Numb is required to prevent p53-dependent senescence following skeletal muscle injury

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Regeneration relies on coordinated action of multiple cell types to reconstitute the damaged tissue. Here we inactivate the endocytic adaptor protein Numb in skeletal muscle stem cells prior to chronic or severe muscle injury in mice. We observe two types of senescence in regenerating muscle; a transient senescence in non-myogenic cells of control and Numb mutant mice that partly depends on INK4a/ARF activity, and a persistent senescence in myogenic cells lacking Numb. The senescence levels of Numb-deficient muscle is reduced to wild type levels by an anti-oxidant treatment or p53 ablation, resulting in functional rescue of the regenerative potential in Numb mutants. Ex vivo experiments suggest that Numb-deficient senescent cells recruit macrophages to sustain inflammation and drive fibrosis, two hallmarks of the impaired muscle regeneration in Numb mutants. These findings provide insights into previously reported developmental and oncogenic senescence that are also differentially regulated by p53.
issue regeneration is characterised by three distinct overlapping phases including inflammation, tissue reconstruction and remodelling. In skeletal muscle, the paired/homeodomain protein Pax7 is a marker of muscle stem (satellite) cells and Pax7-positive cells are critically required for muscle regeneration. Following injury, satellite cells are activated, they proliferate, and some resulting myoblasts differentiate and fuse to form new myofibers, whereas a subset return to quiescence and replenish the stem cell niche. During the expansion of satellite cells, muscle-resident fibroblasts proliferate, provide pro-differentiation signals to myoblasts, and secrete extracellular matrix thereby stabilizing the tissue.

Concomitantly to myogenesis, angiogenesis stimulates myogenic growth. In addition, the inflammatory response that is mediated through the action of macrophages is necessary to repair damaged tissues. Communication between these distinct cell types is crucial during the process of regeneration, as sustained inflammation drives aberrant fibrosis and contributes to pathology.

Senescent cells act in paracrine and via their secretome induce a local inflammatory response leading to their elimination by phagocytosis. Thus, cellular senescence is a mechanism contributing to tissue remodelling, particularly during tumour formation, organogenesis and as reported recently, during the process of wound healing. Paradoxically, senescent cells can be beneficial and detrimental for tissue constitution. Senescent cells share common features such as an irreversible cell cycle arrest, a change in morphology, senescence-associated heterochromatin foci, and a senescence-associated secretory phenotype. In addition, senescent cells can be identified by histochemical detection of β-galactosidase activity under acidic conditions, called senescence-associated β-galactosidase activity (SAβGal; ref. 16). Multiple stresses induce senescence, which is regulated mainly by the tumour suppressors p16, p19, p53 (SAIp16) and Rb, as well as the cyclin-dependent kinase inhibitors p21 and p27.

Studies have focused mainly on the beneficial action of non-myoegenic cells during muscle regeneration, yet it remains unclear to what extent satellite cells and their committed progeny communicate with their environment. The endocytic adaptor Numb possesses multiple protein–protein interaction domains that confer pleiotropic functions including modulation of Notch, Shh and Wnt signalling. Thus, to explore the possibility that Numb can mediate myogenic cell communication in skeletal muscle, we examined the function of this protein specifically in the myogenic lineage following muscle injury where it was reported to control different steps during muscle regeneration. We show that deletion of Numb/Numbl in satellite cells prior to injury lead to impaired regeneration marked by increased inflammation and fibrosis. Importantly we unveiled two types of senescence during regeneration; a transient senescence in non-myoegenic cells in control and Numb mutant mice, which is partially dependent on Ink4A/ARF activity, and a persistent senescence in myogenic cells, exclusively in Numb mutant mice. The latter depends on p53 and is rescued by the administration of anti-oxidant. In vivo and ex vivo experiments further showed that Numb mutant-specific senescent cells are responsible for the impaired regeneration phenotype.

Results
Impaired regeneration in Numb mutants following acute injury.
Numb is widely expressed in different cell types in the muscle and we observed that this protein is expressed in about 85% of both quiescent and ex vivo activated satellite cells (Supplementary Fig.1a–c). To investigate the function of Numb specifically in myogenic cells, we performed a conditional inactivation of Numb using an inducible Tg:Pax7-CreERT2 (hereafter Tg:Pax7CT2) in satellite cells in the context of a constitutive mutant for its paralog Numbl (Supplementary Fig. 1b). Following 4-hydroxytamoxifen (4-OHT) injections, adult Tg:Pax7CT2;NumblF/F:Numbl+/− mice (hereafter Numb:Numbl or mutant) were indistinguishable from adult control mice; 64% of their satellite cells lacked Numb expression at T0, and after 40 h in culture (Supplementary Fig. 1c). We then used R26mT-mC reporter mice to isolate Numb depleted cells. Among the recombined mGFP+ cells, 62% (n = 3) were depleted for Numb protein (data not shown). The Tibialis anterior (TA) muscle was injured with the snake venom cardiotoxin, collected and analysed at different time points during regeneration. Importantly, isolated mGFP+ cells displayed a persistent decrease in Numb transcript levels by about 50% compared with controls at all time points examined during homeostasis, regeneration, and after muscle recovery (Supplementary Fig. 1d). Strikingly, Numb transcript levels in controls increased late in regeneration, suggesting a function for Numb at these stages. At 21 days post-injury (DPI), histological analysis of controls showed centrally localised myonuclei, a hallmark of regenerating myofibers (Fig. 1a, b). In contrast, mutant TA muscles displayed a highly perturbed morphology, including a high number of interstitial cells and heterogenous myofiber sizes, the latter persisting at 60 DPI (Fig. 1b; Supplementary Fig. 1f). Moreover, an inflammatory phenotype was noted by the presence of calcium deposits (Von Kossa staining) and a 1.7-fold increase in the number of macrophages (F4/80+ cells) in mutant TA muscles compared with controls (Fig. 1c–e). In addition, Sirius Red staining on TA muscle revealed increased fibrosis in mutants that was accompanied by a slight increase in the number of fibroblast-like (Tcf4+) cells (Fig. 1f–h). Taken together these results show that in the absence of Numb/Numbl, muscle regeneration was compromised.

Using in vivo and ex vivo approaches, we noted that the ability of mutant cells to proliferate, generate new myofibers, return to quiescence following injury, and differentiate was not overtly compromised in the absence of Numb/Numbl (Fig. 1j–m; Supplementary Figs 1c and 2a–m; see also Supplementary Fig. 3a–d). Notably, the number of Pax7+ cells and the relative levels of Pax7 transcripts in mGFP+ cells were not significantly changed in mutant muscle compared with control muscle at homeostasis and during regeneration (Fig. 1m; Supplementary Fig. 1e). These data contrast with those reported previously showing decreased satellite cell proliferation after injury when Numb was deleted in the Pax7 lineage from embryonic stages to adulthood. Those Numb mutant mice were smaller at 1 month of age and they exhibited smaller fiber size suggesting that Numb plays an important additional role during developmental myogenesis or perinatally. Collectively, these observations suggest that Numb has distinct functions in proliferating muscle progenitors during development, perinatally and in adult satellite cells, as it is the case for Pax7. In addition, the absence of an overt differentiation phenotype strongly suggests that Numb does not inhibit Notch signalling (Supplementary Fig. 2a–i). This point was supported by the observation that the relative levels of Notch target transcripts (Hey1 and HeyL) in isolated mGFP+ cells were similar in mutants and controls during homeostasis, and at different time points following injury (Supplementary Fig. 2n and o).

Lack of Numb exacerbates the muscle dystrophic phenotype.
We then focused on a more physiological model of regeneration by examining DMDmdx/Fgco mice in a Numb:Numbl null mutant background. DMDmdx/Fgco mice constitute a model of muscular dystrophy where dystrophin, a major component of the
Figure 1 | Incomplete regeneration following deletion of Numb:Numbl in satellite cells. (a) Strategy used to study TA muscle regeneration in the context of Numb:Numbl depleted satellite cells. Data were collected from transverse TA muscle cryosections of adult mice. (b) Histological staining with H&E. (c) Histological staining with Von Kossa. Dark precipitates reveal calcium deposits, a sign of chronic inflammation. (d) Immunofluorescence staining for fibroblasts (arrows) using anti-F4/80 antibody. (e) Quantification of the number of macrophages in (d): control: n = 5 mice, 3.2 ± 0.4 cells per unit area; mutant: n = 5 mice, 5.6 ± 0.4 cells per unit area; Mann-Whitney (MW) test P = 0.079. (f) Histological staining with Sirius Red. (g) Quantification of fibrosis based on Sirius Red histology in (f): control: n = 5 TA, 5 mice, 5.6 ± 0.3% fibrosis per unit area; mutant: n = 5 TA, 5 mice, 12 ± 3.5% fibrosis per unit area; MW test P = 0.0397. (h) Immunofluorescence staining for fibroblasts (arrows) using anti-TCF4 antibody. (i) Quantification of the number of fibroblasts in (h): control: n = 4 mice, 14.2 ± 1.4 cells per unit area; mutant: n = 4 mice, 18.8 ± 1.12 cells per unit area; MW test P = 0.0571. (j) Quantification of the numbers of mGFP+ fibres: control: n = 3 TA, 3 mice, 45 ± 3.1 fibres per unit area; mutant: n = 6 TA, 3 mice, 41.4 ± 3.7 fibres per unit area; MW test P = 0.2619. (k) Immunofluorescence staining for quiescent satellite cells (arrows) using anti-CalR receptor antibody. (l) Quantification of quiescent satellite cells in (k): control: n = 4 mice, 2.17 ± 0.15 cells per unit area; mutant: n = 4 mice, 1.83 ± 0.14 cells per unit area; MW test P = 0.2. (m) Quantification of the number Pax7:nGFP+ cells in one diaphragm for quiescent cells: control: n = 8 mice, 60,376 ± 4,038; mutant: n = 7 mice, 65,663 ± 10,106; MW P = 0.6943 and in 2 TA for activated cells (control 5 DPI: n = 3 mice, 174,880 ± 15,190; mutant 5 DPI: n = 3 mice, 170,490 ± 4,747, MW P > 0.99; control 8 DPI: n = 12 mice, 74,306 ± 5,002; mutant 8 DPI: n = 7 mice, 77,852 ± 9,562, MW P = 0.9671; control 21 DPI: n = 3 mice, 59,935 ± 5,284; mutant 21 DPI: n = 4, 36,867 ± 4,276, MW P = 0.0571). Quantifications are presented as mean ± s.e.m. Scale bars: b, c, f: 100 µM; d, h: 25 µM; k: 20 µM. NS, not significant.
architecture of the myofiber, is lacking. In this model, myofibers continuously and asynchronously degenerate, and a proportion of satellite cells is randomly activated to repair the damaged fibers. Moreover, DMDmDmdxgeo mice exhibit chronic inflammation and aberrant fibrosis. Remarkably, at early stages of the disease (8 weeks), Numb:Numbl:DMDmDmdxgeo mice showed significantly higher transcript levels of Collagen1a1 (Fig. 3e). We next tested whether mutant reserve cells could act as myofibroblast marker cells; they were not expressed significantly higher transcript levels of Collagen1a1 compared with DMDmDmdxgeo mutants (Fig. 2e–h). In 1-year-old mice, increased fibrosis was still observed in Numb:Numbl:DMDmDmdxgeo mutants compared with DMDmDmdxgeo mutants, although this was not statistically significant. We thus conclude that loss of Numb/Numbl in the myogenic lineage can exacerbate the muscular dystrophic phenotype.

Self-renewed mutant cells acquire features of senescence. In light of the above findings, we hypothesized that Numb:Numbl mutant satellite cells acquired altered properties at late stages of regeneration during the transition to quiescence or differentiation, resulting in impaired regeneration. To test this notion, we cultured satellite cells and allowed them to fuse and form a network of myotubes. In this model, some cells (called reserve) remain mononucleated and acquire satellite cell properties, including the expression of Pax7 (ref. 33). After 21 days in vitro (DIV), cells were dissociated and mGFP+ mononucleated cells were isolated (Fig. 3a). As expected, most of these cells expressed Pax7 (88 ± 1.3% Pax7+/mGFP+ cells; n = 4 mice), independently of their genotype. Remarkably, 7 days after replating, mutant reserve cells stopped proliferating and displayed altered morphology, in contrast to control cells that continued to proliferate, then fused to form myotubes (Fig. 3b and c). Further analysis showed that some cells in confluent culture of myotubes derived from mutant satellite cells were SAβGal+, a marker of senescence, whereas none were observed in control (Fig. 3b). This was also the case after 7 and 14 DIV (Fig. 3b). In parallel, we showed that this phenotype was not due to replicative senescence as the doubling population rates of control and mutant primary satellite cells were comparable (Supplementary Fig. 3c and d).

We then investigated the acquisition of other senescence features by mutant reserve cells. The change of the nuclear localisation of HP1γ from uniform staining to foci illustrates the formation of the senescence-associated heterochromatin foci. The number of reserve cells harbouring HP1γ in foci 20 h post-plating was significantly higher in mutant compared with control cells (Fig. 3c and d). The green fluorescent protein (GFP) immunostaining clearly demarcated the morphology of mutant reserve cells as that described for senescent cells (Fig. 3c). Accordingly, mutant cells expressed significantly higher transcript levels of the myofibroblast marker Collagen1a1 compared with controls (Fig. 3e). We next tested whether mutant reserve cells could act in paracrine on neighbouring cells. Consistent with this hypothesis, IL6 an identified senescence-associated secretory phenotype component, showed significantly higher transcript levels in mutant reserve cells compared with controls (Fig. 3f). Accordingly, the percentage of reserve cells expressing IL6 protein was significantly higher in mutants compared with controls (Supplementary Fig. 3e–h). Paracrine signalling of reserve cells, was further tested by co-culturing reserve cells (mGFP+ at equal ratio with primary wild-type satellite cells (mGFP–). After 9 DIV, control reserve cells cultured with primary satellite cells contributed to the formation of myofibers (mGFP+/Myosin Heavy Chain+; Fig. 3g). In contrast, the density and the differentiation of the co-culture with mutant reserve cells were severely compromised. Fewer cells differentiated and most of these cells were derived from primary wild-type satellite cells (mGFP–; Fig. 3g). These observations show that the reserve mutant cells act in paracrine to regulate proliferation and differentiation of adjacent wild-type cells in the co-culture assay. These results raise the possibility that Numb mutant-specific senescent cells might recruit, via their secretome, macrophages to induce chronic inflammation. To test this notion, we used a transwell assay to examine the ability of conditioned medium derived from reserve cells to influence the migration of macrophages (Fig. 3h). Following 6 h of culture, the migration of macrophages across the transwell membrane increased when incubated with conditioned medium derived from reserve mutant cells compared with the culture with conditioned medium derived from control reserve cells (Fig. 3h and i). Taken together, these findings show that ablation of Numb/Numbl function in the myogenic lineage results in paracrine signalling that impacts on myogenic and macrophage properties.

We then asked if senescence properties can be identified directly in freshly isolated satellite cells. Satellite cells from 1-year-old DMDmDmdxgeo mice are expected to have undergone multiple rounds of activation and quiescence cycles, thus resembling cultured reserve cells that had returned to quiescence. Notably, five-fold more cells expressed HP1γ in nuclear foci of Numb:Numbl:DMDmDmdxgeo mutants compared with DMDmDmdxgeo mice after 20 h in culture (Fig. 3j and k). In addition, SAβGal+ cells were detected exclusively with Numb:Numbl:DMDmDmdxgeo myogenic cells after 21 DIV (Fig. 3l). In summary, using multiple assays we show that senescence features are induced cell autonomously in satellite cells in the absence of Numb/Numbl during the course of activation and return to quiescence or differentiation, both in vivo and ex vivo.

Increased senescence in the absence of Numb/Numbl. These findings strongly suggest that loss of Numb/Numbl in the Pax7 lineage induces or sustains senescence during regeneration in vivo. Senescence was identified as a cellular mechanism of satellite cell aging in geriatric mice (over 28 months) preventing these cells from activation and self-renewal. Therefore, we evaluated senescence in vivo in young adult mice to distinguish the process of regeneration from ageing. SAβGal analysis was performed on 10 DPI, 21 DPI and 8-week-old DMDmDmdxgeo mice compared with mGFP+/C0 TA muscle from Numb/Numbl and control mice. Strikingly, we noted SAβGal+ cells in controls, as well as in mutants following acute and chronic injury. These cells were not cycling (Ki67−) suggesting that they had undergone senescence (Fig. 4a and b). In the control condition more than half of these cells expressed the endothelial marker Flk-1 (vascular endothelial growth factor receptor 2) and only a subset of SAβGal+ cells expressed the macrophage surface marker F4/80 (Fig. 4b; Supplementary Fig. 4a). Of note, SAβGal+ cells did not express Pax7 (Pax7GFP−), Tcf4, nor the pericyte marker NG2 (Supplementary Fig. 4a). Importantly, we observed a 25% reduction in the number of SAβGal+ cells at 10 DPI in Ink4a/ARF mutant mice compared with controls pointing to a partial role for p16 and/or p19 in the induction of senescence features during regeneration (Fig. 4c). In contrast, no significant difference in the number of SAβGal+ cells was observed in mice mutant for either p21 or p53 at 10 DPI (Supplementary Fig. 4c and d). These findings are reminiscent of the beneficial action of senescent fibroblasts and endothelial cells during wound healing, and that depends on p16/p21 (ref. 13). Consistent with this notion, two observations suggest that these SAβGal+ cells
**Figure 2 | Loss of Numb/Numbl exacerbates the dystrophic muscle phenotype.** Data were collected from transverse TA muscle cryosections from DMDmdx-bgeo mice. 

(a) Scheme of the experiments. (b) Histological staining with H&E. (c) Quantification of fibrosis in mutants and controls in 8-week-old DMDmdx-bgeo mice based on Sirius Red histology (control: n = 8 TA, 5 mice, 10.4 ± 0.8% fibrosis per unit area; mutant: n = 13 TA, 7 mice, 13.5 ± 0.8% fibrosis per unit area; MW P = 0.0303). (d) Histological staining with Sirius Red. (e) Quantification of F4/80⁺ cells (control: n = 4 mice, 9.8 ± 0.9 cells per unit area; mutant: n = 5 mice, 20.8 ± 2.7 cells per unit area; MW P = 0.0159). (f) Immunofluorescence staining for macrophages using anti-F4/80 antibody. (g) Quantification of Tcf4⁺ (control: n = 4 mice, 14.3 ± 0.7 cells per unit area; mutant: n = 4 mice, 19.2 ± 1.0 cells per unit area; MW P = 0.0286). 

(h) Immunofluorescence staining for fibroblasts using anti-Tcf4 antibody. (i) Scheme of the experiments. (j) Histological staining with H&E. (k) Quantification of fibrosis in mutants and controls in 1-year-old DMDmdx-bgeo mice based on Sirius Red histology and (control: n = 3 TA, 3 mice, 9.1 ± 0.4% fibrosis per unit area; mutant: n = 3 TA, 3 mice, 20.1 ± 2.4% fibrosis per unit area; MW P = 0.10). (l) Histological staining with Sirius Red. Quantifications are presented as mean ± s.e.m.. Scale bars b, d, j, l: 100 μM; f, h: 25 μM. NS, not significant.
Figure 3 | Self-renewed mutant satellite cells acquire senescence properties. (a) Strategy used to obtain and examine reserve or self-renewed cells. (b) SAβGal staining on confluent myotubes derived from isolated mGFP+ satellite cells maintained 21 days *in vitro* (21 DIV) and on replated isolated mGFP+ reserve cells after 7 and 14 DIV. Arrowheads point to SAβGal+ cells (dark staining). Windows in left centre and bottom pictures represent high magnifications of the main image highlighting SAβGal+ cells. Data are representative of 3 independent experiments using 2 controls and 2 mutants for each. (c) Immunofluorescence using anti-GFP and anti-HP1γ antibodies on reserve cells 20 h after plating. (d) Quantification of the number of self-renewed cells with HP1γ and HP1β antibodies as opposed to uniform staining at 20 h after plating (control: n = 9 mice, 37.4 ± 2.9%; mutant: n = 9 mice, 56.1 ± 1.2%; MW P < 0.0001). (e,f) Relative levels of Col1a1 (control: n = 13 mice and mutant: n = 13 mice; MW P = 0.0207) and IL6 (control: n = 8 and mutant: n = 8 mice; MW P = 0.0007) mRNA (2–ΔCT) in reserve mutant cells compared with controls. TBP was used as reference gene. (g) Immunofluorescence using anti-GFP and anti-Myosin Heavy Chain (MyHC) antibodies on control and mutant reserve cells (mGFP+) cultured for 9 DIV either alone or mixed at equal ratio with primary isolated satellite cells (mGFP−). Left panels represent for each condition the merge pictures of the right panels. Data are representative of 2 independent experiments using 2 controls and 2 mutants for each. (h) Scheme of the migration assay of the macrophages. (i) Relative levels of Numb:Numbl:DMDmdx−geo mRNA (2–ΔCT) in reserve cells maintained 21 days *in vitro* (21 DIV) and on replated isolated reserve or self-renewed satellite cells. TBP was used as reference gene. (j) Scheme of the migration assay of the macrophages. (k) Quantification of the number of macrophages that migrate through the transwell membrane (control: n = 7 mice and mutant: n = 7 mice; MW P = 0.0186). (l) Scheme used to study DMDmdx−geo satellite cells *in vitro*. (m) Quantification of the number of satellite cells with a nuclear localisation of HP1γ in foci at 20 h after plating (control: n = 2 mice, 2.2 ± 0.3%; mutant: n = 2 mice, 9.9 ± 2.9%). (n) SAβGal staining on satellite cells isolated from 1-year-old DMDmdx−geo mice and cultured 21 DIV. Note extensive SAβGal staining in Numb:Numbl:DMDmdx−geo mutant cells compared with controls. Quantifications are presented as mean ± s.e.m. Scale bars b,g: 50 μM, c: 10 μM, l: 33 μM.

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might play a role in tissue remodelling during muscle regeneration. First, the number of SAβGal+ cells was transient during early stages of regeneration in controls (about 3.5 cells per unit area at 10 DPI compared to 0.15 cells per unit area at 21 DPI; Fig. 4h). Second, no SAβGal+ cells were detected in TA muscle during homeostasis.

Importantly, in all conditions tested, the number of SAβGal+ cells was significantly higher in mutant mice compared with controls (Fig. 4d–h). The most notable difference between both genotypes was a five-fold increase in SAβGal+ cells at 21 DPI when homeostasis was being restored, representing a third of the total population of quiescent cells (see cell numbers in Fig. 1l and Fig. 4h). The level of reactive oxygen species (ROS) can initiate senescence, and treatments with anti-oxidants delay or prevent cellular senescence. We thus tested if reducing ROS levels during regeneration would alter the number of SAβGal+ cells. Mice were exposed to the anti-oxidant N-acetyl cysteine (NAC) from 5–10 DPI and SAβGal+ cells were quantified (Fig. 4i). The number of SAβGal+ cells was similar in controls with or without NAC treatment. In contrast, treatment with NAC resulted in a reduction in the number of senescent cells in mutants to levels observed in controls (Fig. 4j). Strikingly, histological analysis showed that treatment with NAC resulted in a rescue of the regeneration phenotype and tissue architecture in Numb:Numbl null mutants (Fig. 4k).

Figure 4 | Increased senescence in the absence of Numb/Numbl during muscle regeneration. (a) Scheme of the experiments. Data were collected from transverse TA muscle cryosections of adult mice. (b) SAβGal staining combined with immunohistochemistry using anti-Ki67 or anti-GFP antibodies on control (left panel) and Flk-1GFP+ (right panel) mice. Brown arrowheads point to Ki67+ only cells; blue arrowhead points to SAβGal+ only cells and black arrowhead points to GFP+/SAβGal+ cells. (c) Quantification of SAβGal+ cells in INK4a/ARF mutant mice at 10 DPI (control: n=12 TA, 6 mice, 3.7±0.2 cells per unit area; INK4a/ARF mutant mice n=12 TA, 6 mice, 2.72±0.2 cells per unit area; MW P=0.0053). (d, f) Schemes of the experiments. (e, g) SAβGal staining on transverse TA muscle cryosections. (h) Quantification of SAβGal+ cells during regeneration in controls and mutants at 10 DPI, 21 DPI, and in 8-weeks old DMDmdxgeo mice. 10 DPI TgPax7nGFP, n=6 sections, 3 mice, 3.65±0.64 cells per unit area; control: n=5 sections, 4 mice, 3.5±0.26 cells per unit area; mutant: n=9 sections, 3 mice, 5±0.35 cells per unit area; MW TgPax7nGFP versus control P=0.7922, TgPax7nGFP versus mutant P=0.0256, control versus mutant P=0.0040. The number of SAβGal+ cells at 10 DPI is similar in TgPax7nGFP mice compared with controls (TgPax7CT2;Numbfl/fl;Numbl+/+) showing that the tamoxifen treatment or the presence of the Cre recombinase did not interfere with the process. 21 DPI control: n=6 sections, 3 mice, 0.14±0.05 cells per unit area; mutant: n=6 sections, 4 mice, 0.71±0.21 cells per unit area; MW P=0.0043. DMDmdxgeo, n=6 sections, 3 mice, 2.1±0.4 cells per unit area; Numb:Numbl/DMDmdxgeo, n=6 sections, 4 mice, 4.6±1.1 cells per unit area; MW P=0.0411. (i) Scheme of experiment. (j) Quantification of the number of SAβGal+ cells (control: n=6 sections, 3 mice, 3.2±0.1 cells per unit area; control + NAC: n=8 sections, 4 mice, 3.4±0.2 cells per unit area; mutant: n=8 sections, 4 mice, 4.7±0.1 cells per unit area; mutant + NAC: n=8 sections, 4 mice, 3.4±0.5 cells per unit area; MW control versus control + NAC P=0.4136, control versus mutant P=0.0007, control + NAC versus mutant P=0.0006, mutant versus mutant + NAC P=0.0148). (k) Hematoxylin/eosin staining of mutant TA muscles treated or not with NAC. Quantifications are presented as mean ± s.e.m. Scale bars: b: 50 µM, e, g, k: 100 µM. NS, not significant.
(Supplementary Fig. 4f, g). Thus, the absence of Numb/Numbl does not induce an increase of ROS. Importantly, moderate increased levels of ROS appeared during lineage progression (Supplementary Fig. 4f), and this was shown previously to sensitize foetal myogenic and hematopoietic progenitors cells to differentiation35,36. It is thus tempting to speculate that the myogenic population affected by the loss of Numb/Numbl corresponds to a sub-population of cells with increased endogenous ROS levels, and that is committed either to differentiate or to return to quiescence. In summary, we identified two types of senescence; one that is transient during muscle regeneration in wild-type mice, and a second type exclusive to Numb:Numbl mice, that persists until regeneration was virtually completed. The latter was uniquely rescued by anti-oxidant treatment between 5–10 DPI.

**Loss of Numb induces a p53-dependent senescence.** We next aimed at identifying the mediators of senescence that are induced in the absence of Numb/Numbl during muscle regeneration. Immunofluorescence performed on TA muscle revealed that the number of p53+ and p21+ cells increased significantly in Numb:Numbl mutants compared with controls at 10 DPI. Notably, most of the p53+ (>85%) and p21+ (>92%) cells were myogenic independently of their genotype, as they expressed the lineage marker mGFP (Fig. 5a–f). In addition, the small fractions

![Image](attachment://image.png)

**Figure 5 | Loss of Numb/Numbl induces senescence in a p53-dependent manner.** (a) Scheme of the experiment. (b-e) Immunofluorescence on transverse sections using anti-GFP and anti-p53 (b,c) or anti-p21 (d,e) antibodies, arrows point to positive p53 or p21 cells. f' and e' represent higher magnifications of the area highlighted by a square in c and e, respectively. f' and e' are the same images as in c' and e' without the Hoechst and p53 or p21 staining. (f) Quantification of the number of p53+ and p21+ cells. The number of p53+/mGFP+ cells increases in mutant compared with control (control: n = 8 sections, 4 mice, mGFP+ 2.24 ± 0.22 cells per unit area, mGFP– 0.37 ± 0.19 cells per unit area; mutant: n = 8 sections, 4 mice, mGFP+ 4.18 ± 0.48 cells per unit area, mGFP– 0.48 ± 0.06 cells per unit area; MW control mGFP+ versus mutant mGFP+ P = 0.0145). Similarly, the number of p21+/mGFP+ cells increases in mutant compared with control (control: n = 10 sections, 4 mice, mGFP+ 1.24 ± 0.06 cells per unit area, mGFP– 0.1 ± 0.06 cells per unit area; mutant: n = 10 sections, 3 mice, mGFP+ 1.96 ± 0.17 cells per unit area, mGFP– 0.15 ± 0.05 cells per unit area; MW control mGFP+ versus mutant mGFP+ P = 0.0094). (g) Quantification of SAβGal+ cells in control, Numb and compound Numb:p53 mutants. The absence of p53 in Numb mutants reduces the number of SAβGal+ cells down to control levels (control: n = 14 sections, 6 mice, 3.85 ± 0.28 cells per unit area; Numb mutant: n = 12 sections, 3 mice, 5.87 ± 0.63 cells per unit area; Numb:p53 double mutant: n = 16 sections, 4 mice, 4.08 ± 0.63 cells per unit area; MW control versus Numb mutant P = 0.0010, Numb mutant versus Numb:p53 double mutant P = 0.0008). (h) Hematoxylin/eosin stainings of control and mutant TA muscles. (i) Quantification of fibrosis, based on Sirius Red staining (control: n = 8 TA, 6 mice, 18.90 ± 0.33% fibrosis per unit area; Numb null mutant: n = 6 TA, 3 mice, 21.97 ± 0.92; Numb:p53 double mutant: n = 8 TA, 4 mice, 18.63 ± 0.67 cells per unit area; MW control versus Numb mutant P = 0.0205, Numb mutant versus Numb:p53 double mutant P = 0.0401). (j) Sirius Red stainings of control and mutant TA muscles. Quantifications are presented as mean ± s.e.m. Scale bars c,e: 50 μM, c',e': 125 μM; h,j: 100 μM.

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of p53+/−mGFP− cells and p21+/−mGFP− cells remained constant between both control and mutant muscles. These results show that the absence of Numb/Numbl in the myogenic lineage (mGFP+−) results in the cell autonomous induction of the senescent mediators p53 and p21. The findings reported here contrast with the previously described function of Numb in mammary cancer cells where Numb was reported to form a tripartite complex with p53 and the E3 ubiquitin ligase MDM2, hence preventing p53 ubiquitination and further degradation17. Our observations thus suggest that the regulation of p53 levels by Numb during the process of regeneration is indirect. We then determined whether p53 was sufficient to generate supernumerary SAβGal+ cells in Numb mutants by analysing Numb:p53 compound mutants at 10 DPL. Strikingly, the number of SAβGal+ cells in mutants decreased to the levels observed in controls (Fig. 5g) as it was the case for anti-oxidant treatment indicated above. Most importantly, the muscle histological phenotype of Numb mutant TA muscle, including the increased fibrosis, was rescued in the absence of p53 (Fig. 5f–h). We thus identify p53 as a major regulator of a novel type of senescence induced by the absence of Numb during muscle regeneration.

Discussion

Our study shows that cellular senescence participates in the process of skeletal muscle regeneration. We showed in control mice, that some endothelial cells (Flk-1 GFP−− cells) are senescent and that the induction of the SAβGal+ cells partially depends on Ink4a/Arf activity. Our findings together with the described beneficial action of senescent fibroblasts and endothelial cells during wound healing33 suggest that cellular senescence is a common cellular mechanism used during the process of regeneration of distinct organs.

The absence of Numb in the myogenic lineage induced a previously unreported type of senescence that acts in a cell autonomous manner. This persists when regeneration is virtually complete and it requires both p53 and elevated levels of ROS. Importantly, these factors clearly distinguished Numb mutant-specific senescence from the cellular senescence observed during organogenesis and wound healing that was reported to depend on p21 and p16/p21, respectively11−13. Instead, our studies suggest that Numb mutant-specific senescence is more closely related to oncogene-induced senescence models where p38 MAPK activity induced by elevated ROS regulates phosphorylation and activity of p53 (ref. 38). The ability to isolate the population of myogenic cells affected by the loss of Numb will allow the future identification of the underlying mechanisms initiating senescence when the function of Numb is compromised.

Furthermore, our findings underscore the importance of the communication between myogenic cells and their environment during regeneration. The rescue of the impaired regeneration phenotype in Numb mutant mice correlated with the loss of supernumerary SAβGal+ cells (Figs 4j, k and 5g−j). In addition, our ex vivo experiments lead us to propose that Numb mutant-specific senescent cells act in a paracrine manner to recruit macrophages and sustain inflammation, thereby promoting an increase in fibrosis. Chronic inflammation and aberrant fibrosis are also associated with numerous muscle pathologies. In this scenario, we show that in the absence of Numb, the phenotype of dystrophic mice is exacerbated. Therefore, close examination of the properties of activated satellite cells in relation to their environment, such as the identification of secreted factors, could be informative in devising therapeutic strategies for muscle pathologies.

Methods

Mice and breeding. Animals were handled as per European Community guidelines and the ethics committee of the Institut Pasteur (CTEA) approved protocols. TgPax7CT2 mice24 were used to delete Numbfloxed allele in satellite cells. NumbΔδNumbΔδ were obtained from Jackson Laboratories (005384; ref. 25). Numb−/− mutant mice were generated by crossing Numb−/−mice with C57BL/6J females containing the reporter allele R26Rtneg. The genetic background of the resulting mice was mixed (C57BL/6J and 129/Sv). During the course of the experiments we noticed that females were also taken to examine NumbΔαβ compound mutants and control littersmates. DM2αβ; floxed; mGFP− mice were used24. All experiments were carried out on males except females which were also taken to examine NumbΔαβ compound mutants and control littersmates.

Isolation of satellite cells and cell culture. TA muscle (for injured muscle) or hindlimb of one mouse were chopped and divided into 4 samples. Enumeration of GFP+ cells was performed from TA-injured muscles (Fig. 4i, k and 5g–j). In addition, enumeration of GFP+ cells, (hereafter mentioned Numbp53) for easy identification of Pax7+ cells, TgPax7nGFP mice were used40. All experiments were carried out on males except females which were also taken to examine NumbΔαβ compound mutants and control littersmates. DM2αβ; floxed; mGFP− mice were used24. All experiments were carried out on males except females which were also taken to examine NumbΔαβ compound mutants and control littersmates.

Injury and NAC treatment. Mice were anesthetised by intraperitoneal injection of a solution of 0.9% NaCl, 0.5% Imalgene (Merial), 2% Rompun (Bayer; 100 μl per 25 g). TA muscles were injured by injection 20 μl of snake venom notexin (10 mg ml−1) or cardiotoxin (10 mM) (L8104 and L8102, Lotaxan Valence, France, www.lotaxan.com). NAC (A9165 Sigma) was added to the drinking water from 5–10 DPL (1 g per 100 ml). The solution was changed every 2 days.

Isolation of satellite cells and cell culture. TA muscle (for injured muscle) or muscles from the entire hindlimbs and forelimbs (for isolation of quiescent satellite cells) were dissected and placed into cold DMEM. Muscles were then chopped and put into a 50 ml Falcon tube containing 30 ml of DMEM (31966 Gibco, 0.1% Collagenase D (1088866 Roche), 0.25% trypsin (15090-046 Gibco), Dnase 10 μg ml−1 (Roche 1128493201) at 37 °C under gentle agitation for 30 min. Digests were allowed to stand for 5 min at room temperature (RT) and the supernatants were collected in 5 ml of foetal bovine serum (FBS; Gibco) on ice. The digestion was repeated 3–5 times until complete digestion of the muscle. The supernatants were filtered through a 70-μm cell strainer (BD Falcon) before cell sorting. Cells were isolated based on size, granularity and mGFP levels using a FACS MoFlo Legacy or Arios (Beckmann Coulter). Cells were collected after sorting directly in culture media (20% FBS, 1% Penicillin-Streptomycin (15140 Gibco), 2% Ultroser G (Thermo Fisher Scientific) in 50:50 DMEM/F12 (31966 and 31765 Gibco). Cells were plated at low density (3,000 cells cm−2) on glass coverslips that were coated successively with 10 mg ml−1 poly-D-lysine (P6047 Sigma) and 1 mg ml−1 matrigel (354234 BD Biosciences) or directly on regular culture dishes coated with matrigel. For all experiments, control and mutant cells were plated at the same density.

The quantification of Pax7+ cells was performed from TA-injured muscles (activated cells) or diaphragm (quiescent cells) from TgPax7CT2+/+; TgPax7nGFP NumbΔαβ+−; NumbΔαβ−/− (control) and TgPax7CT2+/+; TgPax7nGFP NumbΔαβ+/−; NumbΔαβ−/− (mutant) mice. Enumeration of GFP+ cells was performed with the 123 count eBeads (01-1234 eBiosciences) using a FACS Aria III (BD Biosciences).

Isolation of macrophages and transwell assay. Muscles from forelimbs and hindlimbs of one mouse were chopped and divided into 4 × 50 ml Falcon tubes containing 30 ml of HBSS (24020-091 Gibco), 0.04% Collagenase A (11088793001 Roche), 0.3% Dispase (0494207/8001 Roche), Dnase 10 mg ml−1 (1128493201 Roche) at 37 °C under gentle agitation for 90 min. The supernatants were filtered through a 70-μm cell strainer (BD Falcon) before cell sorting. The pellets were resuspended in 1 ml of HBSS, 2% FBS containing mouse BD-PC, (clone 2A.G2, 553142 BD Pharmingen) and incubated on ice for 30 min. Five volumes of HBSS were added and cells were spun for 15 min, 515 r.c.f. at 4 °C. The pellets were resuspended in HBSS, 2% FBS containing the anti-mouse F4/80-APC (115114.31 M1, 31-18-01, eBioscience) and the anti-mouse CD45.2-eBioscience (clone 1A.2D7, 200 clone, 30F11, 25-0451-81 eBioscience) and incubated on ice for 30 min. Five volumes of HBSS were added and cells were spun for 15 min, 515 r.c.f. at 4 °C. The

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RT-qPCR. Primers were designed using Primer Express software (Applied Biosystems) and were prepared by Eurofins MWG Operon. CD45 and F4/80 were selected as housekeeping genes and expressed at stable levels in all samples. Cloning and verification analysis were performed using GraphPad Prism Software. All data points are presented as mean ± s.e.m. (error bars) except where stated otherwise (see Figure legends). Mann-Whitney test (two-tailed) was applied in all cases (non-significant (NS) P > 0.05; *P < 0.05; **P < 0.01; ***P < 0.001). When quantifications are given per unit area, one unit area is defined by a surface of 75,848.74 μm² (corresponding to a × 40 field acquired with a confocal Leica SP8 microscope). A correction index has been used for the bright field pictures taken with an Nikon Eclipse microscope. A minimum of 10 fields/TA muscle section have been quantified for each marker after immunofluorescence and 40 fields/TA muscle section after SAβGal and Sirius red stainings.

Quantification of fibrosis was performed from Sirius Red staining using ImageJ software. After subtraction of the background from the original picture, the picture was converted to a binary picture. The list on the histogram gives minimum and maximum values corresponding to the area of fibrosis and the area devoid of fibrosis, respectively. Control and mutant sections from one given condition were processed for staining and quantification at the same time.

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Author contributions
I.L.R. and S.T. conceived and designed the experiments and wrote the manuscript. I.L.R. performed the experiments. J.K. performed some of the experiments and analysed the data. I.L.R. provided mice. P.F. performed the histology.

Additional information
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