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C Florian Bentzinger, Yu Xin Wang, Nicolas A Dumont, Michael A Rudnicki
EMBO Reports. doi:10.1038/embor.2013.182
Published online 01.12.2013

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The EMBO Journal. doi:10.1002/embj.201284290
Published online 28.01.2014
Cellular dynamics in the muscle satellite cell niche

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Satellite cells, the quintessential skeletal muscle stem cells, reside in a specialized local environment whose anatomy changes dynamically during tissue regeneration. The plasticity of this niche is attributable to regulation by the stem cells themselves and to a multitude of functionally diverse cell types. In particular, immune cells, fibrogenic cells, vessel-associated cells and committed and differentiated cells of the myogenic lineage have emerged as important constituents of the satellite cell niche. Here, we discuss the cellular dynamics during muscle regeneration and how disease can lead to perturbation of these mechanisms. To define the role of cellular components in the muscle stem cell niche is imperative for the development of cell-based therapies, as well as to better understand the pathobiology of degenerative conditions of the skeletal musculature.

Keywords: skeletal muscle satellite cells; muscle stem cell niche; accessory cell types; myogenic cell types; muscular dystrophy

EMBO reports (2013) 14, 1062–1072; published online 15 November 2013; doi:10.1038/embor.2013.182

See the Glossary for abbreviations used in this article.

Introduction

Homeostatic adult skeletal muscle has a relatively uniform architecture. Bundles of longitudinally aligned muscle fibres surrounded by sheets of extracellular matrix (ECM) are attached to tendons and bone through myotendinous junctions [1]. A dense network of blood vessels supplies the tissue with nutrients and oxygen. Quiescent muscle stem cells, or satellite cells, are found underneath the ECM sheet attached to the muscle fibre plasma membrane [2]. Adult muscle tissue also contains several types of interstitial and vessel-associated cells that show little to no mitotic activity under resting conditions [3–5]. After injury, these cells begin to proliferate and, in conjunction with infiltrating immune cells, disperse throughout the muscle tissue (Fig 1A,B; [3–6]). The cellular dynamics during muscle regeneration are highly complex and occur with distinct temporal and spatial kinetics. In the course of muscle regeneration, satellite cells become activated and some will eventually upregulate transcription factors that trigger the myogenic differentiation programme [7]. Once differentiated into myocytes, the cells will align and form new syncytial muscle fibres or fuse to existing fibres. On completion of this regenerative response, the tissue returns to its homeostatic state and the resident cell populations re-enter a resting state.

Despite there being many differentiating cells, the total number of satellite cells remains constant through multiple rounds of regeneration [8]. This equilibrium is due to the ability of satellite cells to self-renew, which provides progeny for differentiation while uncommitted mother cells are retained [9]. Satellite cell commitment to myogenic differentiation is mediated by the myogenic regulatory factors Myf5 and MyoD. Cre/lox reporter systems show that a subpopulation of about 10% of satellite cells has never expressed Myf5 [10]. Such satellite stem cells can self-renew through asymmetrical cell divisions that give rise to Myf5-positive satellite cells in response to the demand for committed myogenic progenitors. Asymmetric satellite cell division is controlled by the Par complex, which allows activation of p38β MAPK and upregulation of MyoD in the committed daughter cell [11]. Self-renewing satellite cells express higher levels of Pax7 than do cells that are primed for differentiation [12]. Moreover, a subpopulation of MyoD-expressing satellite cells can downregulate this factor to resist differentiation and re-enter quiescence [13]. These mechanisms of self-renewal allow the satellite cell pool to be maintained over multiple rounds of injury and repair, and are ultimately responsible for the outstanding regenerative capacity of muscle tissue.

Self-renewal and the three basic states of satellite cells—quiescence, proliferation and differentiation—are predominantly regulated by extrinsic factors in the local environment, the so-called stem cell niche [14]. These environmental cues include growth factors, cytokines, adhesion molecules and ECM contributed by the various cell types present in regenerating muscle tissue. In addition, satellite cells and their committed progeny actively participate in the remodelling of the niche during regenerative myogenesis. The diverse non-satellite cell types in muscle tissue can be categorized into cells with myogenic potential and into cells with accessory function for muscle regeneration [3–5]. Ablation studies have clearly shown an essential role of many accessory cell types during adult myogenesis [3,5,6]. By contrast, the physiological relevance of non-satellite cell types with myogenic potential is less clear [4]. Importantly, these cell types have unique characteristics that render them suitable for cell therapy approaches directed at treating skeletal muscle diseases.

In this article, we review the literature and present an overview of the cellular dynamics in the muscle stem cell niche in homeostasis, during regeneration and in disease. We introduce the niche...
Notch signals are most likely to be presented by the myofibres. Few heterogeneous cell types under quiescent conditions, regulatory and premature differentiation. Since the satellite cell niche contains tor in the Notch pathway, leads to spontaneous satellite cell activation highly pleiotropic and has important functions throughout development, most satellite cells remain quiescent in the absence of an injury stimulus [7]. Importantly, the composition of the niche and the receptors that allow satellite cells to sense extrinsic signals are fundamentally different in quiescence than in the activated state [14,16]. In the quiescent niche, a few cell types are found in the proximity of satellite cells, for instance vessel-associated cells and muscle fibres (Fig 1B). This environment remains essentially static and imposes signals that promote the quiescent stem cell state.

Several studies have identified the Notch receptors as being critical for the maintenance of satellite cell quiescence [17,18]. Notch proteins are transmembrane receptors that are activated on exposure to ligands presented by juxtaposed cells [19]. The Notch pathway is highly pleiotropic and has important functions throughout development. In adult skeletal muscle, genetic loss of Rbpj, a downstream factor in the Notch pathway, leads to spontaneous satellite cell activation and premature differentiation. Since the satellite cell niche contains few heterogeneous cell types under quiescent conditions, regulatory Notch signals are most likely to be presented by the myofibres.

Quiescent satellite cells express high levels of integrin a7 and b1, as well as dystroglycan [20,21]. These receptors could transduce signals from the laminin-rich ECM that covers the satellite cells on their host muscle fibres [21,22]. Under homeostatic conditions, satellite cells also express M-cadherin and the glycoprotein CD34, which are involved in adhesion to myofibres [23,24]. Moreover, quiescent satellite cells are decorated with the heparan sulphate proteoglycans Sdc-3 and Sdc-4, which serve as co-receptors for integrins and sequester soluble growth factors and ECM in the immediate cellular microenvironment [25,26]. Interestingly, Sdc-3 also binds to Notch in satellite cells, and this interaction is required for self-renewal and reversible quiescence [27].

In contrast to the quiescent state, during muscle regeneration the composition of the niche is in a flux that is regulated by a spectrum of cell types (Fig 2). Satellite cell activation is coupled to the upregulation of specific receptors that integrate these niche signals to trigger the appropriate cellular responses. In the subsequent sections we discuss the different cell types involved in regulation of the niche during muscle repair.

Immune cells are critical effectors of the satellite cell niche

Acute sterile muscle injuries trigger a precisely orchestrated inflammatory process aimed at the removal of damaged cells, coordination of the regenerative response and, ultimately, restoration of tissue homeostasis (Fig 3A; Table 1). The onset, development and resolution of inflammation involve diverse interactions between leukocytes and local cell types, including satellite cells (Fig 3B). In resting conditions, adult skeletal muscle contains different types of resident leukocyte.

Figure 1: Overview of tissue histology during mouse skeletal muscle regeneration. (A) A time course of histological changes in regenerating skeletal muscle. H&E staining of uninjured TA muscles and regenerating TA muscles at 5, 10 and 30 days after intramuscular cardiotoxin injection. Regenerating muscles are reduced to mostly mononuclear cells at day 5, but are able to re-establish multinucleated myofibres by day 10. Notably, the nuclei of uninjured myofibres are located at the periphery, whereas those of regenerating muscle fibres are centrally located. Scale bar, 50 μm. (B) Longitudinal view of whole tissue preparations of uninjured (left) and regenerating (right) skeletal muscle. Immunostaining for the extracellular matrix protein laminin (green) labels the basal lamina surrounding myofibres and capillaries. In regenerating conditions, the proliferation of satellite cells can be observed by the increase in the number of Pax7 (red) expressing cells (arrows). DAPI staining of nuclei (blue) reveals accessory cells in the satellite cell niche. Scale bar, 50 μm. H&E, haematoxylin and eosin; TA, tibialis anterior.
review

The most abundant are mast cells and macrophages. These resident cell types, in conjunction with ‘patrolling’ circulatory monocytes, act as sensors for distress and secrete a number of chemoattractive molecules following muscle injury [28,29]. Particularly, damage-activated mast cells almost instantly begin to secrete TNF-α, histamine and tryptase and then initiate the de novo synthesis of other cytokines, such as interleukin (IL)-6 [30]. At low physiological concentrations, TNF-α, tryptase and IL-6 promote activation and proliferation of satellite cells [31–33]. Moreover, inhibition of mast cell activity leads to reduced leukocyte extravasation and impairs muscle repair [34]. Thus, immune cells contribute substantially to the satellite cell niche in the earliest stages of muscle regeneration.

The initial burst of cytokines and chemokines produced by resident leukocytes, which include TNF-α and MIP-2, along with cellular and extracellular contents released by the damaged tissue, lead to the rapid attraction of circulating granulocytes [35,36]. Neutrophils promote the proinflammatory environment that is necessary for the clearance of cellular debris. Under certain conditions, this cell type has been suspected to transiently aggravate tissue damage [38]. Neutrophils also secrete the chemokines MIP-1α, MCP-1 and others that favour the recruitment of monocytes [39,40]. Beyond the first day after injury, monocytes gradually become the predominant leukocytes in the exudate. Globally, monocytes are divided into two categories: the classic monocytes (Ly6C-), that are dominantly present during the first few days after injury and the non-classical monocytes (Ly6C+) that slowly replace Ly6C- cells as regeneration progresses [41]. Although the origin of this switch in monocyte subpopulations is still debated, distinct functions for both cell types have been established [41,42]. Indeed, Ly6C- monocytes express high levels of anti-inflammatory molecules and growth factors [41]. Importantly, the switch of monocyte subtypes not only influences the general course of inflammation but also is important in the satellite cell niche. The proinflammatory environment established by Ly6C- monocytes promotes the proliferation of myogenic cells and reduces their differentiation and fusion capacity. On the other hand, the anti-inflammatory signals from Ly6C+ monocytes have opposite effects and stimulate differentiation [41]. Therefore, the emergence of Ly6C+ monocytes before Ly6C- monocytes is important to ensure appropriate proliferation of myogenic cells and to prevent their premature differentiation.

Once monocytes have invaded the tissue, they begin to differentiate into macrophages. Macrophages can be divided into several subtypes. Analogous to monocytes, this classification of macrophages during muscle regeneration can be simplified into an initial wave of proinflammatory, or M1, macrophages that is followed by a second wave of anti-inflammatory, or M2, macrophages. These macrophage subsets, however, are not mutually exclusive, and, at a given time point, distinct subtypes can be found in the same regenerating area [43]. Depletion models of different types of acute sterile injury have shown that suppression of M1 macrophages leads to persistence of necrotic cells, impaired myoblast proliferation, increased fibrosis and fat accumulation [44–46]. By contrast, inhibition of the transition from M1 to M2 macrophages in mice negative for IL-10 or the transcription factor C/EBPβ resulted in reduced myogenin expression and fibre growth [47,48]. Therefore, M1 and M2 macrophages stimulate, respectively, the early and the late phases of myogenesis. These results are supported by the observation that in injured human muscle, M1 macrophages are found close to proliferating myogenic cells and M2 macrophages interact with differentiating myocytes [43]. The proximity is important for macrophages to mediate myogenic effects and is favoured by attractive

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**Fig 2** Schematic representation of the various cell types involved in muscle regeneration. Within the complexity of regenerating muscles, satellite cells are subject to a distinct environment determined by the spatial and temporal presence of cytokines, growth factors and other cell types.
During muscle regeneration the extracellular environment in the cell niche during regenerative myogenesis is sensitive to perturbation and efficient muscle repair depends on their precise coordination. For instance, M2 macrophages can secrete anti-inflammatory molecules and pro-resolving mediators [58,59]. Thus, even slight imbalances in immune cell populations due to sustained and successive inflammatory signals in diseased muscles can disrupt the cellular dynamics in the niche and provide inappropriate environmental cues to satellite cells [see below]. Nonetheless, if appropriately synchronized and controlled, immune cells serve as key effectors in the muscle stem cell niche to guide satellite cells through the regeneration process.

Fibrogenic cells remodel the niche during regeneration

During muscle regeneration the extracellular environment in the stem cell niche is dynamically rearranged [60]. The functions of various ECM components being deposited in regenerating tissue are only beginning to be elucidated. Structurally, this transitional fibrillar ECM serves to preserve the gross integrity of the tissue until degenerated fibres have been cleared and innervated young muscle fibres have been formed in the correct anatomical position. The main source of these ECM proteins during muscle regeneration is fibrogenic mesenchymal stromal cells, such as fibroblasts and FAPs. FAPs are mesenchymal stem cells resident in skeletal muscle that have the ability to differentiate into fibroblasts, adipocytes...

**Fig 3** | Participation of non-myogenic cell types in muscle regeneration. (A) The relative presence of immune, fibrotic, vascular and myogenic cell types after muscle injury. (B–D) Immunofluorescence micrographs of tissue sections from regenerating mouse muscles. In their niche, Pax7-positive satellite cells (green) are in close proximity to various non-myogenic cell types (red); (B) CD11b-positive leukocytes; (C) Sca1-positive interstitial cells; and (D) VE-Cad-positive endothelial cells. ECM is shown in orange and nuclei are labelled with DAPI (blue). Scale bar, 10 μm.
The anti-inflammatory properties of these molecules allow M2 anti-inflammatory molecules and pro-resolving mediators [58]. Precise coordination. For instance, M2 macrophages can secrete cell niche and promote self-renewal of satellite cells [56,57].

Stage of macrophage differentiation. For example, M2 macrophages are particularly important to induce proliferative treatment for instance through VCAM1–VLA4, allows macrophages to inhibit cytolysis muscle damage caused by neutrophils and M1 macrophages via their TNF secretion [43,44,47,48,51]. IL-1β signals for instance to recruit cytokine-secreting neutrophils [49]. Indeed, direct physical contact, paracrine signalling [43,51]. IL-1β promotes myoblast proliferation, repress myogenic differentiation [43–46, 49–52].

Table 1 | Cell types in the muscle satellite niche

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Markers</th>
<th>Presence (days after injury)</th>
<th>Effect on myogenic cells</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocyte</td>
<td>CD11b+, CD31+, Gr1+, CD43+</td>
<td>0–3</td>
<td>Indirectly stimulate myogenesis through FAPs</td>
<td>[37,38]</td>
</tr>
<tr>
<td>Monocyte</td>
<td>CD11b+, CD31+, Ly6C(±)</td>
<td>0–3</td>
<td>Promote satellite cell activation and proliferation (Ly6C+) or differentiation (Ly6C−)</td>
<td>[28,41,42]</td>
</tr>
<tr>
<td>M1 macrophage</td>
<td>CD11b+, CD31+, CD68+, iNOS+</td>
<td>Peak at 2–3, return to baseline after 7–9</td>
<td>Promote myoblast proliferation, repress myogenic differentiation</td>
<td>[43–46, 49–52]</td>
</tr>
<tr>
<td>M2 macrophage</td>
<td>CD11b+, CD31+, CD68+, CD206+, CD163+</td>
<td>Peak at 3–4, return to baseline after 8–10</td>
<td>Regulate entry into myogenic differentiation, promote myotube formation</td>
<td>[43,44,47–52,59]</td>
</tr>
<tr>
<td>FAP</td>
<td>CD34+, Pdgfra+, Sca1+(high)</td>
<td>Peak at 3–4, return to baseline after 7–9</td>
<td>Promote myogenic differentiation</td>
<td>[37,61–63,65]</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>Tcf4+, SMAα+, vimentin+, desmin+, FSP1+</td>
<td>Peak at 5–7, persist throughout regeneration</td>
<td>Promote myoblast proliferation, enhance self-renewal</td>
<td>[72]</td>
</tr>
<tr>
<td>Vascular cell</td>
<td>VE-Cad+, SMAα+, CD31+</td>
<td>Peak at 5–7, return to baseline after ~28</td>
<td>Promote myoblast proliferation, enhance self-renewal</td>
<td>[73–78]</td>
</tr>
<tr>
<td>Satellite cell and myoblast</td>
<td>Pax7+, MyoD+, αβTi-1-int+, Sdc4+, VCAM-1+</td>
<td>Peak at 5–7, return to baseline after ~28</td>
<td>Enhance self-renewal of satellite cells</td>
<td>[10,56,57,82]</td>
</tr>
<tr>
<td>Differentiating myocyte</td>
<td>Myogenin+, αβTi-1-int+, Sdc4+, desmin+</td>
<td>Appear around day 4–5 and peak by day 10</td>
<td>—</td>
<td>[124,125]</td>
</tr>
<tr>
<td>Newly formed myofibre</td>
<td>MyHC+</td>
<td>Return to baseline numbers after ~28 (with most myofibres remaining centrally nucleated)</td>
<td>Enhance self-renewal</td>
<td>[10,17,18,85]</td>
</tr>
</tbody>
</table>

*Myoblasts only.

and possibly into bone and cartilage cells, although not into satellite cells or muscle fibres [61–63]. This cell type is marked by the expression of the mesenchymal stem cell surface markers, CD34, Sca-1 and PDGFRα, and by the absence of the haematopoietic markers CD45 and CD31 and of the satellite cell marker integrin α7 [3]. Under quiescent conditions FAPs localize close to blood vessels [64]. On muscle injury, these cells are activated, expand and take over the interstitium, where they have a promyogenic function (Fig 3C; [63]). The number of FAPs increases rapidly and peaks 3–4 days after injury, then returns to baseline levels after 7–9 days (Fig 3A).

FAPs are major contributors to the deposition of several extracellular proteins—for instance, certain collagen isoforms that are abundant in the supportive transitional ECM during muscle regeneration [65]. This cell type also secretes high levels of IL-6, which promotes the differentiation of myogenic cells [63]. Visc versa, myotubes seem to inhibit the differentiation of FAPs into adipocytes [62]. Regulation of cellular crosstalk also involves eicosanoids that are recruited during the early stages of regeneration [37]. Eicosanoids release the cytokines IL-4 and IL-13, which induce the proliferation of FAPs and simultaneously block their adipogenic differentiation. In mice deficient for IL-4 and IL-13, adipocytes accumulated after muscle injury and reparative myogenesis was impeded. Remarkably, FAPs seem to remove necrotic debris from regenerating muscles more efficiently than macrophages [37]. The physiological contribution of these two cell populations to debris clearance, however, remains to be investigated, since their abundance and temporal regulation in regenerating muscle probably differs.

Fibroblasts are elongated cells with extended cell processes and a fusiform or spindle-like shape that are identifiable by high expression levels of the intermediate-filament-associated proteins vimentin, desmin, FSP1 and α-SMA [66,67]. This cell type is of a non-vascular, non-epithelial and non-inflammatory nature. Fibroblasts are heterogeneous, with expression profiles that differ depending on the tissue source [68]. A major function of this cell type is the deposition of fibrillar ECM, such as collagen and fibronectin, and basement membrane constituents [68–70]. Moreover, fibroblasts can actively remodel the ECM by secretion of matrix metalloproteinases [71]. Under homeostatic conditions in adult skeletal muscle, fibroblasts reside in the interstitium between myofibres [72]. After muscle injury, they quickly start to proliferate and become highly abundant. Tissue fibroblast content is greatest at about 5 days after muscle damage and coincides with the peak of satellite cell proliferation (Fig 3A). DTX-driven ablation of a subpopulation of fibroblasts from regenerating muscles, achieved with a tamoxifen-inducible Cre allele driven by the Tcf4 promoter, resulted in premature satellite cell differentiation and impaired regeneration [72]. Similar FAPs, Tcf4-positive fibroblasts express PDGFRα, and it remains to be determined whether the Tcf4-Cre allele is also expressed in the former cell type [61–63,72]. Within the muscle fibroblast population, the Tcf4-Cre allele is only active in about 40% of cells. Despite this low percentage of fibroblasts that were depleted by DTX with this Cre driver, a notable phenotype was observed [72]. These results emphasize the critical role of ECM-producing cell types in the stem cell niche during regenerative myogenesis. A possible interpretation of this study is that fibroblasts allow for transient expansion of satellite cells during muscle regeneration while preventing their differentiation. Interestingly, such a role for fibroblasts during muscle regeneration would be opposed to the effects of FAPs, which seem to have prodifferentiation effects [63].
DTX-mediated ablation of satellite cells with a tamoxifen-inducible Pax7-Cre driver revealed that a proper satellite cell response to an injury stimulus is reciprocally required for a normal fibroblast response [72]. The number of fibroblasts in satellite cell-depleted muscles was reduced by ~50% at the peak of regeneration, 5 days after injury, when the first centrally nucleated fibres are normally formed. This effect on fibroblasts might be due to the absence of a trophic signal from proliferating satellite cells or from the young muscle fibres that cannot be established in satellite cell-depleted muscle. Taken together, the complex role of mesenchymal fibrogenic cells in the muscle stem cell niche only begins to be understood and future studies will be required to explore their interplay with myogenic cells in more detail.

**Endothelial and periendothelial cells in the niche**

Skeletal muscle is laced with a dense microvasculature, and most quiescent satellite cells are found in close proximity to these vessels [73]. During muscle injury, the number of capillaries in the tissue initially increases and then returns to baseline about 4 weeks after injury (Fig 3D; [74,75]). In co-culture experiments, endothelial cells promote the proliferation of satellite cell-derived myoblasts. Reciprocally, differentiating myogenic cells are proangiogenic and increase the formation of capillary-like structures [73]. Endothelial cells secrete a variety of mitogenic and/or antiapoptotic factors, such as VEGF, that influence muscle cells [76]. Intriguingly, differentiating myogenic cells also secrete VEGF and their proangiogenic function mainly depends on this factor [77]. This finding suggests an intricate feed-forward mechanism through which VEGF in the stem cell niche co-regulates both myogenesis and angiogenesis.

In contrast to the predominantly promitotic effects of endothelial cells on myogenic progenitors, cells in the periendothelial position, such as smooth muscle cells and fibrogenic cell types, are crucial for re-entry into quiescence on completion of regeneration [76]. Satellite cells transitioning into quiescence increase expression of the Ang1 receptor Tie-2. Forced expression of Ang1 in mouse muscles increases the number of quiescent cells and inhibition of Tie-2 prevents cell-cycle exit on completion of regeneration [78]. Importantly, periendothelial cells seem to be the major source of Ang1 during muscle regeneration. In summary, vessel cells in the stem cell niche coordinate both the acute satellite cell response and the late stages of muscle regeneration when the tissue returns to homeostasis.

**Regulation of the niche by cells of the muscle lineage**

In many tissues, the committed or differentiated progeny of stem cells become components of the niche where they provide regulatory signals [79]. This feature is the same in the skeletal muscle lineage and, as discussed below, involves cell-cell interactions, as well as the secretion of growth factors and regulatory ECM.

Notch signals originating from differentiating myogenic cells allow for self-renewal of muscle progenitors while suppressing activation of the commitment factor MyoD during development [18,80,81]. This Notch-dependent developmental mechanism is preserved in adult satellite cells. Experiments using the Myf5-Cre-YFP reporter system revealed that during the regenerative response of mature skeletal muscle, committed proliferating YFP+ satellite cells of the Myf5-dependent lineage provide Notch signals to the self-renewing YFP satellite stem cells [10]. Satellite stem cells contain high levels of Notch-3, while their committed Myf5/MyoD-positive progeny express the Notch ligand Delta-1. In asymmetric divisions, Delta-1 localizes to the cell interface with the YFP cell and probably activates self-renewal signals by binding to Notch-3 [10]. Thus, next to its established role in maintaining satellite cell quiescence (see above), Notch also plays a role in activated cells.

When compared with quiescent cells, activated satellite cells and their differentiated progeny express high levels of ECM components and various molecules involved in the remodeling of extracellular space [56,82]. An intriguing role of Notch signalling during myogenic development is the regulation of ECM synthesis by myogenic progenitors [83]. Genetic loss of Rbpj from myogenic cells, whose terminal differentiation is blocked due to knockout of MyoD, leads to an inability to acquire the satellite cell position underneath the basal lamina surrounding the developing muscle fibres. Importantly, the expression of several ECM components is strongly disrupted in such cells, suggesting that they actively contribute to the formation of the basal lamina. Thus, the regulation of ECM synthesis could be another Notch-related developmental process that plays a role in adult myogenesis.

The pool of Myf5-independent satellite stem cells in muscle tissue is critically controlled by Wnt7a, a lipophilic factor that is released into the stem cell niche by newly formed fibres [10,84,85]. Expansion of the satellite cell population is a mechanism that is essential for maintenance of the myogenic progenitor pool after muscle injury. Consequently, knockout of Wnt7a severely reduces overall satellite cell number after regeneration. The effect of Wnt7a on stem cell progenitors depends on the Fzd7–Sdc4 co-receptor complex, the function of which is modulated by fibronectin that is secreted into the niche microenvironment by committed Myf5-positive satellite cells [56]. Binding of Wnt7a and fibronectin to the Fzd7–Sdc4 co-receptor complex allows for downstream GTPase signalling and the induction of symmetric expansion of satellite stem cells. Similar to the loss of Wnt7a, loss of fibronectin from regenerating muscle severely reduces the overall pool of satellite cells. Thus, the release of fibronectin into the stem cell niche represents a feedback mechanism originating from committed satellite cell progeny that, in concert with Wnt7a, modulates the self-renewing stem cell pool. Other cell types in muscle express high levels of fibronectin and, therefore, might also contribute to fine-tuning of the Wnt7a response [68–70].

Another ECM molecule critical to the satellite cell niche is ColVI [57]. Mutations in ColVI are the underlying cause of Bethlem myopathy and Ullrich congenital muscular dystrophy [86]. ColVI knockout mice show deficiency in muscle regeneration and mild myopathy [57,87]. Satellite cells express high levels of ColVI and secrete this factor to autoregulate the softness of their niche. In elegant grafting experiments, wild-type satellite cells ameliorated the degenerative phenotype of ColVI-deficient muscle tissue, which demonstrates a cell-autonomous requirement for this factor. Interestingly, in this mouse model, the ColVI content in the satellite cell niche can also be restored by transplantation of wild-type fibroblasts.

In summary, important regulatory functions of the stem cell niche are controlled by committed satellite cell progeny or by differentiated fibres. Specifically, satellite cells actively autoregulate their immediate microenvironment. The emerging mechanisms that integrate feedback and feed-forward signals within the myogenic lineage are highly complex and further investigation will undoubtedly unravel important new concepts in basic stem cell biology.
The satellite cell niche in ageing and pathology

Chronic degenerative conditions of skeletal muscle can lead to permanent changes within the muscle stem cell niche. Evidence suggests that, under specific conditions, satellite cells can differentiate into brown fat, osteocytes and myofibroblasts [88–90]. Pathological deterioration of the niche or systemic changes can influence these fate decisions and disrupt normal cellular responses to injuries [91,92]. Ultimately, imbalance within the local satellite cell milieu attenuates the formation of new myofibres and leads to the eventual loss of muscle function. Therefore, understanding of pathological conditions of the muscle stem cell niche is important to treat muscle diseases.

Although ageing should not be considered a pathological state, the deterioration in muscle regeneration through this process is directly correlated with changes to local and circulating factors that influence satellite cell function [93]. With use of heterochronic parabiosis to connect the circulatory systems of young and old mice, serum from young mice reduced age-related tissue fibrosis and restored satellite cell function in old mice. Systemically, serum levels of TGF-β1 are increased in elderly humans and mice [94]. This proliferative factor not only stimulates the expansion of tissue-resident fibroblasts but also inhibits the myogenic differentiation of satellite cells, which diminishes the regenerative capacity of ‘old’ muscle. Additionally, altered levels of osteopontin secreted by CD11b+ macrophages that are found in the serum of old mice are associated with reduced proliferative and differentiation capacities of satellite cells [95]. This finding suggests that immune responses to injuries can shift with age. Accordingly, subtle changes to the systemic milieu can affect the cellular response of support cells within the muscle stem cell niche and affect the efficiency of myogenic regeneration. Combined with localized FGF-2 secreted by the myofibres that disrupt the ability of aged muscle stem cells to return to quiescence in old mice, the aged niche creates an unfavourable environment for the proper function of muscle stem cells [96]. The full extent of these gradual changes is not yet known and requires further investigation, along with whether the ‘young’ stem cell niche can be therapeutically restored.

In degenerative muscle diseases, localized muscle pathologies can transform the normal wound-healing programme into a positive feedback loop that prevents the proper function of satellite cells. Notably, the attenuation of muscle repair in most forms of muscular dystrophy is correlated with a build-up of fibrotic scarring, adipose tissue and immune infiltrations [97]. The increased susceptibility of dystrophic muscle fibres to damage leads to cycles of degeneration and regeneration. In most cases, necrotic and regenerating areas occur concurrently throughout dystrophic muscles. Unlike the beneficial effects of transient ECM protein upregulation during normal regeneration, increased inflammation and persistent expression of ECM proteins reduce the differentiation of myoblasts into myofibres [98,99]. Moreover, the altered elasticity of fibrotic muscle tissue is likely to have a negative influence on the self-renewal of satellite cells [57,100]. The lack of efficient myofibre formation in diseased muscle reduces the inhibitory feedback on the fibrogenic and adipogenic differentiation of FAPs [62]. This feedback mechanism persists until anti-myogenic signals accumulate exponentially and muscle regeneration is essentially halted.

Restoration of a functional muscle stem cell niche is an important aspect of treating degenerative muscle diseases. Experimental regulation of supportive cell types during regeneration can optimize the myogenic efficiency of satellite cells [37,63,72]. Therefore, it is conceivable that these cells can be effectively manipulated in disease conditions to combat the loss of muscle stem cell function over time. In agreement with this idea, anti-fibrotic and anti-inflammatory therapies have reduced the progression of Duchenne muscular dystrophy in the short term [101,102]. Furthermore, transplantation of corrective supportive cells can recondition the niche to restore satellite cell function [57,95]. The intricacies of the regenerating environment and altered systemic milieu in specific diseases, however, have made research into this area challenging, and there are many aspects regarding the structural and temporal regulation of the muscle stem cell niche that remain unknown. Thus, future research into the regulation of the stem cell niche in regeneration and disease holds great potential for the therapeutic enhancement or restoration of muscle regeneration.

### Glossary

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer-binding protein</td>
</tr>
<tr>
<td>DTX</td>
<td>diphtheria toxin</td>
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Non-satellite cells with myogenic potential

Various cell types other than satellite cells are able to fuse into differentiating muscle fibres and, in certain cases, can also acquire Pax7 expression and become satellite cells [4]. These properties are attractive for the development of cell-based therapies for muscle diseases. Examples of these cell types include side population cells, PVI1-positive interstitial cells, pericytes, mesoangioblasts, CD133-positive circulating cells and integrin β4-positive interstitial cells [103–108]. The existence of these cell types raises questions of whether a non-satellite cell progenitor could compensate for the role of satellite cells during physiological myogenesis and whether an exogenous stem cell type could feed into the myogenic lineage to generate new satellite cells and replenish the pool of cells available for regenerative myogenesis. Moreover, the niche signals that are required for myogenic conversion of these cell types remain largely unknown. Several groups have started to address this interesting subject by using ablation methods that are coupled to the expression of Pax7 [72,109,110]. These studies have used either a tamoxifen-inducible Pax7-Cre with a stop-flox DTX allele or the human DTX receptor inserted into the Pax7 locus, so that the respective cells can be selectively ablated by exposure to DTX. Targeted elimination of satellite cells with DTX...
Cellular dynamics during muscle regeneration

in these mouse models resulted in a severely impaired regenerative response of muscle tissue. Importantly, in one study, regeneration was induced in muscles that had been depleted of satellite cells 2 weeks earlier, and virtually no fibre formation was observed up to 5 weeks after injury [110]. These results indicate that no other cell type can compensate for the loss of satellite cells by direct differentiation into myofibres, and that no other cell type can replenish the satellite cell pool in the intermediate term after injury. As discussed above, however, signals originating from satellite cells and their committed and differentiated progeny are critical for the function and the recruitment of many different cell types in the niche, for instance fibroblasts, endothelial cells and FAPs [62,72,77]. Therefore, DTX-induced loss of satellite cells and the absence of newly formed muscle fibres on injury could lead to impaired recruitment of myogenic non-satellite cell types due to the absence of a trophic signal. In support of this idea, in vitro, most myogenic non-satellite cell types require coculture and co-differentiation with myoblasts to substantially contribute to the formation of myotubes. In spite of their unclear physiological role in directly contributing to adult myogenesis and to the stem cell niche, non-satellite cells with myogenic potential seem to have tremendous therapeutic value that enables, for instance, systemic delivery or extensive ex vivo expansion [111–122]. Thus, their use for cell therapy might allow bypassing of several problems associated with the isolation and expansion of conventional myogenic cells for transplantation [123]. Importantly, an improved understanding of the niche signals required to recruit these cells to myogenesis will help to advance such therapies.

Conclusion and outlook

On injury, the stem cell niche in muscle transitions from a relatively steady state involving few cell types into an enormously complex environment with spatiotemporally regulated cascades of direct and indirect cellular interactions (Fig 4, Table 1). The sum of these interactions, combined with intrinsic stem cell programming, controls the regenerative dynamics in the tissue and ultimately allows for the re-establishment of muscle structure and function. The study of muscle regeneration has taken us away from a view that is centred on intrinsic satellite cell regulation towards an understanding that integrates the immense relevance of the niche. With the mouse as a versatile model to study the biology of skeletal muscle, it is becoming increasingly apparent how elaborately fine-tuned is the role of the different cell types involved in muscle regeneration, and how detrimental are the consequences of disease-related imbalances in these dynamics.

An integrative understanding of the cellular complexity in the niche will allow for the development of therapeutic strategies targeted to normalize or adapt the global behaviour of specific cell populations rather than single signalling pathways. The field has taken great steps forward due to the development of several important genetic tools allowing the manipulation and observation of specific cell populations in muscle tissue. The further refinement of these tools and the identification of mutually exclusive cellular markers will be crucial to answering many of the outstanding questions (Sidebar A) and to a future holistic understanding of the dynamics of muscle regeneration.
Injury could lead to impaired recruitment of myogenic non-satellite cells from the adult muscle satellite cell niche, non-satellite cells with myogenic potential seem to have tremendous therapeutic value that enables, for instance, systemic transplantation [123]. Importantly, an improved understanding of the niche signals required to recruit these cells to myogenesis will help to establish a functional artificial niche for the expansion of uncommitted satellite cells ex vivo. Thus, their use in these mouse models resulted in a severely impaired regenerative consequence of disease-related imbalances in these dynamics. Remarkably apparent how elaborately fine-tuned is the role of the different m2 macrophage and c-kit+ stem cells from the adult muscle satellite cell niche.

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Cellular dynamics during muscle regeneration


Cellular dynamics during muscle regeneration


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VBP15, a novel anti-inflammatory and membrane-stabilizer, improves muscular dystrophy without side effects

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Keywords: anti-inflammatory; dystrophy; mdx; membrane injury; muscle

Absence of dystrophin makes skeletal muscle more susceptible to injury, resulting in breaches of the plasma membrane and chronic inflammation in Duchenne muscular dystrophy (DMD). Current management by glucocorticoids has unclear molecular benefits and harsh side effects. It is uncertain whether therapies that avoid hormonal stunting of growth and development, and/or immunosuppression, would be more or less beneficial. Here, we discover an oral drug with mechanisms that provide efficacy through anti-inflammatory signaling and membrane-stabilizing pathways, independent of hormonal or immunosuppressive effects. We find VBP15 protects and promotes efficient repair of skeletal muscle cells upon laser injury, in opposition to prednisolone. Potent inhibition of NF-κB is mediated through protein interactions of the glucocorticoid receptor, however VBP15 shows significantly reduced hormonal receptor transcriptional activity. The translation of these drug mechanisms into DMD model mice improves muscle strength, live-imaging and pathology through both preventive and post-onset intervention regimens. These data demonstrate successful improvement of dystrophy independent of hormonal, growth, or immunosuppressive effects, indicating VBP15 merits clinical investigation for DMD and would benefit other chronic inflammatory diseases.

INTRODUCTION

Contraction-induced myofibre injury and inflammation are characteristic features of Duchenne muscular dystrophy (DMD), a fatal genetic muscle disease. We and others have demonstrated that the pro-inflammatory transcription factor NF-κB is active in dystrophin deficient muscle before symptom onset (Chen et al, 2005; Porter et al, 2002, 2003). Pharmacological glucocorticoids (prednisone, deflazacort) are standard of care in DMD, and we hypothesize their primary mechanism of action to be through anti-inflammatory activities via NF-κB pathways (Wissink et al, 1997). However, their harsh side effects in children greatly reduce patient adherence to glucocorticoid regimens and limit their therapeutic window. More general

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immunosuppressive compounds reduce inflammation in DMD but fail to increase patient strength in the same manner as glucocorticoids (Griggs et al, 1993; Kissel et al, 1993), while specific targeting of NF-κB increases strength in animal models (Grounds & Torrisi, 2004; Peterson et al, 2011). These data suggest that the specific mechanism by which glucocorticoids inhibit NF-κB is of particular importance to DMD treatment efficacy. Therapeutics that target this pathway in the absence of side effects may provide a substantial improvement in the treatment of DMD.

At the cellular level, dystrophin deficient muscles show increased susceptibility to stretch-mediated membrane instability and calcium dependent hyper-contracture (BERTORINI et al, 1982; YASUDA et al, 2005), as well as increased oxidative stress (Disatnik et al, 1998; RANDO et al, 1998). Glucocorticoids and other steroidal compounds are multi-mechanistic; in addition to binding hormonal receptors they can interact with the plasma membrane to exert rapid and specific physico-chemical effects (Buttgereit et al, 1999; Lipworth, 2000; Rhen et al, 2003; SHIVAJI & Jagannadham, 1992). These effects can alter membrane fluidity, vesicular fusion (SHIVAJI & Jagannadham, 1992) and ionic flux (Buttgereit et al, 1999), which are important for resistance to and repair of membrane injury. Recently, membrane stabilizing compounds such as poloxamer 188 (SPURNEY et al, 2011; TOWNSEND et al, 2010), MITSUGUMIN 53 (BIRKIN & WUEBBLES, 2012; WEISLEDER et al, 2012) and cromolyn sodium (GRANCHELLI et al, 1996; MARQUES et al, 2008) have shown improvements to pathology, myofibre tension and cardiopulmonary function in dystrophin-deficient mice and dogs. The effects of glucocorticoids on membrane stability, however, have not been reported.

Because glucocorticoids act through multiple mechanisms, it has been unclear and controversial which molecular pathways provide efficacy in DMD and which are simply responsible for detrimental effects. For example, impaired growth is a glucocorticoid side effect for children with asthma (AVIOLI, 1993; WOLThers & PEdersen, 1990), but has been proposed as a pathway of efficacy in DMD by limiting muscle workload and delaying muscle maturation (Grounds & Shavlakadze, 2011). Further, immunotoxic effects contribute to reduced chronic inflammation, but recent evidence suggests anti-inflammatory NF-κB inhibition may be sufficient for efficacy (PETERSON et al, 2011). It is clear, however, that detrimental effects of glucocorticoids currently limit their application; in DMD neonatal screening is not performed, and glucocorticoid regimens are delayed years until after the onset of fairly advanced symptoms. In other forms of muscular dystrophy, glucocorticoids are avoided altogether because the net balance of positive and negative effects is unclear. By investigating the molecular mechanisms of glucocorticoids, we have developed VBP15 as a novel oral drug. This compound is optimized for NF-κB inhibition, membrane insertion and glucocorticoid receptor (GR) specificity. Medicinal chemistry, however, both eliminates key glucocorticoid pathways and provides novel properties. Here, we present the discovery and mechanisms of this drug, then extensively examine efficacy and side effects in mdx muscular dystrophy model mice. We find VBP15 has novel membrane-stabilizing and immunological properties, and shows potent NF-κB inhibition and substantially reduced hormonal effects. To capitalize on this mechanism profile, which targets multiple pre-symptomatic defects, we adopt a prophylactic regimen, beginning dosing before mdx symptom onset in a blinded pre-clinical trial. This strategy would be analogous to a neonatal screening, preventive regimen in the clinic. Another intervention experiment in post-onset adult mdx mice shows repeatable efficacy in a different stage of disease. We find dose-response improvements with successful ablation of growth, bone and immunological toxicities seen with traditional glucocorticoids. These data provide new insights into biological mechanisms of efficacy versus side effects in DMD, identify VBP15 as a novel entity that warrants clinical investigation for DMD, and show therapeutic potential for other disorders of chronic inflammation and membrane instability.

RESULTS

In vitro characterization of VBP15

VBP15 was selected as our lead compound for clinical development from a screening program focused on Δ-9,11 compounds. This Δ-9,11 class is differentiated from glucocorticoids by the key conversion of a hydroxyl group to a carbon-carbon double bond (Fig 1). Preliminary studies suggested these drugs had potential anti-inflammatory effects (BAUDY et al, 2012) but lacked activation of a synthetic GR reporter. Through extensive medicinal chemistry probing the R₉–R₁₀ groups of the D-ring in this steroidal structure to generate a compound library, followed by multiple lines of screening studies focused on 20 candidates, VBP15 was subsequently identified as our lead compound. Selection was based upon its superior profile in an in vitro assay for NF-κB inhibition in myogenic cells, in addition to ligand-induced nuclear translocation of the GR, cytotoxicity, metabolite and pharmacokinetic properties (REEVES et al, 2013). To further screen candidate compounds for target receptor specificity, we performed competitive nuclear hormone receptor binding assays (Fig 1E–H). In these assays, we found that VBP15 shows increased specificity for GR binding in comparison to other Δ-9,11 compounds. For example, VBP15 exhibited an approximately 50-fold greater affinity for the GR than VB3 and, a 64-fold lower affinity for the mineralocorticoid receptor (MR). VBP15 also showed only very low affinity for the androgen receptor (Fig 1G), over 500-fold lower than the control methyltrienolone, and lacked any detectable binding to the oestrogen (Fig 1H) or progesterone (data not shown) receptors in these in vitro assays. From these screening, biochemical and specificity data, VBP15 presented a superior profile for therapeutic development.

Our studies here are benchmarked against prednisolone, the active form of prednisone. Both VBP15 and prednisolone inhibited TNFα-induced pro-inflammatory NF-κB signaling at similar levels in NF-κB reporter assays in C2C12 muscle cells at 1nM or more (Fig 2A). To confirm effects on NF-κB target genes, several inflammatory transcripts known to be induced by TNFα were assayed by qPCR in VBP15- and prednisolone-treated H2K myotubes. We found VBP15 inhibited the TNFα-induced
inflammatory transcripts Cox2, Irf1 and Nos2 (p < 0.005) at potencies similar to prednisolone (Fig 2B).

Both prednisolone and VBP15 are hydrophobic compounds that are expected to have physicochemical effects on lipid bilayers. We compared the effects of VBP15 and prednisolone on membrane injury and repair in live cells using an established laser injury assay (Sharma et al, 2012). Skeletal muscle cells treated with VBP15 showed reduced impact of the injury and enhanced repair in a dose dependent fashion (Fig 2C and D). Cells treated with prednisolone, however, showed greater impact from injury with elevated dye uptake. In this live single-cell injury model, prednisolone exacerbated, while VBP15 protected, injury to the plasma membrane.

GR mediates VBP15 anti-inflammatory effects without inducing classical steroid transactivation

To investigate whether NF-κB inhibition by VBP15 is mediated by the same pathways as glucocorticoids, we examined the effects of the steroid receptor antagonist, RU-486, on NF-κB inhibition. Increasing concentrations of RU-486 from 1 nM to 10 μM ablated NF-κB inhibition by VBP15 in a dose dependent manner, similar to results seen with prednisolone and dexamethasone (Fig 3A). This shows that the anti-inflammatory effects of VBP15, prednisolone and dexamethasone are all mediated through shared steroidal pathways.

A sub-activity of pharmacologic glucocorticoids that is largely separable from NF-κB inhibitor activities is the translocation of ligand-GR complexes to the nucleus where they directly mediate transcriptional pathways via glucocorticoid response elements (GRE) (e.g. classical steroid receptor transactivation or hormonal properties). Both positive- and negative-acting GRE-mediated transcriptional regulation has been described, and both forms of hormonal activities are more often associated with glucocorticoid side effects rather than efficacy, with some of these mediated by the pituitary (Diamond et al, 1990; Drouin et al, 1993; Itani et al, 2002; Meijssing et al, 2009; Yoshiuchi et al, 1998). In AdT-20 pituitary cells, we examined genes regulated by positive and negative GREs. Sgk1, a key mediator of fibrosis, is activated by a positive GRE. Both prednisone and dexamethasone (0.1 μM) showed a greater than 13-fold induction of Sgk1 gene transcription, whereas VBP15 showed no such GRE-mediated transcriptional activity at the same concentration (Fig 3B). At 1.0 and 10 μM, VBP15 began to show some evidence of Sgk1 transcriptional induction, but to a lower degree than traditional glucocorticoids. Adrenocorticotrophic hormone (ACTH), the stimulatory hormone in adrenal steroidogenesis, is negatively regulated by a ligand/GRE interaction (Drouin et al, 1993). Treatment with dexamethasone or prednisolone reduced ACTH secretion in AdT-20 cells to approximately 20% of untreated at all concentrations tested.
VBP15 improves muscular dystrophy

Several mechanisms have been hypothesized for the inhibition of NF-κB by glucocorticoids and the GR. These include GRE-driven transactivation of genes that inhibit NF-κB, direct protein–protein interactions through which the GR may act as a corepressor when bound to NF-κB, and the activation of alternative receptors such as the MR. To investigate the mechanism by which glucocorticoids, VBP15 and/or the activated GR inhibit NF-κB, we performed further experiments in GR mutant cells. First, we tested whether the lack of GR in GRmutant fibroblasts affects the ability of drugs to inhibit inflammatory transcripts, which are predominantly controlled by NF-κB. Absence of the GR in this spontaneous mutant line was previously selected for (Housley & Forsthoefer, 1989) and confirmed here through Western blot (Fig 3D). Cells were then treated with prednisolone or VBP15 and inflammatory transcripts were induced with TNFa. Ablation of GR transactivation functions in GRmutant cells was confirmed through qPCR of Sgk1 transcript levels (Fig 3E). Examining inflammatory transcripts, we found Irf1 (p < 0.0001), Tnfa (p < 0.05) and Il1a (p < 0.05) expression to all be significantly elevated in induced versus non-induced cells. In GR positive cells, both VBP15 and prednisolone inhibited the induction of Irf1 (p < 0.005), Tnfa (p < 0.01) and Il1a (p < 0.05) to levels that were 30–58% of vehicle (Fig 3F). In GRmutant cells, neither drug was able to inhibit the induction of any of these transcripts. This data confirms that ligand-activated GR is essential for the inhibition of predominantly NF-κB driven inflammatory transcripts by both prednisolone and VBP15.

Next, primary splenocytes were harvested from control and GRmutant mice. These mice contain a mutation in the DNA binding domain of the GR (Dahlman-Wright et al, 1991; Reichardt et al, 1998). This mutation prevents the GR from binding to DNA and activating dimer-driven GRE gene transcription, but maintains GR ligand-binding and protein–protein interactions. Here, primary splenocytes were treated with drug and induced with TNFa, then assayed by qPCR. First, we examined the induction of NF-κB inhibitor alpha (Nfkbia, or Irf1), a GRE-activated gene that also encodes an endogenous
inhibitor of NF-κB. In wild type control splenocytes, Nfkbia expression was significantly increased by prednisolone (increase of 45 ± 13%, \( p < 0.005 \)) but not by VBP15 (increase of 16 ± 16%) in comparison to vehicle. No induction was present with either drug in GR\textsuperscript{dim} splenocytes, demonstrating both the absence of GR dimer-driven gene expression in GR dim cells and a lack of induction of NF-κB inhibitory genes. Examining inflammatory transcripts, we found Irf1 (\( p < 0.0001 \)), Tnfα (\( p < 0.0001 \)) and Il6...
(p < 0.05) were significantly elevated within induced versus non-induced primary splenocytes. Consistent with GR positive fibroblasts and H2K myotubes, treatment of wild type splenocytes with both VBP15 and prednisolone successfully inhibited the induction of all three inflammatory transcripts (p < 0.001) to levels that were roughly half those of vehicle. In contrast to GR<sup>mdx</sup> genotype and GRE transcript experiments, we found that inhibition of all three inflammatory transcripts was maintained in the GR<sup>fl/fl</sup> mutant cells. Together, these experiments show that both prednisolone and VBP15 activate the GR to efficiently inhibit inflammatory transcription programs through protein–protein interactions, independent of DNA binding or transactivation of inhibitory genes.

**VBP15 improves dystrophic phenotypes in mice treated before the onset of early necrosis**

The mdx mouse model of DMD shows staged histopathology, with little evidence of dystrophy from 0 to 3 weeks of age, then contrast to GR<sup>mdx</sup> genotype and GRE transcript experiments, we found that inhibition of all three inflammatory transcripts was maintained in the GR<sup>fl/fl</sup> mutant cells. Together, these experiments show that both prednisolone and VBP15 activate the GR to efficiently inhibit inflammatory transcription programs through protein–protein interactions, independent of DNA binding or transactivation of inhibitory genes.

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In histopathology studies, quantitative H&E analysis of mdx diaphragms revealed a clear inflammatory phenotype, with 16-fold higher inflammatory cell counts compared to WT (Fig 4F). Mice treated with VBP15 at 15 and 30 mg/kg displayed 38 and 30% reductions in inflammatory foci compared to vehicle. VBP15 also reduced calcified fibres (Supporting Information Fig 1D). These data are evidence that VBP15 reduces inflammation and improves inflammatory muscle pathology.

**VBP15 improves dystrophic phenotypes in adult mdx mice treated after symptom onset**

In a separate trial, exercised adult mdx mice were treated for 4 months. In agreement with the pre-symptomatic trial above, ProSense600 live animal imaging exhibited a 20% and 13% decrease in muscle inflammation upon VBP15 treatment at 15 and 45 mg/kg (Fig 4G). Isolated EDLs showed a 16% increase in specific force upon treatment with VBP15 at 15 mg/kg (Fig 4H). H&E histology revealed VBP15 significantly decreased diaphragm inflammation (Fig 4I). These data reinforce VBP15 efficacy and indicate both pre-symptomatic and post-onset treatment regimens can benefit disease.

**VBP15 does not display immunotoxicity seen with prednisolone**

Pharmacologic glucocorticoids show immunosuppressive and immunotoxic properties that limit therapeutic windows and long-term prescription. We benchmarked VBP15 against prednisolone to determine if similar sub-activities were seen. Untreated mdx mice showed enlarged spleens and increased numbers of peripheral blood leucocytes (PBLs) compared to WT mice (Supporting Information Fig 2A and B). VBP15 treatment reduced spleen mass and PBL counts in a dose-dependent manner to levels resembling WT. Prednisolone reduced these measures below WT, suggesting immunosuppressive and/or immunotoxic properties. Further, prednisolone significantly decreased viable splenocytes per gram of tissue (p < 0.005), while this was not observed for any VBP15 dose (Fig 5A).

We next examined effects of VBP15 on B and T lymphocytes isolated from mdx spleens at the trial conclusion. Both B lymphocytes and CD4<sup>+</sup> T lymphocytes were depleted by prednisolone but not VBP15, as measured by percent B220<sup>+</sup> and CD4<sup>+</sup> positive splenocytes, respectively (Fig 5B and C). CD4<sup>+</sup> T cell activation was assayed by stimulation of splenocytes with concanavalin A (ConA). Prednisolone treatment significantly
Figure 4. VBP15 improves dystrophic phenotypes of mdx mice in two pre-clinical trials (pre-symptomatic and post-onset treatment regimens).

A–F. Prophylactic treatment of mdx mice beginning at 2 weeks of age showed dose-dependent improvement of clinical and histological endpoints. Mouse limb strength increased upon VBP15 treatment as measured by grip strength of 6 week old mice for both (A) forelimb and (B) hindlimb (n ≥ 12 mice/group). (C) Maximal force exerted by mouse forelimbs increased with VBP15 treatment but decreased with prednisolone treatment, due to prednisolone effects on mouse size (presented later). (D) Specific force of isolated EDL muscle increased with VBP15 treatment (n = 10 mice/group). (E) Live-animal imaging of cathepsin protease activity (ProSense680) shows reduced inflammation and necrosis of the hindlimbs in VBP15-treated mdx mice (E images; E quantitation of fluorescence; n ≥ 6 mice/group). (F) Histology of diaphragm muscle shows a decrease in inflammatory foci from VBP15 treatment at 15 and 30 mg/kg (F representative images, F quantitation; n = 6 mice/group).

G–I. A second pre-clinical trial was performed in exercised adult mdx mice to assess post-onset efficacy. (G) Live-animal imaging of inflammation (ProSense680) showed a significant decrease with VBP15 treatment (G representative images, G quantitation; n ≥ 6 mice/group). (H) Specific force of isolated EDL muscle was measured ex vivo at trial conclusion with 15 mg/kg VBP15 showing an increase consistent with the neonate trial (n ≥ 7 mice/group). (I) Histology of adult diaphragm showed a significant reduction in inflammatory foci upon 45 mg/kg VBP15 treatment (n = 6 mice/group). Values are mean ± SEM. For treatments, the mean percentage of increase or decrease of mdx vehicle values towards WT is provided. (Pred, prednisolone; FL, forelimb; HL, hindlimb; data exceeding 2 SD’s was removed from specific force values as an outlier but included in all statistical analyses; one-tailed t-test of single dose versus vehicle mdx ‘p < 0.05, **p < 0.005, ***p < 0.0005).
VBP15 improves muscular dystrophy

Figure 5. VBP15 does not show immunosuppressive activities shown by prednisolone.
A. Prednisolone significantly reduced the number of viable splenocytes per gram of spleen tissue, whereas VBP15 did not at any dose.
B. The percentage of B lymphocytes, as measured by FACS analysis of B220 positive cells, was reduced in spleens from prednisolone treated mdx mice, while VBP15 showed no decrease in B cells.
C. Spleen CD4^+ T cell numbers were significantly decreased in prednisolone treated mdx spleens, but not by VBP15 treatment.
D. Activation of mdx splenocyte T cells by concavalin A was impaired by prednisolone, but not impaired by VBP15 treatment. Values are mean ± SEM. (Pred, prednisolone; (A) n ≥ 12, (B-D) n = 3–5, *p < 0.05, **p < 0.005).

VBP15 shows a superior side effect profile compared to pharmacological glucocorticoids
Stunted growth is a significant side effect of chronic prednisone use in children (Avioli, 1993; Wolthers & Pedersen, 1990). In our pre-symptomatic mdx study, prednisolone treatment significantly stunted the growth of young mice (Fig 6A). After 5 weeks of treatment, mdx mice receiving prednisolone were significantly shorter (8.6 ± 0.4 cm) than vehicle (9.1 ± 0.3 cm, p < 0.001). No significant reduction in body length was observed for any VBP15 dose.

Chronic treatment with glucocorticoids negatively affects bone growth and development, and can cause osteoporosis (Bircan et al, 1997; Manolagas & Weinstein, 1999). Tibia length was measured to determine if VBP15 inhibited bone growth (Fig 6B). Vehicle mdx mice had tibia lengths of 15.9 ± 0.3 mm, while prednisolone significantly decreased this to 14.8 ± 0.5 mm (p < 0.005). VBP15, however, did not affect tibia length at any concentration. MicroCT was performed on femurs to examine bone density and structure (Fig 6C). Comparison of vehicle, prednisolone and the highest VBP15 dose showed prednisolone to significantly reduce trabecular thickness (p < 0.005) compared to vehicle, while VBP15 did not. Prednisolone thus demonstrated side effects to bone not observed with VBP15 treatment.

We have previously reported deleterious effects of prednisone on increased fibrosis in mdx hearts (Sali et al, 2012). In both pre-clinical trials (pre-symptomatic and adult), we examined cardiac and skeletal muscle for measures of fibrosis. In the pre-symptomatic trial (Fig 6D–F), prednisolone caused a significant elevation of heart mass ratios over vehicle (5.9 ± 0.6 vs. 5.4 ± 0.4, p < 0.05), indicative of cardiac hypertrophy. No increase was present in VBP15 groups. Histologically, clear fibrosis was evident in 50% of young (8 weeks) prednisolone-treated hearts compared to 0% of all other groups. Histological analyses of skeletal muscle (gastrocnemius) also showed increased fibrosis in prednisolone-treated mice (8.1 ± 2.2%, p < 0.05) compared to vehicle-treated (4.2 ± 1.8%), VBP15-treated (3.5 ± 1.2% at 30 mg/kg), and WT (2.0 ± 0.5%) mice (Supporting Information Fig 2C–E). In the adult trial, cardiac findings were consistent with the pre-symptomatic trial (Fig 6G and H). Here as well, prednisolone treatment increased fibrosis and mass ratios of mdx hearts, while VBP15 did not.

DISCUSSION
Development of mechanisms to improve muscular dystrophy in the absence of detrimental hormonal effects will substantially improve DMD patient medical care, could provide a therapy for dystrophies with no current treatment, and could improve care of diverse chronic inflammatory disorders. Here, we describe the development, mechanisms and effects of a novel drug that regulates and optimizes several sub-activities of classic glucocorticoids (Fig 7), demonstrating it is possible to treat muscular dystrophy in the absence of growth, hormonal and immunosuppressive side effects. For one sub-activity, we show VBP15 has protective physicochemical effects on the plasma membrane, protecting cells from injury and promoting membrane repair. This sub-activity is likely to be particularly important in DMD where disease pathogenesis is clearly linked to membrane instability and myofibre injury. For another, we show that a key anti-inflammatory activity, inhibition of TNFs-induced NF-κB, is retained by VBP15. We further show that this mechanism occurs through protein–protein interactions of the VBP15 ligand-activated GR, independently of DNA binding, GRE activation, or upregulation of inhibitory transcripts. We have previously shown that NF-κB activation is among the earliest histological features of DMD neonates (Chen et al, 2005; Porter et al, 2002, 2003), years before symptoms appear. This, coupled with the results of our blinded mdx pre-clinical data here, suggests that very early treatment of DMD patients with VBP15 may prevent or delay the onset of some clinical symptoms. Finally, the well-documented and extensive side effects of other glucocorticoids are avoided with VBP15.
VBP15 improves muscular dystrophy

**DISCUSSION**

The percentage of B lymphocytes, as measured by FACS analysis of B220 expression, was measured to determine if VBP15 inhibited bone growth when compared to 0% of all other groups. Histological results showed a significant increase in trabecular thickness over vehicle (5.9 ± 1.8%), VBP15 did not affect tibia length at any dose.

### Figure 6. VBP15 lacks the side effects of current glucocorticoid regimens in vivo.

**A.** Prednisolone treatment stunted the growth of developing mice in comparison to both vehicle and VBP15 groups. Representative photographs (A) and quantitation of tibia length (B) are provided. Prednisolone decreases tibia length, while VBP15 does not. Taken together, these findings were consistent with the prior reports that NF-κB activation is among the earliest disease mechanisms.

**B.** Bone lengths were reduced upon prednisolone treatment. X-rays (B) of mouse tibias illustrate size differences (scale bars = 2 mm). Prednisolone thus significantly decreased this to 14.8 ± 0.5 mm, while prednisolone showed no decrease in B cells. Prednisolone treatment increased heart mass ratios over vehicle (5.9 ± 1.8%), VBP15 did not. Prednisolone thus significantly reduce trabecular thickness (P<0.005) compared to VBP15.

**C.** MicroCT imaging analysis of femur revealed a significant decrease in trabecular thickness (C') for prednisolone treated mice. Sirius red staining of cardiac muscle shows increased fibrosis in prednisolone-treated mice, but not VBP15 mice. Representative images (D) and digital quantitation of fibrosis (E) are provided. To the right of the image panel is a higher magnification image from the area outlined in box. (F) Heart mass ratios were increased by prednisolone but not by VBP15.

**D-H.** In adult mdx mice as well, increases in cardiac fibrosis (C) and heart mass (C') were observed with prednisolone treatment but not VBP15 treatment. In adult mdx vehicle mice, an expected disease- and age-related increase in fibrosis over WT is seen. Values are mean ± SEM. (n ≥ 12 per group for A,B,E,F; n ≥ 5 for C,D,G,H); "p < 0.05, "p < 0.005, ""p < 0.0005).

**E.** Increases in cardiac fibrosis and heart mass were detected in prednisolone treated mice, suggestive of cardiac damage as a side effect lacking for VBP15.

**F.** Increases in cardiac fibrosis and heart mass were detected in prednisolone treated mice, suggestive of cardiac damage as a side effect lacking for VBP15.

**G.** Heart mass ratios were increased by prednisolone but not by VBP15.

**H.** Heart mass ratios were increased by prednisolone but not by VBP15.

 Steroidal compounds are multi-mechanistic by nature and display physicochemical effects on the plasma membrane (Rhen et al, 2003; Shivaji & Jagannadham, 1992). We find VBP15 and prednisolone differ in their effects on membranes, with VBP15 treatment protecting live cells from laser-induced injury. Membrane-stabilization is a property that is analogous to poloxamer 188, Mitsugumin 53 or cromolyn sodium, which...
Figure 7. Working model of VBP15 and prednisone drug mechanism sub-activity profiles. Steroidal compounds such as glucocorticoids (prednisone) and 3-9.11 compounds (VBP15) are multi-potent drugs. Through dissecting the sub-activities of these compounds, we find that: (1) VBP15 reduces inflammation but does not show the immunosuppressive impairment of lymphocyte viability and function observed for prednisone. (2) Within an environment of plasma membrane disruption, VBP15 helps to promote resistance to and repair of injuries, while prednisone can exacerbate membrane injury. (3) Inside cells, both compounds bind to and activate the GR to potently inhibit inflammatory NF-κB signaling through protein–protein interactions. (4) Though they both bind to the GR, prednisone causes strong induction of hormonal GRE controlled promoter elements, while VBP15 eliminates or greatly reduces these effects.

operate through varying mechanisms and show beneficial effects on dystrophin deficient dog and mouse muscle in vivo (Marques et al, 2008; Townsend et al, 2010; Weisleder et al, 2012). Membrane-stabilizing effects of VBP15, but not prednisolone, are consistent with increases in specific force observed for VBP15 but not for prednisolone. VBP15 effects on membrane stability could be explained by altered compression of phospholipid head groups within the membrane, altered ion balances (Howard et al, 2011), altered membrane or vesicular fusion (Shivaji & Jagannadharm, 1992), or altered oxidative stress at the plasma membrane (Howard et al, 2011; Kavanagh & Kam, 2001; Marques et al, 2008; Saija et al, 2001). With membrane integrity and repair becoming of increasing importance in muscle (Bansal et al, 2003; Jaiswal et al, 2007), cardiovascular (Chase et al, 2009), neurodegenerative (Bazan et al, 2005) and airway (Gajic et al, 2003) disorders, physicochemical properties of VBP15 will be an intriguing area of investigation moving forward.

Chronic treatment with glucocorticoids (prednisone, deflazacort) is the current standard of care for DMD, yet glucocorticoids are well-known to induce muscle atrophy pathways via FOXO1, stunt the growth of paediatric patients, and can suppress the immune system which plays an important role in myofibre repair cycles. Thus, clinical improvements in DMD patients treated with glucocorticoids may be the sum balance of beneficial anti-inflammatory effects and deleterious pathways. Both in vitro and in vivo data presented here are consistent with this model. In mdx mice, we find the net balance of prednisolone treatment increases normalized strength, however at the same time it stunts the growth of mice resulting in lower maximal strength, is immunosuppressive, and increases the presence of muscle damage. We, as well as others in recent reports (Bauer et al, 2009), find that prednisolone increases cardiac fibrosis in mdx mice. Comparable examination of cardiac fibrosis in glucocorticoid treated DMD patients has not been examined directly in the literature, however recent anecdotal cardiac MRI reports show substantial fibrosis, suggesting this may be an intriguing area of investigation moving forward, with a possibility to develop more “heart healthy” treatments. VBP15 however does not stunt the growth of mice, shows no evidence of splenocyte immunotoxicity, and does not increase muscle fibrosis in skeletal or cardiac muscle. In
the absence of these side effects, VBP15 increases strength, increases absolute and specific force measures and decreases muscle inflammation. A comparison of VBP15 and prednisolone mechanistic profiles in the context of these results has several implications. One, stunted growth appears to be a side effect of glucocorticoid treatment in DMD as opposed to a mechanism of efficacy, as has been logically proposed in the past (Grounds & Shavlakadze, 2011) by limiting body size to reduce muscle workload. Here, we see dose-dependent increases in mdx strength in the absence of stunted growth, suggesting the potential to increase patient strength without overt effects to growth and development. Two, immunotoxicity is a potential side effect of glucocorticoid treatment – not the primary cause of efficacy. This is supported by the failure of general immunosuppression to increase DMD patient strength (Griggs et al, 1993; Kissel et al, 1993). Three, NF-κB inhibition is shared by both drugs, supporting our hypothesis that this is a shared pathway of efficacy. In further support of this, peptides or antibodies targeting NF-κB pathways benefit mdx phenotypes (Grounds & Torrisi, 2004; Peterson et al, 2011), while in contrast, constitutive activation of NF-κB causes severe muscle wasting (Cai et al, 2004; Mourkioti et al, 2006). Finally, GRE transactivation appears to be disposable to efficacy in DMD. As GRE-regulated genes have been implicated in a number of glucocorticoid side effects, this is particularly exciting because it provides a clear avenue by which to reduce harsh side effect profiles currently limiting the use of these widely applicable drugs. Indeed, along with a reduction in GRE activity for VBP15, we also see an absence of glucocorticoid side effects in mdx mice. Importantly, efficacy is maintained in the absence of immunosuppression and overt hormonal effects to growth and development, providing insight into mechanisms of glucocorticoids in muscular dystrophy and demonstrating a successful separation of pathways into dystrophy efficacy and side effects.

Exon skipping represents another promising line of therapeutic development for DMD (Hoffman et al, 2011). Short of gene replacement, this may offer the greatest potential to alleviate DMD because it aims to restore expression of disease-causing dystrophin deficiencies. However, isoforms induced by exon skipping, as well as the mini-gene dystrophin constructs envisioned in gene therapy, are also expressed in Becker muscular dystrophy (alleles of dystrophinopathy leading to milder disease). In other words, both exon skipping and gene therapy are expected to mitigate but not cure disease. We and others find exon skipping partially restores specific force deficits in mdx muscle, with ex vivo force contractions typically showing approximately 20% increases over mdx controls (Aoki et al, 2010; Dumonceaux et al, 2010). This likely represents an upper limit to therapeutic strength increases, short of gene replacement. We find VBP15 treatment increases EDL specific force as well, with 15 mg/kg producing 12 and 16% increases in the two trials presented here. Currently, patients with Becker or other milder muscle dystrophies are not routinely administered prednisone (Johnsen, 2001) due to the unclear net balance of detrimental versus beneficial effects it would provide. Evidence here suggests VBP15 could provide a novel therapy for Becker’s and other milder dystrophies, or serve as a valuable combination therapy used with exon skipping to provide efficacy through independent mechanisms.

Currently, glucocorticoid regimens for DMD delay treatment to avoid serious detriment, and many patients eventually discontinue treatment as a result of side effects. A drug lacking such harsh effects has the potential to change physicians’ treatment approaches since it would be more amenable to a chronic treatment regimen, and would enable treatment during pre-symptomatic or late stages when many patients are not taking prednisone. This rationale prompted us to change our approach to mdx preclinical trial design for VBP15, and indeed we saw clear efficacy with an ablation of side effects to mdx growth, bone and muscle. Intriguingly, by enabling treatment of DMD at pre-symptomatic ages, a strong rationale for neonatal screening could be built to move forward towards a preventative medicine approach to treatment, thereby improving the way we diagnose and treat DMD patients.

International consensus has established the mdx mouse as the model of choice for preclinical and proof-of-concept studies because they represent the exact monogenic biochemical defect present in DMD (Nagaraju & Willmann, 2009; Willmann et al, 2009, 2012). However, mdx mice present a milder disease than DMD, with peak severity from approximately 3–8 weeks of age after which they recover substantially until advanced ages. This prompts various strategies to exacerbate the mdx phenotype. One strategy is to introduce additional mutations which exacerbate disease onto the mdx background, examples of which include the mdx:utrophin\(^{-/-}\) (Deconinck et al, 1997; Grady et al, 1997b), mdx:adhn\(^{-/-}\) (Grady et al, 1999), mdx: a7 integrin\(^{-/-}\) (Guo et al, 2006), mdx:PV\(^{-/-}\) (Raymackers et al, 2003) and mdx:MyoD\(^{-/-}\) (Megenev et al, 1999) double knockout models. These provide advantages through increased disease severity and a differing array of symptoms, which allow for more efficient trials utilizing smaller sample sizes without the added need of forced exercise protocols. Several groups have thus utilized mdx:utrophin\(^{-/-}\) double transgenic mice to successfully detect therapeutic efficacy (Defin et al, 2011; Gehrig et al, 2012; Goyenvalle et al, 2010; Wakefield et al, 2000). It is possible for a second mutation to introduce underlying biochemical or biological differences however, for example utrophin\(^{-/-}\) single transgenic mice have an increased susceptibility to seizures (Knuese et al, 2002), along with altered neuromuscular junction folding and altered acetyl choline receptor density (Grady et al, 1997a), which could feasibly affect neuromuscular disease outside of a direct consequence of dystrophin deficiency (Willmann et al, 2009). Here, we chose to use larger sample sizes of monogenic mdx mice to ensure that the efficacy parameters we measured were from phenotypes directly resulting from dystrophin deficiency. To optimize our trial designs, we adopted two strategies to measure mdx phenotypes at points of increased severity, in one trial by assaying young mice during natural peaks in disease severity, and in the other by using forced exercise protocols in adult mice to exacerbate disease. Through both strategies, we consistently detect significant mdx phenotypes and VBP15 efficacy through an improvement of mdx phenotypes towards wild type.
Extension of VBP15 to other clinical disorders of membrane instability and chronic inflammation will require further studies and clinical development. Studies of VBP15 in animal models of arthritis, asthma, multiple sclerosis and inflammatory bowel diseases are currently underway. Through collaboration with the Muscular Dystrophy Association Venture Philanthropy, and the National Institutes of Health Therapeutics for Rare and Neglected Disease (TRND) program, VBP15 is being actively developed for DMD as the initial indication. Participation in these programs has provided repeatability, efficacy and safety through independent trials both in vitro and in vivo. VBP15 shows favourable pharmacokinetic, ADME and metabolite profiles (Reeves et al., 2013). Early safety studies by independent groups suggest a single dose tolerance of at least 500 mg/kg in mice, and a 28 day NOAEL of at least 100 mg/kg in mice, which is more than twice the highest doses (30 and 45 mg/kg) used here to show both efficacy and a clear reduction in side effects in comparison to prednisolone.

Our results demonstrate the successful separation of pathways providing efficacy from side effects in muscular dystrophy. The translation of these into model mice by treatment with a novel, orally available drug, indicates that strength and pathology phenotypes can be improved by treatment without overt hormonal, growth or immunosuppressive effects. VBP15 merits further investigation for efficacy in clinical DMD trials, and is relevant to a diverse group of disorders through shared inflammation or membrane injury molecular pathways. By focusing on DMD as an initial indication, we benefit from having (i) models reproducing the ubiquitous molecular deficit present in all patients, and (ii) a homogenous patient population with strong foundations providing national clinical trial support. Movement into the clinic would improve treatment of human disease, provide further mechanism insight and provide a template for future drug development. With a molecular profile relevant to diverse disorders and DMD amenable to neonatal screening, VBP15 may provide an excellent opportunity to develop an Orphan disease therapy in a way that helps larger groups of more complex disorders.

MATERIALS AND METHODS

NF-κB inhibition

C2C12 cells stably expressing an NF-κB luciferase reporter were cultured and assayed as described previously (Baudy et al., 2009). For GR antagonist experiments, cells were treated with a constant concentration of drug (1 μM) and increasing RU-486 (Sigma) concentrations. In both experiments, cells were pretreated with drug for 1 h, stimulated with TNFa (10 ng/ml) and assayed for luciferase activity 3 h later. H2K myoblasts were cultured with gamma-interferon at 33°C, and differentiated into myotubes in six-well plates with Matrigel at 37°C. Cells were plated 15E per well, treated with drug after 4 days of differentiation, induced with TNFa 24 h later, and RNA harvested the following day.

Pituitary cell assays

For pituitary cell line experiments, A/T-20/D16v-F2 cells (ATCC) were maintained at 37°C, 5% humidity in DMEM with 10% FBS. In Sgh2 studies, cells were plated at 6E5 per well overnight, then serum starved in six-well plates. After 48 h, cells were drug treated for 6 h then lysed for RNA. For ACTH studies, cells were plated at 1.3E6 per T25 flask and treated with drug. Media and drug were changed daily for 5 days, then cells were counted and replated in six-well plates. Twenty-four hours later, media was collected and cells lysed for RNA. ACTH secretion was assessed by lumELISA (Calbiotech).

GR mutant assays

GR<sup>dim/dim</sup> cells and the parental L929 fibroblast line they were derived from (Housley & Forsthoefel, 1989) were cultured in DMEM at 37°C. Protein lysates were obtained from untreated cells using RIPA buffer, separated on 4–15% PAGE gels, and transferred to nitrocellulose membranes, which were immunoblotted with rabbit polyclonal anti-GR (Santa Cruz) and rabbit monoclonal anti-GAPDH (Cell Signaling Technology), followed by HRP-secondary (Bio-Rad). For assays of GRE and inflammatory transcripts, cells were treated with drug for 24 h, then stimulated with TNFa (1 ng/ml) for an additional 24 h, lysed for RNA, and assayed by qPCR.

GR<sup>dim/dim</sup> mice (Reichardt et al., 1998) were obtained from the Deutsches Krebsforschungszentrum (German Cancer Research Center). GR<sup>dim/dim</sup> and wild type control (C57Bl/6) mice were maintained in an animal facility within IACUC guidelines under approved protocols. Spleens were isolated and single cell suspensions generated through homogenization and lysis of red blood cells using ACK lysis buffer (Lonza). Splenocytes were treated with drug for 24 h, then stimulated with TNFa (10 ng/ml) for another 24 h. RNA was extracted from splenocytes, with analysis of GRE and inflammatory transcripts performed by qPCR.

Real-time qPCR

cDNA was produced using the High Capacity CDNA Reverse Transcription Kit (ABI). Transcript levels were analysed via TaqMan qPCR assays (LifeTech). The following assays were used: Sgh2, Mm00441380_m1; Pomp, Mm00435874_m1; Ifi2, Mm01288580_m1; Cx2, Mm00294838_g1; Nos2, Mm00440502_m1; Tnfa, Mm00443258_m1; It1a, Mm00439620_m1; Nfkb1, Mm00477800_g1; Ifi6, Mm00446190_m1. qPCR was performed using TaqMan gene expression master mix and 18s rRNA as a normalization control (ABI).

LASER-MEDIATED WOUNDING OF LIVING CELLS

C2C12 myoblasts were pretreated with drug in growth media for 15 min. Immediately following this, cells were wounded in imaging media ( Hank’s Balanced Salts, 10 mM HEPES, pH 7.4 containing drug or equivalent vehicle, 2 mM Ca<sup>2+</sup> and 2 μg/ml FM1-43 dye (Molecular Probes Inc.) at 37°C. Injuries were performed with a pulsed one-photon laser (Ablate!, Intelligent Imaging Innovations Inc.) and a custom built Olympus IX81 microscope (Olympus America). Wounding was performed with ablation power 116 in a 2 × 2 μm<sup>2</sup> for all injuries. Cells were imaged at 2 s intervals. Initial fluorescence intensity was measured and used to normalize subsequent time points. Fluorescence intensity over time was measured within cell borders using SlideBook 5.0 (Intelligent Imaging Innovations Inc.).

Receptor binding assays

The various steroid receptors (GR, MR, ER, AR and PR) were extracted and incubated with a constant concentration of radiolabeled, high-affinity ligand. Increasing concentrations of unlabeled VBP1, VBP3, 1580
VB15 or high-affinity ligand controls (triacsinolone, spironolactone, 17β-Estradiol, methylthienolone or Promegestone) were added and the percent binding of radiolabeled ligands determined to gauge the affinity of the unlabeled competitors for the steroid receptors.

Animal care and drug dosing
Two separate mdx trials were performed to provide repeatability as well as contrasting treatment and phenotyping regimens. The larger “pre-symptomatic” mdx trial (78 mice total) is presented here as the primary trial. WT (C57BL/10ScSn) and mdx (C57BL/10ScSn-Dmd<mdx>) mice were obtained from Jackson Laboratory (Bar Harbor, ME). All experiments were conducted within IACUC guidelines under approved protocols. PND15 was chosen as the trial start point because it was the earliest age prednisolone could confidently be safely administered (Heine & Rowitch, 2009; Pinsky & Diegert, 1965). At this point, mice were divided into groups of equally matched body mass, which were then blinned to both drug and genotype for subsequent phenotyping and histology experiments. Treatment groups (n = 12–14 per group) consisted of WT vehicle, mdx vehicle, mdx VB15 (5, 15 or 30 mg/kg), and prednisolone (5 mg/kg). Mice received daily AM dosing via cherry syrup vehicle at 1 μl per 1 g body weight. One mouse suffered a head injury during phenotyping and was removed from subsequent experiments. No adverse effects from drug treatment were observed. Functional phenotyping was performed in 5-week-old mice. In vivo imaging was performed in 6–7 week old mice. At 8 weeks of age, terminal assays were performed and tissues harvested.

A separate ‘adult’ mdx trial (48 mice total) was performed during lead compound identification according to established standard operating procedures. In this smaller, open-label experiment, WT and mdx mice (n = 8 per group) received daily PM oral syrup vehicle, prednisolone (5 mg/kg) or VB15 (5, 15 or 45 mg/kg). All mice were subjected to 30-min run on horizontal treadmills at 12 m/min, twice a week except during data collection to unmask the mild phenotype of mdx mice. One death was recorded at VB15 45 mg/kg body weight. Mice were administered drug for 4 months starting at 6 weeks of age.

Motor function
At 5 weeks of age, mice in the neonate trial were assayed for motor function via grip strength measurement. Strength was assessed daily AM for 5 days using a grip strength meter (Columbus Instruments). Data was interpreted as maximum daily values for each of five testing days and averaged over the 5 days. Animals were acclimated for 1 week prior to data collection.

Live imaging
Mice were anaesthetized with isoflurane, and cathepsin caged near-infrared imaging was performed on 6–8 mice per group as described previously (Baudy et al, 2011). Briefly, mice received intraperitoneal (IP) injections of ProSense 680 (Perkin-Elmer) in PBS 24 h prior to imaging within an Optix MX2 Imager (ART). Scans of uninjected mice were performed to obtain baseline optical intensity measurements. Forelimb and hindlimb measurements were made at 0.5 mm resolution and analysed using Optiview software.

Ex vivo force contractions
At trial endpoint, EDL muscle was isolated from live anaesthetized mice and placed in Ringer’s solution (137 mM NaCl, 24 mM NaHCO3, 1 mM D-glucose, 5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, and 0.025 mM tubocurarine chloride) at 25°C bubbled with 95% O2 and 5% CO2. Contractile properties were measured ex vivo according to established methods (Brooks & Faulkner, 1988) using a force apparatus (model 305B, Aurora Scientific). Drop in force was measured after 10 lengthening contractions where each muscle was stretched over 10% of its length.

Immunotoxicity studies
Peripheral blood was obtained via retro-orbital bleed. Following sacrifice, spleens and thymuses were harvested, weighed, and processed to generate single cell suspensions of splenocytes and thymocytes, respectively. Red blood cells in splenocytes and peripheral blood were lysed with 3% acetic acid + methylene blue (Stem Cell Technologies). All leukocytes were quantified via haemocytometer. For lympho-phenotyping studies, splenocytes were stained for FACS with FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8, or APC-conjugated anti-mouse B220 monoclonal antibodies (eBioscience). For CD4+ cell activation studies, splenocytes (5×10⁶ per well) were stimulated in RPMI 1640 + 10% FBS with 5 μg/ml concanavalin A (Sigma–Aldrich) in 48-well plates for 72 h at 37°C. Following stimulation, cells were stained with FITC-conjugated anti-mouse CD4 and APC-conjugated anti-mouse CD25 monoclonal antibodies (eBioscience). All FACS analyses were conducted using a FACSCalibur (BD Biosciences).

Histology
Paraffin cross-sections were made of gastrocnemius, heart and diaphragm muscles and stained with H&E. For gastrocnemius, images were analysed in Image J software (NIH) according to previously established methods (Spurmy et al, 2009). For diaphragm, full tissue sections were scored for inflammation by a trained veterinary immunologist blinded to drug and genotype.

To assay fibrosis, paraffin embedded muscles were cross-sectioned and stained with Sirius Red. Tissue was imaged with a 4× objective, digital captures were made with Olympus software, and fibrotic signal quantified using Image J (NIH). Blood and background were removed from blinded images to prevent false detection of tissue and percent signals when threshold measurements were made during ImageJ quantitative analysis. The percentage fibrotic tissue was calculated as area reaching Sirius Red positive thresholds divided by total tissue area of the section.

X-ray and microCT analysis of bone
Skeletons were harvested at trial endpoint and stored in 10% formalin. X-rays of tibias were obtained using a Cabinet X-Ray System (Faxitron Model 43855) with exposure at 50 kVp for 1.5 min. Magnification error was calculated to be ±0.02 mm. Images were scanned and tibia lengths measured in Adobe Illustrator (v6.0) at 2400% zoom. Measurements of the opposite tibia were also obtained physically with digital calipers during dissection, with results in agreement between methods. MicroCT analysis was performed on harvested femurs using a SkyScan 1172 MicroCT (Bruker, Belgium). Imaging was performed at 40 kV source voltage, 250 μA source current, 295 ms exposure time, and 0.4° rotation step, with a 0.5 mm aluminum filter. The imaging resolution size was 6.2 μm. Three-dimensional reconstructions were performed with SkyScan NRecon and Dataviewer software. Trabecular bone was...
The paper explained

PROBLEM:
Glucocorticoids have been a mainstay in medicine since their discovery over 60 years ago. They are powerful anti-inflammatory drugs used to treat a variety of conditions. However, due to a complex mechanism profile, glucocorticoids also cause harsh side effects such as brittle bones, muscle wasting, stunted growth, adrenal suppression and weight gain. Patients and doctors must therefore manage their net positive and negative effects. This is of particular importance in some chronic or paediatric disorders, where lifelong treatment is required and patients must live with serious side effects. DMD is a lethal genetic muscle disease for which glucocorticoids are the current standard of care. Though glucocorticoids produce established improvements in DMD patient outcome measures, their harsh side effects dramatically affect patients' quality of life. As a result, physicians typically delay treatment in young children until well after disease onset, and many families choose to stop treatment even though there is no alternative currently available in the clinic.

RESULTS:
The discovery that glucocorticoids possess several distinct sub-activities provides an intriguing opportunity to produce drugs that stimulate some of these activities while avoiding others. We discover VBP15 as a novel, orally administered compound that shares specific anti-inflammatory effects with glucocorticoids and also acts to stabilize cell membranes. Importantly, we also find that VBP15 avoids specific activities established to cause glucocorticoid side effects. Translating these findings into mice with muscular dystrophy, we find that both preventive and therapeutic regimens improve muscle strength and disease pathology. Further, this efficacy is displayed in the absence of hormonal, immunological and growth side effects seen in glucocorticoid treated mice.

IMPACT:
There is a clear need for improved treatments in chronic inflammatory diseases such as DMD, where safer drugs would improve quality of life and provide justification for neonatal screening. Data here confirms that small molecules can be produced which separate the sub-activities of glucocorticoids towards fulfilling this need. VBP15 is identified as the lead compound, which is actively being developed towards the clinic. Excitingly, proof-of-principle data shows that this compound provides efficacy in mice with muscular dystrophy while successfully eliminating important side effects. This provides new insight into glucocorticoid sub-activities, and demonstrates the potential to replace glucocorticoids as the standard of care for DMD as well as other chronic inflammatory diseases.

selected for analysis by a polygonal region of interest within the centre of femur, starting at 70 slices (0.43 mm) proximal from the growth plate and extending proximally 200 slices (1.23 mm) further. Trabecular measurements were obtained from 3D analysis of the selected bone using Skyscan CT-analyzer software.

Statistical analyses for animal trials
Unless otherwise noted, normality of each measurement was tested via Shapiro–Wilk normality test and normally distributed measurements were compared between mdx treatment groups using one-way ANOVA. Measurements that were not normally distributed were compared with a non-parametric test. For efficacy studies where mdx treatment comparisons showed a significant overall p-value, post hoc linear tests between each VBP15 dose group and vehicle only were performed and results p-va/adjusted for multiple testing by Sidak method. In side effect assays where comparisons showed a significant overall p-value, a Student's t-test was included between prednisolone and vehicle groups for normally distributed measurements.

Author contributions
CRH designed, performed, and managed in vitro experiments and the in vivo pre-symptomatic trial, analysed data from both trials and wrote the paper. JMD designed and performed immunology and other in vivo experiments, analysed/interpreted data, and is actively participating in preclinical VBP compound development. QY performed several specialized mouse imaging experiments. BCD performed dissection and immunology experiments and is actively participating in preclinical development. TH contributed to the design and interpretation of experiments, and helped to perform in vivo experiments. JHVM performed specialized in vivo muscle physiology experiments. AS, BKM, and AP performed blinded, in vivo experiments in the neonate trial. LS performed initial in vitro injury experiments, provided training, and helped to interpret data. QJ and KT performed blinded histopathology experiments. SJ and SD designed and performed in vivo adult preclinical trial experiments. OCR and CA designed and provided X-ray imaging, services, instruction and data processing for X-ray experiments. MC contributed to histology experiment design and provided automated imaging, blinding and randomization. HGD performed statistical analyses for the two in vivo trials. KJK developed the live cell laser injury technology at CNMC, provided the core equipment/services, provided funding, and participated in experimental design and analysis. EMC and JMM are responsible for identifying and developing the VBP compounds towards clinical development. EPH provided mentorship, funding, input into experimental design, and helped
to write the paper. EKMR helped to identify VBP15, is actively involved in preclinical development, contributed to data analysis/interpretation, provided mentorship and participated in trial and experimental design. KN contributed greatly to experimental designs, provided mentorship, funding, data analysis/interpretation, facilitated the in vivo preclinical trials, and helped to write the paper.

Acknowledgements
The authors would like to thank Brenda Klaunberg and Danielle Donahue along with the NIH Mouse Imaging Facility and Drs. Carsten Bonnemann and Rachael Rooney for a microCT collaboration. We also thank Dr. Gregory Cox and Jackson Laboratories for assistance with mdx mice. QR\textsuperscript{null} fibroblasts were generously donated to us by Dr. Paul Housley, Drs. Alyson Fiorillo and Aurelia Defour, along with Amanda Mullen, Rana Shehata and Beryl Ampong, provided training, technical support and/or supportive efforts for work in the manuscript. X-ray imaging was performed in the Georgetown-Lombardi Preclinical Imaging Research Laboratory. Sinq systems provided automated histology imaging and randomization services. Caliper Life Sciences performed receptor binding assays. These studies were funded in part by the United States Department of Defense CDMRP grants (W81XWH-05-1-0616, W81XWH-09-1-0218, W81XWH-11-1-0754), the Foundation to Eradicate Duchenne, the Muscular Dystrophy Association USA (MDA-VP program), and the National Institutes of Health (R01-AR050478, 1U54HD053177-01A1, Wellstone Muscular Dystrophy Center, RO1AR055686, and NCATS TRND program). Core support was received from NIH P50AR060836 (Center of Research Translation), 2R24HD050846-06 (Center for Medical Rehabilitation) and P30HD046777 (Intellectual and Developmental Disabilities Research Center). CH is funded by a T32 postdoctoral training grant in the Genetics and Genomics of Muscle (5T32AR056993-02). KN is also supported by NIH K26OD011171, the MDA (translational grant), the US Department of Defense (W81XWH-05-1-0659, W81XWH-11-1-0782), and a pilot grant from Parent Project Muscular Dystrophy. Funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Supporting Information is available at EMBO Molecular Medicine Online.

Conflict of interest statement: ReveraGen Biopharma owns method of use intellectual property relating to use of Δ9,11 compounds to treat disease. EMC is CEO of ReveraGen. JMM, EMC, EPH and KN are co-founders of ReveraGen with shares in the company. EKMR, JMD and BCD are employees of ReveraGen.

For more information
Muscular Dystrophy Association: http://mda.org/

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Ret rescues mitochondrial morphology and muscle degeneration of Drosophila Pink1 mutants

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Abstract

Parkinson’s disease (PD)-associated Pink1 and Parkin proteins are believed to function in a common pathway controlling mitochondrial clearance and trafficking. Gial cell line-derived neurotrophic factor (GDNF) and its signaling receptor Ret are neuroprotective in toxin-based animal models of PD. However, the mechanism by which GDNF/Ret protects cells from degenerating remains unclear. We investigated whether the Drosophila homolog of Ret can rescue Pink1 and park mutant phenotypes. We report that a signaling active version of Ret (Ret$^{Akt}$) rescues muscle degeneration, disintegration of mitochondria and ATP content of Pink1 mutants. Interestingly, corresponding phenotypes of park mutants were not rescued, suggesting that the phenotypes of Pink1 and park mutants have partially different origins. In human neuroblastoma cells, GDNF treatment rescues morphological defects of Pink1 knockdown, without inducing mitophagy or Parkin recruitment. GDNF also rescues bioenergetic deficits of Pink knockdown cells. Furthermore, overexpression of Ret$^{Akt}$ significantly improves electron transport chain complex I function in Pink1 mutant Drosophila. These results provide a novel mechanism underlying Ret-mediated cell protection in a situation relevant for human PD.

Keywords Drosophila; neurodegeneration; neurotrophic factors; OXPHOS; Parkinson’s disease

Subject Categories Molecular Biology of Disease, Neuroscience

DOI 10.1002/embj.201284290 | Received 20 December 2012 | Revised 8 November 2013 | Accepted 29 November 2013 | Published online 28 January 2014


Introduction

The etiology of Parkinson’s Disease (PD) is highly complex and largely unknown, involving both environmental and genetic risk factors. Mitochondrial dysfunction, oxidative stress and protein aggregation are believed to be central events in the pathological process, but their interconnection remains unclear (Schapira & Jenner, 2011; Exner et al., 2012; McCoy & Cookson, 2012). The first indications of a role for mitochondria came with the discovery that the toxin 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) causes Parkinsonism in humans and animal models (Burns et al., 1983; Langston et al., 1983). Its active metabolite, 1-methyl-4-phenylpyridinium ion (MPP+), is selectively imported into dopaminergic neurons via the dopamine transporter, and inhibits complex I of the electron transport chain (ETC). Several other mitochondrial toxins, including paraquat and rotenone, generating either mitochondrial reactive oxygen species (ROS) or specifically inhibiting complex I, have been linked to PD in epidemiological studies and animal models (de Lau & Breteler, 2006). Furthermore, patients with sporadic PD can have decreased activity of complex I in brain and other tissues (Schapira et al., 1989; Parker & Swerdlow, 1998), or less complex I proteins in the substantia nigra (Mizuno et al., 1989).

Autosomal recessive PD-associated proteins PARKIN, PINK1 and DJ-1 (OMIM #600116, 605909, 606324) have been shown to have functions related to mitochondrial integrity, (reviewed in Exner et al., 2012; Martin et al., 2011). In three seminal studies, PINK1 mutant Drosophila displayed mitochondrial abnormalities and muscle degeneration in a manner highly similar to park mutants, and Parkin overexpression largely rescued the phenotypes of Pink1 mutants, but not vice versa, suggesting that the two proteins act in a common linear pathway (Clark et al., 2006; Pak et al., 2006; Yang et al., 2006). Manipulation of the mitochondrial remodeling machinery rescues some PINK1 and park mutant phenotypes in Drosophila and in mammalian cell lines. However, while increasing fission rescues the Drosophila phenotypes, shifting the fusion/fission balance in the opposite direction rescues mammalian cell lines, but the underlying mechanisms are not fully understood (Deng et al., 2008; Poole et al., 2008; Lutz et al., 2009). PINK1, a mitochondrial Ser/Thr kinase, and Parkin, an E3 Ubiquitin ligase, were found to...
regulate clearance of damaged mitochondria via mitophagy (Geisler et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010), and microtubular transport (Weihofen et al., 2009; Wang et al., 2011). However, other studies have reported additional functions of Parkin in the regulation of stress response proteins and mitochondrial biogenesis (Bouman et al., 2011; Shin et al., 2011), in promoting NF-κB signaling (Henn et al., 2007; Muller-Rischart et al., 2013), and in controlling cytochrome-c release (Berger et al., 2009). PINK1 also has additional functions, unrelated to recruiting Parkin, such as regulating mitochondrial calcium buffering (Gandhi et al., 2009; Sandebring et al., 2009; Heeman et al., 2011). Furthermore, PINK1 knockout mice have decreased activity of complex I of the ETC (Morais et al., 2009), and overexpression of a yeast substitute for complex I rescued many of the functional impairments of PINK1 mutant flies (Vilain et al., 2012). Additional studies are required to elucidate which of the functions reported for Parkin and PINK1 are critical for causing Parkinson pathology.

The neurotrophic factor Glial cell-derived neurotrophic factor (GDNF) promotes the survival of dopamine neurons (Lin et al., 1993) and protects nigral dopamine neurons from cell death in rodent and primate toxin-models of PD such as 6-hydroxydopamine (6-OHDA) and MPTP (Kearns & Gash, 1995; Sauer et al., 1995; Tomac et al., 1995; Gash et al., 1996). Several clinical trials have been performed with mixed outcomes, but ongoing research and development aims at improving delivery methods of GDNF (Deierborg et al., 2008). GDNF signals via the GPI-anchored co-receptor GFR-α1 and the receptor tyrosine kinase Ret (Airaksinen & Saarma, 2002). Endogenous Ret expression is required for long-term survival of a fraction of nigral dopamine neurons in aged mice (Kramer et al., 2007). Conversely, mice that express a constitutively active Ret receptor in dopamine neurons (Ret\textsuperscript{MEN2B}) show increased numbers of dopamine neurons (Mijatovic et al., 2007). The mechanism by which GDNF/Ret protects dopamine neurons from cell death is not fully elucidated. We hypothesized that Ret-activated signaling pathways converge with functions of proteins associated with familial PD. We recently reported that Ret and DJ-1 double loss-of-function in aged mice exacerbates the neuron loss observed in Ret single mutants (Aron et al., 2010). Here, we investigated whether Ret interacts genetically with park and Pink1 in Drosophila. We found that constitutively active Ret\textsuperscript{MEN2B} specifically rescues phenotypes of Pink1 mutants, including muscle degeneration, mitochondrial morphology and function, whereas park mutants remained unaffected. Moreover, Ret signaling rescued mitochondrial morphological and functional defects of Pink1-deficient human SH-SY5Y cells, without activating mitophagy. Mechanistically, Ret signaling restored the activity of complex I of the ETC, which is reduced in Pink1, but not park mutant flies. Thus our study indicates that Ret signaling can specifically ameliorate Pink1 loss-of-function deficiencies that are relevant to human Parkinson’s disease.

**Results**

**Active Ret rescues Pink1 but not park mutant muscle degeneration**

To study whether Ret can modify Pink1 and park phenotypes, we utilized the Drosophila indirect flight muscles (IFMs) as a model system. Here, Pink1 and park mutants undergo significant muscle degeneration, likely because of the high energy consumption of the IFMs, and display enlarged mitochondria with broken cristae. Late stage pupae display normal muscle morphology, but soon after eclosion, the muscle tissue degenerates (Greene et al., 2003; Clark et al., 2006; Park et al., 2006). In 3- to 5-day-old Pink1 and park mutant animals housed at 18°C, interrupted muscles were found, and one or several of the six muscles displayed degenerated, highly irregular myofibrils with abnormal sarcomere structure, hereafter referred to as “degenerated” (Fig 1I and K) in approximately 65% of the animals as compared to controls, which never displayed this phenotype (Fig 1A, B, E, F, L). To investigate whether Ret signaling could modify muscle degeneration, we utilized the constitutively active version, Ret\textsuperscript{MEN2B}, which has an activating point mutation in the kinase domain (M955T) (Read et al., 2005). In an expression analysis of endogenous Ret by reverse transcriptase PCR (RT-PCR), we detected high levels of Ret mRNA in larvae and pupae, and lower levels in the adult thorax and IFMs (Supplementary Fig S1). To achieve robust overexpression of activated Ret specifically in muscles, we used the UAS-GAL4 system and the Myocyte enhancer factor-2 (Me2) GAL4 driver, which is active in all muscle tissues from the early embryo throughout larval and pupal stages and in the adult fly. Me2>Ret\textsuperscript{MEN2B} overexpression caused lethality at 25°C, but at 18°C, viable progeny eclosed with lower frequency. Surviving transgenic flies displayed mild muscle abnormalities, including deposits of actin dispersed over the muscle tissue, and some abnormally thick and irregular myofibrils (Fig 1C, G, J). A recent RNAi screen for modifiers of muscle development (Schnorrer et al., 2010) identified a large number of lines with a highly reminiscent phenotypic class and designated this “actin blobs”, we therefore refer to this by the same term. When Ret\textsuperscript{MEN2B} was overexpressed in the background of Pink1 mutants, the majority of flies showed significantly improved muscle morphology, with only 12% of flies displaying degenerated myofibrils (Fig 1D and L). The frequency of flies with actin blobs also decreased markedly compared to Ret\textsuperscript{MEN2B} expressing controls, suggesting that Pink1 function may be required for this phenotype. However, in contrast to Pink1 mutants, park mutants overexpressing Ret\textsuperscript{MEN2B} showed no improvement as the frequency of degenerated myofibrils remained unchanged (Fig 1H and L). Expression of the Ret\textsuperscript{MEN2B} protein was examined by Western Blot of thorax homogenates and levels were similar between the Pink1 and park mutants, indicating that differences in transgene expression were not a likely cause of the differential response (Fig 1M). To determine if Ret protein expression or Ret signaling was required for the phenotypic rescue, we overexpressed wild-type (WT) Ret using the same GAL4 driver. We found that Ret\textsuperscript{KT} was unable to modify the phenotype probably because the putative Ret ligand was not present in the IFMs at significant levels at this stage (Supplementary Fig S2). Moreover, the effects of Ret on IFM morphology appeared rather specific, since overexpression of a constitutively active fibroblast growth factor receptor (FGFR), UAS-hFGFR\textsuperscript{KT}, caused a dramatic change in IFM fate (data not shown).

**Rescue of Pink1 mutants is not developmental**

The partial embryonic lethality and appearance of actin blobs by Me2>Ret\textsuperscript{MEN2B} overexpression indicated that high levels of Ret signaling interfered with normal muscle development. Other receptor
Ret signaling rescues Drosophila Pink1 mutants

Figure 1. RetMEN2B overexpression rescues Pink1 but not park mutant muscle degeneration.

A–K Drosophila hemi-thoraces stained with phalloidin at low magnification (upper panels) showing overall indirect flight muscle (IFM) morphology, and at higher magnification (lower panels). High-magnification images of WT sarcomeres (I), sarcomeres with ‘actin blobs’ (J), and degenerated sarcomeres (K). Heterozygous controls (A, E) display normal IFM layout (upper panels), myofibril morphology (lower panels) and sarcomeres (I). Pink1 (B) and park mutants (F) display abnormal morphologies with truncated muscles (yellow arrow heads, upper panels) and disorganized myofibrils (lower panels) with degenerated sarcomere structure (K). Animals overexpressing RetMEN2B (C, G) display normal IFM layout (upper panels), fairly normal myofibril morphology with occasional deposits of mislocated actin filaments, and actin blobs, (red arrow heads, lower panels and J). RetMEN2B overexpression in Pink1 mutants largely rescues the mutant phenotypes, as the majority of animals display normal IFM morphology (D), while park mutants are not rescued (H).

L Percentage of flies with phenotype “wild type” (blue), “actin blobs” (green), “degenerated” (red) or “actin blobs and degenerated” (yellow).

M Western blot analysis of Ret expression in thorax homogenates from w1118 controls, and Pink1, or park mutants overexpressing RetMEN2B, indicating similar levels of Ret overexpression between the two mutant backgrounds. Tissue from three animals per sample. Tubulin was used as a loading control.

N–U Overexpression of UAS-RetMEN2B under control of Mhc-GAL4 and Tub-GAL80ts, pupae were shifted from 18° to 30°C at pupal stage 11, activating expression after muscle formation is completed. Heterozygous controls (N, F) and RetMEN2B late overexpressing animals display normal muscle and myofibril morphologies (N, P, R, S, T). Pink1 (Q) and park mutants (U) display abnormal morphologies with truncated muscles and disorganized myofibrils with degenerated sarcomere structure (lower panels). Late RetMEN2B overexpression in Pink1 mutants (Q) largely rescues the mutant phenotypes, while park mutants (U) are not rescued.

V Percentage of flies with phenotype “wild type” (blue) or “degenerated” (red). Number of animals per genotype as depicted in figure.

Data information: Scale bars: upper panels, 100 µm; lower panels, 10 µm.

Source data are available online for this figure.
tyrosine kinases such as epidermal growth factor receptor (EGFR) and FGFR are known to regulate embryonic myoblast specification via Ras/Erk signaling (Carmena et al., 1998; Halfon et al., 2000), and the insulin receptor controls muscle size (Demontis & Perrimon, 2009). Therefore, it is plausible that active RetMEN2B affects these, or similar developmental processes. To verify that the rescue of the Pnk1 mutants is not a developmental interaction, we utilized the GAL80\(^+\) system which permits transgene expression in a defined time window regulated by temperature. To drive Ret\(^{MEN2B}\) expression, we chose the GAL4 driver, Myosin heavy chain (Mhc) GAL4, which expresses only in differentiated muscles, not in myoblasts, in difference to Mef2-GAL4 and generates higher expression. Unlike Mef2-GAL4, it causes complete lethality when driving Ret\(^{MEN2B}\) from embryonic stages. Flies were crossed at 18°C (non-permissive temperature), after which pupae were shifted to 30°C (permissive temperature) at pharate adult stage P11 \(\pm 3\) h (equivalent of 75 h APF at 25°C) (Flybase FRbv:00005349), a time well after completion of IFM development, but before the onset of apoptotic degeneration in Pnk1 and park mutants (Greene et al., 2003; Clark et al., 2006). Analyses were again performed at 3–5 days post-eclosion. Using this protocol, Pnk1 and park mutants showed degenerated myofibrils with a frequency of approximately 90% and 80% respectively as compared to controls (Fig 1N, O, R, S, V), the higher penetrance being likely due to the increased temperature. Ret\(^{MEN2B}\) overexpressing flies eclosed with Mendelian frequencies and displayed fully normal muscle morphology, without the presence of actin blobs, confirming the hypothesis that the lethality and actin blob phenotypes have developmental origins (Fig 1P, T, V). When Ret\(^{MEN2B}\) was expressed in Pnk1 mutants from this late pupal stage and onwards, it again largely rescued muscle degeneration, indicating that the rescue is not due to a developmental interaction, but a direct protective effect of Ret signaling on degenerating tissue (Fig 1Q and V). Interestingly, park mutants were again not rescued using this expression protocol (Fig 1U and V).

### Ret signaling rescues mitochondrial morphology in flight muscles

One possibility is that Ret\(^{MEN2B}\) inhibits muscle degeneration without directly targeting the primary cause of the Pnk1 phenotype: mitochondrial impairments (Clark et al., 2006). To test this possibility, we analyzed the ultrastructure of mitochondria using transmission electron microscopy. IFMs from control flies showed regular organization of myofibrils and densely packed mitochondria with intact cristae (Fig 2A, E, L, M). Pnk1 and park mutants displayed a heterogeneous population of mitochondria with the majority having significantly enlarged sizes and mild or severe disruption of their cristae structure, when compared to control mitochondria (Fig 2B, F, I–M). Mef2 > Ret\(^{MEN2B}\) overexpression in control flies did not alter normal mitochondria morphology (Fig 2C, G, L, M). However, in Pnk1 mutants, Ret\(^{MEN2B}\) overexpression significantly reduced the fraction of severely impaired mitochondria and increased the fraction of mitochondria with WT-like cristae structure (Fig 2D and L). In contrast, park mutants showed no improvement of structural impairments when Ret\(^{MEN2B}\) was overexpressed (Fig 2H and M).

**Figure 2.** Ret\(^{MEN2B}\) rescues mitochondrial cristae structure of Pnk1 mutants.

A–K Transmission electron microscopy images of indirect flight muscles. Heterozygous controls (A, F) and animals overexpressing Ret\(^{MEN2B}\) (B, I) display normal mitochondria of similar size with highly dense cristae structure. Pnk1 and park mutants have enlarged mitochondria with broken cristae (C, G). Phenotype can vary from mild to severe. High-power images of mitochondria are shown for the categories wild type (I), mild (J), severe phenotype (K). Ret\(^{MEN2B}\) overexpression partially restores mitochondrial size and cristae structure in Pnk1 (D), but not park mutants (H). Scale bar, 2 \(\mu\)m.

L, M Percentages of mitochondria of the indicated categories, 500–800 mitochondria per animal, averages of 6 animals per genotype.
These results demonstrate that RetMEN2B can rescue mitochondrial impairments of pink1 but not park mutants, suggesting that the mitochondrial deficiencies of the two mutant strains have partially different origins.

Ret rescues mitochondrial morphology in dopaminergic neurons

To address whether RetMEN2B also rescues the morphology of mitochondria in dopaminergic neurons, we overexpressed RetMEN2B using TH-GAL4 together with the mitochondrial marker mitoGFP (Pilling et al., 2006). Pink1 and park mutants displayed severely enlarged mitochondria as compared to controls (Fig 3A, B, E, F, I, J). RetMEN2B overexpression in a control background did not significantly alter the normal mitochondrial background (Fig 3C, G, I, J). However, when overexpressed in Pink1 mutants, mitochondrial size was significantly rescued (Fig 3D and I). Quantification of mitochondrial volumes revealed that in the presence of RetMEN2B the abundance of normal mitochondria was increased, while the fraction of enlarged mitochondria decreased to levels similar to those of control flies. Merely, the 4% largest mitochondria were not rescued. In line with the analysis of mitochondria in muscle, mitochondrial morphology in neurons of park mutants was not rescued by RetMEN2B (Fig 3H and J).

GDNF/Ret signaling rescues mitochondrial defects in mammalian cells

In order to assess whether signaling from endogenous Ret can also rescue mitochondrial impairments caused by loss of PINK1 function, we used the human dopaminergic neuroblastoma cell line SH-SY5Y, which expresses endogenous Ret. Acute knock-down of PINK1 in this cell line was previously shown to cause fragmentation of the mitochondrial network (Lutz et al., 2009) (Fig 4A, B, D). Stimulation of Ret by GDNF and soluble GFRA1 rescued mitochondrial fragmentation, demonstrating that endogenous mammalian Ret can rescue mitochondrial impairments (Fig 4C and D). A semi-quantitative RT-PCR analysis of PINK1 mRNA controlled that GDNF/GFRA1 stimulation did not upregulate PINK1 levels (Fig 4E).

Ret rescues mitochondrial morphology independently of Parkin-induced mitophagy

Although the data so far suggested that Ret rescues Pink1-deficient mitochondria independently of Parkin, we cannot exclude that Ret signaling activates Parkin translocation to mitochondria, thus promoting their clearance through mitophagy. To test this hypothesis, we treated SH-SY5Y cells overexpressing Parkin with carbonyl cyanide m-chlorophenyl hydrazone (CCCP) to depolarize mitochondria. CCCP treatment induced recruitment of Parkin to mitochondria (detected 2 h after adding CCCP) followed by the removal of depolarized mitochondria in about 50% of Parkin-expressing SH-SY5Y cells (monitored 24 h later) (Fig 4G and N). Parkin-induced mitophagy required the presence of PINK1, as described previously (Geisler et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010), but was not impaired in cells silenced for Ret expression (Fig 4H, I, J, N, O). Moreover, the overexpression of constitutively active RetMEN2A did not induce Parkin translocation or mitophagy under any condition, including PINK1 knock-down with or without Parkin overexpression (Fig 4K, L, M, N). Similar results were obtained when GDNF and soluble GFRA1 was used to activate signaling via endogenous Ret (Fig 4N). Furthermore, GDNF/GFRA1 treatment also rescued mitochondrial fragmentation induced by PINK1 silencing HeLa cells, a cell type which does not express endogenous Parkin (Denison et al., 2003; Pawlyk et al., 2003), further indicating that Ret signaling rescues PINK1 loss-of-function phenotypes independently of Parkin (Supplementary Fig S3).

Ret signaling rescues impaired bioenergetics of Pink1-deficient cells

It has been reported previously that PINK1 deficiency impairs mitochondrial respiration (Gautier et al., 2008, 2012; Gandhi et al., 2009; Lutz et al., 2009; Morais et al., 2009). We therefore investigated whether activation of Ret signaling via GDNF/GFRA1 treatment could influence this phenotype. We measured mitochondrial function under basal and stress conditions in SH-SY5Y cells silenced for PINK1 expression by using an extracellular oxygen flux analyzer. In comparison to control siRNA-treated cells, PINK1-deficient cells were characterized by a decreased oxygen consumption rate even under basal conditions (Fig 5A). Moreover, the spare respiratory capacity (difference between maximal and basal respiration) was markedly reduced, indicating that the ability of PINK1-deficient cells to respond to an increased energy demand under stress conditions is severely impaired. Remarkably, GDNF/GFRA1 treatment fully rescued basal respiration and increased maximal respiration in PINK1-deficient cells, indicating that the beneficial effect of increased Ret signaling in PINK1-deficient models can be explained by influencing the bioenergetic capacity of mitochondria rather than mitophagy.

Complex I deficiency of Pink1 mutants rescued by Ret signaling

To investigate whether Ret signaling also rescued mitochondrial functionality in Drosophila, we measured ATP content of thoracic homogenates. As previously shown (Clark et al., 2006; Park et al., 2006; Yang et al., 2006; Vos et al., 2012), Pink1 and park mutants showed reduced ATP content in the thorax to approximately 40% of controls, including flies carrying the Mez2-GAL4 driver (Fig 5B and C). Mez2 > RetMEN2B overexpression in control flies caused a slight reduction in ATP as compared to controls, possibly as a result of the mild muscle phenotype. In line with the rescue of myofibril and mitochondrial structures, RetMEN2B overexpression largely rescued ATP levels in Pink1 mutants, while ATP levels of park mutants did not significantly improve (Fig 5B and C). To unravel the underlying mechanism of the improved mitochondrial respiration, we turned our attention to complex I of the ETC. Recent reports have found that Pink1, in contrast to park mutants had decreased activity of the ETC, and specifically of complex I function (Morais et al., 2009; Vilain et al., 2012). For these reasons, we measured complex I activity in RetMEN2B overexpressing Pink1 mutants, by monitoring rotenone-sensitive NADH oxidation by spectrophotometry, normalized to the activity of citrate synthase. As previously observed, Pink1 mutants displayed markedly reduced complex I activity (Fig 5D). Interestingly, RetMEN2B significantly increased complex I activity to levels similar to controls (Fig 5D). In accordance with previously reported data, park mutants showed no decreased complex I activity.
Figure 3. Rescue of Pink1 mutant dopamine neuron mitochondria by RetMEN2B

A–H Confocal maximum projections (left panels) and isosurface renderings (right panels) of dopamine neuron mitochondria in the PPL1 cluster of dopaminergic neurons, visualized by mitoGFP and immunostainings against GFP and TH. Genotypes: All flies contain TH-GAL4 and UAS-mitoGFP and Pink1, park mutant alleles, as well as UAS-RetMEN2B as indicated. Isosurface renderings are color-coded according to volume from 0 to 3 μm^3. RetMEN2B-overexpressing control animals (C, G) display normal mitochondrial morphology as compared to non-transgenic controls (A, E). Pink1 mutants (B) and park mutants (F) display severely enlarged mitochondria, and RetMEN2B partially rescues mitochondrial size in Pink1 mutants (D), but not in park mutants (H). Scale bar, 5 μm.

I, J Mitochondrial volume distributions of (A–D) and (E–H) in categories as indicated. Due to differences in staining and imaging conditions, data between the Pink1 and park datasets cannot be directly compared. n = 8–20 animals per genotype.
activity as compared to controls (Fig 5E). Depleting the complex I subunit (CG11455) from muscles by RNAi abrogated most complex I activity (Fig 5F), and RetMEN2B overexpression was not able to rescue this defect (Fig 5F), suggesting that Ret signaling does not activate alternative means of NADH oxidation as previously shown for the yeast protein Ndi1 (Vilain et al, 2012). The mechanism by which Pink1 controls complex I function is still unknown. Drosophila complex I contains 48 subunits, six of which are mitochondrionally encoded, the rest being nuclear. The supply of commercially available antibodies for Drosophila complex I is limited to the subunit NDUF3, which has recently been shown to be reduced in Pink1 mutants (Liu et al, 2011). By Western blot, we could confirm this reduction of NDUF3, but did not observe an upregulation by RetMEN2B (Supplementary Fig S4A and B). We performed a semiquantitative RT-PCR screen of other complex I subunits in Pink1 mutants compared to RetMEN2B-overexpressing Pink1 mutants. Of 45 subunits analyzed, most were unchanged, but the transcript of CG6485, orthologous to human NDUFV2, was moderately elevated in RetMEN2B-overexpressing Pink1 mutants (Supplementary Fig S4C). Interestingly, when compared to controls, CG6485 mRNA was reduced by 46% in Pink1 mutants, and significantly increased to 117% of controls by RetMEN2B overexpression (Fig 5G and H). This effect may at least in part be responsible for the Ret-mediated rescue of Pink1 deficiency.

Discussion

The receptor tyrosine kinase Ret is already known to be required for long-term survival of nigral dopamine neurons in mice, and stimulation with its ligand GDNF protects dopamine neurons from cell death in a variety of toxin-based rodent and primate models of PD. In the present work, we found that a signaling-active version of the Drosophila homolog of Ret suppresses degeneration of muscle tissue and mitochondrial abnormalities in Pink1 mutants. Interestingly, park mutants were not rescued. In human SH-SY5Y cells, stimulation of endogenous Ret by GDNF rescued both morphological and bioenergetic defects of mitochondria in PINK1-depleted cells. Pink1 and Parkin were previously shown to interact genetically in Drosophila in what was proposed to be a linear pathway, and a significant body of work has described how Pink1 and Parkin function to initiate mitophagy of impaired mitochondria, and arrest of mitochondrial trafficking. However, in our cell culture model, Ret signaling did not induce mitophagy or Parkin recruitment, arguing that Ret rescues PINK1 deficits independently of Parkin. A recent study demonstrated that Pink1 mutants in contrast to park mutants have decreased function of complex I of the electron transport chain, suggesting that Pink1 is required for maintaining efficient complex I enzymatic activity and that this function is upstream of mitochondrial remodeling. We found that Ret rescued both the impairment of complex I activity, and partially the mitochondrial morphology in Pink1 mutants, suggesting that complex I is a target of Ret signaling. Previous studies of complex I inhibition or genetic depletion have shown mild morphological impairments in Drosophila muscle, contrary to the stronger phenotype of Pink1 mutants. Therefore, it was somewhat unexpected that restoring complex I activity would be sufficient to rescue also morphological defects. One interpretation is that the Pink1 mutant morphological phenotype is more severe due to a synergistic effect of deficits in remodeling/mitophagy and complex I activity, which in this study was partially rescued. Another possibility is that Ret signaling not only targets complex I, but also morphology in a Parkin-independent manner.

Extrapolated to mammalian models, our results suggest a novel mechanism by which the GDNF family of neurotrophic factors may promote survival of dopamine neurons in PD. Several of the mammalian models where the neuroprotective effects of GDNF treatment were initially discovered, were in fact models of mitochondrial dysfunction, either directly via complex I inhibition by MPTP treatment (Tomac et al, 1995; Gash et al, 1996), or the more general ROS toxicity of 6-OHDA (Kearns & Gash, 1995; Sauer et al, 1995), which also includes complex I impairments (Glinka et al, 1997). In light of our findings, it would be interesting to investigate whether or not GDNF improves complex I activity in these model systems. GDNF has been tested in models of alpha-synuclein overexpression, a pathology that is not known to cause complex I deficiency, but did not show any neuroprotective effects, fitting with our hypothesis (Lo Bianco et al, 2004; Decressac et al, 2011).

The current findings support recent evidence showing that Pink1 has an important function related to complex I activity, which is independent of its function in recruiting Parkin to the outer mitochondrial membrane upon loss of membrane potential. This model is consistent with a partial rescue of Pink1 deficiencies, e.g. by either overexpressing Parkin or the yeast complex I equivalent NADH dehydrogenase, or, in the current work, RetMEN2B (Clark et al, 2006; Park et al, 2006; Yang et al, 2006; Vilain et al, 2012). In addition, our findings are consistent with a recent study showing that Pink1-deficient flies but not Parkin-deficient flies can be rescued by TRAP1, which also seems to have beneficial effects on complex I activity (Zhang et al, 2013).

The pathways by which Ret signaling targets complex I and rescues Pink1 mutants requires further investigation. Also, the mechanism by which Pink1 regulates complex I remains elusive, it may regulate for example gene expression, phosphorylation status or assembly (Salvi et al, 2005; Pagliarini & Dixon, 2006) (Fig 6). Our gene expression analysis showed that most subunits are unchanged by RetMEN2B, but interestingly one subunit was moderately downregulated in Pink1 mutants and upregulated by RetMEN2B, which may improve function. However, we do not exclude the possibility that Ret signaling targets complex I, and perhaps other metabolic components, by different means.

Brain-derived neurotrophic factor (BDNF) protects mouse cortical neurons against drug-induced excitotoxicity, an effect that was blocked by the complex I inhibitor Rotenone and a MEK1/2 inhibitor, suggesting that BDNF signaling via the Ras/Erk pathway can regulate complex I function (Markham et al, 2012). The signaling properties and functions of Drosophila Ret are not characterized in great detail, but it is structurally homologous to mammalian Ret and can, to some extent, activate the same signaling pathways (Abrescia et al, 2005). Mammalian Ret on the other hand, has been extensively characterized and is known to activate a number of downstream signaling pathways including Ras/Erk, phosphoinositol-3 kinase (PI3K)/Akt, phospholipase C-gamma (PLCγ), Janus kinase (JAK)/STAT, and ERK5, several of which have pro-survival effects, most notably the PI3K/Akt pathway (Sariola & Saarimäki, 2003; Pascual et al, 2011). Recent studies of Pink1 and park mutant Drosophila have indicated that PI3K/Akt signaling or components downstream of this pathway rather exacerbates Pink1 and park
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Ret signaling can rescue phenotypes of Pnk2 mutants. In summary, this work shows that Ret signaling can rescue phenotypes of Pnk2 mutants. Pink1 and Ret regulate complex I activity, and whether this finding is transferrable to mammalian models. Additional studies are required to elucidate the details by which GDNF treatment may provide a new therapeutic strategy.

Immunostaining for TOM20 (white); DAPI (blue) indicates nuclei. Cells silenced for PINK1 expression display increased mitochondrial fragmentation (B).

Ret signaling does not activate mitophagy in control or PINK1-silenced cells (K, L), and does not modulate Parkin translocation or mitophagy in Parkin-overexpressing cells (M).

Immunoblot analysis using SDS-PAGE and blotted according to standard procedures. Protein concentration was determined using the BCA procedure.

Additional studies are required to elucidate the details by which GDNF treatment may provide a new therapeutic strategy.
mutant phenotypes (Tain et al, 2009; Liu & Lu, 2010), making it an unlikely candidate for rescue.

Additional studies are required to elucidate the details by which Pink1 and Ret regulate complex I activity, and whether this finding is transferrable to mammalian models. In summary, this work shows that Ret signaling can rescue phenotypes of Pink1 mutants by restoring mitochondrial respiration and specifically complex I function, and thereby suggests a potential novel mechanism underlying GDNF-mediated protection in mammalian PD models. In the future, screening of PD patients for complex I deficiencies and subjecting specifically those individuals to GDNF treatment may provide a new therapeutic strategy.

Materials and Methods

Fly strains and procedures

Mef2-GAL4;UAS-Res<sup>EN2A</sup> is lethal at 25°C, therefore all crosses were performed at 18°C. All analyses were performed with 2- to 5-day-old flies. In experiments with Mhc-GAL4,Tub-GAL80<sup>UAS</sup>, pupae were shifted from 18 to 30°C at pharate adult stages P11-P12 (Flybase FBdr:00005349) and analyzed at 3–4 days post eclosion. park<sup>C5</sup> [Greene et al, 2003] was provided by Leo Pallanck, park<sup>T</sup> (Cha et al, 2005) and Pink1<sup>IP</sup> (Park et al, 2006) were provided by Jongjeong Chung, Pink1<sup>TC1</sup>:Mef2-GAL4 (Tain et al, 2009) was provided by Alex Whitworth, UAS-Res<sup>EN2A</sup> [Read et al, 2005] was provided by Ross Cagan, TH-GAL4 (Friggi-Grelin et al, 2003), was provided by Hirozu Tanimoto, Mef2-GAL4 (Rangarayakulu et al, 1996), Tub-GAL80<sup>UAS</sup> [McGuire et al, 2003], and UAS-mitoGFP (Pilling et al, 2006) were obtained from the Bloomington stock center, UAS-CG11455<sup>RNAi</sup> (12838) was obtained from Vienna Drosophila RNAi Center. “+” controls express Pink1 and park WT alleles from w<sup>1118</sup> (Bloomington stock #5905). In all histology experiments, flies were genotyped by PCR to assure correct genotypes and control for X-chromosome non-disjunction, for list of primers see Supplementary information.

Myosin heavy chain – GAL4 flies

A 2.5 kb Mhc enhancer was amplified from genomic DNA using primers FS124 (5′-tcagagtacgccccagctctgagaaatgatgatctgC-3′) and FS125 (5′-tcaggcgcggattctgccgcttaaatatttcgctgac-3′) and cloned with Asp718/NotI into a GALA-containing Casper-based P-element transformation vector. Transgenic flies were generated using standard procedures. In contrast to the formerly published GAL4 line (Schuster et al, 1996), which shows a rather weak activity in embryos, larvae and adults, this new Mhc-GAL4 line is very strong and very specifically expressed in differentiated muscles from embryonic stages onwards (FS, unpublished).

Histology, transmission electron microscopy and analysis of mitochondrial morphology

Hemi-thoraces were prepared as described previously (Schnorrer et al, 2010), stained with Phalloidin-Alexa Fluor-568 (Molecular Probes), and single plane images were acquired on an Olympus FV1000 confocal scanning microscope. For transmission electron microscopy, hemi-thoraces were fixed in 2.5% Glutaraldehyde, from which semithin sections were prepared and stained with toluidine blue, subsequently ultrathin serial sections were prepared using a Leica EM UC6 Ultramicrotome. Images at 5,000× magnification were acquired using a JEOL JEM-1230 transmission electron microscope at 80 kV, equipped with a Gatan Orius SC1000 digital Camera. Six TEM Images per animal were acquired from randomly selected regions of the indirect flight muscles. All mitochondria in these images (500–800 per animal) were grouped into three categories, based on the integrity of the cristae structure, with genotypes blinded to the experimenter, using the ImageJ software (NIH). Whole mount immunostaining of fly brains was performed according to standard procedures. The following antibodies were used: rabbit anti-tyrosine hydroxylase (ab)152, Millipore, 1:200) and chick anti-GFP (Abcam ab13970; 1:500). The PPL1 cluster was imaged using an Olympus FV1000 confocal microscope with a 60× NA 1.3 objective with 4× zoom. 52 z-sections of 0.3 μm spacing were acquired and deconvolved by the nearest neighbor algorithm using Meta Morph 7.5 (Molecular Devices). A volume corresponding to 26 × 26 × 15 μm was cropped, subjected to linear rescaling and analyzed in Imaris x64 6.4.2 (Bitplane Scientific Software). Mitochondrial volume was measured by 3D isosurface rendering using a fixed threshold.

Immunoblot analysis

Thoraces from three animals per sample were homogenized in Triton-lysis buffer, protein concentration was determined using the BCA method (BioRad), equal amounts of protein were separated using SDS-PAGE and blotted according to standard procedures.
Ret signaling rescues Drosophila Pink2 mutants

**Figure 5.** Ret signaling rescues mitochondrial respiration and complex I function in PINK1-deficient cells.

A. Oxygen consumption rate in SH-SY5Y cells determined by an extracellular flux analyzer: 1. Injection of the F1F0-ATPase inhibitor oligomycin; 2. injection of the uncoupler FCCP; 3. injection of the complex I inhibitor rotenone and the and complex III inhibitor antimycin A. Under basal conditions, as well as FCCP-evoked respiration of PINK1 knockdown cells with GDNF/GFRα1-α2 and complex III inhibitor antimycin A. Under basal conditions, as well as FCCP-evoked respiration of PINK1 knockdown cells with GDNF/GFRα1-α2.

B. Relative ATP amount (%)

C. Relative ATP amount (%)

D. Activity of Complex I (rotenone sensitive), normalized to citrate synthase activity, percentage of heterozygous controls. Pink1 and park1 mutants have reduced ATP amounts. Pink1 overexpression partially rescues ATP deficiency in Pink1 (B), but not park1 mutants (C). Averages of 6–12 animals per genotype.

E. Relative activity of Complex I (%)

F. Averaged mitochondrial morphology assay of parkin to mitochondria (after 2 h CCCP) and removal of 3-chlorophenylhydrazone (CCCP, Sigma) for 2 or 24 h. Recruitment of PINK1 to mitochondria was determined as the number of co-localization events. Pink1 mutants have reduced complex I activity, which is rescued by RetMEN2B overexpression.

G. Semi-quantitative RT-PCR analysis of complex I subunit CG6485 indicates upregulation by RetMEN2B overexpression in Pink1 mutants. GAPDH was used as a loading control.

H. Quantification of CG6485 mRNA normalized to GAPDH, averages of 3 experiments, RNA from 3 thoraces per sample.
Antibodies used were: panRet (provided by C. Ibanez) and alpha-Tubulin (clone DM1A, Sigma).

**Cell culture, treatments and RNA Interference**

SH-SY5Y (DSMZ number ACC 209) cells were cultivated as described previously (Henn et al., 2005; Schlehe et al., 2008). For acute stimulation of Ret, cells were incubated for 3–4 h with recombinant hGDNF (Shenandoah Biotechnology Inc.) and hGFRα-1 (R&D Systems) at a final concentration of 100 ng/mL. PINK1 and Ret gene silencing was performed with the following stealth siRNA oligos (Invitrogen) using Lipofectamine RNAiMAX (Invitrogen): PINK1 human HSS127945 (SH-SY5Y), Ret human HSS109181.

**Assessment of mitochondrial morphology**

SH-SY5Y: Cells grown on 15-mm glass coverslips were fixed with 3.7% PFA in PBS for 10 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and blocked with 5% BSA in PBS at room temperature. Fixed cells were sequentially incubated with primary antibody diluted in blocking solution (TOM20 pAb, overnight at 4°C) and secondary antibody diluted in blocking buffer (goat anti rabbit Alexa555- conjugated, 2 h at room temperature). Nuclei were counterstained with DAPI. Coverslips were mounted on glass slides and images were acquired with a Zeiss LSM710 confocal microscope equipped with a 63× oil objective (NA 1.4). Cells displaying an intact network of tubular mitochondria were classified as tubular. When this network was disrupted and mitochondria appeared either globular or rod-like they were classified as fragmented. The mitochondrial morphology of the cells was determined in a blinded manner. Quantifications were based on 150 cells from at least 3 independent experiments.

**Assessment of mitophagy**

SH-SY5Y cells were plated on glass coverslips and reversely transfected with siRNA and 24 h later with the indicated DNA plasmid. Human GDNF and GFRalpha were added to the cells 24 h after siRNA transfection and 3 h before CCCP treatment. The next day, cells were treated with 10 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma) for 2 or 24 h. Recruitment of parkin to mitochondria (after 2 h CCCP) and removal of mitochondria (after 24 h CCCP) was detected by indirect immunofluorescence using a monoclonal anti-Parkin antibody (PRK8, Santa Cruz Biotechnology) and a polyclonal antibody against HSP60 (Santa Cruz Biotechnology). Nuclei were stained by DAPI. Cells

**Figure 6. Model of Pink1 and Ret functions.**

Our results suggest a dual role for Pink1. One in recruiting Parkin to the mitochondria and initiating mitochondrial clearance or regulating mitochondrial trafficking, a second in regulating the activity of complex I via an as yet unclear pathway. This could be mediated, for example, via phosphorylation of the protein complex or by regulating expression of complex I components. Loss of Pink1 decreases complex I activity and respiratory function. Ret rescues specifically Pink2 mutants, by restoring complex I activity, respiration and ATP production, in part by upregulating the mRNA levels of the complex I subunit NDUFV2 (CG6485).
were analysed by fluorescence microscopy using a Leica DMRB microscope and confocal images were taken using a Zeiss LSM710 confocal microscope equipped with a 63× oil objective (NA 1.4). Quantifications are based on three independent experiments. At least 1,500 cells were analysed for each condition.

**Real-time RT-PCR, cultured cells**

Knock-down efficiency of PINK1 and Ret was evaluated by real-time RT-PCR with the 7500 Fast Real Time System (Applied Biosystems) as previously described (Bouman et al., 2011). Statistical analysis of RT-PCR data is based on at least four independent experiments with triplicate samples. For list of primers, see Supplementary information.

**Measurement of mitochondrial oxygen consumption**

The oxygen consumption rate was determined using a Seahorse XF 96 analyzer (Seahorse Biosciences). SH-SYSY cells were reversely transfected and plated in a XF 96 cell culture microplate. The next day, fresh medium containing human GDNF/GFRα-1 was added to the wells where indicated. The cells were incubated with low-glucose (1 mM) medium overnight and the sensor cartridge was hydrated overnight according to the manufacturers’ instructions. Measurements were performed 48 h after transfection. The measured values were normalized to protein levels. PINK1 knockdown did not induce apoptosis under these conditions. The cells were washed using the XF Prep Station three times with Seahorse Medium containing 10 mM galactose and 1 mM pyruvate. Mitochondrial function was analyzed using the XF Cell Mito Stress Test Kit (Seahorse Biosciences) and all measurements were carried out at 37°C. The following drugs were diluted in Seahorse Medium and loaded on the sensor cartridge: oligomycin (injection port A), carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP; injection port B), rotenone and antimycin A (both injection port C). The drugs were diluted in Seahorse Medium and loaded on the sensor cartridge. Measured values were normalized to protein levels.

**ATP measurement**

Measurements of thoracic ATP were performed using a luciferase assay as described previously (Park et al., 2006) with some modifications. Briefly, single thoraces from 3-day-old flies with heads and wings removed were homogenized in 50 μl of extraction buffer (100 mM Tris-HCl, 4 mM EDTA pH 7.8) with 6 M Guanidine-HCl using a teflon-on-glass dounce homogenizer. The lysate was boiled for 3 min and cleared by centrifugation at 20,000 g for 1 min. The samples were diluted 1:100 in extraction buffer before analyzing using the ATP determination kit (Invitrogen), according to the manufacturer’s instructions. Values were normalized to total protein content, measured by absorbance at 280 nm using a NanoDrop spectrophotometer. All measurements were performed in triplicate.

**Enzymatic measurements**

Activity of complex I (NADH:ubiquinone oxidoreductase) was assessed by monitoring the oxidation of NADH as previously described (Fischer et al., 1986). Briefly, thoraces from 20 animals were homogenized in 250 mM sucrose, 10 mM Tris pH 7.4, 0.15 mM MgCl2, after which mitochondria were isolated as described previously (Walker et al., 2006). Enzymatic activity of complex I was assessed by NADH oxidation, monitored at A340 nm as described (Bugiani et al., 2004), and rotenone insensitive activity was subtracted. The activity of complex I was normalized to Citrate Synthase activity, which was measured indirectly by AcCoA-SH formation, as described (Ferguson & Williams, 1966).

**RT-PCR, Drosophila complex I subunits**

Thoraces were dissected and snap-frozen, homogenized in RLT buffer (Qiagen) using a rotor-stator homogenizer. Total RNA was prepared using the RNeasy mini kit according to instructions. Samples were treated with DNase1 on-column for 15 min (RNase-free DNase set, Qiagen). RT-PCR analysis was performed using the OneStep RT-PCR kit (Qiagen) using 20 ng of template RNA and 35–40 cycles of PCR amplification depending on signal strength of the primer pair. Primers were designed using the primerBLAST tool (NCBI), and when possible exon-junction spanning primers were used, for list of primers, see Supplementary information. As some of the analyzed transcripts are single-exon, control reactions omitting the reverse transcriptase amplification step were performed to assure that samples were free of contaminating genomic DNA, despite DNase1 treatment.

**Statistical analysis**

Data represent mean ± SEM. Statistical analysis was carried out using analysis of variance (ANOVA) or Student’s t-test; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

**Supplementary information** for this article is available online: http://embj.embopress.org

**Acknowledgements**

We would like to thank Marianne Braun and Ursula Weber for transmission electron microscopy and Pilar Alcalá for molecular biology assistance, the Bloomington stock center for fly strains, Liviu Aron for discussions at early stages of the project and Louise Cañados for critically reading the manuscript. This work was in part supported by the Max-Planck Society, and grants from the German Research Foundation (DFG, the European Union (MOLPARK) and the European Research Council (TOPAC).

**Author contributions**

PK designed, performed and analyzed the majority of the experiments. CS and FS contributed to the design of the fly genetics and analysis of muscle morphology, and FS generated the Mhc-GAL4 line. EM and AKM-R designed, performed and analyzed the SH-SYSY experiments. KFW supervised the cell culture work and contributed to the analysis of the fly data. RK supervised the project, designed experiments and co-wrote the manuscript with PK.

**Conflict of interest**

The authors declare that they have no conflict of interest.
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