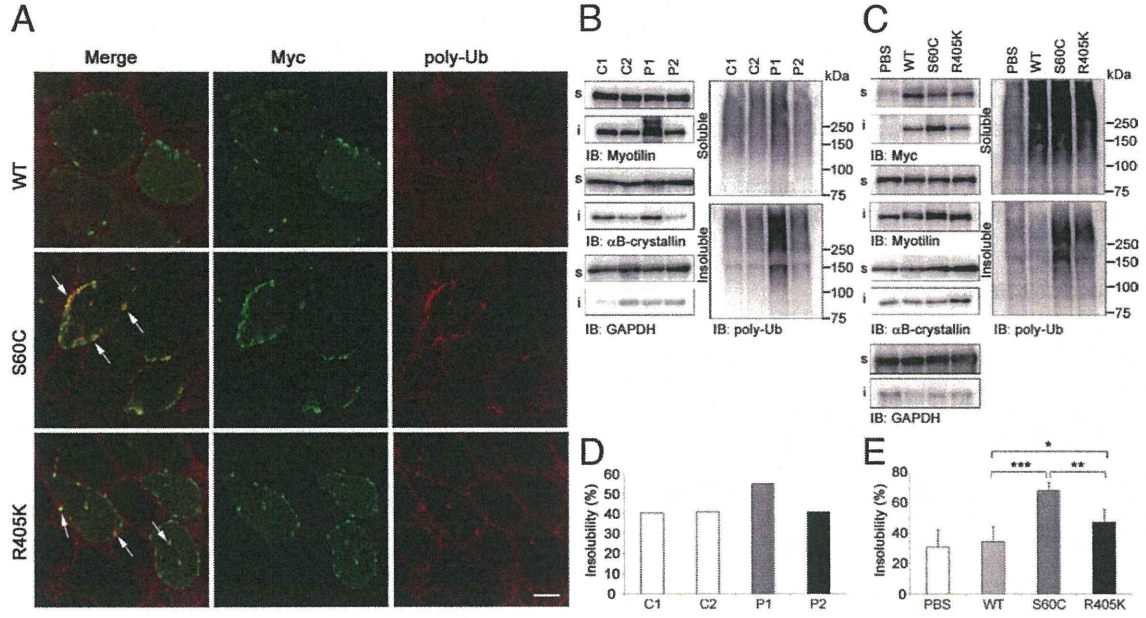

Figure 6. Mutant myotilin displays marked detergent insolubility, along with polyubiquitinated proteins. **A:** At 14 days after electroporation of Myc-wtMYOT (WT) or Myc-mMYOT (S60C or R405K), Myc-mMYOT aggregates, particularly those of S60C, colocalized with polyubiquitin (polyUb) (**arrows**). The WT aggregates rarely costained with polyubiquitin. **B–E** Solubilities of myotilin, polyubiquitinated proteins, and other sarcomeric proteins in muscles from myotilinopathy patients (**B** and **D**) and from electroporated mice (**C** and **E**). GAPDH was used as a loading control. **B:** Immunoblotting of detergent-soluble and detergent-insoluble fractions of muscles from control subjects (C1 and C2) or myotilinopathy patients [P1 (patient 1) and P2 (patient 2)]. In the muscles from P1 with S60C, markedly increasing amounts of myotilin, polyubiquitinated proteins, and αB-crystallin were detected in the insoluble fraction, compared with muscles from control subjects. **D:** Quantification of myotilin insolubilities revealed highest insolubility in P1. **C:** Immunoblotting of detergent-soluble and detergent-insoluble fractions of WT, S60C, or R405K-expressing muscles at 14 days after electroporation. Increasing amounts of insoluble Myc-tagged myotilin proteins and polyubiquitinated proteins were observed in mMYOT-electroporated muscles, compared with WT. Particularly in S60C-electroporated muscles, the amounts of insoluble proteins were notably increased. **E:** Quantification of the insolubilities of electroporated Myc-tagged myotilin in the WT, S60C, and R405K expression groups ($n = 6$ mice per group). Insolubility of endogenous myotilin was measured using PBS-treated mouse muscles. Compared with WT, insolubilities of electroporated Myc-tagged myotilin were significantly increased in S60C and R405K. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Scale bar = 20 μm.

ize with ubiquitin and Z-disk–associated proteins (ie, αB-crystallin, BAG3, actin, desmin, and filamin C) in the muscles of myotilinopathy patients (Figure 1; see also Supplemental Figure S2 at *http://ajp.amjpathol.org*). It has been reported that the myotilin T57I transgenic mice develop progressive myofibrillar changes, including Z-disk streaming and accumulation of mutant myotilin with ubiquitin and Z-disk–associated proteins, similar to those observed in myotilinopathy patients.[28] Expression of mMYOT elicited similar cytoplasmic aggregations in mouse skeletal muscle, and within 2 weeks the aggregates colocalized with polyubiquitin and other Z-disk–associated proteins. Our results indicate that mutant myotilin is able to nucleate aggregations of Z-disk–associated proteins in skeletal muscle.

MFM is a proteinopathy (ie, a protein accumulation disease). In these diseases, protein aggregates are operationally defined by poor solubility in aqueous or detergent solvents.[39,40] Such insoluble protein aggregations are characteristic of many neurodegenerative diseases.[41] In the present study, we discovered that the mutant myotilin S60C protein, along with polyubiquitinated proteins, exhibited marked detergent insolubility in muscles from both the patient and electroporated mice. Mutant myotilin R405K protein showed increased, but lower, detergent insolubility in mice (Figure 6), which may be consistent with the observation that the muscle from the patient with the R405K mutation exhibited only mild protein aggregation (Figure 1). The different detergent insolubilities exhibited by the two *MYOT* mutations may closely correlate with the amounts of protein aggregation. Here, we confirmed the aggregation-prone property of mutant myotilin, which participates in the pathogenesis of myotilinopathy. Using an immunoprecipitation assay, we also showed that electroporated mMYOT was not ubiquitinated in the detergent-soluble fraction (see Supplemental Figure S4 at *http://ajp.amjpathol.org*). A previous study showed that transfected myotilin is degraded by the proteasome system in cultured cells.[42] Our present findings show that ubiquitinated mutant myotilin can form insoluble aggregates. It is also possible that aggregation of insoluble ubiquitinated proteins is induced by the expression of mutant myotilin.

Several causative genes have been identified for MFM; however, in previous studies no mutations were found in nearly half of the MFM patients.[2] To identify the unknown causative genes, easy methods are required for determining the pathogenicity of novel mutations. Some mutant proteins exhibit protein aggregation[43–45] or biological dysfunction, including protein-protein interaction *in vitro*.[23,46–48] However, we could not detect any protein aggregation in mMYOT-expressing cultured cells (Figure 2). The difficulty of *in vitro* investigation may be responsible for the inability to identify Z-disk–associated proteins or mature Z-disk structures. Indeed, myotilin is expressed in later differentiated C2C12 myotubes with

sarcomere-like structures.[49] This suggests that mutant myotilin requires mature Z-disk and/or other sarcomeric proteins to cause aggregations. In such cases, *in vivo* examination is important for evaluating the pathogenicity of mutations. Because *in vivo* electroporation can reproduce the pathological changes observed in MFM patients within a short time, it is a useful and powerful tool for evaluating the pathogenicity of mutations in MFM.

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References

1. Selcen D: Myofibrillar myopathies. Neuromuscul Disord 2011, 21: 161–171
2. Selcen D, Engel AG: Myofibrillar myopathy. (Updated) In GeneReviews. Copyright University of Washington, Seattle. 1997–2012. Available at http://www.ncbi.nlm.nih.gov/books/NBK1499, last revised July 27, 2010
3. OlivÉ M, Odgerel Z, Martínez A, Poza JJ, Bragado FG, Zabalza RJ, Jericó I, Gonzalez-Mera L, Shatunov A, Lee HS, Armstrong J, Maravi E, Arroyo MR, Pascual-Calvet J, Navarro C, Paradas C, Huerta M, Marquez F, Rivas EG, Pou A, Ferrer I, Goldfarb LG: Clinical and myopathological evaluation of early- and late-onset subtypes of myofibrillar myopathy. Neuromuscul Disord 2011, 21:533–542
4. Salmikangas P, Mykkänen OM, Grönholm M, Heiska L, Kere J, Carpén O: Myotilin, a novel sarcomeric protein with two Ig-like domains, is encoded by a candidate gene for limb-girdle muscular dystrophy. Hum Mol Genet 1999, 8:1329–1336
5. Parast MM, Otey CA: Characterization of palladin, a novel protein localized to stress fibers and cell adhesions. J Cell Biol 2000, 150: 643–656
6. Mykkänen OM, Grönholm M, Rönty M, Lalowski M, Salmikangas P, Suila H, Carpén O: Characterization of human palladin, a microfilament-associated protein. Mol Biol Cell 2001, 12:3060–3073
7. Bang ML, Mudry RE, McElhinny AS, Trombitas K, Geach AJ, Yamasaki R, Sorimachi H, Granzier H, Gregorio CC, Labeit S: Myopalladin, a novel 145-kilodalton sarcomeric protein with multiple roles in Z-disc and I-band protein assemblies. J Cell Biol 2001, 153:413–427
8. Faulkner G, Lanfranchi G, Valle G: Telethonin and other new proteins of the Z-disc of skeletal muscle. IUBMB Life 2001, 51:275–282
9. Frank D, Kuhn C, Katus HA, Frey N: The sarcomeric Z-disc: a nodal point in signalling and disease. J Mol Med (Berl) 2006, 84:446–468
10. van der Ven PF, Wiesner S, Salmikangas P, Auerbach D, Himmel M, Kempa S, Hayess K, Pacholsky D, Taivainen A, Schröder R, Carpén O, Fürst DO: Indications for a novel muscular dystrophy pathway. gamma-Filamin, the muscle-specific filamin isoform, interacts with myotilin. J Cell Biol 2000, 151:235–248
11. Gontier Y, Taivainen A, Fontao L, Sonnenberg A, van der Flier A, Carpen O, Faulkner G, Borradori L: The Z-disc proteins myotilin and FATZ-1 interact with each other and are connected to the sarcolemma via muscle-specific filamins. J Cell Sci 2005, 118:3739–3749
12. von Nandelstadh P, Ismail M, Gardin C, Suila H, Zara I, Belgrano A, Valle G, Carpen O, Faulkner G: A class III PDZ binding motif in the myotilin and FATZ families binds enigma family proteins: a common link for Z-disc myopathies. Mol Cell Biol 2009, 29:822–834
13. Witt SH, Granzier H, Witt CC, Labeit S: MURF-1 and MURF-2 target a specific subset of myofibrillar proteins redundantly: towards understanding MURF-dependent muscle ubiquitination. J Mol Biol 2005, 350:713–722
14. Salmikangas P, van der Ven PF, Lalowski M, Taivainen A, Zhao F, Suila H, Schröder R, Lappalainen P, Fürst DO, Carpén O: Myotilin, the limb-girdle muscular dystrophy 1A (LGMD1A) protein, cross-links actin filaments and controls sarcomere assembly. Hum Mol Genet 2003, 12:189–203
15. von Nandelstadh P, Grönholm M, Moza M, Lamberg A, Savilahti H, Carpén O: Actin-organising properties of the muscular dystrophy protein myotilin. Exp Cell Res 2005, 310:131–139
16. Selcen D: Myofibrillar myopathies. Curr Opin Neurol 2008, 21:585–589
17. Olivé M, Goldfarb LG, Shatunov A, Fischer D, Ferrer I: Myotilinopathy: refining the clinical and myopathological phenotype. Brain 2005, 128:2315–2326
18. Foroud T, Pankratz N, Batchman AP, Pauciulo MW, Vidal R, Miravalle L, Goebel HH, Cushman LJ, Azzarelli B, Horak H, Farlow M, Nichols WC: A mutation in myotilin causes spheroid body myopathy. Neurology 2005, 65:1936–1940
19. Hauser MA, Conde CB, Kowaljow V, Zeppa G, Taratuto AL, Torian UM, Vance J, Pericak-Vance MA, Speer MC, Rosa AL: Myotilin mutation found in second pedigree with LGMD1A. Am J Hum Genet 2002, 71:1428–1432
20. Hauser MA, Horrigan SK, Salmikangas P, Torian UM, Viles KD, Dancel R, Tim RW, Taivainen A, Bartoloni L, Gilchrist JM, Stajich JM, Gaskell PC, Gilbert JR, Vance JM, Pericak-Vance MA, Carpen O, Westbrook CA, Speer MC: Myotilin is mutated in limb girdle muscular dystrophy 1A. Hum Mol Genet 2000, 9:2141–2147
21. Penisson-Besnier I, Talvinen K, Dumez C, Vihola A, Dubas F, Fardeau M, Hackman P, Carpen O, Udd B: Myotilinopathy in a family with late onset myopathy. Neuromuscul Disord 2006, 16:427–431
22. Berciano J, Gallardo E, Dominguez-Perles R, Garcia A, Garcia-Barredo R, Combarros O, Infante J, Illa I: Autosomal-dominant distal myopathy with a myotilin S55F mutation: sorting out the phenotype. J Neurol Neurosurg Psychiatry 2008, 79:205–208
23. Shalaby S, Mitsuhashi H, Matsuda C, Minami N, Noguchi S, Nonaka I, Nishino I, Hayashi YK: Defective myotilin homodimerization caused by a novel mutation in MYOT exon 9 in the first Japanese limb girdle muscular dystrophy 1A patient. J Neuropathol Exp Neurol 2009, 68:701–707
24. Reilich P, Krause S, Schramm N, Klutzny U, Bulst S, Zehetmayer B, Schneiderat P, Walter MC, Schoser B, Lochmüller H: A novel mutation in the myotilin gene (MYOT) causes a severe form of limb girdle muscular dystrophy 1A (LGMD1A). J Neurol 2011, 258:1437–1444
25. Mavroidis M, Panagopoulou P, Kostavasili I, Weisleder N, Capetanaki Y: A missense mutation in desmin tail domain linked to human dilated cardiomyopathy promotes cleavage of the head domain and abolishes its Z-disc localization. FASEB J 2008, 22:3318–3327
26. Wang X, Osinska H, Klevitsky R, Gerdes AM, Nieman M, Lorenz J, Hewett T, Robbins J: Expression of R120G-alphaB-crystallin causes aberrant desmin and alphaB-crystallin aggregation and cardiomyopathy in mice. Circ Res 2001, 89:84–91
27. Wang X, Osinska H, Dorn GW 2nd, Nieman M, Lorenz JN, Gerdes AM, Witt S, Kimball T, Gulick J, Robbins J: Mouse model of desmin-related cardiomyopathy. Circulation 2001, 103:2402–2407
28. Garvey SM, Miller SE, Claflin DR, Faulkner JA, Hauser MA: Transgenic mice expressing the myotilin T57I mutation unite the pathology associated with LGMD1A and MFM. Hum Mol Genet 2006, 15:2348–2362
29. Hayashi YK, Matsuda C, Ogawa M, Goto K, Tominaga K, Mitsuhashi S, Park YE, Nonaka I, Hino-Fukuyo N, Haginoya K, Sugano H, Nishino I: Human PTRF mutations cause secondary deficiency of caveolins resulting in muscular dystrophy with generalized lipodystrophy. J Clin Invest 2009, 119:2623–2633
30. Thompson TG, Chan YM, Hack AA, Brosius M, Rajala M, Lidov HG, McNally EM, Watkins S, Kunkel LM: Filamin 2 (FLN2): A muscle-specific sarcoglycan interacting protein. J Cell Biol 2000, 148:115–126
31. Schröder R, Schoser B: Myofibrillar myopathies: a clinical and myopathological guide. Brain Pathol 2009, 19:483–492
32. Liu J, Chen Q, Huang W, Horak KM, Zheng H, Mestril R, Wang X: Impairment of the ubiquitin-proteasome system in desminopathy mouse hearts. FASEB J 2006, 20:362–364
33. Liu J, Tang M, Mestril R, Wang X: Aberrant protein aggregation is essential for a mutant desmin to impair the proteolytic function of the ubiquitin-proteasome system in cardiomyocytes. J Mol Cell Cardiol 2006, 40:451–454
34. Selcen D, Ohno K, Engel AG: Myofibrillar myopathy: clinical, morphological and genetic studies in 63 patients. Brain 2004, 127:439–451

35. Claeys KG, Fardeau M, Schröder R, Suominen T, Tolksdorf K, Behin A, Dubourg O, Eymard B, Maisonobe T, Stojkovic T, Faulkner G, Richard P, Vicart P, Udd B, Voit T, Stoltenburg G: Electron microscopy in myofibrillar myopathies reveals clues to the mutated gene. Neuromuscul Disord 2008, 18:656–666
36. Claeys KG, van der Ven PF, Behin A, Stojkovic T, Eymard B, Dubourg O, Laforêt P, Faulkner G, Richard P, Vicart P, Romero NB, Stoltenburg G, Udd B, Fardeau M, Voit T, Fürst DO: Differential involvement of sarcomeric proteins in myofibrillar myopathies: a morphological and immunohistochemical study. Acta Neuropathol 2009, 117:293–307
37. Olivé M: Extralysosomal protein degradation in myofibrillar myopathies. Brain Pathol 2009, 19:507–515
38. Janué A, Olivé M, Ferrer I: Oxidative stress in desminopathies and myotilinopathies: a link between oxidative damage and abnormal protein aggregation. Brain Pathol 2007, 17:377–388
39. Fink AL: Protein aggregation: folding aggregates, inclusion bodies and amyloid. Fold Des 1998, 3:R9–R23
40. Kopito RR: Aggresomes, inclusion bodies and protein aggregation. Trends Cell Biol 2000, 10:524–530
41. Ross CA, Poirier MA: Protein aggregation and neurodegenerative disease. Nat Med 2004, 10:S10–S17
42. von Nandelstadh P, Soliymani R, Baumann M, Carpen O: Analysis of myotilin turnover provides mechanistic insight into the role of myotilinopathy-causing mutations. Biochem J 2011, 436:113–121
43. Goldfarb LG, Vicart P, Goebel HH, Dalakas MC: Desmin myopathy. Brain 2004, 127:723–734
44. Vicart P, Caron A, Guicheney P, Li Z, Prévost MC, Faure A, Chateau D, Chapon F, Tomé F, Dupret JM, Paulin D, Fardeau M: A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. Nat Genet 1998, 20:92–95
45. Selcen D, Muntoni F, Burton BK, Pegoraro E, Sewry C, Bite AV, Engel AG: Mutation in BAG3 causes severe dominant childhood muscular dystrophy. Ann Neurol 2009, 65:83–89
46. Sharma S, Mücke N, Katus HA, Herrmann H, Bär H: Disease mutations in the "head" domain of the extra-sarcomeric protein desmin distinctly alter its assembly and network-forming properties. J Mol Med (Berl) 2009, 87:1207–1219
47. Bär H, Kostareva A, Sjöberg G, Sejersen T, Katus HA, Herrmann H: Forced expression of desmin and desmin mutants in cultured cells: impact of myopathic missense mutations in the central coiled-coil domain on network formation. Exp Cell Res 2006, 312:1554–1565
48. Bova MP, Yaron O, Huang Q, Ding L, Haley DA, Stewart PL, Horwitz J: Mutation R120G in alphaB-crystallin, which is linked to a desmin-related myopathy, results in an irregular structure and defective chaperone-like function. Proc Natl Acad Sci USA 1999, 96:6137–6142
49. Mologni L, Moza M, Lalowski MM, Carpén O: Characterization of mouse myotilin and its promoter. Biochem Biophys Res Commun 2005, 329:1001–1009