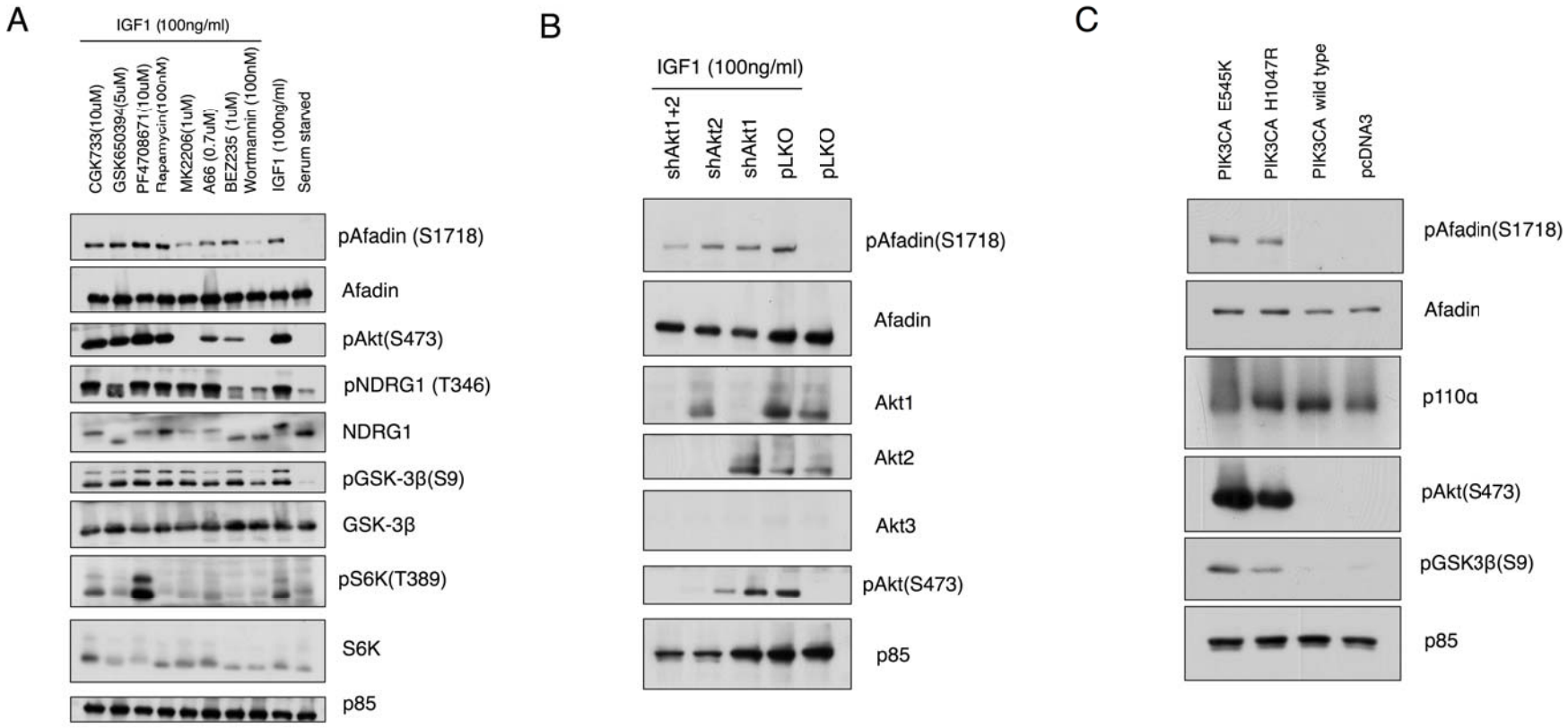


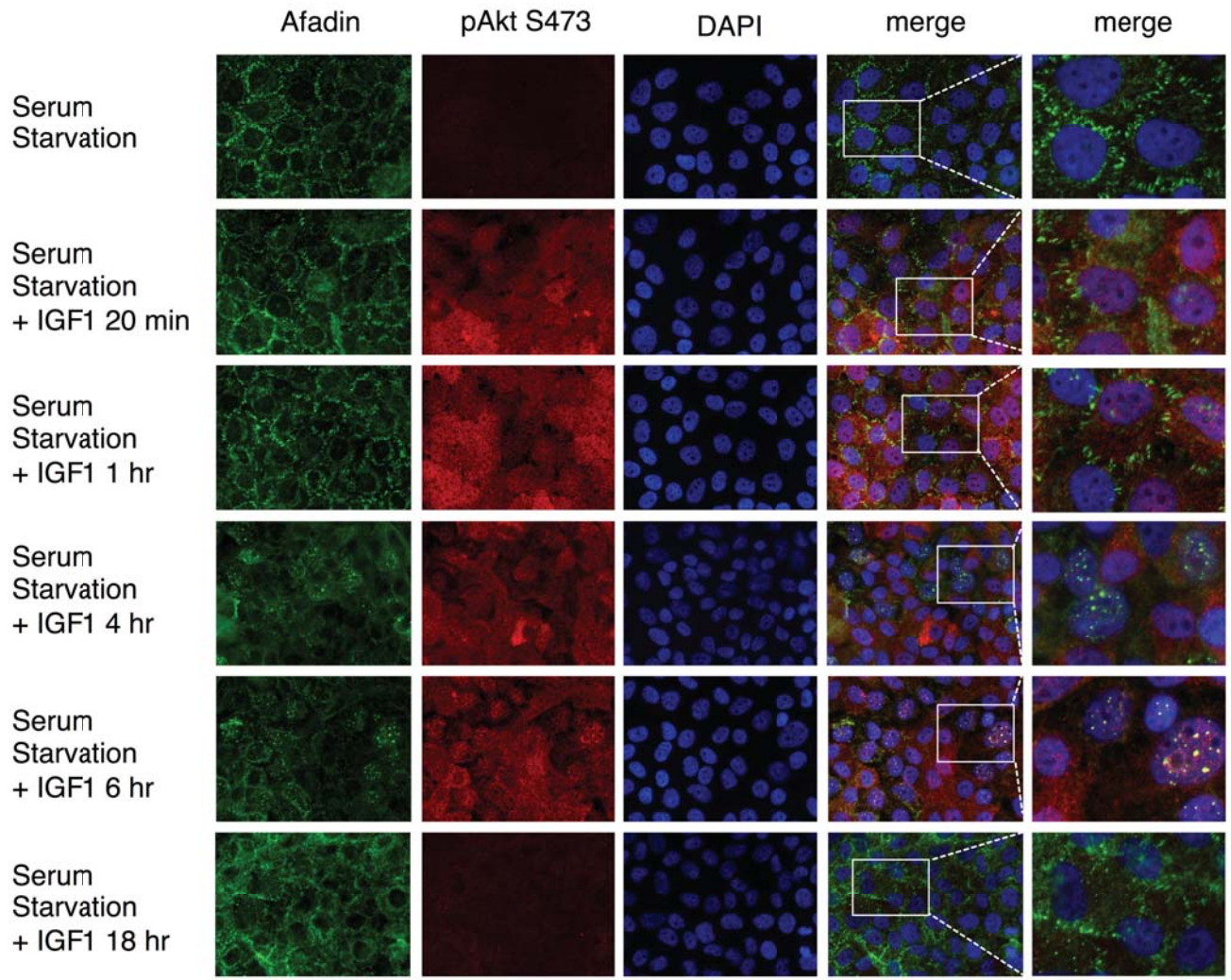
Supplementary Fig. S1:

A. Related to Fig. 1C: HeLa cells were serum starved, pre-treated with the indicated inhibitors and stimulated with IGF-1 for 20 min. In order to assess phosphorylation of Afadin at S1718, whole cell lysates were immunoblotted with the indicated antibodies.

B. Related to Fig. 2A: Akt1 and Akt2 were silenced separately and concomitantly using shRNA in HeLa cells. Phosphorylation of Afadin was assessed. Whole cell lysates were immunoblotted with the indicated antibodies. HeLa cells do not express Akt3.

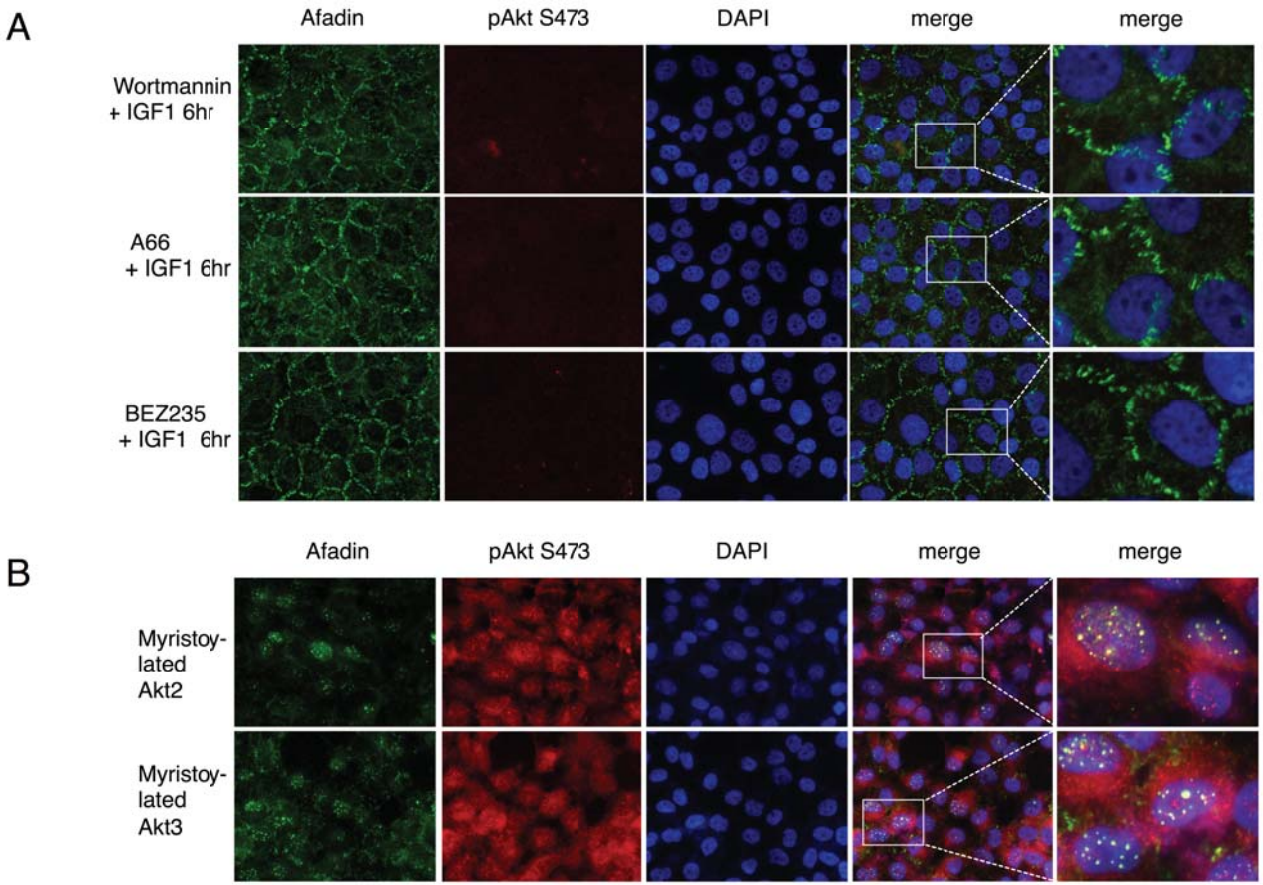
C. Related to Fig. 2D: HeLa cells were transfected with pcDNA3, PIK3CA WT, PIK3CA H1047R, PIK3CA E545K. Cells were serum starved and phosphorylation of Afadin was determined using immunoblotting

Supplementary Fig. S1



Supplementary Fig. S2:
Related to Fig. 3A: MCF10A cells were serum starved and stimulated with IGF-1 for 20 min, 1 hr, 4 hr, 6 hr and 18 hr. Intracellular localization of Afadin was assessed using immunofluorescence. Nuclei were stained with DAPI. Results are representative of multiple fields and at least three independent experiments

Supplementary Fig. S2

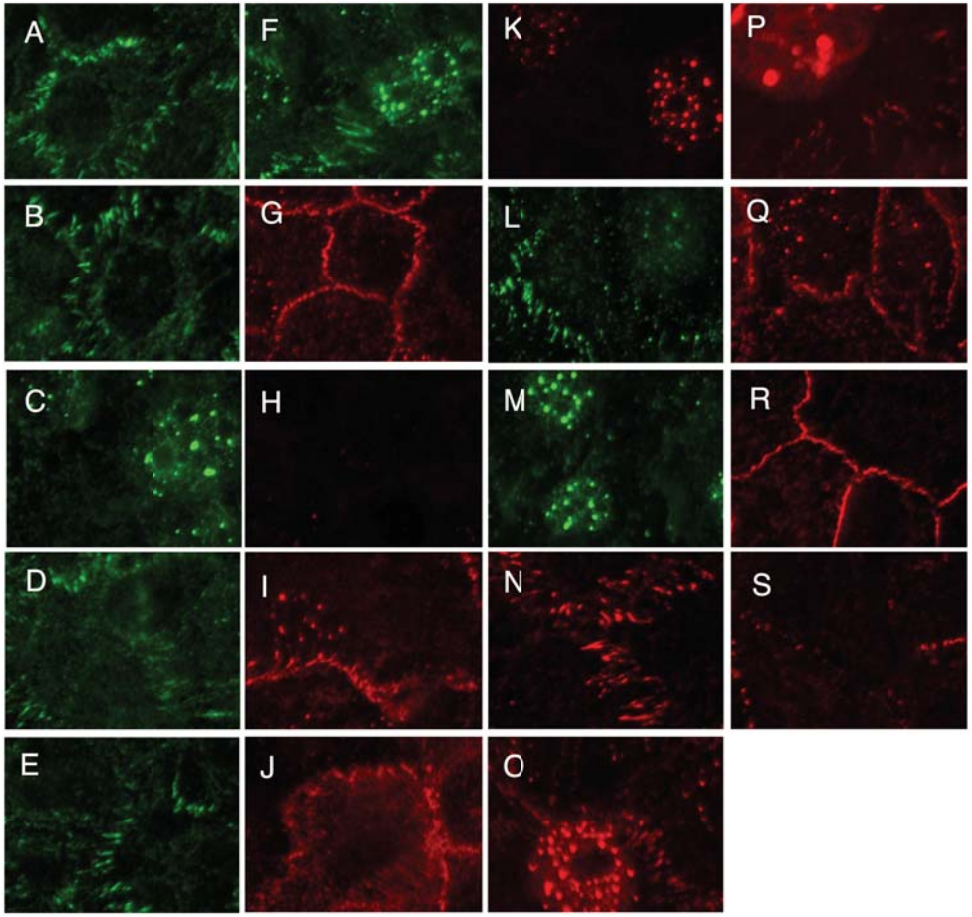


Supplementary Fig. S3:

A. Related to Fig. 3A: MCF10A cells were serum starved and pretreated with the indicated inhibitors, and than stimulated with IGF-1 for 20 min. Intracellular localization of Afadin was assessed using immunofluorescence. Nuclei were staind with DAPI. Results are representative of multiple fields and at least three independent experiments.

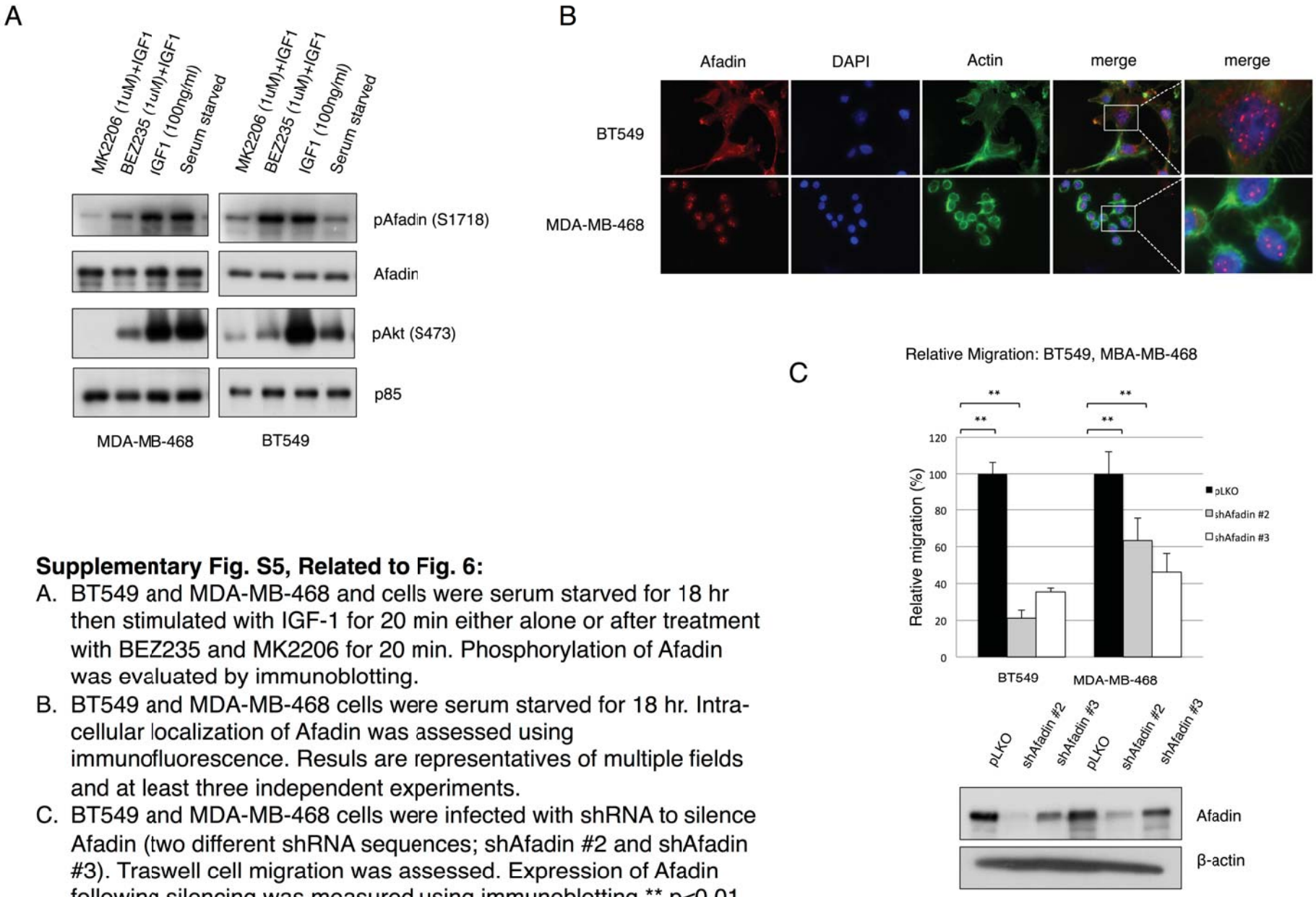
B. Related to Fig. 4B: MCF10A cells transfected with myristoylated Akt2 or Akt3 alleles and immunofluorescence performed with the indicated antibodies. In all cases nuclei were stained with DAPI. Images are representative of multiple fields, and of at least three independent experiments.

Supplementary Fig. S3



Supplementary Fig. S4:
Related to Fig. 3-5: Magnified single channel Afadin staining: **A-D** relate to Figure 3A: A: serum starvation; B: IGF1 1hr; C: IGF1 6 hr; D: MK2206 + IGF1 6 hr. **E-F** relate to Figure 3B: E: serum starvation; F: IGF1 6 hr. **G-K** relate to Figure 4A: G: pLKO; H: shAfadin; I: shAfadin + Afadin WT; J: shAfadin + Afadin S1718A; K: shAfadin + Afadin S1718E. **L-M**: relate to Figure 4B: L: pcDNA3; M: MyrAkt1. **N-S**: relate to Figure 5C: N: Serum starvation; O: IGF1 6 hr; P: IGF1 + CHX 6 hr; Q: Full serum; R: MK2206 6 hr; S: MK2206 + CHX 6 hr.

Supplementary Fig. S4



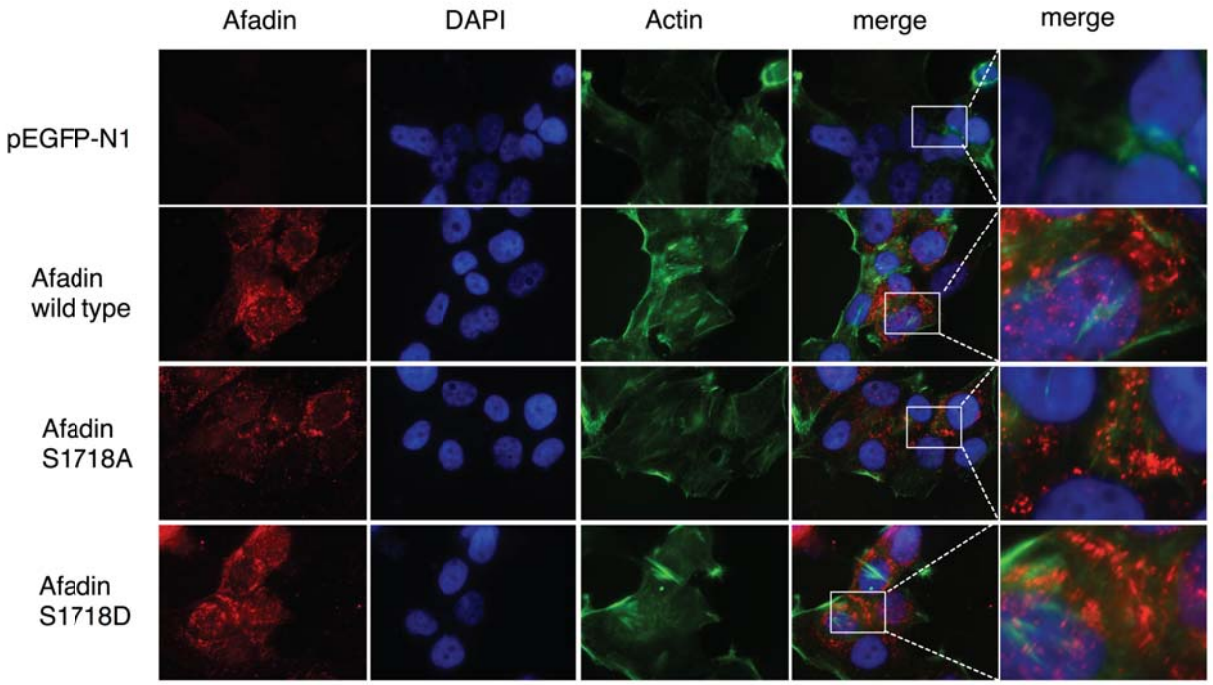
Supplementary Fig. S5, Related to Fig. 6:

A. BT549 and MDA-MB-468 and cells were serum starved for 18 hr then stimulated with IGF-1 for 20 min either alone or after treatment with BEZ235 and MK2206 for 20 min. Phosphorylation of Afadin was evaluated by immunoblotting.

B. BT549 and MDA-MB-468 cells were serum starved for 18 hr. Intracellular localization of Afadin was assessed using immunofluorescence. Resuls are representatives of multiple fields and at least three independent experiments.

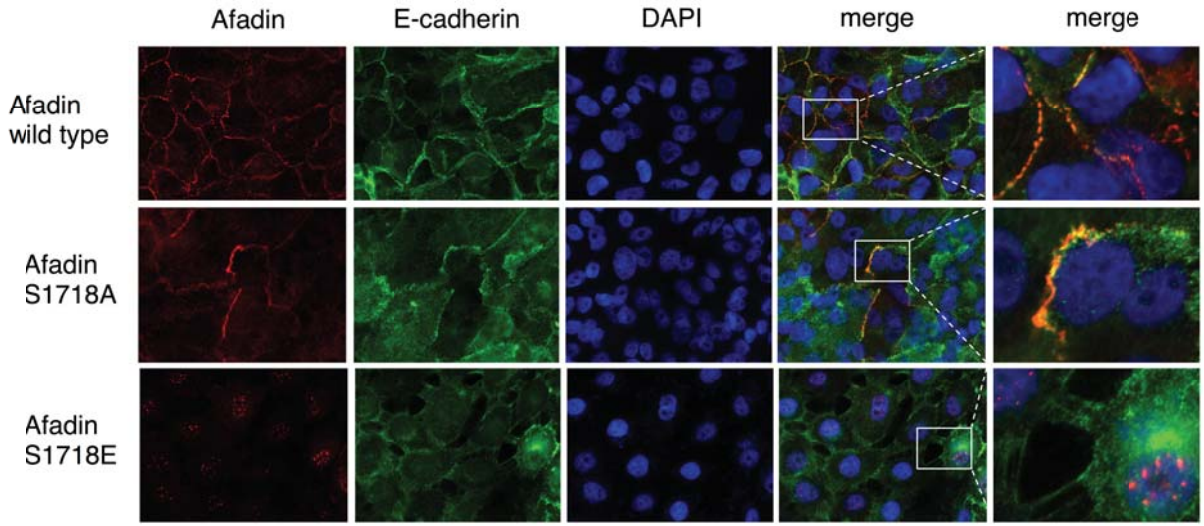
C. BT549 and MDA-MB-468 cells were infected with shRNA to silence Afadin (two different shRNA sequences; shAfadin #2 and shAfadin #3). Traswell cell migration was assessed. Expression of Afadin following silencing was measured using immunoblotting.** $p<0.01$ (Student T-test).

Supplementary Fig. S5



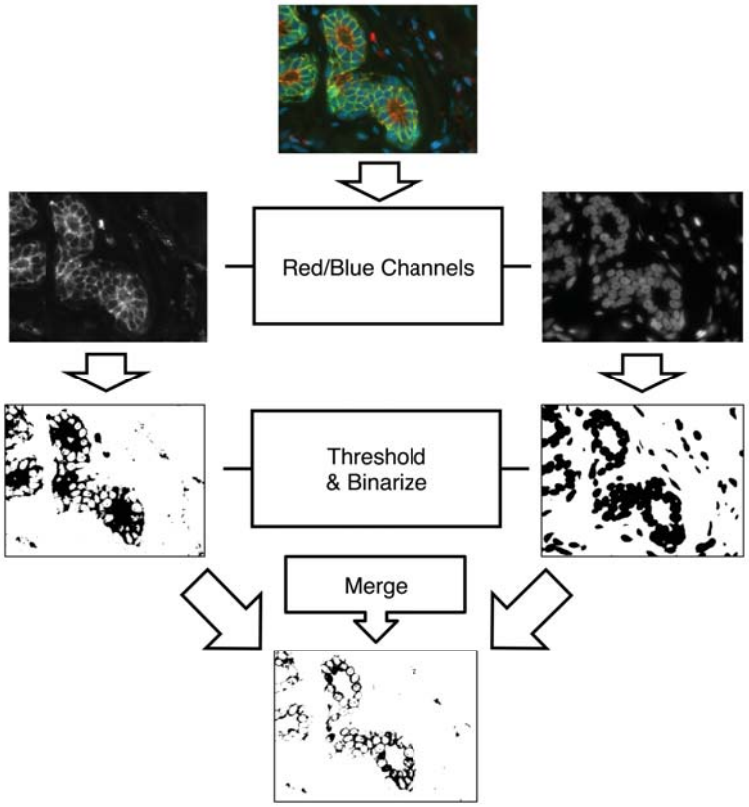
Supplementary Fig. S6 related to Fig. 6:
T47D transfected with pEGFP-N1, wild-type (WT) Afadin, Ser1718Ala (S1718A), Ser1718Asp (S1718D) Afadin. Immunofluorescence staining was performed using the indicated antibodies.Nuclei were stained with DAPI. Results are representative of multiple fields and at least three independent experiments

Supplementary Fig. S6



Supplementary Fig. S7 related to Fig. 6C:
MCF10A cells were transduced with shRNA to silence Afadin and transfected with wild type Afadin wild type, Ser1718Ala Afadin (S1718A) or Ser1718Glu Afadin (S1718E). The cells were evaluated by immunofluorescence with the indicated antibodies. Results are representative of multiple fields and at least three independent experiments

Supplementary Fig. S7



Supplementary Fig. S8 related to Fig. 7
Schematic representation of the quantification of Afadin nuclear localization. To quantitatively assess nuclear localization of Afadin, the immunofluorescence images were split into red, green, and blue channels and background subtraction was performed on the red and blue channels using a sliding paraboloid with a 50 pixel radius. After background correction, we applied an auto-threshold function to create binary images from the red and blue channels. The red channel represents Afadin staining, and the blue channel represents nuclear staining. We computed the total area of red and blue as well as the area of overlap of the red and blue binary images. We computed the Afadin nuclear localization score as the (red and blue overlapping area)/(total red area + total blue area).

Supplementary Fig. S8