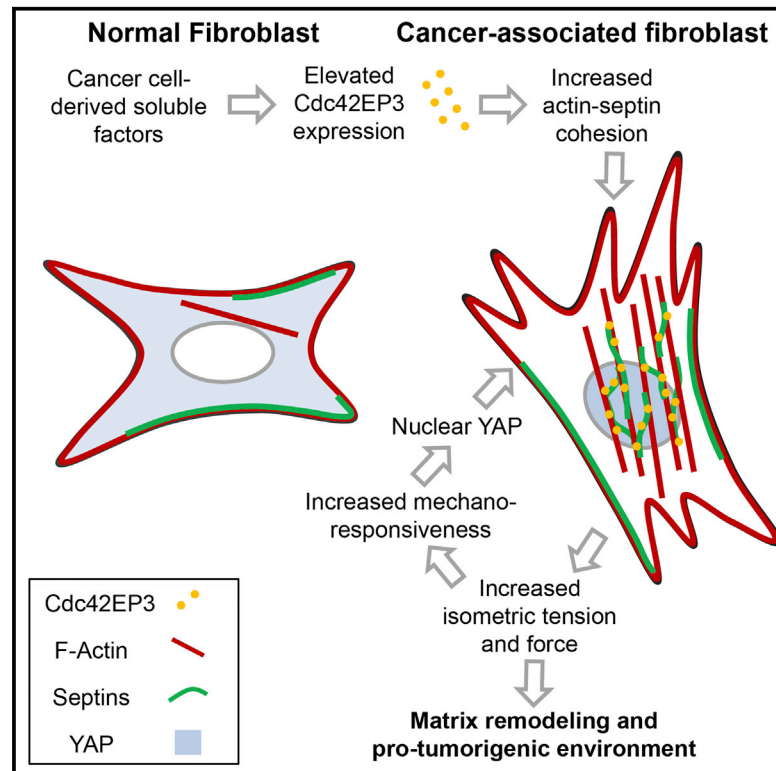


Cdc42EP3/BORG2 and Septin Network Enables Mechano-transduction and the Emergence of Cancer-Associated Fibroblasts

Graphical Abstract



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In Brief

Calvo et al. identify Cdc42EP3 as a regulator of the pro-tumorigenic functions of cancer-associated fibroblasts. Cdc42EP3 is upregulated during fibroblast activation and coordinates actin and septin rearrangements that are required for mechanotransduction and CAF functions.

Highlights

- Cdc42EP3-mediated coordination of actin and septin is required for CAF function
- The septin network is changed in CAFs and is required for their function
- Cdc42EP3 enables responses to changes in matrix stiffness
- Upregulation of Cdc42EP3 is required for the activation of normal fibroblasts



Cdc42EP3/BORG2 and Septin Network Enables Mechano-transduction and the Emergence of Cancer-Associated Fibroblasts

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SUMMARY

Cancer-associated fibroblasts (CAFs) are non-cancerous cells found in solid tumors that remodel the tumor matrix and promote cancer invasion and angiogenesis. Here, we demonstrate that Cdc42EP3/BORG2 is required for the matrix remodeling, invasion, angiogenesis, and tumor-growth-promoting abilities of CAFs. Cdc42EP3 functions by coordinating the actin and septin networks. Furthermore, depletion of SEPT2 has similar effects to those of loss of Cdc42EP3, indicating a role for the septin network in the tumor stroma. Cdc42EP3 is upregulated early in fibroblast activation and precedes the emergence of the highly contractile phenotype characteristic of CAFs. Depletion of Cdc42EP3 in normal fibroblasts prevents their activation by cancer cells. We propose that Cdc42EP3 sensitizes fibroblasts to further cues—in particular, those activating actomyosin contractility—and thereby enables the generation of the pathological activated fibroblast state.

INTRODUCTION

Non-cancerous cells and extracellular matrix molecules within the tumor, collectively defined as the tumor microenvironment (Bhowmick and Moses, 2005; Calvo and Sahai, 2011; Joyce and Pollard, 2009), participate in many hallmarks of cancer (Hannahan and Coussens, 2012). Cancer-associated fibroblasts (CAFs) can provide chemical and physical cues that favor tumor aggressiveness and dissemination (Kalluri and Zeisberg, 2006; Karnoub et al., 2007). CAF-dependent matrix remodeling can lead to the generation of tracks through the extracellular matrix (ECM) that enable subsequent cancer cell invasion (Gaggioli et al., 2007). As a result of increased deposition and remodeling of the ECM by CAFs, most solid tumors are charac-

terized by elevated cellular and tissue tension, and enhanced stiffness (DuFort et al., 2011; Levental et al., 2009; Paszek et al., 2005). These altered physical properties can promote malignancy by enhancing cancer cell growth, survival, and migration (DuFort et al., 2011). CAFs also produce soluble factors that can promote tumorigenesis (Öhlund et al., 2014). Thus, targeting CAFs represents a possibility for therapeutic intervention.

Changes in the actin cytoskeleton, including increased actin stress fibers, α SMA expression, and stronger focal adhesions, is a feature of CAFs (Calvo et al., 2013; Kalluri and Zeisberg, 2006; Öhlund et al., 2014; Sandbo et al., 2011; Xing et al., 2010). Increased actomyosin contractility also leads to activation of the transcriptional regulator YAP1 that is critical for the CAF phenotype (Calvo et al., 2013). Much less is known about alterations to other cytoskeletal networks. Septins are a large conserved family of GTP-binding proteins that participate in cell division, cytoskeletal organization, vesicular transport, cell polarity, and membrane remodeling (Mostowy and Cossart, 2012; Weirich et al., 2008). SEPT2, SEPT6, and SEPT7 can assemble into hetero-oligomeric complexes and higher-order structures, including filaments and rings (Sirajuddin et al., 2007). The adaptor protein Anillin (ANLN) controls actin-septin coordination during cytokinesis, whereas unknown factors mediate this process during interphase (Kinoshita et al., 2002). BORG family proteins, such as Cdc42EP3 and Cdc42EP5, are potential candidates to fulfill this role. These proteins bind active Cdc42 (Bahou et al., 1992; Joberty et al., 1999) and SEPT2, 6, and 7 (Joberty et al., 2001). Overexpression of Cdc42EP3 in fibroblasts can induce the formation of pseudopodia and F-actin-containing structures (Hirsch et al., 2001), while Cdc42EP5 can regulate septin organization within the cell (Joberty et al., 2001; Sheffield et al., 2003). However, the role of BORG proteins in the tumor microenvironment is not well understood. Here, we examine the role of Cdc42EP3/BORG2 and septins in regulating CAF emergence, maintenance, and functions linked to essential rearrangements in the actin and septin network.

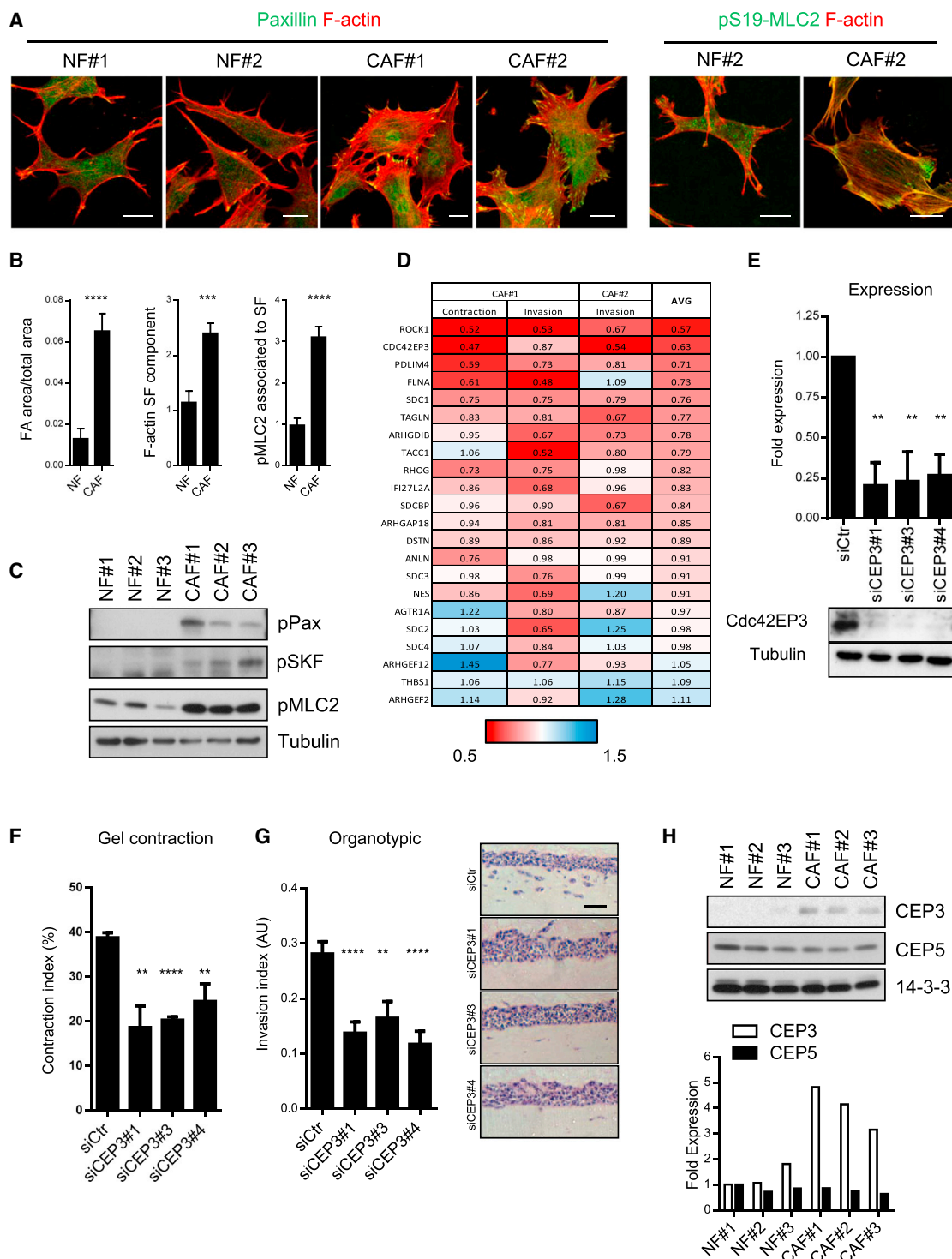


Figure 1. Identification of Cdc42EP3 as a Key Regulator of CAF Functions

(A) The left panels show images of F-actin (red) and paxillin (green) staining in NF#1, NF#2, CAF#1, and CAF#2. The right panels show images of F-actin (red) and pS19-MLC2 (green) staining in NF#2 and CAF#2. Scale bars represent 20 μ m.

(B) Histograms show focal adhesion (FA) area normalized to total cell area (left graph), an F-actin stress fibers (SF) component (middle graph), and pMLC2 associated to SF (right graph) of NF and CAF cells. Bars represent mean \pm SEM. FA measurements, n = 16; measurements were performed on NF#1 and NF#2 (NF) and CAF#1 and CAF#2 (CAF). F-actin and pMLC2 measurements, n = 12 for NF and n = 15 for CAF; measurements were performed on NF#1 and CAF#1.

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RESULTS

Identification of Cdc42EP3 as a Regulator of CAF Functions

To learn about the role of the cytoskeleton in CAFs, we compared its organization in normal fibroblasts (NFs) and CAFs from the MMTV-PyMT mammary tumor model (Calvo et al., 2013). In NFs, F-actin was mainly at the cell cortex and only small focal adhesions were present (Figure 1A; quantification in Figure 1B). In contrast, CAFs had enhanced F-actin stress fibers containing active pS19 myosin light chain 2 (MLC2; phosphorylation on S19 is the major positive regulatory mechanism of myosin activity in non-muscle cells) and abundant paxillin-positive focal adhesions (Figure 1A; quantification in Figure 1B). We confirmed the elevated levels of pS19-MLC2 in CAFs by western blotting (Figure 1C) and also observed elevated levels of active Src-family kinases and increased phosphorylation of paxillin on the FAK and Src-family kinase site, Y118 (Figure 1C) (Deakin and Turner, 2008).

We selected 21 cytoskeletal components that are highly expressed in CAFs compared to NFs based on microarray data (Calvo et al., 2013) and depleted them in CAFs. Following small interfering RNA (siRNA) transfection, the ability of CAF#1 to contract collagen gels and promote the invasion of breast cancer cells in “organotypic” assays was determined. Invasion assays were also performed using CAF#2. Figure 1D shows the effect of gene depletion in each assay, together with a combined metric of “CAF functionality” derived by averaging the individual assay results. Depletion of *Cdc42ep3* reduced CAF function to a similar extent as targeting *Rock1* (Figure 1D), a known regulator of CAF function that was used as a positive control (Gaggioli et al., 2007). Depletion of Cdc42EP3 with three independent siRNA led to reduced ECM remodeling by CAFs (Figures 1E and 1F, confirmed in CAF#2; Figure S1A). There was also a significant reduction in the cancer cell invasion promoting abilities of CAF#1 and CAF#2 after Cdc42EP3 depletion (Figures 1G and S1A). Similar results were obtained in human-patient-derived CAFs (Figures S1B–S1D). Depletion of Cdc42EP3 did not affect the expression of the CAF markers α SMA and FAP (Figure S1E).

Cdc42ep3 encodes a protein of the BORG family of Cdc42 effectors (Joberty et al., 1999). We confirmed that Cdc42EP3 levels were upregulated in three different CAFs when compared to NFs (Figure 1H), whereas levels of other BORG members were unchanged (Figures 1H and S1F). Analysis of expression levels

of BORG genes in patient datasets (Finak et al., 2008; Karnoub et al., 2007) revealed that *CDC42EP3* was consistently upregulated in the stromal compartment of human breast carcinoma compared to normal tissue samples (Figure S1G). These analyses led us to focus on Cdc42EP3 in CAFs.

Altered Septin Networks Are a Feature of CAFs

BORG proteins can regulate the actin and septin cytoskeleton (Joberty et al., 1999, 2001). We therefore investigated the septin network in NFs and CAFs. SEPT2 and SEPT7 formed more extensive networks in CAFs (Figure 2A; quantification in Figure S2A) that generally co-aligned with actin stress fibers (Figure S2B). This was confirmed using a biochemical method based on fractionation of detergent insoluble cytoskeletal components (Posern et al., 2002). In NFs the amount of actin present in the insoluble fraction was low (Figures 2B and S2C). This increased greatly in all four CAFs examined. Importantly, there was a similar shift of both SEPT2 and SEPT7 into the insoluble fraction in CAF#1–4 (Figures 2B and S2C). Time-lapse imaging of CAFs revealed that SEPT2 was highly dynamic, with SEPT2 puncta moving on and off actin stress fibers (Figure S2D; Movie S1). In contrast, SEPT2 localization and dynamics did not show a clear relationship to actin in NFs (Figure S2D; Movie S2). This was reflected in the lower co-localization coefficient of the Lifeact and SEPT2-GFP probes in NFs (Figures S2E and S2F demonstrate that GFP does not co-localize with F-actin). Structured illumination microscopy (SIM) revealed that SEPT2-GFP was present along actin stress fibers, but not always exactly coincident with them (Figure 2C, white arrows). Furthermore, there were wavy filaments that occasionally connected nearby stress fibers (Figure 2C, green arrows). We hypothesized that Cdc42EP3 may coordinate the actin and septin networks. Endogenous Cdc42EP3 partially co-localized with actin stress fibers in CAFs (Figure 2D). Further, SIM revealed that Cdc42EP3-GFP forms an intricate filamentous network in CAFs (Figure 2E). Similar to the SEPT2 network, the Cdc42EP3 network is clearly aligned with, but not exactly coincident with, F-actin (Figure 2E). It often formed filaments close to, or wrapped around, actin stress fibers (Figure 2E, white arrows), while other filaments presented a wavy pattern and did not co-align with F-actin (Figure 2E, yellow arrows). Time-lapse imaging showed that Cdc42EP3 moved both between and along stress fibers, in a manner similar to SEPT2 (Movies S3 and S4). Triple labeling of F-actin, SEPT2-Cherry, and Cdc42EP3-GFP revealed that Cdc42EP3 co-localized with

(C) Western blot showing pY118-paxillin (pPax), pY416-Src (pSFK), and pS19-MLC2 (pMLC2) in three sets of NF and CAFs (NF#1, NF#2, NF#3, CAF#1, CAF#2, and CAF#3). A tubulin blot is also shown.

(D) RNAi-based loss-of-function screen in CAFs. The indicated genes (left column) were knocked down by smart-pool siRNA, and the functional outcomes were evaluated in gel contraction (CAF#1) and organotypic invasion assays (CAF#1 and CAF#2). The values indicate the fold activity normalized to control siRNA-transfected CAFs for each experiment (red is low; blue is high). Genes are ranked on their average score (right column).

(E) Western blot and quantification showing Cdc42EP3 and tubulin expression in CAF#1 after transfection with control and three different Cdc42EP3 siRNA (siCEP3). Bars represent mean \pm SEM (n = 4).

(F) Histogram shows gel contraction by CAF#1 following transfection with control or three different Cdc42EP3 siRNA (siCEP3). Bars represent mean \pm SEM (n = 3).

(G) Images and quantification of 4T1 invasion when co-cultured with CAF#1 transfected with control or three single Cdc42EP3 siRNA (siCEP3). Bars represent mean \pm SEM (n = 14 organotypic assays assessed over three experiments). Scale bar, 50 μ m.

(H) Western blots showing Cdc42EP3, Cdc42EP5, and 14-3-3 levels in three sets of NF and CAFs (NF#1–3, CAF#1–3). The chart shows Cdc42EP3 and Cdc42EP5 fold expression (normalized to 14-3-3).

See also Figure S1.

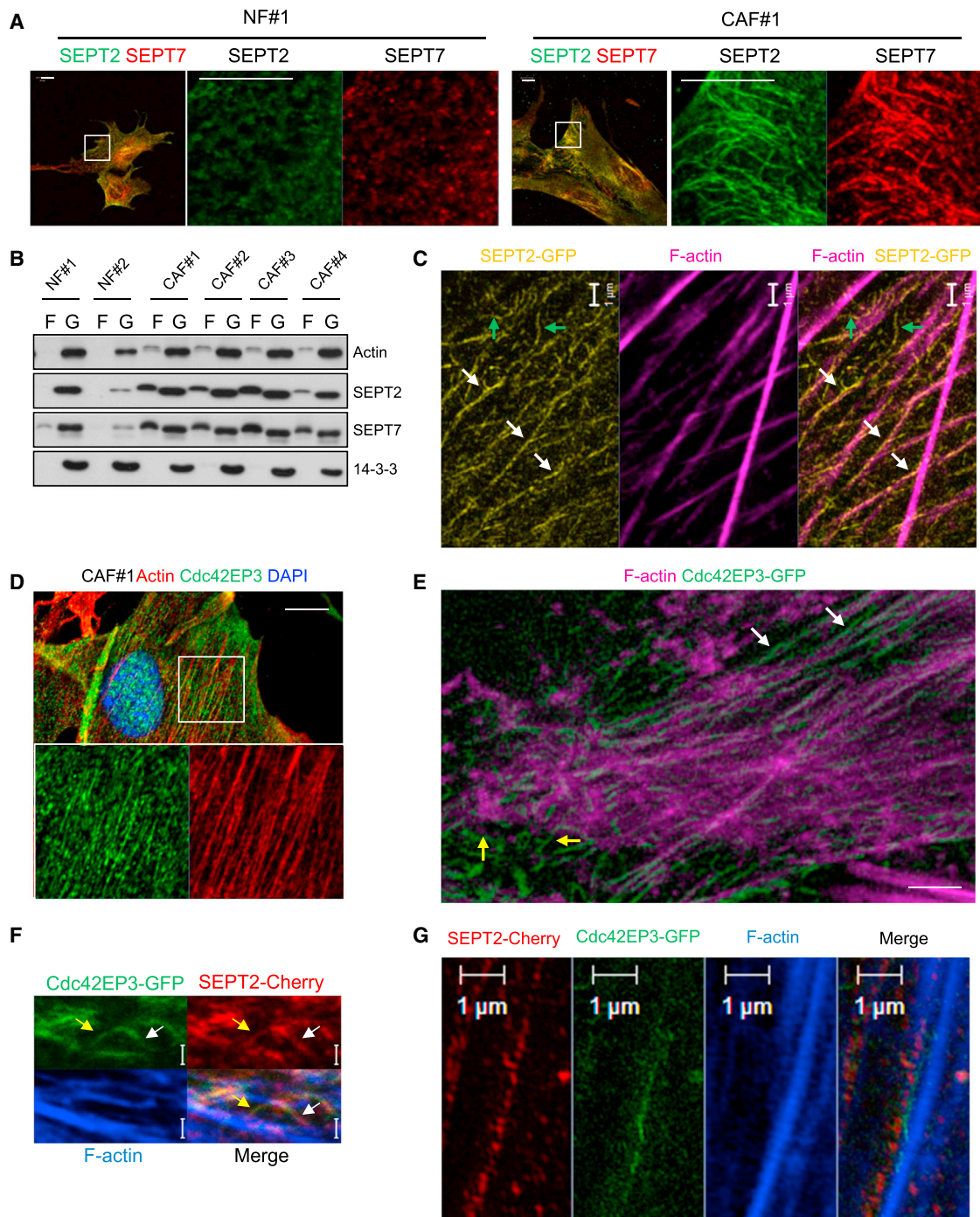


Figure 2. CAFs Have Enhanced Actin and Septin Cytoskeletal Networks

(A) Panels show SEPT2 (green) and SEPT7 (red) staining in NF#1 and CAF#1. Zoom areas are shown for each channel and cell type. Scale bars represent 10 μ m. See Figure S2A for quantification.

(B) Western blot showing the fibrillar (F) and soluble (G) content of actin, SEPT2, and SEPT7 in two NF lines (NF#1-2) and four CAF lines (CAF#1-4). A 14-3-3 blot is also shown as an internal cytosolic control. See Figure S2C for quantification.

(C) Representative SIM images of SEPT2-GFP (yellow) and F-actin (magenta) staining in CAF#1. White arrows indicate SEPT2-GFP filaments along actin stress fibers. The green arrows indicate SEPT2-GFP filaments connecting nearby stress fibers. Scale bars represent 1 μ m.

(D) Panels show actin (red), endogenous Cdc42EP3 (green), and DAPI (blue) staining of CAF#1. Cells were fixed using methanol. Zoom-up area showing actin and Cdc42EP3 is shown. Bar represents 10 μ m.

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SEPT2 filaments (Figure 2F). These filaments exhibited a partial overlap with F-actin stress fibers (Figure 2F, white arrows), but also formed connections between actin fibers (Figure 2F, yellow arrows). Further, SIM imaging revealed that Cdc42EP3 could localize between F-actin fibers and SEPT2 filaments (Figure 2G). Together, these data suggest that Cdc42EP3 links the actin and septin networks.

Depletion of Cdc42EP3 in CAFs resulted in the loss of actin stress fibers and reduced pS19-MLC2 staining (Figure 3A; quantification in Figure S3A). Moreover, the network of SEPT2 and SEPT7 fibers was also disrupted upon Cdc42EP3 depletion (Figure 3B; quantification in Figure S3B, confirmed with two siRNA in Figure S3C). Fractionation confirmed significant reductions in filamentous insoluble levels of F-actin, SEPT2, and SEPT7 in Cdc42EP3-depleted CAFs (Figures 3C and S3D); the total levels of actin, SEPT2, and SEPT7 were unaltered (Figure S3E). These changes were associated with significantly reduced CAF cellular stiffness (Figure 3D).

Gain-of-function experiments demonstrated that overexpression of Cdc42EP3 in NFs was sufficient to induce actin, SEPT2, and SEPT7 polymerization (Figure 3E). The ability of Cdc42EP3 to bind both septins (Joberty et al., 1999, 2001) and the data in Figure S3I showing a direct interaction with F-actin suggest that it may act as a “molecular glue” linking the two networks. To explore this idea, we generated Cdc42EP3 mutants defective in interaction with septins, F-actin, or Cdc42 (Figures S3F–S3H). We mutated key residues in the septin-binding BD3 domain (Cdc42EP3 GPS-AAA mutant [Joberty et al., 1999, 2001]) and the CRIB domain (Cdc42EP3 IS-AA mutant) (Figure S3G). To generate a putative actin-binding defective mutant, we mutated the amino acids KLP in positions 139–141 (Figure S3H). These residues are within a region of the protein with high homology to the actin bundling region of ANLN (Field and Alberts, 1995) and the actin-binding head piece of villin (Friederich et al., 1992). Immunoprecipitation analyses indicated that the GPS mutant of Cdc42EP3 still interacted with actin but had reduced binding to SEPT2 (Figure S3J). On the other hand, Cdc42EP3 KLP-AAA did not interact with actin but maintained the SEPT2 and SEPT7 binding abilities. Unfortunately, the CRIB domain mutant of Cdc42EP3 (Cdc42EP3 IS-AA) was also defective in interaction with actin; because it did not represent a specific loss of function, we excluded this mutant from further analysis.

We assessed the function of these Cdc42EP3 mutants in NFs, which have low Cdc42EP3 expression and relatively low F-actin and filamentous septin levels (Figures 2A and S2B). Overexpression of wild-type Cdc42EP3 led to prominent SEPT2 and SEPT7 filaments (Figure 3F with quantification in Figure 3H) and actin stress fibers (Figures 3G and 3H). As pre-

viously observed in CAFs (Figure 2E), wild-type Cdc42EP3-GFP in NFs formed filamentous structures that co-aligned with septin filaments and actin fibers (Figures 3F and 3G, with quantification in Figure 3H). Neither the GPS nor KLP mutant of Cdc42EP3 could induce the formation of actin or septin fibers (Figures 3F–3H). Further, the localization of both mutants was largely diffuse. These results indicate that Cdc42EP3 needs to directly bind both F-actin and septins to drive both networks into a filamentous state.

The Septin Cytoskeleton Is Required for CAF Functions

To test the role of septins in CAFs, we disrupted the septin network using siRNA-targeting SEPT2. Depletion of SEPT2 led to a loss of actomyosin stress fibers (Figure 4A) and fewer paxillin-containing focal adhesions (Figure 4B). This phenotype was very similar to that observed following Cdc42EP3 depletion or DIAPH1&3 depletion, which is known to block stress fiber formation (Narumiya et al., 2009). Western blotting for pS19-MLC2, active Src-family kinases, and pY118-paxillin confirmed the altered state of the actomyosin cytoskeleton and focal adhesions (Figure 4C). Biochemical fractionation revealed that depletion of either SEPT2 or SEPT7 led to a significant reduction in the level of F-actin in CAFs (Figure 4D). These data establish the inter-relationship of these two networks in CAFs. Next, we investigated the functional importance of the septin network in CAFs. Figure 4E shows that depletion of SEPT2 or SEPT7 significantly reduced the ability of CAF#1 to contract collagen-rich gels (confirmed in other CAFs in Figure S4A). The requirement for both Cdc42EP3 and SEPT2 for effective ECM remodeling was confirmed by staining for the deposition of fibrillar fibronectin and quantification of DQ-collagen I proteolysis (Figures S4B and S4C). Figure 4F further shows that the reduced ECM remodeling was associated with lower matrix stiffness. Moreover, the ability of CAFs to promote cancer cell invasion was reduced following SEPT2 or SEPT7 depletion (Figure 4G; confirmed in other CAFs in Figure S4D).

Next, we sought to test the role of Cdc42EP3 and septins in vivo. CAFs were injected in Matrigel plugs, and matrix remodeling was assessed after 7 days. Control siRNA-transfected CAFs organized collagen into thick bundles that could be visualized by second harmonic imaging and generated elastin fibers that could be detected by their auto-fluorescent properties (Figure S4E). Depletion of either Cdc42EP3 or SEPT2 greatly reduced the formation of thick collagen bundles and elastin fibers in vivo (Figures 4H and S4F). These data establish that Cdc42EP3 and septins are important for the matrix remodeling and invasion-promoting abilities of CAFs.

(E) Representative SIM image of F-actin (magenta) and Cdc42EP3-GFP (green) in CAF#1. Bar represents 1 μ m. White arrows indicate Cdc42EP3-GFP filaments associated to actin stress fibers. Yellow arrows indicate Cdc42EP3-GFP wavy filaments that do not co-align with F-actin.

(F) Single channel and merged confocal images of SEPT2-Cherry (red), Cdc42EP3-GFP (green), and F-actin (blue) in CAF#1. White arrows indicate SEPT2- and Cdc42EP3-containing filaments that co-align with F-actin. Yellow arrows indicate SEPT2- and Cdc42EP3-containing filaments that interconnect F-actin cables. Scale bar represents 1 μ m.

(G) Representative single channel and merged SIM images of SEPT2-Cherry (red), Cdc42EP3-GFP (green), and F-actin (blue) in CAF#1. Scale bars represent 1 μ m.

See also Figure S2 and Movies S1, S2, S3, and S4.

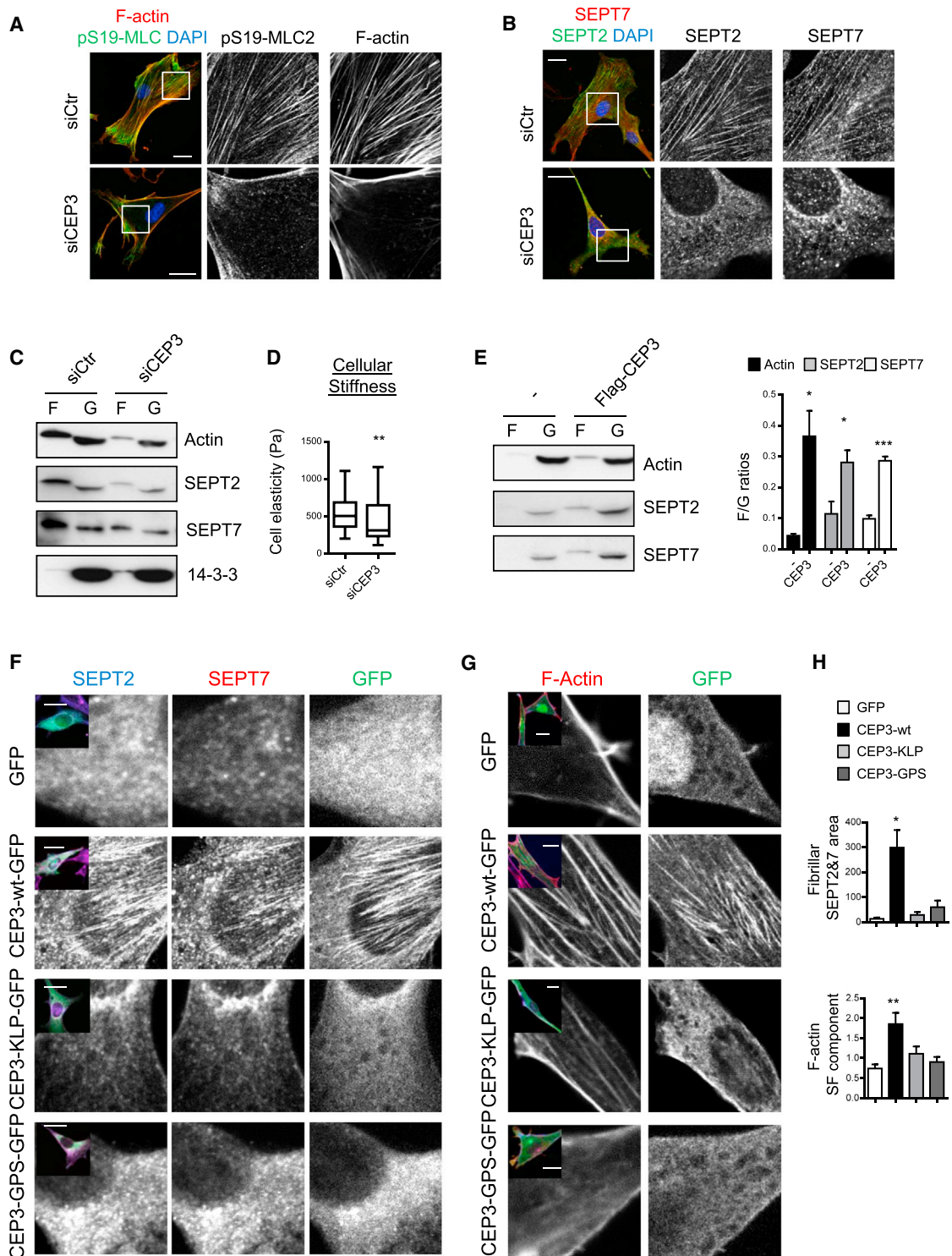


Figure 3. Cdc42EP3 Regulates the Actin and Septin Fibrillar Networks

(A) Images show pS19-MLC2 (green), F-actin (red), and DAPI (blue) staining of CAF#1 following control and Cdc42EP3 siRNA (siCEP3, smart pool). The grayscale panels show individual channel magnifications of the indicated areas. Scale bars, 25 μ m. See Figure S3A for quantification.

(B) Images show SEPT2 (green), SEPT7 (red), and DAPI (blue) staining of CAF#1 following control and Cdc42EP3 siRNA (siCEP3, smart pool). The grayscale panels show individual channel magnifications of the indicated areas. Scale bars, 25 μ m. See Figure S3B for quantification.

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CAFs Require Cdc42EP3 and SEPT2 to Promote Angiogenesis and Tumor Growth

We hypothesized that Cdc42EP3 might also be required for other functions of CAFs besides matrix remodeling. In particular, we focused on angiogenesis and tumor growth. qRT-PCR analysis revealed that Cdc42EP3 is required in CAFs for the expression of the key angiogenic factor, *Vegfa*, and several other genes implicated in angiogenesis, including *Vegfc*, *Tgfb1*, and *Tgfb2* (Figures 5A and S5A). The expression of other factors involved in tumor-stroma crosstalk was not changed following Cdc42EP3 depletion, or in the case of *Vegfb*, *Tgfb3*, and *Ccl2*, modestly increased (Figure S5A). Consistent with the qRT-PCR data, secreted VEGF-A was reduced in CAFs depleted of Cdc42EP3 or SEPT2 (Figure 5B). We tested the ability of CAFs to drive angiogenesis in vivo. Fibroblasts were injected in Matrigel plugs and the extent of angiogenesis evaluated a week later by endomucin staining. Depletion of Cdc42EP3 or SEPT2 dramatically reduced the angiogenic potential of CAFs in vivo (Figures 5C and 5D). To test whether the defective matrix remodeling and angiogenic activity of CAFs lacking Cdc42EP3 might influence tumor growth, we established CAFs that were stably depleted for Cdc42EP3 using two different small hairpin RNA (shRNA) sequences (Figure S5B). These CAFs, or the appropriate control CAFs, were then “admixed” with PyMT breast cancer cells and injected orthotopically in immune-competent FVB mice. Figure 5E shows that tumors containing Cdc42EP3-depleted CAFs were smaller than those tumors with control CAFs. Depletion of Cdc42EP3 in CAFs was also associated with longer survival (Figure 5F). Histological analysis revealed that α SMA-positive cells were visible in both control and Cdc42EP3-depleted tumors (Figure 5G). More interestingly, tumors containing Cdc42EP3-depleted CAFs tended to grow as a viable rim surrounding a necrotic core (visible in the lower magnification images in Figure 5H). This was correlated with reduced blood vessel density in the interior of the tumors, although similar numbers of vessels were present at the margins.

Cdc42EP3 Is Required for Mechano-transduction and YAP Activation

Cdc42EP3 and SEPT2 are important for regulation of the actomyosin cytoskeleton and paxillin phosphorylation (Figures 4A–4C). As these events are responsive to the mechanical properties of the substratum (DuFort et al., 2011), we hypothesized that Cdc42EP3 and septins may be required for responses to

changes in matrix stiffness. Figure 6A shows that GFP-SEPT2 forms fibrillar structures within the cell body in CAFs plated on relatively stiff 12 kPa substrates (Figure 2). However, when the same CAFs are plated on a soft 500 Pa substrate, the distribution of SEPT2 was cortical and fibrillar structures were absent. NFs fail to form SEPT2 fibers in the cell body on both 500 Pa and 12 kPa substrates (Figure 6A). Further, we noted that the localization of Cdc42EP3 in CAFs changed depending on substrate stiffness: it was diffuse on soft substrates and formed fibrillar structures on stiff matrices (Figure S6). These data demonstrate that septin network organization changes in response to substrate stiffness in CAFs, which express high levels of Cdc42EP3.

To test if Cdc42EP3 was causally implicated in the cytoskeletal changes, we undertook a series of gain- and loss-of-function experiments. We observed that, in contrast to control NFs, NFs overexpressing Cdc42EP3 had increased actin stress fibers and focal adhesions on 12 kPa substrates (Figure 6B). We additionally investigated the activity of the mechano-responsive transcriptional co-activator YAP (Calvo et al., 2013; Dupont et al., 2011). Figure 6C shows that Cdc42EP3 overexpression increases the nuclear localization of YAP (quantification in Figure 6D) and leads to a dose-dependent increase in the mRNA levels of two YAP target genes in NFs, *Ctgf*, and *Ankrd1* (Figure 6E). CAFs are highly mechano-responsive; they have few stress fibers or focal adhesions and cytoplasmic YAP on 500 Pa matrices, but on 12 kPa matrices, they have nuclear YAP and form extensive stress fibers and paxillin-containing focal adhesions. In the absence of Cdc42EP3 or SEPT2, these mechano-induced changes do not occur (Figures 6F–6H). We confirmed the reduction in YAP transcriptional activation by measuring *Ankrd1* and *Ctgf* mRNA (Figures 6I). Together, these data show that Cdc42EP3 and the septin network are required for CAFs to respond to changes in matrix stiffness and activation of the mechano-responsive transcriptional regulator YAP.

Cdc42EP3 Is Induced and Required Early in Fibroblast Activation

To explore the chronology of Cdc42EP3 upregulation during the transition from NF to CAF, we evaluated different stages of MMTV-PyMT tumor progression (Calvo et al., 2013). Cdc42EP3 expression is already enhanced in hyperplasia-associated fibroblasts (HpAF) and maintained in adenoma-associated fibroblasts (AdAFs) and CAFs (Figures 7A and 7B). In agreement,

(C) Western blot showing the fibrillar (F) and soluble (G) content of actin, SEPT2, and SEPT7 in CAF#1 following control and Cdc42EP3 siRNA (siCEP3, smart pool). A 14-3-3 blot is also shown as a cytosolic control. See Figure S3D for quantification.

(D) Box and whisker plot showing the Young's elastic modulus of CAF#1 cells with control and Cdc42EP3 siRNA (siCEP3, smart pool). The central box represents the lower to upper quartiles; the middle line represents the mean. The horizontal line extends from the minimum to the maximum value ($n = 61$ single measurements for siCtr; $n = 53$ single measurements for siCEP3).

(E) Western blot showing the fibrillar (F) and soluble (G) content of actin, SEPT2, and SEPT7 in empty vector (–) and Flag-Cdc42EP3 (Flag-CEP3)-transfected NF#1. The histogram shows the F/G ratios of actin, SEPT2, and SEPT7. Bars represent mean \pm SEM ($n = 3$).

(F) Top left inserts show GFP (green), SEPT2 (blue), and SEPT7 (red) staining of NF#1 following transfection with GFP or GFP-tagged Cdc42EP3 (CEP3) proteins. The grayscale panels show individual channel magnifications of perinuclear areas. Scale bars, 20 μ m.

(G) Top left inserts show GFP (green) and actin (red) staining of NF#1 following transfection with GFP or GFP-tagged Cdc42EP3 (CEP3) proteins. The grayscale panels show individual channel magnifications of perinuclear areas. Scale bars, 20 μ m.

(H) Histograms show fibrillar SEPT2&7 area (top) and F-actin stress fiber (SF) component (bottom) for the indicated experimental points. Bars represent mean \pm SEM ($5 < n < 10$ single-cell measurements).

See also Figure S3.

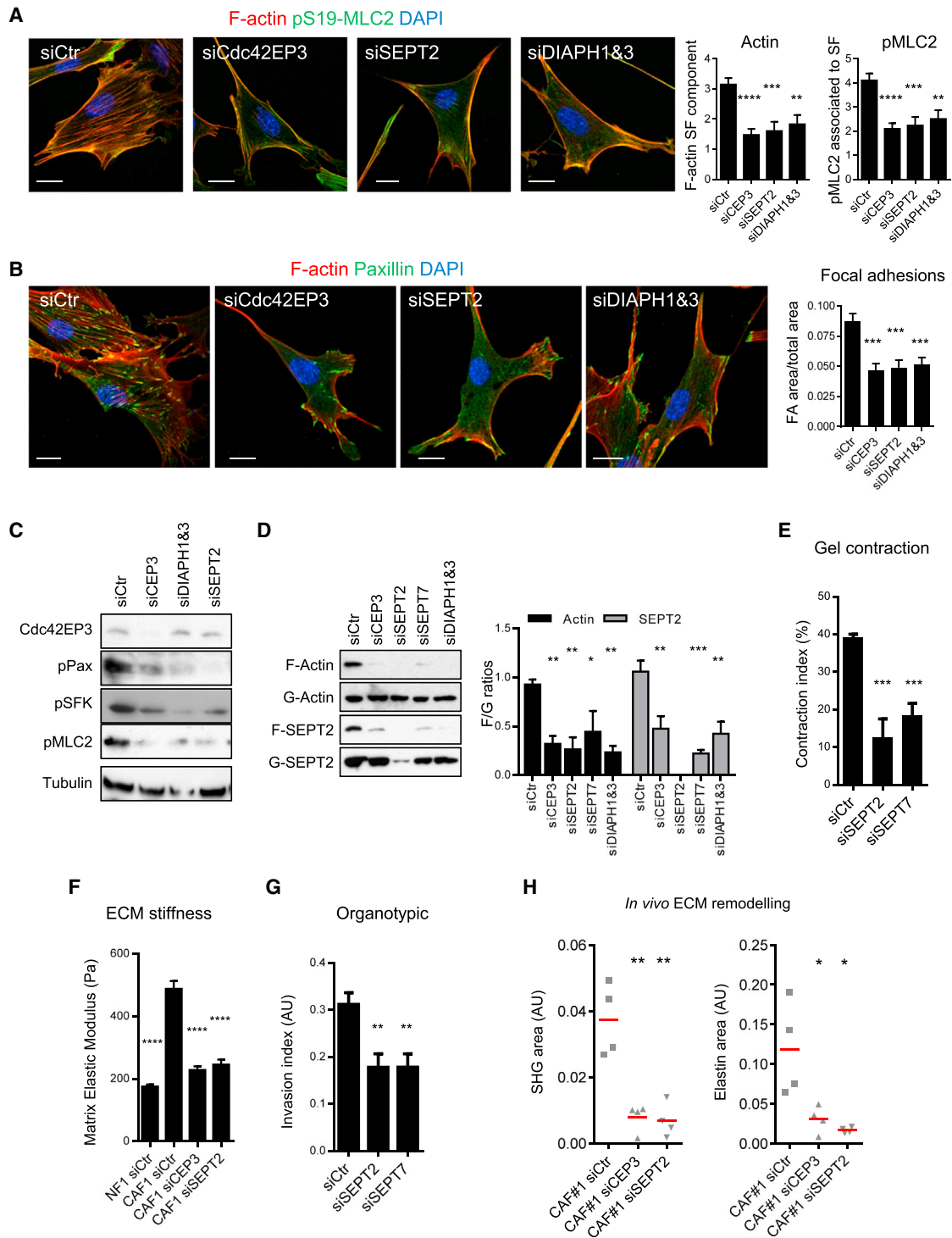


Figure 4. The Septin Cytoskeleton Is Required for CAF Functions

(A) Images showing F-actin (red), pS19-MLC2 (green), and DAPI (blue) staining of CAF#1 following control, Cdc42EP3, SEPT2, and DIAPH1&3 siRNA transfection (smart pools). Scale bars, 20 μ m. Histograms show an F-actin stress fiber (SF) component and pMLC2 associated to SF for the indicated points. Bars represent mean \pm SEM (12 < n < 15).

(B) Images show F-actin (red), paxillin (green), and DAPI (blue) staining of CAF#1 following with control, Cdc42EP3, SEPT2, and DIAPH1&3 siRNA transfection (smart pools). Scale bars, 20 μ m. The histogram shows the focal adhesion (FA) area normalized to total cell area in single cells. Bars represent mean \pm SEM (10 < n < 12).

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increased levels of F-actin and insoluble filamentous septins were also evident in HpAFs and AdAFs (Figure 7C), as well as Src and paxillin phosphorylation (Figure 7D). Interestingly, Cdc42EP3 expression and cytoskeletal rearrangements preceded the strong activation of MLC2 in AdAFs and CAFs. Similarly, YAP activation, as determined by the expression of YAP target genes *Cyr61*, *Amotl2*, *Ankrd1*, and *Ctgf*, was only observed in AdAFs and CAFs (Figure 7E). These data indicate that increased Cdc42EP3 expression is an early event that precedes the highly contractile phenotype and activation of YAP in CAFs.

Many factors, including HGF, TGF β , and SDF-1 α , were able to induce *Cdc42ep3* mRNA in NFs. TGF β and murine breast cancer cell (4T1) conditioned media (CM) yielded the greatest induction of Cdc42EP3 protein expression (Figures 7F and S7A). Consistently, treatment of NFs with either TGF β or 4T1 CM for 48 hr triggered F-actin and septin rearrangements (Figures S7B and S7C), and phosphorylation of MLC2, Src-family kinases, and paxillin (Figure 7F). Furthermore, depletion of Cdc42EP3 prevented conditioned media from causing changes in the F/G ratios of actin and SEPT2, or in MLC2 and Src activation (Figures 7G and 7H). Gain-of-function experiments in NFs demonstrated that increasing the levels of Cdc42EP3 expression enhanced the ability of TGF β and 4T1 CM to induce Src activation and elevated basal pS19-MLC2 levels (Figures S7D and S7E). These changes were associated with increased matrix remodeling after TGF β - and 4T1 CM treatments (Figure S7F). Further, if contractility was boosted by overexpressing MLC2, as occurs in CAFs (Calvo et al., 2013), then a synergistic increase in matrix remodeling was observed in cells with high Cdc42EP3 levels (Figure S7G). These data suggest that elevated expression of Cdc42EP3 sensitizes cells to functional activation by the contractile cytoskeleton. We therefore tested whether elevated Cdc42EP3 levels were required for functional activation of NFs. Depletion of Cdc42EP3 prevented both the activation of collagen gel contraction and cancer cell invasion by NFs in response to cancer cell conditioned media (Figures 7I and 7J). Together, these data demonstrate that Cdc42EP3 is critical for the functional activation of normal fibroblasts by cancer cells.

DISCUSSION

Here, we describe Cdc42EP3 as a key regulator of the conversion of normal fibroblasts into CAFs. CAFs have enhanced stress fibers, α SMA-positive fibers, and focal adhesions (Calvo et al., 2013; Kalluri and Zeisberg, 2006; Öhlund et al., 2014; Sandbo

et al., 2011; Xing et al., 2010). We now describe that CAFs are also characterized by increased levels of filamentous septins. These filaments are partly aligned with F-actin fibers in CAFs (Dolat et al., 2014; Joberty et al., 2001; Kinoshita et al., 1997). However, septin filaments and actin fibers do not precisely colocalize and time-lapse imaging indicated that the actin and septin networks exhibit different dynamic behaviors. During cytokinesis, the adaptor protein ANLN plays a key role in linking the actin and septin networks (Kinoshita et al., 2002), although recent work suggests that septins alone may be sufficient to drive formation of actin ring-like structures (Mavrakis et al., 2014). Our analyses indicate that Cdc42EP3 may play a similar role during interphase in CAFs. Cdc42EP3 can bind both actin and septins and localizes between F-actin fibers and SEPT2 filaments. Dynamic and mutational analysis suggests that Cdc42EP3 can bind to septins independent of binding to F-actin. We propose that dynamic septin filaments associated with Cdc42EP3 are then able to interact with actin filaments. Cdc42EP3 overexpression in NFs induces F-actin and septin polymerization, suggesting that connectivity between the two networks stabilizes them both. Indeed, the “cross brace” pattern of SEPT2 and Cdc42EP3 localization between stress fibers could provide mechanical support for the contractile actin cytoskeleton. This may allow for increased isometric tension in the F-actin network. These cytoskeletal changes are significant for both remodeling of the extracellular environment within tumors and cell signaling within CAFs, such as YAP activation. In the absence of Cdc42EP3, CAFs are unable to effectively remodel the ECM, leading to a reduction in matrix stiffness and less cancer cell invasion. In vivo analysis confirms the importance of Cdc42EP3 for ECM remodeling, as measured by collagen’s second harmonic generation and elastin autofluorescence, and reveals that Cdc42EP3 is required for CAFs to promote angiogenesis. Similar loss-of-function phenotypes are observed when the septin network is depleted. Further, in vivo “admix” experiments with breast cancer cells demonstrate that Cdc42EP3 is required for efficient tumor growth. Our data establish that connectivity between the actin and septin networks is crucial for the function of CAFs from breast and squamous cell carcinoma. It will be interesting to determine the role of actin and septin networks in CAFs from pancreatic cancer, which can have both tumor-promoting and suppressive effects (Özdemir et al., 2014; Rhim et al., 2014).

Intriguingly, Cdc42EP3 upregulation precedes the emergence of highly contractile CAFs and YAP activation. Cdc42EP3 expression is already elevated in fibroblasts at the hyperplasia

(C) Western blot showing phosphorylated levels of pY118-paxillin (pPax), pY416-Src (pSFK), and pS19-MLC2 (pMLC2) in CAF#1 following transfection with control, Cdc42EP3 (CEP3), DIAPH1&3, and SEPT2 siRNA (smart pools). Cdc42EP3 and tubulin blots are also shown.

(D) Western blots showing fibrillar (F) and soluble (G) actin and SEPT2 in CAF#1 following control, Cdc42EP3 (CEP3), SEPT2, SEPT7, and DIAPH1&3 siRNA (smart pools). The histogram shows the F/G ratios of actin and SEPT2. Bars represent mean \pm SEM (n = 5).

(E) Histogram shows gel contraction by CAF#1 following control, SEPT2, and SEPT7 siRNA (smart pools). Bars represent mean \pm SEM (n = 8).

(F) Histogram shows the elastic modulus of matrices remodeled by NF#1 or CAF#1 following control, Cdc42EP3, or SEPT2 siRNA (smart pools). Bars indicate mean \pm SD (n = 167 single measurements for NF-siCtr; n = 142 for CAF-siCtr; n = 173 for CAF-siCEP3; n = 51 for siSEPT2).

(G) Quantification of 4T1 invasion when co-cultured with CAF#1 transfected with control or SEPT2 and SEPT7 siRNA (smart pool). Bars represent mean \pm SEM (n = 9 organotypic assays assessed over two experiments).

(H) Charts show second harmonic generation (SHG) of collagen fibers and elastin-positive areas in the CAF#1 matrix plugs transfected with control, Cdc42EP3 (CEP3), and SEPT2 siRNA. Line and error bars indicate mean \pm SEM (n = 4 plugs from different mice).

See also Figure S4.

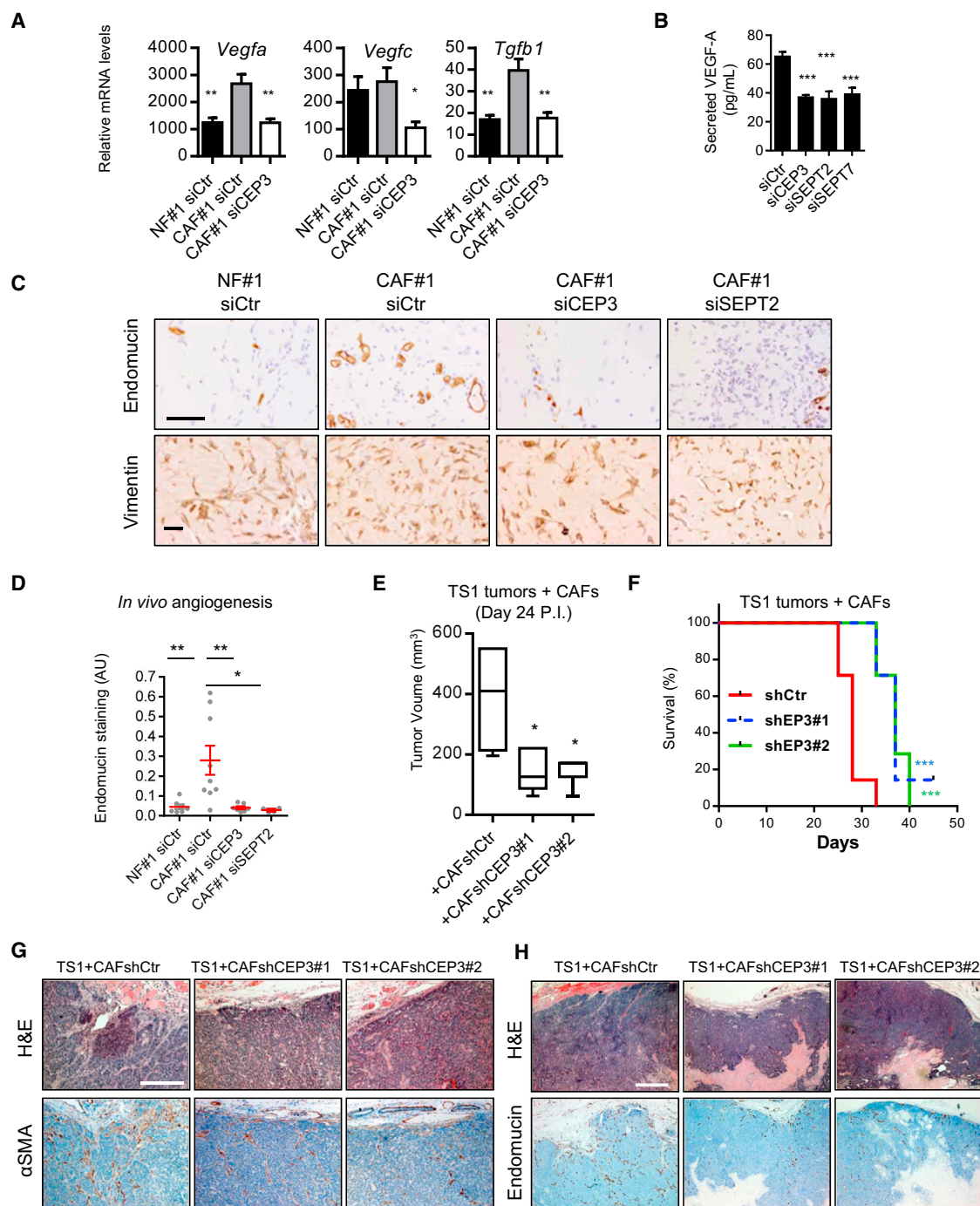


Figure 5. Cdc42EP3 Is Required for the Pro-tumorigenic Activity of CAFs In Vivo

(A) mRNA levels of angiogenic factors *Vegfa*, *Vegfc*, and *Tgfb1* in NF#1 and CAF#1 following transfection with control and Cdc42EP3 siRNA (CEP3, smart pool). Bars represent mean \pm SEM (n = 12).

(B) Secreted levels of VEGF-A on CAF#1 following transfection with control, Cdc42EP3 (CEP3), SEPT2, or SEPT7 siRNA (smart pool). Bars represent mean \pm SEM (n = 6).

(C) Representative images showing endomucin and vimentin staining of matrix plugs with NF#1 or CAF#1 transfected with control, Cdc42EP3 (CEP3), and SEPT2 siRNA (smart pools) and injected sub-cut in mice. Scale bars, 100 μ m.

(D) Chart shows quantification of endomucin staining relative to fibroblast number (vimentin staining). Line and error bars indicate mean \pm SEM (n = 7, except for CAF#1-siCtr that n = 9 and CAF#1-siSEPT2 that n = 5; each experimental point is an individual plug from a different mouse).

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stage. Multiple signaling pathways converge upon *Cdc42EP3* promoter (Esnault et al., 2014; Gomis et al., 2006; McGee et al., 2011; Zhao et al., 2008). Therefore, *Cdc42EP3* expression may be highly responsive to the small perturbations in tissue homeostasis that are likely to occur in hyperplastic tissue. However, this raises the question of why its expression does not lead to fully activated fibroblasts early in disease progression. It has been argued that both chemical and mechanical stimulation are needed for the acquisition of pathological active fibroblasts (Hinz, 2007). We propose that *Cdc42EP3* is located at a node linking both activating processes. Soluble factors—either from hyperplastic epithelia or leukocytes infiltrating the pre-malignant tissue—could induce *Cdc42EP3* expression in normal fibroblasts. This would sensitize the cells to mechanical cues, but in the absence of changes to the physical environment it would not trigger the activation of mechano-responsive signaling pathways. This is consistent with the small effect of *Cdc42EP3* overexpression alone on NF function and the synergy observed when *Cdc42EP3* and *MLC2* are both overexpressed (Figure S7G). Further, when CAFs are grown on low stiffness gels (500 Pa, slightly above normal mouse mammary tissue stiffness) they do not form stress fibers or large focal adhesions and have only modest levels of nuclear YAP (Figures 6E–6G). Full activation of the highly contractile CAF phenotype and YAP activation would require concomitant stiffening of the ECM. The changes in the physical properties of the ECM are likely to happen with slow kinetics and may not coincide with the faster upregulation of *Cdc42EP3* induced by soluble factors. This would explain why full pathological activation of fibroblasts does not occur early in disease progression (Calvo et al., 2013).

To conclude, we demonstrate a key role for coordination between the actin and the septin networks in CAFs. This is required for force-mediated matrix remodeling, promoting cancer cell invasion, angiogenesis, and tumor growth. Further, if coordination of F-actin and septin networks is prevented, then fibroblasts are unable to activate mechano-sensing signaling pathways, including paxillin phosphorylation, Src, and YAP. The elevated expression of *Cdc42EP3* in the stroma of pre-malignant lesions enables the subsequent activation of these pathways and the emergence of fully activated CAFs.

EXPERIMENTAL PROCEDURES

cDNA, siRNA, and Reagents

Murine *Cdc42EP3* cDNA was acquired from Thermo Scientific (pCMV-Sport 6-*Cdc42EP3*) and subcloned into pEGFP.C1 and PKR5.1-Flag. pEGFP-*Cdc42EP3* was used to generate *Cdc42EP3*(IS-AA), *Cdc42EP3*(GPS-AAA), and *Cdc42EP3*(KLP-AAA) and was subcloned in pCSII-IRES to generate sta-

ble cells lines. pEGFP-*MLC2* was a gift from Michael Olson (Beatson Institute). SEPT2-GFP and SEPT2-Cherry were a gift from Elias Spiliotis (Drexel University). MARS-Lifeact was a gift from Michael Sixt (Institute of Science and Technology). siRNA were purchased from Dharmacon and are listed in the Table S1. shRNA-targeting murine *Cdc42EP3* were purchased from Thermo Scientific (pGIPZ backbone). The following growth factors and drugs were used: TGF β (Peprotech), LPA (Sigma), HGF (Peprotech), and SDF-1 (Peprotech). Breast cancer cell conditioned medium was harvested from 4T1 cells in DMEM 1% fetal bovine serum (FBS) for 48 hr and filtered through a 0.22- μ m filter. Fibroblasts were transfected with 100 nM siRNA using DharmaFECT 1 (Dharmacon) and Lipofectamine (Life Technologies) for plasmids.

Cell Lines

Fibroblasts from normal mammary glands (NFs), hyperplastic mammary tissue (HpAFs), mammary adenoma/early carcinoma tissue (AdAFs), and mammary carcinoma (CAFs) have been previously described (Calvo et al., 2013). Fibroblasts were cultured in DMEM (Invitrogen), 10% FBS, and 1% insulin-selenium-transferrin (ITS). 4T1 murine breast cancer cells were used for the organotypic invasion assays and to generate breast cancer cell conditioned media. SCC12 human squamous cell carcinoma cells were used for organotypic invasion assays of HN-CAF and Cer-CAF. MMTV-PyMT TS1 murine breast cancer cells were used to generate orthotopic tumors in syngeneic FVB/n mice. Cell lines stably expressing *Cdc42EP3*-GFP, shRNA control, and shRNA-targeting murine *Cdc42EP3* were generated by lentiviral infection. NF#1 stably expressing *Cdc42EP3*-GFP were sorted into low (NF#1-*Cdc42EP3*-GFP^{low}) and high (NF#1-*Cdc42EP3*-GFP^{high}) expression by fluorescence-activated cell sorting.

ECM Remodeling and Invasion Assays

ECM-remodeling (contraction) assays and human SCC CAF and mouse mammary CAFs organotypic culture systems were as previously described (Calvo et al., 2013). Details are provided in the Supplemental Experimental Procedures.

Screening

Smart-pool siRNA were transiently transfected into two individual CAF lines (CAF#1 and CAF#2). The functional role of each gene was assessed using collagen gel contraction assays and 4T1 breast cancer cell organotypic invasion assays. Each experimental point was normalized against the control siRNA for that experiment.

Biophysical Methods

AFM was performed as described (Calvo et al., 2013), and details are provided in the Supplemental Experimental Procedures. Polyacrylamide gels of defined elastic moduli were made as described (Yeung et al., 2005).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde, except for the analysis of endogenous septins when cells were fixed in ice-cold methanol for 10 min. Cells were permeabilized by incubation in PBS 0.5% NP-40 (Sigma) at 4°C for 20 min (twice), PBS 0.3% Triton 100 (Sigma) at room temperature (RT) for 20 min, and PBS 0.1% Triton 100 at room temperature (RT) for 15 min (twice). Samples were blocked in 4% BSA PBS 0.05% Tween20 (Sigma) and

- (E) Box and whisker plot showing volumes of orthotopic tumors generated by co-injection of TS1 murine MMTV-PyMT breast cancer cells with CAF#3 stably expressing control or two different shRNA targeting *Cdc42EP3*. The central box represents the lower to upper quartiles; the middle line represents the mean; and the horizontal line extends from the minimum to the maximum value ($n = 7$, except for CAF-shCtr that $n = 6$). Tumors were measured at day 24 post-injection.
- (F) Survival curves for orthotopic tumors generated by co-injection of TS1 murine MMTV-PyMT breast cancer cells with CAF#3 stably expressing control or two different shRNA targeting *Cdc42EP3*. The survival curve represents the percentage of animals alive at the indicated time point after injection. Survival curves were estimated by the Kaplan-Meier method and compared among subsets using the log rank test.
- (G) Representative images of orthotopic tumors generated by co-injection of TS1 murine MMTV-PyMT breast cancer cells with CAF#3 stably expressing control or two different shRNA targeting *Cdc42EP3* and stained for H&E or α SMA. Scale bar, 500 μ m.
- (H) Representative images of orthotopic tumors generated by co-injection of TS1 murine MMTV-PyMT breast cancer cells with CAF#3 stably expressing control or two different shRNA targeting *Cdc42EP3* and stained for H&E or endomucin. Scale bar, 200 μ m.
- See also Figure S5.

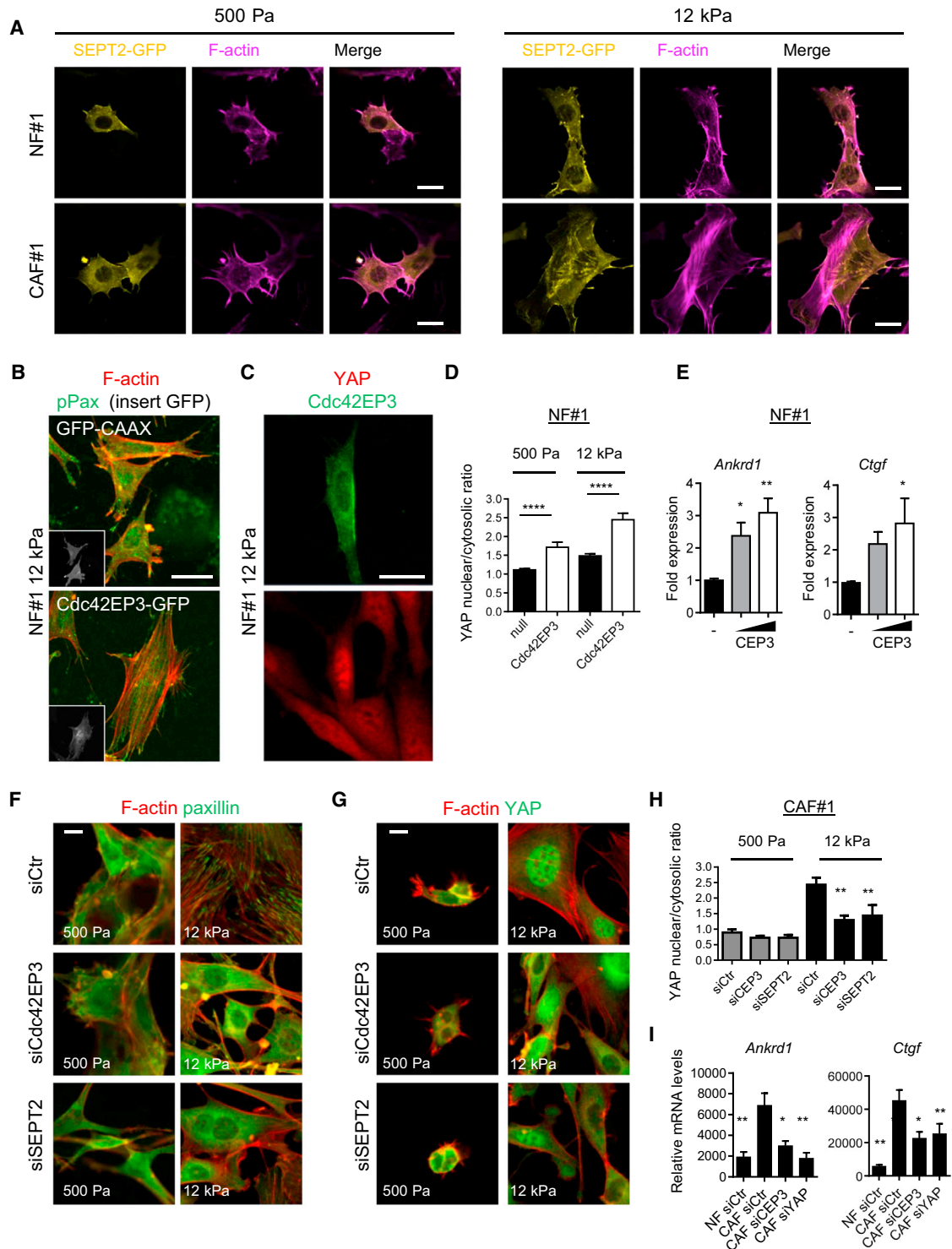


Figure 6. Cdc42EP3 Is Required for Mechano-sensing

(A) Images show single and merged channels of SEPT2-GFP (yellow) and F-actin (magenta) staining of NF#1 and CAF#1 when seeded on soft (~500 Pa) and stiff (~12 kPa) matrices. Scale bar represents 10 μ m.

(B) Images show F-actin (red) and pY118-Paxillin (pPax) staining of NF#1 when seeded on stiff (~12 kPa) matrices following transfection with GFP-CAAX and Cdc42EP3-GFP. Inserts show GFP signal (grayscale). Scale bar represents 10 μ m.

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incubated with primary antibody in blocking solution overnight at 4°C. After three PBS washes, secondary antibody in blocking solution was added for 3 hr. After three PBS washes, samples were mounted and analyzed using an inverted Zeiss LSM780/Leica SP8. For analysis of fibronectin production, cells were kept in 1% FBS for 24 hr before fixing and were permeabilized for only 5 min in 0.1% Triton 100 at RT during the staining procedure to reduce detection of intracellular protein. Antibody description and working dilutions can be found in [Table S2](#). Time-lapse microscopy was performed using a Zeiss LSM780 with cells in CO₂-independent media (GIBCO) containing 10% FBS. The details of the imaging analysis methods are provided in the [Supplemental Experimental Procedures](#).

Structured Illumination Microscopy

Cells were processed for immunostaining as usual and mounted in a 1:1 mixture of glycerol and MOWIOL. Imaging was performed using a Zeiss ELYRA PS1 and data processed using the SIM tools in the Zen 2011 software. Channel alignment was performed with 0.1- μ m TetraSpeck fluorescent microspheres (Life Technologies).

Collagenase Activity Measurements

A thin layer of collagen gel (4.6 mg/ml) containing 50 μ g/ml of D^QFITC-Collagen I from bovine skin (Life Technologies) and 10% FBS was generated. After 1 hr, fibroblasts were seeded on top. After 48 hr, samples were fixed in 4% paraformaldehyde for 1 hr and stained for F-actin. A fluorescein isothiocyanate signal generated by cleaved collagen and F-actin staining was detected using an inverted Leica SP8 microscope.

F-G Actin and Septin Fractionation

We determined the relative proportions of filamentous and globular actin and septins by sedimentation, followed by quantitative analysis by SDS-PAGE. Cells were lysed in warm F-actin stabilization buffer (PIPES 100 mM [pH 6.9], 5 mM MgCl₂, 1 mM EGTA, 30% glycerol, 0.1% Triton X-100, 0.1% NP-40, 0.1% Tween-20, 0.15% 2-mercaptoethanol, 1 mM ATP, and complete EDTA-free cocktail) and homogenized using a 25G gauge. Lysates were incubated for 10 min at 37°C, and cellular debris was removed by centrifugation at room temperature. Supernatants were then subjected to ultracentrifugation at 37°C (1 hr at 100,000 g). Supernatants containing the globular cytosolic fraction (G fraction) were removed. Using a similar volume of 10 mM CaCl₂ 10 μ M Cytochalasin D, the pellet containing the fibrillar fraction (F fraction) was dissolved for 1 hr in ice. Both G and F fractions were mixed with 5 \times sample buffer before western blot analysis. Antibody description and working dilutions can be found in [Table S2](#).

In Vitro F-actin Binding Assay

Human platelet actin (Cytoskeleton, Actin Binding Protein Biochem Kit #BK013) in general actin buffer (5 mM Tris-HCl [pH 8.0] and 0.2 mM CaCl₂, supplemented with 0.2 mM ATP) was polymerized using actin polymerization buffer (50 mM KCl, 2 mM MgCl₂, and 1 mM ATP) at 24°C for 1 hr. 0.3 μ M GST-Cdc42EP3 (Abcam, ab161060) and recombinant 0.3 μ M glutathione

S-transferase (GST) (Sigma-Aldrich; SRP5348) were incubated with 20 μ M F-actin for 1 hr at 24°C and centrifuged at 150,000 g for 90 min at 24°C. Equal volumes of the supernatant and pellet fractions were resolved using a 4%–12% SDS-PAGE gel, stained with InstantBlue (Expedeon) for actin staining, and further processed by western blotting (for GST and GST-Cdc42EP3) analysis.

Immunoprecipitation

GFP-Cdc42EP3 immunoprecipitations were performed in HEK293 cells. Cells were transiently transfected and subsequently lysed in NP-40 lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, and 1% NP-40). Lysates were cleared by centrifugation and supernatants were further cleared by pre-incubating with 25 μ l of G-sepharose. Cleared supernatants were then mixed with 2 μ g of anti-GFP antibody plus 25 μ l of G-sepharose and incubated for 2 hr at 4°C. Immunoprecipitations were then washed four times with NP-40 washing buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP40, and 0.25% gelatin) before adding sample buffer. Antibody details can be found in [Table S2](#).

Western Blotting

Unless stated otherwise, all protein lysates were obtained from cells seeded on top of a thin layer of a mixture of collagen I:Matrigel matrices. Western blot exposures within the dynamic range were obtained on film and quantified by densitometry with the program NIH Image 1.60, except for [Figures S1B, S3I, and S7D](#), which were generated with the Azure c300 scanner. Antibody details can be found in [Table S2](#).

Detection of VEGF-A in Conditioned Media

ELISA was used to assess the levels of secreted murine VEGF-A in accordance with the manufacturers' instructions (R&D; Mouse VEGF DY493-05). 10⁵ cells were seeded on 12-well plates, and 24 hr later cells were washed and 0.6 ml of DMEM supplemented with 0.5% FBS added to each well. Medium was collected and assayed after 24 hr of conditioning.

In Vivo Matrigel Plugs

2 \times 10⁶ fibroblasts were suspended in 100 μ l of PBS and mixed in 300 μ l of Matrigel. Then 300 μ l of the mixture were injected subcutaneously on the mammary fat pad of a 12-week-old FVB/n female. After 7 days, the plugs were fixed in 4% paraformaldehyde. Blood vessel density was calculated by normalizing the area of endomucin staining to the area of vimentin staining of all the slices processed. Antibody details are in [Table S2](#). Collagen second harmonic generation (SHG) and elastin signal were acquired by excitation of sections with an 880-nm pulsed Ti:Sapphire laser and emitted light acquired at 440 nm (SHG) and 535 nm (Elastin) using a Leica SP8 microscope.

Generation of Syngeneic Orthotopic Tumors

MMTV-PyMT TS1 murine breast cancer cells were used to generate orthotopic tumors in wild-type FVB/n mice. 10⁶ TS1 cells and 3 \times 10⁶ CAFs (shRNA control, shRNA-Cdc42EP3#1, or shRNA-Cdc42EP3#2) were suspended in 100 μ l

(C) Images show GFP (green) and YAP (red) staining of NF#1 when seeded stiff (~12 kPa) matrices following transfection with Cdc42EP3-GFP. Scale bar represents 10 μ m.

(D) Histogram shows nuclear YAP fluorescent intensity/cytoplasmic YAP fluorescent intensity of NF#1 when seeded on soft (~500 Pa) and stiff (~12 kPa) matrices following transfection with GFP-CAAX or Cdc42EP3-GFP. The mean and 95% confidence interval are shown (23 < n < 80 cells).

(E) Fold mRNA levels of YAP target genes *Ankrd1* and *Ctgf* in stable cell lines NF#1 null, NF#1-Cdc42EP3^{low}, and NF#1-Cdc42EP3^{high} maintained in 1% FBS for 6 hr. Bars represent mean \pm SEM (n = 5).

(F) Images show F-actin (red) and paxillin (green) staining of CAF#1 when seeded on soft (~500 Pa) and stiff (~12 kPa) matrices following transfection with control, Cdc42EP3, or SEPT2 siRNA (smart pools). Scale bar represents 10 μ m.

(G) Images show F-actin (red) and YAP (green) staining of CAF#1 when seeded on soft (~500 Pa) and stiff (~12 kPa) matrices following transfection with control, Cdc42EP3, or SEPT2 siRNA (smart pools). Scale bar is 10 μ m.

(H) Histogram shows nuclear YAP fluorescent intensity/cytoplasmic YAP fluorescent intensity for the indicated conditions. The mean and 95% confidence interval are shown (23 < n < 80 cells).

(I) Relative mRNA levels of YAP target genes *Ankrd1* and *Ctgf* in NF#1 and CAF#1 following transfection with control, Cdc42EP3 (CEP3), and YAP siRNA (smart pools). Bars represent mean \pm SEM (n = 6).

See also [Figure S6](#).

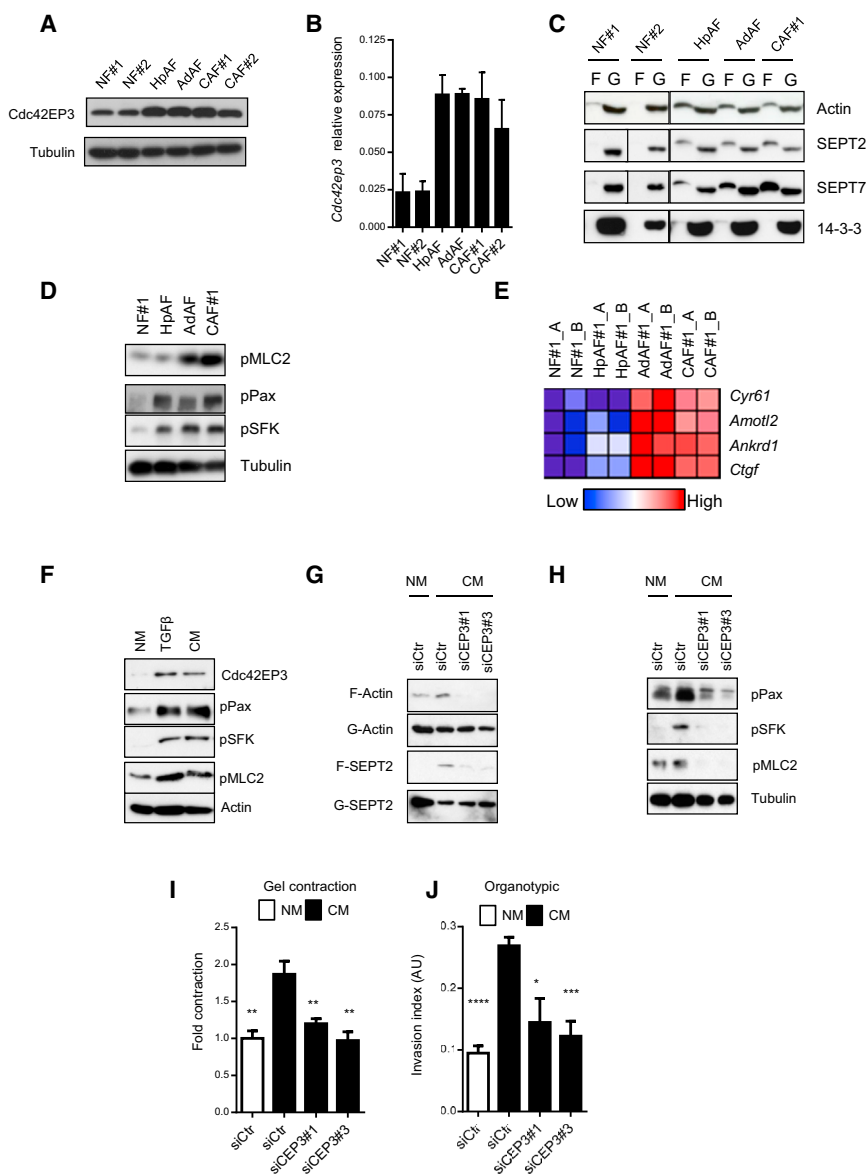


Figure 7. Cdc42EP3 Is Induced and Required Early in Fibroblast Activation

(A) Western blots of Cdc42EP3 in fibroblasts isolated from different disease stages (NF, HpAF, AdAF, and CAF).

(B) Relative levels of *Cdc42ep3* mRNA in fibroblasts from different disease stages. Bars indicate mean \pm SEM (n = 4).

(C) Western blot showing the fibrillar (F) and soluble (G) actin, SEPT2, and SEPT7 in fibroblasts from different disease stages. A 14-3-3 blot is also shown as an internal cytosolic control.

(D) Western blots showing pY118-paxillin (pPax), pY416-Src (pSFK), and pS19-MLC2 (pMLC2) and tubulin levels in fibroblasts isolated from different disease stages.

(E) GSEA-derived heatmap showing the relative levels of mRNA expression of YAP target genes (*Cyr61*, *Amotl2*, *Ankrd1*, and *Ctgf*) in fibroblasts isolated from different disease stages on duplicate. Colors range from dark blue, representing, respectively, the highest and lowest normalized expression of the gene.

(F) Western blots showing pY118-paxillin (pPax), pY416-Src (pSFK), and pS19-MLC2 (pMLC2) in NF#1 subjected to 1% FBS (NM), TGF β (2 ng/ml), and breast cancer cell conditioned media (CM) for 48 hr. Cdc42EP3 and actin blots are also shown.

(G) Western blots of fibrillar (F) and soluble (G) fractions of actin and SEPT2 in NF#1 subjected to 1% FBS (NM) or breast cancer cell conditioned media (CM) following transfection with control and two single Cdc42EP3 (CEP3) siRNA.

(H) Western blots showing tubulin and pY118-paxillin (pPax), pY416-Src (pSFK), and pS19-MLC2 (pMLC2) in NF#1 subjected to 1% FBS (NM) or breast cancer cell conditioned media (CM) following transfection with control and two single Cdc42EP3 (CEP3) siRNA.

(I) Histogram showing gel contraction of NF#1 subjected to normal media (NM) or breast cancer cell conditioned media (CM) following transfection with control and two single Cdc42EP3 (CEP3) siRNA. Bars represent mean \pm SEM (n = 5).

(J) Histogram showing organotypic cancer cell invasion of NF#1 subjected to normal media (NM) or breast cancer cell conditioned media (CM) following transfection with control and two single Cdc42EP3 (CEP3) siRNA. Bars represent mean \pm SEM (n = 5). See also Figure S7.

of PBS:Matrigel (50:50) and injected orthotopically into the mammary fat pad of 6- to 8-week-old females. To calculate tumor volume, the formula $V = (\text{length} \times \text{width}^2)/2$ was used. Tumors were fixed in 4% paraformaldehyde for histological analysis. Antibody description and working dilutions can be found in Table S2. Mice were kept in accordance with UK regulations under project license PPL 70/ 8380.

mRNA Analysis

RNA was isolated using an RNeasy Kit (QIAGEN). Reverse transcription was performed using Precision nanoScript 2 Reverse-Transcription Kit (PrimerDesign), and qPCR was performed using PrecisionPLUS 2x qPCR MasterMix with ROX and SybrGreen (PrimerDesign). Oligonucleotides for qRT-PCR are described in the Table S3. Expression levels are normalized to the expression of *Gapdh*, *Rplp1*, or *Lamc2*. Gene-set enrichment analyses (GSEA) details are provided in the Supplemental Experimental Procedures.

Analysis of Expression in Clinical Samples

mRNA levels of BORG family members in the stromal compartment of human tumors were obtained from GSE9014 (Finak breast dataset) and GSE8977 (Karnoub breast dataset) (Finak et al., 2008; Karnoub et al., 2007). Probes on the Finak dataset were A_23_P166453 (CDC42EP1), A_23_P1602 (CDC42EP2), A_23_P209636 (CDC42EP3), A_23_P66891 (CDC42EP4), and A_24_P44916 (CDC42EP5). Probes on the Karnoub dataset were 204693_at (CDC42EP1), 214014_at (CDC42EP2), 225685_at (CDC42EP3), 218062_x_at (CDC42EP4), and 227850_x_at (CDC42EP5).

Statistical Analyses

Paired or unpaired two-tailed Student's t test, Mann-Whitney's test, and one-way ANOVA with Tukey post-test (for multiple comparisons) were performed using GraphPad Prism (GraphPad Software). Unless stated otherwise, mean values and SEM are shown. Survival curves were estimated based on the

Kaplan-Meier method and compared using a log rank test. p values of less than 0.05 are considered statistically significant: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; ns, non-significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, three tables, and four movies and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.11.052>.

AUTHOR CONTRIBUTIONS

F.C. and E.S. carried out all of the experiments (except those noted otherwise), conceived of the study, and wrote the manuscript. R.R. assisted in qPCR, western blotting, ELISA, in vitro F-actin binding assays, and F-G analyses. S.H. assisted with in vivo experiments. A.J.F. assisted in cloning and the generation of stable cell lines. E.M. and G.C. performed all of the AFM analyses. A.B. and F.B. contributed to the SIM experiments. All authors critically read the manuscript and provided intellectual input.

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