antibodies and when tagged by GFP, endogenous ASC is present in the cytoplasm and the nucleus. Either pool can form specks in HeLa cells (Cheng et al., 2010), although the significance of that and, in particular, whether both nuclear and cytoplasmic specks can induce cell death in vivo, is unclear. To test that, we transiently expressed a nuclear-targeted ASC–mKate2 (NLS-ASC-mKate2) in the Tg(asc:asc-EGFP) line, which allowed us to monitor not only the effect of nuclear ASC but also the endogenous nuclear and cytoplasmic ASC pools. When NLS-ASC-mKate2 formed specks in the nucleus of ASC–GFP-expressing keratinocytes, those cells underwent cell death with the same dynamics as described for keratinocytes overexpressing ASC-mKate2 (Fig. 6). Cell death occurred without the recruitment of the cytoplasmic pool of the endogenous ASC–GFP (Fig. 7A and Video 6). Therefore, the presence of a nuclear speck is sufficient, and neither the depletion of the cytoplasmic pool nor a cytoplasmic speck is required for keratinocyte pyroptosis. However, in cases in which the nuclear envelope became permeable to the endogenous ASC–GFP before death occurred, the cytoplasmic pool of ASC–GFP was also recruited to the nuclear speck (Fig. 7B–E). In cases in which the plasma membrane collapsed before the nuclear-envelope breakdown, cytoplasmic ASC–GFP leaked to the extracellular environment before it was recruited to the nuclear speck (Fig. 7F–I). From 50 cells in 17 larvae from three biological replicates, we observed 27 cases in which the cytoplasmic GFP was lost and 23 in which it was recruited to the speck. We again observed nuclear speck formation by transiently coexpressing ASC–mKate2 with GFP in a transgenic line carrying the flucatin-NLS-tagBFP transgene to label all nuclei (Fig. 7J and Video 6). Specks assembled from either the cytoplasmic or the nuclear pools of ASC, regardless of the compartment in which the speck formed, its assembly led to cell death. This confirms that speck formation in the nucleus is sufficient to trigger pyroptosis in keratinocytes.
Clearance by macrophages of pyroptotic debris containing ASC specks

After macrophages undergo pyroptosis, they leave behind a structure composed of ruptured plasma membrane containing insoluble contents called “pore-induced intracellular traps” (PITs). In culture, neighboring phagocytes clear up PITs through efferocytosis (Jorgensen et al., 2016). There is also evidence that ASC specks are released to the extracellular space and can spread inflammation by recruiting the soluble ASC in the cytoplasm of phagocytes that engulf them (Baroja-Mazo et al., 2014; Franklin et al., 2014). However, whether ASC specks remain trapped in PITs and the rules that determine when engulfed specks induce speck formation and pyroptosis in the phagocyte have yet to be defined. We observed that, after keratinocyte cell death, specks remained enclosed within the cellular debris (Fig. 3, A–C; and Fig. 6 A). To test whether phagocytes could engulf speck-containing cellular debris, we induced ASC–mKate2 expression in the Tg(HSE :asc -mKate2) line, crossed with the macrophage reporter line. Macrophages were indeed capable of engulfing pyroptotic debris with specks (Fig. 8 A and Video 7). Instances of macrophages containing multiple phagosomes with specks suggest there is continuous uptake of speck-containing cellular debris and that engulfed specks do not elicit a pyroptotic response in the macrophages within 2–3 h after engulfment. Instead, the gradual loss of fluorescence from phagocytized ASC–mKate2 suggests that macrophages are capable of digesting specks after engulfment (Fig. 8 B and Video 7). To test whether macrophages themselves form specks during this process, we visualized the engulfment and digestion of specks by inducing the transient expression of ASC–mKate2 in the Tg(asc :asc -EGFP) line, crossed with a line with macrophages carrying a red membrane label. Macrophages digested the engulfed specks, but their endogenous ASC–GFP remained distributed throughout the cytoplasm during that process (Video 7). Thus, the main function of phagocytes that we observed in vivo is to clear speck-containing, pyroptotic cellular debris, and we have seen no incidences of specks triggering further death after engulfment.

Domain requirements for compact speck clustering and efficient cell death

Based on in vitro and cell culture experiments, the PYD and CARD domains of ASC are thought to have distinct roles during speck formation, with PYD\(_A\) assembling into filaments that are cross-linked by interfilament CARD interactions (Dick et al., 2016). To determine each domain’s role in speck assembly and pyroptosis in vivo, we overexpressed the single PYD\(_A\) and CARD\(_A\) fused to mKate2 (PYD\(_A\)–mKate2 and CARD\(_A\)–mKate2, respectively). In muscle cells, PYD\(_A\) most frequently assembled into long, filamentous structures, whereas CARD\(_A\) aggregated into smaller punctate aggregates throughout the cell (Fig. 9 A). In contrast, expression of either domain keratinocytes resulted in the formation of a normal-looking, compact speck, which led to pyroptosis (Fig. S3, A and B; and Video 8). The most likely reason for this difference is the presence of endogenous ASC in keratinocytes. To test that, we repeated those experiments under conditions of asc morpholino knockdown (Fig. S3 C). Although overexpressed ASC*–mKate2 (asc morpholino-resistant) under asc knockdown conditions formed compact specks in keratinocytes and caused cell death (Fig. S3 D and Video 8), as observed in control larvae, overexpressed PYD\(_A\) or CARD\(_A\) failed to do so. Instead, after a slower depletion of the cytoplasmic pool of the protein than that of full-length ASC, the single domains formed aggregates similar to those assembled in muscle cells (Fig. 9, B and C; and Video 8). The formation of these aggregates was not associated with immediate cell death: PYD\(_A\)-expressing epidermal cells died over
2 h after PYD\textsubscript{A} aggregates were first seen, whereas cells with CARD\textsubscript{A} aggregates survived for >10 h after aggregate formation. This differs from the fast response observed within \(\sim 10\) min of ASC–mKate2 speck formation in \(\textit{asc}\) knockdown larvae. PYD\textsubscript{A} is, therefore, both necessary and sufficient for cell death, which suggests that this domain mediates the interaction with downstream elements that trigger pyroptosis.

**PYD-dependent recruitment of Caspa to the ASC speck**

In mammals, the effector domain of ASC for triggering pyroptosis is the CARD, which interacts with the CARD of Caspase-1. For that reason, it is surprising that, in zebrafish, PYD appears to be the effector domain. We, therefore, tested whether caspases were involved in the response to speck formation, and if so, how they interacted with ASC. Treatment of \(Tg(HSE:asc–mKate2)\) larvae with the pan-caspase inhibitor (Q-VD-OPh hydrate) resulted in a significant reduction in cell death, without affecting speck formation (Fig. 10, A and B), showing that caspase activity is required for ASC-dependent pyroptosis. Because caspases are recruited to the speck for autoactivation (Man and Kanneganti, 2016), we tested which caspases could interact with the ASC speck. There are two homologues of mammalian \textit{caspase-1} in zebrafish, \textit{caspa} and \textit{caspb}, both with N-terminal PYD domains. We generated GFP fusions for both caspases, as well as for \textit{casp3a}, the zebrafish orthologue of mammalian Caspase-3, and transiently coexpressed them with ASC–mKate2. Only Caspa was recruited to ASC specks assembled in muscle cells (Fig. 10 C). By expressing the PYD and p20–p10 domains of Caspa (PYD\textsubscript{C} and p20–p10) separately with either the PYD\textsubscript{A} or CARD\textsubscript{A}, we confirmed that the interaction occurs via the PYD domains of both proteins (Fig. 10 D and Fig. S4, A–C).

Transient overexpression of Caspa, unlike that of Caspb or Casp3a, was extremely toxic to epidermal cells (Fig. 10 E). Caspa–GFP-overexpressing embryos lacked normal-looking keratinocytes with homogeneous GFP expression and, instead, had copious green-labeled cellular debris. Even muscle cells, which were not affected by ASC speck formation, displayed signs of damage after Caspa expression (Fig. 10 F). Consider-
Figure 10. **ASC speck formation leads to pyroptosis via activation of Caspa through PYD–PYD domain interaction.** 3-dpf **Tg(HSE:asc-mKate2)** larvae treated with the pan-caspase inhibitor QVD-OpH (100 µM) after or without heat shock were stained with acridine orange at 17 hphs. Acridine orange (AO) spots (A) and specks (B) were quantified. Treatment with QVD-OpH significantly diminished cell death caused by speck formation compared with nontreated controls (one-way ANOVA; ****, P < 0.0001) but did not affect speck formation. (C) Live imaging of transient expression of **HSE:caspa-EGFP**, **HSE:caspb-EGFP**, or **HSE:casp3a-EGFP** with **HSE:asc-mKate2**. Recruitment to the ASC–mKate2 specks only occurs in the case of Caspa–GFP coexpression. Enlarged view of single muscle cells for each case (yellow boxes) are shown below their corresponding row. Live imaging of heat-shock–induced transient expression of **HSE:PYDA-mKate2** or **HSE:CARDA-mKate2** with **HSE:caspa-EGFP** in 3-dpf larvae at 19 hphs (D) with enlarged view of single cells (D’ and D’’). (E) PYDA, but not CARDA, aggregates recruit Caspa-GFP. Live imaging of transient expression of **HSE:caspa-EGFP**, **HSE:caspb-EGFP**, or **HSE:casp3a-EGFP** between 9 and 17 hphs. Vast amounts of epidermal cellular debris are seen only when Caspa–GFP is overexpressed. (F) Single plane of **HSE:caspa-EGFP** transient expression at 17 hphs in muscle cells showing morphological changes upon Caspa-GFP overexpression. caspa antisense wish in 3-dpf larvae (G). Enlarged view shows expression in skin (G’) and ventral fin (G’’). (H) Time-lapse imaging of caspa mutants transiently expressing **HSE:asc-mKate2** with GFP at 3 hphs. Cell death response is severely affected in caspa−/− keratinocytes, with cells dying an apoptotic-like death >7 h after speck formation. Full time-lapses are included in Video 9. Bars: (full larvae) 300 µm; (all others) 40 µm.
ing that endogenous caspa is expressed in the skin (Fig. 10 G and Fig. S4 D), these data strongly suggest that Caspa is the effector caspase that activates pyroptosis in keratinocytes after speck formation and that muscle cells are protected from speck-induced pyroptosis because they do not express it.

To test that hypothesis, we generated a caspa mutant with CRISPR/Cas9 and identified two mutations (caspak−/− and caspaΔ680), which resulted in transcripts with a nonsense codon within the first exon (Fig. S4, E–G). We transiently expressed ASC–mKate2 and GFP in caspa knockout larvae. Speck formation in keratinocytes proceeded normally in those larvae but did not result in pyroptosis, with cells, instead, surviving for hours after speck formation (Fig. 10 H and Video 9). Eventually, keratinocytes with specks displayed cellular blebbing, nuclear condensation, and slowly disintegrated into vesicles strongly reminiscent of apoptotic bodies, suggesting that if Caspa is absent, speck formation results in activation of apoptosis instead of pyroptosis.

Caspase-8–dependent apoptotic death can occur downstream of speck formation via heterologous PYD/DED interaction in the absence of Caspase-1 (Vajjhala et al., 2015). We found that, like Caspa–GFP, transient overexpression of the zebrafish Caspase-8 orthologue (Casp8) was highly toxic to cells (Fig. S4 H). These results establish Caspa as the direct and only downstream effector of ASC speck formation driving immediate pyroptosis in vivo.

Discussion

ASC speck formation is a hallmark of inflammasome activation. The use of cell lines has significantly contributed to dissect the molecular interactions involved in this signaling cascade, but we lack deeper understanding of how inflammasome activation occurs in cells within their native environment. This knowledge gap can be bridged by using models that enable visualization of immune processes in the context of the whole organism (Renshaw et al., 2013; Weinheimer-Haus et al., 2015), and the strong relevance of inflammasome signaling in epithelia. Our work shows that the specific structural mechanisms that lead to ASC assembly into specks are conserved between zebrafish and mammals. First, several different ways of overexpressing ASC in vivo confirmed its high tendency for aggregation, consistent with previous examples showing zebrafish ASC specks in mammalian cells (Masumoto et al., 2003) and in uninfected control zebrafish larvae injected with asc–GFP mRNA (Vincent et al., 2016). Second, the abrogation of speck formation when predicted, conserved phosphorylation sites of zebrafish ASC are mutated suggests conservation of JNK and Syk dependent posttranslational regulatory mechanisms of ASC (Hara et al., 2013; Lin et al., 2015). Last, our CLEM analysis, which constitutes the first structural analysis of in vivo specks, shows their clustered, filamentous nature and confirms the model based on in vitro inflammasome reconstitutions depicting a speck as a three-dimensional, globular ultrastructure composed of multiple, highly intercrossed filaments (Lu et al., 2014).

An important difference between mammalian and zebrafish ASC is the domain that interacts with the effector caspase. In contrast to the mammalian inflammasome, in which Caspase-1 and ASC interact via their CARD domains, zebrafish Caspa, which has an N-terminal PYD instead of a CARD, is recruited to the ASC speck via its PYD domain, in agreement with previous mammalian cell culture experiments (Masumoto et al., 2003). CARDΔ in mammals is located on the surface of ASC filaments, enabling the recruitment of Caspase-1. Because CARD domains can themselves assemble into filaments, as in the case of MAVS in RIG-I antiviral signaling (Cai et al., 2016), the ASC filament domain structure could be inverted in zebrafish, allowing the PYD to interact with Caspa. Both amphibians and teleost fish lack the Gasdermin family genes (Tamura et al., 2007). It remains to be investigated whether members of the closely related DFNA5 family, which is present in zebrafish, act as effectors downstream of Caspa in causing pyroptosis.

Our results on the effects of expressing the individual domains of ASC reveal a correlation between the compaction of the ASC speck and the efficiency with which it leads to cell death. Both PYDΔ and CARDΔ alone have the capacity to aggregate when overexpressed, but neither cluster in a single, compact speck. CARDΔ aggregates have no detrimental effect on cells, but overexpression of only PYDΔ, whose aggregates are able recruit Caspa, results in cell death. Therefore, in this setup, the association of CARD and PYD, the formation of a compact speck, or the bridging of PYD to other molecules via CARD are all unessential for cell death as such. Instead, the PYD-mediated recruitment of Caspa appears to be sufficient. However, the finding that the rate of aggregation and cell death are significantly reduced indicates that CARDΔ is needed for the highly efficient and rapid triggering of pyroptosis. This could be achieved by maximizing speck compaction through filament cross-linking, as shown in cell culture (Dick et al., 2016; Schmidt et al., 2016), which might cause more rapid and efficient nucleation and clustering of Caspa than PYD aggregates achieve, by recruiting additional accessory molecules to the speck that accelerates Caspa activation or through a combination of both mechanisms.

Specks had been shown to remain as stable aggregates in the extracellular space after ASC overexpression in COS-7 cells and in the supernatant of macrophage cell cultures upon exposure to inflammasome-activating stimuli (Balci-Peynircigil et al., 2008; Baroja-Mazo et al., 2014; Franklin et al., 2014). In the Tg(HSE:asc–mKate2) line, ASC specks persist after the death of the cells and appear to remain associated with the pyroptotic cellular debris, which can be readily engulfed by macrophages.
as is the case in culture for in vitro–assembled specks (Franklin et al., 2014) and PITS (Jorgensen et al., 2016). Macrophages in vivo continuously cleared speck-containing cellular debris, and a single macrophage could contain multiple phagosomes with specks. Furthermore, engulfment led to the degradation of the specks within phagosomes. Franklin et al. (2014) reported that macrophages that engulfed in vitro–assembled specks could undergo pyroptosis after the speck was released into the cytosol and nucleated clustering of the phagocytes’ soluble ASC (Franklin et al., 2014), an observation which is supported by recent in vivo data (Sagoo et al., 2016). However, we did not find that a macrophage’s ability to clear debris in vivo diminished or that the macrophage was affected by the engulfment of a speck in the short term. It is possible that specks enclosed within ruptured membranes are less-efficient triggers of the phagolysosomal damage that releases them into the cytosol or that, in vivo, additional conditions are required to activate that mechanism of signaling spreading, such as extraordinarily high or sustained organismal inflammation levels. This would explain why extracellular specks are detected in the case of chronic, but not acute, inflammation (Franklin et al., 2014).

We noticed that not all specks that formed in the epidermis were removed. Keratinocytes belonging to the outer epidermal layer (EVL), marked by the krt4 transgene, were extruded from the epithelium toward the outside of the body. Because they are sloughed off and become separate from the living tissue, macrophages are likely unable to reach and remove their cellular debris.

Recently, speck formation within a tissue was visualized by intravital imaging of macrophages derived from retrovirally transduced, ASC–GFP hematopoietic stem cells in bone marrow chimeric mice (Sagoo et al., 2016). A second study generated a transgenic mouse carrying ASC–citrine, which can be expressed in a lineage-specific manner (Tzeng et al., 2016). Although both studies analyze inflammasome activation within living tissue, they rely on the insertion of an additional copy of ASC–FP expressed under viral promoters for protein visualization; thus, expression levels from the transgene are artificial, and cells that endogenously express asc will have an increased concentration of the protein. These disadvantages are circumvented by endogenous tagging of asc, as in the Tg(asc:asc-EGFP) line, in which ASC–GFP is only present in cells in which it is endogenously expressed and at physiologic levels, thus avoiding activation artifacts. We cannot entirely exclude that the GFP itself influences the behavior of the protein, but that would be a caveat affecting all studies using FPs to visualize ASC live. However, because endogenous inflammasome activation in the context of organismal infection has not been studied live, we believe that the Tg(asc:asc-EGFP) line will prove a valuable tool to address this question in vivo.

Materials and methods

Zebrafish care, transgenic lines, and genotyping

Zebrafish were cared for as described previously (Westerfield, 2007). The chemical 1-phenyl-2-thiourea (PTU; Sigma-Aldrich) was added to E3 medium at a concentration of 0.2 mM to inhibit pigmentation. The Tüpfel long fin (TL) strain was used as the WT. The following transgenic lines were used: mpeg1:EGFP/p62TS (Ellett et al., 2011), spلب :GAL4,UAS:TagRFPPaKTS (Siegler et al., 2012), tyc:DsRed/p62TS (Hall et al., 2007), β-actin-NLS:tagBFP (generated in the lab of D. Gilmour by L. Newton, European Molecular Biology Laboratory Heidelberg, Germany), mfaq4:Tomato-CAAXmTS (Cronan et al., 2016), ktr4:GFP/p62TS, and krtt1c:19e:Tomato-p62TS (Fischer et al., 2014). Lines generated in this study are described below. Genomic DNA (gDNA) was extracted from full larvae or adult fin clips with QuickExtract DNA extraction solution (Epicentre); genotyping was performed with Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). All animal experiments described in the present study were conducted under the rules of the European Molecular Biology Laboratory (EMBL) and the guidelines of the European Commission (Directive 2010/63/EU).

Acridine orange staining

Acridine orange is a live dye that has previously been used to label dying cells in live zebrafish embryos (Peri and Nüsslein-Volhard, 2008). Larvae were stained by immersion for 45 min in a 1:1,500 dilution of a 10 mg/ml stock (Sigma-Aldrich) prepared in E3, rinsed to remove excess dye, anesthetized, mounted, and imaged directly afterward. Because the dye is light sensitive, larvae were kept in the dark during staining.

Chemical and inflammatory treatments

For Caspase inhibition, the pan-caspase inhibitor Q-VD-OPh hydrate (Sigma-Aldrich) was resuspended in DMSO at a stock concentration of 10 mM. For Caspase inhibition, the compound was added directly to the medium at a concentration of 100 µM. For CuSO4 treatment, 3-dpf larvae were treated with for 1 h with copper (II) sulfate (Sigma-Aldrich) at 25 µM. The compound was washed off, and specks were quantified 1 or 3 h after treatment.

Cloning of expression vectors and expression induction

All expression vectors were coinjected with transposase mRNA (100 ng/µl) in embryos at the one-cell stage. For all heat-shock–driven expression, the fusion protein of interest was cloned into a vector backbone containing a bidirectional heat-shock element (HSE) as promoter (Bajoghli et al., 2004), Tol2 sites for transgenesis, and carrying the cmicle2:tagRFP as a transgenic marker (Kwan et al., 2007). To induce expression, injected embryos with red “bleeding heart” expression were heat shocked at 39°C in a heating block at any stage between 2.5 dpf and 3.5 dpf. The Tg(HSE:asc-mKate2)paKTS line, designated Tg(HSE:pyscd-mKate2)paKTS, was generated by raising embryos (F0) carrying the heart marker without exposing them to heat shock. The ubi:LexPR,LexOP:asc-mKate2 vector containing the LexPR/LexOP transactivation system (Emelyanov and Parinov, 2008) was generated via Gateway recombination cloning (Thermo Fisher Scientific) of ubi(p5E)/LexPR,LexOP(pME)/asc-mKate2(p3E). Expression was induced upon addition of 10 µM Mifepristone (RU486; Sigma-Aldrich).

Site-directed mutagenesis

For site-directed mutagenesis of the HSE:asc-mKate2, the QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies) was used, according to manufacturer’s instructions. To make HSE:asc-mKate2 asc ATG morpholinorresistant (HSE:asc-w-mKate2), a total of 6-bp changes were made with two rounds of site-directed mutagenesis; the first introduced the G6A, A9G, and T12A mutations; and the second introduced the G18A, G21A, and G24A mutations (5′-CTCAAAAGCCTCCTG CAGTGGTCTCTTGAATGACTCTTCGATGGTG-3′). Specific primer pairs were used to mutate each phosphorylation site: T38A-CTC AAA AGC CTC CTG, T56A-CTC AAA AGC CTC CTG, Y152F-5′-CAT CAC AAA TGA GGA TTT CTG TAC, and Y158F-5′-CCA AAACTGTGGTCTTGAATGACTCTTCGATGGTG-3′. Specific primer pairs were used to mutate each phosphorylation site: T38A-CTC AAA AGC CTC CTG, T56A-CTC AAA AGC CTC CTG, Y152F-5′-CAT CAC AAA TGA GGA TTT CTG TAC, and Y158F-5′-CCA AAACTGTGGTCTTGAATGACTCTTCGATGGTG-3′. Specific primer pairs were used to mutate each phosphorylation site: T38A-CTC AAA AGC CTC CTG, T56A-CTC AAA AGC CTC CTG, Y152F-5′-CAT CAC AAA TGA GGA TTT CTG TAC, and Y158F-5′-CCA AAACTGTGGTCTTGAATGACTCTTCGATGGTG-3′.
CTCCTCAAAAGAAGATG-3'), and T170A (5'-GAGAGAGTATT AGCAGGCCCAATCAGTG-3').

**Single-guide RNA (sgRNA) and mRNA synthesis**

To synthesize the templates for sgRNAs targeting caspa, the two-oligo PCR method (Shah et al., 2015) was used. For sgRNAs targeting asc, sgRNA-containing plasmids were cloned using oligo annealing (Stemmer et al., 2015). All sgRNAs were transcribed using the MEGAscript T7 transcription kit (Ambion). To synthesize mRNA, linearized pCS2+ DNA vector containing the gene of interest was used as template and transcribed with the mMessage mMach- ine SP6 transcription kit (Ambion). RNA from in vitro transcriptions was purified with the RNA Clean and Concentrator-5 (Zymo Research). mRNAs were injected into embryos at the one-cell stage.

**RNA extraction, cDNA synthesis, and RT-PCR**

Total RNA was extracted from larvae using TriFast (PEQLAB Biotechnologie), according to manufacturer’s instructions. To prevent contamination from gDNA, samples were treated with RNase-Free DNase (Promega) and were then repurified using TriFast. To generate first-strand cDNA from the total, extracted RNA was generated using the Superscript III Reverse transcription enzyme (Thermo Fisher Scientific). The cDNA obtained was used directly for RT-PCR using Plusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). The following primers were used: asc (forward, 5'-AGTACGAGATGATCTTTGAGG-3'; reverse, 5'-AGAGCATCATAAAGAGTCCCTTCC-3'), caspa (forward, 5'-AGTCAGCAGCCTGGAGCTAAACATG-3'; reverse, 5'-TCACTAGTGCTGAGCCTTCCG-3'), and efla (forward, 5'-CTTCTCAGGCTGACTGTC-3'; reverse, 5'-CCGCTAGCTTTACCCT-3').

**Whole-mount, in situ hybridization; plastic embedding; and sectioning**

In situ hybridization was performed essentially as described previously (Thisse and Thisse, 2008). Antisense and sense probes for asc and caspa coding DNA sequence (CDS) were transcribed in vitro from linearized pCS2+ DNA vector containing the entire CDS of each gene with the DIG RNA labeling kit (Roche) and purified with SigmaSpin Post-Reaction Clean-Up columns (Sigma-Aldrich). BM Purple AP substrate (Roche) was used for staining. Whole-mount, in situ samples were sectioned using the Historesin-embedding kit (Leica Biosystems), as tiles and later stitched.

**ASC polyclonal antibody production**

ASC polyclonal antibody was generated from the full-length, recombinant ASC, purified from a bacterial expression system. Antigen production and antibody purification were performed by the Protein Expression and Purification Core Facility at EMBL. The rabbit immunization procedure and all animal handling were performed by the Polyclonal Antibody Service at the EMBL Laboratory Animal Resources. Antibody specificity was confirmed with preimmunization serum as a negative control and in the immunostaining pattern in asc morphant embryos.

**Immunostaining**

Two variants of immunostainings were used, depending on the tissue of interest. Immunostainings of myeloid cells were performed, as previously described (Varela et al., 2014). To visualize keratinocyte stainings, a less-abrasive protocol, lacking methanol dehydration, proteinase K treatment, and postfixation steps, was used for epidermis preservation. The following primary antibodies were used: antiASC (1:10^3 dilution), antiGFP (1:10^3 dilution; Santa Cruz Biotechnology, Inc.), or antiLamin B2 (1:200 dilution; Thermo Fisher Scientific). Secondary antibodies (Thermo Fisher Scientific) were coupled to Alexa Fluor 488, 568, and 647 (1:500, 1:500, and 1:300 dilutions, respectively).

**Protein extraction and Western blotting**

To obtain whole-embryo protein lysates, embryos were sonicated in fresh buffer (10 mM Hepes, pH 7.5, 100 mM KCl, 2 mM MgCl_2, 0.1 mM CaCl_2, 5 mM EGTA, pH 8.0, 1 mM NaF, 1 mM Na_3VO_4, 0.5% Triton X-100, and protease inhibitor cocktail tablets [1 tablet/10 ml; Roche]). Lysate was cleared by centrifugation, and supernatant was collected and stored after addition of 5X SDS sample buffer (10% SDS, 20% glycerol, 0.2 M Tris-HCl, pH 6.8, 0.05% Bromophenol blue, and 10% β-mercaptoethanol added right before use). Prepared protein samples were separated by SDS-PAGE with the Mini-PRO TEAN vertical electrohoresis cell system (Bio-Rad Laboratories), transferred to a polyvinylidene difluoride membrane (Immobilon-P) in a semidy transfer cell (Bio-Rad Laboratories), probed with anti-ASC (1:10^4 dilution) or anti-GFP (1:10^4 dilution; Santa Cruz Biotechnology, Inc.), and developed with the corresponding HRP-coupled secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Detection was performed using Luminata Crescendo Western HRP substrate (EMD Millipore).

**Imaging**

For confocal microscopy, larvae were anesthetized with ethyl m-amino benzoate methanesulfonate by adding the compound to the medium at a concentration of 40 µg/ml and mounting it in 1.3% low–melting-point agarose (PEQLAB Biotechnologie). Imaging of immunostainings was performed with a Leica Biosystems SP8 TCS confocal microscope using dry 20×/0.8 or water 40×/1.1 objectives. Live imaging was performed using Zeiss Biosystems LSM 780 confocal microscope at RT. For time-lapse imaging of epidermal and muscle cells, a 40× water objective was used (LD C-Apochromat 40×/1.1 W Corr M27 or C-Apochromat 40×/1.2 W Corr M27; Zeiss Biosystems). Whole larvae were imaged using a 5× (Plan-Apochromat 5×/0.16 M27; Zeiss Biosystems) or 10× (Plan-Apochromat 10×/0.45 M27; Zeiss Biosystems) as tiles and later stitched.

**asc knockdown**

Design and synthesis of asc ATG morpholino (5'-GCTGCTCTTTGA AAGATTCGCAAT-3') was performed by Gene Tools, LLC. Stock morpholino was diluted in nuclease-free H₂O to a concentration of 3 mM and stored at RT. For knockdown experiments, morpholino was injected at a concentration of 0.6 mM. Morpholino was validated by immunostaining and, for in vivo experiments, by a loss of fluorescence after injection in homozygous Tg(ascasc;EGFP)embryos.

**Generation of the Tg(ascasc;EGFP)embryos line**

For sgRNA design, guide RNAs that targeted the last exon of asc (ENS DARG000000040076) were designed using the CRISPR/Cas9 target online predictor CCTop (http://crispr.cox.uni-heidelberg.de; Stemmer et al., 2015). Two suitable hits, guide 1 (5′-ATTCTCTGATGGATGA CCTTG-3′) and guide 2 (5′-ATCTCTCACTGAGCTATCTC-3′), were synthesized using the oligo-annealing method into vector DR274. DR274 was a gift from K. Joung (Addgene, Cambridge, MA; plasmid 42250; Hwang et al., 2013). For sgRNA in vivo validation and to test whether sgRNAs guides 1 and 2 targeted the region of interest in vivo, the guides were individually injected with varying concentrations (15–150 ng/µl) together with 1 µl of Cas9 protein (4 mg/ml) complemented with ~150 mM KCl into fertilized eggs at the one-cell stage of the zebrafish TL strain. Successful knockdown was verified by sequencing of a 1.3-kb PCR product from the targeted region of asc (forward,
5'-CCTGTCATGACCATGTAACCT-3'; reverse, 5'-TTAGCA TTGGCTCTATGCGCAAC-3'. Donor vectors were constructed via Golden GATEway cloning (Kirchmaier et al., 2013). In short, 50 ng of entry vector (EV) plasmids, numbered 1–6, and a vector backbone were digested with 0.5 µl of Bsal (Fast Digest; Thermo Fisher Scientific) and ligated with 0.5 µl of T4 DNA ligase (30 U/µl; Thermo Fisher Scientific) in several rounds in one continuous reaction of 10 cycles, consisting of 30 min at 37°C and 20 min at 16°C, followed by 5 min of 50°C and 5 min of 80°C to inactivate both enzymes. EV1 included a donor plasmid-specific target site for in vivo plasmid linearization (5′-GGC GAGGGGATGCCACCTAAGG-3′; Stemmer et al., 2015). EV3 contained an EGFP CDS with a flexilinker for tagging of asc. EV4 was empty, and EV6 contained a stop codon. Homology 5′ and 3′ flanks of different lengths (1 kb for 5′ and 1 or 2 kb for 3′) were amplified from zebrafish gDNA and cloned into empty EV2 and EV5. Flanks were amplified and designed according to the specific Cas9 cleavage sites for guides 1 and 2, as previously reported (Hisanso et al., 2015), to increase chances of precise integration. All vectors whose cloning is not mentioned were provided by the J. Withbrod laboratory (Heidelberg University, Heidelberg, Germany). For homologous recombination, the asc sgRNA guides 1 or 2 (120 ng/µl) and a corresponding donor vector (20–50 ng/µl) were injected into a donor-specific sgRNA for donor in vivo plasmid linearization (150 ng/µl) and 1 µl of Cas9 protein (4 mg/ml) in a solution complemented with ~150 mM KCl. Larvae were screened at 2 dpf for GFP expression. We observed greater successful recombination rates when using asc guide 2 and a donor vector with 5′ and 3′ homology flanks of 1 and 2 kb, respectively. However, the number of positive embryos was low and highly variable, ranging from 1 in 40 to 1 in 200 injected embryos. In total, 18 positive F0 larvae were raised into adulthood and screened for positive integration in the germline by outcrossing with WT fish. One founder, whose F1 progeny carried an allele with a correct insertion of linker-EGFP cassette at a rate of 30%, was found. Successful integration was confirmed by amplification of the targeted region in the asc locus by PCR and sequencing (SP4). Heterozygous asc-EGFP/+ embryos were raised and incrossed to obtain homozygous asc-EGFP embryos. The official designation of the Tg(asc:asc-EGFP)dab00 line is Tg(py7card:py7card-EGFP)dab00).

Generation of caspa mutant

For sgRNA design, sgRNAs targeting the first exon of the zebrafish gene caspa (ENSDARG00000008165) were designed using the tool at crispr.mit.edu (Hsu et al., 2013) and were selected, as reported (Shah et al., 2015). To test whether sgRNAs were targeting the region of interest in vivo, sgRNAs were injected in varying concentrations (120–275 ng/µl), together with 1 µl of in-house (Protein Expression and Purification Facility, EMBL, Heidelberg, Germany), synthesized Cas9 protein (4 mg/ml), complemented with ~150 mM KCl, into fertilized eggs at the one-cell stage of the zebrafish TL strain. Successful knockdown was verified by sequencing an 800-bp PCR product from the targeted region of caspa (forward, 5′-TGGTATTACGGAAGTAGGGAAGG-3′; reverse, 5′-AGGTTTCACAGACAGATGCTG-3′), or reverse 5′-CCACATCGGAGGGTTGAA-3′). To screen, embryos were injected with the most-efficient sgRNA (5′-GGAGGCCTTTAG TAATTTGGG-3′) and raised to adulthood to obtain the F0. At 6 wk after fertilization, F0 fish were genotyped by fin clipping. F0 fish showing successful targeting were incrossed, and the F1 generation was raised to adulthood. Through genotyping of the F1 adults, two knockout alleles were found: the caspa<sup>−/−</sup> (caspare<sup>dab02</sup>) allele, carrying a 5′-AAATAATAAA-3′ insertion at the expected Cas9 cleavage site, resulting in two stop codons, and the caspa<sup>−/−</sup> (caspare<sup>dab03</sup>), carrying a deletion of ~800 bp, including most of the first exon and part of the first intron, which resulted in a nonsense mutation. Heterozygous F1 fish carrying both alleles were incrossed to obtain homozygous mutants with either the caspa<sup>−/−</sup> or the caspa<sup>dab00</sup> deletion allele.

CLEM

For CLEM analysis, the embryos were high-pressure frozen (HPM010; ABRA Fluid), using 20% dextran or 20% Ficoll as cryoprotectant. The embryos were pierced with a needle in a cryo-microtome chamber (EM FC6; Leica Biosystems) at −160°C to facilitate freeze sublimation (Eltsov et al., 2015). Embryos were then freeze-substituted (EM-AFS2; Leica Biosystems) with 0.1% uranyl acetate in acetone at −90°C for 48 h. The temperature was then raised to −45°C at 3.5°C/h, and samples were further incubated for 5 h. After rinsing in acetone, the samples were infiltrated in Lowicryl HM20 resin, and the temperature was raised to −25°C and left to polymerize under UV light for 48 h at −25°C and for further 9 h, the temperature was gradually raised to 20°C (5°C/h). Thick sections (300 nm) were cut from the polymerized resin block and picked up on carbon-coated mesh grids. The imaging of sections by fluorescence microscopy was performed, as previously described (Kukulski et al., 2011; Avinoam et al., 2015) using a widefield fluorescence microscope (Ti-E; Nikon). Images were collected with mCherry-specific settings, as well as transmitted light.

TEM tomography was acquired with a FEI Tecnai F30 electron microscope. Dual-axis tomograms were obtained using SerialEM (Manostrane, 2005) and reconstructed in cifom, part of the IMOD software package (Kremer et al., 1996). Correlation between light and electron micrographs was performed with the plugin ec-CLEM (http://icy.bioimageanalysis.org/plugin/ec-CLEM) of the software platform Icy (de Chaumont et al., 2012). Features visible in both the light and electron microscopy images were manually assigned by clicking. The coordinates of pairs in the two imaging modalities were used to calculate a linear transformation, which allowed mapping of the coordinates of the fluorescent spot of interest (red channel) and to overlay it on the electron micrograph. The tomograms were threshold- and mCherry-specific settings, as well as transmitted light.

Online supplemental material

Fig. S1 shows ASC expression pattern during zebrafish early development and generation of Tg(asc:asc-EGFP) line. Fig. S2 shows ASC misexpression in vivo, which results in speck formation and leads to keratinocyte cell death. Fig. S3 shows that, in the presence of endogenous ASC, PYDA<sup>Δ</sup> or CARD<sup>Δ</sup> overexpression leads to speck formation. Fig. S4 shows the consequences of Caspa overexpression and generation of a <i>caspa</i> mutant. Table S1 shows the results of INK and Syk kinase-specific phosphorylation site prediction in zebrafish ASC by the online software GPS version 2.1.1. Video 1 shows time-lapse imaging of endogenous speck formation in the Tg(asc:asc-EGFP) line. Video 2 shows time-lapse imaging of spec formation induced by transient overexpression of NLR or ASC. Video 3 shows time-lapse imaging of speck formation in full larva and single cells
in the Tg(HSE:asc-mKate2) line. Video 4 shows a TEM tomography stack of specks with superposition of manual tracings of individual ASC filaments and their rotation. Video 5 shows time-lapse imaging of induced speck formation in single-muscle cells, EVL keratinocytes, and keratinocytes with plasma membrane labeling. Video 6 shows time-lapse imaging of nuclear speck formation. Video 7 shows time-lapse imaging of macrophages engulfing and digesting specks. Video 8 shows time-lapse imaging of PYD\(_{\perp}\) or CARD\(_{\perp}\) transient expression in wild-type and asc morphant larvae. Video 9 shows time-lapse imaging of transient expression of ASC in wild-type and caspa mutant larvae.

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