

# Reduced Granulation Tissue and Wound Strength in the Absence of $\alpha 11\beta 1$ Integrin

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Previous wound healing studies have failed to define a role for either  $\alpha 1\beta 1$  or  $\alpha 2\beta 1$  integrin in fibroblast-mediated wound contraction, suggesting the involvement of another collagen receptor in this process. Our previous work demonstrated that the integrin subunit  $\alpha 11$  is highly induced during wound healing both at the mRNA and protein level, prompting us to investigate and dissect the role of the integrin  $\alpha 11\beta 1$  during this process. Therefore, we used mice with a global ablation of either  $\alpha 2$  or  $\alpha 11$  or both integrin subunits and investigated the repair of excisional wounds. Analyses of wounds demonstrated that  $\alpha 11\beta 1$  deficiency results in reduced granulation tissue formation and impaired wound contraction, independently of the presence of  $\alpha 2\beta 1$ . Our combined *in vivo* and *in vitro* data further demonstrate that dermal fibroblasts lacking  $\alpha 11\beta 1$  are unable to efficiently convert to myofibroblasts, resulting in scar tissue with compromised tensile strength. Moreover, we suggest that the reduced stability of the scar is a consequence of poor collagen remodeling in  $\alpha 11^{-/-}$  wounds associated with defective transforming growth factor- $\beta$ -dependent JNK signaling.

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## INTRODUCTION

In the dermis, fibroblast interactions with the collagen network are important in maintaining skin homeostasis (Sorrell and Caplan, 2004) and become essential during repair of skin lesions (Liu *et al.*, 2010). During wound healing, numerous complex cell–matrix interactions occur. Keratinocytes migrate on fibronectin in the provisional matrix to seal the wound toward the outside (Margadant *et al.*, 2009). Dermal fibroblasts migrate into the wound, proliferate, and differentiate into myofibroblasts, which produce granulation tissue (GT) rich in collagen I (Hinz, 2007; Driskell *et al.*, 2013). Myofibroblasts, which express  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), use cytoskeleton-driven forces to contract the wound (Tomasek *et al.*, 2002; Wipff *et al.*, 2007). Finally, the con-

tracted GT is remodeled to restore normal tissue architecture. Although the importance of cell–matrix interactions for wound contraction seems to be deductive, the identity of participating receptors in the dermis has been ambiguous.

In the skin, the collagen-binding integrins  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  are present on fibroblasts and on microvasculature, but only  $\alpha 2\beta 1$  is expressed on basal keratinocytes (Gardner *et al.*, 1999; Grenache *et al.*, 2007; Zweers *et al.*, 2007). Wound healing studies in  $\alpha 1\beta 1$  or  $\alpha 2\beta 1$  integrin-deficient mice have surprisingly failed to define a role for either receptor in fibroblast-mediated wound contraction (Gardner *et al.*, 1999; Grenache *et al.*, 2007; Zweers *et al.*, 2007), suggesting that additional collagen receptor(s) are involved in this process. One possible candidate is  $\alpha 11\beta 1$ , which in the skin is exclusively expressed on fibroblasts (Velling *et al.*, 1999; Zhang *et al.*, 2006).

Early remodeling of collagen matrices mediated by fibroblasts *in vitro* has been shown to be essentially an arginine-glycine-aspartic acid-independent but  $\beta 1$  integrin-dependent process (Gullberg *et al.*, 1990). Data collected so far on fibroblasts have identified integrins  $\alpha 2\beta 1$  (Klein *et al.*, 1991; Zhang *et al.*, 2006) and  $\alpha 11\beta 1$  (Popova *et al.*, 2007) as being involved in collagen remodeling.  $\alpha 2\beta 1$  has been shown to contribute to collagen gel remodeling in fibroblasts of different origin, including the dermis, whereas functional studies on  $\alpha 11\beta 1$  have so far been limited to mouse embryonic fibroblasts (MEFs) and periodontal ligament fibroblasts (Popova *et al.*, 2007; Barczyk *et al.*, 2013).

In the present study, we demonstrate that repair of skin wounds is compromised in mice lacking integrin  $\alpha 11\beta 1$ ,

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Abbreviations:  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; GT, granulation tissue; MEF, mouse embryonic fibroblast; TGF- $\beta$ , transforming growth factor- $\beta$

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characterized by diminished wound contraction, reduced formation of GT, and altered scar stability. Our combined *in vivo* and *in vitro* data show that myofibroblast differentiation and collagen remodeling are impaired in the absence of  $\alpha 11\beta 1$ . Hence, we demonstrate that efficient collagen remodeling requires both  $\alpha 11\beta 1$  and non-canonical transforming growth factor- $\beta 1$  (TGF- $\beta 1$ )-dependent JNK signaling.

## RESULTS

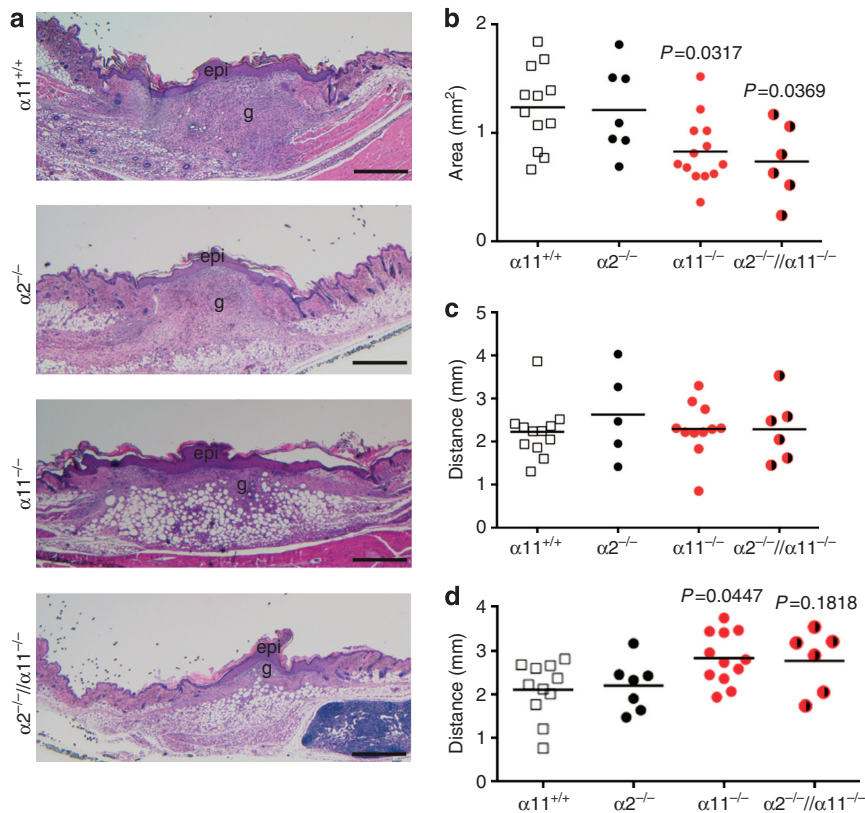
### Impaired wound healing in $\alpha 11\beta 1$ -deficient mice

In previous studies, we showed that expression levels of the integrin subunits  $\alpha 2$  and  $\alpha 11$  are elevated during the course of wound healing, with a peak of integrin  $\alpha 11$  at 7 days after injury (Zweers *et al.*, 2007). To dissect whether these two integrins have distinguishable functions during tissue regeneration, we investigated wound repair in mice that are deficient of  $\alpha 2\beta 1$ ,  $\alpha 11\beta 1$  or both integrins. Specifically, we analyzed the formation of GT and contraction of full-thickness skin wounds 7 days after lesion. Sections through the middle of such wounds are illustrated in Figure 1a, showing GT of C57BL/6 wild-type mice and their integrin-deficient littermates. As expected, the amount of GT developed in  $\alpha 2$ -null mice did not differ from wild-type mice (Zweers *et al.*, 2007); however, significantly less GT was developed by  $\alpha 11$ -null mice ( $P=0.0317$ ) and by double mutants ( $P=0.0369$ ;

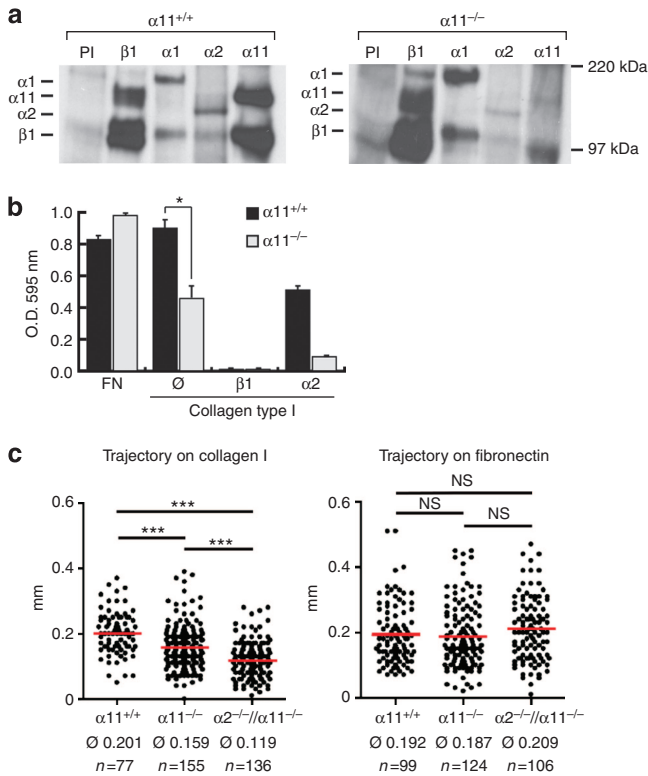
Figure 1b). As there was no difference between the single  $\alpha 11$ -null and the double  $\alpha 2/\alpha 11$ -null mutant wounds, we conclude that the effect seen in the double mutant is attributed to the absence of  $\alpha 11\beta 1$  integrin. Differences in wound area were not reflected by the scab size, which was comparable in all 3 mutants and wild-type mice (Supplementary Figure S1A and B online). However, histology revealed that the distances between wound edges—which serve as an indicator of wound contraction—were significantly increased in integrin  $\alpha 11\beta 1$ -deficient wounds ( $P=0.0447$ ; Figure 1d). The reduced wound contraction was not caused by an impaired function of the panniculus carnosus muscle that participates in the contraction, as it was equally contracted in all genotypes (Figure 1c). As we identified the lack of  $\alpha 11$  as responsible for reduced GT formation and impaired wound contraction, we limited our further analysis to comparison of wounds in  $\alpha 11\beta 1$ -deficient versus littermate control mice.

### Impaired migration of fibroblasts lacking $\alpha 11\beta 1$

As fibroblasts are the main cells that produce GT during wound healing, we aimed to determine the impaired fibroblast function that could be responsible for the  $\alpha 11^{-/-}$  wound healing phenotype. For this purpose, we isolated dermal fibroblasts from murine newborn skin. We determined expression levels of collagen-binding integrins by co-precipitation



**Figure 1. Ablation of  $\alpha 11\beta 1$  impairs wound healing.** (a) Hematoxylin and eosin staining of mid-wound sections from  $\alpha 11^{+/+}$ ,  $\alpha 2^{-/-}$ ,  $\alpha 11^{-/-}$  and  $\alpha 2^{-/-}/\alpha 11^{-/-}$  mice at day 7 after wounding. (b) Histomorphometry of granulation tissues. (c) Distance between panniculus carnosus edges and (d) left and right wound edges. Each symbol represents one wound and 3–5 sections/wound were analyzed. Data were evaluated by one-way analysis of variance and Tukey’s multiple comparison test. epi, new epidermis; g, granulation tissue. Scale bar = 500  $\mu$ m.



**Figure 2. Cell migration is impaired in  $\alpha 11^{+/+}$  fibroblasts.** (a)  $^{35}\text{S}$  metabolic labeling and immunoprecipitation of integrins in murine dermal fibroblasts. (b) Contribution of  $\alpha 11$  integrin to dermal fibroblast adhesion. Integrin blocking antibodies (anti- $\beta 1$  or anti- $\alpha 2$ ) were added ( $10 \mu\text{g ml}^{-1}$ ).  $\emptyset$  represents untreated cells. Adhesion to fibronectin was used as control. (c) Random migration of dermal fibroblasts was analyzed on type I collagen or fibronectin. Cell motility was quantified by measuring the cell trajectory of single cells; each symbol represents one cell. Average trajectories ( $\emptyset$ ) were calculated for each condition. (\* $P < 0.05$ ; \*\*\* $P < 0.001$ ; NS, not significantly different; mean  $\pm$  SD). O.D. optical density.

with the  $\beta 1$  subunit and indeed detected high levels of co-precipitating  $\alpha 11$  but not of  $\alpha 2$  integrin subunit (Figure 2a). Dermal fibroblast interactions with collagen I appeared to exclusively depend on integrins, as antibodies to integrin  $\beta 1$  completely blocked adhesion of wild-type cells on collagen I. Interestingly, adhesion to collagen I was strongly reduced in dermal  $\alpha 11^{-/-}$  fibroblasts, whereas antibodies to  $\alpha 2$  reduced attachment of  $\alpha 11^{+/+}$  dermal fibroblasts by only 50% (Figure 2b).

As an effective healing process relies on fibroblast migration, we investigated the importance of  $\alpha 11\beta 1$  and  $\alpha 2\beta 1$  for cell migration on collagen I and fibronectin (Figure 2c). Trajectory path length of individual fibroblast was measured, revealing a significant reduction in migration when  $\alpha 11$  was absent. This effect was even more pronounced when  $\alpha 2^{-/-}/\alpha 11^{-/-}$  fibroblasts were analyzed. As expected, migration on fibronectin was not affected by the absence of both  $\alpha 2\beta 1$  and  $\alpha 11\beta 1$  (Figure 2c).

To test whether compromised migration of fibroblasts into the wound might be responsible for the impaired formation of GT, the total number of cells in the GT was estimated by

assessment of DAPI-stained nuclei per unit area. However, the total number of cells in  $\alpha 11\beta 1$ -deficient GTs was not reduced, indicating that impaired migration is not responsible for the diminished GT area (Supplementary Figure S1C and E online). Furthermore, we were able to exclude the possibility that  $\alpha 11$  deficiency might impair proliferation and thus offer an explanation to the comprised formation of GT, as we did not observe a proliferation defect in the absence of  $\alpha 11$  either in the wounds (Ki67 staining, Supplementary Figure S1F and G online) or in the cultured dermal fibroblasts (Supplementary Figure S1H online).

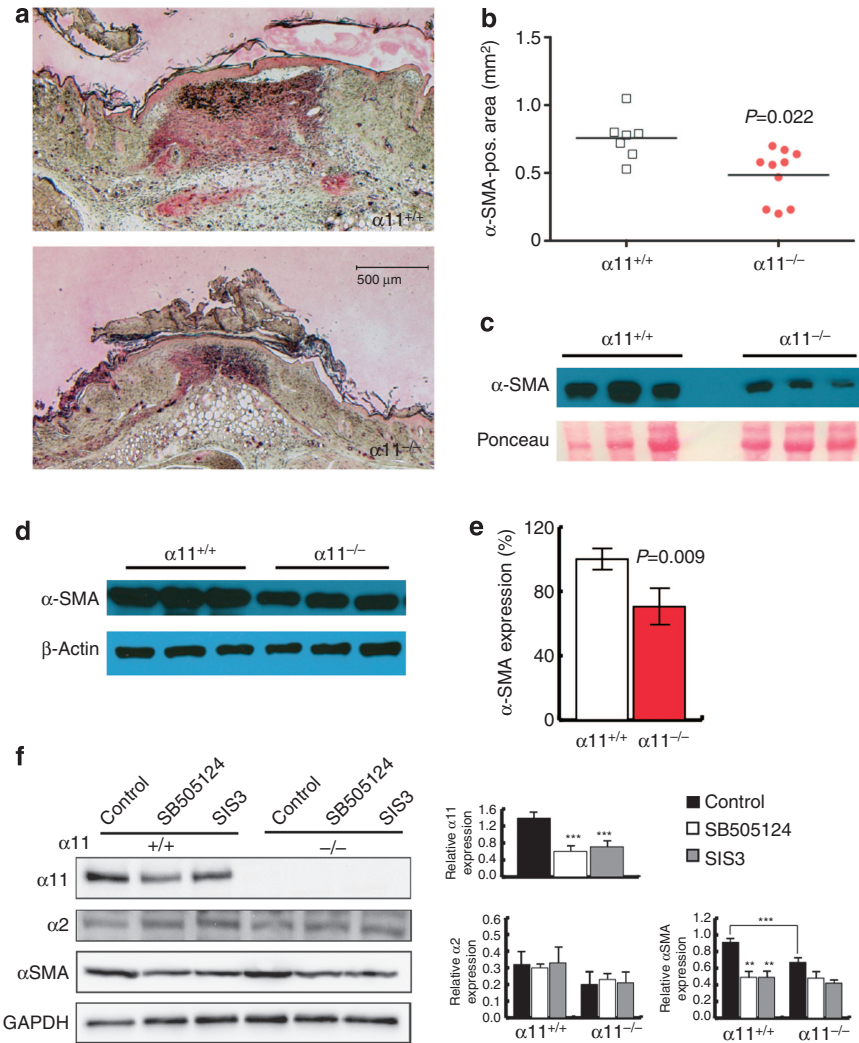
### Reduced myofibroblast differentiation in the absence of $\alpha 11\beta 1$

During wound healing, fibroblasts can become activated by mechanical tension and differentiate into  $\alpha$ -SMA-expressing myofibroblasts, which secrete ECM proteins to form the GT and contract the wound (Van De Water *et al.*, 2013). Immunohistochemical staining revealed that the  $\alpha$ -SMA-positive area was significantly smaller in  $\alpha 11^{-/-}$  wounds compared with control littermates ( $P = 0.022$ ; Figure 3a and b). In the skin,  $\alpha$ -SMA is expressed by myofibroblasts and vascular smooth muscle cells. As vascularization was not affected (Supplementary Figure S1C and D online), we concluded that reduced levels of  $\alpha$ -SMA in GTs of  $\alpha 11$ -deficient mice were caused by a reduced number of myofibroblasts. Western blotting of wound extracts confirmed reduced  $\alpha$ -SMA levels in  $\alpha 11^{-/-}$  wound lysates (Figure 3c).

*In vitro*, myofibroblast differentiation can be induced by using attached collagen lattices in which embedded fibroblasts experience mechanical resistance (Tomasek *et al.*, 2002; Grinnell and Petroll, 2010). Hence,  $\alpha 11^{+/+}$  dermal fibroblasts expressed high levels of  $\alpha$ -SMA when they were subjected to the mechanical strain of an attached collagen gel, indicating that they had differentiated into myofibroblasts. However,  $\alpha 11^{-/-}$  fibroblasts embedded in attached collagen lattices under identical conditions displayed reduced  $\alpha$ -SMA induction, clearly pointing to defective myofibroblast differentiation (Figure 3d and e).

Myofibroblast differentiation is not only dependent on mechanical forces but also requires TGF- $\beta$  (Van De Water *et al.*, 2013). When we treated fibroblasts *in vitro* with either an TGF- $\beta$  receptor type I inhibitor or a reagent inhibiting Smad3 signaling, we found that—similar to  $\alpha$ -SMA—expression of the integrin subunit  $\alpha 11$  depends on TGF- $\beta$  and downstream Smad signaling (Figure 3f). It is notable that in turn TGF- $\beta$ -dependent Smad3 activation is not impaired by  $\alpha 11$ -deficiency (Supplementary Figure S2D online). Although treatment of fibroblasts with TGF- $\beta$  resulted in elevated levels of the integrin  $\alpha 11$ -subunit, it did not influence expression of  $\alpha 2$  (Supplementary Figure S2B online), which was also unaffected by inhibition of TGF- $\beta$ RI and Smad3 (Figure 3f). To exclude that impaired myofibroblast differentiation of  $\alpha 11^{-/-}$  fibroblasts could be due to an impaired ability to activate TGF- $\beta$ , fibroblasts were co-cultured with mink lung endothelial cells (TMLC), which express luciferase when they are exposed to active TGF- $\beta$ . Using this approach, we showed that activation of TGF- $\beta$  is not affected by the presence of integrin  $\alpha 11\beta 1$  (Supplementary Figure S2C online).





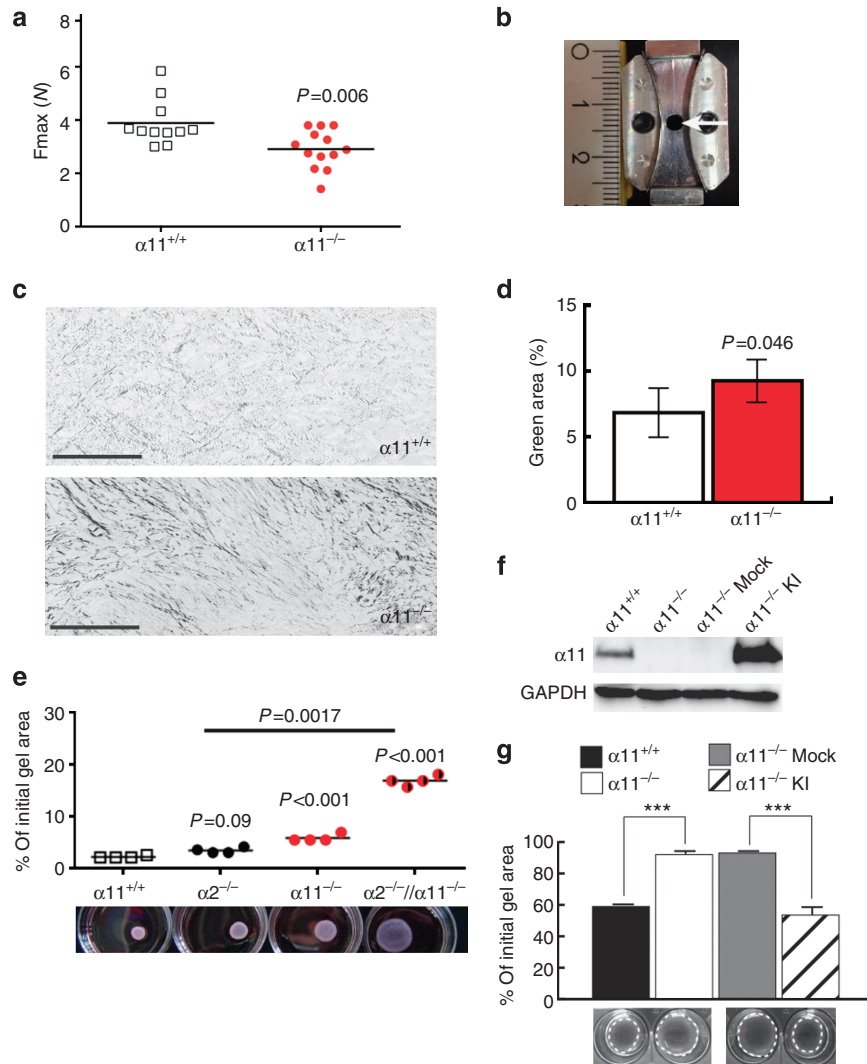
**Figure 3. Ablation of  $\alpha 11\beta 1$  results in significant reduction in myofibroblast differentiation.** (a) Immunohistochemical staining of mid-wound sections at 7 days after injury with antibodies directed to  $\alpha$ -SMA (red). Scale bar = 500  $\mu$ m. (b)  $\alpha$ -SMA-positive area was quantified by histomorphometry. Each symbol represents one wound, and 3–5 sections per wound were evaluated. (c) Western immunoblotting of wound extracts harvested 7 days after injury (4 mm punch biopsies from the wound center) indicating levels of  $\alpha$ -SMA. (d)  $\alpha$ -SMA expression by primary fibroblasts embedded in attached collagen lattices was analyzed by western immunoblotting. (e) Quantification of  $\alpha$ -SMA signals in (d). (f) Primary fibroblasts on collagen I (100  $\mu$ g ml<sup>-1</sup>) were treated with SB505124 (10  $\mu$ M) or SIS3 (10  $\mu$ M) for 48 hours. Protein expression was analyzed by western immunoblotting and quantified by densitometry.  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin.

### Defect of collagen remodeling in $\alpha 11$ -null mice

$\alpha$ -SMA-expressing myofibroblasts are the major source of fibrillar collagens, which are essential for producing a functional scar, thereby terminating the repair process (Klingberg *et al.*, 2013). Therefore, we next addressed the question whether the impaired formation of GT would still give rise to a fully functional scar. For this purpose, we inflicted incisional wounds on the backs of  $\alpha 11^{-/-}$  and control mice, which heal at a faster rate compared with excisional wounds and allowed us to assess the tensile properties of the developed scar (Wu *et al.*, 2003). Thus, incisional wounds were removed together with cranial and caudal skin (Figure 4b) at 16 days after injury and placed in a material testing device, which determines the tensile strength. Scars of  $\alpha 11^{-/-}$  mice ruptured at lower force applied ( $P=0.0063$ ),

indicating that expression of  $\alpha 11\beta 1$  integrin confers stability to cutaneous scars (Figure 4a). To investigate whether reduced scar stability was a consequence of an impaired formation of the reconstituted collagen matrix, collagens within the GT were stained with Sirius red. Polarized light microscopy allowed allocation of the anisotropic thin (green) and thick (red) collagen fibrils (Junqueira *et al.*, 1979). This analysis revealed a significant increase in thinner fibrils in the GT of integrin  $\alpha 11$ -deficient mice (Figures 4c and d), demonstrating an abnormal composition of the provisional collagen matrix.

To verify that  $\alpha 11^{-/-}$  fibroblasts had reduced capacity to reorganize the collagen environment, floating collagen lattices were used as a suitable model for early GT contraction (Dallon and Ehrlich, 2008; Grinnell and Petroll, 2010). Fibroblasts were embedded in collagen lattices and allowed



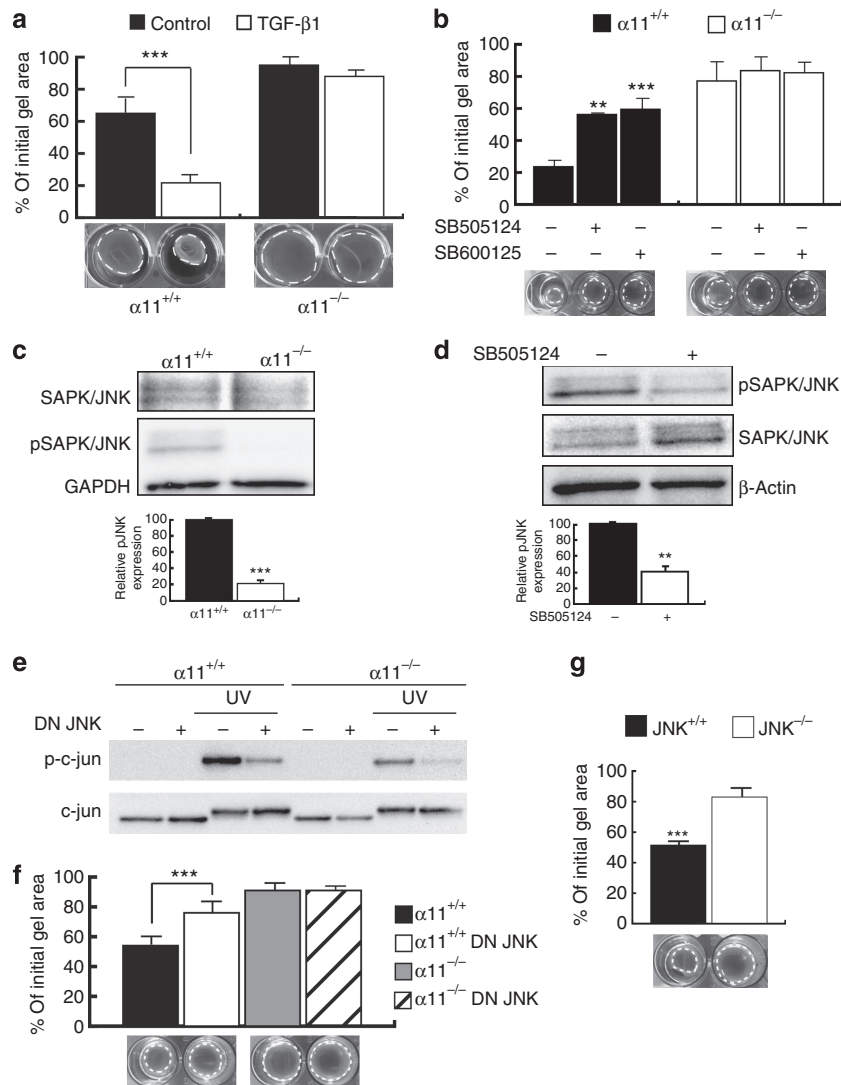
**Figure 4. Ablation of  $\alpha 11\beta 1$  impairs collagen remodeling.** (a) Tensile strength of incisional wounds determined at 16d after injury. Fmax depicts ultimate force applied to skin strips before the scar ruptured. Each symbol represents one incisional wound. (b) Skin strips were excised using the punch with the indicated shape. Arrow indicates position of the scar. (c) Sirius red staining of GTs analyzed by polarized light microscopy. Colors were inverted and split into single channels to separately assess the amount of green (thin) fibrils. (d) Quantification of the green fibrils from (c). (e) Fibroblasts were allowed to remodel collagen for 72 h. (f) Western immunoblotting of  $\alpha 11$  expression after  $\alpha 11$  knock-in (KI) in  $\alpha 11^{-/-}$  fibroblasts. (g) Effect of  $\alpha 11$  knock-in on collagen remodeling (\*\* $P < 0.001$ ; mean  $\pm$  SD). GT, granulation tissue; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

to reorganize the matrix, which was quantified by measuring gel diameters over time and calculating gel area. As illustrated in Figure 4e, over a time course of 72 h of contraction,  $\alpha 11^{-/-}$  fibroblasts displayed significantly less remodeling of the collagen matrix compared with  $\alpha 11^{+/+}$  and  $\alpha 2^{-/-}$  cells. To exclude the involvement of collagen integrin bridging that might assist in integrin collagen interaction (Zeltz *et al.*, 2013), we limited the following contraction experiments to 24 hours. Under these conditions, antibodies to the  $\beta 1$  integrin subunit almost completely blocked fibroblast-mediated collagen gel contraction and  $\alpha 11$ -deficiency considerably reduced collagen remodeling (Supplementary Figure S2E online), implicating the integrin  $\alpha 11\beta 1$  as a crucial receptor in this process. Importantly, overexpression of  $\alpha 11$  in  $\alpha 11^{-/-}$

fibroblasts ( $\alpha 11^{-/-}$ -KI, Figure 4f) rescued the  $\alpha 11^{-/-}$  phenotype (Figure 4g).

#### Collagen remodeling requires $\alpha 11\beta 1$ and TGF- $\beta$ -dependent JNK signaling

Our previous collagen remodeling experiments were assessed in the presence of 2% serum, as unstimulated murine dermal fibroblasts (no serum) displayed minimal basal remodeling activity (Figure 5a). To understand why  $\alpha 11^{-/-}$  cells fail to reorganize collagen lattices, we investigated the soluble autocrine factor(s) responsible for initiation of collagen remodeling. On the basis of its documented importance for collagen remodeling, TGF- $\beta 1$  was again a good candidate (Montesano and Orci, 1988). The cytokine TGF- $\beta 1$  is known



**Figure 5. Collagen remodeling is dependent on TGF- $\beta$ -mediated JNK signaling.** (a) Fibroblasts were incorporated into collagen lattices and allowed to contract for 24 hours in serum-free DMEM or in the presence of TGF- $\beta 1$  (5 ng ml $^{-1}$ ). (b) Effect of signaling inhibitors (SB505124, 10  $\mu$ M or SP600125, 25  $\mu$ M) on collagen remodeling stimulated with 2% serum. (c) JNK activation was evaluated during collagen remodeling. (d) Effect of SB505124 on JNK phosphorylation in  $\alpha 11^{+/+}$  fibroblasts during collagen remodeling. (e) c-jun activation was assessed after fibroblast co-transfection with DN JNK constructs. UV light was used to activate the JNK pathway. (f) Effect of DN JNK on collagen remodeling stimulated with 2% serum. (g) JNK $^{-/-}$  MEFs were allowed to remodel floating collagen gels for 24 hours in the presence of 2% serum (\*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; mean  $\pm$  SD). DN, dominant negative; TGF- $\beta$ , transforming growth factor- $\beta$ .

to enhance the capacity of fibroblasts to remodel collagen matrices (Poon *et al.*, 2009), which was confirmed when the cytokine was applied to collagen gels with embedded  $\alpha 11^{+/+}$  dermal fibroblasts. Interestingly, the TGF- $\beta 1$  stimulating effect on collagen remodeling was lost when added to collagen gels containing  $\alpha 11^{-/-}$  fibroblasts (Figure 5a).

TGF- $\beta$  exerts its effects via two major signaling pathways, the canonical pathway, which involves Smad signaling, or alternatively by a non-canonical pathway, which leads to activation of MAPK signaling by a mechanism that includes JNK and p38. As our data obtained with chemical inhibitors indicated that  $\alpha 11$  expression was regulated via canonical Smad signaling, we investigated whether this

pathway was also involved in the  $\alpha 11$ -dependent collagen remodeling. For this purpose, we silenced Smad4, a cofactor of Smad2 and Smad3, using siRNA (Supplementary Figure S3A online). Knock-down efficiency was confirmed by western blotting showing reduced  $\alpha 11$  expression by 50%, similar to the reduction that we previously obtained with the Smad3 inhibitor. However, the Smad4 knock-down did not impair collagen reorganization (Supplementary Figure S3B online), indicating that the canonical Smad pathway was not involved in this process.

We next investigated the non-canonical pathway. Inhibition of p38-MAPK by SD203580 did not impair collagen reorganization (Supplementary Figure S3C online). In contrast,

inhibition of JNK by SP600125 and of the upstream MKK1 using PD098059 significantly delayed  $\alpha$ 11-mediated collagen remodeling (Figure 5b and Supplementary Figure S3C online). We furthermore observed a defect of JNK phosphorylation in  $\alpha$ 11-deficient cells during collagen gel contraction (Figure 5c). However, JNK could also be activated through the soluble integrin- and growth factor-activated tyrosine kinase, FAK. To discriminate between JNK signaling as result of integrin/FAK activation versus non-canonical TGF- $\beta$  signaling in  $\alpha$ 11<sup>+/+</sup> fibroblasts, phospho-JNK levels were determined in fibroblasts embedded in contracting collagen gels following treatment with FAK inhibitor PF-573228 or TGF- $\beta$  receptor type I inhibitor SB505124. Despite inhibition of collagen remodeling, PF-573228 did not affect JNK phosphorylation (Supplementary Figure S3D and E online). Conversely, SB505124 significantly inhibited both JNK phosphorylation (Figure 5d) and collagen remodeling (Figure 5b), indicating involvement of the non-canonical TGF- $\beta$  signaling pathway in  $\alpha$ 11-mediated collagen remodeling.

To further ascertain the role of JNK in collagen remodeling, fibroblasts were co-transfected with the dominant negative form of JNK1 and JNK2. The efficiency of the dominant negative JNK variants was confirmed by reduced levels of activated c-jun, a downstream effector of JNK (Figure 5e). Dominant negative JNK-transfected fibroblasts embedded in collagen lattices showed a reduced ability to reorganize the matrix (Figure 5f). Moreover, MEFs isolated from JNK1/2-null mouse embryos also presented a defect in collagen remodeling (Figure 5g). Together these results clearly demonstrate that fibroblast-mediated collagen remodeling requires  $\alpha$ 11 $\beta$ 1 integrin-mediated contact with collagen and the presence of TGF- $\beta$  that elicits a non-canonical signal pathway involving JNK.

## DISCUSSION

In the present study, we addressed the functional relevance of the  $\alpha$ 11 $\beta$ 1 integrin during the repair process of skin wounds and described specific functions exerted by  $\alpha$ 11 $\beta$ 1 but not by other collagen-binding integrins such as  $\alpha$ 2 $\beta$ 1. Using targeted deletion of  $\alpha$ 11, we show that development of GT and formation of mechanically stable scar tissue critically rely on the presence of  $\alpha$ 11 $\beta$ 1. Our *in vitro* results strongly suggest that  $\alpha$ 11 $\beta$ 1-deficient fibroblasts exhibit a reduced ability to differentiate into myofibroblasts. The resulting reduction in myofibroblast number causes an impaired tissue restoration and compromised wound contraction *in vivo*. Finally, remodeling of collagenous tissues to a mature and strong scar depends almost exclusively on  $\alpha$ 11 $\beta$ 1, supported by the *in vitro* finding that lattice contraction by  $\alpha$ 11<sup>-/-</sup> fibroblasts is similarly abolished as reported for fibroblasts deficient in  $\beta$ 1 integrins (Liu *et al.*, 2010).

Our *in vitro* data further show that  $\alpha$ 2 $\beta$ 1 and  $\alpha$ 11 $\beta$ 1 cooperate in murine fibroblast adhesion and cell migration on collagen I-coated two-dimensional surfaces and confirm our earlier results obtained using  $\alpha$ 2<sup>-/-</sup> dermal fibroblasts (Zhang *et al.*, 2006). Despite these findings, the overall cell number in wounds did not differ from controls, maybe reflecting the ability of  $\alpha$ 11-null fibroblasts to migrate on

fibrin and other components of the provisional wound matrix (Reyhani *et al.*, 2014). We recently suggested compensatory/complementary cellular interactions with collagen integrin bridging molecules (Zeltz *et al.*, 2013). A prominent collagen integrin bridging molecule in the dermis is periostin (Egbert *et al.*, 2014), which is important for fibroblast migration into the wounds (Elliott *et al.*, 2012). The integrin-mediated periostin-dependent adhesion might provide an indirect link of fibroblasts to fibrillar collagen and is one potential mechanism to compensate for the lack of  $\alpha$ 11 $\beta$ 1 (Zeltz *et al.*, 2013).

Integrin  $\alpha$ 11 $\beta$ 1 can regulate myofibroblast differentiation in corneal and cardiac fibroblasts (Carracedo *et al.*, 2010; Talior-Volodarsky *et al.*, 2012). Here we showed that  $\alpha$ 11 and  $\alpha$ -SMA are regulated in a similar manner by TGF- $\beta$  signaling in primary dermal fibroblasts and that the absence of  $\alpha$ 11 leads to reduced myofibroblast numbers in GT *in vivo* and impaired ability to remodel collagen lattices *in vitro*. Addition of recombinant TGF- $\beta$ 1 failed to rescue the ability of  $\alpha$ 11<sup>-/-</sup> fibroblasts to contract/remodel lattices.

Although both canonical and non-canonical TGF- $\beta$  receptor-mediated induction of myofibroblast differentiation has been described,  $\alpha$ -SMA can be induced in wounds in the absence of TGF- $\beta$  type II receptor (Martinez-Ferrer *et al.*, 2010), indicating that fibroblasts can activate parallel/compensatory mechanisms.

Integrins are also dynamically regulated in both two-dimensional and three-dimensional contexts. In the three-dimensional environment, integrin  $\alpha$ 2 is upregulated in floating collagen lattices (Klein *et al.*, 1991) and both  $\alpha$ 2 and  $\alpha$ 11 are upregulated in attached collagen gels (Klein *et al.*, 1991; Carracedo *et al.*, 2010). We think that the low levels of  $\alpha$ 2 expression in mouse dermal fibroblasts are, however, sufficient to provide cell attachment and migration but not to efficiently remodel collagen at early time points (24 hours). Expression of  $\alpha$ 2 is upregulated over time by fibroblasts in collagen lattices, which leads to more efficient collagen remodeling, as we observed at 72 hours. As only  $\alpha$ 11 appears to induce  $\alpha$ -SMA levels, we assume that differential signaling is active during myofibroblast differentiation and also during collagen remodeling.

Our results demonstrate that both TGF- $\beta$  and  $\alpha$ 11 $\beta$ 1 integrin have important roles in collagen matrix reorganization by activation of the JNK signaling pathway. Blocking JNK has been shown to inhibit TGF- $\beta$ -induced collagen remodeling (Shi-Wen *et al.*, 2009) and myofibroblast differentiation (Shi-Wen *et al.*, 2006). A previous report showed that FAK is essential for TGF- $\beta$ -induced JNK activation in MEFs (Liu *et al.*, 2007). Although FAK is also necessary for collagen reorganization in our model, we could not demonstrate its role in JNK phosphorylation. Cross talk between non-Smad TGF- $\beta$  signaling and integrins has already been described (Mu *et al.*, 2012). A tempting speculation is that part of the non-canonical TGF- $\beta$  signaling might depend on  $\alpha$ 11 $\beta$ 1 but not on FAK.

In summary, our data demonstrate that  $\alpha$ 11 $\beta$ 1 is the major collagen receptor present on dermal fibro-



blasts essential in collagen remodeling. We also demonstrate that  $\alpha$ 11 $\beta$ 1 is involved in myofibroblast differentiation and GT formation following injury to ensure scar tissue strength. As myofibroblast differentiation and collagen dynamics are at the core of fibrotic processes,  $\alpha$ 11 $\beta$ 1 is an interesting collagen receptor to investigate further in pathological fibrosis.

## MATERIALS AND METHODS

Detailed materials and methods are provided in Supplementary Data online.

### Mice

C57BL/6 mice with targeted deletion of *Itga2* or of *Itga11* gene as previously described (Holtkotter *et al.*, 2002; Popova *et al.*, 2007) were used in this study. Compound  $\alpha 2^{-/-} // \alpha 11^{-/-}$  mutant mice were generated by intercrossing the single mutants. Wild-type and compound  $\alpha 2^{-/-} // \alpha 11^{-/-}$  mutant mice were crossed with the immorto mouse carrying the Simian virus 40 large T antigen under control of the temperature-sensitive H-2Kb-tsA58 promoter (Jat *et al.*, 1991; kindly provided by U Mayer, University of East Anglia, UK), generating immorto mice carrying at least one copy of the H-2Kb-tsA58 transgene. Animals were housed in specific pathogen-free facilities. PCR genotyping was performed on DNA extracted from tail tip biopsies as described (Holtkotter *et al.*, 2002; Popova *et al.*, 2007). All animal experiments were approved by the local veterinary authorities (LANUV NRW, Germany or the Norwegian Animal Research Authority).

### Cell culture

Mouse dermal fibroblasts were isolated as previously described and used in experiments up to passage 2 (Zweers *et al.*, 2007). Briefly, the entire trunk skin of postnatal day 3 C57BL/6 mice,  $\alpha 11^{-/-}$  mutant mice or compound  $\alpha 2^{-/-} // \alpha 11^{-/-}$  mutant mice was removed and incubated overnight at 4 °C with trypsin-EDTA (0.05%-0.02%, PAA Laboratories, Pasching, Austria). Upon manual detachment from the epidermis, the dermis was minced and incubated with Dulbecco's modified essential medium (DMEM, Gibco Invitrogen, Oslo, Norway) containing 400 U ml<sup>-1</sup> of collagenase I (CLS-1, Worthington, Lakewood, NJ) for 1 hour at 37 °C. Dermal fibroblasts were cultured in DMEM supplemented with 10% fetal calf serum (PAA Laboratories) and 1% penicillin and streptomycin (Sigma-Aldrich, Oslo, Norway). Immorto mouse dermal fibroblasts were generated as previously described (Whitehead and Joseph, 1994). Immortalized cells showed collagen-binding integrin expression levels similar to primary cells (Supplementary Figure S2A online). Immortalized fibroblasts were used in experiments leading to the following results: Figure 2c, 4f and g, 5e and f. Integrin  $\alpha 11$  was rescued in Simian virus 40-immortalized  $\alpha 11^{-/-}$  fibroblasts ( $\alpha 11^{-/-}$ -KI) by viral transfection with the full-length of mouse *Itga11* cDNA (Lu *et al.*, 2014). *jnk1^{-/-} // jnk2^{-/-}* MEFs are described in Schumacher *et al.* (Schumacher *et al.*, 2014), originally isolated by Prof. Erwin Wagner (Javelaud *et al.*, 2003).

### Wounding and staining of wound tissues

Excisional and incisional full-thickness wounds were inflicted on the shaved backs of 10-week-old female mice as described (Zweers *et al.*, 2007). Incisional wounds of 2.5 cm were placed on the lower back

crossing the midline, sutured in the center, and harvested at 16 days after injury.

For histology, wounds were bisected, and either fixed for 2 hours in 4% paraformaldehyde and then processed for paraffin embedding or frozen unfixed in O.C.T. compound (Sakura, Staufen, Germany). Paraffin sections were stained with hematoxylin and eosin or Sirius red according to standard procedures. Vascular structures were visualized by immunofluorescence staining of cryosections, acetone fixed, and incubated overnight at 4 °C with an antibody against CD31 (MEC13.3, BD Biosciences, Heidelberg, Germany; 1:1,000 in (phosphate buffered saline) PBS/1% bovine serum albumin (BSA)), followed by incubation with a Cy3-conjugated antibody directed against  $\alpha$ -SMA (4A1, Sigma-Aldrich; 1:500 in PBS/1% BSA). Secondary IgG1 $\kappa$ , Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes, Darmstadt, Germany; 1:500 in PBS/1% BSA) was applied for 1 hour at room temperature. Sections were counterstained with DAPI and mounted.

Myofibroblasts were visualized by immunohistochemical staining with an antibody directed to  $\alpha$ -SMA (Tomasek *et al.*, 2005). Paraffin sections were dewaxed in xylol and rehydrated, blocked in 10% normal goat serum for 30 minutes (endogenous biotin was blocked using the Biotin Blocking System (Dako, Hamburg, Germany)), incubated with primary fluorescein-conjugated antibody against  $\alpha$ -SMA (1:250 in 1% BSA), followed by rabbit anti-fluorescein antibody (1:750, Molecular Probes A889) and subsequently by biotinylated goat anti-rabbit IgG (Vectastain ABC kit, Vector Laboratories, Peterborough, UK). Complex was detected by incubation with the ABC-alkaline phosphatase complex (Vector Laboratories), and color was developed with Vector red alkaline phosphatase substrate (Vector Laboratories).

### Collagen type I gel contraction

Collagen gel contraction was performed as previously described (Barczyk *et al.*, 2013). Briefly, each ml contained the following: 500  $\mu$ l of 2  $\times$  DMEM containing 1.6  $\times$  10<sup>5</sup> cells per ml, 10  $\mu$ l 200 mM Glutamine (Cambrex Bioscience, Stockholm, Sweden), 10  $\mu$ l antibiotics, 100  $\mu$ l 0.2M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma-Aldrich) pH 8.0, and 400  $\mu$ l collagen type I (PureCol, Advanced Biomatrix, Carlsbad, CA). A total of 300  $\mu$ l of this mixture was added into each well of a 24-well plate and were allowed to polymerize (~90 minutes) at 37 °C. In order to obtain floating conditions, gels were poured into wells that had been previously coated overnight with 5% BSA in sterile PBS. Once the cell-containing collagen mixture had polymerized, DMEM supplemented with 2% fetal calf serum was added. Recombinant human TGF- $\beta$ 1 (PeproTech, Stockholm, Sweden; 5 ng ml<sup>-1</sup>), SB505124 (Sigma-Aldrich; 10  $\mu$ M), SP600125 (Sigma-Aldrich; 25  $\mu$ M), SB203580 (Sigma-Aldrich; 10  $\mu$ M), PD098059 (Sigma-Aldrich; 5  $\mu$ M), PF-573228 (Sigma-Aldrich; 10  $\mu$ M) or  $\beta$ 1 integrin antibody Ha 2/5 (BD Biosciences) were used as indicated. After 24 hours of collagen remodeling, we did not notice significant differences in cell number between  $\alpha 11^{+/+}$  and  $\alpha 11^{-/-}$  fibroblasts. Results are expressed as the mean from three independent experiments  $\pm$  SD, each condition at least performed in triplicates per experiment.

### Statistical analysis

Results are expressed as the mean  $\pm$  SD of at least three replicates and are representative of three independent experiments. Statistical



significance was assessed using unpaired Student's *t*-tests unless stated differently, with *P*<0.05 being considered significant. Gaussian distribution was verified by the Kolmogorov-Smirnov test. Calculations were performed using GraphPad Prism (GraphPad software, La Jolla, CA).

#### CONFLICT OF INTEREST

The authors state no conflict of interest.

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#### SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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