subunits. Thus, introduction of bulky amino acids narrows the channel pore, while introduction of smaller amino acids widens the channel pore. Our analysis first disclosed that the M3 domain backs up the channel-lining pore that is composed by the M2 transmembrane domains and has sterochemical effects on channel gating kinetics [51].

FCCMS can be effectively treated with anticholinesterases and 3,4-diaminopyridine. The mechanism of action of anticholinesterases is described in the section devoted to endplate AChR deficiency. The drug 3,4-diaminopyridine blocks the presynaptic potassium channel, which slows the repolarization of the action potential delivered to the nerve terminal [67]. The enhanced nerve action potential stimulates the presynaptic voltage-gated P/Q-type and N-type Ca^{2+} channels and increases Ca^{2+} influx to the nerve terminal, which then enhances synaptotagmin and the SNARE complex to facilitate the fusion of ACh vesicles to the presynaptic membrane. This increases the amount of ACh released by a single nerve stimulus and enhances AChR channel openings.

Other phenotypes associated with AChR mutations and a single nucleotide polymorphism

Mutations or a single nucleotide polymorphism (SNP) in muscle nicotinic AChR subunits also give rise to phenotypes other than CMS.

The first phenotype is fetal akinesia deformation sequence (FADS). Mutations in the AChR subunit genes cause neuromuscular transmission defects in embryos and restrict intrauterine movements. As human embryos use the fetal γ-AChR by 33 weeks of gestation [68], mutations in *CHRNG* [69,70], as well as in *CHRNA1* and *CHRND* [27], cause FADS.

The second phenotype is early onset myasthenia gravis [71]. Promiscuous expression of a set of self-antigens occurs in medullary thymic epithelial cells to impose T-cell tolerance and to provide protection against autoimmune disorders. The AChR α1 subunit is one of those self-antigens. A SNP in the promoter region of *CHRNA1* compromises expression of the α1 subunit in thymic epithelial cells, which increases the chance of developing myasthenia gravis 2.01- to 2.35-fold in individuals carrying the SNP.

Conclusions

We addressed three types of CMS that are caused by mutations in the AChR subunit genes.

Congenital deficiency of endplate AChRs is caused by mutations in *CHRNA1*, *CHRNB1*, *CHRND*, and *CHRNE* encoding the AChR α1, β1, δ, and ε subunits, respectively. The mutations are classified into two groups. The first group includes mutations in *CHRNE* that nullify or significantly reduce the expression of the ε subunit. Patients survive with embryonic γ-AChR even when the adult-type ε-AChR is lacking. Null mutations in the other AChR subunit genes are likely to be fatal, which supports a general notion that we have no chance to identify mutations that result in lethal phenotypes. The second group of mutations includes missense mutations of *CHRNA1*, *CHRNB*, and *CHRND*. These mutations compromise the expression level of the mutant subunit and/or the assembly of AChRs, but do not completely abolish the expression of AChRs. Differences between mutations in *CHRNE* and those in *CHRNA1*, *CHRNB*, and *CHRND* are the tolerance to low expression of the affected subunit. As in autoimmune myasthenia gravis, endplate AChR deficiency is well controlled by anticholinesterases.

The slow channel congenital myasthenic syndrome (SCCMS) is an autosomal dominant disorder, in which a gain-of-function mutation causes prolonged AChR channel openings and increases the synaptic response to ACh. In SCCMS, neuromuscular transmission defects are caused by (i) staircase summation of endplate potentials, (ii) excessive desensitization of AChRs, and (iii) endplate myopathy caused by excessive influx of extracellular calcium. SCCMS mutations cause neuromuscular transmission defects either by increasing the affinity of AChR for ACh binding or by retarding the channel closing rate α and variably enhancing the channel opening rate β. SCCMS can be effectively treated with conventional dosages of long-lived open channel blockers of AChR, such as the antiarrhythmic agent quinidine and the antidepressant fluoxetine.

The fast channel congenital myasthenic syndrome (FCCMS) is caused by loss-of-function missense mutations in the AChR subunit genes. The mutations render the AChR resistant to be transferred to an open state and prematurely coming back to a closed state. Detailed kinetic analyses of FCCMS mutations have unmasked yet uncharacterized

molecular architectures of the AChR subunits especially in the third transmembrane domain and in the long cytoplasmic loop. FCCMS can be effectively treated with anticholinesterases and 3,4-diaminopyridine.

Two more clinical phenotypes are associated with variations of the AChR subunit genes. Mutations in *CHRNG* encoding the AChR γ subunit cause another phenotype FADS by restricting intrauterine movement of an embryo. A SNP in the promoter region of *CHRNA1* compromises expression of the α1 subunit in thymic epithelial cells, and increases the chance of developing myasthenia gravis.

Acknowledgements

Works in our laboratories were supported by Grants-in-Aid from the MEXT and the MHLW of Japan (to K.O.), and by the NIH grant (NS6277) and the Muscular Dystrophy Association grant (to A.G.E.).

References

1. Ohno, K., Hutchinson, D.O., Milone, M., Brengman, J.M., Bouzat, C., Sine, S.M. and Engel, A.G. 1995, Proc. Natl. Acad. Sci. U. S. A., 92, 758.
2. Sine, S.M., Ohno, K., Bouzat, C., Auerbach, A., Milone, M., Pruitt, J.N. and Engel, A.G. 1995, Neuron, 15, 229.
3. Ohno, K., Engel, A.G., Shen, X.-M., Selcen, D., Brengman, J., Harper, C.M., Tsujino, A. and Milone, M. 2002, Am. J. Hum. Genet., 70, 875.
4. Milone, M., Shen, X.M., Selcen, D., Ohno, K., Brengman, J., Iannaccone, S.T., Harper, C.M. and Engel, A.G. 2009, Neurology, 73, 228.
5. Huze, C., Bauche, S., Richard, P., Chevessier, F., Goillot, E., Gaudon, K., Ben Ammar, A., Chaboud, A., Grosjean, I., Lecuyer, H.A., Bernard, V., Rouche, A., Alexandri, N., Kuntzer, T., Fardeau, M., Fournier, E., Brancaccio, A., Ruegg, M.A., Koenig, J., Eymard, B., Schaeffer, L. and Hantai, D. 2009, Am. J. Hum. Genet., 85, 155.
6. Chevessier, F., Faraut, B., Ravel-Chapuis, A., Richard, P., Gaudon, K., Bauche, S., Prioleau, C., Herbst, R., Goillot, E., Ioos, C., Azulay, J.P., Attarian, S., Leroy, J.P., Fournier, E., Legay, C., Schaeffer, L., Koenig, J., Fardeau, M., Eymard, B., Pouget, J. and Hantai, D. 2004, Hum. Mol. Genet., 13, 3229.
7. Chevessier, F., Girard, E., Molgo, J., Bartling, S., Koenig, J., Hantai, D. and Witzemann, V. 2008, Hum. Mol. Genet., 17, 3577.
8. Beeson, D., Higuchi, O., Palace, J., Cossins, J., Spearman, H., Maxwell, S., Newsom-Davis, J., Burke, G., Fawcett, P., Motomura, M., Muller, J.S., Lochmuller, H., Slater, C., Vincent, A. and Yamanashi, Y. 2006, Science, 313, 1975.
9. Hamuro, J., Higuchi, O., Okada, K., Ueno, M., Iemura, S., Natsume, T., Spearman, H., Beeson, D. and Yamanashi, Y. 2008, J. Biol. Chem., 283, 5518.
10. Tsujino, A., Maertens, C., Ohno, K., Shen, X.-M., Fukuda, T., Harper, C.M., Cannon, S.C. and Engel, A.G. 2003, Proc. Natl. Acad. Sci. U. S. A., 100, 7377.
11. Ohno, K., Brengman, J., Tsujino, A. and Engel, A.G. 1998, Proc. Natl. Acad. Sci. U. S. A., 95, 9654.

12. Ohno, K., Brengman, J.M., Felice, K.J., Cornblath, D.R. and Engel, A.G. 1999, Am. J. Hum. Genet., 65, 635.
13. Kimbell, L.M., Ohno, K., Engel, A.G. and Rotundo, R.L. 2004, J. Biol. Chem., 279, 10997.
14. Ohno, K., Tsujino, A., Brengman, J.M., Harper, C.M., Bajzer, Z., Udd, B., Beyring, R., Robb, S., Kirkham, F.J. and Engel, A.G. 2001, Proc. Natl. Acad. Sci. U. S. A., 98, 2017.
15. Lang, B. and Vincent, A. 2009, Curr. Opin. Pharm., 9, 336.
16. Hoch, W., McConville, J., Helms, S., Newsom-Davis, J., Melms, A. and Vincent, A. 2001, Nat. Med., 7, 365.
17. Cole, R.N., Reddel, S.W., Gervasio, O.L. and Phillips, W.D. 2008, Ann. Neurol., 63, 782.
18. Higuchi, O., Hamuro, J., Motomura, M. and Yamanashi, Y. in press, Ann. Neurol.,
19. Keramidas, A., Moorhouse, A.J., Schofield, P.R. and Barry, P.H. 2004, Prog. Biophys. Mol. Biol., 86, 161.
20. Mihailescu, S. and Drucker-Colin, R. 2000, Arch. Med. Res., 31, 131.
21. Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C. and Sakmann, B. 1986, Nature, 321, 406.
22. Lee, W.Y., Free, C.R. and Sine, S.M. 2009, J. Neurosci., 29, 3189.
23. Mukhtasimova, N., Lee, W.Y., Wang, H.L. and Sine, S.M. 2009, Nature,
24. Ohno, K., Anlar, B. and Engel, A.G. 1999, Neuromuscul. Disord., 9, 131.
25. Ohno, K., Quiram, P.A., Milone, M., Wang, H.-L., Harper, M.C., Pruitt, J.N., 2nd, Brengman, J.M., Pao, L., Fischbeck, K.H., Crawford, T.O., Sine, S.M. and Engel, A.G. 1997, Hum. Mol. Genet., 6, 753.
26. Engel, A.G., Ohno, K., Bouzat, C., Sine, S.M. and Griggs, R.C. 1996, Ann. Neurol., 40, 810.
27. Michalk, A., Stricker, S., Becker, J., Rupps, R., Pantzar, T., Miertus, J., Botta, G., Naretto, V.G., Janetzki, C., Yaqoob, N., Ott, C.E., Seelow, D., Wieczorek, D., Fiebig, B., Wirth, B., Hoopmann, M., Walther, M., Korber, F., Blankenburg, M., Mundlos, S., Heller, R. and Hoffmann, K. 2008, Am. J. Hum. Genet., 82, 464.

28. Ohno, K., Engel, A.G., Brengman, J.M., Shen, X.-M., Heidenrich, F.R., Vincent, A., Milone, M., Tan, E., Demirci, M., Walsh, P., Nakano, S. and Akiguchi, I. 2000, Ann. Neurol., 47, 162.
29. Croxen, R., Hatton, C., Shelley, C., Brydson, M., Chauplannaz, G., Oosterhuis, H., Vincent, A., Newsom-Davis, J., Colquhoun, D. and Beeson, D. 2002, Neurology, 59, 162.
30. Croxen, R., Newland, C., Beeson, D., Oosterhuis, H., Chauplannaz, G., Vincent, A. and Newsom-Davis, J. 1997, Hum. Mol. Genet., 6, 767.
31. Engel, A.G., Ohno, K., Milone, M., Wang, H.L., Nakano, S., Bouzat, C., Pruitt, J.N., 2nd, Hutchinson, D.O., Brengman, J.M., Bren, N., Sieb, J.P. and Sine, S.M. 1996, Hum. Mol. Genet., 5, 1217.
32. Wang, H.-L., Auerbach, A., Bren, N., Ohno, K., Engel, A.G. and Sine, S.M. 1997, J. Gen. Physiol., 109, 757.
33. Vohra, B.P., Groshong, J.S., Maselli, R.A., Verity, M.A., Wollmann, R.L. and Gomez, C.M. 2004, Ann. Neurol., 55, 347.
34. Ohno, K., Wang, H.-L., Shen, X.-M., Milone, M., Brengman, J.M., Bernasconi, L., Sine, S.M. and Engel, A.G. 2000, Neurology, 54 (Suppl 3), A183 (abstract).
35. Abdelgany, A., Wood, M. and Beeson, D. 2003, Hum. Mol. Genet., 12, 2637.
36. Milone, M., Wang, H.L., Ohno, K., Fukudome, T., Pruitt, J.N., Bren, N., Sine, S.M. and Engel, A.G. 1997, J. Neurosci., 17, 5651.
37. Shen, X.M., Deymeer, F., Sine, S.M. and Engel, A.G. 2006, Ann. Neurol., 60, 128.
38. Gomez, C.M., Maselli, R., Staub, J., Day, J.W., Cens, T., Wollmann, R.L. and Charnet, P.C. 1998, Soc Neurosci Abstr, 24, 484 (abstract).
39. Gomez, C.M., Maselli, R., Gammack, J., Lasalde, J., Tamamizu, S., Cornblath, D.R., Lehar, M., Mcnamee, H. and Kuncl, R.W. 1996, Ann. Neurol., 39, 712.
40. Shen, X.M., Ohno, K., Milone, M., Brengman, J.M., Tsujino, A. and Engel, A.G. 2003, Mol. Biol. Cell, 14 (Suppl), 223a (abstract).
41. Gomez, C.M., Maselli, R.A., Vohra, B.P., Navedo, M., Stiles, J.R., Charnet, P., Schott, K., Rojas, L., Keesey, J., Verity, A., Wollmann, R.W. and Lasalde-Dominicci, J. 2002, Ann. Neurol., 51, 102.

42. Shelley, C. and Colquhoun, D. 2005, J Physiol, 564, 377.
43. Hatton, C.J., Shelley, C., Brydson, M., Beeson, D. and Colquhoun, D. 2003, J Physiol, 547, 729.
44. Shen, X.M., Ohno, K., Fukudome, T., Tsujino, A., Brengman, J.M., Engel, A.G., DeVivo, D.C. and Packer, R.J. 2002, J. Neurol. Sci., 199 (Suppl 1), S96 (abstract).
45. Fidzianska, A., Ryniewicz, B., Shen, X.M. and Engel, A.G. 2005, Neuromuscul. Disord., 15, 753.
46. Outteryck, O., Richard, P., Lacour, A., Fournier, E., Zephir, H., Gaudon, K., Eymard, B., Hantai, D., Vermersch, P. and Stojkovic, T. 2009, J. Neurol. Neurosurg. Psychiatry, 80, 450.
47. Ohno, K., Milone, M., Brengman, J.M., LoMonaco, M., Evoli, A., Tonali, P.A. and Engel, A.G. 1998, Neurology, 50 (Suppl. 4), A432 (abstract).
48. Gomez, C.M. and Gammack, J.T. 1995, Neurology, 45, 982.
49. Shen, X.-M., Ohno, K., Tsujino, A., Brengman, J.M., Gingold, M., Sine, S.M. and Engel, A.G. 2003, J. Clin. Invest., 111, 497.
50. Webster, R., Brydson, M., Croxen, R., Newsom-Davis, J., Vincent, A. and Beeson, D. 2004, Neurology, 62, 1090.
51. Wang, H.-L., Milone, M., Ohno, K., Shen, X.-M., Tsujino, A., Batocchi, A.P., Tonali, P., Brengman, J., Engel, A.G. and Sine, S.M. 1999, Nat. Neurosci., 2, 226.
52. Shen, X.M., Fukuda, T., Ohno, K., Sine, S.M. and Engel, A.G. 2008, J. Clin. Invest., 118, 1867.
53. Brownlow, S., Webster, R., Croxen, R., Brydson, M., Neville, B., Lin, J.P., Vincent, A., Newsom-Davis, J. and Beeson, D. 2001, J. Clin. Invest., 108, 125.
54. Ohno, K., Wang, H.-L., Milone, M., Bren, N., Brengman, J.M., Nakano, S., Quiram, P., Pruitt, J.N., Sine, S.M. and Engel, A.G. 1996, Neuron, 17, 157.
55. Shen, X.-M., Ohno, K., Milone, M., Brengman, J.M., R., S.P. and Engel, A.G. 2001, Neurology, 56 (Suppl 3), A60 (abstract).
56. Sine, S.M., Shen, X.-M., Wang, H.-L., Ohno, K., Lee, W.-Y., Tsujino, A., Brengmann, J., Bren, N., Vajsar, J. and Engel, A.G. 2002, J. Gen. Physiol., 120, 483.

57. Milone, M., Wang, H.-L., Ohno, K., Prince, R., Fukudome, T., Shen, X.-M., Brengman, J.M., Griggs, R.C., Sine, S.M. and Engel, A.G. 1998, Neuron, 20, 575.
58. Wang, H.-L., Ohno, K., Milone, M., Brengman, J.M., Evoli, A., Batocchi, A.P., Middleton, L.T., Christodoulou, K., Engel, A.G. and Sine, S.M. 2000, J. Gen. Physiol., 116, 449.
59. Shen, X.-M., Ohno, K., Sine, S.M. and Engel, A.G. 2005, Brain, 128, 345.
60. Abicht, A., Stucka, R., Song, I.-H., Karcagi, V., Kugler, K., Baumgarten-Walczak, A., Stier, C., Pongratz, D., Mortier, W., Müller-Felber, W., Rüdel, R. and Lochmüller, H. 2000, Acta Myologica, 19, 23.
61. Groshong, J.S., Spencer, M.J., Bhattacharyya, B.J., Kudryashova, E., Vohra, B.P., Zayas, R., Wollmann, R.L., Miller, R.J. and Gomez, C.M. 2007, J. Clin. Invest., 117, 2903.
62. Fucile, S., Sucapane, A., Grassi, F., Eusebi, F. and Engel, A.G. 2006, J Physiol, 573, 35.
63. Di Castro, A., Martinello, K., Grassi, F., Eusebi, F. and Engel, A.G. 2007, J Physiol, 579, 671.
64. Fukudome, T., Ohno, K., Brengman, J.M. and Engel, A.G. 1998, Neuroreport, 9, 1907.
65. Harper, C.M. and Engel, A.G. 1998, Ann. Neurol., 43, 480.
66. Harper, C.M., Fukodome, T. and Engel, A.G. 2003, Neurology, 60, 1710.
67. Wirtz, P.W., Titulaer, M.J., Gerven, J.M. and Verschuuren, J.J. 2010, Expert Rev Clin Immunol, 6, 867.
68. Hesselmans, L.F., Jennekens, F.G., Van den Oord, C.J., Veldman, H. and Vincent, A. 1993, Anat. Rec., 236, 553.
69. Morgan, N.V., Brueton, L.A., Cox, P., Greally, M.T., Tolmie, J., Pasha, S., Aligianis, I.A., van Bokhoven, H., Marton, T., Al-Gazali, L., Morton, J.E., Oley, C., Johnson, C.A., Trembath, R.C., Brunner, H.G. and Maher, E.R. 2006, Am. J. Hum. Genet., 79, 390.
70. Hoffmann, K., Muller, J.S., Stricker, S., Megarbane, A., Rajab, A., Lindner, T.H., Cohen, M., Chouery, E., Adaimy, L., Ghanem, I., Delague, V., Boltshauser, E.,

Talim, B., Horvath, R., Robinson, P.N., Lochmuller, H., Hubner, C. and Mundlos, S. 2006, Am. J. Hum. Genet., 79, 303.

71. Giraud, M., Taubert, R., Vandiedonck, C., Ke, X., Levi-Strauss, M., Pagani, F., Baralle, F.E., Eymard, B., Tranchant, C., Gajdos, P., Vincent, A., Willcox, N., Beeson, D., Kyewski, B. and Garchon, H.J. 2007, Nature, 448, 934.

Journal of the Neurological Sciences 309 (2011) 9–11

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

A novel mutation in the calcium channel gene in a family with hypokalemic periodic paralysis

Makito Hirano [a], Yosuke Kokunai [b], Asami Nagai [c], Yusaku Nakamura [a], Kazumasa Saigoh [d], Susumu Kusunoki [d], Masanori P. Takahashi [b,*]

[a] Department of Neurology, Sakai Hospital Kinki University Faculty of Medicine, Sakai, Osaka, Japan
[b] Department of Neurology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[c] Department of Internal Medicine, Tohokashiba Hospital, Japan
[d] Department of Neurology, Kinki University, Faculty of Medicine, Osakasayama, Osaka, Japan

ARTICLE INFO

Article history:
Received 10 June 2011
Received in revised form 23 July 2011
Accepted 26 July 2011
Available online 19 August 2011

Keywords:
Hypokalemic periodic paralysis
Hyperkalemia
Calcium channel
Voltage sensor
Diagnostic difficulty
Increased CK

ABSTRACT

Hypokalemic periodic paralysis (HypoPP) type 1 is an autosomal dominant disease caused by mutations in the Ca(V)1.1 calcium channel encoded by the *CACNA1S* gene. Only seven mutations have been found since the discovery of the causative gene in 1994. We describe a patient with HypoPP who had a high serum potassium concentration after recovery from a recent paralysis, which complicated the correct diagnosis. This patient and other affected family members had a novel mutation, p.Arg900Gly, in the *CACNA1S* gene.

1. Introduction

Hypokalemic periodic paralysis (HypoPP) is an autosomal dominant disease caused by mutations in either the Ca(V)1.1 calcium channel encoded by the *CACNA1S* gene (HypoPP type 1) or the Na(V)1.4 sodium channel encoded by the *SCN4A* gene (HypoPP type 2) [1]. HypoPP type 1 is more common than HypoPP type 2 (1:5–8). In HypoPP type 1, only seven mutations have been found since the discovery of the causative gene in 1994 [2]. However, the prevalence of HypoPP is not extremely low (1:100,000), suggesting that a few common mutations affect many families [3]. The Ca(V)1.1 channel consists of four domains, and each domain has S1 to S6 transmembrane segments. All mutations but one rare one (p.Val876Glu) [4] are involved in arginine residues in the S4 segments of the calcium channel [2,3,5–7]. A recent study proposed a gating pore cation leak current caused by loss of a positive charge of arginine in S4 voltage sensors as a common pathomechanism of HypoPP [1,8].

We describe a patient with HypoPP who had a high serum potassium concentration after recovery from paralysis, which complicated the correct diagnosis. This patient had a novel mutation, p.Arg900Gly, in the *CACNA1S* gene.

2. Patients

The proband (patient 1) was a 41-year-old man who started to have periodic episodes of paralysis, occurring about five times a year, since the age of 21 years. Each episode lasted 12 hours to 2 days. Hard physical exercise seemed to induce paralysis and was therefore avoided. Overeating, but not coldness, also induced paralysis. On a careful, detailed interview, he recalled that he had had a history of hypokalemia at the time of the previous episode of paralysis, but the value was not currently available since the clinic he had visited had permanently closed. He also reported having received potassium supplements at that time, which seemed effective, but were soon discontinued for no apparent reason. A family tree (Fig. 1A) suggested a pattern of autosomal dominant inheritance (Fig. 1A). Two days after a recent episode of paralysis he visited our clinic. Muscle strength was normal, with no muscular atrophy. Laboratory tests showed increased levels of potassium (5.4 mEq/l, normal 3.3–5.0 mEq/l), CK (3233 IU/ml, normal 45–190 IU/ml), AST (104 IU/l, normal 10–40 IU/ml), and ALT (54 IU/ml, normal 5–45 IU/ml). Blood sugar was only slightly decreased to 69 mg/dl (normal 70–139 mg/dl). Other data, including thyroid function, were normal: Na 144 mEq/l, Cl 104 mEq/l, TSH 0.89 mU/ml (normal 0.34–3.88), fT3 3.8 pg/ml (normal 2.1–4.1 pg/ml), and fT4 1.5 ng/ml (normal 1.0–1.7 ng/ml). Electrocardiography and chest radiography showed normal findings. Electromyography showed no myotonic discharges or myogenic changes during non-

* Corresponding author at: Department of Neurology Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita 565-0871, Osaka, Japan.
E-mail address: mtakahas@neurol.med.osaka-u.ac.jp (M.P. Takahashi).

0022-510X/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jns.2011.07.046

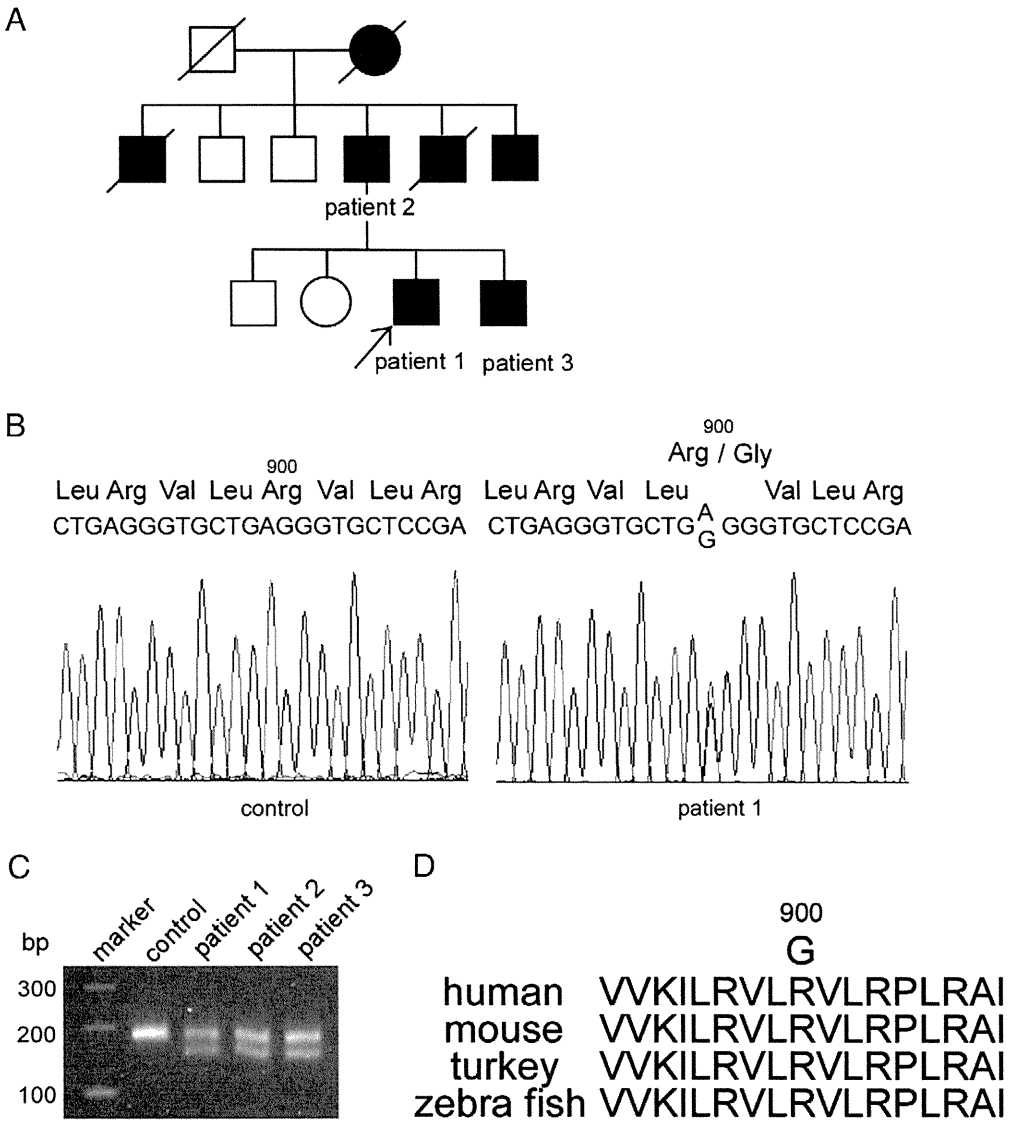

Fig. 1. (A) A family tree of the patient 1 (arrow). This family tree suggests an autosomal dominant pattern of inheritance. (B) The patient had a heterozygous A-to-G transition, resulting in the substitution of Arg by Gly at the 900 residue. (C) PCR-restriction fragment length analyses with a mismatch primer confirmed that Patients 1–3 were heterozygous for the p.Arg900Gly mutation. (D) The Arg at the 900 position is phylogenetically conserved.

paralytic periods. After 19 days, all abnormal blood test values had normalized.

Patient 2 (69 years), the father of the proband, had paralytic attacks since the age of 13 years. Most attacks, occurring 5 times/year, were mild or partial, but severe episodes occurred 10 times over the course of 37 years. Paralysis was induced by hard physical exercise and overeating, but not coldness. He had not been examined in any medical institutions during paralysis. He has had no attacks since the age of 50 years. Patient 3 (35 years), a younger brother of the proband, started to have mild paralytic attacks since the age of 13 years and severe ones since age 15. He also recalled that he had had a history of hypokalemia during paralysis, but the value was not currently available. Recently, he had severe attacks 10 times/year, induced by hard exercise and overeating, but not by coldness.

After obtaining written informed consent from Patient 1, a genetic analysis was performed as described previously [2,3,5]. Patients 2 and 3 agreed to genetic testing, but the mother of the proband declined. A novel heterozygous A-to-G transition was identified in the *CACNA1S* gene (c.2698A>G), resulting in a missense change (p.Arg900Gly, Fig. 1B). This mutation was confirmed by PCR-restriction fragment length analyses as follows. The forward primer carried two nucleotide substitutions (ex21mF: 5′-GTGCCATCTCCGTGGTGAAGAcCaT-GAGGGTGCT-3′, lower case letters mean substituted nucleotides) to create the endonuclease *Xcm*I (CCANNNNNNNNNTGG) site in a PCR fragment only from a mutant allele. The reverse primer was 5′-GGTCCCAGCCATGGCTGGGCTGA-3′. The PCR-amplified mutant fragment (179 bp) was digested into two fragments (29 and 150 bp), though only the larger fragment was visible. The normal fragment remained undigested. This mutation was present in Patients 1–3, but not in 100 control chromosomes (Fig. 1C). This Arg900 residue is highly conserved among humans, mice, turkeys, and zebra fish (Fig. 1D).

3. Discussion

We found a novel mutation in a patient with HypoPP type 1. This mutation in the S4 segment of the domain III affects the same residue as the previously identified mutation p.Arg900Ser [3]. Similar to previous other mutations involving arginine residues, the positive charge of arginine was abolished by a neutral amino acid, glycine. The Arg900 residue is phylogenetically conserved. Thus, this novel

p.Arg900Gly mutation may cause a gating pore current leak and is most likely causative for HypoPP.

Our patient had a high serum potassium concentration at the visit to our clinic after recovery from paralysis, which complicated the correct diagnosis. However, such hyperkalemia during recovery periods in HypoPP has been described previously, although mutations in either the calcium channel or sodium channel were not specified [9]. To our knowledge, mutations in the *CACNA1S* gene have not previously been associated with hyperkalemic periodic paralysis. Consistent with this, our patient presented with previous episodes of hypokalemia. Although hyperkalemia might have simply been a rebound response to hypokalemia, another possible explanation is that hypokalemia during paralysis may have destabilized muscular membranes, releasing intramuscular potassium into serum. This hypothesis may be supported by the temporal elevation of CK in our patient.

A previous study reported at least one patient with p.Arg900Ser mutation, a mutation occurring in the same residue as ours. The patient had a typical HypoPP phenotype, although detailed clinical information was not provided [3]. Attacks began to occur in the second decade and were associated with low potassium levels or with provocative factors that reduced serum potassium levels. In our patients, disease developed at 13 to 21 years of age and was accompanied by typical HypoPP episodes. Thus, mutations involving the Arg900 residue seem to be associated with a typical phenotype.

In summary, we found a novel mutation in the *CACNA1S* gene, which further supports the gating pore current hypothesis as a pathomechanism of HypoPP. Because this is only the eighth mutation identified over the course of 17 years, further discovery of mutations may provide further insight into voltage-gated channels as well as the pathomechanism of HypoPP.

Acknowledgments

This study was supported partly by Research Grants for Intractable Disease from the Ministry of Health, Labour and Welfare (to MPT).

References

[1] Cannon SC. Voltage-sensor mutations in channelopathies of skeletal muscle. J Physiol 2010;588(Pt 11):1887–95.
[2] Ptacek LJ, Tawil R, Griggs RC, Engel AG, Layzer RB, Kwiecinski H, et al. Dihydropyridine receptor mutations cause hypokalemic periodic paralysis. Cell 1994;77(6):863–8.
[3] Matthews E, Labrum R, Sweeney MG, Sud R, Haworth A, Chinnery PF, et al. Voltage sensor charge loss accounts for most cases of hypokalemic periodic paralysis. Neurology 2009;72(18):1544–7.
[4] Ke T, Gomez CR, Mateus HE, Castano JA, Wang QK. Novel CACNA1S mutation causes autosomal dominant hypokalemic periodic paralysis in a South American family. J Hum Genet 2009;54(11):660–4.
[5] Jurkat-Rott K, Lehmann-Horn F, Elbaz A, Heine R, Gregg RG, Hogan K, et al. A calcium channel mutation causing hypokalemic periodic paralysis. Hum Mol Genet 1994;3(8):1415–9.
[6] Elbaz A, Vale-Santos J, Jurkat-Rott K, Lapie P, Ophoff RA, Bady B, et al. Hypokalemic periodic paralysis and the dihydropyridine receptor (CACNL1A3): genotype/phenotype correlations for two predominant mutations and evidence for the absence of a founder effect in 16 caucasian families. Am J Hum Genet 1995;56(2):374–80.
[7] Chabrier S, Monnier N, Lunardi J. Early onset of hypokalaemic periodic paralysis caused by a novel mutation of the CACNA1S gene. J Med Genet 2008;45(10):686–8.
[8] Sokolov S, Scheuer T, Catterall WA. Gating pore current in an inherited ion channelopathy. Nature 2007;446(7131):76–8.
[9] Lehmann-Horn F, Rudel R, Jurkat-Rott K. Nondystrophic myotonias and periodic paralyses. In: Engel AG, editor. Myology. McGraw Hill Medical Publishing Division; 2003.

Misregulation of miR-1 processing is associated with heart defects in myotonic dystrophy

Frédérique Rau[1–4], Fernande Freyermuth[1–4], Charlotte Fugier[1–4], Jean-Philippe Villemin[5], Marie-Christine Fischer[1–4], Bernard Jost[1–4], Doulaye Dembele[1–4], Geneviève Gourdon[6–8], Annie Nicole[6–8], Denis Duboc[8,9], Karim Wahbi[10–13], John W Day[14], Harutoshi Fujimura[15], Masanori P Takahashi[16], Didier Auboeuf[10], Natacha Dreumont[1–4], Denis Furling[10–13] & Nicolas Charlet-Berguerand[1–4]

Myotonic dystrophy is an RNA gain-of-function disease caused by expanded CUG or CCUG repeats, which sequester the RNA binding protein MBNL1. Here we describe a newly discovered function for MBNL1 as a regulator of pre-miR-1 biogenesis and find that miR-1 processing is altered in heart samples from people with myotonic dystrophy. MBNL1 binds to a UGC motif located within the loop of pre-miR-1 and competes for the binding of LIN28, which promotes pre-miR-1 uridylation by ZCCHC11 (TUT4) and blocks Dicer processing. As a consequence of miR-1 loss, expression of GJA1 (connexin 43) and CACNA1C (Cav1.2), which are targets of miR-1, is increased in both DM1- and DM2-affected hearts. CACNA1C and GJA1 encode the main calcium- and gap-junction channels in heart, respectively, and we propose that their misregulation may contribute to the cardiac dysfunctions observed in affected persons.

Myotonic dystrophy, which is the most common muscular dystrophy in adults, is characterized by multiple symptoms that include muscle weakness, myotonia, cardiac defects, cataracts, insulin resistance and neuropsychiatric impairment. Myotonic dystrophy type 1 (DM1) is caused by an expansion of CTG repeats located within the 3′ noncoding region of the *DMPK* gene[1–3], whereas myotonic dystrophy type 2 (DM2) is caused by an expansion of CCTG repeats located within the first intron of the *ZNF9* (also known as *CNBP*) gene[4]. The mutant RNA, which contains 100 to several thousand CUG or CCUG repeats, is retained in nuclear aggregates that sequester the MBNL1 RNA binding protein[5–8]. Furthermore, the expression of CUGBP1(also known as *CELF1*) is increased in some tissues of DM1-affected people[9,10]. MBNL1 and CUGBP1 are splicing regulators, and their alterations in people with myotonic dystrophy result in misregulation of the alternative splicing of several pre-mRNAs[11–13], including those encoded by the muscle chloride channel gene *CLCN1* and the insulin receptor gene *INSR*, resulting in myotonia and insulin resistance, respectively[14–17]. However, the molecular mechanisms underlying the cardiac defects, which affect 80% of people with DM1 and DM2 and represent the second most common cause of their death, are unclear. Cardiac involvements in affected persons[18–21] and in mouse models[22,23] are characterized by nonspecific changes such as interstitial fibrosis and fatty infiltration, which lead to degeneration of the conduction system and to fatal atrioventricular blocks. Furthermore, people with myotonic dystrophy are also characterized by atrial and ventricular tachyarrythmias of unknown causes, which may explain the occurrences of sudden cardiac death observed in these people despite pacemaker implants[18–21].

MicroRNAs (miRNAs) are small, conserved, noncoding RNAs that are important components of post-transcriptional gene regulation and are involved in the control of many fundamental processes, including cardiac development and function. In particular, the expression of the miR-1 family, which comprises miR-1-1 and miR-1-2, is altered in mouse models and people with heart diseases[24–27]. Furthermore, overexpression of miR-1 in normal or infarcted rat heart exacerbates electrophysiological abnormalities[26], whereas ablation of the *miR-1-2* (*Mir1a-2*) gene in mice results in arrhythmias, cardiac conduction defects and sudden cardiac death[27], demonstrating that the heart needs tight regulation of miR-1. Here we set out to test whether or not miRNAs are misregulated in myotonic dystrophies.

RESULTS

The processing of miR-1 is altered in myotonic dystrophy

In a preliminary profiling analysis of the expression of miRNAs in primary differentiated muscle cells isolated from unaffected persons and those with DM1 (**Supplementary Fig. 1a,b** and **Supplementary Data**), we identified a robust misexpression of miR-1. Because inactivation of *miR-1-2* in mice did not result in an overt phenotype in

[1]Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Illkirch, France. [2]Institut National de la Santé et de la Recherche Médicale (INSERM) U964, Illkirch, France. [3]Centre National de la Recherche Scientifique (CNRS) UMR7104, Illkirch, France. [4]Strasbourg University, Illkirch, France. [5]Institut National de la Santé et de la Recherche Médicale U590, Lyon, France. [6]Necker Hospital, Paris, France. [7]Institut National de la Santé et de la Recherche Médicale U781, Paris, France. [8]Université Paris 5, Paris, France. [9]Cochin Hospital, Paris, France. [10]Université Pierre et Marie Curie (UM76), Paris, France. [11]Institut de Myologie, Paris, France. [12]Institut National de la Santé et de la Recherche Médicale U974, Paris, France. [13]Centre National de la Recherche Scientifique UMR7215, Paris, France. [14]University of Minnesota, Minneapolis, Minnesota, USA. [15]Department of Neurology, Toneyama Hospital, Osaka, Japan. [16]Department of Neurology, Osaka University Graduate School of Medicine, Osaka, Japan. Correspondence should be addressed to N.C.-B. (ncharlet@igbmc.fr).

Received 14 September 2010; accepted 7 April 2011; published online 19 June 2011; doi:10.1038/nsmb.2067

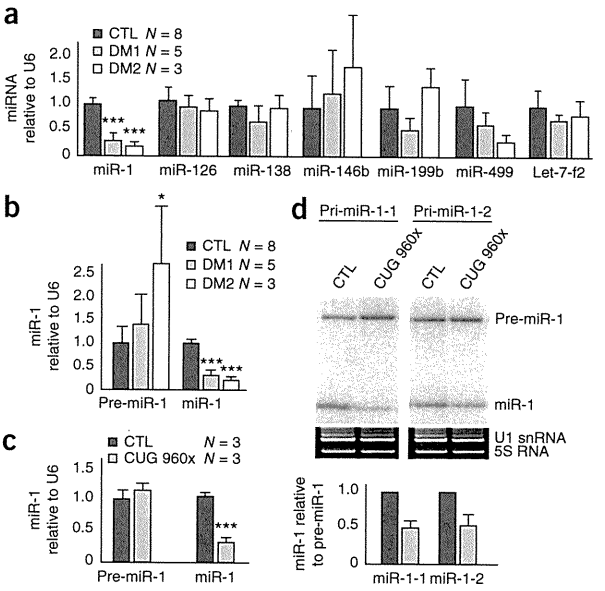

Figure 1 The processing of miR-1 is altered in myotonic dystrophies. (**a**) Quantitative real-time PCR (qRT-PCR) analysis of the expression of mature miR-1, miR-126, miR-138, miR-199 and miR-499 relative to the U6 small nuclear (sn) RNA in heart samples of adults with DM1 or DM2 and in control (CTL) heart samples from 2 unaffected persons, 1 person with dilated cardiomyopathy and 5 people with ALS. $^{***}P < 0.001$. (**b**) qRT-PCR analysis of the expression of pre-miR-1 and mature miR-1 relative to the U6 snRNA in heart samples of control (2 unaffected, 1 with dilated cardiomyopathy and 5 with ALS), DM1-affected and DM2-affected subjects. $^{*}P < 0.05$, $^{***}P < 0.001$. (**c**) qRT-PCR analysis of the expression of pre-miR-1 and mature miR-1 in H9C2 rat cardiomyocytes differentiated for 6 days and infected with recombinant adenovirus (MOI 100) expressing GFP (CTL) or 960 CUG repeats (CUG). The mean of at least three independent infections is depicted as the percentage of mature or pre-miR-1 relative to the U6 snRNA. Error bars indicate s.d. $^{***}P < 0.001$. (**d**) 5 μg of total RNA extracted from HeLa cells co-transfected with ectopic pri-miR-1-1 or pri-miR-1-2 minigenes and a plasmid expressing either no (CTL) or 960 CUG repeats (CUG) were analyzed by northern blot analysis using an antisense miR-1 [γ-^{32}P]–labeled probe. The mean of at least three independent transfections is depicted as the percentage of mature miR-1 relative to pre-miR-1. Ethidium bromide staining demonstrates equal loading. Error bars indicate s.d.

nonregenerating skeletal muscle but did cause cardiac dysfunction[27], we quantified miR-1 expression in heart samples from people with DM1 and in unaffected individuals. Quantitative RT-PCR demonstrated a significant reduction in expression of miR-1 ($P = 1.8 \times 10^{-6}$) in DM1 heart tissue (**Fig. 1a**), whereas the expression of miR-126, miR-138, miR-199, miR-208, miR-499 and Let-7-f2 was not altered. Additional northern blot analysis confirmed decreased expression of miR-1 in DM1 heart tissue (**Supplementary Fig. 1c**). Next, miR-1 expression was examined in myotonic dystrophy type 2, which undergoes cardiac alterations similar to those in DM1 (ref. 28). Quantitative RT-PCR demonstrated a significant ($P < 0.001$) reduction in expression of miR-1 in heart samples from people with DM1 and DM2 but no alteration in heart samples from those with amyotrophic lateral sclerosis (**Fig. 1a**), suggesting that the expression of miR-1 is specifically altered in both forms of myotonic dystrophy.

miRNAs are initially transcribed by RNA polymerase II as primary miRNAs (pri-miRNAs), which are processed into precursor miRNAs (pre-miRNAs) by Drosha complexed with DGCR8 (also called Pasha)[29–31] and exported from the nucleus to the cytoplasm by exportin 5 (XPO5)[32,33]. Pre-miRNAs are hairpin-shaped RNAs of approximately 70 nucleotides (nt) that are processed into mature miRNAs by Dicer[34,35], a cytoplasmic endoRNase of the RNase type III family. Expanded CUG or CCUG repeats interfere *in trans* with the alternative splicing of other pre-messenger RNAs[14–17]. To investigate whether expanded CUG or CCUG repeats also interfere *in trans* with the processing of miR-1 in myotonic dystrophic heart tissues, we quantified the expression of pre-miR-1 and mature miR-1. Whereas the levels of pre-miR-1 were normal or slightly increased, the quantities of mature miR-1 were decreased in heart samples of people with DM1 and people with DM2 (**Fig. 1b**), suggesting that pre-miR-1 is normally produced but incorrectly processed in individuals with myotonic dystrophy. In contrast, the expression of pre- and mature miR-138 and pre- and mature Let7-f2 were normal, indicating that the misregulation of the processing of pre-miR-1 was specific and was not the result of a global alteration of miRNA biogenesis (**Supplementary Fig. 1d**).

To test whether the expression of expanded CUG repeats directly altered the processing of pre-miR-1, we engineered an adenovirus expressing 960 expanded CUG repeats and infected rat H9C2 cardiomyocytes, which express endogenous miR-1. RNA FISH coupled to immunofluorescence analysis confirmed that >90% of the cardiomyocytes were infected and expressed expanded CUG repeats, which sequestered endogenous Mbnl1 within nuclear aggregates (**Supplementary Fig. 1e**). In CUG-infected cardiomyocytes, the expression of mature miR-1 was decreased, whereas that of pre-miR-1 was not altered (**Fig. 1c**). This misregulation of pre-miR-1 processing was specific, as the expression of mature and pre-miR-138 or of mature and pre-Let7-f2 was normal (**Supplementary Fig. 1f**). The miR-1 family includes two paralogs expressed in heart tissue, miR-1-1 and miR-1-2, encoded by two different genes but expressing identical mature miRNAs, which cannot be differentiated by northern blot or quantitative PCR with reverse transcription (RT-qPCR) (**Supplementary Fig. 1g**). To rule out any transcriptional regulation and to determine whether expanded CUG repeats alter the processing of pre-miR-1-1, pre-miR-1-2 or both, we cloned human pri-miR-1-1 and pri-miR-1-2 under the control of a cytomegalovirus (CMV) promoter. Coexpression of ectopic pri-miR-1-1 or pri-miR-1-2 minigene in HeLa cells, which do not express endogenous miR-1, with a vector expressing expanded CUG repeats altered both pre-miR-1-1 and pre-miR-1-2 processing, resulting in decreased expression of mature miR-1-1 and miR-1-2 (**Fig. 1d**). As a negative control, expression of expanded CUG repeats had no substantial effect on the processing of the ectopically expressed pre-miR-16 (**Supplementary Fig. 1h**). Overall, these data demonstrate that the processing of the miR-1 family is altered in the hearts of people with DM1 or DM2, and that expression of expanded CUG repeats is sufficient to trigger that alteration.

MBNL1 regulates the processing of miR-1

Because expanded CUG repeats alter MBNL1 and CUGBP1 functions, we tested whether these proteins regulate the processing of pre-miR-1. Depletion of MBNL1 by short hairpin RNA (shRNA) mimicked the action of CUG repeats and altered the processing of ectopically expressed pri-miR-1-1 or pri-miR-1-2 minigenes, resulting in lower quantities of mature miR-1-1 or miR-1-2 (**Fig. 2a** and data not shown). These results were confirmed with two other shRNAs directed against *MBNL1* (**Supplementary Fig. 2a**). In contrast, overexpression or depletion of CUGBP1 had little effect on

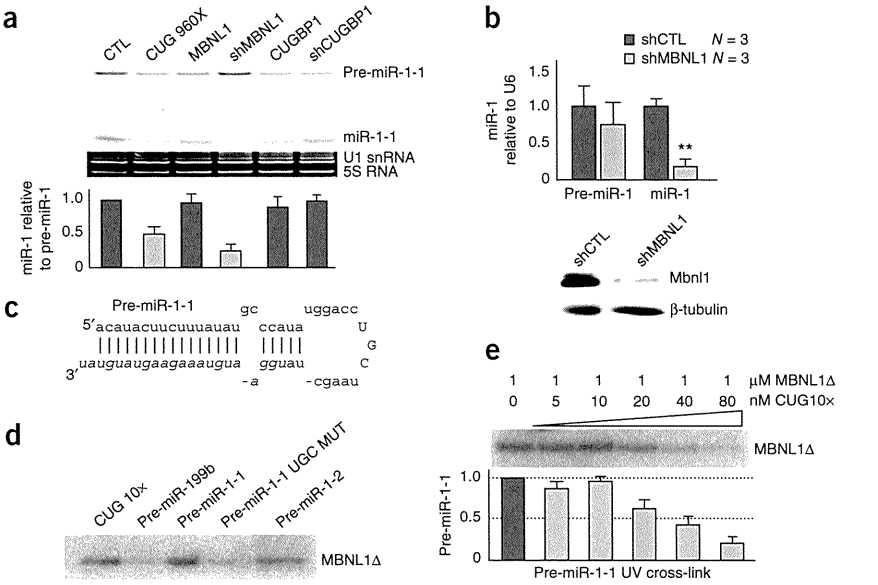

Figure 2 MBNL1 regulates the processing of miR-1. (**a**) Northern blot analysis of RNA extracted from HeLa cells co-transfected with ectopic pri-miR-1-1 minigene and a plasmid expressing either no CTG (CTL), 960 CUG repeats (CUG), MBNL1, CUGBP1 or shRNA directed against *MBNL1* (shMBNL1 #1) or against *CUGBP1* (shCUGBP1). The mean of at least three independent transfections is depicted as the percentage of mature miR-1 relative to pre-miR-1. Error bars indicate s.d., and ethidium bromide staining demonstrates equal loading. (**b**) Top, qRT-PCR analysis of the expression of endogenous pre-miR-1 and mature miR-1 in H9C2 rat cardiomyocytes infected with recombinant adenovirus expressing an shRNA against *LacZ* (shCTL) or against *Mbnl1* (shMBNL1). The mean of at least three independent infections is depicted as the percentage of mature or pre-miR-1 relative to the U6 snRNA. Error bars indicate s.d. $^{**}P < 0.01$. Bottom, Mbnl1 depletion was confirmed by western blotting. (**c**) Sequence of the wild-type human pre-miR-1-1. Mature miR-1 is indicated in italic. The UGC motif mutated in UAC is indicated in upper case. (**d**) UV-cross-linking analysis of pre-miR-1-1, pre-miR-1-2 and mutated pre-miR-1-1 (UGC MUT) using purified bacterial recombinant GST-MBNL1ΔCter and uniformly [α-^{32}P]CTP-labeled RNAs. (**e**) UV-cross-linking binding of GST-MBNL1ΔCter to uniformly [α-^{32}P]CTP-labeled pre-miR-1-1 RNA is competed by increasing amounts of unlabeled RNA composed of 10 CUG repeats. The mean of at least three independent experiments is depicted as the binding of MBNL1 to pre-miR-1. Error bars indicate s.d.

pre-miR-1 processing, suggesting that the sole depletion of MBNL1 is sufficient to mimic the action of expanded CUG repeats and to alter the processing of pre-miR-1. To test whether MBNL1 regulates the processing of endogenous pre-miR-1, we engineered an adenovirus expressing an shRNA directed against *Mbnl1* to transduce rat H9C2 cardiomyocytes. Depletion of Mbnl1 in cardiomyocytes resulted in a significantly decreased quantity of mature miR-1 ($P < 0.01$), whereas the expression of pre-miR-1 was not altered (**Fig. 2b**), suggesting that MBNL1 regulates the processing of endogenous pre-miR-1. Levels of endogenous miR-126, miR-138, miR-199 and Let-7-f2, which were tested as negative controls, were not altered, suggesting that MBNL1 specifically regulates the processing of pre-miR-1 (**Supplementary Fig. 2b**). Next, we found that recombinant purified glutathione *S*-transferase (GST)-tagged MBNL1 binds to pre-miR-1-1 and to pre-miR-1-2 RNAs, and—consistent with previous studies[36,37]—mutation of a UGC motif (**Fig. 2c**), which is located within the loop of pre-miR-1 and is conserved among species and miR-1 family members (**Supplementary Fig. 2c**), was sufficient to reduce MBNL1 binding in UV-cross-linking experiments (**Fig. 2d**), and in gel-shift analysis (**Supplementary Fig. 2d**). Finally, the binding of MBNL1 to pre-miR-1-1 RNA was outcompeted by an excess of unlabeled expanded CUG repeats (**Fig. 2e**), in accordance with the proposed model[14–17] of myotonic dystrophy pathogenesis, in which an excess of expanded CUG repeats reduces the quantity of free MBNL1 and consequently the binding of MBNL1 to its physiological targets.

LIN28 regulates the processing of miR-1

To determine the mechanism underlying the alteration of pre-miR-1 processing, we examined pre-miR-1 localization and cleavage but found that neither expanded CUG repeats nor MBNL1 had a detectable effect on pre-miR-1 export or on Dicer localization and activity (**Supplementary Fig. 3a** and data not shown). Recent reports demonstrated that the LIN28 and LIN28B RNA binding proteins regulate the expression of the Let-7 family of miRNA[38–41], raising the question of whether LIN28 may also regulate miR-1. Overexpression of LIN28 inhibited the processing of ectopically expressed pre-miR-1-1 or pre-miR-1-2, resulting in lower expression of mature miR-1-1 and miR-1-2 (**Fig. 3a**). Similar experiments conducted in the presence of actinomycin D, which inhibited *de novo* production of pre-miR-1, suggested that LIN28 blocked the processing of pre-miR-1 at the Dicer step (**Supplementary Fig. 3b**). To test whether LIN28 regulates the processing of endogenous pre-miR-1, rat H9C2 cardiomyoblastes were infected with lentiviral particles expressing an shRNA directed against *Lin28B* (*Lin28b*). Depletion of Lin28B stimulated the expression of endogenous mature miR-1, whereas the expression of pre-miR-1 was not substantially altered (**Fig. 3b**), confirming that LIN28 regulates the processing of pre-miR-1. Effect of Lin28B depletion was also tested on the expression of endogenous Let-7-f2 and miR-199, which were used as positive[38,39] and negative controls, respectively (**Supplementary Fig. 3c**). Similar to pre-Let7 miRNAs[40,41], recombinant purified histidine (His)-tagged LIN28 promoted *in vitro* uridylation of pre-miR-1 by immunoprecipitated Flag-tagged ZCCHC11 (also called TUT4) (**Fig. 3c** and **Supplementary Fig. 3d**). Consistent with the discovery that uridylation of pre-Let-7 blocks Dicer processing[41], uridylated pre-miR-1 was not cleaved by immunoprecipitated Myc-tagged Dicer, whereas control nonuridylated pre-miR-1 was processed into mature miR-1 (**Fig. 3d**). Next, using UV-cross-linking assays (**Fig. 3e**) and gel-shift analysis, we found that recombinant purified His-tagged LIN28 protein binds to pre-miR-1-1 and to pre-miR-1-2 RNAs, (**Supplementary Fig. 3e**). The pre-miR-1-1 loop possesses G- and A-rich motifs similar to the sequences recognized by LIN28 in the pre-Let-7-A1 loop[38], and mutation of an AAG motif in the pre-miR-1-1 loop reduced LIN28 binding (**Fig. 3e**). These data suggest that LIN28 and MBNL1 may bind the pre-miR-1 loop in a mutually exclusive pattern.

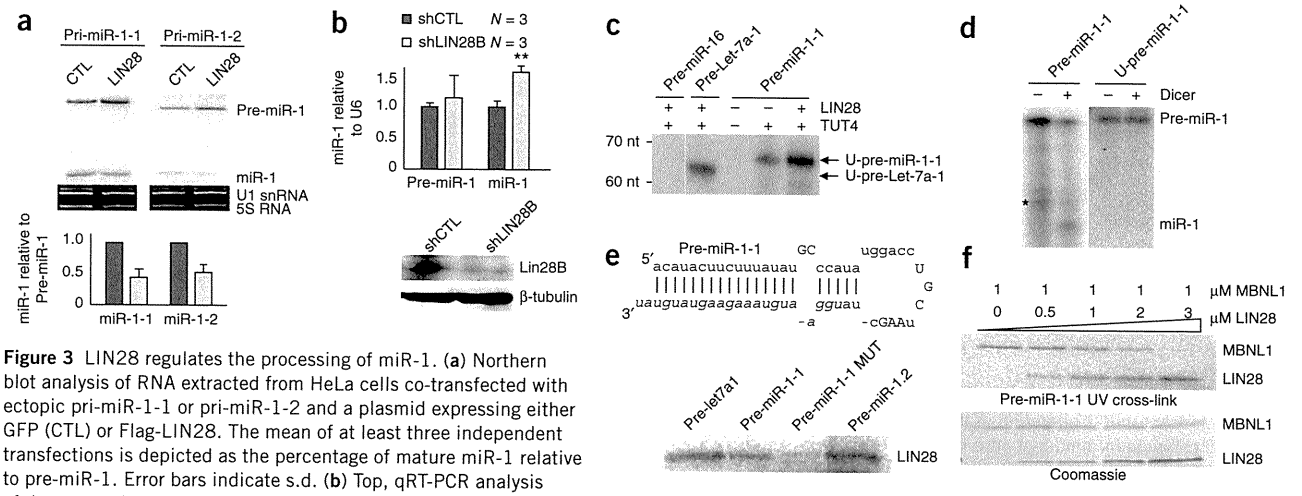

Figure 3 LIN28 regulates the processing of miR-1. (**a**) Northern blot analysis of RNA extracted from HeLa cells co-transfected with ectopic pri-miR-1-1 or pri-miR-1-2 and a plasmid expressing either GFP (CTL) or Flag-LIN28. The mean of at least three independent transfections is depicted as the percentage of mature miR-1 relative to pre-miR-1. Error bars indicate s.d. (**b**) Top, qRT-PCR analysis of the expression of endogenous pre-miR-1 and mature miR-1 in undifferentiated H9C2 rat cardiomyoblastes infected with recombinant lentivirus expressing an shRNA against *Gapdh* (shCTL) or against *Lin28B* (shLIN28B). The mean of at least three independent infections is depicted as the percentage of mature or pre-miR-1 relative to the U6 snRNA. Error bars indicate s.d. $^{**}P < 0.01$. Bottom, Lin28B depletion was confirmed by western blotting. (**c**) Immunoprecipitated Flag-TUT4 uridylates pre-miR-1-1 in presence of [α-^{32}P]UTP and 100 nM of His-LIN28. Pre-miR-16 and pre-Let-7-A1 are negative and positive controls, respectively. (**d**) Immunoprecipitated Myc-Dicer processes [γ-^{32}P]–labeled pre-miR-1 into mature miR-1 but does not cleave pre-miR-1 that was previously uridylated by TUT4 and LIN28. The asterisk (*) indicates a nonspecific degradation product. (**e**) Top, sequence of human pre-miR-1-1. Mature miR-1 is indicated in italics. Mutated UGC and AAG motifs are indicated in upper case (MUT). Bottom, UV-cross-linking analysis of pre-miR-1-1, pre-miR-1-2 and mutated pre-miR-1-1 (MUT) was conducted using purified His-LIN28 and uniformly [α-^{32}P]CTP-labeled RNAs. (**f**) UV-cross-linking binding of GST-MBNL1ΔCter to uniformly [α-^{32}P]CTP-labeled pre-miR-1-1 RNA is competed by the indicated increasing amounts of His-LIN28.

We confirmed this hypothesis and found that increasing amounts of recombinant purified LIN28 outcompeted MBNL1 binding to pre-miR-1-1 RNA (**Fig. 3f**). Both LIN28 and MBNL1 have been reported to be expressed in adult heart tissue[42,43], suggesting that competition may arise *in vivo*. Finally, we found that regulation of the processing of pre-miR-1 is a physiological process, which is controlled during rat H9C2 cardiomyocytes differentiation and correlates with a decreased expression of Lin28B (**Supplementary Fig. 3f**). This is consistent with splicing misregulation in myotonic dystrophy, which copies a undifferentiated state. Together, these data suggest that the LIN28 and ZCCHC11 pathway regulates the expression of miR-1 and that LIN28 and MBNL1 compete for binding to pre-miR-1-1, which implies that a reduction of free MBNL1 in myotonic dystrophy would enable LIN28 to bind and to downregulate pre-miR-1.

Targets of miR-1 are upregulated in myotonic dystrophy

Finally, miR-1 regulates the post-transcriptional expression of various targets[26,44–47], which brings into question the consequences and importance of misregulation of miR-1 for myotonic dystrophy. Analysis with miRanda (http://www.microrna.org/) predicted various targets of miR-1, including the cardiac L-type calcium channel gene *CACNA1C* (*CAV1.2*) (**Fig. 4a**). CACNA1C is the main calcium channel in heart tissue, and gain-of-function mutations in the *CACNA1C* gene result in arrhythmias and sudden death[48]. We confirmed that CACNA1C is regulated by miR-1, as co-transfection of miR-1 reduced the expression of a luciferase reporter plasmid containing part of the 3′ UTR sequences of *CACNA1C* (**Fig. 4b**). In addition, overexpression of miR-1 in H9C2 cardiomyoblasts reduced the level of endogenous CACNA1C protein, whereas an antisense RNA oligonucleotide directed against miR-1

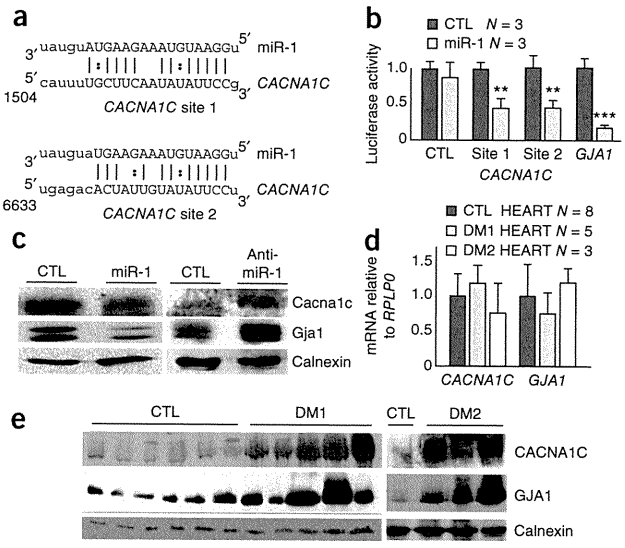

Figure 4 Targets of miR-1 are upregulated in myotonic dystrophies. (**a**) Sequence alignments between miR-1 and the 3′ UTR of human *CACNA1C*. (**b**) Luciferase activity of HeLa cells co-transfected with either no (CTL) or 40 nM of miR-1 mimic and a plasmid expressing no, *CACNA1C* or *GJA1* miR-1 binding sites (3×) cloned within the *Renilla* luciferase 3′ UTR. The mean of at least three independent transfections is depicted as the luciferase activity. Error bars indicate s.d. $^{**}P < 0.01$, $^{***}P < 0.001$. (**c**) Western blot analysis of the expression of endogenous Cacna1c, Gja1 and calnexin in H9C2 rat cardiomyoblasts transfected with 50 nM of miR-1 mimic or 50 nM of anti-miR-1. (**d**) qRT-PCR analysis of the expression of *CACNA1C* and *GJA1* relative to *RPLP0* mRNA in heart samples of control (2 unaffected, 1 with dilated cardiomyopathy and 5 with ALS), DM1- and DM2-affected subjects. Error bars indicate s.d. (**e**) Western blot analysis of the expression of CACNA1C, GJA1 and calnexin in membrane extracts from heart samples of control (2 unaffected, 1 with dilated cardiomyopathy and 5 with ALS), DM1- and DM2-affected subjects.

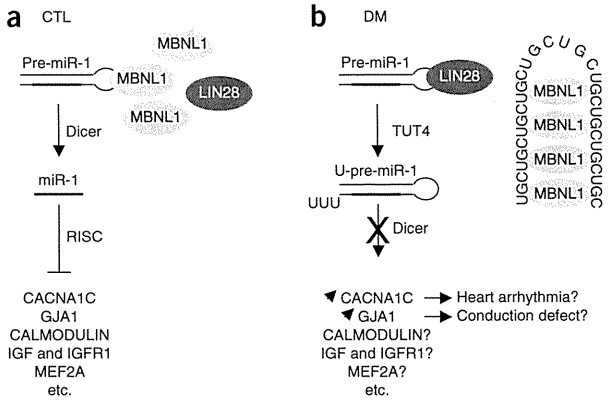

Figure 5 Model of miR-1 alteration in myotonic dystrophies. (**a**) MBNL1 and LIN28 compete for binding to pre-miR-1 loop. In presence of MBNL1, the processing of pre-miR-1 in mature miR-1 is favored and results in regulated expression of miR-1 targets. (**b**) In people with myotonic dystrophy, the sequestration of MBNL1 by expanded CUG or CCUG repeats allows LIN28 or LIN28B to bind to pre-miR-1, which promotes its consequent uridylation by TUT4. Uridylated pre-miR-1 is resistant to Dicer cleavage, which results in lower amounts of miR-1 and increased levels of its targets, GJA1 and CACNA1C.

increased the expression of endogenous CACNA1C (**Fig. 4c**). Notably, expression of CACNA1C protein was upregulated in DM1 and DM2 heart samples compared to control, whereas *CACNA1C* mRNA levels were not altered (**Fig. 4d,e**). Similarly, the expression of connexin 43 (GJA1), which is a known target of miR-1 (ref. 26) and is responsible for intracardiomyocyte conductance, was also upregulated at the protein level in heart samples of persons with DM1 or DM2 (**Fig. 4d,e**). These data demonstrate a post-transcriptional upregulation of miR-1 targets GJA1 and CACNA1C in people with myotonic dystrophy, which is consistent with a downregulation of the inhibitor, miR-1.

DISCUSSION

Our results suggest that mutant RNAs containing expanded CUG or CCUG repeats, which are known to modify the maturation of several pre-mRNAs, can also alter the processing of a pre-miRNA. Furthermore, we describe a previously unidentified function for MBNL1 as a cytoplasmic regulator of the biogenesis of pre-miR-1. Whether MBNL1 regulates the processing of other miRNAs remains to be determined, but we noted that UGC motifs are conserved in the loop of various pre-miRNAs (**Supplementary Table 1**). Furthermore, consistent with a report of LIN28 presence in the adult heart[42], our data suggest that the LIN28 and ZCCHC11 pathway regulates the processing of miR-1. We propose a model (**Fig. 5**) in which depletion of free MBNL1 by expanded CUG or CCUG repeats enables LIN28 and ZCCHC11 to uridylate pre-miR-1, which blocks Dicer processing, resulting in decreased expression of mature miR-1 and increased levels of its targets, including CACNA1C and GJA1, in the heart of people with myotonic dystrophy. GJA1 and CACNA1C encode the main gap junction and calcium channels in the heart, respectively, and we propose that their misregulation may contribute to the cardiac conduction defects and arrhythmias observed in people with DM1 or DM2 (**Fig. 5**). Whether other known (calmodulin[44], MEF2A[44], PP2A[45], IGF1 (ref. 46), TWF1 (ref. 47), etc.) or predicted (KCNA5, triadin, CUGBP2, etc.) targets of miR-1 are also upregulated in people with myotonic dystrophy remains to be determined. Furthermore, the miR-1 family regulates muscle-cell differentiation[49–53], which is delayed in the severe congenital DM1 form[54,55], suggesting that the alteration of pre-miR-1 processing may be involved in the manifestation of other symptoms of myotonic dystrophy. Finally, this work opens the possibility that the processing of specific miRNAs, which is highly regulated[56–59], can be altered and is involved in the development of human genetic diseases.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/nsmb/.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

ACKNOWLEDGMENTS

We thank T. Cooper (Baylor College of Medicine) for the gift of the DT960 and tgCUGBP1 plasmids, C. Branlant (National Center of Scientific Research) for the pGEX-MBNL1-Δ101 (MBNL1ΔCter) vector, M. Swanson (University of Florida) for the pGEX-6P-MBNL1-His vector, C. Thornton (University of Rochester) for the polyclonal antibody against MBNL1, N. Kim (University of Seoul) for the pri-miR-16, Flag-TUT4, His-LIN28 and Flag-LIN28 plasmids, P. Provost (Université Laval) for the gift of the Myc-Dicer plasmid and all members of the French Myotonic Dystrophy Network for fruitful discussion. This work was supported by Institut National de la Santé et de la Recherche Médicale (INSERM) AVENIR (N.C.-B.), Agence nationale de la recherche (ANR) GENOPAT P007942 (N.C.B.), Association Française contre les Myopathies (AFM) MNM1-12982 (N.C.B.), the UPMC-emergence program (D.F), US National Institutes of Health (NIH) P30 AR057220 (J.W.D.), Muscular Dystrophy Center Core Laboratories (J.W.D.), the Ministère de l'enseignement supérieur et de la recherche and AFM (F.R., F.F.) and the University of Strasbourg (F.R., F.F.).

AUTHOR CONTRIBUTIONS

Experiments were conducted by F.R., F.F., C.F., J.-P.V., D.D., N.D., M.-C.F., A.N., D.A. and B.J. Clinical samples and patient data were obtained from J.W.D., D.D., K.W., D.F., G.G., H.F., D.D., M.P.T. and from the Research Resource Network supported by the Research Grant for Nervous and Mental Disorders from the Ministry of Health, Labour and Welfare, Japan. The study was designed and coordinated by N.D., D.F. and N.C.-B. The paper was written by N.C.-B.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/nsmb/.
Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Brook, J.D. *et al.* Molecular basis of myotonic dystrophy: expansion of a trinucleotide (CTG) repeat at the 3′ end of a transcript encoding a protein kinase family member. *Cell* **68**, 799–808 (1992).
2. Fu, Y.H. *et al.* An unstable triplet repeat in a gene related to myotonic muscular dystrophy. *Science* **255**, 1256–1258 (1992).
3. Mahadevan, M. *et al.* Myotonic dystrophy mutation: an unstable CTG repeat in the 3′ untranslated region of the gene. *Science* **255**, 1253–1255 (1992).
4. Liquori, C.L. *et al.* Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. *Science* **293**, 864–867 (2001).
5. Miller, J.W. *et al.* Recruitment of human muscleblind proteins to (CUG)n expansions associated with myotonic dystrophy. *EMBO J.* **19**, 4439–4448 (2000).
6. Kanadia, R.N. *et al.* A muscleblind knockout model for myotonic dystrophy. *Science* **302**, 1978–1980 (2003).
7. Ho, T.H. *et al.* Muscleblind proteins regulate alternative splicing. *EMBO J.* **23**, 3103–3112 (2004).
8. Kanadia, R.N. *et al.* Reversal of RNA missplicing and myotonia after muscleblind overexpression in a mouse poly(CUG) model for myotonic dystrophy. *Proc. Natl. Acad. Sci. USA* **103**, 11748–11753 (2006).
9. Philips, A.V., Timchenko, L.T. & Cooper, T.A. Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy. *Science* **280**, 737–741 (1998).
10. Kuyumcu-Martinez, N.M. *et al.* Increased steady-state levels of CUGBP1 in myotonic dystrophy 1 are due to PKC-mediated hyperphosphorylation. *Mol. Cell* **28**, 68–78 (2007).
11. Mankodi, A. *et al.* Myotonic dystrophy in transgenic mice expressing an expanded CUG repeat. *Science* **289**, 1769–1773 (2000).
12. Lin, X. *et al.* Failure of MBNL1-dependent post-natal splicing transitions in myotonic dystrophy. *Hum. Mol. Genet.* **15**, 2087–2097 (2006).
13. Kalsotra, A. *et al.* A postnatal switch of CELF and MBNL proteins reprograms alternative splicing in the developing heart. *Proc. Natl. Acad. Sci. USA* **105**, 20333–20338 (2008).

14. Savkur, R.S. *et al.* Aberrant regulation of insulin receptor alternative splicing is associated with insulin resistance in myotonic dystrophy. *Nat. Genet.* **29**, 40–47 (2001).
15. Mankodi, A. *et al.* Expanded CUG repeats trigger aberrant splicing of ClC-1 chloride channel pre-mRNA and hyperexcitability of skeletal muscle in myotonic dystrophy. *Mol. Cell* **10**, 35–44 (2002).
16. Charlet-B., N. *et al.* Loss of the muscle-specific chloride channel in type 1 myotonic dystrophy due to misregulated alternative splicing. *Mol. Cell* **10**, 45–53 (2002).
17. Wheeler, T.M. *et al.* Correction of ClC-1 splicing eliminates chloride channelopathy and myotonia in mouse models of myotonic dystrophy. *J. Clin. Invest.* **117**, 3952–3957 (2007).
18. Groh, W.J. *et al.* Electrocardiographic abnormalities and sudden death in myotonic dystrophy type 1. *N. Engl. J. Med.* **358**, 2688–2697 (2008).
19. Pelargonio, G. *et al.* Myotonic dystrophy and the heart. *Heart* **88**, 665–670 (2002).
20. Phillips, M.F. & Harper, P.S. Cardiac disease in myotonic dystrophy. *Cardiovasc. Res.* **33**, 13–22 (1997).
21. Lazarus, A. *et al.* Long-term follow-up of arrhythmias in patients with myotonic dystrophy treated by pacing: a multicenter diagnostic pacemaker study. *J. Am. Coll. Cardiol.* **40**, 1645–1652 (2002).
22. Wang, G.S. *et al.* Elevation of RNA-binding protein CUGBP1 is an early event in an inducible heart-specific mouse model of myotonic dystrophy. *J. Clin. Invest.* **117**, 2802–2811 (2007).
23. Mahadevan, M.S. *et al.* Reversible model of RNA toxicity and cardiac conduction defects in myotonic dystrophy. *Nat. Genet.* **38**, 1066–1070 (2006).
24. Sayed, D. *et al.* MicroRNAs play an essential role in the development of cardiac hypertrophy. *Circ. Res.* **100**, 416–424 (2007).
25. Carè, A. *et al.* MicroRNA-133 controls cardiac hypertrophy. *Nat. Med.* **13**, 613–618 (2007).
26. Yang, B. *et al.* The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2. *Nat. Med.* **13**, 486–491 (2007).
27. Zhao, Y. *et al.* Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1–2. *Cell* **129**, 303–317 (2007).
28. Schoser, B.G. *et al.* Sudden cardiac death in myotonic dystrophy type 2. *Neurology* **63**, 2402–2404 (2004).
29. Lee, Y. *et al.* The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415–419 (2003).
30. Denli, A.M. *et al.* Processing of primary microRNAs by the Microprocessor complex. *Nature* **432**, 231–235 (2004).
31. Han, J. *et al.* The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev.* **18**, 3016–3027 (2004).
32. Lund, E. *et al.* Nuclear export of microRNA precursors. *Science* **303**, 95–98 (2004).
33. Yi, R. *et al.* Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev.* **17**, 3011–3016 (2003).
34. Zhang, H. *et al.* Single processing center models for human Dicer and bacterial RNase III. *Cell* **118**, 57–68 (2004).
35. Chendrimada, T.P. *et al.* TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436**, 740–744 (2005).
36. Goers, E.S. *et al.* MBNL1 binds GC motifs embedded in pyrimidines to regulate alternative splicing. *Nucleic Acids Res.* **38**, 2467–2484 (2010).
37. Yuan, Y. *et al.* Muscleblind-like 1 interacts with RNA hairpins in splicing target and pathogenic RNAs. *Nucleic Acids Res.* **35**, 5474–5486 (2007).
38. Heo, I. *et al.* Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol. Cell* **32**, 276–284 (2008).
39. Rybak, A. *et al.* A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. *Nat. Cell Biol.* **10**, 987–993 (2008).
40. Hagan, J.P. *et al.* Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells. *Nat. Struct. Mol. Biol.* **16**, 1021–1025 (2009).
41. Heo, I. *et al.* TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell* **138**, 696–708 (2009).
42. Yang, D.H. & Moss, E.G. Temporally regulated expression of Lin-28 in diverse tissues of the developing mouse. *Gene Expr. Patterns* **3**, 719–726 (2003).
43. Kalsotra, A. *et al.* A postnatal switch of CELF and MBNL proteins reprograms alternative splicing in the developing heart. *Proc. Natl. Acad. Sci. USA* **105**, 20333–20338 (2008).
44. Ikeda, S. *et al.* MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes. *Mol. Cell. Biol.* **8**, 2193–2204 (2009).
45. Terentyev, D. *et al.* miR-1 overexpression enhances Ca^{2+} release and promotes cardiac arrhythmogenesis by targeting PP2A regulatory subunit B56α and causing CaMKII-dependent hyperphosphorylation of RyR2. *Circ. Res.* **104**, 514–521 (2009).
46. Elia, L. *et al.* Reciprocal regulation of microRNA-1 and insulin-like growth factor-1 signal transduction cascade in cardiac and skeletal muscle in physiological and pathological conditions. *Circulation* **120**, 2377–2385 (2009).
47. Li, Q. *et al.* Attenuation of microRNA-1 derepresses the cytoskeleton regulatory protein twinfilin-1 to provoke cardiac hypertrophy. *J. Cell Sci.* **123**, 2444–2452 (2010).
48. Splawski, I. *et al.* $Ca_V1.2$ calcium channel dysfunction causes a multisystem disorder including arrhythmia and autism. *Cell* **119**, 19–31 (2004).
49. Hirai, H. *et al.* MyoD regulates apoptosis of myoblasts through microRNA-mediated down-regulation of Pax3. *J. Cell Biol.* **191**, 347–365 (2010).
50. Chen, J.F. *et al.* microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. *J. Cell Biol.* **190**, 867–879 (2010).
51. Cacchiarelli, D. *et al.* MicroRNAs involved in molecular circuitries relevant for the Duchenne muscular dystrophy pathogenesis are controlled by the dystrophin / nNOS pathway. *Cell Metab.* **12**, 341–351 (2010).
52. Chen, J.F. *et al.* The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat. Genet.* **38**, 228–233 (2006).
53. Yan, D. *et al.* MicroRNA-1/206 targets c-Met and inhibits rhabdomyosarcoma development. *J. Biol. Chem.* **284**, 29596–29604 (2009).
54. Furling, D., Lemieux, D., Taneja, K. & Puymirat, J. Decreased levels of myotonic dystrophy protein kinase (DMPK) and delayed differentiation in human myotonic dystrophy myoblasts. *Neuromuscul. Disord.* **8**, 728–735 (2001).
55. Furling, D. *et al.* Defective satellite cells in congenital myotonic dystrophy. *Hum. Mol. Genet.* **10**, 2079–2087 (2001).
56. Guil, S. & Cáceres, J.F. The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. *Nat. Struct. Mol. Biol.* **14**, 591–596 (2007).
57. Davis, B.N. *et al.* Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha. *Mol. Cell* **39**, 373–384 (2010).
58. Trabucchi, M. *et al.* The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. *Nature* **459**, 1010–1014 (2009).
59. Yang, W. *et al.* Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. *Nat. Struct. Mol. Biol.* **13**, 13–21 (2006).

ONLINE METHODS

Samples. All samples were from heart left ventricles. Heart samples from unaffected persons (CTL #1 and #2) were purchased at Ambion and Stratagene, respectively. CTL #3 was from a person suffering from familial dilated cardiomyopathy. CTL #4–8 were from ALS-affected subjects described previously[60]. DM1 #1 was from a 45-year-old patient suffering from DM1 with an expansion of 166 CTG repeats in the blood who underwent a cardiac transplantation for end-stage heart failure and dilated cardiomyopathy with conduction system disease. DM1 #2–5 were from previously described subjects (DM1 #9–11 in ref. 60) with expansion of 4,300 (female, 58 years), 4,800 (male, 63 years), 5,800 (female, 56 years) and ~6,000 CTG repeats in the heart. Subjects with DM2 were described previously[28].

RT-qPCR. miRNAs RT-qPCR analyses were conducted using the miScript reverse transcription kit (Qiagen), the specific miScript primer and precursor assays (Qiagen) and an miScript Sybr green PCR kit (Qiagen) in a Lightcycler 480 (Roche) for 15 min at 94 °C, followed by 50 cycles of 15 s at 94 °C, 20 s at 55 °C and 20 s at 72 °C. U6 snRNA was used as the standard. *GJA1* (fwd: 5′-TTTCCATCCACTTGCACAATA3′, rev: 5′-GGCTTGATTCCCTGACT-3′) and *CACNA1C* (fwd: 5′-TTCGTGTCCCTCTTCAACC-3′, rev: 5′-GTTGCT CAAGGAGTTCCAGTA-3′) RT-qPCR analyses were conducted using the Transcriptor Reverse Transcriptase Kit (Roche) and the QuantiTect SYBR Green PCR kit (QIAGEN) in a Lightcycler 480 (Roche) for 15 min at 94 °C, followed by 50 cycles of 15 s at 94 °C, 20 s at 58 °C and 20 s at 72 °C. *RPLP0* (fwd: 5′-GAAGTCACTGTGCCAGCCCA-3′, rev: 5′-GAAGGTGTAATCCGTCTCCA-3′) was used as the standard. Data were analyzed using Lightcycler 480 analysis software and the 2ΔCt method.

Northern blotting. HeLa cells were co-transfected using JetPei (Polyplus) in DMEM, 1 g l^{-1} glucose, 5% fetal calf serum and gentamycin at 37 °C, 5% (v/v) CO_2. 24 h after transfection, total RNA was extracted using Tri Reagent, treated with DNase, extracted with phenol-chloroform and precipitated with ethanol. 5 µg of RNA were electrophoresed on a 12% acryl-urea gel, transferred on nylon membrane, prehybridized 90 min at 42 °C in 6× SSPE, 0.1% (v/v) SDS, 2× Denhardt, hybridized overnight at 42 °C with 50 ng of antisense miR-1 (5′-ATACATACTTCTTTACATTCCA-3′) or U6 snRNA (5′-ATA TGGAACGCTTCACGAATT-3′) [γ-^{32}P]ATP-labeled probe, washed three times in SSPE 6×, SDS 0.1% (v/v), and revealed using a Typhoon scanner.

Lentiviral and adenoviral infection of H9c2 cells. Lentivirus expressing an shRNA against LIN28B were ordered from OpenBiosystems (V3LHS_327847) H9C2 cells were grown in DMEM, 1 g l^{-1} glucose, 10% (v/v) fetal calf serum and gentamycin at 37 °C, 5% (v/v) CO_2 and infected at a multiplicity of infection (MOI) of 20 during 48 h. Adenovirus expressing shMBNL1 or 960 CUG repeats were cloned into pAD-DEST (Invitrogen) and produced according to manufacturer's instructions. H9C2 cells were grown in DMEM, 1 gl^{-1} glucose, 10% fetal calf serum and gentamycin at 37 °C, 5% (v/v) CO_2. Confluent H9C2 cells were differentiated for 6 d in DMEM, 1 gl^{-1} glucose, 2% v/v) horse serum and gentamycin at 37 °C, 5% (v/v) CO_2 and transduced with recombinant CUG)960x or shMBNL1 adenovirus (MOI 100). Medium was replaced 24 h after infection, and cells were analyzed 48 h after infection.

***In vitro* uridylation.** HeLa cells were collected 24 h after transfection with Flag-TUT4, incubated at 4 °C in 300 mM Nacl, 10 mM Tris, pH 8.0, 1% (v/v) Triton X-100 for 20 min, sonicated and centrifuged 10 min at 21,000*g* at 4 °C. Supernatant was incubated with 30 µl of anti-Flag M2 affinity gel (Sigma) with constant rotation for 2 h at 4 °C. Beads were washed 3 times in 200 mM KCl, 10 mM Tris, pH 8.0, and incubated with 5 pmol of synthetic pre-miR-16, pre-miR-1-1 or pre-Let-7a-1 RNA (Sigma), 3.2 mM $MgCl_2$ and 125 nM [α-^{32}P]UTP at 37 °C for 1 h and analyzed on 12% (v/v) acryl, 8 M urea gel.

***In vitro* Dicer processing.** HeLa cells were harvested 24 h after transfection with Myc-Dicer, incubated at 4 °C in lysis buffer (150 mM NaCl, 10 mM Tris, pH 8.0, 0.1% (v/v) Triton X-100) for 20 min, sonicated and centrifuged for 10 min at 21,000*g*. Supernatant was incubated with 30 µl of anti-Myc antibody bound to protein G–Sepharose with constant rotation for 2 h at 4 °C. Beads were washed three times in lysis buffer and incubated with [γ-^{32}P]ATP-labeled pre-miR-1-1 or previously uridylated pre-miR-1-1 in 6.4 mM $MgCl_2$, 1 mM DTT, for 30 min at 37 °C and analyzed in 12% (v/v) acrylamide, 8 M urea gel.

Luciferase assays. 40-nt fragments encompassing miR-1 binding sites within the 3′ UTR of human *CACNA1C* or *GJA1* were inserted in triplicate within the XbaI-NotI sites of pRLTK. HeLa cells were plated in a 24-well plate and transfected with 100 ng of luciferase vectors and 20 pmol of mimic miR-1 (Qiagen), using Lipofectamine2000 and assayed for luciferase activities 24 h after transfection, using the Dual Luciferase Reporter Assay kit (Promega).

Western blotting. Total- and membrane-protein extraction were done as previously described[17], and the product was separated on 7% (v/v) or 10% (v/v) SDS-PAGE gel, transferred onto nitrocellulose membranes, blocked with 5% (v/v) nonfat dry milk (NFM) in TBS, incubated with GJA1 (rabbit polyclonal, Sigma, 1:20,000), CACNA1C (rabbit polyclonal, Alomone, 1:100), MBNL1 (rabbit polyclonal, 1:100, gift from C. Thornton), LIN28B (rabbit polyclonal ab71415, Abcam) or calnexin (rabbit polyclonal, Stressgen, 1:1,000) antibody overnight in TBS and 5% (v/v) NFM, washed and incubated with Donkey anti-rabbit peroxidase antibody (Jackson Immunoresearch, 1:10,000) for 1 h in TBS and 5% (v/v) NFM, 0.1% (v/v) Tween 20, which was followed by autoradiographic revelation.

Additional methods. Information on miRNA expression profiling, bioinformatic analysis, RNA fluorescence *in situ* hybridization (FISH), immunofluorescence, recombinant protein production and purification, UV-cross-linking assays, gel-shift assay constructions and primer sequences is available in the **Supplementary Methods**.

60. Nakamori, M. *et al.* Aberrantly spliced α-dystrobrevin alters α-syntrophin binding in myotonic dystrophy type 1. *Neurology* **70**, 677–685 (2008).

doi:10.1038/nsmb.2067