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Genome editing in the unicellular holozoan *Capsaspora owczarzaki* suggests a premetazoan function for the Hippo pathway in multicellular morphogenesis

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ABSTRACT

Animal development is mediated by a surprisingly small set of canonical signaling pathways such as Wnt, Hedgehog, TGF-beta, Notch and Hippo pathways. Although once thought to be present only in animals, recent genome sequencing has revealed components of these pathways in the closest unicellular relatives of animals. These findings raise questions about the ancestral functions of these developmental pathways and their potential role in the emergence of animal multicellularity. Here, we provide the first functional characterization of any of these developmental pathways in unicellular organisms by developing techniques for genetic manipulation in *Capsaspora owczarzaki*, a close unicellular relative of animals that displays aggregative multicellularity. We then use these tools to characterize the *Capsaspora* ortholog of the Hippo signaling nuclear effector YAP/TAZ/Yorkie (coYki), a key regulator of tissue size in animals. In contrast to what might be expected based on studies in animals, we show that coYki is dispensable for cell proliferation but regulates cytoskeletal dynamics and the three-dimensional (3D) shape of multicellular structures. We further demonstrate that the cytoskeletal abnormalities of individual coYki mutant cells underlie the abnormal 3D shape of coYki mutant aggregates. Taken together, these findings implicate an ancestral role for the Hippo pathway in cytoskeletal dynamics and multicellular morphogenesis predating the origin of animal multicellularity, which was co-opted during evolution to regulate cell proliferation.

INTRODUCTION

The vast morphological diversity observed in animals is generated by developmental programs mediated by a surprisingly small set of conserved signaling pathways (Pires-daSilva and Sommer, 2003). An exciting and unexpected finding from comparative genomic analysis indicates that many components of these pathways are present in the closest unicellular relatives of animals (King et al., 2003; Ros-Rocher et al., 2021). This raises the question of whether these pathways show similar functions in animals and their unicellular relatives, and, if not, what the ancestral

functions of these pathways were, and how they evolved to function in multicellular processes in animals. Answering this question could illuminate the roots of animal multicellularity and provide novel perspective on how to manipulate the activity of these biomedically important pathways.

One such signaling pathway is the Hippo pathway, which coordinates cell proliferation, differentiation, and survival in animals. Initially defined as a tumor suppressor pathway that restricts tissue growth in *Drosophila* development, Hippo signaling plays a conserved role in organ size control and regeneration in mammals (Davis and Tapon, 2019; Harvey and Hariharan, 2012; Moya and Halder, 2018; Yu et al., 2015; Zheng and Pan, 2019). This pathway comprises a core kinase cascade involving sequential activation of two kinases, Hpo/MST and Wts/LATS, which culminates in the phosphorylation and inactivation of the potent growth-stimulatory transcriptional coactivator YAP/TAZ/Yorkie. This core kinase cascade is in turn regulated by diverse upstream inputs, most notably mechanical force, the state of the actin cytoskeleton, and cell-cell or cell-substrate adhesion as part of a mechanotransduction pathway that relays mechanical and architectural cues to gene expression. Echoing its pervasive function in metazoan growth control, the Hippo pathway is required for contact-inhibition of proliferation in cultured mammalian cells, and defective Hippo signaling leading to YAP/TAZ oncogene activation is a major driver of human cancers (Davis and Tapon, 2019; Harvey and Hariharan, 2012; Moya and Halder, 2018; Yu et al., 2015; Zheng and Pan, 2019).

The Hippo pathway was at one point thought to be specific to animals. However, genome sequencing efforts have revealed that a core Hippo pathway and many upstream regulators are encoded in the genomes of the closest unicellular relatives of animals such as choanoflagellates and filasterians (Figure 1A) (Sebe-Pedros et al., 2012). The choanoflagellates are flagellated filter feeders that can form multicellular rosette structures by incomplete cytokinesis (Fairclough et al., 2010), whereas filasterians are amoeboid organisms characterized by actin-rich filopodial

projections (Figure 1, B and C) and represent the most basal known unicellular organism encoding all core components of the Hippo pathway, including upstream kinases and downstream transcriptional machinery (Sebe-Pedros et al., 2012). *Capsaspora owczarzaki* is a filasterean originally isolated as a putative endosymbiont in the freshwater snail *B. glabrata* that can attack and kill the parasitic schistosome *S. mansoni* (Stibbs et al., 1979). *Capsaspora* can be cultured easily in axenic growth medium containing protein source and serum in adherent or shaking culture and can be transiently transfected, allowing for the examination of protein localization using fluorescent fusion proteins (Parra-Acero et al., 2018). Although *Capsaspora* tends to grow as a unicellular organism in laboratory culture conditions, under certain conditions *Capsaspora* cells can adhere to each other to form multicellular aggregates (Figure 1, D to F and Figure S1, (Sebe-Pedros et al., 2013)), suggesting that multicellular behaviors may have been selected for in the lineage leading to *Capsaspora*. Previously, we have shown that overexpression of *Capsaspora* Hippo pathway components such as coYki and coHpo in *Drosophila* elicits similar phenotypes as their *Drosophila* orthologs (Sebe-Pedros et al., 2012), suggesting that a functional Hippo signaling pathway may have evolved well before the emergence of Metazoa. The *Capsaspora* genome also encodes homologues of other key regulators of animal development such as components of the integrin adhesome (Sebe-Pedros et al., 2010), cadherins (Nichols et al., 2012), tyrosine kinases such as Src and Abl (Suga et al., 2012), NF- κ B (Williams and Gilmore, 2020) and p53 (Sebe-Pedros et al., 2017). Thus, *Capsaspora* offers a unique opportunity to elucidate the ancestral function of these important developmental regulators in a close unicellular relative of animals.

RESULTS

Development of genome editing tools in *Capsaspora owczarzaki* enables genetic disruption of a YAP/TAZ/Yorkie ortholog

In an effort to make *Capsaspora* a tractable system for evolutionary cell biology studies, we developed genetic tools for overexpression and loss-of-function analysis. We first established a method for engineering stable *Capsaspora* cell lines by transfecting a vector encoding the fluorescent protein mScarlet and resistance to the antibiotic Geneticin (GenR). After transfection, antibiotic selection, and generation of clonal lines by serial dilution, we were able to generate uniformly mScarlet-positive clonal populations of cells (Figure S2A). Transfection of *Capsaspora* with genes encoding resistance to nourseothricin (NatR) or hygromycin (HygR) followed by selection with the respective antibiotic could also generate drug-resistant transformants (Figure S2B). These results demonstrate that stable *Capsaspora* cell lines expressing multiple transgenes can be generated.

We next sought to develop tools for loss-of-function analysis in *Capsaspora*. After unsuccessful attempts at CRISPR-Cas9-mediated genome editing, which may be due to the lack of nonhomologous end joining machinery components in *Capsaspora*, we used PCR-generated targeting constructs to achieve gene disruption by homologous recombination, replacing each allele of a diploid *Capsaspora* gene with a distinct selectable antibiotic marker (Figure 1G). This strategy allowed us to generate a cell line in which both *coYki* alleles were disrupted (Figure S2 C-E), with no detectable expression of the deleted region of *coYki* (Figure 1, H to J). These results demonstrate the generation of a homozygous *coYki* disruption mutant (subsequently *coYki* $-/-$) and establish a method for the generation of mutants by gene targeting in *Capsaspora*.

coYki is dispensable for cell proliferation but regulates the 3D shape of multicellular structures

To determine the effect of *coYki* on cell proliferation, we examined the proliferation of *Capsaspora* cells in adherent or shaking culture. In contrast to what may be expected based on previous

studies in animal cells, we observed no significant difference in cell proliferation between WT and *coYki* Δ cells in either condition (Figure S3). We next sought to examine cell proliferation within *Capsaspora* aggregates. Previously, aggregates have been generated by gentle shaking of cell cultures (Sebe-Pedros et al., 2013). To make aggregates more amenable to extended imaging *in situ*, we developed a condition that induced robust aggregate formation by plating *Capsaspora* cells in low-adherence wells (Figure 1, D to F). Under this condition, most cells coalesced into aggregates over 2-3 days, leaving few isolated cells in the culture. Treatment with the calcium chelator EGTA resulted in rapid dissociation of the aggregates (Figure S1), suggesting that calcium-dependent cell adhesion mediates aggregate integrity. We used EdU incorporation to label proliferating cells in aggregates, and observed no significant difference in the percent of EdU-positive cells between WT and *coYki* Δ genotypes (Figure 2, A and B). Unlike clones of cultured mammalian cells that often display increased cell proliferation at the clonal boundary (Fisher and Yeh, 1967), distribution of EdU+ cells was homogeneous within *Capsaspora* aggregates (Figure 2A), suggesting that a mechanism akin to contact-mediated inhibition of proliferation is absent in *Capsaspora*. Together, these results suggest that the rate of proliferation of *Capsaspora* is independent of *coYki* or contact inhibition.

While examining *Capsaspora* aggregates, we observed a profound phenotype in the shape of the *coYki* Δ aggregates. After aggregate induction by plating cells in low-adherence conditions, WT cell aggregates showed a round morphology (Figure 2C). In contrast, *coYki* Δ aggregates were asymmetric and less circular than WT aggregates (Figure 2D). Computational analysis of aggregate morphology and size revealed that WT and *coYki* Δ aggregate size was not significantly different (Figure 2E), whereas *coYki* Δ aggregates were significantly less circular than WT aggregates (Figure 2F). Optical sectioning of aggregates expressing mScarlet showed that WT aggregates were thicker perpendicular to the culture surface and more spherical than

coYki Δ aggregates (Figure 2G). Time-lapse microscopy of aggregation over a period of six days showed that, whereas WT cells accreted into round aggregates that fused with other aggregates when in close proximity but never underwent fission, *coYki* Δ aggregates were dynamically asymmetrical, less circular than WT, and sometimes underwent fission (Video S1). Taken together, these findings uncover a critical role for *coYki* in 3-dimensional (3D) shape of aggregates, but not cell proliferation, in *Capsaspora*.

Loss of *coYki* affects cell-substrate adhesion but not cell-cell adhesion

Cell-cell adhesion and cell-substrate adhesion affect 3D shape of tumor cell aggregates (Blandin et al., 2016; Saias et al., 2015). We therefore tested whether cell-cell adhesion and cell-substrate adhesion differ in WT and *coYki* Δ cells. A prediction of differential cell-cell adhesion is that mosaic aggregates of WT and *coYki* Δ cells may show cell sorting within aggregates (Foty and Steinberg, 2005). To test this prediction, we induced aggregate formation after mixing mCherry-labeled WT or *coYki* Δ cells with unlabeled WT cells at 1:9 ratio. The distribution of mCherry-labeled WT or *coYki* Δ cells within such mixed aggregates was similar and no cell sorting was evident (Figure 3, A to D), indicating that individual *coYki* Δ cells are competent to adhere to and integrate within aggregates and suggesting that the aberrant morphology of *coYki* Δ aggregates is an emergent property of a large number of *coYki* Δ cells.

To further test whether cell-cell adhesion differs in WT and *coYki* Δ cells, we vigorously vortexed *Capsaspora* cultures to separate individual cells, allowed cell clusters to form by cell-cell adhesion under gentle rotation, and then counted the number of cells in each cluster. The number of cells per cluster was similar for WT and *coYki* Δ cells (Figure 3E), suggesting that the occurrence of cell-cell adhesion is similar in these genotypes. We next tested whether cell-substrate adhesion differed in WT and *coYki* Δ cells by agitating cultures of adherent cells and counting the number of cells that disassociate from the culture surface. After agitation, significantly more *coYki* Δ cells

remained attached to the culture surface compared to WT cells (Figure 3F), indicating that coYki negatively regulates cell-substrate adhesion. Together, our data suggest that coYki affects the morphology of cell aggregates by affecting cell-substrate adhesion but not cell-cell adhesion.

coYki *-/-* cells show aberrant cytoskeletal dynamics and bleb-like protrusions

To characterize properties of coYki *-/-* cells that may contribute to abnormal aggregate morphology, we used time-lapse microscopy to examine the dynamic morphology of individual WT and coYki *-/-* cells in adherent culture. Interestingly, the cortices of coYki *-/-* cells were much more dynamic than those of WT cells. Whereas coYki *-/-* cells display extensive membrane protrusions and retractions, such dynamic membrane structures were rarely observed in WT cells (Video S2). To characterize the differences in cell membrane dynamics, we quantified the occurrence of cell protrusions in WT cells, coYki *-/-* cells, and coYki *-/-* cells expressing a coYki rescue transgene. WT cells rarely showed protrusions. In contrast, coYki *-/-* cells showed an average of 3.1 protrusions per minute (Figure 4A), a phenotype that was rescued in coYki *-/-* cells expressing a coYki transgene (Figure 4A, Video S3). These results suggest that coYki affects the cortical dynamics of *Capsaspora* cells.

In principle, the extensive membrane protrusions made by coYki *-/-* cells may represent F-actin-rich pseudopodia, which drive amoeboid motility, or, alternatively, F-actin-poor blebs, which are actin-depleted extensions of plasma membrane generated by disassociation of the membrane from the cell cortex or cortical rupture (Charras and Paluch, 2008). To distinguish between these possibilities, we stably expressed a fusion protein of the F-actin-binding Lifeact peptide (Riedl et al., 2008) with mScarlet in WT and coYki *-/-* cells and examined F-actin dynamics by time-lapse microscopy. At the basal side of WT cells, Lifeact-mScarlet was often enriched at the cell edge corresponding to the direction of cell movement, suggesting that *Capsaspora* undergoes F-actin-mediated amoeboid locomotion (Video S4). As cells move, some Lifeact-mScarlet signal appears

as stationary foci on the basal surface, suggesting the existence of actin-rich structures that mediate cell-substrate interaction in *Capsaspora* (Figure 4B, Video S4). Optical sections at mid-height of WT cells showed that F-actin was enriched at a circular cell cortex, where transient bursts of small F-actin puncta were often observed (Figure 4B, Video S5). In contrast, cortical shape was less circular in *coYki* $-/-$ cells, and F-actin concentration along the cortex was less uniform (Figure 4B). During the formation of the membrane protrusions produced by *coYki* $-/-$ cells, F-actin was initially depleted as compared to the rest of the cell (Figure 4C, Video S6). After the membrane protrusions had fully extended, F-actin accumulated first at the distal end and then along the entire cortex of the protrusion, followed by the regression of the protrusion. A similar sequence of cytoskeletal events occurs in blebbing mammalian cells (Charras et al., 2006). These results suggest that the protrusions observed in *coYki* $-/-$ cells are actin-depleted blebs, and that after bleb formation, F-actin re-accumulates within the bleb leading to cortical reformation and bleb regression.

To determine whether the abnormal cortical cytoskeleton is specific to *coYki* $-/-$ cells in adherent culture, we examined *coYki* $-/-$ cells within aggregates by time-lapse microscopy. Lifeact-mScarlet-expressing cells were mixed with cells stably expressing the green fluorescent protein Venus so that Lifeact-mScarlet-expressing cells were well-spaced and able to be imaged individually. As in adherent cells, *coYki* $-/-$ cells within aggregates showed dynamic formation of actin-depleted blebs (Video S7). Thus, *coYki* $-/-$ cells show aberrant cortical cytoskeleton and excessive membrane blebbing in both isolated adherent cells and within aggregates.

Treatment of *coYki* $-/-$ cells with blebbistatin rescues both membrane blebbing and aberrant shape of multicellular aggregates

Loss of *coYki* causes both bleb-like protrusions and abnormal aggregate morphology, suggesting that the cellular phenotypes observed in *coYki* $-/-$ cells (membrane blebbing and related cortical

cytoskeletal defects) may underlie the aberrant aggregate morphology. We tested this hypothesis by treating *coYki* $-/-$ cells with Blebbistatin, a myosin II inhibitor named after its bleb-inhibiting activity in mammalian cells (Straight et al., 2003). Strikingly, not only did low concentrations of Blebbistatin suppress the formation of blebs in *coYki* $-/-$ cells (Figure 4D, Video S8), Blebbistatin also rescued the abnormal morphology of *coYki* $-/-$ aggregates (Figure 4E). Whereas *coYki* $-/-$ cell aggregates were asymmetric and showed low circularity (Figure 4E'''), *coYki* $-/-$ aggregates treated with blebbistatin were circular and resembled WT aggregates (Figure 4E'''' and 4F). These results suggest a causal link between the membrane/cytoskeletal defects observed in individual *coYki* $-/-$ cells and the abnormal morphology of *coYki* $-/-$ aggregates.

Gene expression changes in *coYki* $-/-$ cells support a role for *coYki* in cytoskeletal functions

To characterize the transcriptional targets of *coYki* that may underlie the morphological/cytoskeletal defects of *coYki* $-/-$ cells, we performed RNAseq on WT and *coYki* $-/-$ cells in adherent culture. This analysis revealed 1205 differentially expressed genes, including 397 downregulated and 808 upregulated genes in the *coYki* mutant. Functional enrichment analysis of these two gene sets revealed distinct enrichment of functional categories (Figure 5, A and B). Genes predicted to encode actin-binding proteins were enriched in the set of genes upregulated in the *coYki* $-/-$ mutant, suggesting that these genes may play a role in the aberrant cytoskeletal dynamics observed in *coYki* $-/-$ cells. To further inquire into the potential function of *coYki*-regulated genes, we searched the 1205 differentially expressed genes against a previously reported *Capsaspora* phylome (Sebe-Pedros et al., 2016) to identify 638 *Capsaspora* genes with predicted human or mouse orthologs. Ingenuity Pathway Analysis of this mammalian ortholog set showed that the two most significantly enriched functional categories corresponded to cell movement and cell migration (Figure 5C). Notably, regulation of cell migration was reported as the most enriched functional category among YAP target genes in glioblastoma cells (Stein et al.,

2015), and cell motility was an enriched category in an integrative analysis of gene regulatory networks downstream of YAP/TAZ utilizing transcriptomic and cistromic data from multiple human tissues (Paczkowska et al., 2020). However, in contrast to functional enrichment studies in metazoan systems examining genes regulated by YAP/TAZ/Yorkie (Ikmi et al., 2014; Stein et al., 2015; Zanconato et al., 2015), no enrichment was detected in functional categories of cell proliferation or the cell cycle (Figure 5C). This finding is consistent with the lack of a proliferative phenotype in *coYki* Δ cells.

DISCUSSION

Given that all current biological phenomena are the product of a long and complex evolutionary process, understanding the evolutionary history of biological systems, such as signaling pathways, can give important insight into their current properties and functions. The presence of complete core Hippo pathway components in the close unicellular relatives of animals (Sebe-Pedros et al., 2012) provides the opportunity to elucidate the premetazoan function and evolutionary history of this pathway. By developing tools for genome editing in *Capsaspora*, we demonstrate that *coYki* regulates cytoskeletal dynamics at the cell cortex but is dispensable for the proliferation of *Capsaspora* cells. Such *coYki*-dependent cortical cytoskeletal function, in turn, regulates the 3D shape of *Capsaspora* aggregates in a manner that may involve tuning the strength of cell-substrate adhesion. Investigating the Hippo pathway in other unicellular relatives of animals such as choanoflagellates and ichthyosporeans, which are now amenable to transgenic studies (Booth and King, 2020; Booth et al., 2018; Suga and Ruiz-Trillo, 2013), will further clarify the ancestral function of this pathway predating the emergence of animal multicellularity.

Our findings implicate cytoskeletal regulation as an ancestral function of the Hippo pathway predating the origin of Metazoa, and that this pathway was later co-opted to control cell proliferation after the unicellular-to-multicellular transition in the lineage leading to animals. This co-option likely occurred early in the animal lineage, as in early-diverging ctenophores and cnidarians Hippo pathway activity is enriched in proliferating tissues (Coste et al., 2016). Given that the cortical cytoskeleton is both an important upstream regulator and a downstream effector of Hippo signaling in animal cells (Deng et al., 2020; Fernandez et al., 2011; Gaspar and Tapon, 2014; Sun and Irvine, 2016), the co-option of the Hippo pathway for cell proliferation control in early metazoans may have provided a convenient mechanism that couples cell proliferation with cytoskeletal and mechanical properties of cells.

Our study suggests that the abnormal 3D shape of *coYki* ^{-/-} aggregates is due to increased cell-substrate adhesion without affecting cell-cell adhesion. *Capsaspora* encodes an integrin adhesome (Sebe-Pedros et al., 2010), and *Capsaspora* cell-substrate adhesion is mediated at least in part by integrins (Parra-Acero et al., 2020). Indeed, our RNA-seq data shows that two integrin- β genes (CAOG_05058 and CAOG_01283) are upregulated in *coYki* ^{-/-} cells, suggesting a potential contribution by integrins in this phenotype. Given that the strength of integrin-mediated focal adhesions can be regulated by cytoskeletal contractility (Dumbauld et al., 2010), the aberrant cytoskeletal properties of *coYki* ^{-/-} cells may also contribute to their abnormal cell-substrate adhesion. This possibility is consistent with our data showing that blebbistatin, which inhibits myosin and thus perturbs cytoskeletal contractility, can rescue the aberrant morphology of *coYki* ^{-/-} aggregates.

A dedicated role for *coYki* in regulating the 3D shape but not the size of *Capsaspora* aggregates may reflect the different selection pressures on animals versus their close unicellular relatives. It has been suggested that the emergence of multicellularity is driven by phagotrophic predators of

unicellular organisms, as multicellular aggregates are less likely to be consumed by phagotrophic predators and thus are selectively favored (Stanley, 1973). A round aggregate shape ensures maximum contact between cells within the group, reducing the chance of a cell disassociating and thus being vulnerable to predation. Interestingly, a YAP loss-of-function mutation in medaka fish causes a tissue flattening phenotype (Porazinski et al., 2015) similar to that observed in *coYki* *-/-* *Capsaspora* aggregates (Figure 5D). This suggests that the regulation of 3D tissue architecture is an ancient and conserved function of YAP/TAZ/Yorkie predating the origin of animal multicellularity. We suggest that YAP/TAZ/Yorkie may have first been selected during evolution to regulate the 3D shape of multicellular aggregates in unicellular ancestors of animals, and evolved later to also control organ size in order to maintain appropriate scaling between the size of an organ and the size of the body as a whole.

Besides implicating an ancestral function of the Hippo pathway in pre-metazoan multicellular morphogenesis, the genetic tools we have developed for modifying the *Capsaspora* genome provide a valuable opportunity to interrogate the premetazoan functions of other developmental regulators that are present in the genomes of close unicellular relatives of animals. Future functional studies in *Capsaspora* and other close unicellular relatives of animals should clarify the evolutionary history of these important signaling pathways and further elucidate the nature of the unicellular ancestor of animals and the emergence of animal multicellularity.

METHODS

Cell culture

Capsaspora cells were maintained in ATCC Medium 1034 (modified PYNFH medium) supplemented with 25 µg/mL Ampicillin (referred to below as “growth medium”) in a 23°C incubator. Cells were kept in either 25 cm² cell culture flasks in 8 mL medium or in 75 cm² cell culture flasks in 15 ml medium. To induce cell aggregation, cells were resuspended to 7.5 x 10⁵ cells/ml in growth medium, and 1 ml of this cell suspension was added per well to a 24-well ultra-low attachment plate (Sigma CLS3473). To test the effect of blebbistatin on aggregate morphology, a 20 mM stock solution of blebbistatin (Sigma B0560) in DMSO was made, and cells were resuspended to 7.5 x 10⁵ cells/ml in growth medium with 1 µM blebbistatin or DMSO only as a vehicle control. Aggregates were then generated as described above and imaged with a Nikon Eclipse Ti inverted microscope with NIS-Elements acquisition software.

To characterize cell proliferation during growth on a solid substrate, *Capsaspora* cells were diluted to 1 x 10⁵ cells/ml in growth medium, and 800 µl of this dilution was pipetted into multiple wells in a 24-well polystyrene cell culture plate. To prevent edge effects, wells at the edge of the plate were not used and were instead filled with 800 µl of water. The plates were then kept in in a 23°C incubator. Each day, 1.5 µl of a 500 mM EDTA solution was added to a well, and cells in the well were resuspended by pipetting up and down for one minute. Resuspended cells were then transferred to a 1.5 mL tube, vortexed, and then the cell density was determined by hemocytometer. To characterize cell proliferation in shaking culture, cells were diluted to 1 x 10⁵ cells/ml in growth medium, and 20 ml of this dilution was added to a 125 ml Erlenmeyer culture flask. Cultures were then incubated in an orbital shaker at 150 rpm and 23°C. Cell density was determined daily by transferring 200 µl of culture to a 1.5 ml tube, adding 1.5 µl of 500 mM EDTA solution to disassociate any aggregated cells, vortexing, and counting by hemocytometer.

Fixation and staining of aggregates

To fix and stain aggregates, aggregates were generated as described above, and then aggregates were collected from a 24-well plate by gentle pipetting. 500 µl of aggregate suspension was added dropwise to 9 ml of PM PFA (100 mM PIPES pH 6.9, 0.1 mM MgSO₄, 4% PFA filtered through a 0.45 µm syringe filter) in a 15 mL polystyrene tube, and tubes were left undisturbed for 1 hour to allow fixation. Aggregates were then centrifuged at 1000 x g for 3 minutes and resuspended in 500 µl of PEM buffer (100 mM PIPES pH 6.9, 1 mM EGTA, 0.1 mM MgSO₄). After transferring aggregates to a microcentrifuge tube, aggregates were centrifuged for 2000 x g for 2 minutes, the supernatant was removed, and aggregates were resuspended in 300 µl of PEM.

As we found that aggregates were fragile during continued centrifugation-resuspension cycles, we developed a protocol where solution changes could be done without centrifugation using 24-well plate inserts with an 8 micron pore membrane at the insert base (Corning 3422). Before use with aggregates, 100 µl PEM buffer was added to a well in a 24-well plate, 300 µl of PEM was added to a membrane insert, and the insert was placed in the well and allowed to drain (contact between the membrane and the solution within the well is critical for draining). Aggregates in 300 µl of PEM were added to the insert, and the insert was moved to a new well with 100 µl of PEM and allowed to drain. As a wash step, the membrane insert was then moved to a well containing 1200 µl of PEM, allowing the membrane insert to fill with PEM buffer, and after a 5 minute incubation, the membrane was moved to a new well with 100 µl of PEM and allowed to drain. The membrane insert was then moved to a well containing 1200 µl of PEM block/permeabilization solution (100 mM PIPES pH 6.9, 1 mM EGTA, 0.1 mM MgSO₄, 1% BSA, 0.3% Triton X-100) and incubated for 30 minutes at room temperature. The membrane insert was moved to a well with 100 µl of PEM and allowed to drain and then moved into a well containing 1 mL PEM with DAPI

(2 µg/mL) and Phalloidin (0.1 µM) and incubated for 15 minutes at room temperature. Aggregates were then washed once more in 1200 µl PEM. To mount aggregates for imaging, the membrane insert was placed in a new well with 100 µl of PEM buffer and was allowed to drain, aggregates were resuspended in the residual buffer (approximately 50 µl) by gentle pipetting, and then aggregates were pipetted onto a microscope slide and allowed to settle for 5 minutes. Kimwipes were then placed in contact with the edge of the liquid on the slide, allowing for removal of excess buffer by capillary action. 15 µl of Fluoromount G (SouthernBiotech) was pipetted onto the aggregates, which were then covered with a 22 x 40 mm cover glass and sealed with nail polish. Aggregates were subsequently imaged with a Zeiss LSM 880 confocal microscope. Staining of adherent cells with phalloidin was done as previously described (Parra-Acero et al., 2020).

EdU labeling of aggregates

To examine cell proliferation within aggregates by EdU labeling, aggregates were generated in ultra-low adherence 24-well plates as described above. 3 days after aggregation was initiated, 100 µl of growth medium containing EdU was added to aggregates to result in a 150 µM EdU concentration. Aggregates were incubated with EdU for 4 hours and then fixed and resuspended in 300 µl PEM buffer as described above. Aggregates were then processed for imaging following the manufacturer's protocol (ThermoFisher C10339) using the 24-well plate membrane insert method described above to transfer aggregates between solutions. For all steps, wells with 1 mL of solution were used for washing or staining cells in membrane inserts, and wells with 100 µl of solution were used to drain wells in preparation for transfer into the next solution.

Molecular Biology

For the sequences of synthesized DNA fragments and oligonucleotides used in this study, see Data S1 and S2, respectively. For a list of plasmids generated for this study, see Data S3. To generate a vector for mScarlet expression in *Capsaspora* (pJP71), a DNA construct containing

an open reading frame encoding the mScarlet protein (Bindels et al., 2017) codon-optimized for expression in *Capsaspora* and under control of the previously described EF1- α promoter and terminator from the pONSY-mCherry vector (Parra-Acero et al., 2018) was constructed by gene synthesis (sJP1) and cloned into the EcoRV site of the pUC57-mini vector (Genscript). A KpnI site and an AflII site were incorporated at the beginning and end of the coding sequence, respectively, allowing for the construction of N or C-terminal protein fusion constructs by Gibson assembly. To generate a plasmid for expression of mScarlet and a Geneticin resistance gene (pJP72), the NeoR coding sequence from the *Dictyostelium* pDM323 vector (Veltman et al., 2009) was codon optimized for *Capsaspora*, the optimized sequence was synthesized with the promoter and terminator from the *Capsaspora* actin ortholog gene CAOG_06018, and the synthesized fragment (sJP2) was cloned into the SmaI site of pJP71 by Gibson assembly. To generate plasmids expressing mScarlet and either nourseothricin resistance (pJP102) or hygromycin resistance (pJP103), an identical strategy to that used in constructing pJP72 was used, except either a synthesized codon-optimized nourseothricin acetyltransferase gene (sJP3) based on the sequence of pUC18T-mini-Tn7T-nat (Lehman et al., 2016) or a synthesized codon-optimized hygromycin B phosphotransferase gene (sJP4) based on the sequence of *Dictyostelium* vector pDM358 (Veltman et al., 2009) was used.

To generate a plasmid encoding coYki and Hygromycin resistance for rescue of *coYki* $-/-$ phenotypes (pJP119), a DNA fragment encoding coYki and a c-terminal OLLAS epitope tag (Park et al., 2008) was generated by PCR from a synthesized a gene encoding the coYki protein (sJP5) using primers oJP101 and oJP102. To limit recombination of the transgene with the endogenous coYki loci, we recoded the coYki reading frame using synonymous codon replacement, resulting in a coYki gene with 73 percent nucleotide similarity to the WT coYki gene but encoding an identical polypeptide. This PCR product was then cloned into a KpnI and AflII digest of pJP103 using Gibson assembly.

424
425 To generate a plasmid encoding a Lifeact-mScarlet fusion protein and hygromycin resistance
426 (pJP118), a DNA fragment optimized for expression in *Capsaspora* encoding the Lifeact peptide
427 (Riedl et al., 2008) (sJP6) was synthesized and cloned into the KpnI site of pJP103 by Gibson
428 assembly. To generate a plasmid expressing Venus and Geneticin resistance (pJP114), the
429 Venus reading frame was amplified from p_{EF1 α} -CoH2B:Venus (Addgene #111877, (Parra-Acero
430 et al., 2018)) using primers oJP103 and oJP104 and cloned into a KpnI and AflII digest of pJP72
431 by Gibson assembly.

432
433 *Capsaspora* Transfection
434 24-well plates were prepared for transfection by inserting one sterile 12mm circular glass coverslip
435 in each well to aid in cell adhesion during transfection. *Capsaspora* cells at exponential growth
436 phase were collected by pipetting medium over attached cells, resuspended to 7.5×10^5 cells/ml
437 in growth medium, and then 800 μ l of this cell culture was added per well to the prepared 24-well
438 plate. Cells were incubated at 23°C overnight. The following day growth medium was removed
439 from cells and was replaced by 800 μ l of transfection medium (Scheider's *Drosophila* Medium
440 (ThermoFisher 21720024) with 10% FBS (ThermoFisher 26140079) supplemented with 25 μ g/mL
441 Ampicillin). After a 10-minute incubation at room temperature, transfection medium was removed
442 from the cells and replaced with 500 of fresh transfection medium. Transfection mixes were then
443 prepared: 100 μ l of Opti-MEM I Reduced Serum Medium (ThermoFisher) was added to a 1.5ml
444 tube, and 1 μ g of transfecting DNA was added to this medium (for multiple plasmids, an equivalent
445 amount of each plasmid by mass was used). 3 μ l of *TransIT*-X2[®] transfection reagent (Mirus Bio)
446 was then added to the tube, and the solution was immediately mixed by pipetting up and down.
447 Transfection mixes were incubated at room temperature for 5 minutes, and then 70 μ l of the
448 transfection mix was added dropwise to one well in the 24-well plate. Cells were incubated at
449 23°C for 24 hours, and then the medium was removed and replaced with 800 μ l of growth medium.

To image fluorescent transient transfectants, cells were then resuspended by pipetting up and down, and 300 µl of resuspended cells in growth medium were transferred to a well of an 8-well chambered coverglass slide (Nunc). Cells were then imaged 48 hours after addition of growth medium using a Zeiss LSM 880 confocal microscope.

Stable expression of transgenes in clonal *Capsaspora* cell lines

Transforming plasmids were linearized by digestion with either *ScaI*-HF® or *Asel* restriction enzymes (NEB), which cut within the ampicillin resistance gene, and then purified from solution and resuspended in nuclease-free water. *Capsaspora* cells were transfected with the linearized plasmids using the *TransIT*-X2® transfection reagent as described above. Two days after growth medium was added to cells following transfection, the growth medium was removed and replaced with 800 µl of growth medium supplemented with selective drugs. Due to observations of variability in cell viability between transfections, three drug concentrations were tested in parallel for each transfection: for Geneticin (ThermoFisher), cells were treated at 40, 60, or 80 µg/ml; for Nourseothricin (GoldBio), cells were treated at 50, 75, or 100 µg/ml; for Hygromycin B (Sigma), cells were treated at 150, 200, or 250 µg/ml. Cells were grown in selective medium for 2 weeks, and medium was changed every 3 days. Following selection, clonal cell populations were generated by resuspending cells in growth medium by pipetting, diluting cells to 3 cells/mL in growth medium, and adding 100 µl of this dilution to 200 µl of growth medium per in a well in a 48-well plate (this procedure generated approximately 10 wells with cell growth for each 48-well plate). To image fluorescence in stably transfected lines, clonal cell populations were transferred into wells in a 4-well glass bottom chamber slide (Nunc), incubated at 23°C for 24 hours, and cells were then imaged using a Nikon Eclipse Ti inverted microscope with NIS-Elements acquisition software.

Disruption of the *Capsaspora coYki* gene by gene targeting

To disrupt *coYki*, we attempted to delete a 228 bp segment of the *coYki* open reading frame that encodes the predicted Tead-binding domain (Sebe-Pedros et al., 2012) using a PCR-generated gene targeting construct. We designed a pair of primers that amplify the drug resistance markers described above, including the actin (CAOG_06018) promoter and terminator, with 90 bp of homology adjacent to the sequence targeted for deletion at the 5' end of the primers (oJP105 and oJP106). These primers were used to amplify the Geneticin resistance cassette from pJP72 by PCR, the resulting PCR product was gel purified and resuspended in nuclease-free water, and WT *Capsaspora* cells were transfected with this DNA as described above. Clonal populations of drug-resistant transformants were generated as described above, each clone was grown to confluency in one well in a 24-well plate, cells were collected by pipetting up and down, and genomic DNA was prepared following the protocol described below. To genotype potential mutants, we performed diagnostic PCRs on genomic DNA: to test for presence of the WT allele, we used a forward primer with homology to the genome 5' of the *coYki* sequence targeted for deletion (oJP107) and a reverse primer with homology within the sequence targeted for deletion (oJP108). To detect successful deletion, we used oJP107 as a forward primer and a reverse primer with homology to the geneticin resistance gene (oJP109). 40 percent of analyzed clones (6 of 15 tested clones) showed a PCR product indicative of disruption of the *coYki* allele (Figure S2D). However, all clones showed a band indicative of an intact WT *coYki* gene. We therefore reasoned that, at least for the culture conditions used during transfection, *Capsaspora* cells may be diploid.

After the isolation of clonal lines that were heterozygous for *coYki* disruption with the Geneticin resistance marker as indicated by diagnostic PCR, we attempted to disrupt the remaining *coYki* allele using nourseothricin resistance as a marker. pJP102 was used as a template for PCR using primers oJP105 and oJP106 to generate a nourseothricin resistance cassette flanked by homology arms targeting *coYki* for disruption. This construct was used to transfect a Geneticin-

resistant heterozygous *coYki* disruption mutant, and drug selection of transfectants was done as described above using simultaneous selection with 60 µg/ml Geneticin and 75 µg/ml nourseothricin. After generating clonal populations of drug-resistant transformants, diagnostic PCR was done to detect the intact *coYki* allele as described above or a deletion allele containing the nourseothricin resistance gene using primers oJP107 and oJP110. Two cell lines that showed absence of the WT *coYki* allele and presence of both disruption alleles encoding either Geneticin or nourseothricin resistance were used for subsequent phenotypic studies.

To quantify *coYki* gene expression in a putative homozygous *coYki* disruption mutant (*coYki*^{-/-}) by qPCR, 5 x 10⁶ WT or *coYki*^{-/-} cells were collected from culture flasks while in growth medium by pipetting up and down, and RNA was isolated using a RNeasy Mini Plus kit (Qiagen). cDNA was made using the iScript cDNA Synthesis Kit (BioRad), qPCR reactions were made using iQTM SRBR[®] Green Supermix (BioRad), and qPCR was performed using a CFX96 Touch Real-time PCR detection system (BioRad) with the following primers: oJP111 and oJP112 to detect GAPDH, oJP113 and oJP114 to detect a region of *coYki* within the sequence targeted for deletion, and oJP115 and oJP116 to detect a region *coYki* 3' of the sequence targeted for deletion.

Preparing *Capsaspora* genomic DNA

Capsaspora genomic DNA was prepared for PCR analysis following a procedure previously developed for *Dictyostelium discoideum* (Charette and Cosson, 2004). Cells grown in a 24-well or 48-well plate in growth medium were collected by pipetting up and down, pipetted into a 1.5 mL tube, centrifuged at 4000 x g for 5 minutes, and resuspended in 20 µl of nuclease-free water. 20 µl of Lysis buffer (50 mM KCl, 10 mM TRIS pH 8.3, 2.5 mM MgCl₂, 0.45% NP40, 0.45% Tween 20, and 800 µg/ml Proteinase K added fresh from a 20 mg/ml stock) was added to cells, which were then incubated at room temperature for 5 minutes. Tubes were then placed at 95°C for 5

minutes. After cooling to room temperature, 1 μ l of this sample was then used as a template in a 20- μ l diagnostic PCR.

Quantification of aggregate size and circularity

ImageJ was used to quantify aggregate size and circularity using aggregate images. Images were converted to 8-bit format, processed twice using the “Smooth” function, and a Threshold was adjusted for each individual image. The Analyze Particles command was then run with a gate for particle size at 400-infinity micron² and the “exclude on edges” option selected. The returned values for aggregate area and circularity were then used for further analysis. Values corresponding to more than one adjacent aggregate interpreted by the algorithm as a single particle were discarded.

Assaying cell-cell adhesion and cell-substrate adhesion

To examine cell-cell adhesion, cells were collected from a culture flask by pipetting growth media over the culture surface, diluted to 1×10^6 cells/mL in growth medium, and vortexed to disrupt cell clusters. 1 mL volumes of cell culture were then transferred to 1.5 mL tubes and incubated on a Labquake™ rotator (Thermo) for one hour at room temperature. Cultures were then examined by hemocytometer, and the number of cells in each cluster of cells was counted for at least 35 cell clusters for each independent experiment.

To examine cell-substrate adhesion, cells were diluted to 5×10^5 cells/mL in growth medium, and 3 mL volumes of culture were transferred per well to 6-well tissue culture-treated polystyrene plates (Sigma CLS3506) and incubated at 23°C for 48 hours. For agitation, plates were then placed on an orbital shaker and shaken at 140 RPM for 10 minutes, and then the medium from each well (“disassociated fraction”) was transferred to a separate tube and 3 mL of new growth medium was added to each well. 3 μ L of 500 mM EDTA was then added to each well to detach

cells from the culture surface, medium was pipetted up and down over the culture surface 20 times, and this resuspension (“adherent fraction”) was transferred to a separate tube. Cell densities of the collected fractions were determined by hemocytometer counts, total cell amounts were calculated by adding the cell numbers for disassociated and adherent fractions for each condition, and the percent of adherent cells for each condition was then calculated. As a control to examine cell-substrate adhesion in the absence of agitation, the above protocol was followed, except the orbital shaker agitation step was omitted.

Time-lapse Microscopy

To image cells by time-lapse transmitted light or fluorescence microscopy, cells were resuspended to 1×10^8 cells/ml in growth medium, and 10 μ l of this cell suspension was added as a spot in the center of a well in a 4-well chambered coverglass slide (Nunc). After a 1 hour incubation at room temperature to allow the cells to settle, the 10 μ l volume of medium was removed, and 1 ml of growth medium was added to the well, resulting in a spot of cells in the center of the well. Cells were incubated at room temperature for 24 hours, a field of view with well-spaced cells was located, and then cells were imaged by time-lapse microscopy using a Nikon Eclipse Ti inverted microscope with NIS-Elements acquisition software. To image the effect of blebbistatin on individual cells, similar imaging of cells was done, except that cells were treated with either DMSO or 1 μ M blebbistatin (Sigma B0560) for one hour before imaging. To image aggregates by time-lapse microscopy, aggregates were generated in ultra-low attachment plates as described above. During aggregate formation, 4-well chambered coverglass slides (Nunc) were coated with UltraPure agarose (ThermoFisher) by making a 1% agarose in nuclease-free water mixture, microwaving to dissolve the agarose, adding 800 μ l of molten agarose per well, and removing the molten agarose after 10 seconds by aspiration. Coated wells were then allowed to dry at room temperature in a cell culture hood, resulting in a thin coating of agarose. This coating functions to prevent aggregate adhesion to the glass surface. After aggregate formation,

aggregates were resuspended in the well by gentle pipetting, and then an 800 µl volume of resuspended aggregates was transferred into one agarose-coated well in a 4-well chambered coverglass slide. Aggregates were then imaged using a Zeiss LSM 880 confocal microscope.

RNA-seq

To perform RNA-seq on *Capsaspora* cells, WT or *coYki* ^{-/-} cells were collected from growth flasks by removing growth medium, adding fresh growth medium, and resuspending cells attached to the flask by pipetting the medium over the surface. Cells were then diluted to 2 x 10⁵ cells/mL, and for each genotype two 75 cm² culture flasks were prepared by adding 16 mL of culture dilution to each flask. Culture flasks were incubated at 23°C for 2 days, and then cells were collected by pipetting growth medium over attached cells. Cells were collected by centrifugation at 2100 x g for 5 minutes, and then all cells for each genotype were combined, resuspended in 500 µl of growth medium, and transferred to a microcentrifuge tube. RNA was then prepared with the RNEasy Plus Mini Kit (Qiagen) using the QIAshredder spin column (Qiagen) to homogenize the lysate. RNA samples were run on an Agilent Tapestation 4200 to determine level of degradation, thus ensuring only high quality RNA was used (RIN Score 8 or higher). A Qubit® 4.0 Fluorimeter (ThermoFisher) was used to determine the concentration prior to starting library prep. One microgram of total DNase-treated RNA was then prepared with the TruSeq Stranded mRNA Library Prep Kit (Illumina). Poly-A RNA was purified and fragmented before strand specific cDNA synthesis. cDNA was then A-tailed and indexed adapters were ligated. After adapter ligation, samples were PCR-amplified and purified with AmpureXP beads, then validated again on the Agilent Tapestation 4200. Before being normalized and pooled, samples were quantified by Qubit then run on an Illumina NextSeq 500 using V2.5 reagents. Three biological replicates were sequenced for each genotype, with 22-31 million reads generated per sample. The fastq files were checked for quality using fastqc (v0.11.2)(Andrews, 2010) and fastq_screen (v0.4.4)(Wingett, 2011). Fastq files were mapped to GCF_000151315.2 reference assembly

using STAR(Dobin et al., 2013). Read counts were then generated using featureCounts(Liao et al., 2014). Trimmed Mean of M-values normalization and differential expression analysis were performed using edgeR(Robinson et al., 2010) (false discovery rate (FDR) ≤ 0.05 , absolute $\log_2(\text{fold change}) \geq 0.5$, $\log(\text{counts per million}) \geq 0$). Phylogenetic trees were downloaded from PhyloDB(Huerta-Cepas et al., 2014) corresponding to *Capsaspora* (PhyID 101) and pairwise distances for each gene were extracted using an R package ape (Paradis and Schliep, 2019). Closest Human and mouse orthologues were then extracted and used to annotate the *Capsaspora* genes. These annotated gene names were then used with IPA (Kramer et al., 2014) (QIAGEN Inc., <https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis>) to get significantly enriched pathways in “Diseases and Bio Functions” category. To identify functional enrichment in the sets of genes upregulated or downregulated in *coYki* Δ cells, DAVID Functional Annotation Tool (Huang et al., 2009) was used to identify functional categories with FDR < 0.05 using all *Capsaspora* genome genes as the gene population background.

Statistics

All statistics were done using Prism (GraphPad software, San Diego, CA). Student’s t-test was done to compare differences between two groups, and one-way analysis of variance (ANOVA) with a post-hoc test was used to compare differences among groups greater than two.

Gene nomenclature

In the manuscript we use italicized text to refer to a gene (e.g., *coYki*), unitalicized text to refer to a protein (e.g., coYki), “ Δ ” to indicate a homozygous deletion mutant (e.g., *coYki* Δ), and “ Δ ” to indicate a promoter driving the expression of a gene (e.g., *EF1* Δ *coYki*).

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Author Contributions: JEP and DP conceived the study and designed experiments. JEP and MS performed experiments. JEP, MK, and CX analyzed RNAseq data. JEP, DP, and MK wrote the original manuscript draft. JEP, MS, DP, MK, and CX revised and edited the manuscript.

Competing interests: Authors declare that they have no competing interests.

Data and materials availability: RNAseq data generated in this study have been deposited into the NCBI SRA (Bioproject PRJNA759885). The following plasmids have been deposited at Addgene: pJP72 (#176479), pJP102 (#176480), pJP103 (#176481), and pJP118 (#176494).

REFERENCES

- Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data. . Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>.
- Bindels, D.S., Haarbosch, L., van Weeren, L., Postma, M., Wiese, K.E., Mastop, M., Aumonier, S., Gotthard, G., Royant, A., Hink, M.A., *et al.* (2017). mScarlet: a bright monomeric red fluorescent protein for cellular imaging. *Nat Methods* 14, 53-56.
- Blandin, A.F., Noulet, F., Renner, G., Mercier, M.C., Choulier, L., Vauchelles, R., Ronde, P., Carreiras, F., Etienne-Selloum, N., Vereb, G., *et al.* (2016). Glioma cell dispersion is driven by alpha5 integrin-mediated cell-matrix and cell-cell interactions. *Cancer Lett* 376, 328-338.
- Booth, D.S., and King, N. (2020). Genome editing enables reverse genetics of multicellular development in the choanoflagellate *Salpingoeca rosetta*. *Elife* 9.
- Booth, D.S., Szmidt-Middleton, H., and King, N. (2018). Transfection of choanoflagellates illuminates their cell biology and the ancestry of animal septins. *Mol Biol Cell* 29, 3026-3038.
- Charette, S.J., and Cosson, P. (2004). Preparation of genomic DNA from *Dictyostelium discoideum* for PCR analysis. *Biotechniques* 36, 574-575.
- Charras, G., and Paluch, E. (2008). Blebs lead the way: how to migrate without lamellipodia. *Nat Rev Mol Cell Biol* 9, 730-736.
- Charras, G.T., Hu, C.K., Coughlin, M., and Mitchison, T.J. (2006). Reassembly of contractile actin cortex in cell blebs. *J Cell Biol* 175, 477-490.
- Coste, A., Jager, M., Chambon, J.P., and Manuel, M. (2016). Comparative study of Hippo pathway genes in cellular conveyor belts of a ctenophore and a cnidarian. *Evodevo* 7, 4.
- Davis, J.R., and Tapon, N. (2019). Hippo signalling during development. *Development* 146.
- Deng, H., Yang, L., Wen, P., Lei, H., Blount, P., and Pan, D. (2020). Spectrin couples cell shape, cortical tension, and Hippo signaling in retinal epithelial morphogenesis. *J Cell Biol* 219.
- Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15-21.

673 Dumbauld, D.W., Shin, H., Gallant, N.D., Michael, K.E., Radhakrishna, H., and Garcia, A.J.
674 (2010). Contractility modulates cell adhesion strengthening through focal adhesion kinase and
675 assembly of vinculin-containing focal adhesions. *J Cell Physiol* 223, 746-756.

676 Fairclough, S.R., Dayel, M.J., and King, N. (2010). Multicellular development in a
677 choanoflagellate. *Curr Biol* 20, R875-876.

678 Fernandez, B.G., Gaspar, P., Bras-Pereira, C., Jezowska, B., Rebelo, S.R., and Janody, F.
679 (2011). Actin-Capping Protein and the Hippo pathway regulate F-actin and tissue growth in
680 *Drosophila*. *Development* 138, 2337-2346.

681 Fisher, H.W., and Yeh, J. (1967). Contact inhibition in colony formation. *Science* 155, 581-582.

682 Foty, R.A., and Steinberg, M.S. (2005). The differential adhesion hypothesis: a direct evaluation.
683 *Dev Biol* 278, 255-263.

684 Gaspar, P., and Tapon, N. (2014). Sensing the local environment: actin architecture and Hippo
685 signalling. *Current opinion in cell biology* 31, 74-83.

686 Harvey, K.F., and Hariharan, I.K. (2012). The hippo pathway. *Cold Spring Harb Perspect Biol* 4,
687 a011288.

688 Huang da, W., Sherman, B.T., and Lempicki, R.A. (2009). Bioinformatics enrichment tools:
689 paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res* 37, 1-
690 13.

691 Huerta-Cepas, J., Capella-Gutierrez, S., Pryszcz, L.P., Marcet-Houben, M., and Gabaldon, T.
692 (2014). PhylomeDB v4: zooming into the plurality of evolutionary histories of a genome. *Nucleic*
693 *Acids Res* 42, D897-902.

694 Ikmi, A., Gaertner, B., Seidel, C., Srivastava, M., Zeitlinger, J., and Gibson, M.C. (2014).
695 Molecular evolution of the Yap/Yorkie proto-oncogene and elucidation of its core transcriptional
696 program. *Mol Biol Evol* 31, 1375-1390.

697 King, N., Hittinger, C.T., and Carroll, S.B. (2003). Evolution of key cell signaling and adhesion
698 protein families predates animal origins. *Science* 301, 361-363.

699 Kramer, A., Green, J., Pollard, J., Jr., and Tugendreich, S. (2014). Causal analysis approaches
700 in Ingenuity Pathway Analysis. *Bioinformatics* 30, 523-530.

701 Lehman, S.S., Mladinich, K.M., Boonyakanog, A., Mima, T., Karkhoff-Schweizer, R.R., and
702 Schweizer, H.P. (2016). Versatile nourseothricin and streptomycin/spectinomycin resistance
703 gene cassettes and their use in chromosome integration vectors. *J Microbiol Methods* 129, 8-
704 13.

705 Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program
706 for assigning sequence reads to genomic features. *Bioinformatics* 30, 923-930.

707 Moya, I.M., and Halder, G. (2018). Hippo-YAP/TAZ signalling in organ regeneration and
708 regenerative medicine. *Nature reviews Molecular cell biology*.

709 Nichols, S.A., Roberts, B.W., Richter, D.J., Fairclough, S.R., and King, N. (2012). Origin of
710 metazoan cadherin diversity and the antiquity of the classical cadherin/beta-catenin complex.
711 *Proc Natl Acad Sci U S A* 109, 13046-13051.

712 Paczkowska, M., Barenboim, J., Sintupisut, N., Fox, N.S., Zhu, H., Abd-Rabbo, D., Mee, M.W.,
713 Boutros, P.C., Drivers, P., Functional Interpretation Working, G., *et al.* (2020). Integrative
714 pathway enrichment analysis of multivariate omics data. *Nat Commun* 11, 735.

715 Paradis, E., and Schliep, K. (2019). ape 5.0: an environment for modern phylogenetics and
716 evolutionary analyses in R. *Bioinformatics* 35, 526-528.

717 Park, S.H., Cheong, C., Idoyaga, J., Kim, J.Y., Choi, J.H., Do, Y., Lee, H., Jo, J.H., Oh, Y.S., Im,
718 W., *et al.* (2008). Generation and application of new rat monoclonal antibodies against synthetic
719 FLAG and OLLAS tags for improved immunodetection. *J Immunol Methods* 331, 27-38.

720 Parra-Acero, H., Harcet, M., Sanchez-Pons, N., Casacuberta, E., Brown, N.H., Dudin, O., and
721 Ruiz-Trillo, I. (2020). Integrin-Mediated Adhesion in the Unicellular Holozoan *Capsaspora*
722 *owczarzaki*. *Curr Biol* 30, 4270-4275 e4274.

723 Parra-Acero, H., Ros-Rocher, N., Perez-Posada, A., Kozyczowska, A., Sanchez-Pons, N.,
724 Nakata, A., Suga, H., Najle, S.R., and Ruiz-Trillo, I. (2018). Transfection of *Capsaspora*
725 *owczarzaki*, a close unicellular relative of animals. *Development* 145.

726 Pires-daSilva, A., and Sommer, R.J. (2003). The evolution of signalling pathways in animal
727 development. *Nat Rev Genet* 4, 39-49.

728 Porazinski, S., Wang, H., Asaoka, Y., Behrndt, M., Miyamoto, T., Morita, H., Hata, S., Sasaki,
729 T., Krens, S.F.G., Osada, Y., *et al.* (2015). YAP is essential for tissue tension to ensure
730 vertebrate 3D body shape. *Nature* 521, 217-221.

731 Riedl, J., Crevenna, A.H., Kessenbrock, K., Yu, J.H., Neukirchen, D., Bista, M., Bradke, F.,
732 Jenne, D., Holak, T.A., Werb, Z., *et al.* (2008). Lifeact: a versatile marker to visualize F-actin.
733 *Nat Methods* 5, 605-607.

734 Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for
735 differential expression analysis of digital gene expression data. *Bioinformatics* 26, 139-140.

736 Ros-Rocher, N., Perez-Posada, A., Leger, M.M., and Ruiz-Trillo, I. (2021). The origin of
737 animals: an ancestral reconstruction of the unicellular-to-multicellular transition. *Open Biol* 11,
738 200359.

739 Saias, L., Gomes, A., Cazales, M., Ducommun, B., and Lobjois, V. (2015). Cell-Cell Adhesion
740 and Cytoskeleton Tension Oppose Each Other in Regulating Tumor Cell Aggregation. *Cancer*
741 *Res* 75, 2426-2433.

742 Sebe-Pedros, A., Degnan, B.M., and Ruiz-Trillo, I. (2017). The origin of Metazoa: a unicellular
743 perspective. *Nat Rev Genet* 18, 498-512.

744 Sebe-Pedros, A., Irimia, M., Del Campo, J., Parra-Acero, H., Russ, C., Nusbaum, C., Blencowe,
745 B.J., and Ruiz-Trillo, I. (2013). Regulated aggregative multicellularity in a close unicellular
746 relative of metazoa. *Elife* 2, e01287.

747 Sebe-Pedros, A., Pena, M.I., Capella-Gutierrez, S., Anto, M., Gabaldon, T., Ruiz-Trillo, I., and
748 Sabido, E. (2016). High-Throughput Proteomics Reveals the Unicellular Roots of Animal
749 Phosphosignaling and Cell Differentiation. *Dev Cell* 39, 186-197.

750 Sebe-Pedros, A., Roger, A.J., Lang, F.B., King, N., and Ruiz-Trillo, I. (2010). Ancient origin of
751 the integrin-mediated adhesion and signaling machinery. *Proc Natl Acad Sci U S A* 107, 10142-
752 10147.

753 Sebe-Pedros, A., Zheng, Y., Ruiz-Trillo, I., and Pan, D. (2012). Premetazoan origin of the hippo
754 signaling pathway. *Cell reports* 1, 13-20.

755 Stanley, S.M. (1973). An ecological theory for the sudden origin of multicellular life in the late
756 precambrian. *Proceedings of the National Academy of Sciences of the United States of America*
757 70, 1486-1489.

758 Stein, C., Bardet, A.F., Roma, G., Bergling, S., Clay, I., Ruchti, A., Agarinis, C., Schmelzle, T.,
759 Bouwmeester, T., Schubeler, D., *et al.* (2015). YAP1 Exerts Its Transcriptional Control via
760 TEAD-Mediated Activation of Enhancers. *PLoS Genet* 11, e1005465.

761 Stibbs, H.H., Owczarzak, A., Bayne, C.J., and DeWan, P. (1979). Schistosome sporocyst-killing
762 Amoebae isolated from *Biomphalaria glabrata*. *J Invertebr Pathol* 33, 159-170.

763 Straight, A.F., Cheung, A., Limouze, J., Chen, I., Westwood, N.J., Sellers, J.R., and Mitchison,
764 T.J. (2003). Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor.
765 *Science* 299, 1743-1747.

766 Suga, H., Dacre, M., de Mendoza, A., Shalchian-Tabrizi, K., Manning, G., and Ruiz-Trillo, I.
767 (2012). Genomic survey of premetazoans shows deep conservation of cytoplasmic tyrosine
768 kinases and multiple radiations of receptor tyrosine kinases. *Sci Signal* 5, ra35.

769 Suga, H., and Ruiz-Trillo, I. (2013). Development of ichthyosporeans sheds light on the origin of
770 metazoan multicellularity. *Dev Biol* 377, 284-292.

771 Sun, S., and Irvine, K.D. (2016). Cellular Organization and Cytoskeletal Regulation of the Hippo
772 Signaling Network. *Trends in cell biology* 26, 694-704.

773 Veltman, D.M., Akar, G., Bosgraaf, L., and Van Haastert, P.J. (2009). A new set of small,
774 extrachromosomal expression vectors for *Dictyostelium discoideum*. *Plasmid* 61, 110-118.

775 Williams, L.M., and Gilmore, T.D. (2020). Looking Down on NF-kappaB. *Mol Cell Biol* 40.

776 Wingett, S. (2011). FastQ Screen: quality control tool to screen a library of sequences in FastQ
777 format against a set of sequence databases. . Available online at:
778 http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen.

779 Yu, F.X., Zhao, B., and Guan, K.L. (2015). Hippo Pathway in Organ Size Control, Tissue
780 Homeostasis, and Cancer. *Cell* 163, 811-828.

781 Zanconato, F., Forcato, M., Battilana, G., Azzolin, L., Quaranta, E., Bodega, B., Rosato, A.,
782 Bicciato, S., Cordenonsi, M., and Piccolo, S. (2015). Genome-wide association between
783 YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. *Nat Cell Biol* 17, 1218-1227.

784 Zheng, Y., and Pan, D. (2019). The Hippo Signaling Pathway in Development and Disease. *Dev*
785 *Cell* 50, 264-282.
786
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Figure 1. Disruption of a YAP/TAZ/Yorkie ortholog in *Capsaspora*. (A) Phylogenetic tree showing conservation of the Hippo pathway in close unicellular relatives of animals. (B) A solitary *Capsaspora* cell showing thin F-actin-enriched projections. Phalloidin and DAPI are used to stain

F-actin and DNA, respectively. **(C)** Cells at higher densities can contact other cells through projections. Scale bar is 5 microns. **(D-E)** *Capsaspora* cells were inoculated into either a tissue-culture treated plate **(D)** or a low-adherence plate **(E)**. At 3 days, cells in standard plates grow as a monolayer whereas cells in low-adherence plates form round aggregates. **(F)** An aggregate stained for F-actin shows actin-rich connections between cells within an aggregate. Scale bar is 10 microns. **(G)** Strategy for disrupting the *coYki* gene by homologous recombination. GenR, geneticin resistance cassette; TBD, Tead-binding domain; WW, WW domain. **(H)** Strategy for demonstrating disruption of *coYki* by qPCR. Amplicon 1 corresponds to a region of the *coYki* gene that should be absent in a homozygous mutant, whereas Amplicon 2 corresponds to a segment of the gene 3' of the region targeted for deletion. **(I-J)** qPCR of Amplicons 1 and 2 in WT and putative *coYki* ^{-/-} cells. The differences between WT and *coYki* ^{-/-} for both Amplicons 1 and 2 are significant (t-test).

See also Figure S1 and S2.

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812 **Figure 2. Loss of *coYki* results in no apparent effect on cell proliferation but alters the**

813 **morphology of multicellular aggregates. (A)** To examine proliferation within aggregates, EdU

814 was used to label proliferating cells within aggregates grown in low-adherence plates. **(B)** The

815 percent of cells positive for EdU within aggregates was quantified. Each red circle indicates

816 measurement results for a single aggregate, and mean $\pm$ SEM is shown in black. The difference

817 between WT and *coYki* $-/-$ cells is not significant (t-test). **(C-D)** WT or *coYki* $-/-$ cells were inoculated

818 into low-adherence plates, and cell aggregates were imaged at 5 days. Scale bar is 75 microns.

819 **(E-F)** Aggregate size and circularity was measured from aggregate images using ImageJ. The

820 difference in aggregate circularity between WT and *coYki* $-/-$ is significant (t-test). Bars indicate

821 the mean $\pm$ SEM (n=3 with 15 aggregates measured for each independent experiment), and dots

822 indicate values for individual aggregates. **(G)** Orthogonal views of WT and *coYki* $-/-$ aggregates

823 are shown. Cell aggregates stably expressing mScarlet were imaged live by confocal microscopy.

824 Red lines show the location of the culture surface. Scale bar is 50 microns.

825 **See also Figure S3.**

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Figure 3. Loss of *coYki* affects cell-substrate adhesion but not cell-cell adhesion. (A-D)

Cells expressing mScarlet in the WT background (A-B) or in the *coYki*^{-/-} mutant background (C-D) were allowed to aggregate as a homogeneous population (A, C) or were mixed with 90% WT cells and allowed to aggregate (B, D). Individual mScarlet-labeled *coYki*^{-/-} cells associate with WT cells within aggregates (D') in an organization like that of cells from the WT background labeled with mScarlet (B'). Scale bar is 75 microns. (E) To examine cell-cell adhesion, cells in suspension were gently rotated for an hour to stimulate cluster formation through cell-cell adhesion. Cultures were then examined by hemocytometer and the number of cells per cluster was counted. Each red circle indicates the number of cells in a single cluster, and error bars

839 indicate the mean $\pm$ SEM of the average number of cells per cluster from 3 independent
 840 experiments. Absence of error bars indicates that error is smaller than the plot symbol. The
 841 difference in average number of cells per cluster between WT and *coYki* $-/-$ is not significant (t-
 842 test). **(F)** To examine cell-substrate adhesion, adherent cell cultures were either agitated on a
 843 rotary shaker for 10 minutes or left untreated, and then the number of adhered and unadhered
 844 cells in each culture was counted and the percent of cells adhered to the culture substrate was
 845 calculated.
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Figure 4. Loss of coYki causes bleb-like protrusions at the cell cortex. (A) *Capsaspora* cells on a glass surface were imaged by time-lapse microscopy and individual cells were scored for number of protrusions per minute. A protrusion is defined as a localized extension of the cell boundary that disrupts a previously inert region of the cortex and/or changes the direction of movement of the cell. Differences between WT and coYki^{-/-} are significant (one-way ANOVA, Tukey's test). Values from 3 independent experiments with 20 cells measured for each condition per experiment are shown. Red dots indicate the measurement from an individual cell, and black

bars indicate the mean $\pm$ SEM of the means from each independent experiment. **(B)** Cells on a glass surface stably expressing Lifeact-mScarlet were imaged focusing on the base of the cell at the substrate (“basal”) or at the mid-height of the cell (“medial”). Arrowhead shows an example of Lifeact signal at the base of the cell that remains stationary as the cell moves (see Video S4). Scale bar is 5 microns. **(C)** Time series of a *coYki* $-/-$ cell stably expressing Lifeact-mScarlet on a glass surface. White arrows indicate Lifeact-depleted bleb-like protrusions, and yellow arrowheads indicate areas of cell cortex constriction, which correlate with increased Lifeact signal. Scale bar is 5 microns. **(D)** Cells on a glass surface were treated with DMSO or 1 μ M blebbistatin for 1 hour, and then cells were imaged by time lapse microscopy and the number of protrusions per cell was quantified. **(E)** Cells were inoculated into low-adherence plates with DMSO (**E’,E’’**) or 1 μ M blebbistatin (**E’’’,E’’’’**), and aggregates were imaged after 5 days. Scale bar is 75 microns. **(F)** Circularity of aggregates was measured with ImageJ using images of aggregates from the indicated conditions. Black bars indicate the mean $\pm$ SEM (n=4 with 15 aggregates measured for each independent experiment), and red dots indicate the measurements for individual aggregates. Differences in circularity between WT and *coYki* $-/-$ aggregates treated with DMSO are significant (one-way ANOVA, Dunnett’s test), whereas differences between WT aggregates treated with DMSO and *coYki* $-/-$ aggregates treated with blebbistatin are not. “Bleb” indicates blebbistatin.

Figure 5. Functional enrichment of genes differentially expressed in *coYki* ^{-/-} cells. Top enriched categories from the sets of genes significantly downregulated (**A**) or upregulated (**B**) in *coYki* ^{-/-} cells compared to WT are shown. (**C**) Ingenuity Pathway Analysis was performed on a set of predicted human/mouse orthologs of *Capsaspora* genes with significant changes in expression in *coYki* ^{-/-} cells. Enriched diseases and biological functions with false discovery rate (FDR) ≤ 0.05 are shown. (**D**) A schematic showing the flattened body phenotype of *YAP* ^{-/-} medaka fish embryos (top) and the flattened 3D shape of *coYki* ^{-/-} *Capsaspora* aggregates (bottom).

SUPPLEMENTAL INFORMATION

Figure S1. Calcium is required for adhesion in *Capsaspora* cell aggregates, related to Figure 1. *Capsaspora* aggregates were treated with water (vehicle control), the calcium chelator EGTA, or EGTA with an excess of calcium. At 30 minutes aggregates treated with EGTA had begun to disassociate, with further disassociation evident at 24 hours. Simultaneous addition of an excess of calcium blocked this disassociation.

Figure S2. Stable transgene expression and confirmation of gene knockout in *Capsaspora*, related to Figure 1.

(A) WT *Capsaspora* cells and a clonal population of cells stably expressing mScarlet were imaged to show fluorescence. Scale bar is 20 microns. **(B)** Transfection of *Capsaspora* with genes encoding antibiotic resistance markers results in viable cell populations after drug treatment. Cells were transfected with plasmids encoding mScarlet with no antibiotic marker (pJP71), GenR

(pJP72), NatR (pJP102), or HygR (pJP103) genes and were treated with the indicated antibiotics 2 days after transfection for the indicated time period. Representative images of the population are shown. Round, phase-bright cells are visible in populations transfected with resistance markers but not cells transfected with mScarlet. **(C-E)** Diagnostic PCR of putative *coYki* deletion clonal cell lines. **(C)** Diagram of primer pairs used to evaluate the presence of the WT *coYki* allele (*coYki* WT), a deletion of a region of *coYki* using the GenR marker ($\Delta coYki::GenR$), or a deletion of a region of *coYki* using the NatR marker ($\Delta coYki::NatR$). TBD, Tead-binding domain; WW, WW domain; GenR, Geneticin resistance; NatR, nourseothricin resistance. **(D)** WT cells were transfected with a gene-targeting construct encoding GenR designed to delete the TBD in the *coYki* gene by homologous recombination (see figure 1G in main text). Following transfection and drug selection, clonal populations of transfectants were generated and analyzed by diagnostic PCR using the indicated primer pairs. “*” indicates a clone showing a PCR product indicative of heterozygous *coYki* disruption. **(E)** A clonal cell line with a diagnostic PCR result indicating the disruption of a *coYki* allele with GenR was transfected with a gene-targeting construct encoding NatR designed to delete the TBD in the *coYki* gene. After selection with both Geneticin and nourseothricin, clonal populations of transfectants were generated and analyzed by diagnostic PCR using the indicated primer pairs. “*” indicates a clone showing a PCR product indicative of homozygous *coYki* disruption.

Figure S3. WT and *coYki* -/- cells proliferate at a similar rate, related to Figure 2.

(A) To examine proliferation on a solid substrate, cells were grown in wells in a 24-well plate, and each day cells within one well were resuspended and counted. **(B)** To examine proliferation in shaking culture, cells were grown in flasks on an orbital shaker, and an aliquot of cells was collected and counted daily. Values are mean $\pm$ SEM ($n \geq 3$). Absence of error bars indicates that error is smaller than the plot symbol.

937 **Video S1. Aggregation of WT or *coYki* $-/-$ cells over 6 days on a low-adherence surface.**

938 Time units are given in days.

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940 **Video S2. WT and *coYki* $-/-$ cells on a glass surface.**

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942 **Video S3. *coYki* $-/-$ cells expressing a *coYki* rescue transgene.**

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944 **Video S4. Basal region of *Capsaspora* cells expressing Lifeact-mScarlet.**

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946 **Video S5. Medial region of WT or *coYki* $-/-$ cells expressing Lifeact-mScarlet.**

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948 **Video S6. Actin dynamics in a single *coYki* $-/-$ cell expressing Lifeact-mScarlet.**

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950 **Video S7. WT or *coYki* $-/-$ cells expressing Lifeact-mScarlet within an aggregate.**

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952 **Video S8. Treatment of *coYki* $-/-$ cells expressing Lifeact-mScarlet with blebbistatin**
 953 **reduces blebbing.**

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956 **Data S1. Sequences of synthesized gene fragments used in this study**

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958 **Data S2. Sequences of oligonucleotides used in this study**

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960 **Data S3. Plasmids constructed for this study**

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