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Journal

Molecular Biology of the Cell, 25(5)

ISSN

1059-1524

Authors

Lee, Ting-Hein
McKleroy, William
Khalifeh-Soltani, Amin
et al.

Publication Date

2014-03-01

DOI

10.1091/mbc.e13-07-0382

Peer reviewed

Functional genomic screen identifies novel mediators of collagen uptake

Ting-Hein Lee[a,b], William McKleroy[a,b], Amin Khalifeh-Soltani[a,b], Stephen Sakuma[a,b], Stanislav Lazarev[b,c], Kirsi Riento[d], Stephen L. Nishimura[e], Ben J. Nichols[d], and Kamran Atabai[a,b,c]

[a]Cardiovascular Research Institute, [b]Department of Medicine, [c]Lung Biology Center, and [e]Department of Pathology, University of California, San Francisco, San Francisco, CA 94158; [d]MRC Laboratory of Molecular Biology, Cambridge CB2 0QH, United Kingdom

ABSTRACT **Tissue fibrosis occurs when matrix production outpaces matrix degradation. Degradation of collagen, the main component of fibrotic tissue, is mediated through an extracellular proteolytic pathway and intracellular pathway of cellular uptake and lysosomal digestion. Recent studies demonstrate that disruption of the intracellular pathways can exacerbate fibrosis. These pathways are poorly characterized. Here we identify novel mediators of the intracellular pathway of collagen turnover through a genome-wide RNA interference screen in *Drosophila* S2 cells. Screening of 7505 *Drosophila* genes conserved among metazoans identified 22 genes that were required for efficient internalization of type I collagen. These included proteins involved in vesicle transport, the actin cytoskeleton, and signal transduction. We show further that the flotillin genes have a conserved and central role in collagen uptake in *Drosophila* and human cells. Short hairpin RNA–mediated silencing of flotillins in human monocyte and fibroblasts impaired collagen uptake by promoting lysosomal degradation of the endocytic collagen receptors uPARAP/Endo180 and mannose receptor. These data provide an initial characterization of intracellular pathways of collagen turnover and identify the flotillin genes as critical regulators of this process. A better understanding of these pathways may lead to novel therapies that reduce fibrosis by increasing collagen turnover.**

Monitoring Editor
Tamotsu Yoshimori
Osaka University

Received: Jul 22, 2013
Revised: Dec 24, 2013
Accepted: Dec 26, 2013

INTRODUCTION

Fibrosis is a common pathological response that leads to significant morbidity and mortality by distorting normal tissue architecture and resulting in impaired organ function (Friedman *et al.*, 2013). Although fibrosis develops in response to a variety of stimuli, the persistence of fibrotic tissue can serve as a nidus that promotes further matrix deposition through stiffness-mediated activation of collagen production (Liu *et al.*, 2010; Zhou *et al.*, 2013). Fibrosis is also commonly associated with malignancies, where it is believed to promote tumor progression in part through signals generated by the mechanical stiffness characteristic of areas of fibrosis (Levental *et al.*, 2009; Calvo *et al.*, 2013).

Collagen is the main component of fibrotic tissue. Although collagen is continuously produced and degraded, the rates of production and degradation are precisely balanced to prevent the development of fibrosis (Barnes *et al.*, 1970; Bradley *et al.*, 1975) and preserve normal tissue architecture. With tissue injury there is an increase in the rates of both collagen production and degradation (Atabai *et al.*, 2009). An imbalance favoring collagen production is a prerequisite for the development of fibrosis (McKleroy *et al.*, 2013). Although many of the molecular pathways that induce excess collagen production have been described (Ratziu *et al.*, 1998; Munger *et al.*, 1999; Tager *et al.*, 2008; Wilson *et al.*, 2010; Li *et al.*, 2011), there is a limited understanding of the degradation pathways that normally serve to limit or prevent the development of fibrosis (McKleroy *et al.*, 2013).

The degradation of fibrillar collagens that predominate in areas of fibrosis is a two-step process that involves proteolytic cleavage (Pardo and Selman, 2006) in the extracellular space, followed by cell-mediated uptake of collagen fragments for lysosomal digestion

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E13-07-0382) on January 8, 2014.

Address correspondence to: Kamran Atabai (Kamran.Atabai@UCSF.edu).

Abbreviations used: ANOVA, one-way analysis of variance; DAVID, Database for Annotation, Visualization and Integrated Discovery; dsRNA, double-stranded RNA; DSS, disuccinimidyl suberate; FAP, fibroblast activation protein; FITC, fluorescein isothiocyanate; FL, full length; FLOT1, flotillin-1; FLOT2, flotillin-2; MR, mannose receptor; MRC, Medical Research Council; RNAi, RNA interference; S2, Schneider 2; shRNA, short hairpin RNA; TB, trypan blue.

(Everts *et al.*, 1996). Although the proteolytic enzymes that cleave collagen in the extracellular matrix have been extensively studied (Zuo *et al.*, 2002; Hayashidani *et al.*, 2003; Buhling *et al.*, 2004; Yamashita *et al.*, 2011), relatively little is known about the molecular pathways that underlie cell-mediated collagen turnover. The efficient removal of collagen fragments from the extracellular compartment is important in preventing acute inflammation secondary to recruitment of neutrophils (Riley *et al.*, 1988; Weathington *et al.*, 2006) and chronic inflammation secondary to induction of autoimmune disease (Trentham *et al.*, 1978; Schrier *et al.*, 1982). The importance of the intracellular pathways in limiting the severity of fibrosis is apparent in work showing that mice deficient in the collagen endocytic receptor uPARAP/Endo180 (Endo 180) and the collagen opsin Mfge8 develop exaggerated lung fibrosis after injury due to impaired cell-mediated collagen turnover (Atabai *et al.*, 2009; Bundesmann *et al.*, 2012).

We were interested in better understanding the molecular pathways that govern cell-mediated collagen turnover. We therefore designed a high-throughput screen examining the effect of RNA interference (RNAi)–mediated silencing of *Drosophila* genes that have metazoan orthologues on the internalization of a fluorescein isothiocyanate (FITC)–conjugated collagen type I by *Drosophila* S2 cells. In this work, we identify a number of genes that are required for efficient internalization of type I collagen. We further explore the mechanism by which the flotillin genes regulate collagen turnover in *Drosophila* and human cells.

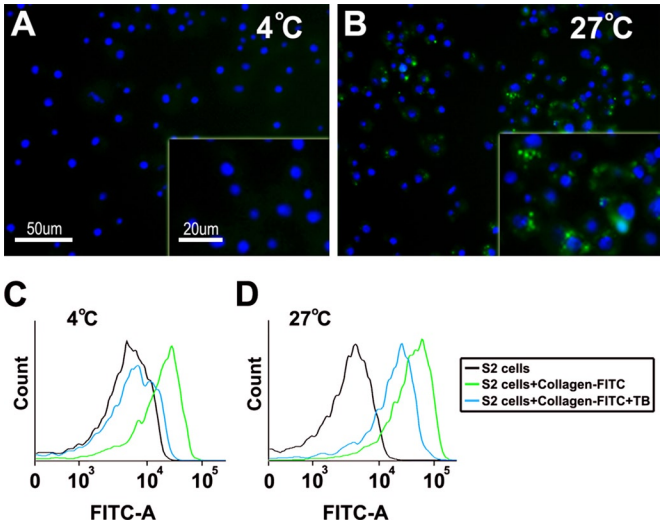

FIGURE 1: Flow cytometry–based collagen uptake assay in *Drosophila* S2. (A–D) S2 cells incubated with FITC-conjugated collagen type I at 4°C (A, C) or 27°C (B, D) for 3 h with the addition of trypan blue (TB) before fluorescence microscopy (A, B) or flow cytometry (C, D).

RESULTS

As an unbiased approach to identifying novel mediators of the intracellular pathways of collagen turnover, we designed an RNAi-based genomic high-throughput screen of collagen internalization using flow cytometry. We chose S2 cells because of their similarity with mammalian phagocytes and the efficiency of gene knockdown by double-stranded RNA (dsRNA) in these cells (Stroschein-Stevenson *et al.*, 2006). The RNAi library consisted of dsRNAs targeting 7505 *Drosophila* genes with murine and human orthologues and has been previously described (Foley and O'Farrell, 2004; Cheng *et al.*, 2005; Stroschein-Stevenson *et al.*, 2006; D'Ambrosio and Vale, 2010). We selected the *Drosophila* system because we were interested in identifying conserved pathways of collagen turnover. The screen consisted of coculturing RNAi-treated S2 cells with a FITC-conjugated collagen type I and measuring internalized collagen by flow cytometry (Figure 1, A–D, and Supplemental Figure S1, A–C) after quenching the FITC signal of bound (but not internalized collagen) with trypan blue.

In the preliminary screen, 4.8% (365/7505) of silenced genes caused a 1.5-SD decrease in collagen uptake (Supplemental Table S1). The absolute reduction in collagen uptake for the majority of these hits ranged from 20 to 40% (Supplemental Table S1). To further validate the ability of the screen to distinguish biological signals from background noise, we used the DAVID Bioinformatics program (Huang da *et al.*, 2009). We evaluated for enrichment of functionally related gene groups in the 365 hits as compared with a similar number of randomly selected genes from the screen. As shown in Supplemental Table S2, there were multiple gene groups with high enrichment scores in the 365 genes identified by the screen as compared with the control genes. This analysis indicated that the screen was sufficiently optimized to identify functionally related gene groups rather than a random group of genes.

We next set out to rescreen a selected number of the genes identified in the preliminary screen. We excluded 163 genes from further analysis because of their marked effects on cell shape or growth or because they were involved in cell cycle, transcription, or translation processes (Supplemental Table S3). We designed new dsRNA sequences for the remaining 202 genes. We then repeated the collagen uptake assay three independent times in S2 cells treated with the new dsRNA sequences, as well as with a number of control sequences that did not affect collagen uptake (Supplemental Table S1). We identified candidate genes as those that resulted in >25% reduction in collagen uptake after the rescreening process. Twenty-two genes met these criteria, including genes involved in vesicle function, signal transduction, actin binding, and proteolysis (Figure 2, A and B).

We then selected several genes for further follow-up in human cells. We used short hairpin RNA (shRNA) to knock down these genes in human U937 cells and quantified the effect on collagen uptake (Supplemental Figure S2, A–C, and Supplemental Table S4). From this analysis, we chose the flotillin genes for more in-depth analysis. Flotillins are a family of two cytosolic proteins, flotillin-1 and 2, that are associated with the inner plasma membrane, as well as with the Golgi and endosomal vesicles, and have roles in protein trafficking (Otto and Nichols, 2011; Pust *et al.*, 2013). Flotillin-1 and 2 have been reported to regulate podosome-mediated matrix degradation through interactions with the kinesin motor family member KIF9 (Cornfine *et al.*, 2011). Flotillins are also overexpressed in malignancies, and flotillin-2–deficient mice are protected from breast cancer cell metastases to the lung in vivo (Berger *et al.*, 2013).

shRNA knockdown of flotillin-1 and 2 resulted in a marked reduction in collagen uptake by U937 cells (Figure 3A and Supplemental Figure S2). The reduction in collagen uptake was of the same magnitude as that observed with shRNA-mediated knockdown of the collagen endocytic receptor Endo180 (Figure 3A and Supplemental Figure S2; Engelholm *et al.*, 2003; Curino *et al.*, 2005; Madsen *et al.*, 2011). Given that flotillins regulate receptor recycling and stability (Solis *et al.*, 2012), we evaluated the effect of flotillin knockdown on protein expression of Endo180. Both flotillin-1 and flotillin-2 knockdown caused a marked reduction in Endo180 protein expression without affecting Endo180 transcript levels in U937 cells (Figure 3, B–D). To ensure that the effects on Endo180 were not the result of

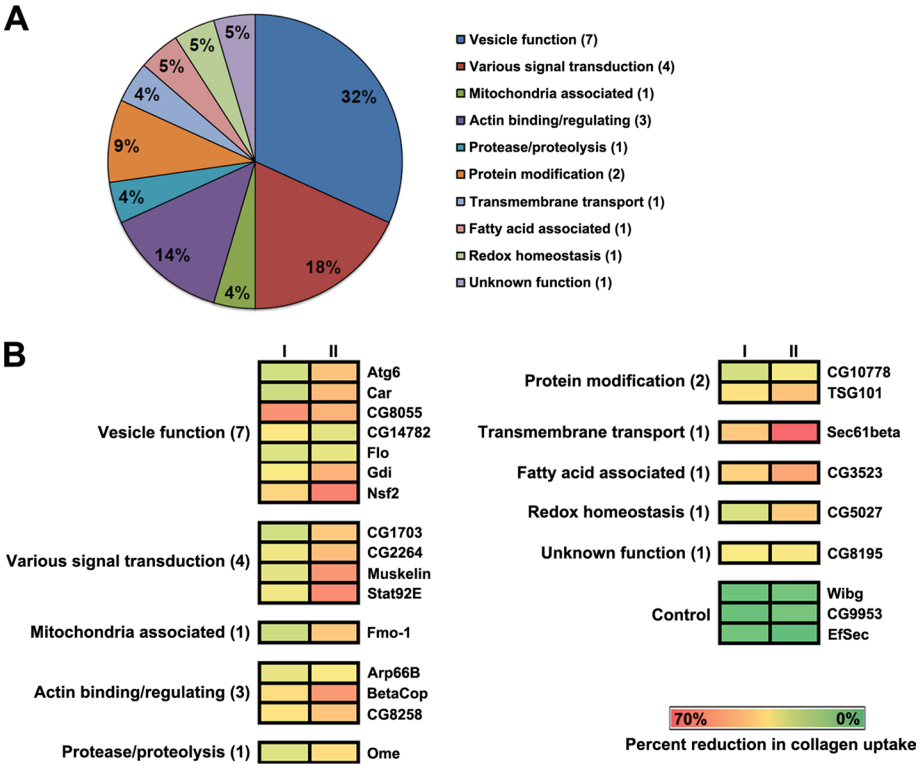

FIGURE 2: Identification of genes that when silenced reduce collagen uptake. (A) Twenty-two *Drosophila* genes that when silenced resulted in >25% reduction in collagen uptake. (B) Percentage reduction in collagen uptake relative to control for each gene using two independent dsRNA sequences (I and II). Each experiment was repeated three independent times.

off-target effects of flotillin-1 or 2 shRNA, we created myc-tagged flotillin-1 and 2 "rescue" cDNA constructs with mutations that prevented recognition of these constructs by the flotillin-1 and 2 shRNAs (Supplemental Table S4) we were using. We then expressed the flotillin-1 rescue construct in flotillin-1 shRNA–treated U937 cells and the flotillin-2 rescue construct in flotillin-2 shRNA–treated U937 cells. These constructs successfully increased flotillin-1 and 2 protein expression and rescued the decrease in Endo180 protein expression and collagen uptake (Figure 3, E–G).

We were next interested in determining whether the effect of flotillins was specific for collagens or could be generalized to other matrix molecules. As with collagen type I, knockdown of flotillin-1 or 2 reduced uptake of type IV collagen, and this effect was rescued by expressing the "rescue" cDNAs (Figure 3H). Of interest, flotillin-1 or 2 knockdown did not affect uptake of fibronectin or laminin, suggesting that this pathway is specific for collagens (Figure 3, I and J).

In addition to macrophages, fibroblasts are the main cell type responsible for collagen turnover (Everts *et al.*, 1996). We therefore examined whether shRNA-mediated knockdown of flotillin-1 and 2 in primary human lung fibroblasts modulated collagen turnover. Flotillin-2 knockdown reduced collagen uptake and Endo180 protein expression in primary fibroblasts (Figure 4, A and B). The reduction in collagen uptake was rescued by expression of "rescue" flotillin-2 cDNA (Figure 4C). Of interest, flotillin-1 knockdown did not significantly reduce collagen turnover or Endo180 protein expression in primary lung fibroblasts (Figure 4, A and B).

Flotillins-1 and 2 can form hetero- or homo-oligomers. Gene silencing of either flotillin has been reported to reduce expression of the other flotillin (Babuke *et al.*, 2009; Ludwig *et al.*, 2010; Otto and Nichols, 2011). We therefore evaluated whether the inability of flotillin-1 knockdown to reduce collagen uptake in fibroblasts while efficiently reducing collagen uptake in U937 cells was related to differential effects of flotillin-1 knockdown on flotillin-2 protein expression in each cell type. Of interest, flotillin-1 caused a greater reduction of flotillin-2 levels in U937 cells as compared with fibroblasts (Figure 4D). These data suggest that the residual flotillin-2 protein in fibroblasts after flotillin-1 knockdown was functionally sufficient to coordinate efficient collagen turnover. To determine whether flotillin-2 existed as homo-oligomers after flotillin-1 knockdown, we incubated U937 cells with the cross-linking agent disuccinimidyl suberate (DSS) and evaluated the size of flotillin-2 bands by gel electrophoresis and Western blot. We found a predominant band at ~200 kDa, indicating the presence of flotillin-2 homo-oligomers (Figure 4E).

We next set out to determine whether the differential effect on collagen uptake and Endo180 protein expression after shRNA-mediated flotillin-1 knockdown in primary fibroblasts and U937 cells was apparent in cells from mice deficient in flotillin-1 (*flotillin-1*$^{-/-}$). We evaluated collagen uptake and Endo180 expression in *flotillin-1*$^{-/-}$ mouse embryonic fibroblasts and splenic monocytes. Consistent with our findings with shRNA knockdown, *flotillin-1*$^{-/-}$ monocytes (Figure 5, A–C) but not *flotillin-1*$^{-/-}$ fibroblasts (Figure 5, D and E) had a reduction in collagen uptake and Endo180 expression.

To determine whether flotillin knockdown affected collagenase activity, we measured MMP2 and MMP2 transcript levels. Flotillin knockdown did not alter expression of either enzyme in U937 cells or fibroblast (Supplemental Figure S3, A and B). Furthermore, we did not find differences in enzymatic activity by zymography (Supplemental Figure S3, C and D). Procollagen I and III transcripts were also unaffected by flotillin knockdown in U937 cells or fibroblasts (Supplemental Figure S3, E–G).

To demonstrate an intracellular association between flotillin-2 and Endo180, we overexpressed a Myc-tagged flotillin-2 construct in primary human lung fibroblasts. We then coimmunoprecipitated Endo180 protein after pulling down flotillin-2 with an anti-Myc antibody (Figure 6A). We also colocalized the two proteins by immunofluorescence staining (Figure 6B). To determine whether flotillin-2 accompanied Endo180 in collagen-containing cytoplasmic vesicles, we costained primary human lung fibroblasts after incubation with FITC-conjugated collagen type I for flotillin-2 and Endo180. Collagen-containing vesicle stained positively for flotillin-2 and Endo 180 (Figure 6C).

Endo180 is part of the macrophage mannose receptor family, which includes a second collagen endocytic receptor named the mannose receptor (MR; Engelholm *et al.*, 2003; Napper *et al.*, 2006; Madsen *et al.*, 2011). To determine whether flotillin-2 also regulated MR receptor expression, we evaluated the effect of flotillin-2 knockdown on MR protein. Similar to Endo180, flotillin-2 knockdown reduced MR protein in both U937 cells and fibroblasts (Figure 6D). MR

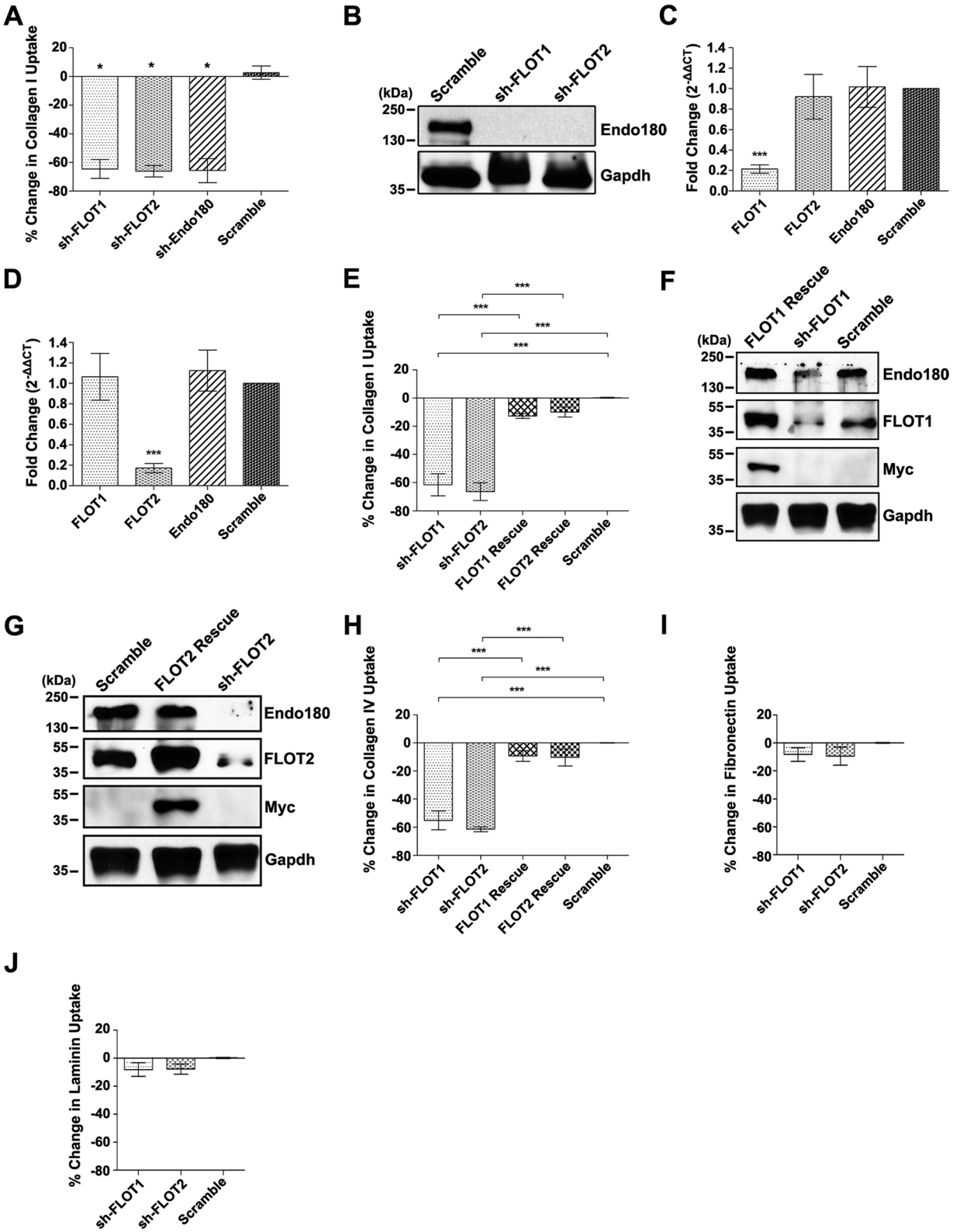

FIGURE 3: Flotillin-1 and 2 regulate collagen uptake in U937 cells. (A) Collagen uptake assay in U937 treated with shRNAs targeting flotillin-1 (sh-FLOT1), flotillin-2 (sh-FLOT2), and Endo180 (sh-Endo180). $N = 3$. (B) Representative Western blot for Endo180 expression in U937 cells treated with sh-FLOT1 or sh-FLOT2. (C, D) Quantitative PCR for target genes after sh-FLOT1 or sh-FLOT2 treatment of U937 cells. $N = 3$. (E–G) Collagen uptake assay (E) and Western blots (F, G) in U937 cells treated with sh-FLOT1 (E, F) or sh-FLOT2 (E, G) and transfected with "rescue" cDNA of myc-tagged FLOT1 (E, F) or FLOT2 (E, G) construct resistant to sh-FLOT1 or sh-FLOT2 shRNA, respectively. $N = 3$. (H–J) Uptake of FITC-conjugated collagen IV (H), fibronectin (I), and laminin (J) after treatment of U937 cells with sh-FLOT1 or sh-FLOT2. $N = 3$. Data are expressed as mean ± SD. *$p < 0.05$; ***$p < 0.001$.

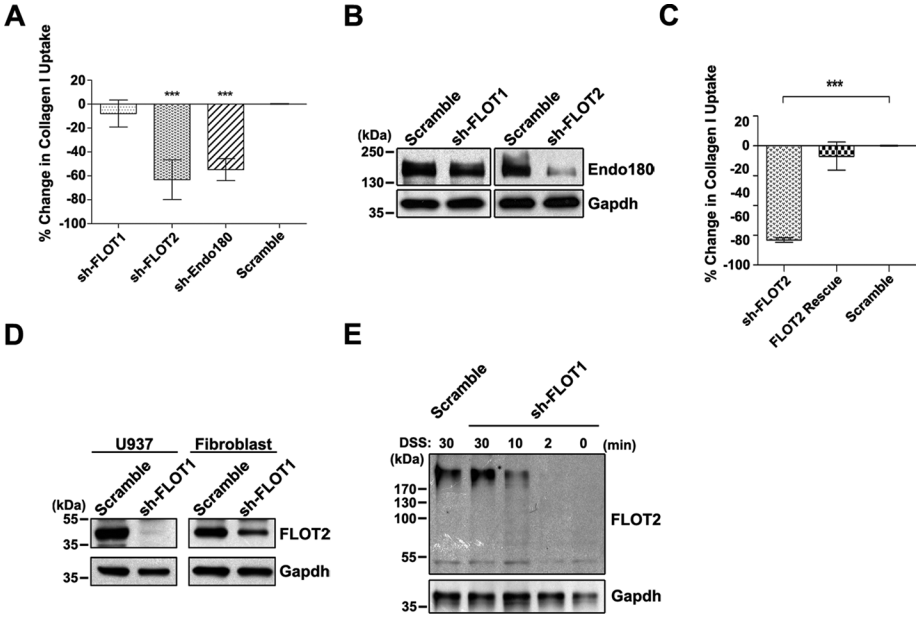

FIGURE 4: Flotillin-2 regulates collagen uptake in fibroblasts. (A) Collagen uptake assay in sh-FLOT1–, sh-FLOT2–, and sh-Endo180–treated fibroblasts. $N = 3$. (B) Representative Western blot for Endo180 expression in fibroblasts treated with sh-FLOT1 or sh-FLOT2. (C) Collagen uptake after expression of myc-tagged FLOT2 "rescue" cDNA construct in sh-FLOT2-treated fibroblasts. $N = 3$. (D) Representative Western blot for flotillin-2 expression in U937 cells and fibroblasts after sh-FLOT1 treatment. (E) Western blot of flotillin-2 expression in U937 cells after treatment with DSS. Data are expressed as mean ± SD. ***$p < 0.001$.

also coimmunoprecipitated with flotillin-2 (Figure 6E). Of note, flotillin-2 knockdown did not affect protein expression of the collagen receptor α2β1 or DDR1 (Supplemental Figure S4, A and B), suggesting that this pathway is specific for the macrophage mannose receptor family of collagen receptors.

The lack of any effect of flotillin shRNA knockdown on Endo180 transcript expression suggested a role for the flotillins in stabilizing Endo180 protein. We therefore set out to determine whether flotillins prevented degradation of Endo180 and MR protein. We first evaluated lysosomal degradation by incubating shRNA treated cells with chloroquine. Chloroquine increased Endo180 (Figure 6F) and MR (Figure 6G) protein levels in U937 cells treated with shRNA targeting flotillin-2. The proteosome inhibitor MG132 had no effect on Endo180 protein expression (Figure 6H). These data indicate that flotillin-2 stabilizes Endo180 and MR expression by preventing the targeting of these receptors for lysosomal degradation.

DISCUSSION

Our work identifies a number of *Drosophila* genes with roles in intracellular collagen turnover. The lack of fibrillar collagens in *Drosophila* indicates that the candidate genes discovered in our screen using type I collagen, a fibrillar collagen, are likely to be part of conserved pathways that regulate matrix internalization. This is exemplified by our discovery of the role of flotillin genes in collagen turnover. Flotillins are evolutionarily conserved and are present in both *Drosophila* and humans. We show that in mammalian cells, flotillins target stabilization of the collagen endocytic receptors Endo180 and MR, neither of which have orthologues in the *Drosophila* genome. By identifying the flotillins, our genomic screen elucidates an intracellular pathway regulating the only known family of collagen endocytic receptors in mammalian cells. Our studies in *Drosophila* therefore can facilitate dissection of conserved intracellular pathways of collagen turnover in mammalian cells.

The exact cellular function of flotillins is unclear. Flotillins are lipid microdomain scaffolding proteins that can be identified at the cell surface, in endosomal compartments, in the Golgi, in the nucleus, and in the phagosome (Otto and Nichols, 2011). Flotillins mediate clathrin-independent and -dependent endocytosis (Glebov *et al.*, 2006; Otto and Nichols, 2011; Ren *et al.*, 2013), cell surface receptor stabilization (Pust *et al.*, 2013), and intracellular targeting of cargo (Massol *et al.*, 2004). Our data indicate that loss of flotillin leads to lysosomal degradation of Endo180 and MR, with subsequent reduction of collagen internalization. Therefore it appears that flotillin regulation of these collagen endocytic receptors occurs either by stabilization at the cell surface or targeting to the cell surface. Support for the former mechanism comes from recent work demonstrating that flotillins, especially flotillin-2, stabilize expression of ErbB2 at the cell surface and that depletion of flotillins led to the destabilization, internalization, and degradation of ErbB2 (Pust *et al.*, 2013).

These data also suggest a potential mechanism for some of the other reported functions for the flotillin genes. Cornfine *et al.* (2011) reported that RNAi-mediated knockdown of either flotillin in human macrophages resulted in reduced degradation of a gelatin matrix. The work focused on the role of the kinesin KIF9 in mediating macrophage podosome formation and podosome-mediated matrix degradation (Cornfine *et al.*, 2011). Even though the authors demonstrated a direct interaction between KIF9 and flotillin-2, flotillin knockdown only affected matrix degradation, whereas KIF9 knockdown affected matrix degradation and podosome formation. They speculated that the flotillins might deliver or regulate the activity of metalloproteinases at sites of contact between podosomes and the matrix. Our work suggests that stabilization and delivery of Endo180 and/or MR to the podosome cell surface may be the mechanism by which flotillins regulate matrix degradation. Other studies showed that both flotillin-2 and Endo180 are overexpressed in a number of malignancies, including breast cancer and squamous cell carcinomas of the head and neck, in which they promote tumor progression and metastases (Hazarika *et al.*, 2004; Curino *et al.*, 2005; Sulek *et al.*, 2007; Rickman *et al.*, 2008; Berger *et al.*, 2013). Our data suggest that the role of flotillins in malignancies may in part be due to stabilization of Endo180 protein, leading to enhanced collagen degradation and tumor progression.

The relative contribution of fibroblasts and macrophages to collagen degradation is unknown. Both cell types express high concentrations of proteolytic enzymes, and both have been shown to take up collagen for intracellular degradation (Everts *et al.*, 1996; Atabai *et al.*, 2009). Whereas fibroblasts are the main cell type that produces collagen, macrophages are the primary cell type that mediates uptake of cellular and molecular debris. Although Endo180 protein is expressed in both cell types, the predominant receptor for macrophage-mediated collagen endocytosis has been proposed to be the mannose receptor (Madsen *et al.*, 2011). Our data suggest that regulation of the endocytic collagen receptors by flotillins is different in each cell type. Whereas flotillin-2 knockdown reduced collagen

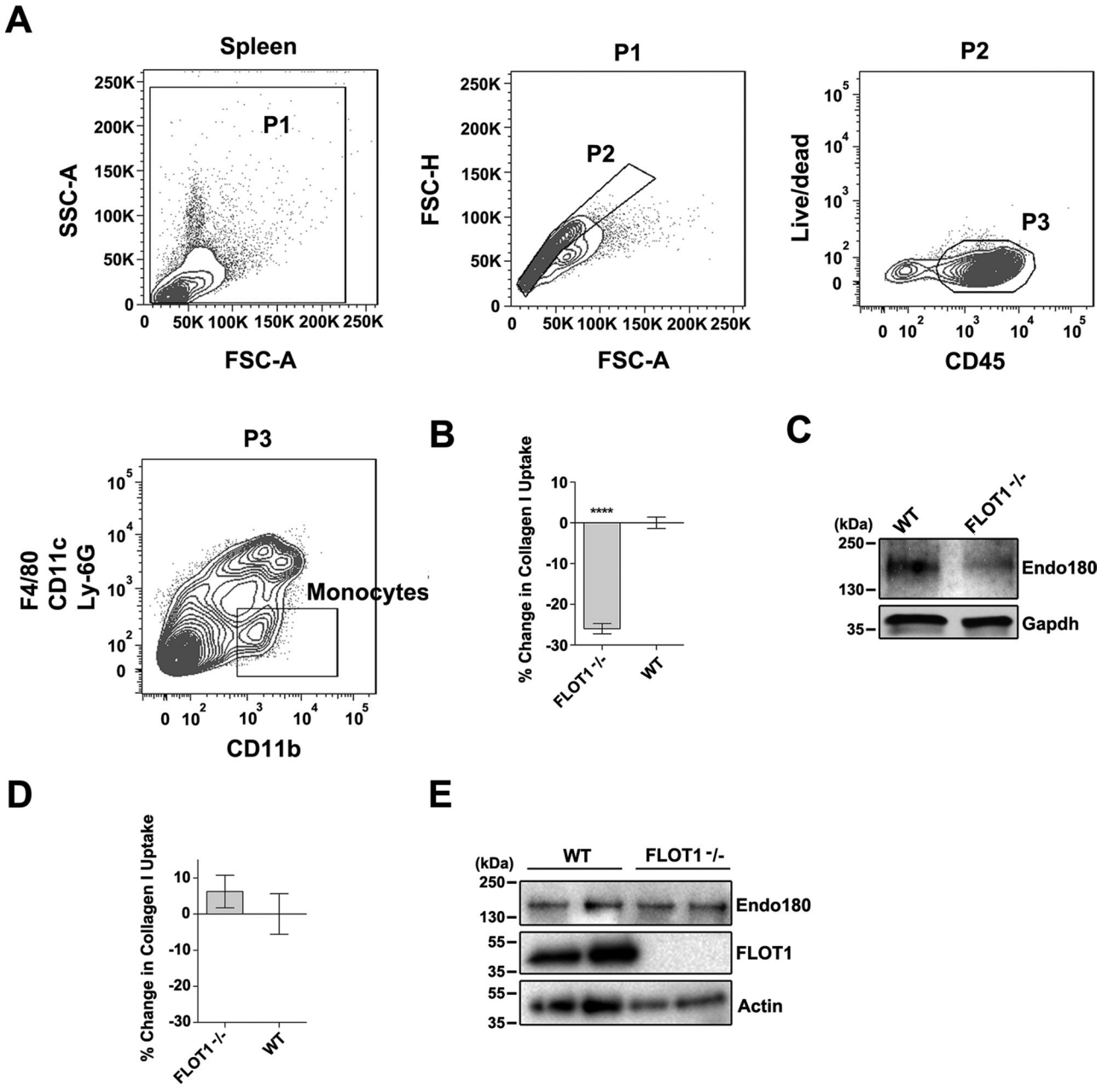

FIGURE 5: *flotillin-1*$^{-/-}$ monocytes have impaired collagen uptake. (A) Splenic monocytes were isolated and sorted by flow cytometry after staining for CD45, F4/80, CD11b, Ly-6G, and CD11c. Splenic monocytes were identified as CD45+, F4/80/CD11c/Ly-6G low, and CD11b high. (B, C) Collagen uptake (B, $N = 3$) and Endo180 protein expression (C) in WT and *flotillin-1*$^{-/-}$ monocytes. (D, E) Collagen uptake (D) and Endo180 protein expression (E) in WT and *flotillin-1*$^{-/-}$ fibroblasts.

turnover in both monocytes and fibroblasts, flotillin-1 knockdown only reduce collagen turnover in monocytes. Furthermore, this effect appeared to be secondary to the degree of flotillin-2 protein reduction with flotillin-1 knockdown in each cell type, with a relatively greater effect of knockdown on flotillin-2 monocytes.

The screen also identifies a number of interesting candidate genes in addition to the flotillins. Omega, for example, is the *Drosophila* orthologue of fibroblast activation protein (FAP). FAP is an integral membrane protein with gelatinase activity that is expressed in fibroblasts associated with tissue remodeling and malignancies (Mathew *et al.*, 1995). FAP expression is also found in hepatic stellate cells found in areas of hepatic cirrhosis (Levy *et al.*, 2002). ATG6 (Beclin1 in mammals) is an autophagy-related protein that plays a central role in coordinating formation of the autophagosome important in degradation organelles and long-lived proteins (Kang *et al.*, 2011). We also identified candidate genes of unknown function (CG2264, CG8258, CG5027, CG8195) that were previously identified in screens of endocytosis, as well as genes with a more generalized role in endocytosis. These candidates are the focus of ongoing investigations focused on understanding the mechanism by which they regulate the intracellular pathway of collagen turnover.

Despite the effect of fibrosis on essentially every organ system, there are no therapies that successfully treat established fibrotic

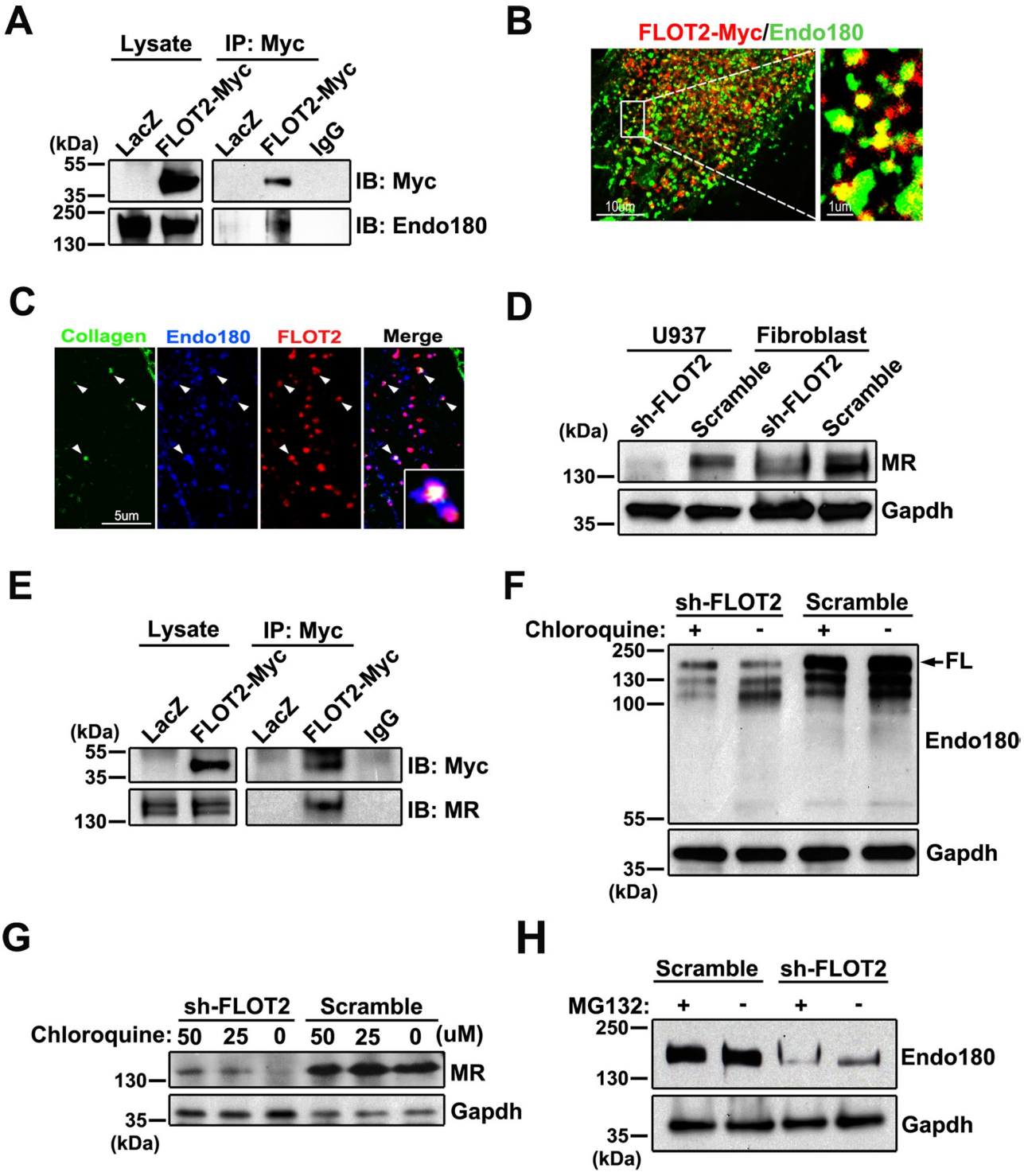

FIGURE 6: Flotillin-2 prevents lysosomal degradation of Endo180 and MR. (A) Coimmunoprecipitation of Endo180 after overexpression of a Myc-tagged flotillin-2 in fibroblasts. (B) Colocalization by immunofluorescence of flotillin-2 (red) and Endo180 (green) in fibroblasts. (C) Immunofluorescence staining of flotillin-2 (red) and Endo180 (blue) after incubation of fibroblasts with FITC-conjugated collagen. (D) Western blot of MR expression in U937 cells and fibroblasts after treatment with sh-FLOT2. (E) Interaction of flotillin-2 with MR by coimmunoprecipitation. (F, G) Western blot of Endo180 (F) and MR (G) protein expressions in U937 cells treated with sh-FLOT2 and chloroquine. (H) Western blot of Endo180 expression after treatment with sh-FLOT2 and the proteasome inhibitor MG132 in U937 cells.

disease. In several disease processes, including idiopathic pulmonary fibrosis (Montano *et al.*, 1989; Selman *et al.*, 2000), acute respiratory distress syndrome (Armstrong *et al.*, 1999), and scleroderma (Brady, 1975), there is evidence for impaired collagen degradation in vivo, suggesting that strategies that increase collagen degradation may be of potential therapeutic benefit. Manipulation of the

extracellular pathways of collagen degradation by releasing proteolytic enzymes into the extracellular compartment is fraught with the potential for serious adverse consequences secondary to uncontrolled destruction of normal tissue. Disruption of the intracellular pathways is exacerbated in experimental injury models (Atabai *et al.*, 2009; Bundesmann *et al.*, 2012). Manipulation of these pathways may be an attractive potential therapy if it can lead to measured resorption of fibrotic tissue without destruction of normal architecture. This approach may be especially effective if polymorphisms and/or deficiencies in specific components of the intracellular pathways can be identified in patient populations suffering from fibrosis. A better understanding of these pathways is needed to address their therapeutic potential. This work is an important step toward understanding the molecular mechanisms that regulate collagen turnover.

MATERIALS AND METHODS

All animal experiments were performed in compliance with the United Kingdom Home Office and Medical Research Council Laboratory of Molecular Biology Ethical Review Committee and by the University of California, San Francisco, Institutional Animal Care and Use Committee in adherence to National Institutes of Health guidelines and policies. Experiments using human fibroblasts were approved by the University of California, San Francisco, Committee on Human Research.

Statistics

All data shown as mean ± SD. One-way analysis of variance (ANOVA) was used to make comparisons between multiple groups. When the ANOVA comparison was statistically significant ($p < 0.05$), further pairwise analysis was performed using a post hoc Bonferroni test. Student's *t* test was used for comparisons between two groups. For collagen uptake data, statistics were performed on the raw data (FITC signal) after subtracting out background (cold control), and data were expressed in graphical form after normalizing to control values (scrambled shRNA). GraphPad (San Diego, CA) Prism 6.0 was used for statistical analysis.

Cell culture

Drosophila S2 cells (Invitrogen, Carlsbad, CA) were maintained in Schneider media (Invitrogen) with 10% fetal bovine serum (FBS), human U937 cells were grown in RPMI1640 with 10% FBS, and primary human lung fibroblasts were grown in DMEM with 10% FBS. Mouse embryonic fibroblasts and splenic monocytes were isolated from *flotillin-1*$^{-/-}$ mice (Ludwig *et al.*, 2010). For studies using chloroquine or MG132, U937 cells were grown on 100-mm dishes and treated with 50 μM or different concentrations of chloroquine (Sigma-Aldrich, St. Louis, MO) as indicated in Figure 4 for 12 h or 20 μM of MG132 (Sigma-Aldrich) for 24 h before immunoblot analysis. For studies using DSS (Thermo Scientific, Waltham, MA), U937 cells were grown on six-well plates and treated with DSS for different periods as indicated in Figure 4E.

Primary human lung fibroblast isolation

Adult lung parenchyma was collected from lobectomy specimens from resections performed on primary lung cancer or normal lungs not used for transplantation. Lung tissue was considered "normal" if the pulmonary function was normal. Informed consent was obtained from all surgical participants as part of an approved ongoing research protocol by the University of California, San Francisco, Committee on Human Research in full accordance with the Declaration of Helsinki principles. Lung fibroblasts were isolated by explant technique (Finkbeiner, 1997) and used from passage 1 to passage 4.

Splenic monocyte isolation by flow cytometry

Anti–F4/80-PE (clone BM8; BioLegend, San Diego, CA), anti–CD11c-PE (clone N418; BioLegend), anti–Ly6G-PE (clone 1A8; BioLegend), anti–CD11b-PerCP/Cy5.5 (clone M1/70; BioLegend), anti–CD45-Pacific Blue (30-F11; BioLegend), Fc-blocking purified anti-CD16/32 antibody (clone 93; BioLegend), and LIVE/DEAD fixable aqua dead cell stains (Invitrogen) were used for the flow cytometry analyses. Splenic monocytes were identified as $CD45^{+}$ (F4/80/CD11c/Ly-6G)^{low} $CD11b^{high}$. The monocytes were sorted on a BD FACSAriaII (BD Biosciences, Franklin Lakes, NJ) and then collected for further studies including collagen uptake and Western blot analysis.

dsRNA-mediated gene silencing

The *Drosophila* dsRNA library (version 1, V1) used for the initial screen was created and generously provided by the laboratory of Ron Vale (Goshima and Vale, 2003). The different version of dsRNAs (version 2, V2) used in rescreening assay were synthesized by 5X MEGAscript T7 Kits (Ambion, Austin, TX) and verified by 2% agarose gel electrophoresis. The sequence information of V2 dsRNA is available at http://rnai.ucsf.edu/mitoscreendb/general.php?tablename=*Drosophila*%20genes&num_p_r=12&gen_curr_page=0&user=guest&pwd=guest&javascript_enabled=true. Before dsRNA treatment, S2 cells were washed with fresh, serum-free Schneider media twice, resuspended at 5×10^5 cells/ml in serum-free Schneider media, and then plated into 96-well culture plates (50 μl/well). One hour after addition of 2 μl of dsRNA, 20% FBS-containing media (50 μl/well) was added into each well, and plates were placed at 27°C in a tissue incubator. Four days after dsRNA treatment, S2 cells were used for collagen uptake.

High-throughput screen

We coated 96-well plates with dsRNA and added cells in serum-free media for 60 min to allow for uptake of the dsRNA. Serum was subsequently added to a final concentration of 10%, and cells were allowed to grow for 4 d at 27°C, after which the media was replaced with fresh media before the collagen uptake assay. As a control for successful dsRNA uptake, cells were incubated with a sequence silencing the *beta tubulin* gene, which led to growth and morphometric defects that were visible by light microscopy (Supplemental Figure S1, A and B). After 4 d of treatment with dsRNA, the collagen uptake assay was performed in three 96-well plates/day. The FITC signal was measured from within a standard gate (G1 gate, Supplemental Figure S1C) that excluded cells with marked differences in forward or side scatter characteristics due to a particular dsRNA. The SD was calculated for the 288 genes examined on a given day. Genes that when silenced resulted in a 1.5-SD decrease in collagen uptake were considered positive hits for the preliminary screen. dsRNA targeting three genes that had no effect on collagen uptake (identified from a set of preliminary plates examined when setting up the screen) were used as negative controls. The FITC signal of the 288 experimental genes evaluated per day were expressed relative to the average of the FITC signal of negative controls run simultaneously in 96-well plates.

shRNA-mediated gene knockdown and rescue

The sequences of shRNA used to silence the selected human genes (Supplemental Table S4) were subcloned into pSicoR-mCherry or pSicoR-puromycin vectors for lentiviral generation by UCSF

ViraCore. To generate flotillin-1 and flotillin-2 rescue constructs (FLOT1, NM_005803.2; and FLOT2, NM_004475.2) resistant to shRNA knockdown (Supplemental Table S4), mutations of wild-type FLOT1 and FLOT2 with a Myc-tagged C-terminus were performed using a QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) as per the manufacturer's directions. After sequence conformation, all of the mutants were subcloned into a new pENTR backbone (Invitrogen). All constructs were then incorporated into lentivirus per manufacturer's specifications (Invitrogen). shGFP or a scrambled flotillin-2 shRNA sequence was used as the control shRNA. Lentiviral infection at 10 multiplicity of infection (MOI) was performed in cells overnight, and medium was replaced with fresh complete medium the next day. The infected cells were allowed to grow and then selected by flow cytometry based on expression of mCherry or by resistance to puromycin (0.5 µg/ml for U937; 0.3 µg/ml for primary human lung fibroblasts; Sigma-Aldrich). mCherry-positive or puromycin-selected cells were collected for quantitative real-time PCR, collagen uptake, and Western blotting.

Quantitative real-time PCR

Total RNA from cells was isolated using TRIzol (Invitrogen). Total RNA from each sample was reversed transcribed with a First Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCRs were detected with different sets of quantitative PCR primers (Supplemental Table S4) and SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) on a CFX384 Real-Time PCR Detection System (Bio-Rad). Quantitative real-time PCR was analyzed from cells treated with target RNAi as compared with control treatment. Each quantitative real-time PCR was performed at least three times, and representative results were shown as fold change relative to the control by the standard $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), where *Ct* represents the number of cycles required to reach threshold for the target gene subtracted from the number of cycles required to reach threshold for a control housekeeping gene (in this case glyceraldehyde-3-phosphate dehydrogenase [GAPDH]).

Collagen, fibronectin, and laminin uptake

dsRNA-treated S2 cells were cultured for 4 d in 96-well culture plates (5×10^5 cells) for the high-throughput screen. shRNA-transduced human cells (U937 cells and fibroblasts) were cultured in 24-well culture plates ([0.5–1.5] $\times 10^5$ cells/well) in complete medium overnight. On the day of the collagen uptake assay, fresh media was added for each cell type. Cells were then placed in the cold room (4°C) for 30 min. FITC-conjugated collagen type I (25 µg/ml final concentration; Invitrogen) was added to the cells, which were protected from light for the rest of the experiment. Thirty minutes later, cells were placed at 27°C for S2 cells or 37°C for human cells in a tissue culture incubator. S2 cells, U937 cells, or trypsinized/resuspended fibroblasts were placed on ice, and trypan blue (0.02% final concentration) was added to quench the extracellular FITC signal from collagen immediately before flow cytometry. Data were acquired on an LSRII or FACSAriaII (BD Biosciences) and analyzed with FlowJo, version 7.6.1 (Tree Star, Ashland, OR). For the preliminary screen, three 96-well culture plates coated with dsRNA were run simultaneously per day. Collagen uptake was quantified by flow cytometry measuring the signal of cells cultured with FITC-conjugated collagen type I at 27°C or at 37°C after the addition of trypan blue subtracted from the FITC signal of cells cultured with FITC-conjugated collagen type I at 4°C throughout the protocol and also after the addition of trypan blue (Figure 1, A–D). S2 and U937 cells typically had a threefold to fourfold increase in collagen uptake relative to control. For collagen type IV, fibronectin and laminin uptake studies, FITC-conjugated collagen type IV (25 µg/ml final concentration; Invitrogen), HiLyte Fluor 488–labeled fibronectin (20 µg/ml final concentration; Cytoskeleton, Denver, CO), and HiLyte Fluor 488–labeled laminin (20 µg/ml final concentration; Cytoskeleton) were substituted for type I collagen.

Zymography

Homogenized cell lysates or concentrated media were electrophoresed on 10% polyacrylamide precast gel with gelatin (Bio-Rad). After running, gels were incubated in the zymogram renaturation buffer for 30 min at room temperature and in the zymogram development buffer (Bio-Rad) overnight at 37°C. After staining with 0.5% Coomassie blue R-250, gels images were captured by scanners.

Immunoblotting

Cells were homogenized with cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA) supplemented with protease and phosphatase inhibitor cocktail (Thermo Scientific). Cell lysates were cleared by centrifugation at 12,000 rpm for 15 min at 4°C, then electrophoresed on 4–15% or 10% precast gels (Bio-Rad or Invitrogen) and transferred to nitrocellulose. The membranes were incubated with antibody to flotillin-1 (1:1000; Cell Signaling, Beverly, MA), flotillin-2 (1:1000; Cell Signaling), Myc (1:1000; Cell Signaling), Endo180 (1:200; R&D), MR (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), or GAPDH (1:6000, Sigma-Aldrich) overnight at 4°C, followed by horseradish peroxidase–conjugated secondary antibodies (1:5000; Santa Cruz Biotechnology) for 1 h at room temperature. Blots were developed using enhanced chemiluminescence (Thermo Scientific).

Coimmunoprecipitation

To overexpress flotillin-2 (National Center for Biotechnology Information accession number NM_004475.2) in primary human lung fibroblasts, adenovirus harboring C-terminal Myc-tagged flotillin-2 (FLOT2-Myc) or LacZ was generated by ViraPower adenoviral expression system (Invitrogen). The titration was done by adenovirus titration kits (Clontech, Mountain View, CA) according to the manufacturer's instructions. FLOT2-Myc and LacZ control were overexpressed by adenovirus at 100 MOI. At 48 h after transduction, cells were washed with cold phosphate-buffered saline (PBS) three times and homogenized with cold lysis buffer (0.1% Triton-X-100, 15 mM Tris-HCl, pH 7.5, 50 mM KCl, 1.5 mM EDTA, 10% glycerol, and protease and phosphatase inhibitor cocktail) for 30 min. Supernatants were mixed with anti-Myc antibody or immunoglobulin G control overnight at 4°C. Protein G–Sepharose (GE Healthcare, Little Chalfont, United Kingdom) was then added for 1–2 h at 4°C. After washing three times, associated proteins were eluted by boiling for 5 min and analyzed by immunoblotting.

Confocal fluorescence imaging

Primary human lung fibroblasts overexpressing flotillin-2–Myc or LacZ were fixed with cold 2% paraformaldehyde in PBS for 15 min. For localization of Endo180 and FLOT2, the internalized collagen study, primary human lung fibroblasts were incubated with FITC-conjugated collagen type I (25 µg/ml final concentration; Invitrogen) for 3 h at 37°C before fixation. Cells then were permeabilized with 0.1% Triton-X 100 in PBS (PBS-T) for 10 min, blocked with 5% normal goat serum in PBS-T, and incubated with primary antibodies to Myc (1:100) and Endo180 (1:50) or FLOT2 (1:100) and Endo180 (1:50) overnight at 4°C, followed by Alexa Fluor 488– and Alexa

Fluor 594– or Alexa Fluor 647– and Alexa Fluor 594–conjugated secondary antibodies (1:100; Invitrogen) for 1 h at room temperature. Confocal images were captured by a Leica point scanner using a Leica TCS SPE confocal microscope with an ACS APO 63× Oil CS objective lens at room temperature with Leica Type F Immersion Liquid (11513859). Images were processed with Photoshop CS6 software (Adobe, San Jose, CA).

ACKNOWLEDGMENTS

This research was supported by National Institutes of Health Grants HL104009 (K.A.), HL107972 (K.A.), HL113032 (S.L.N.), and HL063993 (S.L.N.) and a Nina Ireland Lung Disease Program grant (K.A.). We thank Khoa Nguyen and YiFu Qiu for help with flow cytometry and coimmunoprecipitation studies, James Smyth and TingTing Hong for assistance with adenovirus generation, and Ron Vale and Nico Stuurman for providing the dsRNA library.

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