Article

FSHD muscular dystrophy region gene 1 binds Suv4-20h1 histone methyltransferase and impairs myogenesis

Maria Victoria Neguembor1,2, Alexandros Xynos1, Maria Cristina Onorati3, Roberta Caccia1, Sergio Bortolanza1, Cristina Godio1, Mariaelena Pistoni1, Davide F. Corona3, Gunnar Schotta4, and Davide Gabellini1,∗

1 Dulbecco Telethon Institute and Division of Regenerative Medicine, San Raffaele Scientific Institute, 20132 Milano, Italy
2 Università Vita-Salute San Raffaele, 20132 Milano, Italy
3 Dulbecco Telethon Institute, Università degli Studi di Palermo, Dipartimento STEMBIO – Sezione Biologia Cellulare, 90128 Palermo, Italy
4 Munich Center for Integrated Protein Science and Adolf Butenandt Institute, Ludwig Maximilians University, 80336 Munich, Germany
* Correspondence to: Davide Gabellini, E-mail: gabellini.davide@hsr.it

FSHD muscular dystrophy (FSHD) is an autosomal dominant myopathy with a strong epigenetic component. It is associated with deletion of a macrosatellite repeat leading to over-expression of the nearby genes. Among them, we focused on FSHD region gene 1 (FRG1) since its over-expression in mice, Xenopus laevis and Caenorhabditis elegans, leads to muscular dystrophy-like defects, suggesting that FRG1 plays a relevant role in muscle biology. Here we show that, when over-expressed, FRG1 binds and interferes with the activity of the histone methyltransferase Suv4-20h1 both in mammals and Drosophila. Accordingly, FRG1 over-expression or Suv4-20h1 knockdown inhibits myogenesis. Moreover, Suv4-20h1 KO mice develop muscular dystrophy signs. Finally, we identify the FRG1/Suv4-20h1 target Eid3 as a novel myogenic inhibitor that contributes to the muscle differentiation defects. Our study suggests a novel role of FRG1 as epigenetic regulator of muscle differentiation and indicates that Suv4-20h1 has a gene-specific function in myogenesis.

Keywords: D4Z4, copy number variation, muscle differentiation, H4K20, chromatin, SUV4-20H1, KMT5B

Introduction

Facioscapulohumeral muscular dystrophy (FSHD, OMIM 158900) is the third most common myopathy and exhibits autosomal dominant inheritance, with no effective treatment currently available (Cabianca and Gabellini, 2010). FSHD typically arises with a reduction of facial and shoulder girdle muscle mass. The disease may extend to abdominal and pelvic girdle muscles impairing the ability to walk. Although FSHD is primarily a disease of skeletal muscle, up to 75% of FSHD patients also present with vascular defects (Fitzsimons et al., 1987; Padberg et al., 1995; Osborne et al., 2007).

FSHD is characterized by extreme variability. Asymmetric distribution of muscle wasting and gender differences in the severity of the phenotype are often observed (Zatz et al., 1998; Tonini et al., 2004). Besides, onset, progression and severity of the phenotype differ dramatically among patients, even between individuals carrying the same genetic mutation. Notably, discordant disease progression has been reported in several monozygotic twins (Tawil et al., 1993; Griggs et al., 1995; Hsu et al., 1997; Tupler et al., 1998). Although the molecular basis of this heterogeneity is not fully understood, there is increasing evidence that it derives from the interplay of complex genetic and epigenetic events (Neguembor and Gabellini, 2010).

FSHD is associated with reduction in the copy number of the D4Z4 macrosatellite repeat that is located at the 4q35 subtelomere (Wijmenga et al., 1992). In healthy individuals, the number of repeats varies between 11 and 100, while FSHD patients carry 1–10 repeats (van Deutekom et al., 1993). The reduction in D4Z4 copy number causes a Polycomb/Trithorax switch leading to the over-expression of several genes within the FSHD region (Cabianca et al., 2012). The unusual nature of the mutation that causes FSHD and its complex effect on chromatin surrounding the 4q35 region makes it highly unlikely that the root cause of the disease can be attributed to a single gene. Since the expression of multiple genes is affected, the molecular pathogenesis of FSHD has been challenging to untangle, and as yet no therapy is available. The two most important FSHD candidate genes are the D4Z4 repeat gene double homeobox 4 (DUX4) (Snider et al.,...
2009, 2010; Lemmers et al., 2010) and the proximal gene FSHD region gene 1 (FRG1) (Gabellini et al., 2002). DUX4 transgenic mice have been recently described (Krom et al., 2013). Despite displaying a DUX4 expression pattern and an alteration of DUX4 target genes similar to FSHD patients, these mice do not show any obvious muscle phenotype (Krom et al., 2013). On the contrary, FRG1 transgenic mice develop muscular dystrophy (Gabellini et al., 2006). In addition, studies conducted in X. laevis and C. elegans revealed that frg1 is required for normal muscle development and its over-expression causes muscle defects and vascular abnormalities correlated with the clinical findings from FSHD patients (Hanel et al., 2009; Wuebbles et al., 2009; Liu et al., 2010).

FRG1 is a dynamic nuclear and cytoplasmic shuttling protein that, in skeletal muscle, is also localized to the sarcomere (Hanel et al., 2011). Interestingly, over-expressed FRG1 is almost completely nuclear and is localized in nucleioli, Cajal bodies, and actively transcribed chromatin (van Koningsbruggen et al., 2004; Sun et al., 2011). Although it has been associated with RNA biology (van Koningsbruggen et al., 2004, 2007; Gabellini et al., 2006; Sun et al., 2011), the molecular and cellular mechanism that follows FRG1 over-expression leading to muscular defects is currently unknown.

Here, we show that FRG1 directly binds to suppressor of variegation 4–20 homolog 1 (Suv4-20h1), a histone methyltransferase involved in constitutive heterochromatin formation (Schotta et al., 2004; Gonzalo et al., 2005; Benetti et al., 2007). Our data indicate that Suv4-20h1 is required for myogenic differentiation and FRG1 over-expression interferes with its function. Finally, we show that EP300 interacting inhibitor of differentiation 3 (Eid3) is an FRG1/Suv4-20h1 epigenetic target. Based on these findings, we propose that FRG1 and Suv4-20h1 are novel epigenetic regulators of muscle differentiation.

Results
FRG1 directly interacts with the histone methyltransferase Suv4-20h1
To address the molecular mechanism that follows FRG1 over-expression, we identified potential interaction partners using an unbiased yeast two-hybrid screening. In accordance with van Koningsbruggen et al. (2007), we identified karyopherin alpha 2 (KPN2A) as first interactor (30% of positive clones). The second interactor identified (18.8% of positive clones) corresponded to the C-terminus region (517–885) of human suppressor of variegation 4–20 homolog 1 (Suv4-20h1), a histone methyltransferase responsible for the di- and tri-methylation of lysine 20 of histone 4 (H4K20me2 and H4K20me3) (Schotta et al., 2004). These epigenetic modifications play a crucial role in the control of repressive heterochromatin (Schotta et al., 2004). Interestingly, there are several indications that H4K20me3 is implicated in muscle differentiation (Biron et al., 2004; Terranova et al., 2005; Tsang et al., 2010). The levels of H4K20me3 dramatically increase during muscle differentiation (Biron et al., 2004; Terranova et al., 2005) and it has been suggested that this could act as a switch in the myogenic program (Tsang et al., 2010). Therefore, the interaction of FRG1 with Suv4-20h1 has appeared potentially relevant as it could provide a molecular clue in the myogenic defects associated with FRG1 over-expression.

We first investigated the FRG1/Suv4-20h1 interaction in vivo by co-immunoprecipitation and found that endogenous Suv4-20h1 interacts with over-expressed FRG1 (Figure 1A). To confirm it, we used co-immunoprecipitation with epitope-tagged proteins and showed that FRG1 binds to both human and murine Suv4-20h1 (Supplementary Figure S1A and B). Next, we performed in vitro pull-down assays using purified, recombinant full-length proteins as well as the C-terminus region of Suv4-20h1. We established that FRG1 and Suv4-20h1 interact in a direct manner and that the binding occurs through the C-terminal region of the protein (Figure 1B), validating our yeast two-hybrid results. In particular, from a panel of truncated forms of Suv4-20h1 (Figure 1C), we found that the Suv4-20h1(509–630) region was sufficient for FRG1 binding in vitro (Figure 1D) and in vivo (Figure 1E).

We sought to investigate the FRG1 and Suv4-20h1 interaction at the cellular level. Figure 2 shows that, when expressed singularly, the two proteins displayed distinct localizations in line with previous reports (Schotta et al., 2004; van Koningsbruggen et al., 2004, 2007; Hanel et al., 2011). Suv4-20h1 was enriched in heterochromatic regions (Schotta et al., 2004), while FRG1 was broadly distributed in the nucleus with nucleolar enrichment (van Koningsbruggen et al., 2004, 2007; Hanel et al., 2011). Strikingly, in cells over-expressing FRG1, Suv4-20h1 was de-localized from heterochromatin, displayed a wider nucleoplasmic distribution, and co-localized with FRG1 (Figure 2A). Several controls support the specificity of these results. Firstly, the result was independent from the position or the nature of the tag fused to Suv4-20h1 (data not shown). Secondly, the localization of a Suv4-20h1 isoform lacking the FRG1-binding domain (Suv4-20h1.2) (Tsang et al., 2010) was not altered in cells over-expressing FRG1 (Figure 2B). Finally, Suv4-20h2, a heterochromatin-enriched histone methyltransferase that shares the enzymatic activity of Suv4-20h1 (Schotta et al., 2004), was unaffected by FRG1 over-expression (Figure 2C).

Collectively, our results suggest that FRG1 over-expression specifically alters Suv4-20h1 sub-nuclear distribution, titrating it away from some target loci.

The functional interaction between FRG1 and Suv4-20h1 is evolutionarily conserved in Drosophila
Since the Drosophila homolog of Suv4-20h1, dSuv4-20, was identified as a dominant suppressor of position effect variegation (PEV) (Schotta et al., 2004), we investigated whether dFRG1, the Drosophila homolog of FRG1, could also have an effect on PEV. While no dFRG1 mutant is available, we took advantage of available dFRG1 RNAi flies (Vienna Drosophila RNAi Stock center). As previously done (Schotta et al., 2004), PEV analyses were conducted on the T(2;3)Sb strain background where the dominant negative marker Stubble (Sb), which gives rise to short bristles, is translocated close to pericentric heterochromatin, being hence subjected to PEV-dependent silencing (Moore et al., 1983; Sinclair et al., 1983). When crossed into the T(2;3)Sb strain background, Suv4-20h1 RNAi mutation leads to de-repression of the dominant Stubble allele (Supplementary Figure S2A; Fisher’s exact test: P < 0.0001, n = 400 from 20 flies), as previously reported (Schotta et al., 2004). Conversely, UAS-FRG1 RNAi (FRG1 RNAi) flies showed stronger silencing of pericentric heterochromatin compared with the control (Supplementary Figure S2A; Fisher’s exact test: P < 0.0001, n = 400 from 20 flies). To investigate the molecular mechanism, we
monitored the levels of the dSuv4-20-associated repressive histone mark H4K20me3 on chromosomal spreads from salivary glands. As previously reported (Schotta et al., 2004), Suv4-20h1*GB00814 mutation leads to a decrease in H4K20me3 compared with controls (Supplementary Figure S2C and D; unpaired t-test: \( P < 0.0001 \), \( n = 5 \)). On the contrary, FRG1 RNAi flies displayed increased H4K20me3 levels (Supplementary Figure S2C and D; unpaired t-test: \( P < 0.0001 \), \( n = 5 \)). These data indicate that the interaction between FRG1 and Suv4-20h1 is evolutionarily conserved and suggest that the regulation of Suv4-20 function is part of the normal FRG1 activity in Drosophila.

**FRG1 over-expression or Suv4-20h1 knockdown inhibits myogenic differentiation of C2C12 muscle cells**

The regulation of H4K20 methylation has been associated with muscle differentiation (Biron et al., 2004; Terranova et al., 2005).

Besides, over-expression of Suv4-20h proteins can enhance myogenic differentiation (Tsang et al., 2010). Based on our results, we reasoned that over-expression of FRG1 could interfere with Suv4-20h1 function. To verify this, we investigated the myogenic differentiation of C2C12 muscle cells over-expressing FRG1 or knockdown for Suv4-20h1. Both FRG1 over-expression and Suv4-20h1 knockdown, using three independent shRNAs, reduced the myogenic differentiation ability of C2C12 cells (Figure 3A–D; paired t-test: \( P = 0.0067 \), \( n = 3 \) and one-way ANOVA test: \( P < 0.0001 \), \( n = 3 \), respectively). Noteworthily, low levels of FRG1 over-expression and a partial Suv4-20h1 knockdown were sufficient to observe this phenotype (Figure 3E and F). These results indicate that appropriate expression levels of both proteins are required for muscle differentiation in C2C12 cells.
Our data suggest that the interference with Suv4-20h1 function is an important mechanism through which FRG1 over-expression affects myogenic differentiation. Based on this, we reasoned that over-expression of Suv4-20h1 in FRG1 over-expressing cells could rescue their myogenic defect. Since the constitutive over-expression of Suv4-20h1 is not well tolerated by C2C12 myoblasts (Tsang et al., 2010), we used an inducible Suv4-20h1_ERα fusion, allowing the translocation of the protein to the nucleus upon 4-hydroxytamoxifen (4-OHT) treatment. By performing differentiation experiments, we observed a partial but significant amelioration of the phenotype in Suv4-20h1_ERα/FRG1 over-expressing cells treated with 4-OHT compared with control cells (FRG1) (Figure 4A–C; two-way ANOVA test, \( P = 0.0406; n = 3 \)). Overall, these results indicate that FRG1 over-expression inhibits muscle differentiation at least in part by interfering with Suv4-20h1 function.

Muscle-specific Suv4-20h knockout mice develop muscular dystrophy signs

Suv4-20h1 and the related enzyme Suv4-20h2 share functional redundancy in muscle (Schotta et al., 2008). To further investigate the role of Suv4-20h on muscle biology, we crossed Suv4-20h1−/−/Suv4-20h2−/− mice with transgenic mice expressing cre recombinase selectively in the skeletal muscles.
muscle to obtain Suv4-20h1_Suv4-20h2 muscle-specific double knockout (mDKO) mice. Unfortunately, we obtained only a partial excision of the Suv4-20h1\textsuperscript{floox} allele and a partial reduction of Suv4-20h1 expression (Figure 5A and B), resulting in significant residual H4K20me3 levels in the skeletal muscle (Figure 5C). Nevertheless, mDKO mice displayed several signs of muscular dystrophy, including necrosis (Figure 5D and E; Mann–Whitney test: \( P = 0.0079, n = 5 \)) and an increased number of centrally nucleated myofibers (Figure 5D and F; Mann–Whitney test: \( P = 0.0079, n = 5 \)). Collectively, these results suggest that Suv4-20h1 activity plays a relevant role in muscle biology and the interference with Suv4-20h1 function might contribute to the muscular dystrophy signs associated with FRG1 over-expression.

The novel inhibitor of differentiation Eid3 is an FRG1/Suv4-20h1 target involved in the myogenic defects caused by FRG1 over-expression

Based on our results, we hypothesized that FRG1 could repress myogenesis at least in part by binding to Suv4-20h1 and interfering with its function. While Suv4-20h1 has been mainly associated with establishment and maintenance of constitutive heterochromatin (Schotta et al., 2004), we found no evidence of global changes in H4K20me3 in FRG1 over-expressing cells and a slight reduction in Suv4-20h1 knockdown cells compared with controls (Supplementary Figure S3). This result is expected since Suv4-20h2 can compensate for the lack of Suv4-20h1 in pericentric heterochromatin regions that constitute the major target of Suv4-20h proteins (Schotta et al., 2008). Given this, we reasoned...
that FRG1 could act at a gene-specific level by hindering the recruitment of Suvs4-20h1 to a subset of its targets. For example, the over-expression of FRG1 could prevent the silencing of ‘myogenic inhibitors’ by Suvs4-20h1. To test our hypothesis, we focused on the differential expression of differentiation inhibitor genes in skeletal muscles from FRG1 mice compared with WT controls (Xynos et al., 2013). Among the differentially expressed genes, DNA microarray and qRT–PCR validation (Supplementary Figure S4) identified the up-regulation of Eid3 (Bavner et al., 2005). Despite its name, no information is available with regard to the biological function of Eid3. To understand if Eid3 could play a role in myogenic differentiation, we first analysed its expression in both primary and C2C12 muscle cells and we found that Eid3 is normally silenced during myogenic differentiation (Figure 6A and B; unpaired t-test: \( P < 0.0001, n = 3 \) and one sample t-test: \( P = 0.0052, n = 3 \), respectively). To assess whether its repression is required for muscle differentiation, we generated stable Eid3 over-expressing C2C12 cells (pH-Eid3) where we observed that Eid3 over-expression reduces myogenic differentiation compared with control cells (pFH) (Figure 6C–E; paired t-test: \( P = 0.0057, n = 3 \)). This suggests that Eid3 acts as an inhibitor of muscle differentiation.

Interestingly, we found that Eid3 expression remains significantly higher in muscles and C2C12 cells over-expressing FRG1 (Figure 7A and B; paired t-test: \( P = 0.0039, n = 5 \) and one sample t-test: \( P = 0.0025, n = 4 \), respectively). Importantly, increased Eid3 expression was already present in young, pre-dystrophic FRG1 mice indicating that altered Eid3 expression is not simply secondary to muscle wasting (Figure 7A; paired t-test: \( P = 0.0039, n = 5 \)). Moreover, Eid3 over-expression was significantly higher in muscles that are highly affected in FRG1 mice (vastus lateralis) compared with mildly affected muscles (biceps brachii), suggesting that deregulation of Eid3 expression correlates with the severity of the disease in different muscles (Figure 7C; paired t-test: \( P = 0.0086, n = 3 \)). Eid3 expression was also significantly increased in muscles from mDKO mice and in C2C12 muscle cells knocked down for Suvs4-20h1 (Figure 7D and E; unpaired t-test: \( P = 0.0019, n = 5 \) and one sample t-test: \( P = 0.0039, n = 4 \), respectively), suggesting that FRG1 over-expression affects Eid3 expression through Suvs4-20h1. Intriguingly, we observed that Eid3 expression was significantly up-regulated in biopsies of FSHD patients compared with healthy subjects (Figure 7F; one-way ANOVA test: \( P = 0.0029, n = 7 \), while Eid3 levels were normal in patients affected by other types of muscular dystrophy (Figure 7F, \( n = 8 \)). Similar results were obtained for FRG1 (Figure 7G, one-way ANOVA test: \( P = 0.0013, n = 7–8 \)). However, Suvs4-20h1 and β-glucuronidase (GUS), a gene with stable expression in FSHD (Krom et al., 2012), were not significantly altered in FSHD patients compared with controls (Supplementary Figure S5), indicating that FRG1 and Eid3 up-regulation is not a general feature of muscular dystrophies. Notably, Eid3 up-regulation was significantly correlated with increased FRG1 levels (Figure 7H, Pearson’s test: \( R^2 = 0.6611, P < 0.0001, n = 22 \)).
To determine whether the aberrant Eid3 up-regulation could be attributed to a lack of epigenetic silencing by Suv4-20h1, we investigated the levels of the Suv4-20h1-associated repressive histone mark, H4K20me3, at the Eid3 promoter. Chromatin immunoprecipitation revealed that H4K20me3 levels were significantly reduced at the Eid3 genomic regions spanning −6 to −2 kb from the transcription start site, both in FRG1 over-expressing and Suv4-20h1 knockdown C2C12 muscle cells (Figure 7I and J; two-way ANOVA test: \( P = 0.0099 \) and \( P = 0.0043 \), respectively). These data suggest that FRG1 over-expression might interfere with Suv4-20h1-dependent H4K20 trimethylation of the Eid3 promoter, potentially resulting in its aberrant up-regulation.

To investigate whether the lack of repression of Eid3 plays a role in the FRG1-associated phenotype, we down-regulated Eid3 expression in C2C12 cells over-expressing FRG1. Down-regulation of Eid3 was able to significantly rescue the myogenic defect of FRG1 over-expressing cells compared with controls (Figure 8A–C; paired t-test: \( P = 0.0009 \), \( n = 5 \)). Collectively, our results suggest that the over-expression of FRG1 interferes with the repressive activity of Suv4-20h1 leading to aberrant Eid3 up-regulation and myogenic defects.

**Discussion**

In this study, we focused on the largely unexplored role of FRG1 in muscle biology. We have recognized Suv4-20h1 as a direct FRG1 interactor and revealed that it is aberrantly localized upon FRG1 over-expression, suggesting that over-expression of FRG1 could interfere with Suv4-20h1 function. Accordingly, the lack of Suv4-20h1 reproduces phenotypes similar to the FRG1 over-expression while its over-expression ameliorates FRG1-associated...
myogenic defects. Altogether, these results indicate that the interference with Suv4-20h1 activity might play a relevant role in the myogenic defects associated with FRG1 over-expression. Notably, similar mechanisms might govern differentiation in other contexts. For example, it has been recently reported that the differentiation of postnatal spermatogonial progenitor cells (SPCs) is regulated by physical interaction and altered localization of the essential factors Sall4 and Plzf (Hobbs et al., 2012). Similarly to FRG1, Plzf is localized to euchromatic regions and nuclear speckles (Melnick et al., 2000). On the contrary, Sall4 is associated with DAPI-dense pericentric heterochromatin like Suv4-20h1 (Sakaki-Yumoto et al., 2006; Yamashita et al., 2007). When its expression increases in postnatal testis, Plzf binds Sall4 sequestering it away from heterochromatin. This allows the expression of Sall1, a gene repressed by Sall4, and the inhibition of SPCs differentiation. Thus, it is tempting to speculate that the regulation of differentiation through altered localization of a heterochromatin-associated protein could be a more general mechanism used by other proteins.

Suv4-20h1 has been traditionally considered to be involved in the structural maintenance of constitutive heterochromatin (Schotta et al., 2004; Gonzalo et al., 2005; Benetti et al., 2007). Instead, our data show that Suv4-20h1 plays a relevant role in muscle biology and uncover a novel function for Suv4-20h1 as a gene-specific repressor required for myogenic differentiation. In particular, our results suggest that Eid3 (Bavner et al., 2005) is a novel inhibitor of differentiation and a Suv4-20h1 target. We found that Eid3 expression is normally silenced upon induction of myogenic differentiation, but its silencing fails in C2C12 muscle cells over-expressing FRG1 or knocked down for Suv4-20h1. Based on our results, we propose that FRG1 over-expression might sequester Suv4-20h1 away from its epigenetic targets leading to their inappropriate de-repression (Figure 8D). Accordingly, we found that FRG1 over-expression and Suv4-20h1 knockdown are both associated with an epigenetic de-regulation of the Suv4-20h1 enzymatic product, the repressive mark H4K20me3, at the Eid3 promoter.

Despite its extensive study, FSHD pathogenesis remains unclear and controversial. All current models predict that deletion of D4Z4 repeats results in the de-regulation of a candidate gene(s) located in the FSHD region, leading to disease (Cabianca and Gabellini, 2010; van der Maarel et al., 2011). While the two most accepted FSHD candidate genes are DUX4 and FRG1, the molecular and cellular mechanism following their de-regulation and finally causing the disease remains elusive. Furthermore, FSHD is characterized

Figure 6 Eid3 is down-regulated upon muscle differentiation and behaves as a myogenic inhibitor gene. (A) qRT–PCR shows that WT primary myoblasts express significantly higher Eid3 levels than myotubes (unpaired t-test: P < 0.0001, n = 3, mean ± SEM). (B) qRT–PCR shows that C2C12 myoblasts express significantly higher Eid3 levels compared with myotubes (one sample t-test: P = 0.0052, n = 3, mean ± SEM). Immunofluorescence for Mhc (C) and fusion index analysis (D) show that pH-Eid3 over-expressing cells display a significantly decreased fusion index compared with control (pFH) (paired t-test: P = 0.0057, n = 3, mean ± SEM). (E) Immunoblotting performed on pFH and pH-Eid3 myoblasts (anti-HA and anti-Tubulin).
Figure 7 The myogenic inhibitor gene Eid3 is specifically over-expressed in FSHD and is an FRG1/Suv4-20h1 target. (A–F) qRT–PCR for Eid3 in several biological samples. (A) Eid3 is significantly up-regulated in vastus from asymptomatic 3-week-old FRG1 mice compared with WT controls (paired t-test: \( P = 0.0039, n = 5 \), mean ± SEM). (B) Eid3 levels are significantly increased in C2C12 myotubes over-expressing FRG1 compared with empty vector controls (one sample t-test: \( P = 0.0025, n = 4 \), mean ± SEM). (C) Eid3 expression is preferentially altered in severely affected muscles (vastus lateralis) compared with mildly affected muscles (biceps brachii) (paired t-test: \( P = 0.0086, n = 3 \), mean ± SEM). (D) Eid3 is significantly more abundant in mDKO mice than WT controls (unpaired t-test: \( P = 0.0019, n = 5 \), mean ± SEM). (E) Eid3 is significantly up-regulated in C2C12 muscle cells knockdown for Suv4-20h1 compared with non-silencing control cells (one sample t-test: \( P = 0.0039, n = 4 \), mean ± SEM). (F) Eid3 levels are significantly increased in FSHD muscle biopsies compared with healthy and other muscular dystrophy controls (one-way ANOVA test: \( P = 0.0029, n = 7–8 \), mean ± SEM). (G) qRT–PCR for FRG1 in human muscle biopsies shows that FRG1 is specifically over-expressed in FSHD patients compared with healthy and other muscular dystrophy controls (one-way ANOVA test: \( P = 0.0013, n = 7–8 \), mean ± SEM). (H) Pearson’s correlation analysis shows that FRG1 and Eid3 expression levels are highly correlated (\( R^2 = 0.6611; P < 0.0001, n = 22 \)). (I and J) Chromatin immunoprecipitation, using total H4, H4K20me3, and IgG. H4K20me3 is significantly reduced at the Eid3 genomic region spanning −6 to −2 kb from TSS in FRG1 over-expressing (I; two-way ANOVA test: \( P = 0.0099 \), representative experiment, mean ± SEM) and Suv4-20h1 knockdown (J; two-way ANOVA test: \( P = 0.0043 \), representative experiment, mean ± SEM) C2C12 myotubes. H4K20me3 and IgG levels are relative to H4 and normalized by the H4K20me3 − 6 kb region enrichment levels of control samples (pFH and non-silencing, respectively).
Epigenetic deregulation by FRG1

Figure 8 Eid3 knockdown rescues the myogenic capability of FRG1 over-expressing cells. Immunofluorescence for Mhc (A) and fusion index analysis (B) show that Eid3 knockdown significantly ameliorates the differentiation capability of pFH-FRG1 over-expressing cells (Eid3 siRNA) compared with non-silencing control (control siRNA) (paired t-test: \( P = 0.0009, n = 5, \text{mean} \pm \text{SEM} \)). (C) qRT–PCR analysis for Eid3 in pFH-FRG1/ Eid3 siRNA cells displays a partial Eid3 knockdown compared with pFH-FRG1/control siRNA cells (paired t-test: \( P = 0.0016, n = 5, \text{mean} \pm \text{SEM} \)). Scale bars, 200 \( \mu \text{m} \). (D) Proposed model for the effects of FRG1 over-expression.

by an extreme variability in disease onset, progression and severity. This heterogeneity in disease manifestation could reflect heterogeneity in gene expression of FSHD candidate gene(s). An interesting possibility, therefore, is that the complexity of FSHD could be explained envisaging that the epigenetic alteration of DUX4, FRG1, and other potential genes could collaborate to determine the final phenotype. In this context, it is relevant to investigate the biological role of these players and address how each could contribute to the different aspects of the disease such as the muscle differentiation defects described in FSHD (Tupler et al., 2003; Celegato et al., 2005). We found that Eid3 is up-regulated in affected muscles of FRG1 over-expressing or Suv4-20h knock out mice and Eid3 levels are inappropriately increased in biopsies of FSHD patients. Our results suggest that FRG1 and Eid3 up-regulation is not a general feature of muscular dystrophies but selectively found in FSHD patients when compared with other muscular dystrophy patients. Importantly, we have observed that Eid3 over-expression causes muscle differentiation defects while its knockdown rescues the myogenic defects in FRG1 over-expressing cells. Overall, these data promote Eid3 as a novel myogenic inhibitor that might explain, at least in part, the muscle differentiation defects associated to FRG1 over-expression.

Materials and methods

Ethics statement

All procedures involving human samples were approved by the Fondazione San Raffaele del Monte Tabor Ethical Committee. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Fondazione San Raffaele del Monte Tabor and were communicated to the Ministry of Health and local authorities according to Italian law.

Yeast two-hybrid screening

MATCHMAKER two-hybrid system 3 (Clontech) was used for this study. DNA-BD/FRG1 and AD/HeLa cDNA plasmid library (3.5 x 10^6 independent clones) were co-transformed in yeast and plated onto high stringency SD/- Ade/-His/-Leu/-Trp/3-AT/X-a-Gal plates. 20 x 10^6 clones were screened corresponding to ~6-fold coverage of the HeLa cDNA library. AD/libraries plasmids were isolated from positive clones, rescued via transformation of Escherichia Coli, and sequenced.

Constructs and cloning procedures

Primers employed for cloning are listed in Supplementary Table S1. PCR amplifications were performed with Pfx50 DNA polymerase (Invitrogen) or GoTaq polymerase (Promega). PCR products were digested with the restriction enzymes (Takara) listed in Supplementary Table S1. For pCMV-myc-FRG1, FRG1 coding sequence was excised with PstI from pGBKT7-FRG1, blunted with T4 DNA polymerase (Fermentas), and digested with SflI. pCMV-myc (Clontech) was digested with Xhol, blunted with Klenow Fragment (Fermentas), and digested with SflI. pDEST15GW-Suv4-20h1, pGEX-6P1GW-Suv4-20h1(385-874 aa), and pEGFP-N1-Suv4-20h1 were previously described (Schotta et al., 2004). pLKO.1 lentiviral vectors expressing control shRNA or shRNAs for Suv4-20h1 and packaging constructs were purchased from Open Biosystems. pBABE-SUV4-20H1_ERa plasmid was a kind gift from Dr Holger Bierhoff. For pIRE3neo3-HA-Eid3 (pFH-Eid3), Eid3 was first amplified from C2C12 cDNA and cloned
into pCMV-HA (Clontech). The HA-Eid3 sequence was excised with StuI and NotI and ligated into pIRESneo3 (Clontech).

**Protein purification**

6 × His-FRG1 and GST-Suv4-20h1 proteins were expressed in Rosetta2(DE3)pLys E.coli (Novagen), at 0.4–0.6 OD with 1 mM IPTG (Biosciences) for 3 h at 37°C (or 8 h at 30°C for GST-Suv4-20h1 full-length). Bacterial pellets were resuspended in PBS and Protease Inhibitor cocktail (PI; Sigma) or in Lysis Buffer (50 mM NaH2PO4, 250 mM NaCl, pH 8.0, plus PI) for GST- and His-tagged proteins, respectively. Bacteria were sonicated (Bandelin), incubated by gentle rotation for 15 min at 4°C after adding Triton X-100 (1%; Sigma), and centrifuged at 19000 rpm at 4°C for 20 min. Supernatants were incubated for 1 h at 4°C in batch with Glutathione-Agarose beads (Sigma) or HIS-Select Nickel Affinity gel beads (Sigma). Beads were washed on column by gravity flow with PBS-Triton X-100 (1%) plus PI or Lysis Buffer with 10 mM imidazole (Fluka), for GST- and His-tagged proteins, respectively. Proteins were eluted with 50 mM Tris–HCl (pH 9.0), 10 mM reduced l-glutathione (Sigma) plus PI or 50 mM NaH2PO4, 250 mM NaCl, 250 mM imidazole (pH 8.0) plus PI, for GST- and His-tagged proteins, respectively. Proteins were dialyzed overnight at 4°C in Slide-A-Lyzer Dialysis Cassettes (Thermo scientific) in 50 mM Tris–HCl (pH 9.0) or in 50 mM NaH2PO4, 250 mM NaCl (pH 8.0), for GST- and His-tagged proteins, respectively. After dialysis, the latter were supplemented with 10% glycerol.

**Co-immunoprecipitation assays**

For Figure 1A, HEK293T cells were transfected with pCMV-Myc-FRG1. For Supplementary Figure S1A and B, HEK293T cells were co-transfected in a 1:1 ratio with pCMV-HA-Suv4-20h1/pCMV-HA-Suv4-20h1(509–630)/pCMV-HA or pEGFP-N1-Suv4-20h1/pEGFP-N3 and pCMV-Myc-FRG1/pCMV-Myc with Lipofectamine LTX (Invitrogen). Cells were collected after 36 h from transfection. Co-immunoprecipitation (co-IP) assays were performed as described previously (van Koningsbruggen et al., 2007) with mouse anti-HA clone 16B12 (MMS-101R, Covance), rabbit anti-GFP (A11122, Molecular Probes), rabbit anti-Myc (Calbiochem) and rabbit anti-GST (G-8035, Jackson Immunoresearch). Input (0.1% or 3%) and Bound (20%) fractions of the co-IP were analyzed by immunoblotting with the above-mentioned primary antibodies at 1/500 dilution for anti-SUV4-20h1 and 1/1000 dilution for anti-HA, anti-GFP, and mouse anti-c-Myc clone 9E10 (MMS-150R Covance).

**GST and histidine pull-down assays**

Pull-down assays were performed by incubating, overnight at 4°C, equal molar amounts (10–50 picomoles) of GST-tagged and His-tagged proteins in cold CHAPS buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% CHAPS (3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; Fluka] and PI (Sigma) plus 5 mM Imidazole for His pull-downs. Twenty microliters of beads slurry were incubated for 1 h at 4°C, washed 4 times with 1 ml cold CHAPS buffer and once with cold 50 mM Tris–HCl (pH 7.4) plus PI, and boiled in 50 μl of 1× Laemmlı buffer at 95°C for 8 min. Input (1%) and Bound (20%) fractions were analyzed by immunoblotting with mouse anti-GST (G1160, Sigma; dilution 1/10000), mouse anti-6× His (#631212, Clontech; dilution 1/50000) and anti-mouse IgG HRP-conjugated secondary antibody (#715-035-150, Jackson Immunoresearch; dilution 1/20000).

**FRG1 and Suv4-20H localization analysis**

C2C12 cells were seeded on glass coverslips and were co-transfected 24 h later with pCMV-Myc-FRG1 or pCMV-Myc and pEGFP-C1-SUV4-20H1_i1, pEGFP-C1-SUV4-20H1_i2, pEGFP-C1-SUV4-20H2, kindly provided by Alan Underhill (Tsang et al., 2010), or pEGFP-N3 (Clontech) in a 1:1 ratio (500 ng each) using Lipofectamine LTX. Cells were fixed in 4% paraformaldehyde (PFA, Electron Microscopy Science) 36 h post-transfection and immunostained with mouse anti-c-Myc clone 9E10 (MMS-150R, Covance; dilution 1/5000) and Alexa Fluor 555 goat anti-mouse (Molecular Probes; dilution 1/500) for secondary detection. Samples were mounted in aqueous medium and acquired using a DeltaVision Restoration Microscopy System (Applied Precision) built around an Olympus IX70 microscope equipped with a mercury-arc illumination CoolSnap_Hq/ICX285 CCD camera. Images of 0.1 μm sections were collected with an Olympus 60X/1.4 NA Plan Apo oil immersion objective lens and deconvolved with SoftWoRx 3.5.0 (Applied Precision) by the constrained iterative algorithm using 10 iterations and standard parameters. Representative pictures of three independent experiments are shown.

**Drosophila PEV analysis**

Flies were raised at 25°C on K12 Medium (USBiological). All crosses were conducted at 25°C. Suv4-20h1Δ (Bloomington, stock # 12510), UAS-FRG1RNAi (Vienna Drosophila RNAi Stock Center, stock # v23447), w118 (Bloomington, stock # 3605) and T(2;3)SB′/TM3, Ser kindly provided by Sergio Pimpinelli) fly strains were employed for this study. T(2;3)SB′ translocation juxtaposes the Sb mutation and the centric heterochromatin of the second chromosome, resulting in mosaic flies with both Sb and WT bristles. Ten pairs of major dorsal bristles of 20 flies were analyzed assigning an Sb− or Sb+ phenotype to each bristle. The extent of Sb variegation was expressed as the mean of Sb and WT bristles per strain.

**Immunofluorescence on Drosophila polytene chromosome spreads**

Larval salivary glands were dissected from third-instar larvae grown at 25°C. Immunofluorescence on polytene chromosome spreads were conducted as previously described (Burgio et al., 2008) with rabbit anti-H4K20me3 (ab9053, Abcam; dilution 1/500). Images were acquired with a Leica DM4000B microscope and densitometric analysis was performed with LAS AF Software (Leica). Quantification was carried out by calculating the intensity ratio between FITC (H4-K20me3) and DAPI channels. Five chromosomal spreads were analyzed per strain.

**Cell line generation, cell culture and differentiation**

HEK293T and Phoenix-Eco cells were cultured at 37°C in a 5% CO2 incubator in DMEM, 10% FBS and 1% penicillin/streptomycin. C2C12 cells were cultured at 37°C in a 5% CO2, 5% O2 incubator in DMEM, with 10% FBS and 1% penicillin/streptomycin.

pFH-FRG1 and pH C2C12 cells were previously described (Gabellini et al., 2006).

pLKO.1 C2C12 cells expressing the non-silencing shRNA control or shRNAs specific for Suv4-20h1 were generated by lentiviral transduction according to the manufacturer’s instructions (Open Biosystems) and maintained as polyclonal populations under 0.5 μg/ml puromycin selection.
Epigenetic deregulation by FRG1

pBABE-SUV4-20H1_ERα/pFH-FRG1 over-expressing cells were generated by retroviral transduction of pFH-FRG1 myoblasts. Retroviral particles were prepared from Phoenix-Eco cells (a gift from Dr Gary Nolan) following the Nolan Laboratory protocol (http://www.stanford.edu/group/nolan/protocols/pro_helper_dep.html). Transduced cells were maintained as a polyclonal population under G418 (0.5 μg/ml) and puromycin (0.5 μg/ml) selection. The translocation of SUV4-20H1-ERα to the nucleus was induced with 500 nM 4-OHT (H7904, Sigma) for 72 h prior to differentiation and was maintained during differentiation.

pH-Eid3 cells were generated by transfecting C2C12 cells with linearized pH-Eid3 or pH using Lipofectamine LTX. G418-resistant cells were maintained as a pool and grown under constant selection.

Eid3 knockdown/pFH-FRG1 over-expressing cells were generated by transfecting, 72 h prior to differentiation, pFH-FRG1 myoblasts with 50 nM siRNAs against Eid3 (L-046381-01, ON-TARGETplus SMARTpool, Mouse 1700027M21R, Thermo Scientific) or non-silencing control (D-001810-10, ON-TARGETplus Non targetting pool, Thermo Scientific) following the manufacturer’s instructions.

Protein over-expression and down-regulation were evaluated by immunoblotting with mouse anti-FRG1 (sc-101050, Santa Cruz; dilution 1/500) for pFH-FRG1, mouse anti-HA clone 16B12 (MMS-101R, Covance; dilution 1/500) for pFH-FRG1 and for pH-Eid3, rabbit anti-SUV4-20H1 (ab18186, Abcam; dilution 1/1000) for pLKO.1 SUV4-20H1 knockdown, rabbit anti-ERα (sc-543, Santa Cruz; dilution 1/500) for pBABE-SUV4-20H1-ERα cells and mouse anti-Tubulin (T9026, Sigma; dilution 1/40000) for normalization.

Global H4K20me3 levels were evaluated by immunoblotting with rabbit anti-H4-20me3 (kindly provided by Dr Thomas Jenuwein, dilution 1/300) and rabbit anti-H4 (#62-141-13, Millipore; dilution 1/3000). Histone extracts were obtained following the histone extraction protocol from Abcam (http://www.abcam.com/index.html?pageconfig=resource&rid=11410).

For differentiation experiments, C2C12 cells were plated at confluence in collagen-coated dishes and were differentiated for 3 days in DMEM containing 2% donar horse serum (EuroClone).

For fusion index quantification, cells were fixed in 4% PFA and immunostained with mouse MF20 antibody (Developmental Studies Hybridoma Bank; dilution 1/2) followed by Alexa Fluor 488 goat anti-mouse (Molecular Probes; dilution 1/500) and Hoechst (1 mg/ml; Sigma; dilution 1/2000). Samples were visualized using Observer.Z1 (N-Achroplan 10 x /0.25 NA Ph1) microscope (Zeiss). Fusion index analysis was performed with ImageJ by counting the number of nuclei belonging or not to myotubes (myosin positive syncytia containing at least 3 nuclei). At least three independent differentiation experiments were performed; for each experiment at least 6 fields were analyzed, counting at least 1000 nuclei for each cell type.

Human samples

Muscle biopsies from healthy donors, FSHD patients, and patients affected by other muscular dystrophies (Supplementary Table S2) were obtained from the Italia Telethon Network of Genetic Biobanks (http://www.biobanknetwork.org).

Mouse handling

FRG1-high mice (Gabellini et al., 2006) and control C57BL/6J littersmates were maintained at Charles River (Calco, Italy). To obtain muscle-specific Suv420h1−/−_Suv420h2−/− double knockout mice, Suv420h1−/−Rox and Suv420h2−/− mice (Schotta et al., 2008) were bred with HSA-cre mice, in which the cre recombinase gene is driven by the human alpha-skeletal actin (HSA) promoter. Mice at 3–18 weeks of age were sacrificed for this study.

Primary muscle cell cultures and muscle histology

Cell preparations were obtained from vastus lateralis muscles of 4-week-old males as previously described (Xynos et al., 2011) and were plated in collagen-coated dishes after pre-plating for 1 h in uncoated dishes. Primary myoblasts were grown in nutrient mixture F-10 Ham (Sigma) with 20% PBS (Hyclone) and 5 μg/ml bFGF (Peprotech) for 1–5 days and differentiated in Dulbecco’s modified Eagle medium (DMEM; EuroClone) with 5% donor horse serum (EuroClone) for 1–2 days. Vastus lateralis and tibialis anterior muscles were dissected, frozen in isopentane cooled in liquid nitrogen, and cryosectioned (8-μm thick). Gomori-trichrome staining was performed as previously described (Dubowitz, 1985; Xynos et al., 2011). For H4K20me3 immunofluorescence, tissue sections were fixed in 4% PFA for 10 min at RT and incubated with rabbit anti-H4K20me3 (ab9053, Abcam; dilution 1/200).

Real-time PCR analysis

Total RNA from primary cells and tissues was extracted and treated with DNase 1, using the RNAqueous-4PCR kit (Ambion) and RNeasy Fibrous Tissue Kit (Qiagen), respectively. cDNA was synthesized using Invitrogen’s SuperScript III First-Strand Synthesis Super-Mix. Genomic DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen). qPCRs (for primers see Supplementary Table S2) were performed with SYBR GreenER qPCR SuperMix Universal (Invitrogen) using CFX96 Real-Time PCR Detection System (Bio-Rad). Relative quantification was calculated using CFX Manager Software V1.6. Validation of the differential expression of genes identified by a DNA microarray was performed using TaqMan gene expression assays with custom-made TaqMan array microfluidic cards (Applied Biosystems). Relative quantification was calculated with qBasePLUS V1.5 using Gapdh, Ppia and 18S rRNA as reference genes.

Chromatin immunoprecipitation

Cells were fixed for 10 min in 1% formaldehyde in PBS. After formaldehyde quenching with Glycine (final concentration 125 mM) for 5 min, cells were washed with PBS, harvested by scraping and pelleted. The pellet was lysed in 50 mM HEPES–KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40, and 0.25% Triton X-100 for 10 min on ice. Nuclei were pelleted and subsequently lysed in 10 mM Tris–HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, and 0.5 mM EGTA with a gentle swirl for 10 min. Next, samples were centrifuged and the resulting pellets were resuspended in 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, and 0.5% N-laurylsarcosine. Chromatin was sonicated using Bioruptor (Diagenode) and Triton X-100 was added to lysates (1% final concentration). Ten micrograms of chromatin were used for each immunoprecipitation and pre-cleared for 3 h at 4°C with 20 μl of Protein G dynabeads (Invitrogen). Immunoprecipitations were carried out at 4°C overnight with 50 μl of beads previously bound for 3 h at 4°C with 5 μg of the following antibodies: rabbit anti-H4 (#62-141-13, Millipore), rabbit anti-H4K20me3 (pAb-057-050, Diagenode) and whole molecule rabbit IgG (#011-000-003, Jackson Immunoresearch). Immunoprecipitated

Journal of Molecular Cell Biology | 305
chromatin was washed extensively with 50 mM HEPES-KOH (pH 7.6), 500 mM LiCl, 1 mM EDTA, 1% NP-40, and 0.7% Na-Deoxycholate, and protein–DNA cross-links were reverted overnight at 65°C in TE buffer with 2% SDS. DNA was purified with the QiAquick PCR Purification Kit (Qiagen) and qPCRs were performed with a custom-made ChampionChip PCR Array (SA Biosciences) using an Applied Biosystems VIIa 7 Real-Time PCR System.

**Statistical analysis**

All statistical analyses were two-tailed tests and performed using GraphPad Prism 5.0a (GraphPad Software). The type of statistical test, P-value, number of independent experiments, mean, and standard error of the mean are provided for each dataset in the corresponding figure legends.

**Supplementary material**

Supplementary material is available at Journal of Molecular Cell Biology online.

**Acknowledgements**

We thank G. Cossu (University College London, UK), T. Jenuwein (Max-Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany), and J. Teodorò (Department of Biochemistry, McGill University, Montreal, Quebec, Canada) for helpful discussions. We are grateful to H. Bierhoff (German Cancer Research Center, Heidelberg, Germany) for SUV4-2OH-ER plasmids and D.A. Underhill (University of Alberta, Canada) for plasmids of SUV4-2OH isoforms. C. Covino and M. Ascani (San Raffaele Alembic Bioimaging Center, Milan, Italy) are acknowledged for their excellent technical assistance. Maria Victoria Neguembor conducted this study as partial fulfillment of her PhD in Molecular Medicine, Program in Cellular and Molecular Biology, San Raffaele University, Milan, Italy. Davide Gabellini is a Dulbecco Telethon Institute Assistant Scientist.

**Funding**

This work was supported by the European Research Council (grant number 204279), the Italian Epigenomics Flagship Project, the Italian Ministry of Health (grant number GR-2008-1134796), the Italian Telethon Foundation (grant number S05001TELA), and the FSHD Global Research Foundation.

**Conflict of interest:** none declared.

**References**


Epigenetic deregulation by FRG1


