Daam1 negatively regulates USP10 activity

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Abstract

The differentiation of fibroblasts into pathological myofibroblasts during wound healing is in part characterized by increased cell surface expression of α v-integrins. Our previous studies found that the deubiquitinase (DUB) USP10 removes ubiquitin from α v-integrins, leading to cell surface integrin accumulation, subsequent TGF β 1 activation, and myofibroblast differentiation. In this study, a yeast-two hybrid screen elucidated a novel binding partner for USP10, the formin, Daam1. The USP10/Daam1 interaction was also supported by proximity ligation assay (PLA) activity. Treatment with TGF β 1 significantly increased USP10 and Daam1 protein expression, PLA signal, and co-localization to actin stress fibers. Furthermore, Daam1 siRNA knockdown significantly reduced a) co-precipitation of USP10 and Daam1 on purified actin stress fibers, and b) β 1- and β 5-integrin ubiquitination resulting in increased α v-, β 1-, and β 5-integrin total protein levels, α v integrin recycling to the cell surface, and extracellular fibronectin (FN) organization. Together, our data suggest that Daam1 negatively regulates USP10's DUB activity and subsequently maintains integrin protein homeostasis.

Introduction:

Corneal tissue has been used extensively as a model system to study scarring as it is transparent and thus scars in the cornea lead to visual disability and blindness (Stepp et al., 2014; Whitcher et al., 2001). Given the readily available access to non-transplantable donor tissue, primary human corneal cells (HCFs) are used as a model system to discover new pathways that lead to scarring outcomes and these results may be generalized to scarring in other tissues (Bukowiecki et al., 2017; Wilson et al., 2017). Corneal myofibroblasts arise from resident corneal stromal cells termed keratocytes, circulating, bone marrow-derived fibrocytes which readily infiltrate the cornea, as well as through the epithelial-mesenchymal transition of cells within the site of injury (Lassance et al., 2018; Shu and Lovicu, 2017). These cells are important to the wound healing process and normally will have apoptosed in a regeneratively healed wound. However, scar tissue is characterized by the persistence of pathological myofibroblasts

(Desmouliere et al., 1995; Wilson, 2012). Myofibroblasts have pronounced actin stress fiber cytoskeleton, characterized by organized α -smooth muscle actin (α -SMA). These prominent stress fibers facilitate myofibroblast-mediated contraction of extracellular matrix (ECM), important in the process of ECM remodeling during wound healing. However, persistent myofibroblasts overly contact tissue, secrete fibrotic extracellular matrix proteins, and promote an autocrine loop of TGF β activity (Lorenzo-Martin et al., 2019; Massoudi et al., 2016; Pakshir and Hinz, 2018; Sandbo and Dulin, 2011), creating scar tissue.

Integrins also play important roles in wound healing, physically coupling the ECM to the intracellular actin network through focal adhesion complex formation, as well as initiating signaling cascades that induce actin stress fiber contraction and rearrangement in cytoskeletal architecture (Schiller and Fassler, 2013; Thannickal et al., 2003). In regard to fibrosis, the α v-integrins (α v β 1, α v β 3, α v β 5, α v β 6, and α v β 8) are essential to myofibroblast differentiation (Asano et al., 2006; Henderson et al., 2013; Lygoe et al., 2004), with particular α v-heterodimers having more critical roles than others in differing forms of fibrosis (Chang et al., 2017; Horan et al., 2008; Reed et al., 2015; Sarrazy et al., 2014). Our work demonstrated that α v β 1 and especially α v β 5 are most strongly-associated with corneal scarring (Gillespie et al., 2017; Wang et al., 2012). While direct inhibition or knockdown of α v-integrins sequesters myofibroblast differentiation and fibrosis (Chang et al., 2017; Sarrazy et al., 2014), it may also compromise wound healing by interfering with re-epithelialization (Blanco-Mezquita et al., 2011; Duperret et al., 2016).

Previous work by our group identified an alternate strategy for targeting α v-integrins and preventing myofibroblast-induced scar tissue formation. We found that the deubiquitinase (DUB), USP10, is upregulated during myofibroblast differentiation. USP10 deubiquitinates the

$\beta 1$ and $\beta 5$ subunits of αv -integrin heterodimers, protecting them from degradation and leading to an increase in cell surface expression of αv -dimers. In addition, overexpression of USP10 in HCFs was sufficient to increase the activation of local TGF $\beta 1$, induce α -SMA-positive stress fiber formation, and lead to an increase in cellular FN accumulation (Gillespie et al., 2017). In contrast, siRNA knockdown of USP10 significantly reduced the formation of scar tissue in an organ culture wound healing model (Castro et al., 2019; Gillespie et al., 2017), and in vivo in rabbits (Boumil et al., 2020) suggesting that USP10 represents a novel target for intervention in scarring outcomes and perhaps more broadly fibrotic diseases.

The present study further elucidates the regulation of USP10-induced αv -integrin accumulation in myofibroblasts. A yeast 2-hybrid screen, using USP10 as bait, identified novel protein-protein interactions with USP10. Daam1 (Diaphanous-associated activator of morphogenesis 1) was the strongest confidence novel interacting protein with USP10. Daam1 is a formin protein with a diverse set of functions within cells. Formins, including Daam1, are responsible for actin cytoarchitectural arrangement, facilitating such functions as actin filament polymerization (Yang et al., 2007) and severing (Harris et al., 2004), as well as cross-linking actin into bundles (Esue et al., 2008; Jaiswal et al., 2013). Our data suggest that Daam1 negatively regulates USP10, by sequestering the complex to actin stress fibers. Overall, we found that the Daam1/USP10 axis is a novel post-translational regulator of integrin protein levels. These results provide insights into an additional layer of control over the integrin life-cycle.

Results:

Yeast 2-hybrid screen reveals novel Daam1-USP10 protein-protein interaction

In order to further characterize the mechanisms by which USP10 promotes integrin recycling and aids in the differentiation of myofibroblasts, a yeast 2-hybrid screen (Hybrigenics)

was carried out using USP10 as bait, to identify novel targets for study. The screen produced both established and novel hits (Fig 1A). The established hits included both G3BP proteins; G3BP stress granule assembly factor 1 (G3BP1; NCBI Ref # NM_005754.2) and G3BP stress granule assembly factor 2 (G3BP2; NCBI Ref # NM_203504 and NM_012297). These were considered strong positive controls for this screen. The strongest confidence novel interaction identified by our yeast 2-hybrid screen for USP10 binding partners was with the formin protein Disheveled-associated activator of morphogenesis 1 (Daam1; NCBI Ref # NM_001270520.1), the primary subject of this study. This interaction was denoted as “Very Strong” confidence, as determined by a proprietary calculation developed by Hybrigenics to screen out putative false positive interactions. Of note was the USP10 interaction signal from the protein Fascin-1 (NCBI Ref # NM_003088), another actin-associated protein that directly interacts with Daam1 on actin bundles in lamellipodia (Jaiswal et al., 2013). These are thought to be structural interactions as using USP10 as bait did not reveal known enzymatic interactions in which USP10 acts as a DUB such as p53 or integrins (Gillespie et al., 2017; Yuan et al., 2010).

Daam1 is composed of 4 primary functional domains: GTPase-Binding Domain/Formin Homology Domain 3 (GBD/FH3), Formin Homology Domain 2 (FH2), Formin Homology Domain 1 (FH1), and the Diaphanous Autoregulatory Domain (DAD), as well as a coiled-coil (CC) structural domain between the GBD/FH3 domain and the FH2 domain. FH2 domain was obtained as the site of interaction in all readable hits (Fig 1B). The yeast 2-hybrid screen provides information about the fragment of the mRNA which generates the interaction. Of the 15 hits for Daam1, 12 gave readable sequence data. Eight hits were specific to a fragment of the parent Daam1 mRNA containing only the formin homology domain 2 (FH2), while 2 fragments

were specific to the FH2 domain and the C-terminal diaphanous auto-regulatory domain. The remaining 3 fragments were not capable of providing sequence related information.

Next, we determined if Daam1 is expressed in HCFs by western blot. Daam1 was detected in lysates from cultured HCFs at a characteristic 123 kDa band, confirming its expression by these cells (Fig 1C). In addition, incubation of HCFs in 2ng/mL of TGF β 1 for 3 days significantly increased Daam1 expression by 1.8-fold ($p<0.001$), a similar result to what was observed in myofibroblasts in lung samples of idiopathic pulmonary arterial hypertension patients (Yanai et al., 2017), as well as total tissue gene expression analysis of kidney samples following injury (Saito et al., 2015). TGF β 1-induced differentiation of HCFs into myofibroblasts was confirmed by a well-characterized increase in expression of α -SMA (4.7-fold $p<0.05$). Our previously-published finding that USP10 expression increases in myofibroblasts was also found to be reproducible (1.9-fold increase, $p<0.05$; (Gillespie et al., 2017)).

Finally, because our model system includes treatment of HCFs with TGF β 1 to enhance protein expression and elucidate protein interactions, we investigated the effect of TGF β 1 treatment of integrin expression in HCFs over time (between 24 and 72hr; Fig 1D). Expression of α v, β 5, α -SMA were all found to significantly increase over this time course (2.1-fold, $p<0.01$; 2.8-fold, $p<0.05$; and 5.3-fold, $p<0.05$, respectively), whereas β 1 trended towards increase but was not significant. β 3-integrin expression did not change over this same time, as has been previously observed (Gillespie et al., 2017). Interestingly, α 5-integrin levels were reduced at 72hrs by 32% ($p<0.01$).

TGF β 1 treatment promotes Daam1-USP10 Proximity

To support the results of the yeast 2-hybrid screen, proximity ligation assay (PLA) was carried out in cultured HCFs either untreated or treated with 2ng/ml TGF β 1 for 3 days (Fig 2). After optimization of assay conditions, positive signal between USP10 and Daam1 was achieved in untreated cells, which appeared as bright, randomly dispersed puncta present through the cytoplasmic space (Fig 2A). Puncta are defined as a positive indicator of interaction of target proteins with a spatial resolution of <30-40 nm (Zatloukal et al., 2014). Signal was quantified by puncta density to analyze the change in interaction frequency. The USP10/Daam1 PLA pair increased in signal density between untreated and TGF β 1 treated cells by 1.2-fold ($p<0.05$) (Fig 2B). PLA signal was significantly reduced by omission of either primary antibody, suggesting the signal was not due to nonspecific interaction by oligo-conjugated secondary antibodies. The USP10/G3BP2 PLA pair in cells treated with TGF β 1 (which served as a positive control for the assay) produced the highest signal, with a 1.4-fold ($p<0.001$) higher puncta density than the USP10/Daam1 PLA signal without TGF β treatment and 1.2-fold ($p<0.05$) higher density than the USP10/Daam1 PLA signal in cells treated with TGF β . Overall, these data demonstrate that USP10 is in proximity to both Daam1 and G3BP2 and that TGF β increases the USP10/Daam1 interaction.

TGF β 1 drives USP10 and Daam1 localization to actin stress fibers

To determine the localization of Daam1 and USP10 in HCFs, cells were cultured without or with TGF β 1. Untreated HCFs immunostained with Daam1 antibody resulted in a diffuse and randomly-organized punctate fashion, scattered throughout the cell (Fig 3A). Following TGF β 1 treatment, as expected α -SMA antibody labeled cells demonstrated a characteristic filamentous stress fiber phenotype. Daam1 likewise exhibited a “ribbed” or filamentous pattern that corresponded with the presence of α -SMA stress fibers in cells. Omission of Daam1 primary

antibody abolished 488-fluorescence, suggesting staining is not due to non-specific secondary antibody labeling.

Treated and untreated HCFs were then immuno-labelled with USP10 antibody (with phalloidin used as a counter stain because of IgG host restrictions; Fig 3B). Like Daam1, USP10 was observed in a diffuse manner throughout the cytoplasm of untreated HCFs, with a slight perinuclear bias in intensity. Following TGF β 1 treatment, USP10 also underwent a shift in localization, with an increased coincidence with actin stress fibers.

We quantified this transition of USP10 and Daam1 fluorescence by measuring fluorescence intensity profiles across the perinuclear cytoplasm (Fig 3C), defining a threshold fluorescence level which segregated the cytoplasmic space from actin stress fibers as labeled by phalloidin, and then binning the corresponding fluorescence intensities in the Daam1 and USP10 channels into either “Cytoplasmic” or “Stress Fiber” group and averaging them. The Stress Fiber bin was divided by the Cytoplasmic bin to generate a Stress Fiber-to-Cytoplasmic intensity ratio for each cell, and averaged across all cells for statistical analysis (Fig 3D). We predicted that a random distribution of Daam1 and USP10 would result in Stress Fiber-to-Cytoplasmic ratios with values closer to 1 (meaning equal average intensity associated with stress fibers and cytoplasm), and that TGF β 1 would increase these values based on our observations. Daam1 stress fiber-to-cytoplasmic ratio was 1.22 in untreated cells, TGF β 1 treatment increased this ratio to 1.45 ($p < 0.001$). Thus, Daam1 transitioned from a distribution that was, on average, 1.22-fold higher at stress fibers than in cytoplasmic areas, to a distribution where Daam1 intensity was 1.45-fold higher at stress fibers than in the cytoplasm. USP10 exhibited a similar shift, changing from 0.97 in untreated cells to 1.25 in TGF β 1 treated cells ($p < 0.001$). Phalloidin, which by

definition should be much greater than 1 as it was used to label stress fibers, likewise experienced a shift of 2.85 to 4.11 ($p < 0.001$).

Daam1 siRNA reduces Daam1 and USP10 binding to stress fibers

In order to confirm the observation from the immunocytochemical analyses that Daam1 and USP10 associate with actin stress fibers, we utilized a protocol to isolate intact stress fibers from fibroblasts (Fig 4). Our hypothesis was two-fold: 1) if Daam1 and USP10 stress fiber localization is due to physical cytoskeleton-associated complex formation, both proteins should be coprecipitated with actin stress fibers, and 2) if Daam1 (a canonical actin binding protein) recruits USP10 to stress fibers, Daam1 knockdown will result in a concomitant reduction in USP10-stress fiber coprecipitation.

HCFs were transfected with either non-RISC associating siRNA (siGLO) or a pool of siRNAs against human Daam1 (siDaam1), and cultured with TGF β 1 for 3 days (to promote stress fiber formation). Images were taken of the cultures as they underwent washes in buffers with increasingly strong detergents to monitor extraction progress (Fig 4A). Cells became increasingly difficult to observe by phase contrast microscopy as the extraction process progressed, suggesting a decrease in cell density as non-stress fiber associated components were stripped away. Stress fibers remained visible and intact throughout the process, indicating proper preservation of this cytoskeletal element. Following collection of the stress fibers and enrichment by ultracentrifugation, precipitates were processed and analyzed by western blot (Fig 4B). Quantification is expressed as fold-changes after normalization to β -actin. The precipitates from both siGLO and siDaam1 transfections were rich in both β -actin and α -SMA, which developed with ≤ 1 second exposure times, suggesting proper enrichment of the actin

cytoskeleton. siDaam1 did not induce any significant changes in α -SMA levels as compared to siGLO ($p>0.05$). Conversely, the normally heavily abundant proteins GAPDH and α -tubulin was barely (or not at all) detectable, even with abnormally long exposure times (>30 sec, as compared to ~ 1 sec for total cell lysates in previous experiments). Precipitates from siGLO transfected cultures stained strongly for both Daam1 and USP10, suggesting they are physically associated with stress fibers. Transfection of siDaam1 reduced Daam1 co-precipitation with stress fibers by 87.0% ($p<0.001$). Further, siDaam1 transfection also resulted in a 60.1% reduction in USP10 coprecipitation with stress fibers ($p<0.001$). siDaam1 treatment did not significantly affect total USP10 levels in unfractionated lysates from HCFs (Fig 4C; $p>0.05$). These data suggest that Daam1 may either recruit USP10 to stress fibers or is at least in part necessary for its association.

Daam1 knockdown promotes integrin protein accumulation with reduced integrin ubiquitination

Because TGF β is a fibrotic growth factor, and we know from our work and others that USP10 and Daam1 are increased under fibrotic conditions, we reasoned that Daam1 was a positive regulator of USP10's DUB activity. However, in the following experiments, we found that the opposite result, our data support the idea that Daam1 negatively regulates USP10's DUB activity.

To assess the biological function of USP10-Daam1 interaction in HCFs, we analyzed integrin subunit protein levels without or with Daam1 knockdown by immunoblot analysis (Fig 5A), with quantification of densitometry normalized to GAPDH loading controls. In this experiment we continued to use TGF β 1 treatment of HCFs to increase total integrin protein

levels. siDaam1 transfection resulted in a 69.8% reduction in Daam1 levels vs siGLO ($p<0.001$). Daam1 knockdown increased total α v-, β 1-, and β 5-integrin levels in HCFs by 1.6-fold, 1.4-fold, and 1.4-fold, respectively, vs. siGLO controls ($p<0.05$ for each). Daam1 knockdown also elevated α 5-integrin levels by 1.3-fold, however this change was not significant ($p=0.065$), and no effect was observed on β 3-integrin levels, as had been seen previously on USP10's effect on β 3-integrin (Gillespie et al., 2017). Collectively, these results demonstrate that Daam1 regulates total levels of α v, β 1, and β 5 integrin protein expression. These data support the idea that Daam1 is negatively regulating USP10's DUB activity on integrins. We propose that knocking down Daam1 releases USP10 activity, leads to less ubiquitination and degradation of integrins, and therefore an increase in total integrin protein.

To test this, we utilized a sensitive ubiquitin capture ELISA (Ubiquant™), with this assay, treatment with TGF β was not necessary to obtain significant results. HCFs were transfected with either siGLO, siDaam1 siRNA, or as a positive control, human USP10 targeting siRNA (siUSP10) (Gillespie et al., 2017), and cultured for 3 days (Fig 5B). Compared to siGLO, siDaam1 transfection resulted in a 62.1% decrease in β 1-integrin ubiquitination ($p<0.05$), conversely, siUSP10 increased β 1-integrin ubiquitination by 1.2-fold ($p<0.05$). Similarly, daam1 knockdown decreased β 5-integrin ubiquitination by 87.9% ($p<0.001$), whereas USP10 knockdown increased β 5-integrin ubiquitination by 1.3-fold ($p<0.05$). The results with USP10 siRNA mirrored our previous published results (Gillespie et al., 2017). These data suggest that less Daam1 expression (siDaam1) leads to increased USP10 activity (removes more ubiquitin) and reduced integrin ubiquitination. Knockdown of USP10 (siUSP10) has the opposite effect. Together our data suggest that Daam1 is a negative regulator of USP10's DUB activity on integrins. To test the effect of siDaam1 on integrin gene expression, HCFs were transfected with

siGLO or siDaam1 and treated with TGF β for 3 days prior to processing for qPCR. The relative expression of Daam1, integrin α V, β 1, and β 5 is shown as compared to GAPDH. Whereas Daam1 knocked down by 50.7% ($p < 0.01$), the other relationships were not significant (Fig 5C), supporting the idea that the effect of Daam1 on integrins is post-translational.

Daam1 knockdown results in α v integrin and FN cell surface accumulation.

To further link the Daam1/USP10 interaction to integrins, we tested if Daam1 knockdown would increase integrin recycling, and a functional consequence of increase integrin recycling, FN recycling. For the integrin recycling assay, 48 hrs post-transfection cells were blocked and treated with Ab for 30 minutes prior to cell surface stripping for 30 sec. Cells were further incubated for 90 min prior to incubation with 2° Ab-488 for 30 min (Fig 6A). Using live cell confocal microscopy, cell surface integrin signal was imaged. In parallel with our western blot findings, whereas there was a significant increase in α v integrin recycling (2.1 fold $p < 0.05$), α 5 β 1 integrin recycling was not significantly different between conditions (Fig 6B,C).

Our recent work demonstrates that increased USP10 overexpression that leads to the accumulation of cell surface integrins, (Gillespie et al., 2017) also results in the integrin-mediated accumulation of extracellular FN. (Phillips et al., 2021) Furthermore, that in cell culture, approximately 1/3 of the deposited extracellular FN was derived from recycled FN (Phillips et al., 2021). To determine if Daam1 knockdown affects FN recycling, HCFs were transfected with siGLO or siDaam1. After 24 hours cells were treated with biotinylated-FN for 3 hours, prior to trypsinization to remove uninternalized biotinylated-FN and reseeding. After 48 hours, external FN was detected with Streptavidin-488 (green), and associated cells were detected with SiR-actin (red). In line with an increase in total and recycled integrin, FN

accumulation increased significantly in the siDaam1 condition, 1.4 fold $p < 0.01$ (Fig 6D,E). Fig 6F demonstrates that equal numbers of cells survived the siGLO and siDaam1 transfections.

Discussion

Integrins are dynamic molecules, and in general cells must have the ability to rapidly fine tune the localization or strength of focal contacts to carry out a diverse array of functions (Huttenlocher and Horwitz, 2011; Schmidt and Friedl, 2010). In order to achieve this, integrins are continuously in a state of flux, internalized into endosomes and either degraded by the endolysosomal system or shuttled back to the cell membrane in a regulated manner (Bridgewater et al., 2012). Our previous work identified USP10 as a critical regulator in the dynamic control of integrins, shifting the balance from intracellular degradation to cell surface accumulation after wounding (Gillespie et al., 2017). The present study further expands on that work by providing an additional point of control on integrin turnover through the novel interaction of USP10 with Daam1.

The formin, Daam1, has been well characterized as an effector of the actin cytoskeleton, influencing such processes as ciliogenesis (Corkins et al., 2019), filopodia extension (Jaiswal et al., 2013), and cell polarity (Ju et al., 2010; Nishimura et al., 2016), to name a few. Daam1 has been found to be upregulated in idiopathic pulmonary fibrosis (IPF) (Rydell-Tormanen et al., 2016), likely due to increased WNT signaling during the wound healing process (Konigshoff et al., 2008; Newman et al., 2016), linking Daam1 to fibrotic conditions. In addition, 4 SNP's have been identified in GWAS of Caucasian patients with IPF near the Daam1 gene, providing further evidence for a connection to fibrosis (Manichaikul et al., 2017). Furthermore, Daam1 may influence wound healing by promoting haptotaxis (Zhu et al., 2012) whereby epithelial cells and

fibroblasts migrate to the site of injury to repopulate and repair a wound (Basan et al., 2013; Blanco-Mezquita et al., 2013).

Lead by a yeast-two hybrid study using USP10 as bait, here we demonstrate a novel role for Daam1 as a binding partner of USP10 (Fig 1A), likely through an interaction with Daam1's FH2 domain (Fig 1B). Daam1 has numerous binding partners critical to its function, including the integrin-associated kinase Src (Aspenstrom et al., 2006), the Wnt-pathway associated protein Disheveled (Dvl) to the DAD of Daam1, and Rho-GTPase association with the GBD/FH3 subdomain (Liu et al., 2008). However, FH2 domain is canonically thought to directly associate with actin, and to facilitate actin polymerization (Higgs, 2005; Higgs and Peterson, 2005). Thus, our data suggests that Daam1 has a non-canonical role as a part of an USP10-Daam1 FH2 axis, linking Daam1 to integrin turnover and the ubiquitin system. This interaction was supported by proximity ligation assay data (Fig 2). We found that treatment with TGF β 1 enhanced the proximity between USP10 and Daam1 and promoted co-localization with stress fibers (Fig 2 and 3). Daam1 and USP10 localization in the absence of TGF β 1 was observed to be diffuse throughout the cell. USP10's localization was also perinuclear, an observation we confirm through quantification of the ratio fluorescence associated with stress fibers over the unassociated (cytoplasmic) with stress fibers. One interpretation of this result is that this interaction reflects a spatial aspect of USP10's function in integrin recycling, perhaps implicating localization to the perinuclear recycling compartment (PNRC). Another possibility is that USP10 associates with early (sorting) endosomes, responsible for facilitating the recycling of a number of membrane proteins, such as CFTR and integrins (Bomberger et al., 2009; Jonker et al., 2018)

The stress fiber localization of USP10 and Daam1 proteins was supported and expanded on by co-precipitation of these proteins during biochemical isolation of actin stress fibers from cells (Fig 4). siDaam1 knockdown reduced Daam1 and USP10 precipitation with stress fibers (Fig 4B), despite having no effect on total USP10 levels (Fig 4C) suggesting that Daam1 may recruit USP10 to stress fibers. siDaam1 treatment did not have a significant effect on α -SMA composition of stress fibers, suggesting changes in USP10 were not due to gross defects in stress fiber assembly. It also may be possible that other actin-associated proteins, such as Fascin-1 (Fig 1A), are in this complex with Daam1, tethering USP10 to actin stress fibers.

From the data in Figs 1-4, we postulated that Daam1 was necessary to “activate” USP10 because TGF β 1 treatment, a typical wounding model, increased association and our previous data showed that wounding and TGF β 1 treatment increased USP10 expression. We hypothesized that Daam1 was structurally necessary for USP10’s DUB activity. However, data in Fig 5 countered that model. Indeed, we found that in fact Daam1 is a “brake” for USP10 negatively regulating its activity. USP10 overexpression post-translationally increases integrin protein levels with no effect on gene expression (Gillespie et al., 2017). USP10 removes ubiquitin from the integrin β 1 and β 5 subunit and thus USP10 overexpression decreases integrin ubiquitination and degradation and integrins accumulate in the cell (Gillespie et al., 2017). Here, we reasoned that if Daam1 is a brake for USP10, that reducing Daam1 would increase USP10 activity, removing ubiquitin leading to increased protein levels (Fig 5A) and decreased ubiquitination of integrins β 1 and β 5 (Fig 5B). Daam1 knockdown had no effect on β 3-integrin, a result also found during the previous investigation into USP10’s effect on integrin levels (Gillespie et al., 2017). The integrin α v subunit was not tested because it is not ubiquitinated (Hsia et al., 2014; Lobert and Stenmark, 2010). In Fig 5C, we demonstrated that by qPCR after Daam1

knockdown, integrin subunits are not significantly changed, although they are trending towards an increase. These data suggest that the effect of siDaam1 in HCFs is primarily post-translational (through USP10's DUB activity), however it is possible that there is an indirect effect on gene transcription with siDaam1 (less Daam1 > more USP10 activity > more TGF β activity) (Gillespie et al., 2017) which eventually leads to an increase in integrin gene expression (Munger and Sheppard, 2011). Taken together, these data (Fig 5) support the hypothesis that Daam1/USP10 interaction is inhibitory to USP10's ability to deubiquitinate β 1- and β 5-integrin subunits.

Daam1 serving as a negative regulator of USP10 is further supported by an increase in integrin and FN recycling (Fig 6) after Daam1 knockdown. FN undergoes an extracellular stepwise integrin-dependent polymerization to generate fibrils from soluble, monomeric FN (Mao and Schwarzbauer, 2005; Pankov et al., 2019; Schwarzbauer and Sechler, 1999; Wierzbicka-Patynowski et al., 2004). α 5 β 1 and α v integrins recognize the common integrin-binding motif (RGD) in FN (Benito-Jardon et al., 2020; Danen and Sonnenberg, 2003; Huveneers et al., 2008) and coordinate to achieve efficient FN binding (Benito-Jardon et al., 2021; Bharadwaj et al., 2017). Using a live cell recycling assay, we demonstrated an increase in α v integrin α 5 β 1 integrin recycling, and integrin-mediated FN recycling after USP10 overexpression (Phillips et al., 2021). Here we find that after Daam1 knockdown, similar to USP10 overexpression and in line with integrin accumulation (Fig 5), an increase in α v integrin and extracellular FN recycling (Fig 6). We did not find a significant increase in α 5 protein levels (Fig 5A) or α 5 β 1 integrin recycling (Fig 6B) suggesting perhaps that the Daam1/USP10 axis more directly effects α v integrins.

In Fig 7, we propose a model that fits our data. First, although TGF β is frequently used as a wounding model, in general it upregulates protein expression of many proteins. In this context, both USP10 and Daam1 were upregulated with TGF β treatment. However, we found that Daam1 is a brake for USP10-mediated integrin protein accumulation, allowing for an additional layer of homeostatic control over total integrin levels in a wounding environment, possibly by creating a USP10 “sink” on stress fibers. TGF β treatment of HCFs appears to have elucidated this part of the USP10 life cycle.

In terms of positive regulators of the USP10/integrin axis, although it is currently unknown, studies indicate that the G3BP proteins could be activators of USP10 activity on integrins. In terms of G3BP/USP10 binding, in addition to our yeast-two hybrid study and PLA (Fig 1A, Fig 2), it was previously demonstrated that USP10 binds to G3PB2 in the cytosol, inducing p53 cytoplasmic localization, ubiquitination, and degradation (Takayama et al., 2018). Connecting G3BP to integrins is the finding that G3BP knockdown inhibits Scr/FAK/ERK signaling, and human lung cancer cell migration and invasion, suggesting a USP10/integrin/G3BP complex and coordination between these proteins (Zhang et al., 2013). Further studies will elucidate these interactions. In summary, our studies indicate that DUBs are a key point of regulation in the integrin life-cycle and may be therapeutic targets for a broad range of integrin-mediated pathologies.

Materials and Methods:

Cell Culture

Human cadaver corneas from unidentifiable diseased subjects were obtained from the Syracuse Eye Bank, Syracuse, NY and The Eye-Bank for Sight Restoration, New York City,

NY. The SUNY Upstate Medical University Institutional Review Board has informed us that, as described under Title 45 CFR Part 46 of the Code of Federal Regulations unidentifiable cadaver tissue does not constitute research in human subjects. Hence, the experiments performed in this report do not require their approval or waiver. Human corneal fibroblasts (HCFs) were isolated from cadaver corneas as previously described (Bernstein et al., 2007; Gillespie et al., 2017). HCFs were maintained with complete DMEM/F12 media (Gibco; Waltham, MA; Cat # 11330-032) with addition of 10% Fetal Bovine Serum (Atlanta Biologicals; Flowery Branch, GA) and 1X Antibiotic-Antimycotic (Sigma-Aldrich; St. Louis, MO). To prevent mycoplasma contamination, HCFs were also initially treated with Mycoplasma Removal Agent (Bio-Rad; Hercules, CA) after thawing from liquid nitrogen, and routinely with 10 µg/ml Plasmocin (Invivogen; San Diego, CA).

For all experiments, cells were passaged with TrypLE (Gibco; Waltham, MA), counted, and plated at either 2.5×10^4 cells/well on glass coverslips in 24 well plates, 1×10^5 cells per glass-bottom dish (for live cell imaging; MatTek; Ashland, MA) or 1.2×10^6 cells per 10 cm dish. Glass and plastic dishes were treated with 10 µg/ml Type I bovine collagen (Advanced BioMatrix; San Diego, CA) in PBS for >1hr at 37°C, then washed once before addition of HCFs. HCFs were then cultured in supplemented serum-free media (SSFM; 1 mM sodium pyruvate (Lonza; Basel, Switzerland), 2mM L-glutamine (Sigma-Aldrich; Waltham, MA), 1X MEM essential vitamin mixture (Gibco; Waltham, MA), 1X nonessential amino acid solution (Gibco; Waltham, MA), Insulin-Transferin-Selenium solution (Gibco; Waltham, MA), and 1X Antibiotic-Antimycotic solution, in DMEM/F12 media). TGFβ1 (R&D Systems; Minneapolis, MN) concentration for all relevant experiments was 2 ng/ml.

Plasmid transfection and siRNA treatment

All transfections (2 μ g cDNA or 15 pmol siRNA) were carried out using the Lonza Nucleofector X-module using 1×10^6 - 1.3×10^6 cells per transfection. Transfections were conducted with Lonza's P3 solution in 100 μ l cuvettes using program EN-130. Cells were then plated on collagen-coated 10 cm dishes for biochemical analyses or MatTek glass bottom dishes.

Immunocytochemistry

Cells were fixed using a protocol which is known to aid in the preservation of cytoskeletal morphology (Lunn et al., 1997; Smith-Clerc and Hinz, 2010). PHEM buffer (60mM PIPES, 25mM HEPES, 10mM EGTA, 2mM MgCl₂, pH 6.9) and 3% PFA in PBS were warmed to 37°C before fixation. Cells were briefly rinsed in PHEM buffer twice and then fixed in 3% PFA for 15 min. Cells were then washed 3 times for 5 minutes in PBS at room temperature to remove PFA, and blocked in 10% goat serum (Thermo Fisher; Waltham, MA), 0.2% Triton X-100 in PBS for 1hr at room temperature. Cultures were then stained with a solution of primary antibody in 2% goat serum, 0.2% Triton X-100 in PBS for 2hr at room temperature. Primary antibodies and their concentrations were as follows: 1:1000 mouse-anti- α -SMA (Millipore Sigma; Burlington, MA; clone ASM-1/1A4), 1:300 rabbit-anti-Daam1 (Proteintech; Rosemont, IL; Cat # 14876-1-AP), and 1:300 mouse-anti-USP10 (Novus Biologicals; Centennial, CO; clone OTI2E1). For some experiments, Alexa Flour 555-conjugated phalloidin (Cytoskeleton Inc.; Denver, CO) was utilized as a counterstain at a concentration of 14 nM. After primary incubation, cells were again washed 3 times in PBS for 5 min per wash. Alexa Fluor 488-conjugated goat-anti-rabbit (1:500) and Alexa Fluor 647-conjugated goat-anti-mouse (1:800) secondary antibodies (Jackson ImmunoResearch; West Grove, PA) were diluted in a solution consisting of 2% goat serum, 0.2% Triton X-100 in PBS and incubated on cultures for 1hr at

room temp. Cultures were then washed 3 times in PBS, and coverslips were mounted with ProLong Gold mounting media with DAPI (Invitrogen) to glass slides. Following curing of mounting media, coverslips were sealed with clear nail polish and prepared for imaging.

Proximity Ligation Assay

Duolink proximity ligation assay (Sigma) was carried out largely in congruence with manufacturer's protocol, with some adjustments made after reaction optimization. Cultures were washed and fixed as described above. Before blocking, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes, and blocked with the kit blocking solution supplemented with an addition of 10% goat serum, 0.2% Triton X-100, and 2 ng/ μ L herring sperm DNA (ThermoFisher; Waltham, MA) for 1hr at 37°C. Cultures were incubated with primary antibodies diluted in the proprietary Antibody Diluent solution plus addition of 2% goat serum and 0.2% Triton X-100. For comparison of cultures either untreated or treated with 2ng/ml TGF β 1, 1:300 rabbit-anti-USP10 (Cell Signaling Technologies; Danvers, MA; Clone D7A5) and 1:300 mouse-anti-Daam1 (Novus Biologicals; Centennial, CO; Cat # NP_055807) were utilized. For comparison of Daam1-USP10 interaction to positive control (G3BP2), the following additional antibodies were used: 1:300 mouse-anti-USP10 (Novus Biologicals; Centennial, CO; clone OTI2E1) and 1:300 rabbit-anti-G3BP2 (Novus Biologicals; Cat # NBP1-82977). Cultures were incubated in primary antibody solution at 4°C overnight. The following day, cultures were washed 3 times with Duolink wash buffer A for 5 minutes per wash. Secondary antibody diluted as per manufacturer's instructions (with addition of 2% goat serum and 0.2% Triton X-100) was applied and cultures incubated at 37°C for 1hr. Ligation and amplification/probe hybridization steps were carried out as per manufacturer's instructions. Following amplification/probe

hybridization step, cultures were washed with 1X Duolink wash buffer B for 10 minutes, and then twice with wash buffer A for 2 minutes each. HCFs were counterstained with 14nM Alexa Fluor-conjugated phalloidin in PBS for 1hr at room temperature, then washed twice with Wash Buffer A for 1 minute each. Cells were then briefly rinsed in 0.01X Wash Buffer B, and mounted as described above.

Cell Imaging and Analysis

Epifluorescent imaging was carried out using a Nikon Eclipse Ni upright fluorescence microscope with an Andor Zyla camera and NIS-Elements software (Nikon Instruments Inc.; Melville, NY). Live cell imaging was carried out using a Zeiss LSM780 confocal microscope, outfitted with a temperature and humidity-controlled chamber. Confocal images were acquired using ZEN software (Zeiss; Oberkochen, Germany). Image analysis, including immunoblots, was carried out using FIJI (ImageJ) software.

qPCR

Cells were collected from culture dishes using trypsin. Total RNA was extracted from the corneal fibroblasts using the PureLink RNA kit (Invitrogen) according to manufacturer's recommendations and was added as a template into a one-step multiplex qRT-PCR assay using Quanta qScript XLT ToughMix with ROX dye (VWR). For that, 1uL of total RNA was mixed with the reagent and primer-probe mixes for human Daam1, α v, β 1, β 5, and reference gene GAPDH in a 10uL reaction. The cycling parameters were as recommended by Quanta.

Immunoblotting

HCFs grown on 10cm tissue-culture dishes were washed once in PBS and scraped from plates in RIPA buffer (0.1% SDS, 0.15 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100 in 0.05 M Tris (pH 6.8)) or a general solubilization buffer (2% SDS in 25 mM Tris (pH 8.8)) with 2mM PMSF and cOmplete protease inhibitor tablet. Lysates were then homogenized through a 26-gauge syringe 3 times and protein content measured by BCA (ThermoFisher; Waltham, MA). Samples were then diluted in 4X Sample Buffer (2% SDS, 10% glycerol, 1% β -mercaptoethanol, 12.5 mM EDTA, 0.02 % bromophenol blue in 50mM Tris (pH 6.8)), boiled at 95°C for 5 minutes, and loaded in pre-cast, 10% polyacrylamide gels (ThermoFisher; Waltham, MA). SDS-PAGE was carried out at 100V for 20 minutes for stacking phase and then 200V until completion.

Upon completion, polyacrylamide gels were transferred to Polyvinylidene fluoride (PVDF) membranes at 100V for 1hr. Membranes were then blocked for 1hr in tris-buffered saline with Tween-20 (TBST; Tris, sodium chloride, 0.1% Tween) with 5% goat serum and 1% bovine serum albumin (BSA) with gentle agitation. Following blocking, membranes were incubated in primary antibody solution consisting of 2% goat serum, 0.4% BSA in TBST, overnight at 4°C on a rocker. Antibodies used in this study are as follows: 1:1000 mouse-anti-USP10 (Novus Biologicals; Centennial, CO; clone OT12E1), 1:1000 rabbit-anti-Daam1 (Proteintech; Rosemont, IL; Cat # 14876-1-AP), 1:2000 rabbit-anti-GAPDH (Cell Signaling Technologies, 14C10), 1:2000 mouse-anti- α -tubulin (Cell Signaling Technologies, Danvers, MA; clone DM1A), 1:1000 mouse-anti- α -SMA (Millipore; Burlington, MA; Clone ASM-1), 1:2000 mouse-anti- β -actin (Cell Signaling Technologies; Danvers, MA; cat #: 3700), 1:1000 rabbit-anti- α v-integrin (Cell Signaling Technologies; Danvers, MA; cat #: 4711), 1:1000 rabbit-anti- α 5-integrin (Cell Signaling Technologies; Danvers, MA; cat #: 4705), 1:1000 rabbit-anti- β 1-

integrin (Cell Signaling Technologies, Danvers, MA; cat #: 4706), 1:1000 rabbit-anti- β 3-integrin (Cell Signaling Technologies, D7X3P-XP), and 1:1000 rabbit-anti- β 5-integrin (Cell Signaling Technologies; Danvers, MA; clone D24A5). Subsequently, membranes were washed 3 times in TBST. Secondary antibodies were diluted in TBST with 2% goat serum, 0.4% BSA and incubated on membranes for 1hr at room temperature. Secondary antibodies were 1:3000 dilutions of horseradish peroxidase-conjugated goat-anti-mouse (Jackson ImmunoResearch; West Grove, PA) and goat-anti-rabbit (EMD-Millipore; Burlington, MA). Membranes were washed 3 final times with TBST, and then incubated with chemiluminescent substrate (Thermo Fisher; Waltham, MA) briefly before imaging with a Chemidoc (Bio-Rad; Hercules, CA). Relative protein intensities were measured by ImageJ software.

Stress Fiber Isolation

Stress Fiber Isolation protocol was based on previously published work by other groups (Kato et al., 1998). Cells were washed briefly with chilled PBS and incubated in a solution of 2.5mM triethanolamine (pH 8.1) with cOmplete protease inhibitor and 2mM phenylmethylsulfonylfluoride (PMSF) for 20 minutes on ice and with gentle rocking. This solution was aspirated and replaced with 0.05% NP-40 in PBS with cOmplete protease inhibitor and 2mM PMSF for 10 min. Finally, this solution was aspirated and replaced with 0.5% Triton X-100 in PBS with cOmplete protease inhibitor and 2mM PMSF for 5 min. The last wash was aspirated and replaced with 200 μ L of fresh 0.5% Triton buffer, and the stress fibers scraped and collected in microcentrifuge tubes. The lysates were passed 3 times through 26-gauge syringes and centrifuged at 100,000 x g for 1hr at 4°C. The supernatants were discarded and the pellets resuspended in solubilization buffer (2% SDS, 8M urea in 25mM Tris (pH 8.8) with 2mM PMSF

and cOmplete protease inhibitor tablet). Final lysates were subjected to 1 freeze-thaw cycle before use in BCA and western blot, which aided the homogenization of cytoskeletal components. Lysates were then analyzed by immunoblot as described above.

Ubiquant™ Ubiquitin Capture ELISA

Ubiquant™ analysis was carried out as described previously (Gillespie et al., 2017). HCFs were transfected with 150 pmol of siGLO nontargeting control siRNA (Dharmacon; Lafayette, CO), siDaam1, or siUSP10 (both: Santa Cruz Biotechnology; Dallas, TX) and incubated in SSFM on collagen-coated 10cm cell culture treated dishes for 24hrs. HCFs were then switched into SSFM with 5 μ M MG132 and 10 μ M chloroquine for 8hrs. Cultures were then scraped in RIPA buffer (as described above) plus complete protease inhibitor tablet (Roche; Basel, Switzerland), PMSF (Thermo Fisher; Waltham, MA), 2mM NEM (Pierce; Waltham, MA) and 10 μ g/ml PR-619 (Lifesensors; Malvern, PA). Protein content determined by BCA assay and samples were diluted in RIPA buffer to 0.4 μ g/ μ L.

100 μ L of lysate was added to each well (experiments carried out in triplicate) and incubated on a rocker at room temperature for 1hr. Wells were then washed 4 times with TBST and then incubated in 1x Blocking Buffer in PBS with a 1:10,000 dilutions of either β 1- (Assay Biotechnology; San Francisco, CA; catalogue #: R12-2927) or β 5-integrin (Assay Biotechnology; San Francisco, CA; catalogue #: F-5) primary antibody for 1 hr at room temperature. Wells were washed again 4 times with TBST and incubated in the same buffer with a 1:15,000 dilution of goat anti-rabbit HRP-conjugated IgG for 1hr. Wells were washed a final 4 times, with 100 μ L Developing Solution added to each well, and luminescence read by Epoch II plate reader and Gen5 software (BioTek Instruments; Winooski, VT).

Biotinylated-FN Recycling Assay

HCFs were transfected (see above) with 150 pmol of either siGLO or siDaam1, then seeded in DMEM/F12 and 1% serum plus antibiotic-antimycotic solution. 24 hours post transfection, the cells were loaded with 10ug biotinylated-FN (Tang and Saito, 2017) for 3 hours. Cells were then passaged with trypsin and plated on 35mm glass bottom dishes in DMEM/F12 and 1% serum. After 48 hours, cells were washed 3 times for 30 minutes each prior to imaging with the following procedure: 1% PBSA in PHEM, 150mM Sodium Azide in PHEM, and 1:100 streptavidin-488 in PHEM. Images were analyzed using ImageJ's 3D Object Counter plugin.

Integrin Recycling Assay

HCFs were transfected (see above) with 150 pmol of either siGLO or siDaam1, then seeded in DMEM/F12 and 1% serum plus antibiotic-antimycotic solution 48 hrs post-transfection cells were blocked and treated with Ab against $\alpha 5\beta 1$ (Novus, 2-52680) and αv (Cell Signaling 4711) at 10ug/ml for 30 minutes prior to cell surface stripping (0.2M acetic acid, 0.5M NaCl) for 30 sec. Cell were incubated for 90 min prior to incubation with 2° Ab-488 for 30 min. Live cells were imaged (Zeiss LSM 780 confocal) and analyzed using ImageJ's Analyze Particles.

Data and Statistical Analyses

All data was collected using Microsoft Excel software and graphs generated using GraphPad Prism software. Student's unpaired, 2-tailed t-tests, logarithmic transformation, one-way ANOVA were calculated using Prism. Replicate number (n) refers to individual biological repeats (cell lines) derived from distinct human cadaver corneas. P-values: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, **** $p < 0.0001$.

Competing Interests

No competing interests declared.

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Figure 1 – Yeast 2-Hybrid screen reveals Daam1 as a novel USP10-binding partner. A) Yeast 2-hybrid data (Hybrigenics), human placental mRNA library, and human USP10 as bait. B) Daam1 subdomain schematic and yeast 2-hybrid fragment data. C) Immunoblot with quantification of untreated and TGFβ1 treated HCFs (72 hrs). Quantification is expressed as the mean fold change after normalization to GAPDH. D) Comparison of HCF lysates treated for 24 and 72hrs with TGFβ1. N=3. Statistical significance was calculated using an unpaired t-test +/- SEM.

Figure 2 – Proximity Ligation Assay supports USP10-Daam1 interaction in HCFs. A) Representative images of untreated and TGFβ1 treated HCFs (PLA, green), counterstained for actin (phalloidin, red) and nucleus (DAPI, blue). B) Quantification of puncta density in each condition. Stroked portions of the merged channels are magnified (Merge Magnified). Bar=50μm. A total of 15 cells were analyzed for each condition from 3 independent experiments. Statistical significance was calculated using one-way ANOVA after log transformation of the data +/- SEM.

Figure 3 – TGFβ1 Drives Daam1 and USP10 Localization Toward Actin Stress Fibers. A) Analysis of untreated and TGFβ1-treated HCFs, with DAPI (blue), α-SMA (red), and Daam1 (green) immunostaining. TGFβ1 treatment drives Daam1 immunofluorescence from a diffuse, punctate distribution to a stress fiber pattern. IgG control abolished immunofluorescence signal. B) Immunostaining for USP10 (violet), phalloidin (red), Daam1 (green). Like Daam1, TGFβ1 treatment drives USP10 immunofluorescence from a diffuse pattern to a stress fiber pattern. C) Fluorescence intensity profiling across representative cells (from panel B) in scatterplot format. D) Quantification of multiple fluorescence intensity profiles, comparing untreated (black) and TGFβ1-treated cells (grey), expressed as a ratio of fluorescence intensity at stress fibers to the intensity between stress fibers (cytoplasmic). An increase in stress fiber association with TGFβ1 was observed for all three proteins. Statistical significance was calculated using an unpaired t-test +/- SEM. Bar= 50μm. N=3

Figure 4 –Daam1 and USP10 precipitate with stress fibers. A) Representative phase-contrast images of TGFβ1 treated HCFs subjected to a series of buffer washes with increasing concentration of detergent. Bar=200μm B) Immunoblot analysis of stress fiber isolates generated from TGFβ1 treated cells transfected with nontargeting siGLO or siDaam1. Daam1 knockdown also reduced USP10 association with stress fibers. C) Immunoblot analysis of total protein from HCFs transfected with siGLO or siDaam1. siDaam1 did not significantly affect total USP10 levels. Statistical significance was calculated using an unpaired t-test +/- SEM. N=3

Figure 5 – Effect of Daam1 knockdown on integrins A) HCFs were transfected with siDaam1 or siGLO and treated with TGFβ for 3 days to increase integrin expression. siDaam1 transfection resulted in increased expression of αv-, β1-, β5-integrins. N=6. B) HCFs were transfected with siDaam1 or siGLO. After 3 days cells were lysed and subjected to Ubiquant™ ubiquitin capture ELISA. siDaam1 reduced, whereas siUSP10 increased ubiquitination of β1 and β5. N=4. (C) HCFs were transfected with siGLO or siDaam1 and treated with TGFβ for 3 days prior to processing for qPCR. The relative expression of Daam1, integrin αV, β1, and β5 is shown as

compared to GAPDH. Statistical significance was calculated using an unpaired t-test
Bar=200 μ m. N=3.

Figure 6 – Knockdown of Daam1 increases integrin and fibronectin (FN) recycling. A) Live cell integrin recycling assay. 48 hrs post-transfection cells were blocked and treated with Ab against $\alpha 5\beta 1$ or αv at 10ug/ml for 30 minutes prior to cell surface stripping for 30 sec. Cells were incubated for 90 min prior to incubation with 2° Ab-488 for 30 min. Bar=50 μ m. Quantification of recycled (B) Integrin $\alpha 5\beta 1$ and (C) Integrin αV , N=3 with a total of 15 images analyzed for each condition. (D) Live cell FN recycling assay. HCFs were transfected as stated above. 24hrs post transfection HCFs were loaded with biotinylated-FN for 3 hours. After trypsinization (to separate cells from extracellular, non-internalized FN), HCFs were replated and imaged 48 hours post transfection. Prior to imaging, cells were incubated with streptavidin-488 to detect only recycled biotinylated-FN. Imaged by live cell confocal. Bar= 50 μ m. N=3. D) Quantification of extracellular FN. A total of 15 cells were analyzed for each condition from 3 independent experiments. E) Cell numbers 2 days after transfection for each condition are not significantly different. N=3. Image analysis was done using ImageJ's Analyze Particles function. Statistical significance was calculated using an unpaired t-test +/- SEM.

Figure 7 – Working model. In response to stress-induced TGF β 1 release, both USP10 and Daam1 expression is increased. Daam1 sequesters USP10 to stress fibers, and USP10's DUB activity on integrins is inhibited by Daam1. This may act as a level of control over USP10 activity in myofibroblasts. We further hypothesize that the known interaction of USP10 and G3BP1/2 proteins (also shown in Fig 1) positively regulates USP10's DUB activity on integrins leading to integrin accumulation. The previously known roles of G3BP1/2 in integrin signaling support this concept.

A.

Name	NCBI Ref #	Protein Full Name	# Hits	Confidence	Function
G3BP1	NM_005754.2	Ras GTPase-activating protein-binding protein 1	78	Very High (78/78)	Stress granule formation Endoribonuclease activity Helicase activity
G3BP2	NM_203504	Ras GTPase-activating protein-binding protein 2	207	Very High (207/207)	Stress granule formation mRNA-binding
G3BP2 var 2	NM_012297	Ras GTPase-activating protein-binding protein 2 variant 2	12	High (12/12)	Stress granule formation mRNA-binding
Daam1	NM_001270520.1	Disheveled-associated Activator of Morphogenesis 1	15	Very High (13/15)	Actin elongation, organization, bundling Stress fiber Formation
FSCN-1	NM_003088	Fascin-1	2	Good (2/2)	Actin bundling

B.

Transcript Nucleotide #	Corresponding Amino Acid	Domains	Hits/Total
1830-3998	623-1068	FH2, DAD, and 3'UTR (Transcript)	(1/15)
1977-3174	611-1010	FH2	(1/15)
2106-3184	654-1013	FH2	(8/15)
2229-3187	695-1014	FH2	(1/15)
2232-3503	696-1068	FH2, DAD, and 3'UTR (Transcript)	(1/15)

C.

2 ng/ml TGFβ1: - +

D.

Post-TGFβ: 24 72 hr

Figure 1

Figure 2

Figure 3

Figure 4

A. **B.** **C.** **Figure 5**

Figure 6

Figure 7