The mechanosensor Filamin A/Cheerio promotes tumourigenesis via specific interactions with components of the cell cortex

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Introduction

Development and maintenance of epithelial tissues rely on mechanical cues that, in concert with chemical signals, orchestrate cellular behaviours at the level of individual cells as well as cell populations. Epithelial cells exert mechanical forces primarily through the contractile activity of the cellular cortex composed of...
a thin but dense network of actin filaments (F-actin) associated with myosin motors and various actin-binding and membrane tethering proteins underneath the plasma membrane. Myosin-generated forces and the composition and organization of the cell cortex have been recognized as key regulators of cortical tension and contractility gradients underlying cell shape changes during tissue morphogenesis [1]. In contrast, altered contractility and tensional homeostasis have been linked to malignant transformation, driving tumour growth and invasiveness [2,3].

Actin-binding and crosslinking proteins of the Filamin family represent key regulators of cellular mechanics integral to cortex architecture, as well as mechanotransduction by converting mechanical force into the biochemical response. Filamins are large, evolutionarily conserved proteins composed of an N-terminal actin-binding domain (ABD) followed by immunoglobulin-like repeats (IgFLN), which can unfold under force [4,5]. While the ABD is crucial and sufficient for F-actin binding, the C-terminus facilitates Filamin homodimerization, which is pivotal for the actin-crosslinking [6]. In addition, Filamins also act as scaffolds interacting with various intracellular signaling molecules, ion channels, membrane receptors, enzymes, and transcription factors. Importantly, in vitro studies demonstrated that binding affinities for the partners can be modulated by force-induced conformational changes in the mechanosensory region (MSR) composed of the C-terminal IgFLN domains [7,8]. The force-regulated differential binding thus represents the basis for initiating the cascade of biochemical events, ultimately altering cellular behaviour [5,9].

It has become evident that tumour cells can take over the mechanosensory machinery. Filamins have been found overexpressed in multiple human cancer types [10]. We have shown previously that in the established *Drosophila* epithelial tumour model, in which malignancy arises from a cooperation of the oncogenic Ras (RasV12) and the loss of key polarity proteins of the Scribble (Scrib) complex [11,12], the levels of the *Drosophila* Filamin A (FLNA), known as Cheerio (Cher), are highly elevated [13]. When deprived of Cher, these rasV12scrib1 tumour clones lose their proliferative and invasive capacity, which coincides with reduced expression of genes downstream of the Hippo (Hpo) signaling pathway. We further demonstrated that in the tumour context, Cher physically and genetically interacts with Zipper (Zip), the *Drosophila* ortholog of the nonmuscle myosin heavy chain (Mhc) subunit and promotes myosin II (MyoII) activity [13]. While defined as a central scaffolding and mechanotransduction element of the cytoskeleton in cells and developing tissues [14], the roles and the requirement for the Filamin MSR domain and its interactors in the tumour context remain only partially understood.

Using the *Drosophila* epithelial tumour model, we provide primary genetic evidence that the conformation of the Cher MSR is integral to its tumour-promoting functions. We show that the loss of cytoskeletal contractility and tumour growth caused by cher deficiency can be rescued by stimulating MyoII activity. Within the Cher interactome, we identify several known proteins of the cell cortex, which interact with Cher in an MSR-dependent and independent manner. Finally, we demonstrate that the Cher-interacting proteins Big bang (Bbg), Karst (Kst), and 14-3-3e modulate MyoII activity and act as tumour suppressors.

**Results**

**The conformation of the Cher MSR is pivotal to tumour growth**

To determine the requirement of the MSR and its conformation for the tumour-promoting activity of Cher, we utilized the mosaic analysis with a repressible cell marker (MARCM) technique [15] to induce RasV12 overexpressing, polarity deficient (rasV12scrib1) clones in the eye/antennal imaginal discs (EADs) that carried either a wild type allele of *cher* (cherwt) or were homozygous for three different mechanosensing mutant alleles cheropen, cherclosed, and cherAMSR. These cher alleles were generated by reintroducing the GFP-tagged wild type or respective mutant gene segments into the endogenous *cher* locus [14] (Fig. 1A,B). Compared to Cherwt, Cherclosed, and Cheropen mutant proteins require more or less force, respectively, to change the MSR conformation. The CherAMSR variant lacks the MSR domain completely [14]. Examination of the rasV12scrib1 clonal cells bearing the different GFP-tagged cher alleles revealed enrichment of Cherwt and Cheropen proteins in the cell cortex, while CherAMSR and Cherclosed variants were also found in the cytoplasm (Fig. 1C–F). Importantly, rasV12scribcherwt clones carrying the GFP-tagged cherwt allele recapitulated the overgrowth and invasive phenotype of rasV12scribcherwt tumours containing the unmodified cher locus (Fig. 1G–J,N,O). Interestingly, the presence of cheropen or cherAMSR variants did not compromise tumour growth or invasiveness (Fig. 1K,L,N,O). For both mutant variants, the percentage of clonal cells reached the numbers observed for the cherwt allele or was even slightly higher compared to rasV12scribcherwt tumours bearing the endogenous cher locus (Fig. 1N). In contrast,
the cher\textsuperscript{closed} allele markedly reduced the clone size and the capacity of GFP\textsuperscript{+} cells to spread into the ventral nerve cord, exceeding the antitumour effect of cher\textsuperscript{1} deficiency (Fig. 1I–O). The force-sensitive MSR domain thus represents an important part of the Cher protein, which conformation affects its function and localization.

\textbf{Cherclosed variant restrains tumour cell proliferation while promoting cell death}

The strong negative impact of the cher\textsuperscript{closed} allele on growth and invasiveness of ras\textsuperscript{V12scrib}\textsuperscript{1} clones compared to the cher\textsuperscript{1} allele (Fig. 1H, I, M–O) was unexpected and intriguing. Although both alleles reduced clonal cell proliferation compared to ras\textsuperscript{V12scrib}\textsuperscript{1} and ras\textsuperscript{V12scrib}/cher\textsuperscript{wt} tumours and nonclonal cells [13] (Fig. 2A–D), cher\textsuperscript{closed} but not cher\textsuperscript{1} sensitized tumour cells to death as indicated by increased Dcp-1 signal, particularly at the clone boundaries (Fig. 2E–H) [13]. The cher\textsuperscript{1} mutants have been shown to lack the long 240 kDa Filamin isoform (Cher240) due to a 3.5 kb long deletion affecting the coding exons 2 and 3 (Fig. 1A), but they retain a short 90 kDa isoform (Cher90) [16]. While Cher240 plays a key role in organizing filamentous actin networks utilizing calponin-homology ABDs [14, 16], the Cher90 isoform lacks the ABD, hence actin crosslinking activity (Fig. 1B). Both isoforms, however, can act as scaffolds facilitating spatially localized protein-protein interactions [17]. Importantly, unlike cher\textsuperscript{1} mutants, the GFP-tagged cher alleles lack the Cher90 isoform as the alternative promoter controlling its expression has been deleted during the gene targeting (Fig. 1A) [14]. While the absence of the Cher90 protein did not compromise the growth and invasiveness of ras\textsuperscript{V12scrib}/cher\textsuperscript{wt}, ras\textsuperscript{V12scrib}/cher\textsuperscript{open}, and ras\textsuperscript{V12scrib}/cher\textsuperscript{AMSR} tumours, it is plausible that its deficiency could contribute to the detrimental effect of the Cher\textsuperscript{closed} protein variant, which requires more force to change the MSR conformation.

\textbf{Stimulating intracellular tension restores the growth of cher-deficient tumours}

Our previous work demonstrated that in contrast to the ras\textsuperscript{V12scrib}\textsuperscript{1} clones, which display increased intracellular tension, the impaired growth and invasiveness of ras\textsuperscript{V12scrib}/cher\textsuperscript{1} tumours coincided with reduced MyoII activity (Fig. 3A) [13]. Given the aggressive tumour phenotype, we hypothesized that neither the absence of the Cher MSR domain nor its open conformation would interfere with enhanced actomyosin contractility within the clonal tissue. Indeed, similar
to ras\textsuperscript{V12scrib}\textsuperscript{1} and ras\textsuperscript{V12scrib}/cher\textsuperscript{wt} EADs, ras\textsuperscript{V12scrib}/cher\textsuperscript{AMSR} and ras\textsuperscript{V12scrib}/cher\textsuperscript{open} cells showed accumulation of a phosphorylated myosin regulatory light chain Spaghetti squash (p-Sqh) protein, which marks sites of MyoII activity (Fig. 3B–E). This sharply contrasted with ras\textsuperscript{V12scrib}/cher\textsuperscript{closed} cells, which showed no p-Sqh enrichment relative to the nonclonal neighbours (Fig. 3B,F). These results indicate that similar to the loss of Cher240 (Fig. 3A) [13], the closed conformation of its MSR domain might hinder the activation of the actomyosin network and the reinforcement of mechanical tension within the invasive tumours.

As increased intracellular tension has been shown sufficient to promote cell proliferation and migration [3, 18], we tested whether stimulating actomyosin contractility could restore malignancy of ras\textsuperscript{V12scrib}/cher\textsuperscript{1} tumours. To this end, we overexpressed the activated form of Rho-associated kinase (Rok\textsuperscript{CAT}) that promotes MyoII activity by phosphorylating Sqh [19], or suppressed a regulatory subunit of the protein phosphatase 1 (PP1) Sds22 that inhibits MyoII activation [20]. Indeed, expression of either rok\textsuperscript{CAT} or sds22\textsuperscript{RNAi} restored the growth and invasiveness of ras\textsuperscript{V12scrib}/cher\textsuperscript{1} clones (Fig. 4A, C, G, I) and the enrichment of p-Sqh levels (Fig. 4J–M). Notably, overexpression of rok\textsuperscript{CAT} further augmented the growth of ras\textsuperscript{V12scrib}\textsuperscript{1} tumours bearing the endogenous cher locus (Fig. 4D–F, H). These results demonstrate that increasing intracellular tension through stimulating myosin activity is sufficient to promote malignancy and overcome the tumour-suppressive effect of cher240 deficiency. On the other hand, they further support the notion that Cher acts as an integral regulator of actomyosin contractility promoting tumourigenesis.

\textbf{Cher protein interactome comprises components of the cell cortex}

To address how Cher and its MSR might regulate intracellular tension and cell growth, we focused on its role as a molecular scaffold [7] by performing an unbiased search for Cher interactors. To this end, we immunoprecipitated proteins from lysates of mosaic ras\textsuperscript{V12scrib}\textsuperscript{1} EADs using a Cher240-specific antibody [13] and analysed them by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Samples prepared from cher\textsuperscript{1} homozygous mutant EADs served as controls to exclude proteins that bound unspecifically to IgG and beads. Out of 499 protein groups detected, 307 proteins were significantly enriched [false discovery rate (FDR) < 0.025] in EADs carrying ras\textsuperscript{V12scrib}\textsuperscript{1} tumours compared to cher\textsuperscript{1} samples (Fig. 5A and
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Dataset S1A,B). Consistent with its functional involvement in the cell cortex, the Cher interactome in rasV12-scrib+ tumours contained proteins associated with the organization and functions of the actin and microtubule cytoskeleton, plasma membrane, and adherens junctions. However, we also found an overrepresentation of ribosomal proteins and factors linked to translation, ribonucleoprotein (RNP) complex biogenesis, the regulation of protein metabolism, gene expression, and chromatin organization, as well as components of the chaperonin containing T-complex polypeptide-1 (CCT) known to assist in the folding of actin and tubulin (Fig. 5C,D andDatasets S2A,B and S3A,B).

Importantly, besides high enrichment of a known Cher binding partner Zip (Fig. 5A and Dataset S1A) [13], the Cher interactome comprised further components of the nonmuscle MyoII complex, including the essential and regulatory myosin light chains Mlc-c and Sqh, respectively (Fig. 5A and Dataset S1A), supporting the notion that Cher may directly regulate MyoII in the tumour context.

To address the contribution of the MSR domain to Cher tumour-specific interacting network, a comparison of Cherwt and Cherclosed binding partners would be the most interesting given the strong tumour-suppressive effect of the cherclosed allele (Fig. 1N,O). As the small number of GFP-positive cells in rasV12-scribcherclosed mosaic EADs limited direct analyses, we instead sought to determine which interactions are dependent on the Cher MSR by comparing proteins pulled down from rasV12-scribcherwt, rasV12-scribcherwt, and rasV12-scribcherMSR mosaic EADs. Strikingly, Bbg was the only significantly enriched protein in rasV12-scribcherwt and rasV12-scribcherwt samples compared to rasV12-scribcherMSR (Fig. 5B and Dataset S4A,B). On the other hand, ChemSR precipitated more of the cytoskeletal linker protein Short stop (Shot) and Myosin heavy chain (Mhc) (Fig. 5B and Dataset S4A,B). The proteomic results were further validated by independent IP experiments. Consistent with MS data, an intermediate Bbg isoform (Bbg-M), which predominates in EADs, was pulled down from rasV12-scribcherwt samples but much less efficiently from rasV12-scribcherMSR and control lysates (Fig. 5E).

In contrast, 14-3-3ε, like Zip, coprecipitated with all tested Cher variants (Cher, Cherwt, and ChemSR) from rasV12-scribcherwt tumour-bearing EADs (Fig. 5E). Taken together, our unbiased proteomic approach identified known, as well as novel Cher interacting partners potentially relevant for tumour development and progression. Moreover, we demonstrate that efficient Cher-Bbg binding requires the MSR while the interaction with Zip and 14-3-3ε is MSR-independent.
Cher interacting partners Bbg, Kst, and 14-3-3 act as tumour suppressors

To determine the contribution of Cher interactors to the malignancy of ras^{V12, scrib}\(^{1}\) clonal tumours, we selected three candidates, Bbg, Kst, and 14-3-3, for further functional analysis. We focused on these proteins as they have been shown to interact with or regulate MyoII activity, developmental tissue growth, and in the case of Bbg, also differentially bind to Cher\(^{wt}\).

**Fig. 2.** Closed Cher conformation inhibits proliferation and compromises the survival of tumour cells. (A–H) Green eyFLP MARCM technique was used to generate GFP-labelled clones (green) in the EADs of the indicated genotypes. (A–D) Compared to mosaic ras^{V12, scrib}\(^{1}\)cher\(^{wt}\) EADs where proliferation occurred in clonal and non-clonal cells (A, B), the proliferation of ras^{V12, scrib}\(^{1}\)cher\(^{closed}\) clonal cells was markedly reduced, while the number of surrounding pH3-positive wild type cells increased (C, D). (E–H) In ras^{V12, scrib}\(^{1}\)cher\(^{wt}\) mosaic EADs, apoptosis frequently occurred nonautonomously, although clonal cell death was also observed (E, F). In contrast, in EADs bearing ras^{V12, scrib}\(^{1}\)cher\(^{closed}\) clones, apoptosis occurred primarily in the clonal cells, particularly those located at clonal boundaries (white arrows), facing their phenotypically wild type neighbours (G, H). Micrographs show EADs 7 days AEL either as projections of multiple (A, C, E, G) or single confocal sections (B, D, F, H). EADs are outlined by white dotted lines based on DAPI staining, scale bars: 50 \(\mu\)m (A, C, E, G) and 20 \(\mu\)m (B, D, F, H).
and Cher\textsuperscript{AMSР} proteins. The 14-3-3 proteins are a family of small evolutionarily conserved factors that, through binding to a broad spectrum of proteins, control numerous cellular processes, and signaling pathways vital for normal growth and development \cite{21}. Besides binding to Cher (Fig. 5A,E) and Cofilin \cite{22}, the physical interaction between 14-3-3 and MyoII appears important for myosin turnover in the cell cortex, hence implicating 14-3-3 proteins in modulating cortical tension and cell mechanics \cite{23,24}. While 14-3-3 isoforms are often found dysregulated in human cancers, their role in tumour malignancy appears context-dependent \cite{21,25,26}. Bbg, on the other hand, is a large scaffolding protein with three PDZ domains. Bbg has been implicated in maintaining cell shape and junctional tension in the wing imaginal epithelium by binding to the \(\beta\)-heavy Spectrin Kst \cite{27,28}. However, unlike Kst, which restrains growth \cite{29}, Bbg stimulates it by promoting F-actin accumulation and MyoII activity \cite{28}. Based on these data, we hypothesized that silencing Kst could further enhance the growth of \(\text{ras}^{V_{12}}\text{scrib}\) clones and restore the malignancy of those deficient for cher. Conversely, the knockdown of Bbg would have the opposite effect. While blocking Kst in \(\text{ras}^{V_{12}}\text{scrib}\) cells indeed expedited clonal expansion (Fig. 6A,B,K), bbg silencing, to our surprise, mimicked the phenotype of \(kst\) deficiency. Rather than reducing tumour burden, \(\text{ras}^{V_{12}}\text{scrib}\) \(\text{bbg}_{\text{RNAi}}\) mosaic EADs contained more GFP\textsuperscript{+} cells than EADs bearing \(\text{ras}^{V_{12}}\text{scrib}\) clones (Fig. 6C-E,K). In contrast, knockdown of 14-3-3e did not significantly change the amount of \(\text{ras}^{V_{12}}\text{scrib}\) clonal cells (Fig. 6F,K). Of note, neither Bbg, Kst, nor 14-3-3e silencing hindered the accumulation of p-Sqh in \(\text{ras}^{V_{12}}\text{scrib}\) tumours (Fig. 6N-Q).

Intriguingly, of the three investigated interactors the knockdown of Kst or 14-3-3e partially restored the growth of \(\text{ras}^{V_{12}}\text{scrib}^{+}\text{cher}^{+}\) clones (Fig. 6G-I,L), which coincided with recovered MyoII activity compared to their nonclonal neighbours and \(\text{ras}^{V_{12}}\text{scrib}^{+}\text{cher}^{+}\) clones (Fig. 6R-T). However, only 14-3-3e silencing was able to reinduce invasiveness (Fig. 6M). In contrast, blocking bbg rescued neither growth, invasiveness nor p-Sqh enrichment in \(\text{ras}^{V_{12}}\text{scrib}^{+}\text{cher}^{+}\) tumours (Fig. 6J,L,M, U).

Taken together, these results demonstrate that in \(\text{ras}^{V_{12}}\)-driven clonal epithelial tumours with compromised polarity Bbg and Kst harbor tumour-suppressive activity containing clonal growth. The 14-3-3e and Kst, on the other hand, emerge as negative regulators of growth in tumours lacking Cher, as their inhibition restored myosin activity and promoted the expansion of \(\text{ras}^{V_{12}}\text{scrib}^{+}\text{cher}^{+}\) tumours. The fact that the loss of Kst was sufficient to rescue growth but not invasiveness of \(\text{ras}^{V_{12}}\text{scrib}^{+}\text{cher}^{+}\) tumours further suggests that malignant cell spreading requires increased actomyosin contractility but also a support of the spectrin cytoskeleton that links the cortical actin network to integral plasma membrane proteins.

**Overexpression of 14-3-3e suppresses tumour growth and invasiveness due to apoptosis**

Our functional analysis highlighted 14-3-3e as an interesting candidate for further investigation as its knockdown restored the MyoII activity and growth but also the invasiveness of \(\text{ras}^{V_{12}}\text{scrib}\) \(\text{cher}^{+}\) clones. Considering the capacity of Cher to bind 14-3-3e we hypothesized that \(\text{ras}^{V_{12}}\text{scrib}\) tumours might exploit Cher upregulation to mute the negative impact of 14-3-3e on MyoII activity and growth. In the absence of Cher, on the other hand, 14-3-3e would be free to act as a tumour suppressor accelerating MyoII turnover, thereby decreasing cortical tension. To test whether increasing 14-3-3e levels would be sufficient to override Cher and inhibit tumourigenesis, we overexpressed 14-3-3e in \(\text{ras}^{V_{12}}\text{scrib}\) tumours. Strikingly, excess 14-3-3e significantly reduced clonal size (Figs 6K and 7A,C), which was accompanied by increased apoptosis of clonal cells relative to the nonclonal and \(\text{ras}^{V_{12}}\text{scrib}\) cells (Fig. 7A-D). Importantly, the surplus 14-3-3e blocked the invasiveness of \(\text{ras}^{V_{12}}\text{scrib}\) tumour cells (Fig. 6M) and allowed all tumour-bearing larvae to eclose as adult flies, albeit their eyes were enlarged compared to control, containing surplus ommatidia and patches of undifferentiated tissue (Fig. 7E,F). Altogether, these data demonstrate that the gain of 14-3-3e restrains tumourigenesis, manifested by the reduction of growth and malignancy of \(\text{ras}^{V_{12}}\text{scrib}\) tumour clones.

**Discussion**

The mechanical properties of cells are dictated by the architecture and dynamics of the actomyosin cytoskeleton that generates and transmits force by engaging a set of mechanosensors. Aberrant cytoskeletal organization and intracellular tension have been linked to tumour formation and aggressiveness. Here, we show that the upregulation of the actin crosslinking protein Cher (FLNA) and the conformation of its MSR is essential for the growth and malignancy of the polarity-compromised Ras-driven tumours in *Drosophila* epithelial tissue. We identify the Cher interactome and demonstrate that Cher promotes tumourigenesis by stimulating actomyosin contractility and...
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interactions with components of the cell cortex. Based on our results, we propose that besides its role in actin crosslinking, Cher supports tumour cell mechanics (a) through interactions with and regulation of myosin motors and (b) through association with 14-3-3e, Kst, and Bbg, hindering their capacity to inhibit myosin activity (Fig. 8). We further show that reinstating actomyosin contractility in cher-depleted rasV12;scrib1 tumours is sufficient to rescue tumour growth, while invasive cell migration also requires coupling between the actomyosin and spectrin network.

**Filamin as an MSR-dependent mechanosensor**

Filamins are evolutionarily conserved actin organizers and mechanosensors involved in crosslinking actin filaments into orthogonal networks and stabilizing the plasma membrane through their capacity to unfold in response to mechanical stress [30,31]. Cher/FLNA levels have been found upregulated in Drosophila epithelial tumours [13] and in various human cancers, and were associated with poor prognosis and invasiveness [10,32]. Conversely, loss of Cher/FLNA has been shown to restrain rasV12;scrib1 tumours in Drosophila [13], K-Ras driven lung tumours in mice and to alleviate metastases of xenograft cancer cells in nude mice [33,34]. As cancer cells experience the buildup of shear stress when transiting through circulation, it is conceivable that the MSR of Cher/FLNA, which conveys mechanosensitivity, might contribute to tumour malignancy. Here, we show that the MSR and its conformation affected Cher localization in the rasV12;scrib1 tumour cells of the larval EADs. Unexpectedly, only Cher<sup>closed</sup> variant suppressed growth and invasiveness of rasV12;scrib1 tumours by reducing their proliferation and survival while Cher<sup>open</sup> and Cher<sup>AMSR</sup> did not impair tumour progression. These data argue that unlike in developing Drosophila ovariess where the MSR is required for the maturation of the actin-rich ring canals [14], its absence does not hinder tumourigenesis. The strong tumour-suppressive effect of Cher<sup>closed</sup>, however, supports the notion that the MSR, if present, might facilitate specific, conformation-dependent interactions [7,8,35,36, and this study].

Importantly, our study demonstrated that expression of Cher<sup>closed</sup> variant in rasV12;scrib1 clones exceeded tumour-suppressive effect of cher<sup>1</sup> allele, suggesting a possible context-dependent contribution of a short, actin-binding incapacitated Cher90 isoform to the behaviour of rasV12;scrib1 tumour. While dispensable for animal viability, a recent study implicated Cher90 in postsynaptic membrane growth by orchestrating the recruitment of the small GTPase Ral and glutamate receptor composition at fly larval neuromuscular junctions [17]. Intriguingly, a naturally occurring 100 kDa fragment of human FLNA (IgFLNa 16–24), generated through proteolytic cleavage, has been found in the nucleus participating in the androgen receptor-mediated transcriptional repression [37,38]. Whether and how the Cher90 isoform contributes to the malignancy of rasV12;scrib1 cells remains to be elucidated.

**Filamin tumour- and MSR-specific interactome**

Exploiting mass spectrometry, we provide an unbiased comprehensive snapshot of proteins interacting with Cher and its MSR in the tumour context. The Cher interactome was enriched for proteins instrumental for cytoarchitecture and cell motility but also for factors linked to translation, gene expression, and RNP complex assembly (Fig. 5C,D, Datasets S2A,B and S3A, B). While we did not identify the major regulators of myosin-mediated contractility such as Rho GTPases.
(Rho, Rac, Cdc42) or the ROCK kinase (Rok) that are known to bind FLNA [6,39], several myosin types (Zip, Jar (MyoVI), Myo10A (MyoXV), Didum (MyoV), Ck (MyoVIIA), Mhc), myosin light chains (Sqh, Mlc-c, Mlc1) and associated proteins such as Tropomyosin 1 (Tm1) precipitated with Cher (Dataset S1A). These results together with the genetic data strongly imply that Cher increases intracellular tension by scaffolding myosin subunits, which could potentiate the ATPase activity that drives actomyosin contractility (Fig. 8). Significantly, Cher also coprecipitated three proteins which have been shown to positively (Bbg) or negatively (14-3-3, Kst) regulate MyoII activity [23,24,28,29,40]. In polarized imaginal epidermis, Bbg has been shown to promote F-actin accumulation, MyoII activity, and growth [27,28]. In contrast,
**Fig. 4.** Stimulating myosin activity restores malignancy of cher-deficient tumours. (A–M) Green eyFP MARCM technique was used to generate GFP-labelled clones (green) in the EADs of the indicated genotypes. (A–F) Compared to large rasV12scribcher clones (D), rasV12scribcher clones (A) were markedly smaller, but their size was rescued by overexpression of an activated Rho-associated kinase rokCAT (B) or RNAi knockdown of the PP1 subunit sds22 (C). Overexpression of rokCAT (E) but not sds22 knockdown (F) exacerbated the growth of rasV12scribcher clones. Micrographs show EADs 7 days AEL as projections of multiple confocal sections. EADs are outlined by white dotted lines based on DAPI staining, scale bars: 50 µm. (G, H) Quantification of GFP+ cells by FACS sorting on day 7 AEL revealed that expression of rokCAT or sds22RNAi in rasV12scribcher clones significantly increased the percentage of GFP+ cells within the EADs (G). RokCAT overexpression further exacerbated the growth of rasV12scribcher GFP+ cells (H). Data are means with SD; n = 5 (G) n ≥ 8 (H). The one-way ANOVA with Tukey’s multiple comparison test was used to determine statistical significance, **P < 0.01, n.s., non-significant. (I) Overexpression of rokCAT and RNAi silencing of sds22 restored invasiveness of rasV12scribcher tumours. Four grades of invasiveness were scored based on spreading of the clonal GFP+ cells into larval brains dissected on day 8 AEL. Results show the percentage of brains falling into each category. Statistical significance was determined using a Chi-square test. ** **P < 0.0001, n.s., non-significant. (J–M) Similar to rasV12scribcher clones (J), p-Sqh signal accumulated in rasV12scribcher rokCAT (L) and rasV12scribcher sds22RNAi clones (M), while no enrichment was found in rasV12scribcher clones (K). Clonal cells are outlined by white dotted lines based on the GFP signal (J–M). Note a positive correlation between GFP and p-Sqh signal intensities in rasV12scribcher (J), rasV12scribcher rokCAT (L), and rasV12scribcher sds22RNAi (M) EADs. In contrast, in rasV12scribcher (K) clones, the p-Sqh intensity remained at levels comparable to wild type neighbours. Intensity profile plots for GFP (green) and p-Sqh (black) signals (J′–M′) were generated along the white dashed arrowed lines shown in J–M. The x-axis represents the distance along the line and the y-axis shows the pixel intensities. Micrographs are single confocal sections of EADs 7 days AEL. Scale bars: 20 µm.

The Bbg binding partner Kst inhibits growth [29] by altering the actomyosin network and reducing active Sqh levels [40]. Congruent with these findings, bbg mutant tissues are smaller and contain fewer cells [27,28], while Spectrin deficient cells mildly overproliferate [29,40,41]. We show that the Cher-Bbg binding partners tumours recapitulating the effect of Kst knockdown. The function of Bbg in growth regulation is apparent when the Cher/FLNA also a possible key determinant.

Finally, we define Kst and 14-3-3e as binding and genetic interaction partners of Cher and potent tumour suppressors. We demonstrate that blocking Kst or 14-3-3e restored growth and MyoII activity of rasV12scribcher tumours recapitulating the effect of Kst knockdown. The function of Bbg in growth regulation thus appears context dependent with cellular polarity being a possible key determinant.

Interestingly, silencing of YWHAE, the human orthologue of 14-3-3e, induced cell invasion and migration in all gastric cancer cell lines tested [44], and high expression levels of YWHAE are associated with favourable prognosis for patients with endometrial cancer (https://www.proteinatlas.org), indicating its tumour-suppressive role. Based on our results, we propose that Cher upregulation might serve to sequester 14-3-3e and/or restrain its activity, resulting in higher cortical tension and growth due to reduced 14-3-3-mediated MyoII turnover. At the same time, it is tempting to speculate that less 14-3-3 would be available to curb the activity of critical growth regulators such as Yki, allowing its nuclear translocation and expression of tumour-promoting genes as observed in rasV12scribcher clones [13,45,46]. In the absence of Cher or in tumour cells with 14-3-3 excess, 14-3-3 is free to expedite MyoII turnover and restrain Yki activity, resulting in reduced tumour growth and apoptosis. Together, these results highlight the function of Cher both as a scaffold protein that interacts with a multitude of cytoskeletal factors and as a mechanosensor that responds to changes in cellular tension. The cooperation of these functions makes Cher/FLNA also a likely target in cancer cells that are exploited to propel tumour growth via the dysregulation of the actomyosin network.

**Materials and methods**

**Sequencing of the cher mutants**

The cher gene locus was amplified from the genomic DNA with Phusion HS II polymerase (ThermoFisher Scientific,
Cheerprotein interactome comprises components of the cell cortex. (A) Cher interactome was analysed by quantitative LC-MS/MS analysis of Cher-IP samples obtained from lysates of mosaic rasV12scrib1 and cher1 homozygous EADs. The significantly enriched proteins (FDR < 0.025) are marked in orange and those discussed in the text are highlighted in green. The FDR was estimated by a permutation-based approach (s0 = 0.1, # of permutations: 500) to 2.5%. (B) The volcano plot depicts results of comparative LC-MS/MS analysis showing differences in proteins pulled down with a Cher-specific antibody from lysates of EADs bearing rasV12scrib1cherWT and rasV12scrib1cherDMSR clonal tumours. Significantly enriched proteins (P < 0.05) are labelled in orange and dark green. A two-sided t-test assuming equal variances was used to determine statistical significance. (C, D) The bar graphs depict GO clusters ‘Biological Process’ (C) and ‘Cellular Component’ (D) overrepresented with fold enrichment FE > 4 and adjusted P-value, Padj < 0.005, among the Cher-interacting proteins in rasV12scrib1 compared to cher1 EADs. The fold enrichment is noted within the bars. The GO clusters related to cytoskeleton organization and function are highlighted in dark green. The grey bars depict those linked to ribosome and translation. The complete list of GO clusters can be found in Datasets S2A,B and S3A,B. AARS: aminoacyl tRNA-synthetase, cell.: cellular, cont.: containing, cpx.: complex, cytopl.: cytoplasmic, elf3: eukaryotic translation initiation factor 3, est.: establishment, maint.: maintenance, metab.: metabolic, neg.: negative, NMB: non-membrane bound, org.: organization, proc.: process, reg.: regulation, RNP: ribonucleoprotein. (E) The immunoblots (left) show the levels of Cher, Zip, Bbg and 14-3-3 proteins in lysates from EADs bearing clones of the indicated genotypes. Note the two Bbg protein isoforms (Bbg-L and Bbg-M) detected in all EAD samples and enrichment of Cher protein in all rasV12scrib1 samples relative to control. Immunoprecipitation with the anti-Cher antibody (upper right) recovered more Cher and Zip from rasV12scrib1, rasV12scrib1cherWT and rasV12scrib1cherDMSR samples compared to control. On the other hand, Bbg-M protein was enriched in rasV12scrib1 and rasV12scrib1cherWT relative to control and rasV12scrib1cherDMSR samples. Despite the similar efficiency of 14-3-3c pull down with an anti-14-3-3c antibody (lower right), Cher protein was only detected in rasV12scrib1, rasV12scrib1cherWT and rasV12scrib1cherDMSR samples. Controls without primary antibodies (-antibody) confirmed the specificity of the interactions.
Schwerte, Germany, Cat# F549L). Purified PCR fragments were subjected to Sanger sequencing. PCR and sequencing primers are listed in Table 1.

**Fly strains and clonal analysis**

The following fly strains were used: (a) FRT82B (RRID: BDSC_2035), (b) UAS-ras12V (RRID: BDSC_64196), (c) FRT82B scrib' [11], (d) cher<sup>ect</sup>:GFP, (e) cher<sup>ppm</sup>:GFP, (f) cher<sup>closed</sup>:GFP, (g) cher<sup>MSR</sup>:GFP [14], (h) cher' [47], (i) UAS-rok<sup>CAT</sup> (RRID: BDSC_6668), (j) UAS-sdz2<sup>RNAi</sup> (VDRC; ID42051), (k) UAS-bbg<sup>RNAi</sup> (VDRC; ID15975), (l) UAS-bbg<sup>RNAi</sup> (VDRC; ID15974), (m) UAS-kdb<sup>RNAi</sup> (RRID: BDSC_50556), (n) UAS-I4-3-3<sup>ε</sup><sup>RNAi</sup> (VDRC; ID108129), and (o) UAS-I4-3-3<sup>ε</sup> (FlyORF, F001332). Mosaic clones were generated in the EADs using the MARCM technique [15] as described [48] by crossing ey-FLP1; act>γ-GAL4, UAS-CD8-mCherry; FRT82B, tub-GAL80 (Red eyFLP MARCM, this study) or ey-FLP1; act>γ-GAL4, UAS-GFP; FRT82B, tub-GAL80 (Green eyFLP MARCM) [12] females to males of the appropriate genotypes (Table 2). All crosses were carried out at 25°C on our standard food media [49].

**Tissue immunostaining**

Dissected EADs were processed as described previously [48]. The following primary antibodies were used at the indicated dilutions: rabbit anti-p-Myosin Light Chain 2 (Ser19) (p-Myosin, 1: 250; Cell Signaling Technology, Frankfurt am Main, Germany). Cat#: 3671, RRID: AB_264820), and rabbit anti-phospho-histone H3 (rabbit, 1: 500; Cell Signaling Technology, Darmstadt, Germany, Cat#: 3671, RRID: AB_59060). After washing, tissue samples were incubated with a corresponding secondary antibody coupled to Cy3 or Cy5 (1: 500; Jackson ImmunoResearch Laboratories, Ely, UK, Cat#: 111-165-152 and 712-175-153, RRID: AB_2307443, and RRID: AB_2340672) for 2 h. Samples were counterstained with DAPI (1 µg/mL, Roth, Cat#: 6335) and Phalloidin (1: 500; Sigma–Aldrich, Darmstadt, Germany, Cat#: 65906) to visualize nuclei and F-actin, respectively, and mounted in DABCO-Mowiol 4-88 medium (Sigma–Aldrich, Cat#: D2522 and Cat#: 81381).

**Quantification of EAD cells by flow cytometry**

Dissected EADs were prepared as previously described [50]. For each sample (n ≥ 4 per genotype), 30 000 events were counted on LSR Fortessa Flow Cytometer (BD Biosciences, Heidelberg, Germany). Data were analysed with FACSDIVA™ V8.0.1 software (BD Biosciences) using gates set to distinguish GFP<sup>+</sup>, GFP<sup>−</sup>, and Propidium Iodide (PI<sup>−</sup>) cells. Statistical analysis and plotting were carried out with GRAPHPAD Prism (GraphPad, RRID: SCR_002798) using ordinary one-way ANOVA with Tukey’s multiple comparisons test.

**Quantification of tumour invasiveness**

Tumour invasiveness was quantified 8 days after egg laying (AEL) as described previously [48]. For each genotype, at least 25 EAD/brain complexes were analysed. Statistical analysis and plotting were carried out with GRAPHPAD Prism (GraphPad, RRID: SCR_002798) using the Chi-square test.

**Image acquisition and processing**

Confocal images and stacks were acquired with an FV1000 confocal microscope (Olympus, RRID:SCR_020337) equipped with 20X UPlan S-Apo (NA 0.85), 40X UPlan FL (NA 1.30), and 60X UPlanApo (NA1.35) objectives. Maximum projections were generated using FLUOVIEW FV10-ASW Software (Olympus, Hamburg, Germany, RRID: SCR_014215) and Fiji (Fiji, RRID: SCR_002285). Final image processing including panel assembly, brightness, and contrast adjustments were performed in Photoshop CS5.1 (Adobe Systems, Inc., RRID: SCR_014199). White outlines of the EADs and clones shown in the figures were drawn based on DAPI staining and GFP signal, respectively.

Imaging of adult eyes was performed with an Olympus SXZ-16 microscope fitted with a DP72 camera. Images were captured with the cellSens standard v1.11 software (Olympus, RRID: SCR_014551). The model was created with BioRender.com (Biorender, RRID:SCR_018361).

**Measurement of p-Sqh and Cher::GFP signal intensities**

The images analysed were acquired with identical intensity and gain settings on an FV1000 confocal microscope (Olympus, RRID: SCR_020337) with a 60X UPlanApo (NA1.35) objective. Further processing and measurements were carried out in Fiji (Fiji, RRID: SCR_002285). For each image, the biggest possible selection rectangle was drawn in an EAD clone that did not include nonclonal tissue, and the average p-Sqh signal intensity within the rectangle was measured. Next, the same selection was moved to a nonclonal region and the average intensity was measured again. The ratio was calculated by dividing the clonal to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal angle was measured. Next, the same selection was moved to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal angle was measured. Next, the same selection was moved to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal angle was measured. Next, the same selection was moved to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal angle was measured. Next, the same selection was moved to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal angle was measured. Next, the same selection was moved to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal angle was measured. Next, the same selection was moved to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal angle was measured. Next, the same selection was moved to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal to a nonclonal region and the average intensity was measured. The ratio was calculated by dividing the clonal angle was measured.
Cheerio promotes cancer via actomyosin cortex

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containing clones of the indicated genotypes generated using the Green eyFLP MARCM technique. Like 

EADs were lysed and processed as described previously [13]. For LC-MS/MS analysis, 1000 µg of total protein lysates were incubated overnight with 50 µL of equilibrated Dynabeads Protein G (Invitrogen, Cat# 10003D) and rat anti-Cher antibody (1:200) [13]. Samples for LC-MS/MS were washed five times with lysis buffer (50 mM Tris (pH 7.8), 0.1 mM EDTA (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.01% Igepal CA-630, protease inhibitor cocktail (Roche, Cat# 11873580001), three times with 50 mM TRIS (pH 7.5) and finally solubilized in 50 µL 6 M urea/2 M thiourea (Sigma–Aldrich, Cat# U5378 and T7875) in 10 mM Hepes buffer (pH 8.0). For profiling of Cher interactome, the experiments were performed with three independent biological replicates of rasV12scrib1 EADs and homozygous

**Fig. 6.** Cher interacting partners Bbg, Kst and 14-3-3ε act as tumour suppressors. (A–J) Representative examples of mosaic EADs containing clones of the indicated genotypes generated using the Green eyFLP MARCM technique. Like rasV12scrib1 clones (A) those expressing kstRNAi (B), single independent (C, D) or combined bbg RNAi lines (E) or 14-3-3ε RNAi (F) overgrew most of the EAD tissue. Knockdown of kst (H) or 14-3-3ε (I) but not bbg (J) restored the growth of rasV12scrib1cher1 clones (G). Micrographs show EADs 7 days AEL as projections of multiple confocal sections. EADs are outlined by white dotted lines based on DAPI staining, scale bars: 50 µm. (K, L) Quantification of GFP+ cells by FACS sorting on day 7 AEL revealed that silencing of kst and bbg in rasV12scrib1 clones further increased the amount of GFP+ cells in the mosaic EADs while 14-3-3ε overexpression had the opposite effect (K). 14-3-3ε RNAi as well as kstRNAi significantly increased the percentage of rasV12scrib1cher1 clonal cells (L). Data are means with SD; n ≥ 4 (K) and n ≥ 12 (L). One-way ANOVA with Tukey’s multiple comparison was used to determine statistical significance, *P < 0.05, **P < 0.01, ****P < 0.0001, and n.s., non-significant. (M) Knockdown of 14-3-3ε but not kst or bbg restored the invasive capacity of cher-deficient rasV12scrib1 tumours while 14-3-3ε overexpression completely blocked invasiveness of rasV12scrib1 tumours. Four grades of invasiveness were scored based on the spreading of clonal GFP+ cells into larval brains dissected on day 8 AEL. Results are the percentage of brains falling into each category. Statistical significance was determined using a Chi-square test, ****P < 0.0001, and n.s., non-significant. (N–U) Similar to rasV12scrib1 clones (N), rasV12scrib1kstRNAi (O), rasV12scribbbgRNAi (P), and rasV12scrib114-3-3εRNAi (Q) clones showed enrichment of p-Sqh signal compared to the surrounding wild type tissue. Silencing of kst (S), 14-3-3ε (T) but not bbg (U) in rasV12scrib1cher1 cells resulted in cortical enrichment of p-Sqh relative to the nonclonal cells and rasV12scrib1cher1 clones (R). Intensity profile plots for GFP (green) and p-Sqh (black) signals (N–U) were generated along the white dashed arrowed lines shown in N–U. The x-axis represents the distance along the line and the y-axis shows the pixel intensities. Micrographs show single confocal sections of EADs 7 days AEL. Clones are outlined by white dotted lines based on GFP signal, scale bars: 20 µm.

**Fig. 7.** 14-3-3ε overexpression induces apoptosis of tumour cells. (A–D) Green eyFLP MARCM technique was used to generate GFP-labelled clones (green) in the EADs of the indicated genotypes. Compared to rasV12scrib1 mosaic EADs (A), the clones overexpressing 14-3-3ε were markedly smaller (C) while the amount of apoptotic Dcp-1-positive cells increased (A’, C’). In rasV12scrib1 tumour-bearing EADs, apoptosis frequently occurred nonautonomously, although clonal cell death was also observed (B’). In contrast, 14-3-3ε overexpression caused a dramatic increase of the Dcp-1 signal in clonal cells (D’). Micrographs show EADs 7 days AEL either as projections of multiple (A, C) or single confocal sections (B, D). White dotted lines outline EADs based on DAPI staining (A, C). Scale bars: 50 µm (A, C), 20 µm (B, D). (E, F) While animals bearing rasV12scrib1 EAD tumours die as giant larvae, 100% of those overexpressing 14-3-3ε in rasV12scrib1 tumours eclosed as adult flies. In contrast to control (E), mosaic rasV12scrib114-3-3ε adult eyes are larger, containing surplus ommatidia and patches of undifferentiated tissue (F).

**LC-MS/MS sample preparation**

EADs were lysed and processed as described previously [13]. For LC-MS/MS analysis, 1000 µg of total protein lysates were incubated overnight with 30 µL of equilibrated Dynabeads Protein G (Invitrogen, Cat# 10003D) and rat anti-Cher antibody (1:200) [13]. Samples for LC-MS/MS were washed five times with lysis buffer (50 mM Tris (pH 7.8), 0.1 mM EDTA (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.01% Igepal CA-630, protease inhibitor cocktail (Roche, Cat# 11873580001), three times with 50 mM TRIS (pH 7.5) and finally solubilized in 50 µL of 6 M urea/2 M thiourea (Sigma–Aldrich, Cat# U5378 and T7875) in 10 mM Hepes buffer (pH 8.0). For profiling of Cher interactome, the experiments were performed with three independent biological replicates of rasV12scrib1 EADs and homozygous
cher<sup>1</sup> EAD/brain complexes, or ras<sup>V12scrib<sup>1</sup>, ras<sup>V12scrib<sup>1</sup>cher<sup>wt</sup>, and ras<sup>V12scrib<sup>1</sup>cher<sup>MSR</sup></sup> mosaic EADs.

**In-solution digestion and LC–MS/MS**

Immunoprecipitated proteins were reduced and alkylated by 10 mM DTT (Dithiothreitol, Applichem, Darmstadt, Germany, Cat# 3483-12-3) for 30 min and 55 mM IAA (Iodacetamide; Merck, Darmstadt, Germany, Cat# 8.04744.0025) in the dark. One microliter of a 0.5 µg/µL Lys-C (FUJIFILM, Wako, Neuss, Germany, Cat# 129-02541) solution was added to the sample and incubated at room temperature for 2 h. Then, the urea concentration was diluted using 50 mM ammonium bicarbonate (Sigma–Aldrich, Cat# 09830) to 2 M, and 1 µL trypsin (0.5 µg/µL<sup>−1</sup>, Serva, Heidelberg, Germany, Cat# 9002-07-7) was added. After overnight digestion, samples were acidified by adding trifluoroacetic acid (TFA, Thermo Scientific, Cat# 85183) to a final concentration of 0.2%. Generated peptides were desalted with the StageTip technique [51].

For LC–MS/MS analysis, an EASY nLC 1000 (Thermo Scientific, Cat# LC120) was coupled to the quadrupole-based Q Exactive Plus (Thermo Scientific, RRID: SCR_020556) instrument by a nano-spray ionization source. Peptides were separated on a 50 cm in-house packed column using a two solvent buffer system: buffer (A) 0.1% formic acid and (B) 0.1% formic acid in acetonitrile, as described previously [52]. The content of buffer B was increased from 7% to 23% within 40 min and followed by an increase to 45% in 5 min and a washing and re-equilibration step prior next sample injection. The mass spectrometer operated in a Top10 data-dependent mode using the following settings: MS1 – 70 000 (at 200 m/z) resolution, 3e6 AGC target, 20 ms maximum injection time, 300–1750 scan range and MS2 – 35 000 (at 200 m/z) resolution, 5e5 AGC target, 120 ms maximum injection time, 1.8 Th isolation window, and 25 normalized collision energy.

**MaxQuant analysis and bioinformatics**

All raw files were processed by MaxQuant 1.5.3.8 [53] (RRID:SCR_014485) and the implemented Andromeda search engine [54] for correlation of acquired MS/MS spectra against the *Drosophila* reference 2017 Uniprot proteome. Default mass tolerance and modification settings were used. The minimal peptide length was set to seven amino acids and N-terminal acetylation, as well as oxidation at methionine residues were defined as variable modifications. Cysteine-carbamidomethylation was set as a fixed modification. The iBAQ quantification approach and match-between-runs were...
Table 1. Summary of oligonucleotides.

Primers used (5′-3′ sequence)

| Amplification of cher locus fragment containing the deletion | Sequencing primers |
|-------------------------------------------------------------|-------------------|
| cher deletion for | cher deletion rev |
| cher1 deletion for | cher1 deletion rev |
| cher1 seqI for cher1 | cher seqI for |
| cher seqII for cher1 | cher seqII for |

Table 2. Summary of fly crosses.

Mosaic analysis

♀ eyFLP; act-γ’ > GAL4, UAS-GFP/CyO; FRT82B, tub-GAL80/TM6B
d w; FRT82B
d w; UAS-rasV12; FRT82B, scrib1/TM6B
d w; UAS-rasV12; FRT82B, scrib1, cher1/TM6B
d w; UAS-rasV12; FRT82B, scrib1, cherWT::GFP/TM6B
d w; UAS-rasV12; FRT82B, scrib1, UAS-14-3-3/TM6B
d w; UAS-rasV12; FRT82B, scrib1, cheropen::GFP/TM6B
d w; UAS-rasV12; FRT82B, scrib1, cherclosed::GFP/TM6B
d w; UAS-rasV12; FRT82B, scrib1, FRT82B, scrib1, cherWT::GFP/TM6B
d w; UAS-rasV12; FRT82B, scrib1, FRT82B, scrib1, UAS-14-3-3/TM6B
d w; UAS-rasV12; FRT82B, scrib1, cheropen::GFP/TM6B
d w; UAS-rasV12; FRT82B, scrib1, UAS-14-3-3/TM6B
d w; UAS-rasV12; FRT82B, scrib1, FRT82B, scrib1, cheropen::GFP/TM6B
d w; UAS-rasV12; FRT82B, scrib1, FRT82B, scrib1, cherclosed::GFP/TM6B
d w; UAS-rasV12; FRT82B, scrib1, FRT82B, scrib1, UAS-14-3-3/TM6B

Immunoprecipitation from EADs

A 500 µg of total protein lysates were incubated overnight with 15 µL of equilibrated Dynabeads Protein G (Invitrogen, Cat# 10003D) and rat anti-Cher antibody (1 : 200) [13] or guinea pig anti-14-3-3ε antibody (1 : 250) [57] at 4 °C. The IP samples were washed three times in lysis buffer containing 150 mM NaCl. Proteins were recovered by heating in 1× Laemmli Buffer [2% SDS, 10% glycerol, 60 mM Tris–HCl (pH 6.8), 0.01% bromophenol blue, and 2.5% β-mercaptoethanol] or in two consecutive elution steps each with 50 µL of 0.1 M glycine–HCl (pH 3.0) for 5 min and neutralization with 20 µL of 0.5 M Tris–HCl (pH 7.8) and 0.5 mM NaCl. Proteins were subjected to SDS-PAGE and detected by immunoblotting with rat anti-Cher antibody (1 : 2000) [13], rabbit anti-Bbg antibody (1 : 10 000) [28], guinea pig anti-14-3-3ε antibody (1 : 2000) [57], and chicken anti-Zip antibody (1 : 5000, a gift from Eric Wieschaus) followed by incubation with corresponding HRP-conjugated secondary antibodies (1 : 5000; Jackson ImmunoResearch Labs, Cat# 712-035-098) and chemiluminescent signal was captured using ImageQuant LAS4000 reader (GE Healthcare, Solingen, Germany).

Gene ontology analysis

The GO analysis of the Cher-interacting proteins enriched in rasV12;scrib1 versus cher1 EADs with FDR ≤ 0.025, was performed using the Protein Analysis Through Evolutionary Relationships classification system (PANTHER, RRID: SCR_004869). Only GO terms with a fold enrichment > 4 and an adjusted P-value Padj < 0.005 were taken into account. For visualization, GO terms were clustered with the help of REVIGO [58] (RRID: SCR_005825) using medium (0.7) or small (0.5) settings to remove redundancies. A complete list of all enriched GO clusters ‘Biological Function’ and ‘Cellular Component’ can be found in Datasets S2A,B and S3A,B.

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Conflict of interest
The authors declare no conflict of interest.

Author contributions
EK, MK, and GC designed and performed most experiments and analysed the data. HN helped with the experimental design, analyses, and presentation of the proteomic data, and methods. TB helped with the establishment of genetic stocks and FACS experiments. EK, MK, and GC wrote the draft of the manuscript. MU conceptualized the study, supervised the project, performed experiments, analysed the data, wrote the manuscript, and secured financial support.

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Data Availability Statement
The data that supports the findings of this study are available in the supplementary material (Datasets S1–S4) of this article.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Dataset S1.** (A) Comparative LC–MS/MS analysis of Cher-IP samples obtained from ras<sup>V12</sup>scrib<sup>-</sup> mosaic and cher<sup>-</sup> EADs. (B) Peptide counts for Dataset S1A.

**Dataset S2.** (A) Gene Ontology enrichment analysis of ‘Biological Process’ of immunoprecipitated proteins enriched in ras<sup>V12,scrib</sup> mosaic EADs versus cher<sup>-</sup> EADs. (B) Clustering of enriched ‘Biological Process’ GO terms for Dataset S2A.
**Dataset S3.** (A) Gene Ontology enrichment analysis ‘Cellular component’ of immunoprecipitated proteins enriched in ras\(^{V12}\)scrib\(^{1}\) mosaic EADs versus cher\(^{1}\) EADs. (B) Clustering of enriched ‘Cellular component’ GO terms for Dataset S3A.

**Dataset S4.** (A) Comparative LC–MS/MS analysis of Cher-IP samples obtained from ras\(^{V12}\)scrib\(^{1}\)cher\(^{wt}\), ras\(^{V12}\)scrib\(^{1}\)cher\(^{AMSR}\), and ras\(^{V12}\)scrib\(^{1}\) mosaic EADs. (B) Peptide counts for Dataset S4A.