MCAD-MEDIATED INTERCELLULAR INTERACTIONS ACTIVATE SATELLITE CELL DIVISION

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SUMMARY

The adult stem cells of the muscle and their committed myogenic precursors, commonly referred to as the satellite cell population, are involved in both muscle growth after birth and regeneration after damage. It has been previously proposed that, under these circumstances, satellite cells first become activated, divide and differentiate, and only later fuse to the existing myofiber through M-cadherin-mediated intercellular interactions. Our data shows that satellite cells fuse with the myofiber concomitantly to cell division, and only when the daughter cells’ nuclei are inside the myofiber, do they complete the process of differentiation. In here we demonstrate that M-cadherin plays an important role in cell-to-cell recognition and fusion, and that is critical for cell division activation. Treatment of satellite cells with M-cadherin in vitro stimulates cell division while addition of anti M-cadherin antibodies reduces the cell division rate. Our results suggest an alternative model for the contribution of satellite cells to muscle development, which might be useful to understand muscle regeneration, as well as muscle-related dystrophies.
INTRODUCTION

The muscle is the one of the few tissues composed of cells with various nuclei. Based on transmitted electron microscope observations, initially it was postulated that endoreplication and amitotic divisions model syncytium formation during early muscle development (Boudjelida and Muntz, 1987). Later, several studies demonstrated the inability of differentiated muscle cells to proliferate and that post-mitotic mononuclear myocytes fuse among themselves, giving rise to the syncitium (Hamilton, 1969; Hilfer et al., 1973; Muntz, 1975; Goldspink, 1979; Kielbowna and Daczewska, 2005). This primary musculature models the type, form, and location of the fibers. Subsequently, satellite cells (Mauro, 1961) contribute to increase the diameter and length of the existing fibers, as they are the main source of myogenic cells after birth and during regeneration (Snow et al., 1978; Cusella-De Angelis et al., 1994; Edom-Vovard et al., 1999; Schmalbruch and Lewis, 2000; Seale et al., 2000; Chanoine et al., 2003; Gargioli and Slack, 2004; Spalding et al., 2005; Chen et al., 2006; Mochii et al., 2007; Tseng and Levin, 2008, Cavaco et al., 2012). It was initially described (Moss and Leblond 1971) and widely assumed (Grounds et al. 2002; Chargé and Rudnicki, 2004) that satellite cells first become activated, divide and differentiate and only later do they fuse to the existing myofibers.

The physiology of satellite cells is tightly controlled by a complex network of transcription factors. Pax7 is the most frequently used marker to label satellite cells both in quiescent and activated states (Seale et al., 2000; Zammit et al., 2006). This transcription factor modulates the activation of satellite cells by controlling the induction of specific myogenic regulatory factors (MRFs) (Sassoon, 1993; Cornelison and Wold, 1997; Nicolas et al., 1998; McKinnell et al., 2008). Myf5 and MyoD are the first transcription factors expressed in committed myogenic progenitors (Davis et al., 1987; Hopwood et al., 1989, Kuang et al 2007), while myogenin and MRF4 are the last genes to be induced during the differentiation of the satellite cells to myoblasts, just before specific muscle proteins, such as myosin, become expressed (Rhodes and Konieczny, 1989; Wright et al., 1989; Jennings, 1992; Cooper et al., 1999).

Although initially described as a homogenous population, nowadays it is widely accepted that satellite cells (Pax7+) are a heterogeneous population composed of adult stem cells (Pax7+/Myf5-) and committed myogenic progenitors (Pax7+/Myf5+) that
coexist at different levels of differentiation. Pax7 positive cells can divide symmetrically, giving rise to identical daughter cells, or asymmetrically, giving rise to different daughter cells (Conboy and Rando, 2002, Shinin et al., 2006; Kuang et al., 2007). Moreover, satellite cell coexist in the same anatomical location together with satellite-cell-derived myoblasts (Pax7-/MyoD+/myogenin+) (Zammit et al., 2004; Kuang et al., 2007; Zammit, 2008, Day et al., 2009). Although myogenin is expressed before committed myoblasts fuse with myofibers, a minor percentage of this population still retains proliferative capacity (Andres et Walsh, 1996; Zammit et al., 2004). In here both populations, satellite cells and derived myoblasts, are referred as to myogenic cells.

The transcription factor Mef2 (Myocite enhancer factor-2) has been described as a cofactor of the MRFs, driving myoblasts differentiation together with MyoD and myogenin (Wong et al., 1994; Molkentin et al., 1995; Molkentin and Olson, 1996; Black and Olson, 1998). Desmin (a muscle specific intermediate filament protein) and p21 (a regulator induced in post-mitotic myoblasts bringing about withdrawal from the cell cycle) are other markers of terminally differentiated myocytes (Lazarides and Hubbard, 1976; Guo et al., 1995; Halevy et al., 1995).

Another marker found in both quiescent and activated satellite cells is M-cadherin (Mcad), a calcium-dependent homophilic cell-to-cell adhesion molecule (Donalies et al., 1991; Irintchev et al., 1994; Rose et al., 1994, Cooper et al., 1999). The intercellular interaction between two sets of Mcad is necessary for the fusion between embryonic myoblasts (Zeschnigk et al., 1995; Kaufman et al., 1999) as well as for the fusion of derived myoblasts with the existing myofiber (Wernig et al., 2004). However, mice with a null mutation in Mcad show no muscle defects, probably due to compensation by the other cadherins (Hollnagel et al. 2002). It has been described that the cytoplasmatic tails of Mcad interacts with Beta-catenin (Bcat), and that both proteins are essential for the differentiation of the fusing myoblasts (Kuch et al., 1997; Wróbel et al., 2007). Moreover, different studies suggest a role for Bcat in controlling the transcription of targets genes in proliferating satellite cells, in addition to its role in myoblast fusion. It has been described that in non proliferating satellite cells, Bcat is located in a membrane associated-region, whereas in proliferating cells it translocates to the cytoplasm and the nucleus where it functions as a coactivator for the Wnt canonical signaling pathway (Otto et al., 2008; Zammit et al., 2008).
The muscle niche plays a critical role in the physiology of satellite cells (Velleman, 2002; Collins et al., 2005; Kuang et al., 2008). The main components of the satellite cells niche are the associated microvasculature, extracellular matrix and muscle fibers. Proteins like dystrophin, a key component of a multiprotein complex located in the plasmatic membrane of the myofiber, play a prominent role in the crosstalk between the extracellular matrix and the muscle fiber. In addition dystrophin provides tensile strength to the muscle fiber and acts as a scaffold for several signaling molecules (Hoffmaen et al., 1987; Peng and Chen, 1992; Rando, 2001).

As opposed to the prevailing model, our study shows that activated satellite cells fuse with the myofibers while they are still in mitosis. The analysis of different muscle samples by electron microscopy revealed the presence of satellite cells with condensed chromosomes and without a plasma membrane in the region of contact with the myofiber. Further histochemical analysis showed activated satellite cells with an intimate contact zone with the myofiber where dystrophin and the plasma membrane were absent. These observations prompted us to perform a thorough analysis of satellite cell division in neonatal mice musculature, due the high ratio of cell divisions and the possibility to study such mechanisms in vivo and in vitro. In addition to the electron and confocal microscopy study, we confirmed that Mcad-mediated intercellular interactions are critical to stimulate satellite cell division in vitro. Furthermore, we show that the inhibition of cell fusion in vivo reduces the cycling of satellite cells. Our results suggest that Mcad-facilitated intercellular interactions play a fundamental role in the proliferation of muscle satellite cells both in vitro and in vivo.
RESULTS

Muscle fiber dystrophin is undetectable in areas close to dividing satellite cells in different models

We analyzed, by immunohistochemistry, muscle samples obtained during normal growth (Fig 1A) and regeneration after tail amputation in Xenopus tadpoles (Fig 1B), as well as normal growth (P7 mice Fig 1C, P30 mice Fig 1D) and regeneration after cardiotoxin induced injury in mice (3 months old mice, Fig 1E). The presence of satellite cells was determined using the marker Pax7 and activated satellite cells using markers of cell division, PCNA (proliferating cell nuclear antigen, a marker for entrance into the S phase and maximally expressed in that phase of the cell cycle) (Johnson et Allen, 1993) or PHH3 (phosphohistone H3, a marker for entrance into the M phase) (Gurley et al., 1978). In order to clearly identify the myofiber we performed colocalization with dystrophin, which is located at the muscle fiber membrane.

In all cases we observed that dividing satellite cells were always located in close physical proximity with the myofiber and over a contact zone in which dystrophin staining in the myofiber was almost undetectable. The absence of dystrophin in the area of the muscle fiber that was in contact with the dividing satellite cells suggested a potential disruption of the fiber’s plasma membrane and fusion with the satellite cell. Moreover, electron microscopy revealed signs of cell fusion between dividing satellite cells and the muscle fiber (Figure 1F, G, H), without the presence of plasma membranes or cytoplasmatic continuity in the contact zone in the different musculatures studied.

The fusion of satellite cell with the myofiber occurs simultaneously to cell division in neonatal mouse muscle

In order to gain details about the contact zone between the muscle fiber and activated satellite cell, we analyzed ultra-thin sections from the musculature of one week old mice by electron microscopy. As expected, myogenic cells were shown to be located between the plasma membrane of the muscle fiber and its basal lamina, with clumps of highly condensed heterochromatin in their nuclei (Mauro, 1961, Hawke and Garry, 2001, Seale et al., 2000). When sections were analyzed at higher magnification, we observed about
80% (see Table S1 in Supplementary Material) of total myogenic cells with areas of fusion with the myofiber, as judged by the presence of cytoplasmatic continuity between both cells, disappearance of the plasma membrane, imprecise boundaries and amorphous material (Fig. 2A). These characteristics have been previously described during the last stages of the fusion process (Santander et al. 1993). In some cases, we observed only one edge of the contact region fused, whereas at the other edge the two plasma membranes and the corresponding intercellular space could be clearly identified (Fig. 2B). However, in the areas where the two plasma membranes were clearly visible we could observe fusion intermediates as aligned vesicles, plaques (Fig. 2C) and pores (Fig. 2D) suggesting that the fusion process was in progress (Shimada, 1971, Kalderon and Gilula, 1979, Doberstein et al., 1997, Chen et al., 2007). We also noticed the presence of cytoplasmatic extensions of the myofiber that tend to surround the myogenic cell, fading at the point of contact, as previously described as cytoplasmatic flaps by Santander et al. (1993).

Some of the cells that showed signs of fusion were undergoing cell division (16% of fusing myogenic cells). According to previous descriptions of the mitotic cell ultrastructure, (appearance of the nuclear membrane, mitotic spindle and degree of chromatin condensation, or the presence of two cells with a mid body between them (Robbins and Gonatas, 1964, Wendell et al., 1993)) we observed two satellite cells in prophase (Fig. 2E), five in metaphase (Fig. 2F) and six in telophase. Interestingly, the degree of fusion in the contact zone between the satellite cell and myofiber increased as the cell cycle progressed, being complete in cells in metaphase. In three out of the six telophases both daughter cells were totally fused to the myofiber, indicating that probably both daughter cells would stay inside the myofiber after division (Fig. 2G). In contrast, in the other three telophases, only one of the two daughter cells was fused to the myofiber while the other had a clear plasma membrane and intercellular space between itself and the myofiber, indicating that probably only one of the cells would stay inside the myofiber after division (Fig. 2H).

Since mitotic myogenic cells show clear fusion regions with the myofibers, we conclude that cell fusion takes place while the cell is still dividing. The rest of the cells that show fusion with interphasic nuclei (84% of the fusing satellite cells) could correspond to post-mitotic myogenic cells undergoing differentiation or activated cells in a pre-mitotic phase.
In all cell cycle phases satellite cells are over the myofiber contact zone with undetectable dystrophin.

To figure out if the main population of satellite cells observed with signs of cell fusion were differentiating or activated cells, samples were analyzed by immunohistochemistry and confocal microscopy. Paraffin sections of one week old mice musculature were stained for Pax7, PCNA and dystrophin. We observed a vast population of Pax7+/PCNA+ satellite cells (63% of total immunodetected cells, Supplementary Material Table S2). Interestingly, these cells had a contact zone with the myofiber with a fainter and discontinuous or totally absent dystrophin staining (undetectable dystrophin) (Fig. 3A), whereas the cells just adjacent to the myofiber perimeter presented a continuous and thick dystrophin staining (detectable dystrophin) (Supplementary Material Movie S1A, B). The highest magnification and axial resolution of the confocal microscope (HCX PL APO lambda blue 63.0x1.40 OIL UV objective and pinhole aperture 1 Airy, z-sections of 0.5 µm) was needed to observe the delimited gap in the dystrophin organization, thus the z-planes over or below Pax7 cells presented a detectable dystrophin, as reported by Zhang and McLenan (1994). No similar gaps were observed in the rest of the myofiber without satellite cells. In some cases myofibers showed extended irregular regions without dystrophin staining in all the z-planes observed, though this seems more likely to be an artifact of the sectioning.

Similarly, we identified a small fraction of Pax7-/PCNA+ cells (16% of total cells) comparable in terms of morphology and location to the Pax7+ population, which was localized above a contact region with undetected dystrophin (Fig. 3A, red arrow). As expected, the percentage of total activated cells detected by immunohistochemistry in close physical proximity with the myofiber and showing a contact zone with undetected dystrophin, was similar to the percentage (80%) of myogenic cells initiating fusion with the myofiber identified by electron microscopy.

To further characterize cells undergoing cell division, we used an anti pH3 antibody, which labels mitotic cells (Fig. 3B). Pax7+/pHH3+ cells (Supplementary Material Table S3), as well as a few Pax7-/pHH3+ cells, were mostly located in the contact zone where dystrophin was absent. It is worth mentioning that Pax7+/PCNA+ satellite cells were located mainly in areas where dystrophin was partially undetected, whereas Pax7+/pHH3+ cells were exclusively found in areas where dystrophin was
totally absent. This observation suggests that dystrophin expression decreases as satellite cells progress through the cell cycle, which is consistent with our previous observations using electron microscopy. Similar results were obtained by using a third proliferation marker, Ki67. About 66% of all Ki67+ cells detected had a contact zone with undetectable dystrophin (Fig. 3C, Supplementary Material Table S4) and 58% of Ki67 positive cells were also positive for Pax7 (Fig. 3D, Supplementary Material Table S5).

These data point out that in the neonatal mouse muscle most satellite cells are activated and present a contact zone with the myofiber with undetectable dystrophin.

**Contact zones without dystrophin do not show plasma membrane staining**

We then decided to stain the plasma membranes with wheat germ agglutinin (WGA). WGA co-localized with dystrophin along the myofibers. However, we observed discordant patterns, but only in the contact zones between activated satellite cells and the myofiber. In a few cases (8% of total cells, Supplementary Material Table S6) we observed detectable dystrophin staining, which colocalized with WGA staining (Fig. 3Ea), demonstrating that we were able to stain the plasma membrane in the narrow space between the two cells. In most cases we detected contact zones with undetectable dystrophin, with clear WGA staining (45% of total cells) (Fig. 3F) or with weaker or totally absent WGA staining (Fig.3Eb) (47% of total cells). We did not observe contact zones with clear dystrophin without WGA staining, demonstrating the reliability of the WGA as plasma membrane staining. This data suggests that there is a progressive process just in the contact zone between the activated satellite cell and myofiber, in which there is dystrophin and WGA staining first, then the dystrophin disorganizes and disappears and finally the WGA staining disappears between the two cells.

As previously highlighted, in this case it was also necessary to use the highest magnification and axial resolution to observe the gap in WGA staining between the Pax7+ cell and myofiber, whereas the rest of the myofiber perimeter showed a continuous WGA staining (Supplementary Material Movie S2A, B).

In agreement with the electron microscopy results, we conclude that most of the satellite cells that are activated do not show a continuous plasma membrane in the contact zone with the myofiber.
Two different activated myogenic cell subpopulations have a myofiber contact zone without dystrophin and WGA

We next used the marker laminin to confirm that myogenic cells undergoing cell division in intimate contact with the myofiber were in the internal face of the basal lamina. We identified mostly Pax7+/PCNA+ or pHH3+ satellite cells (Fig. 3G, H) and a smaller population of Pax7-/PCNA+ or pHH3+ cells surrounded by laminin (Supplementary Material Table S7, S8).

In an effort to characterize the Pax7-/PCNA+ or pHH3+ myogenic subpopulation, antibodies against myogenesis markers such as MyoD, Myf5 and myogenin were used. It is worth mentioning that when using different methodologies to detect these two markers (MyoD and Myf5), the gaps in the dystrophin staining just below the myogenic cells also appear, as with the paraffin embedding.

We found MyoD+ cells over undetectable dystrophin (Fig. 4A) but we did not observe MyoD-/PCNA+ cells. Even though we found a large number of Myf5+/PCNA+ cells located just over areas where the dystrophin was undetectable (Fig. 4B), most Myf5 positive cells outside the myofiber were also positive for Pax7 (Supplementary Material Table S9, S10 and S11). These results suggest that neither MyoD nor Myf5 exclusively mark the subpopulation of Pax7-/PCNA+ or pHH3+ cells.

Cells positive for myogenin were mainly located under the laminin (Fig. 4C and Supplementary Material Table S12). Myogenin+/PCNA+ cells (21% of total cells, Fig. 4D and Supplementary Material Table S13) or myogenin+/pHH3+ cells (4% of total cells, Fig. 4E and Supplementary Material Table S14) were always located over undetectable dystrophin and surrounded by a large population of PCNA+ or pHH3+ cells that were negative for myogenin (76% and 15% of total cells in each immunodetection, respectively). Triple staining for Pax7, myogenin and PCNA (Fig. 4F) showed that all PCNA+ cells in intimate contact with the myofiber were positive for either Pax7 or myogenin and that these two markers were never co-expressed (Supplementary Material Table S15).

Similar to the Pax7+ cells, myogenin+/PCNA+ cells showed a myofiber contact zone with undetectable dystrophin, whereas the rest of the myofiber showed a clear and continuous staining. Similarly, we observed some myogenin+ cells that showed WGA staining in the myofiber contact zone (Fig. 4G) and others that presented altered or
absent WGA staining in that area (Fig. 4H), while the rest of the fiber showed a continuous staining.

Our results suggests that two activated sub-populations of myogenic cells, positive for Pax7 (satellite cells) or myogenin (derived myoblasts), present a myofiber contact zone with altered or absent dystrophin and some without WGA staining.

**Mead and Bcat expression precedes the disorganization of dystrophin and decreases during cell cycle progression**

The described observations prompted us to investigate if Mcad could be localized in the contact zone with undetectable dystrophin. First, we confirmed that other cadherins, such as E-cadherin or N-cadherin, were not expressed between the muscle fiber and satellite cells (data not shown). Next, we analyzed whether both sub-populations of myogenic cells, Pax7 or myogenin positive cells, express Mcad in quiescent and activated states (Fig. 5) as already described for Pax7+ cells (Irintchev et al., 1994, Rose et al., 1994). A few Pax7+ cells were negative for the proliferation marker Ki67 and positive for Mcad (Fig. 5A). Most Pax7+ cells were positive for Ki67, some of them (60%) also positive for Mcad (Fig. 5B and Table S16), and the rest negative for Mcad. The same study using an anti myogenin antibody gave rise to similar results, with a few myogenin+ cells negative for Ki67 and positive for Mcad (Fig. 5C), and a majority of cells positive for Ki67, some of them (60%) also positive for Mcad (Fig. 5D and Supplementary Material Table S17), and the rest negative for Mcad. This data suggests that satellite cells express Mcad before (Mead+/Ki67-) and during cell proliferation (Mead+/Ki67+) and that its expression goes down concomitantly with cell cycle progression (Mead-/Ki67+). In agreement with this, we observed a clear reduction of Mcad signal in mitotic Mcad+/pHH3+ cells (Fig. 5E).

Staining with anti Mcad and anti dystrophin antibodies (Supplementary Material Table S18) showed a few cells with perfect colocalization of the two cell markers at the contact region (10%, Fig. 5F), whereas 76% showed Mcad staining over discontinuously detectable dystrophin (Fig. 5G) and 14% showed Mcad over undetectable dystrophin (Fig. 5H).

Since it has been described that Mcad interacts with Bcat (Kuch et al., 1997; Wróbel et al., 2007) we decided to analyze potential correlations between the
expression of Bcat, Mcad, Ki67 and dystrophin. We found that Bcat and Mcad always colocalize at the contact area between the myofiber and quiescent (Fig 5I) and activated (Fig 5J) satellite cells, and follows the same expression pattern, with most Bcat+ cells (50%) also positive for Ki67 and located over a contact zone with discontinuously detectable dystrophin (70%) (Fig. 5K-L; Supplementary Material Table S19-20).

This data points out that Mcad+/Bcat+ quiescent cells are located over a detectable dystrophin contact zone, whereas the main population of Mcad+/Bcat+ activated cells are situated over discontinuous or undetectable dystrophin, before the disappearance of both proteins at the contact region.

**Satellite cells undergo terminal differentiation inside the myofiber**

To further confirm that the main population of myogenic cells observed by electron microscopy with signs of fusion were not differentiated cells, we used antibodies against four proteins of fully differentiated myogenic cells: Mef2, desmin, p21 and macroH2A2 (mH2A2). The histone variant mH2A2 expression has been described to be high in terminally differentiated cells (Pehrson et al., 1997) and during adult stem cell differentiation (Barrero et al., 2013).

When we combined Mef2, PCNA and dystrophin staining, we observed a large number of Mef2+ nuclei inside the myofiber (Fig. 6A and Supplementary Material Table S21), a few Mef2+ cells outside the myofiber that were likely to be smooth muscle cells from the vasculature (Black and Olson, 1998), and a small percentage of Mef2+/PCNA+ cells in areas where the dystrophin expression was altered (Fig. 6B). These Mef2+/PCNA+ cells are likely to be myogenin+ cells, since we could not detect any colocalization between Pax7 and Mef2 signals (Fig. 6C and Supplementary Material Table S22). On the contrary, we detected colocalization between Mef2 and myogenin+/PCNA+ cells (Fig. 6D and Table S23). Importantly, we could not detect any significant pool of Mef2 cells surrounding the myofiber before fusion.

Desmin signal was observed only inside the myofiber (Fig. 6E), where there were no mononuclear derived myoblasts with desmin+ cytoplasms outside the area with dystrophin staining (Fig. 6F). mH2A2+ nuclei (Fig. 6G) and p21+ nuclei were also in intimate physical contact with the myofiber, never colocalized with PCNA+ nuclei (Fig.
6H), or Pax7+ cells (Fig. 6I) and were always located under the dystrophin signal (Fig. 6J).

These observations indicate that satellite cells and derived myoblasts that initiate their fusion with the myofiber are activated cells, and that final myogenic differentiation occurs once the myogenic nuclei are inside the myofiber.

**Mcad increases the myogenic cell division rate in vitro**

Taking into account the in vivo results, we decided to study mouse myogenic cell division in culture. Following the prevailing model, we expected to see individual Pax7 positive proliferating cells, and some groups of Pax7 negative derived myoblasts, initiating their fusion via Mcad. Surprisingly, immunocytochemical analyses revealed a significant proportion of proliferating Pax7+ (Fig. 7A) or myogenin+ (Fig. 7B) cells that were in intimate physical contact expressing Mcad between them (80% of total Pax7+Ki67+ cells; and 89% of total Myogenin+Ki67+ cells respectively; Supplementary Material Table S24 and S25), suggesting a potential role for Mcad in cell division, in addition to cell recognition and fusion. No E-cadherin or N-cadherin signal was detected (data not show), and Mcad signal colocalized with Bcat in the contact regions of Ki67 positive cells (Fig. 7C).

To study live myogenic cell division, we transduced cells with a GFP-PCNA encoding vector and performed time lapse experiments. While constitutive expression of GFP-PCNA was observed as homogeneous nuclear staining, clear replication foci could be detected from the beginning and throughout the S phase (Leonhardt et al., 2000; Kisielewska et al., 2005). Therefore, we were able to detect the activation of myogenic cells through changes in the GFP signal, as well as mitotic cells by transmitted light imaging (Supplementary Material Movie S3A and 3B). Moreover, we were capable of measuring the duration between two clear changes in the GFP signal: from the appearance of the first replication foci, to the breakdown of the nuclear membrane characterized by a decrease in the GFP signal (transition time, \( t_T \)).

Interestingly, we observed cells dividing in intimate physical contact with a two nuclei cell that was likely to be an incipient myotube (Fig. 7D and Supplementary Material Movie S5). Some other cells divided in a way that strongly reminded us of mouse embryo division (Fig. 7E and Supplementary Material Movie S6). These
observations prompted us to think that cell-to-cell contact mediated by Mcad could activate myogenic cell proliferation, in a similar way to E-cadherin in mouse blastomeres (Shapiro et al., 1995; Stockinger et al., 2001). Experiments by Gonzalez and colleagues (2011) showed that the adhesion of chimeric E-cadherin to single blastomeres recreates the signaling of the neighboring blastomere increasing their division rate. In a similar way, and to confirm our hypothesis, we tested the effects of stimulating or blocking the Mcad response (by using Mcad recombinant protein or specific antibodies, respectively) on myogenic cell division in 15 hour time lapse experiments.

In the absence of an Mcad recombinant protein or antibody (control conditions) we detected 14% of the cells activating S phase, 20% in mitosis and a tT of 6h and 42min (n=336 cells; Supplementary Material Movie S4). Addition of Mcad recombinant protein to the culture medium (Mcad-treatment) resulted in an increase in the number of activated myogenic cells (65% of total cells) and detected mitosis (64%) compared to the control. Interestingly, tT was reduced to 4h and 38min. (n= 296 cells; Supplementary Material Movie S7, 8A, 8B). When an antibody against the extracellular domain of the Mcad was added to the medium (Ab 1 -treatment) we observed an increase in the percentage of activated myogenic cells (28%), but a clear decrease in the number of mitotic cells (12%) compared to the control and the tT was extended to 7h 24min long (n=448 cells). The delay in tT was evident in some videos, where myogenic cells take more time to undergo complete cell cycle in the presence of anti-Mcad (Supplementary Material Movie S9A, B), and this is probably the cause behind observing more activated cells than in the control.

This growth retardation is very similar to what has been described in cells overexpressing E-cadherin (Stockinger et al., 2001). In these cells, overexpressed E-cadherin prevents the translocation of Bcat to the nucleus, resulting in cell cycle arrest. To test if blocking Mcad with antibodies resulted in changes in Bcat driven transcriptional activity, we performed qRT-PCR to evaluate the expression of Bcat target genes (Supplementary Material Graphic S1A S4). Interestingly, we found a statistically significant reduction in the Mcad transcripts in the Ab-1 treated cells, as well as a significant reduction in c-Myc expression levels, a Bcat target gene (He et al., 1998).
Growth curves comparing control versus Mcad or Ab 1- treatment further confirmed that recombinant Mcad addition stimulates the proliferation of myogenic cells. Moreover, treatment with an anti Mcad antibody raised against the cytoplasmatic domain of the protein (Ab 2-treatment) had no significant effects on growth rates (Supplementary Material Graphic S1B).

To further prove the differences in the three experimental conditions, we performed an EdU incorporation test (pulse of 45 min) 5 hours after starting the treatment. Dot plots and histograms (Fig. 7F) show an increase in the levels of incorporated EdU in Mcad treated cells and a decrease in anti-Mcad Ab-1 treated cells, compared to the control. Accordingly, we detected (Supplementary Material Graphic S1C-E) clear differences in the mean of intensity of fluorescence (MIF) suggesting that the Mcad-treated cells were incorporating EdU faster, whereas the anti Mcad Ab-1 treated cells were replicating their DNA slower than the control. Although we did not detect significant differences in the percentage of EdU+ cells (S-phase) among the three groups, an increase in the total number of cells was observed after Mcad treatment, as well as an increase in the number of cells in G2/M after the Ab-1 treatment.

When EdU pulses were longer (15h as in the time lapse studies) no significant differences in the cell cycle profile between control and Mcad treated cells were observed (Fig. 7G). However, in the presence of Mcad we detected a significant increase in the intensity of the EdU signal in the presence of Mcad. Moreover, we detected (Supplementary Material Graphic S1F-H) double the number of cells after Mcad treatment, which is consistent with our observation in in vitro cultured cells that were able to undergo two rounds of cell division during the course of the time lapse (15h) (see supplementary Movie S10). After anti Mcad-Ab-1 treatment the differences were notable, with an increase in the number of cells that did not undergo replication (EdU-) and a decrease in the EdU+ cell fraction and MIF value, compared to the control. These effects were not observed after the treatments of mouse embryonic fibroblast (MEFs).

These results suggest that Mcad mediated cell interactions work as a trigger to boost cell cycle progression in cultured myogenic cells.

*The inhibition of myogenic cells fusion in vivo reduces cell proliferation*
In order to investigate if the cell-to-cell interaction of myogenic cells with the myofiber mediated by Mcad affects their proliferation capacity in vivo, we analyzed the effect of blocking Mcad interactions by two strategies: injecting antibodies against Mcad (using the same antibodies as in the in vitro study), as well as injecting lentiviruses encoding shRNAs against Mcad directly into the muscle of neonatal mice.

48h after injecting Ab 1 in neonatal mice legs (Supplementary Material Graphic S2A) the number of total and activated Pax7+ cells became dramatically reduced compared to Ab 2 injected and control legs. Accordingly, we detected a clear reduction of Mef2+ nuclei inside the myofiber in the Ab 1 injected legs compared to Ab 2 injected and control legs.

We next tested the effects of knocking down the expression of Mcad in vivo using lentiviruses encoding shRNAs against Mcad. In order to confirm the efficiency of the shRNAs, we infected myogenic cells in culture with four different shRNAs against Mcad. Growth curves (Supplementary Material Graphic S2B) showed a clear reduction in the ratio of cell division in cells infected with the anti-Mcad shRNAs (LV-ShMcad) compared to those infected with the empty viruses (LV-ShCtrol). From the four tested shRNAs, shRNA1 showed more conspicuous effects and efficiently reduced the expression of Mcad as judged by immunodetection (Supplementary Material Graphic S2C) and by qPCR (Supplementary Material Graphic S2D). Therefore, we used this lentivirus to inject neonatal mouse muscles. 72h after the injection (Supplementary Material Graphic S2E) a clear reduction of the total and activated Pax7+ cells, as well as Mef2+ nuclei inside the myofiber, was observed in muscles injected with LV-shMcad compared to LV-shCtrol injected and non injected (control) legs.

Due to methodological difficulties in using antibodies or shRNAs in adult Xenopus, we performed a cell fusion inhibition experiment in tadpoles using tunicamycin (Supplementary Material Graphic S2F). Previous findings revealed that tunicamycin inhibits myoblast fusion by blocking the synthesis of UDP-N-acetyl-glucosamine, reducing the number of nuclei inside the muscle fibers (Gilfix and Sanwal, 1980). After 48 hours of tunicamycin treatment, we observed a clear reduction in the number of total and activated Pax7+ cells in the body musculature. We also detected a decrease in Mef2+ nuclei inside the myofiber.
We thus concluded that inhibition of satellite cells fusion in vivo blocks their proliferation and potentially their differentiation.

DISCUSSION.

Here we have explored the mechanisms of myogenesis in neonatal mouse and Xenopus tadpoles. We found that mouse myogenic cells interact via Mcad with the myofiber in a contact zone where dystrophin expression is absent. Our observations in cultured mouse myogenic cells demonstrate that such cell-to-cell interaction activates and accelerates their cell division. Although more studies should be performed in order to reveal the mechanistic nature of these effects, our data suggest that the Mcad-Mcad interactions could be regulating cell proliferation through Bcat transcriptional activity, and thus likely via the canonical Wnt signaling pathway. The fact that we could not detect nuclear Bcat accumulation in our samples is in agreement with other reports that describe changes in Bcat transcriptional activity in the absence of detectable changes in nuclear localization, since low levels of nuclear Bcat were found to be sufficient to stimulate the transcription of its target genes (Stockinger et al. 2001).

Taking into account our observations, we propose a revised model for myogenic cell division during neonatal myogenesis (Fig. 8). Quiescent Pax7+ or Myogenin+ myogenic cells express Mcad and Bcat in their contact zone with the myofiber, which still shows detectable dystrophin. Once the muscle growth process starts, the dystrophin in the contact zone starts to disorganize and myogenic cell proliferation becomes activated, followed by its fusion initiation with the myofiber. Subsequently, the dystrophin completely disappears from the contact zone where Mcad, Bcat and plasma membranes are still present. Finally, the plasma membrane disappears between both cells while the myogenic cell is still in mitosis. After telophase, one daughter cell stays outside the myofiber to replace the Pax7 positive cell pool while the other one stays inside the myofiber and differentiates, or both daughter cells differentiate.

We conclude that the alteration of dystrophin, which gives resistance and rigidity to the myofiber in an organized state, is related to the activation of the myogenic cells and its fusion with the myofiber. This alteration seems to confer plasticity to the plasma membrane of the myofiber, as judged by the presence of cytoplasmatic extensions or flaps that surround part of the myogenic cell during cell fusion. It is also likely that the
disorganization of the dystrophin is necessary for the cell-to-cell interaction of the satellite cells in humans, since it has been described that dystrophin becomes narrow in areas where activated satellite cells reside after intense exercise (Lindström et al., 2010).

We propose a model in which proliferation and differentiation of satellite cells take place in physical contact with the myofiber. Therefore, the muscle fiber would play a critical role in satellite cell self-renewal and differentiation regulation. It has been described that changes in the muscle niche, rather than modifications of the satellite cells themselves, appear to be the main factor responsible for the declining regenerative response of old muscle (Carlson and Faulkner, 1989). In fact, aged satellite cells transferred into a younger muscle can divide at the same ratio as young satellite cells (Shefer et al., 2006; Carlson and Conboy, 2007).

Another aspect of our model that differs from the prevailing model is the final differentiation of the adult stem cells or the myogenic progenitors. Our results agree with the study done by Schulze (Schulze et al., 2005), in which mesenchymal stem cells only differentiate to myocytes once they fuse with them. In fact, it has been postulated that the direct interaction between differentiated muscle cells and stem cells is a prerequisite for their differentiation (Nunes et al., 2007; Boonen and Post, 2008).

Previous studies have already linked cell proliferation and fusion, suggesting that embryonic myoblasts can fuse among themselves or with the myofiber only just after mitosis, during the G1 phase (Bischoff, 1990; Bischoff et al., 1969). According to our model, the fusion of myogenic cells start at the interphase of the mother cell and finishes just after a cell division in the G1 phase of the daughter cell. The data here reported are thus in agreement with those previously described by Bischoff and colleagues (Bischoff, 1990; Bischoff et al., 1969; Ishikawa, 1968, and besides providing new insights towards a better understanding of vertebrate, myogenesis, they may serve as the basis for the development of new to induce muscle regeneration in human muscle dystrophies.
MATERIAL AND METHODS

Animal husbandry.
Body musculature of *Xenopus* at stage 54 and thigh muscle of B6 mice at p7 were used in this study. All animal experiments were done following experimental protocols previously approved by the Institutional Ethics Committee on Experimental Animals, in full compliance with Spanish and European laws and regulations.

Transmission electron microscopy.
Samples were fixed with 2.5% glutaraldehyde, 2 h at 4ºC and post-fixed in 1% osmium tetroxide (2 h at 4ºC), dehydrated with ethanol and embedded in EPOXY. Samples were examined with a JEOL 1011 transmission electron microscope (Tokyo, Japan).

Immunofluorescence analysis on *Xenopus* tadpoles, P7 mice and cultured satellite cells.
Samples were fixed with 4% paraformaldehyde for 2 h at 4ºC for tadpoles and 24 h at 4ºC for mice samples. Cultured satellite cells were fixed with 2% paraformaldehyde for 20 min at room temperature. Paraffin samples were processed with antigen retrieval in the Dako Pascal system (Glostrup, Denmark), and treated with 0.5% Triton X-100 and 3% donkey serum for 30 min. Primary antibodies (see Table S26 in SM) were incubated overnight at 4ºC, and secondary antibodies (see Table S27 in SM) for 2 h at 37ºC. To use the Myf5 antibody, samples were processed with OCT. For MyoD antibody, samples were frozen with isopentane, sectioned with a cryostat, and fixed with paraformaldehyde for 10 min at room temperature. mH2A2 antibody was previously described (Buschbeck et al., 2009). Images were taken using a Leica TCS SP5 microscope (Wetzlar, Germany).

Isolation and culture of mouse satellite cells
Satellite cells were isolated from p7 mice as previously described (Perdiguero et al., 2007). Briefly, thighs were mechanically dissected, digested using 1 % pronase, and satellite cells were recovered by centrifugation on a Percoll density gradient. Cells were cultured in collagen coated dishes in the presence of Ham’s F-10 containing 20% FBS, X mM Glutamax, and 0.1 µl/ml FGF. A phenotyping of the cell culture was performed prior to each experiment (see Table SM 26 for antibodies used) by flow cytometry.
(Beckman Coulter, Indianapolis, IN, USA), giving similar results to what has been published for satellite cells in culture (Ieronimakis et al., 2010).

**Viral transduction**

The vector pEGFP-PCNA-IRES-puro2b from Addgene (26461) was digested with XhoI and BamH and the insert was subcloned into pMSCVpuro (Clontech). Retroviral particles were obtained by transfection into Phoenix Amphotropic cells (ATCC). Viral supernatants were harvested on two consecutive days every 24h and filtered through a 0.45µm PVDF filter (Millipore). 100,000 satellite cells were spinfected (at 750 g for 45 min) twice at 24h intervals with fresh retroviral preparations in the presence of polybrene. Transduced cells were selected with puromycin.

**Time lapse captions**

Cells were filmed for 15h in a Leica SP5 inverted microscope with an *in vivo* system, with a 63x objective, taking one image every 6 min, in Mat-tect plates. 10 fields were detected in the same caption by the motorized stage. Each condition was repeated at least twice.

**M-Cadherin and antibody treatment**

Recombinant Mcad (12µmg/ml; R&D 4096-MC-050; Minneapolis, MN, USA), an antibody-1 against the extracellular domain of the Mcad (1:50, Santa cruz; sc-81471; Santa Cruz, California, USA), or an antibody-2 against the cytoplasmic domain (1:50, Santa Cruz; sc-374093) were added to satellite cell culture for 5h or 15h. At least three replicates of each condition were analyzed, and the experiment was repeated at least twice.

**EdU treatment and detection.**

Cells were incubated in 6-well plates with 1 µmM EdU for 45 min or 15 h and analyzed using the Click-iT EdU cell proliferation assay kit from Invitrogen (Carlsbad, California, USA), detected by a Moflo flow cytometer, (Beckman Coulter, Indianapolis, IN, USA).

**Real Time RT-PCR**

Isolation of total RNA from was performed using Trizol reagent (Invitrogen, Carlsbad, CA). All samples were treated with TURBO DNase inhibitor (Ambion) to remove any
residual genomic DNA and 2 ug of RNA was used to synthesize cDNA using the Invitrogen SuperScript II Reverse Transcriptase kit. 25ng of cDNA were used to quantify gene expression by Quantitative RT-PCR using primers described in Table S28 in SM.

**Lentiviruses production.**

The recombinant lentiviruses were produced by transient transfection of HEK293T cells cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). At 60-70% confluence the cells were co-transfected with 4 µg of pCMV VSV-G, 8 µg psPAX2, carrying lentiviral envelope protein or lentiviral packaging proteins, respectively, and 12 µg of lentiviral pLKO.1-puro shRNA plasmids carrying interferential sequences to knock down Cadherin 15 (MISSION shRNA NM_007662, TCRN0000094574-77. Sigma-Aldrich, St. Louis, MO, USA). The transfection was done using Fugene 6 transfection reagent (Promega Corporation, Madison WI, USA) following the manufacturer’s instructions. 24 hours and 48 hours post-transfection, medium containing viruses was collected, pooled, centrifuged at 3500 rpm at RT for 5 min and filtered through a 0.45 μm PVDF-filter. The viruses were concentrated by centrifugation at 19600 rpm at 22°C for 140 min. The pellet was resuspended in phosphate-buffered saline (PBS) and aliquoted viral stocks were stored at -80°C.

**In vivo inhibition of satellite cell fusion.**

2.5 µg of Ab-1 or Ab-2 against Mcad diluted in 25 µl of NaCl were injected in the thighs of P7 mice with a Hamilton syringe. 2.5 µl of LV-shCtrol or LV-shMcad-1 (TCRN0000094574) preparations (10⁹ IU) were injected in the thighs of P7 mice with a Hamilton syringe. Tunicamycin (Sigma; Ref: T7765; St. Louis, MO. USA) stock solution (1mg/ml) was prepared in DMSO, and a 1/10 dilution in mQ H₂O was injected in the body musculature of stage 54 tadpoles. Animals were sacrificed at 24 h and 48 h post injection for Ab and tunicamycin treatments, 48h and 72h post injection for lentivirus experiments.
**Counting methods.**

In the muscle sections we used images that had been obtained with the 63x objective, taking images of at least 8 fields in each individual. For the study of dystrophin organization and Mcad distribution, an over zoom was needed. In the cultured cells we took 10 random fields in each slide with the 20x objective. The percentages for the different satellite cell sub-populations were expressed over the total number of cells immunodetected with each antibody combination. For the cell culture, the percentage was calculated over the total number of fields. In the flow cytometer assay, evaluation of cycling cells was based on the MIF, measured as signal to noise ratio (MIF from the EdU positive cell fraction / MIF from the EdU negative cell). In the *in vivo* experiments 10 fields from three different sections were taken randomly, and the percentage was calculated over the total myofiber number. We processed the images using the Metamorph software (Molecular Devices, Sunnyvale, California, USA). The statistical analysis was carried out using the T-Student test.

**AUTHORS' CONTRIBUTIONS**

MM designed the study, made the observations and wrote the manuscript. NM performed the cell culture studies, participated in the experiment design and helped with manuscript writing. CP performed the electron microscopy study. LM performed all immunohistochemistries. LMS assisted with the *in vitro* cultures and lentiviral infections. ACR performed the tunicamycin experiments in *Xenopus*. JAV performed the flow cytometry study. BK performed the viral transduction and lentiviral infections. CM assisted with the observations, counting processes and time lapse experiments. MJB performed the differentiation study, helped with the experiment design and reviewed the manuscript. JCIB participated in design of the study, financial support and reviewed the manuscript. All authors read and approved the final manuscript.

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FIGURE LEGENDS.

Fig. 1. Dystrophin is undetectable in the contact zone between the myofiber and dividing satellite cells. (A) *Xenopus* body musculature stage 54. PCNA positive cell (red) over a region without dystrophin (cyan). Insert: detail of the dystrophin gap in the area of contact. Scale bar: 25 microns. (B) *Xenopus* tail stage 53; 3 days after tail amputation. pH3 (red) positive cell in close contact with the myofiber over a region without dystrophin (cyan). Insert: detail of the dystrophin gap in the contact area (small arrow). Scale bar: 25 microns. (C) Mouse muscle 7 days after birth. Pax7 (green) PCNA (red) positive cell over undetectable dystrophin (cyan). Insert: detail of the dystrophin gap in the contact area (small arrow). Scale bar: 10 microns. (D) Mouse muscle 30 days after birth. Pax7 (green) PCNA (red) positive cell over undetectable dystrophin (cyan). Insert: detail of the dystrophin gap in the contact area (small arrow). Scale bar: 10 microns. (E) 3 month old mouse muscle, 6 days after cardiotoxin induced injury. Pax7 (green) PCNA (red) positive cell over undetectable dystrophin (cyan). Insert: detail of the dystrophin gap in the contact area (small arrow). Scale bar: 25 microns. (F) *Xenopus* limb bud, stage 57. Area without a plasma membrane between the satellite cell chromosomes and the sarcomeres (arrow). Insert: lower magnification showing the chromosomes of the satellite cell and the area observed (small arrow). Scale bar: 0.2 microns. (G) *Xenopus* tail stage 53; 14 days after amputation. Contact area between one satellite cell and the myofiber, without a plasma membrane between them (arrow). Insert: lower magnification showing the chromosomes of the satellite cell and the area observed (small arrow). Scale bar: 0.2 microns. (H) Mouse muscle 7 days post birth. Area without a plasma membrane between the satellite cell cytoplasm and myofiber cytoplasm. Part of the myofiber cytoplasm, with a clear plasma membrane, surrounds the satellite cell (red arrow). Insert: lower magnification showing the chromosomes of the satellite cell and the area observed (small arrow). Scale bar: 0.5 microns.

Fig. 2. Satellite cells fuse to the myofiber during cell division in neonatal mouse muscle (A) Detail of one edge of the myofiber (MF) - satellite cell (SC) contact zone where neither a plasma membrane or intercellular space could be seen (black arrows). (B) Detail of the other edge of the myofiber contact zone in the same satellite cell, under the basal lamina (black arrow), with a clear plasma membrane and intracellular space. At the myofiber side there are a lot of aligned vesicles (white arrows). (C) Detail of an
adherent structure (black arrow) between the plasma membranes of the satellite cell and myofiber. (D) Detail of a fusion point between a satellite cell and myofiber. (E) Satellite cell in prophase with one edge of the myofiber with a clear plasma membrane, digital projections, and intercellular space (left panel, black arrows) and the other edge of the contact zone fused (right panel, black arrows) (F) Satellite cell in metaphase fused with the myofiber (right panel, black arrows). Note the cytoplasmatic extension of the myofiber or flaps around the satellite cell with a clear plasma membrane between them (left panel, red arrow). (G) Satellite cell in telophase with both daughter cells fused to the myofiber (black arrows). (I) Satellite cell in telophase. One daughter cell shows a clear plasma membrane and intercellular space at the contact zone (black arrows), and the other daughter cell is fused to the myofiber (white arrows). Scale bar in B-H are 2 µm. In all the high magnification details and in A, scale bars correspond to 0.2 µm.

**Fig. 3. Activated Pax7+ cells are situated over regions without dystrophin or plasma membrane staining.** (A) Immunohistochemistry of neonatal mouse muscle sections show a Pax7 (green) PCNA (red) positive cell, as well as a Pax7 negative PCNA positive cell (red arrow), that have a myofiber contact zone with undetectable dystrophin (white arrows, cyan). (B) Pax7 (green), pH3 (red) positive cell over the myofiber contact zone with absent dystrophin staining (white arrow, cyan). (C) Ki67 (red) positive cell over a myofiber contact zone with absent dystrophin staining (white arrow, cyan). Note the dystrophin positive cytoplasmatic extension over the satellite cell (red arrow). (D) Pax7 (green) Ki67 (red) positive cell in close contact with the sarcomeric actin of the myofiber (cyan). Note the myofiber extension over the satellite cell (red arrow). (E) Two PCNA (cyan) positive cells, one (a, left panel) with clear WGA (red) staining in the myofiber contact zone colocalizing with dystrophin staining (green) and the other one (b, right panel) without dystrophin nor WGA staining in the myofiber contact zone (arrows). (F) A PCNA positive cell (cyan) with clear WGA staining (red) in the myofiber contact zone, were no dystrophin (green) is detected. (G) A Pax7 (green) PCNA (red) positive cell under intact (white arrow) laminin (cyan). (H) A Pax7 (green) pH3 (red) positive cell under intact (white arrow) laminin (cyan).

Scale bars in A, C, D and G correspond to 10µm, in B and H to 25 µm and in E and F to 75 µm. The center panel shows merged images and the side panels show the enlargement of the indicated areas.
Fig. 4. Myogenin + derived myoblasts are situated over regions without dystrophin or plasma membrane staining (A) Immunohistochemistry of neonatal mouse muscle sections shows a MyoD (green) and PCNA (red) positive cell with a contact zone without dystrophin staining (white arrow, cyan). (B) A Myf5 positive nuclei (green) inside the myofiber and another (yellow) positive nuclei for Myf5 and PCNA (red) over a contact zone with undetectable dystrophin staining (white arrow, cyan). (C) A myogenin positive cell (green) and another cell (yellow) positive for myogenin and PCNA (red) both under laminin (arrow, cyan). (D) A myogenin (green) and PCNA (red) positive cell over a contact zone with undetectable (white arrow) dystrophin (cyan). (E) A myogenin (green) and pHH3 (red) positive cell with a myofiber contact zone without dystrophin staining (white arrow, cyan). (F) Two Pax7 positive cells (green) and one myogenin positive cell (red), all of them positive for PCNA (blue). Dapi has been removed in the merged image. (G) A myogenin (green) and PCNA (cyan) positive cell with clear WGA staining (red) in the myofiber contact zone (white arrow). (H) A myogenin (green) and PCNA (cyan) positive cell without WGA staining (red) in the myofiber contact zone (white arrow). Scale bar in C corresponds to 7.5 µm and in all others to 10 µm. The center panel shows merged images and the side panels show the enlargement of the indicated areas.

Fig. 5. Mcad is present in quiescent and activated myogenic cells

(A) Immunohistochemistry of neonatal mouse muscle sections show a Pax7 (green) positive and Ki67 (red) negative cell expressing Mcad (white arrow, cyan). (B) A Pax7 (green) and Ki67 (red) positive cell expressing Mcad (white arrow, cyan), as well as a Pax7 negative and Ki67 positive cell (red arrow). (C) A myogenin (green) positive and Ki67 (red) negative cell expressing Mcad (white arrow, cyan). (D) A myogenin (green) and Ki67 (red) positive cell expressing Mcad (white arrow, cyan). (E) Reduction of Mcad expression (green) in pHH3 (red) positive cells. (F) Mcad (red) expression colocalizing with detectable dystrophin (green). (G) Mcad (red) expression in an area with discontinuously detectable dystrophin (green). (H) Mcad (red) expression in an area of undetectable dystrophin (green). (I) Bcat (green) expression colocalizing with Mcad (green) in a contact zone between a Ki67 negative cell and the myofiber. (J) Bcat (green) expression colocalizing with Mcad (green) in a contact zone between a Ki67 positive cell and the myofiber. (K) Bcat (green) expression in two contact zones, over intact dystrophin (red, upper cell) or discontinuously detectable dystrophin (lower cell).
(L) Bcat (green) expression in an area of undetectable dystrophin (red). Scale bars in A to D, F, and I-L correspond to 10 µm, in E to 25 µm G to 7.5 µm, in H to 5 µm. The center panel shows merged images and the side panels show the enlargement of the indicated areas.

**Fig6. Only the nuclei that are inside the myofiber show total differentiation.**

(A) Immunohistochemistry of neonatal mouse muscle sections shows a Mef2 positive nucleus (green) located inside the myofiber (arrow), whereas a Mef2-/PCNA+ cell (red) is over undetectable dystrophin (cyan). (B) A Mef2 (green) and PCNA (red) positive nucleus located over a myofiber contact zone without dystrophin (white arrow, cyan). (C) Mef2 positive nucleus (green) do not colocalize with Pax7 signal (red), but can stain positive (magenta) for PCNA (blue). Dapi has been removed in the merged image. (D) A Mef2 positive nucleus (green) that colocalizes with myogenin (red) and PCNA (blue) signal. Dapi has been removed in the merged image. (E) Mononucleated cells positive for both desmin (green) and Mef2 (red) are not detected. (F) Desmin (green), PCNA (red) and dystrophin (cyan) staining shows that the desmin signal is always inside the dystrophin barrier. (G) A mH2A2 positive nucleus (green) negative for PCNA (red) and located below the dystrophin staining (cyan). (H) p21 positive nucleus (green) do not colocalize with PCNA nuclei (red). (I) p21 positive nucleus (green) do not colocalize with Pax7 nuclei (red). (J) p21 positive nucleus (green) is located below the dystrophin signal (red). Scale bars in A to D and G to J correspond to 10 µm, in E to 50 µm and in F to 7.5 µm. The center panel shows merged images and the side panels show the enlargement of the indicated areas.

**Fig. 7. Satellite cells in in vitro culture**

(A) Immunocitochemistry showing three cells expressing Mcad (cyan) between them. Two of them (yellow) are positive for Pax7 (green) and Ki67 (red). Dapi has been removed in the merged center image. (B) Myogenin+ (green) cells positive or negative for ki67 (red), expressing Mcad (cyan) between them. Dapi has been removed in the merged central image. (C) Bcat (green) and Mcad (red) expression colocalizing between two cells positives for Ki67 (blue). Dapi has been removed from the merged image. (D) Different frames of the Supplementary Material Movie S5, showing a satellite cell in intimate contact with a two nuclei cell that looks like an immature
myotube. Myogenic cell starts their replication and mitosis still in intimate contact with it. One daughter cell stays totally connected to the myotube, whereas the other daughter cell stays outside. (E) Different frames of Supplementary Material Movie S6, showing two myogenic cell that divide in a way that is reminiscent of mouse embryo division, from two-cells to four-cells. **Satellite cell culture pulsed 45 min with EdU after 5h of treatment: control, in the presence of Mcad protein (+Mcad), or anti-Mcad antibody (+Anti-Mcad Ab).** (F) Representative dot plot showing the distribution of myogenic cells along with their DNA content (Dapi) vs incorporated EdU, and an illustrative histogram showing cell distribution according EdU incorporation and indicating the average of signal to noise ratio, in the three conditions. **Satellite cell and MEFs culture pulsed 15 h with EdU during 15h of treatment: control, in the presence of Mcad protein (+Mcad), or anti-Mcad antibody (+Anti-Mcad Ab).** (G). Representative dot plot showing the distribution of satellite cells along with their DNA content (Dapi) vs incorporated EdU, and an illustrative histogram showing cell distribution according EdU incorporation and indicating the average of signal to noise ratio, in the three conditions.

**Fig. 8. Scheme of our proposal.**

Quiescent myogenic cells express Mcad over detectable dystrophin and plasma membranes. Then, dystrophin starts to disorganize in the contact zone between the satellite cell and myofiber, and the myogenic cell enters the cell cycle. Simultaneously, the fusion process starts at some regions of the contact zone. Subsequently, the dystrophin disappears from the contact area, where Mcad and the plasma membrane are still present. Finally, both cells completely fuse at the contact region, while the satellite cell is still in mitosis. After telophase one daughter cell stays outside the myofiber to renew the Pax7 positive cell pool, whereas the other one stays inside the myofiber and differentiates. Alternatively, both daughter cells could differentiate.
SUPPLEMENTARY DATA:

Movie S1A) 3D reconstruction of a xyz series by average projection, showing the gap of the dystrophin (green) only in the contact zone of the Pax7+ (red) cell with the myofiber. Movie S1B) Idem video showing only the dystrophin.

Movie S2A) 3D reconstruction of a xyz series by average projection, of a xyz series showing the gap of the WGA staining (red) only in the contact zone of the Pax7+ cell (green). Movie S2B) Idem video showing only the WGA staining.

Movie S3A) Time lapse caption of myogenic cells in culture with GFP/PCNA vector infection showing the changes in the distribution of replication foci as described by Leonhardt et al. (2000): fine punctuate pattern at frame 3, perinucleolar pattern at frame 6, first few and large foci at frame 10, redistribution of the GFP signal at the cytoplasm at frame 51. Caption of 6 h 36 min in 6-min intervals. Movie S3A) Transmitted light caption of the same video. Notice that at frame 51 the myogenic cell is in mitosis.

Movie S4) Time lapse caption of myogenic cells in culture with transmitted light and GFP/PCNA vector detection, taking one image every 6 min for 15 h. 5 mitoses can be detected.

Movie S5) Time lapse caption of myogenic cells in culture with transmitted light and GFP/PCNA vector detection, taking one image every 6 min for 16 h. The myogenic cell of the right side show replication foci meanwhile is in intimate physical contact with the two nuclei myotube (frame 60). Subsequently myogenic cell undergo mitosis (frame 118), and one daughter cell stay in intimate physical contact with the myotube. Details in figure 7C.

Movie S6) Time lapse caption of myogenic cells in culture with transmitted light and GFP/PCNA vector detection, taking one image every 6 min for 11 h 24 min. One of a pair cell in contact starts its cell division, and subsequently the other does, without loss of contact in any moment. This cell division is reminiscent of mouse embryo division from 2-cells to 4-cells. Details in figure 7D.

Movie S7) Time lapse caption of myogenic cells in culture with transmitted light and GFP/PCNA vector detection, taking one image every 6 min for 15 h, 1h after Mcad recombinant protein addition.17 mitoses can be detected.
**Movie S8A)** Detail of a time lapse caption of myogenic cells in culture with GFP/PCNA vector detection, taking one image every 6 min for 16 h, in a Mcad recombinant protein treated cell culture. The two activated myogenic cells undergo cell division, and subsequently the 4 daughter cells initiate cell activation (notice the fine punctuate pattern of replication foci in them). tT of the cell in the center of the image: 43 frames, from 6 to 49. **Movie S8B** same detail of a time lapse caption of myogenic cells in culture with transmitted light detection.

**Movie S9A)** Detail of a time lapse caption of myogenic cells in culture with GFP/PCNA vector detection, taking one image every 6 min for 16 h, in an anti-Mcad antibody treated cell culture. Two myogenic cells undergo cell activation and both divide, but in a slower manner compared to control. tT of the lower cell: 105 frames, from 55 to 160. **Movie S9B** same detail of a time lapse caption of myogenic cells in culture with transmitted light detection.

**Movie S10** Detail of a time lapse caption of myogenic cells in culture with transmitted light and GFP/PCNA-vector detection, taking one image every 6 min for 15 h, 1h after Mcad recombinant protein addition. Two rounds of cell divisions can be detected in the same cell.

**Graphic S1 (S1A)** Mcad, Bcat and c-myc mRNA levels determined by qPCR after Mcad and antibody treatment. Levels were normalized to GAPDH in three replicates for each group (* p≤0.05). **(S1B)** Growth curve of myogenic cells after 36h of treatment with Mcad, Ab1 or Ab2, compared to the control. **(S1C)** Bar graph showing the average of total counted cells per ml in the three conditions; the graph shows the mean and standard deviation of 4 replicates in each group (* p≤0.05). **(S1D)** Percentages of the different populations G0/1, G2/M and S phase of the myogenic cells *in vitro*, in the three conditions. The graph shows the mean and standard deviation of 4 replicates in each group (* p≤0.05). **(S1E)** Bar graph showing MIF value calculated as signal/noise ratio in the three conditions. The graph shows the mean and standard deviation of 4 replicates in each group (* p≤0.05). **(S1F)** Bar graph showing the average of total counted cells per ml in the three conditions; the graph shows the mean and standard deviation of 4 replicates in each group (* p≤0.05). **(S1G)** Percentages of the different populations EdU- G0/1, EdU- G2/M and EdU+ of the myogenic cells *in vitro*, in the three conditions. The graph shows the mean and standard deviation of 4 replicates in
each group (* p≤0.05). (S1H) MIF value calculated as signal/noise ratio in the three conditions. The graph shows the mean and standard deviation of 4 replicates in each group (* p≤0.05).

Graphic S2). (S2A) Pax7, PCNA, and Mef2, positive and negative cell count in tissue sections of mice treated with Ab-1 and Ab-2 for 48 hours and control mice legs. The graph shows the mean and standard deviation of 3 replicates in each group (* p≤0.05). (S2B) Growth curve of the cells infected with the 4 different shMcad tested and LV-shCtro (S2C) myogenic cells infected with LV-ShMcad-1 expressed less Mcad (red) between them than the ones infected with LV-ShCtro, and a decrease in the number of Ki67+ positive cells (cyan) was clear (S2D) A decrease was seen in Mcad transcripts in the myogenic cells infected with LV-shMcad1 versus the cells infected with the LVshCtro. Analyses performed by means of RTq PCR (S2E) Pax7, PCNA, and Mef2, positive and negative cell count in tissue sections of mice treated with LV-shMcad and LV-shCtro for 48 hours, and control mice legs. The graph shows the mean and standard deviation of 3 replicates in each group (* p≤0.05). (S2F) Pax7, PCNA, and Mef2 positive and negative cell count in tissue sections of Xenopus treated with tunicamycin for 48 hours. The graph shows the mean and standard deviation of 4 replicates in each group (* p≤0.05).

Table S1. Percentage of fused and not fused cells. 103 cells observed.

Table S2. Percentage of activated Pax7 cells over undetected dystrophin. 309 cells observed.

Table S3. Percentage of mitotic Pax7 cells over undetected dystrophin. 397 cells observed.

Table S4. Percentage of Ki67+ cells over undetected dystrophin. 420 cells observed.

Table S5. Percentage of Ki67+Pax7+. 451 cells observed.

Table S6. Percentage of colocalization between WGA and dystrophin. 88 cells observed.
Table S7. Percentage of activated Pax7 cells situated under the laminin. 422 cells observed.

Table S8. Percentage of mitotic Pax7 cells situated under the laminin. 294 cells observed.

Table S9. Percentage of activated Myf5 cells situated over undetectable dystrophin. 339 cells observed.

Table S10. Percentage of Myf5+Pax7+ cells. 664 cells observed.

Table S11. Percentage of Myf5+Myogenin+ cells. 742 cells observed.

Table S12. Percentage of activated myogenin cells situated under the laminin. 475 cells observed.

Table S13. Percentage of activated myogenin cells situated over undetectable dystrophin. 333 cells observed.

Table S14. Percentage of mitotic myogenin cells situated over undetectable dystrophin. 185 cells observed.

Table S15. Percentage of Myogenin+Pax7+ cells. 450 cells observed.

Table S16. Percentage of activated Pax7 cells with Mcad expresion. 171 cells observed.

Table S17. Percentage of activated Myogenin cells with Mcad expresion. 208 cells observed.

Table S18. Percentage of Mcad+ cells over undetectable dystrophin. 143 cells observed.

Table S19. Percentage of Bcat+ Ki67+. 347 cells observed.

Table S20. Percentage of Bcat+ cells over undetectable dystrophin. 147 cells observed.

Table S21. Percentage of Mef2+ cells over undetectable dystrophin. 1246 cells observed.

Table S22. Percentage of activated Mef2+Pax7+ cells. 1579 cells observed.
Table S23. Percentage of activated Mef2+Myogenin+ cells. 1355 cells observed.

Table S24. Percentage of activated Pax7 cells in group. 588 cells observed.

Table S25. Percentage of activated Myogenin cells in group. 5 cells observed. Table S26: Primary antibodies used in this study.

Table S27: Secondary antibodies.

Table S28: Primers sequences.