1). It should be noted, however, that subcellular organelles underwent a profound reorganization during myogenic differentiation, and their localization patterns and architectures differed remarkably between undifferentiated mononuclear myoblasts and differentiated multinuclear myotubes (8,61). However, the amount of endogenous sortilin seems to be insufficient for conferring any apparent insulin responsiveness in terms of glucose uptake in C2C12 myotubes, since insulin stimulation of 2-deoxy[^{3}H]glucose uptake was marginal (Fig 4B), due to a relatively high level of basal glucose uptake mediated through GLUT1. Consistent with previous studies using fibroblastic cell types (12,48), over-expression of sortilin in myocytes apparently produced insulin-responsiveness as assessed by both the 2-deoxy[^{3}H]glucose uptake assay (Fig. 4) and the Myc Ab uptake assay (Fig. 5). These results support the suggestion of Kandror and colleagues that sortilin plays an essential role in the development of GLUT4 storage vesicles and responsiveness to insulin (12,48).

However, our aforementioned novel observation that sortilin over-expression significantly potentiates myogenesis sets the stage for an alternative hypothesis that sortilin participates in development of the entire insulin-responsive glucose transport system, being involved in more than just GLUT4 storage compartments, and that this is achieved, at least in part, via its myogenic stimulatory actions. This hypothesis is supported by evidence that sortilin over-expression strongly stimulates expression of the GLUT4 gene, while inhibiting that of the GLUT1 gene, leading to opposite changes in GLUT4 and GLUT1 abundance (Fig. 6), which thereby produces lower basal glucose uptake and greater augmentation of glucose uptake in response to insulin (Fig. 4). In addition, emergence of the insulin responsive glucose uptake in skeletal muscle cells by sortilin over-expression appeared to have resulted, at least in part, from the marked reduction in basal glucose uptake (Fig. 4) due to significantly reduced GLUT1 contents (Fig. 6). In this regard, we observed the half-lives of GLUT proteins to be reciprocally regulated in sortilin-overexpressing C2C12 cells (Fig. 6C). Thus, these data indicate sortilin to be involved not only in generating insulin-responsive GLUT4 storage vesicles, but also in elaborating the entire glucose transport system exhibiting enhanced insulin responsiveness via regulation of the processes of myogenesis including expressions of GLUT proteins and also perhaps various other proteins involved in the development of insulin responsiveness.

Another interesting observation presented in this study is that sortilin over-expression resulted in significant increases in the contents of Ubc9 and SUMOylated proteins in C2C12 cells (Fig. 7). Ubc9 is the only protein serving as an E2-type SUMO-conjugating enzyme in vertebrates, and most of the well known Ubc9 interacting proteins are nuclear or are translocated to the nucleus (62,63). Intriguingly, however, Ubc9 has been shown to interact directly with GLUT1 and GLUT4, and to modulate their membrane expression levels in opposite directions through a post-translational mechanism (45). Namely, over-expression of Ubc9 in the L6 skeletal muscle cell line results in a marked decrease in the cellular GLUT1 content and an increase in the GLUT4 content (45), which is in excellent agreement with our present results, shown in Fig. 6 and Fig. 7. Thus, the effects of sortilin over-expression on the significant reduction in cellular GLUT1 content, despite similar expression levels of its gene (Fig. 6A and B, *Day 4* and *Day 7*), may have resulted from the post-translational regulation of GLUT1 governed by the accumulated Ubc9 (Fig. 7). Although possible involvement of the accumulated Ubc9 in the increase in GLUT4 protein is not clear at this time since it coincides with up-regulation of GLUT4 gene expression in response to sortilin over-expression (Fig. 6 A and B, *right panels*), a recent report ~~also~~ demonstrated that Ubc9 over-expression results in the inhibition of GLUT4 degradation and promotes its targeting to insulin-responsive GLUT4 storage compartments (64). However, another recent report demonstrated that Ubc9 is also directly involved in the myogenic differentiation of C12C12 cells (65). Thus, Ubc9 ~~has~~ seems to have dual functions and our observations suggest that the sortilin-induced opposite changes in GLUT4 and GLUT1 abundance might reflect a combination of the consequences of the accumulation of Ubc9, which may regulate the half-lives of GLUT proteins and also further participate in the potentiation of

myogenesis. Although the degree to which sortilin actions on myogenesis or the opposite changes in GLUT1 and GLUT4 abundance, possibly mediated through Ubc9 accumulation, contributes to enhanced insulin responsiveness is an important question warranting further research, our findings clearly provide novel insights into the functional roles of sortilin in development of the insulin-responsive glucose uptake system in muscle cells.

REFERENCES

1. DeFronzo, R. A., Jacot, E., Jequier, E., Maeder, E., Wahren, J., and Felber, J. P. (1981) *Diabetes* **30**(12), 1000-1007
2. Nuutila, P., Knuuti, M. J., Raitakari, M., Ruotsalainen, U., Teras, M., Voipio-Pulkki, L. M., Haaparanta, M., Solin, O., Wegelius, U., and Yki-Jarvinen, H. (1994) *Am J Physiol* **267**(6 Pt 1), E941-946
3. Koistinen, H. A., and Zierath, J. R. (2002) *Ann Med* **34**(6), 410-418
4. Mueckler, M. (1990) *Diabetes* **39**, 6-11
5. Mitsumoto, Y., Burdett, E., Grant, A., and Klip, A. (1991) *Biochem. Biophys. Res. Commun.* **175**, 652-659
6. Shimokawa, T., Kato, M., Ezaki, O., and Hashimoto, S. (1998) *Biochem Biophys Res Commun* **246**(1), 287-292
7. Rodnick, K. J., Slot, J. W., Studelska, D. R., Hanpeter, D. E., Robinson, L. J., Geuze, H. J., and James, D. E. (1992) *J Biol Chem* **267**, 6278-6285
8. Ralston, E., and Ploug, T. (1996) *J Cell Sci* **109**, 2967-2978
9. Rudich, A., and Klip, A. (2003) *Acta Physiol Scand* **178**(4), 297-308.
10. Garcia de Herreros, A., and Birnbaum, M. J. (1989) *J. Biol. Chem.* **264**, 9885-9890
11. Haney, P. M., Slot, J. W., Piper, R. C., James, D. E., and Mueckler, M. (1991) *J Cell Biol* **114**, 689-699
12. Shi, J., and Kandror, K. V. (2005) *Dev Cell* **9**(1), 99-108
13. Baumann, C. A., Ribon, V., Kanzaki, M., Thurmond, D. C., Mora, S., Shigematsu, S., Bickel, P. E., Pessin, J. E., and Saltiel, A. R. (2000) *Nature* **407**, 202-207
14. Chiang, S. H., Baumann, C. A., Kanzaki, M., Thurmond, D. C., Watson, R. T., Neudauer, C. L., Macara, I. G., Pessin, J. E., and Saltiel, A. R. (2001) *Nature* **410**(6831), 944-948.
15. Nielsen, M. S., Madsen, P., Christensen, E. I., Nykjaer, A., Gliemann, J., Kasper, D., Pohlmann, R., and Petersen, C. M. (2001) *Embo J* **20**(9), 2180-2190
16. Lefrancois, S., Zeng, J., Hassan, A. J., Canuel, M., and Morales, C. R. (2003) *Embo J* **22**(24), 6430-6437
17. Petersen, C. M., Nielsen, M. S., Nykjaer, A., Jacobsen, L., Tommerup, N., Rasmussen, H. H., Roigaard, H., Gliemann, J., Madsen, P., and Moestrup, S. K. (1997) *J Biol Chem* **272**(6), 3599-3605
18. Lin, B. Z., Pilch, P. F., and Kandror, K. V. (1997) *J Biol Chem* **272**(39), 24145-24147
19. Morris, N. J., Ross, S. A., Lane, W. S., Moestrup, S. K., Petersen, C. M., Keller, S. R., and Lienhard, G. E. (1998) *J Biol Chem* **273**(6), 3582-3587
20. Nielsen, M. S., Jacobsen, C., Olivecrona, G., Gliemann, J., and Petersen, C. M. (1999) *J Biol Chem* **274**(13), 8832-8836
21. Mazella, J., Zsurger, N., Navarro, V., Chabry, J., Kaghad, M., Caput, D., Ferrara, P., Vita, N., Gully, D., Maffrand, J. P., and Vincent, J. P. (1998) *J Biol Chem* **273**(41), 26273-26276
22. Nykjaer, A., Lee, R., Teng, K. K., Jansen, P., Madsen, P., Nielsen, M. S., Jacobsen, C., Kliemannel, M., Schwarz, E., Willnow, T. E., Hempstead, B. L., and Petersen, C. M. (2004) *Nature* **427**(6977), 843-848
23. Teng, H. K., Teng, K. K., Lee, R., Wright, S., Tevar, S., Almeida, R. D., Kermani, P., Torkin, R., Chen, Z. Y., Lee, F. S., Kraemer, R. T., Nykjaer, A., and Hempstead, B. L. (2005) *J Neurosci* **25**(22), 5455-5463
24. Mazella, J. (2001) *Cell Signal* **13**(1), 1-6
25. Bronfman, F. C., and Fainzilber, M. (2004) *EMBO Rep* **5**(9), 867-871
26. Seidl, K., Erck, C., and Buchberger, A. (1998) *J Cell Physiol* **176**(1), 10-21
27. Erck, C., Meisinger, C., Grothe, C., and Seidl, K. (1998) *J Cell Physiol* **176**(1), 22-31
28. Reddypalli, S., Roll, K., Lee, H. K., Lundell, M., Barea-Rodriguez, E., and Wheeler, E. F. (2005) *J Cell Physiol* **204**(3), 819-829
29. Shibata, H., Suzuki, Y., Omata, W., Tanaka, S., and Kojima, I. (1995) *J Biol Chem* **270**, 11489-11495
30. Yaffe, D., and Saxel, O. (1977) *Nature* **270**(5639), 725-727
31. Onishi, M., Kinoshita, S., Morikawa, Y., Shibuya, A., Phillips, J., Lanier, L. L., Gorman, D. M., Nolan, G. P., Miyajima, A., and Kitamura, T. (1996) *Exp Hematol* **24**(2), 324-329
32. Kanzaki, M., Furukawa, M., Raab, W., and Pessin, J. E. (2004) *J Biol Chem* **279**(29), 30622-30633
33. Mizuguchi, H., and Kay, M. A. (1998) *Hum Gene Ther* **9**(17), 2577-2583
34. Mizuguchi, H., and Kay, M. A. (1999) *Hum Gene Ther* **10**(12), 2013-2017
35. Nedachi, T., and Kanzaki, M. (2006) *Am J Physiol Endocrinol Metab* **291**(4), E817-828
36. Tortorella, L. L., and Pilch, P. F. (2002) *Am J Physiol Endocrinol Metab* **283**(3), E514-524
37. Shibata, H., Omata, W., Suzuki, Y., Tanaka, S., and Kojima, I. (1996) *J Biol Chem* **271**, 9704-9709
38. Zeng, J., Hassan, A. J., and Morales, C. R. (2004) *Mol Reprod Dev* **68**(4), 469-475
39. Peters, E. M., Stieglitz, M. G., Liezman, C., Overall, R. W., Nakamura, M., Hagen, E., Klapp, B. F., Arck, P., and Paus, R. (2006) *Am J Pathol* **168**(1), 221-234
40. Yamashita, T., and Tohyama, M. (2003) *Nat Neurosci* **6**(5), 461-467

41. Passino, M. A., Adams, R. A., Sikorski, S. L., and Akassoglou, K. (2007) *Science* **315**(5820), 1853-1856
42. Kanzaki, M., and Pessin, J. E. (2001) *J Biol Chem* **276**(45), 42436-42444.
43. Mitsumoto, Y., and Klip, A. (1992) *J. Biol. Chem.* **267**(7), 4957-4962
44. Santalucia, T., Camps, M., Castello, A., Munoz, P., Nuel, A., Testar, X., Palacin, M., and Zorzano, A. (1992) *Endocrinology* **130**, 837-846
45. Giorgino, F., de Robertis, O., Laviola, L., Montrone, C., Perrini, S., McCowen, K. C., and Smith, R. J. (2000) *Proc Natl Acad Sci U S A* **97**(3), 1125-1130.
46. Kanzaki, M. (2006) *Endocr J* **53**(3), 267-293
47. Walker, P. S., Ramlal, T., Sarabia, V., Koivisto, U. M., Bilan, P. J., Pessin, J. E., and Klip, A. (1990) *J Biol Chem* **265**, 1516-1523
48. Shi, J., and Kandror, K. V. (2007) *J Biol Chem*
49. Florini, J. R., Magri, K. A., Ewton, D. Z., James, P. L., Grindstaff, K., and Rotwein, P. S. (1991) *J Biol Chem* **266**(24), 15917-15923
50. Florini, J. R., Ewton, D. Z., and Coolican, S. A. (1996) *Endocr Rev* **17**(5), 481-517
51. Rende, M., Brizi, E., Conner, J., Treves, S., Censier, K., Provenzano, C., Taglialatela, G., Sanna, P. P., and Donato, R. (2000) *Int J Dev Neurosci* **18**(8), 869-885
52. Takano, H., Komuro, I., Oka, T., Shiojima, I., Hiroi, Y., Mizuno, T., and Yazaki, Y. (1998) *Mol Cell Biol* **18**(3), 1580-1589
53. Charrasse, S., Meriane, M., Comunale, F., Blangy, A., and Gauthier-Rouviere, C. (2002) *J Cell Biol* **158**(5), 953-965
54. Nishiyama, T., Kii, I., and Kudo, A. (2004) *J Biol Chem* **279**(45), 47311-47319
55. Charrasse, S., Comunale, F., Grumbach, Y., Poulat, F., Blangy, A., and Gauthier-Rouviere, C. (2006) *Mol Biol Cell* **17**(2), 749-759
56. Castellani, L., Salvati, E., Alema, S., and Falcone, G. (2006) *J Biol Chem* **281**(22), 15249-15257
57. Hermans-Borgmeyer, I., Hermey, G., Nykjaer, A., and Schaller, C. (1999) *Brain Res Mol Brain Res* **65**(2), 216-219
58. Shiba, T., Takatsu, H., Nogi, T., Matsugaki, N., Kawasaki, M., Igarashi, N., Suzuki, M., Kato, R., Earnest, T., Nakayama, K., and Wakatsuki, S. (2002) *Nature* **415**(6874), 937-941
59. Watson, R. T., Khan, A. H., Furukawa, M., Hou, J. C., Li, L., Kanzaki, M., Okada, S., Kandror, K. V., and Pessin, J. E. (2004) *Embo J* **23**(10), 2059-2070
60. Li, L. V., and Kandror, K. V. (2005) *Mol Endocrinol* **19**(8), 2145-2153
61. Ralston, E. (1993) *J Cell Biol* **120**(2), 399-409
62. Melchior, F. (2000) *Annu Rev Cell Dev Biol* **16**, 591-626
63. Wilson, V. G., and Rangasamy, D. (2001) *Exp Cell Res* **271**(1), 57-65
64. Liu, L. B., Omata, W., Kojima, I., and Shibata, H. (2007) *Diabetes*
65. Riquelme, C., Barthel, K. K., Qin, X. F., and Liu, X. (2006) *Exp Cell Res* **312**(11), 2132-2141

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FOOTNOTES

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[1]The abbreviations used are: p75NTR, p75 neurotrophin receptor; VPS, vacuolar protein sorting; GGA, (Golgi-localizing γ-adaptin ear homology domain, ARF-binding proteins); ARF, ADP-ribosylation factor; siRNA, small interfering RNA; SUMO, small ubiquitin-related modifier; NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; MHC, myosin heavy chain

FIGURE LEGENDS

Fig. 1. Protein expression and subcellular localization of sortilin during C2C12 differentiation. **A)** C2C12 myoblasts were differentiated in conventional differentiation medium [D-medium: DMEM containing 5 mM glucose + 2% calf serum (CS)]. The D-medium was changed every 24 h. Whole cell lysates were obtained daily until day 8 of differentiation. Total protein extracts (22.5 μg/lane) were subjected to SDS-PAGE followed by western blot analysis using anti-sortilin (*upper panel*), anti-myogenin (*middle panel*), and anti-myosin heavy chain (MHC: *lower panel*) antibodies. **B)** Differentiated C2C12 myotubes (Day 6) were fixed and subjected to immunofluorescent staining using rabbit anti-sortilin antibody (green) with either mouse monoclonal anti-myc (*panels a* and *b*), anti-CI-M6PR (*panels c* and *d*), anti-syntaxin 6 (*panels e* and *f*), or anti-p115 (*panels g* and *h*) antibodies (*red*). For examining co-localization between sortilin and GLUT4 (*panels a* and *b*), myc-GLUT4-ECFP was transiently expressed by infecting C2C12 cells at day 4 of differentiation with the adenovirus containing the myc-GLUT4-ECFP gene and then subjecting these cells to immunofluorescent analysis at 2 days after infection (*Day 6* of differentiation). Fluorescent signals were detected under confocal microscopy. The squares indicated in *panels a, c, d* and *g* were magnified and are shown in the *right panels*. Images are representative field-of views from 3 independent experiments. Scale bar = 10 μm.

Fig. 2. Sortilin over-expression induces spontaneous myogenic differentiation of C2C12 cells. **A)** Parental (WT, *panel a*), empty-vector-expressing (*panel b*) and sortilin-over-expressing (Sort10, *panel c;* Sort15, *panel d)* C2C12 cells were grown to confluence (*Day 0*). Myotubes are indicated by *arrowheads*. Images are representative field-of views from 3 independent experiments. Scale bar = 300 μm. Exogenously expressed sortilin in each C2C12 cell culture was detected by western blotting analysis (*right panel*). **B)** WT- (*panels a-c*), empty-vector- (*panels d-f*), and Sort10 (*panels g-i*)-C2C12 cells at *Day 0* were fixed and subjected to immunofluorescent staining using mouse monoclonal anti-myogenin (*panels a, d* and *g*), anti-MHC (*panels b, e* and *h*) and anti-titin (*panels c, f* and *i*) antibodies, which were visualized with Alexa488-conjugated secondary antibody (*green*). Nuclei were stained by DAPI (*blue*). Images are representative field-of views from 3 independent experiments. **C)** Whole cell lysates were obtained from WT- (*lanes 1, 3* and *5*) and Sort10- (*lanes 2, 4* and *6*) C2C12 cells on the indicated days after differentiation and total protein extracts (25 μg/lane) were subjected to western blot analysis using antibodies against sortilin, MHC, sarcomeric α-actinin, troponin T and myogenin. β-actin was used as a loading control. These are representative immunoblots obtained from 3 independent experiments.

Fig. 3. Involvement of sortilin-p75NTR-proNGF autocrine loop in the process of C2C12 differentiation. **A)** WT- (*open bars*) and Sort10- (*solid bars*) C2C12 cells on Day -1 with the addition of either control IgG, anti-p75NTR-neutralizing antibody (*left panel*), or anti-NGF-neutralizing antibody (*right panel*), followed by culture for an additional 24 hours. The cells were then fixed and subjected to immunofluorescent staining using mouse monoclonal anti-myogenin visualized with Alexa594-conjugated secondary antibody. Nuclei were stained by DAPI, and the number of myogenin positive nuclei was counted. The ratio of myogenin-positive nuclei out of total nuclei was expressed as the mean ± SEM of 4 independent experiments. *, $p<0.05$; **, $p<0.01$. **B)** Empty-vector- (*panels a* and *b*) or Sort10- (*panels c* and *d*) C2C12 cells seeded in an 8-well slide chamber were treated with 10 μg/ml of anti-p75NTR antibody or mouse IgG before they reached confluence, followed by culture for an additional 24 hours. To detect incorporated IgGs, the cells were subjected to immunostaining with anti-mouse IgG (*red*) and anti-sortilin antibody (*green*), as described in A). **C)** WT-C2C12 cells (day -1) were transfected with siRNA oligos targeting sortilin (*lanes 1, 2* and *3*) or control scrambled siRNA oligo (*lane 4*) by Oligofectamine for 24 hours and then cultured in D-medium for an additional 3 days. Total protein extracts (135 μg/lane) were subjected to western blot analysis using anti-sortilin (*upper panel*), anti-

myogenin (*middle panel*) or anti-β-actin (*lower panel*) antibodies. **D)** Confluent WT-C2C12 cells were cultured in D-medium in the absence or presence of 10 μg/ml of either control IgG, anti-p75NTR- or anti-NGF-antibodies for 48 hours. The ratio of myogenin-positive nuclei was assessed by immunostaining analysis as described in A). **E)** Empty-vector- (*upper panel*) or Sort10- (*lower panel*) C2C12 cells were incubated with or without the indicated concentrations of Y27632 for 24 h. The whole cell lysates were then subjected to western blotting analysis using anti-myogenin antibody.

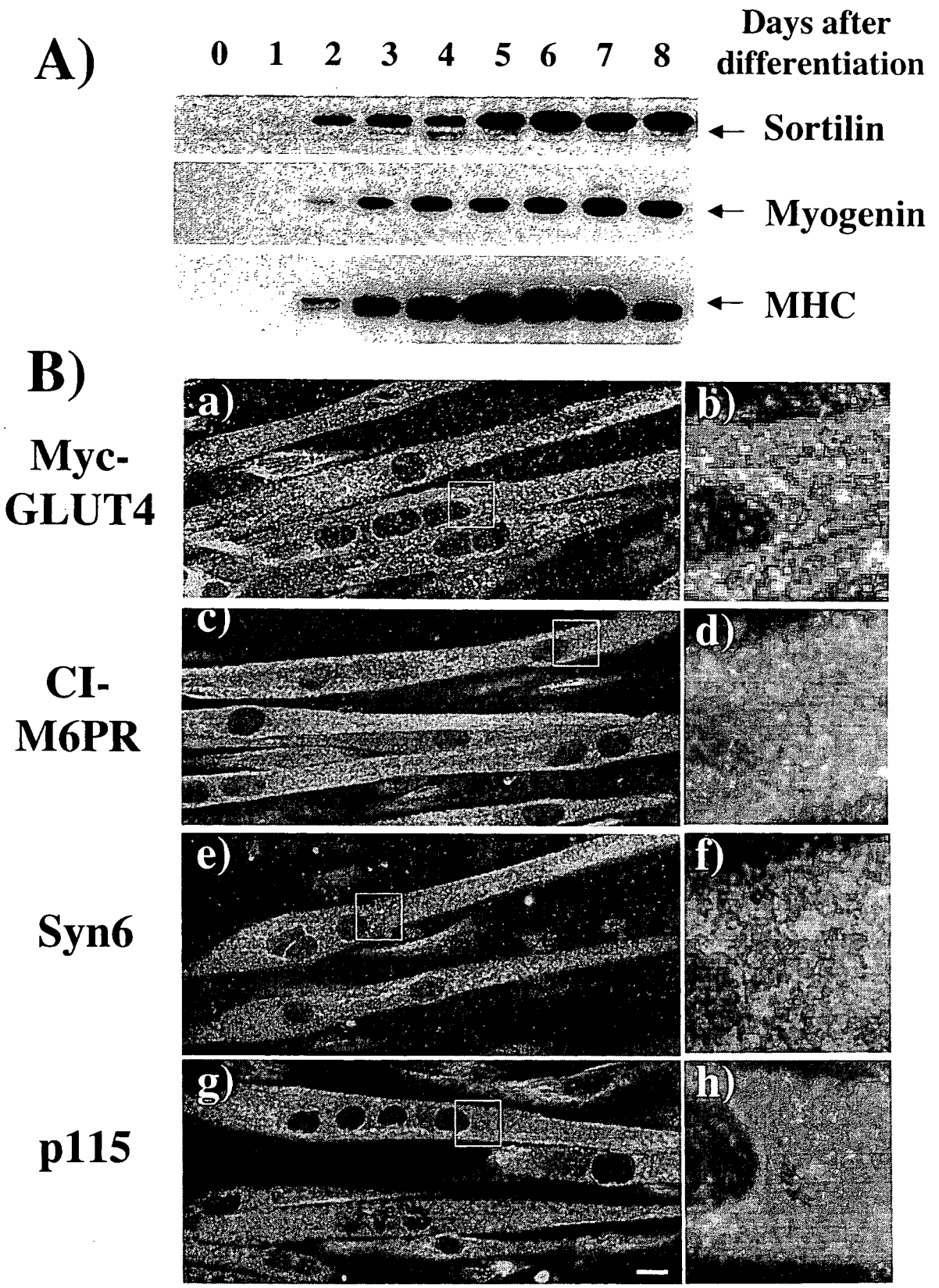
Fig. 4. Effects of sortilin over-expression on basal and insulin-stimulated 2-deoxy[^{3}H]glucose uptake in C2C12 cells. **A)** Undifferentiated WT- (*open bars, WT*) and Sort10- (*solid bars, Sort*) C2C12 myoblasts were infected with adenovirus containing the myc-GLUT4-ECFP gene (*G4*) or no insert. Thirty-six hours after infection, the cells were serum starved for 4 hours, followed by pre-incubation with KRPH buffer for 10 minutes, and then stimulated with or without 100 nM insulin for 60 min. The cells were then subjected to the 2-deoxy[^{3}H]glucose uptake assay as described in "*Materials and Methods*". Results were expressed as the mean ± SEM of 3 independent experiments. *, $p<0.05$; **, $p<0.01$. **B)** WT- (*open bars, WT*) and Sort10- (*solid bars, Sort*) C2C12 myotubes at *Day 5* of differentiation were infected with adenovirus containing the myc-GLUT4-ECFP gene (*G4*) or no insert. Thirty-six hours after infection, the cells were subjected to the 2-deoxy[^{3}H]glucose uptake assay as described in (A). Results were expressed as the mean ± SEM of 3 independent experiments. *, $p<0.05$; **, $p<0.01$

Fig. 5. Effect of sortilin over-expression on insulin-induced GLUT4 translocation in C2C12 cells. **A)** Differentiated WT- (*open bars*) and Sort10- (*solid bars*) C2C12 myotubes at *Day 5* were infected with adenovirus containing the myc-GLUT4-ECFP gene. Thirty-six hours after infection, the cells were serum starved for 4 hours, followed by stimulation with or without 100 nM insulin for 60 minutes. During the insulin stimulation, 4 μg of anti-Myc antibody were added to the culture. After insulin stimulation, amounts of associated/internalized antibody (*upper panel*) were analyzed by western blotting. The total amount of myc-GLUT4-ECFP (*lower panel*) was also analyzed by western blotting for normalization. **B)** The results of 3 independent experiments were quantified using Image J software. Summarized results were expressed as the mean ± SEM. *, $p<0.05$; **, $p<0.01$.

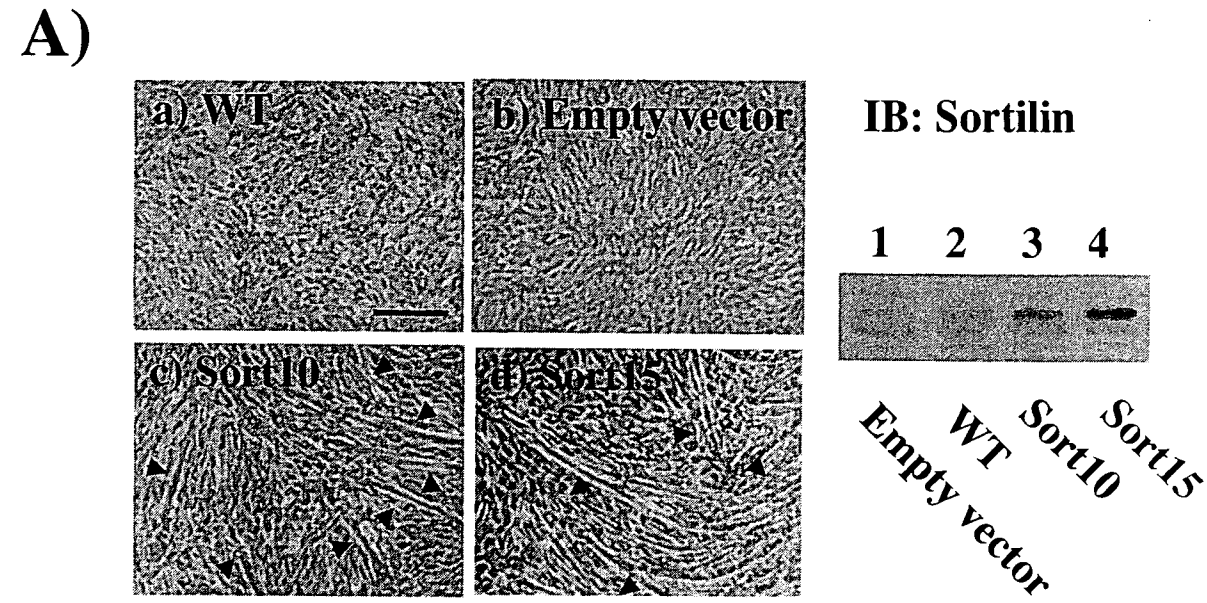
Fig. 6. Effect of sortilin over-expression on the protein and mRNA levels of GLUT1 and GLUT4 in C2C12 cells. **A)** Total membrane fractions were obtained from WT-C2C12 cells (*lanes 1, 3* and *5*) and Sort10-C2C12 cells (*lanes 2, 4* and *6*) at *Day 0* (*lanes 1* and *2*), *Day 4* (*lanes 3* and *4*) and *Day 7* (*lanes 5* and *6*) of differentiation, as described in "*EXPERIMENTAL PROCEDURES*". Protein extracts from total membranes (120 μg/lane) were subjected to SDS-PAGE followed by western blotting using antibodies against GLUT1 (*left panel*) or GLUT4 (*right panel*). **B)** Total RNA was isolated from WT-C2C12 cells (*open bars*) and Sort10-C2C12 cells (*solid bars*) on the indicated day after differentiation. The relative abundances of mRNAs for GLUT1 (*left graph*) and GLUT4 (*right graph*) were evaluated by real-time PCR analysis. Data normalized using the GAPDH transcript were averaged over 3 independent experiments and are shown as fold changes over *Day 0* in WT-C2C12 cells. **C)** Time-dependent changes in protein amounts of GLUT1 (*left panels*) and myc-GLUT4-ECFP (*right panels*) after addition of 10 μg/ml of cycloheximide were monitored in WT- (*open circles*) and Sort10- (*closed circles*) C2C12 cells by western blotting analysis.

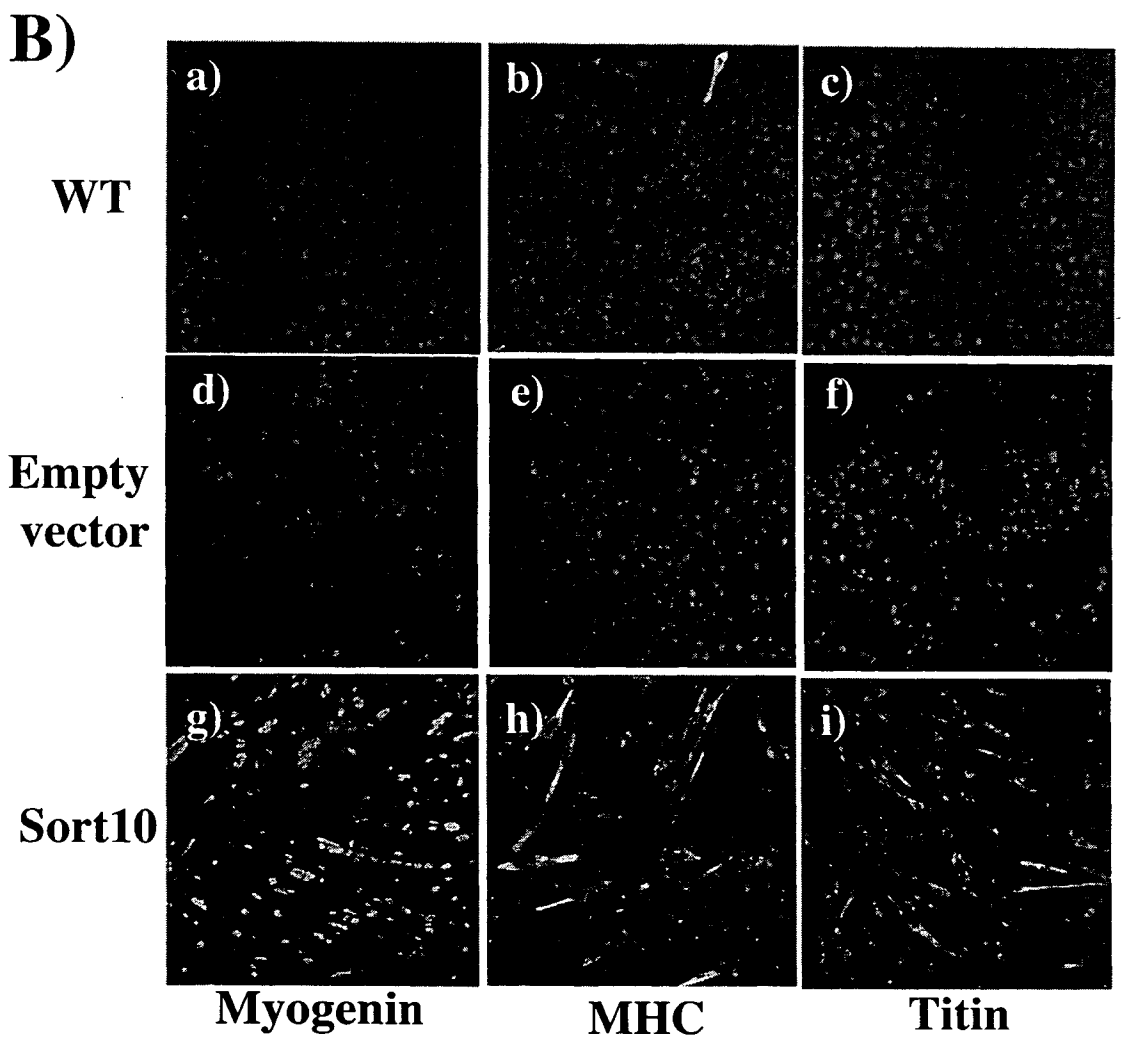
Fig. 7. Sortilin over-expression increases Ubc9 resulting in the accumulation of SUMOylated proteins in C2C12 cells. **A)** Whole cell lysates were obtained from WT-C2C12 cells (*lanes 1, 3* and *5*) and Sort10-C2C12 cells (*lanes 2, 4* and *6*) at *Day 0* (*lanes 1* and *2*), *Day 4* (*lanes 3* and *4*) and *Day 7* (*lanes 5* and *6*) of differentiation. Protein extracts (25 μg/lane) were subjected to SDS-PAGE followed by western blotting using antibodies against anti-Ubc9 (*upper panel*), anti-GMP1 (SUMO-1) (*middle panel*) or anti-β-actin (*lower panel*). **B)** Total RNA was isolated from WT-C2C12 cells (*open bars*) and Sort10-C2C12 cells (*solid bars*) on the indicated day after differentiation. The relative abundance of mRNAs for Ubc9

was evaluated by real-time PCR analysis. Data normalized using the GAPDH transcript were averaged over 3 independent experiments and are shown as fold changes over *Day 0* in WT-C2C12 cells.



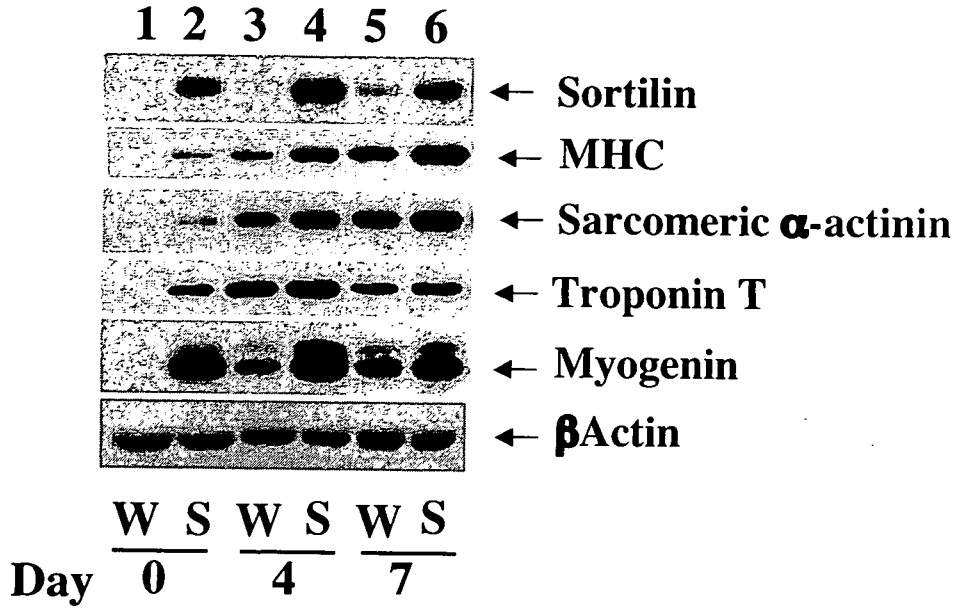
Ariga, M. *et al.* Figure 1 AB





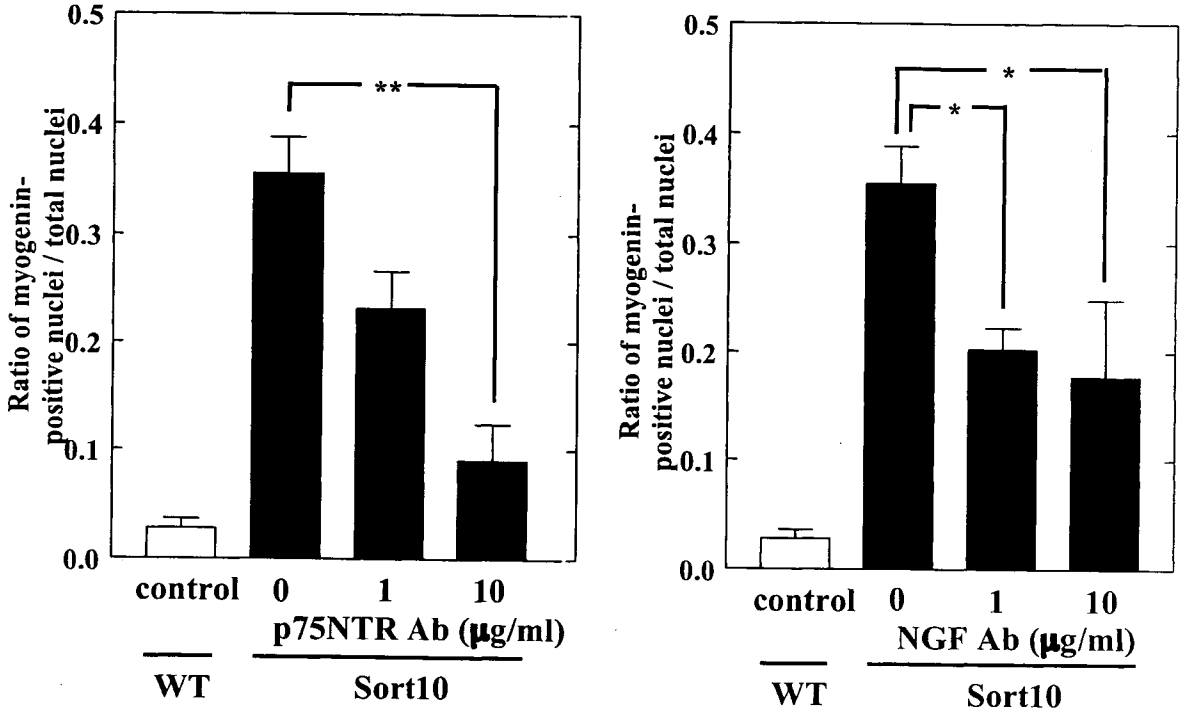
Ariga, M. *et al.* Figure 2AB

C)

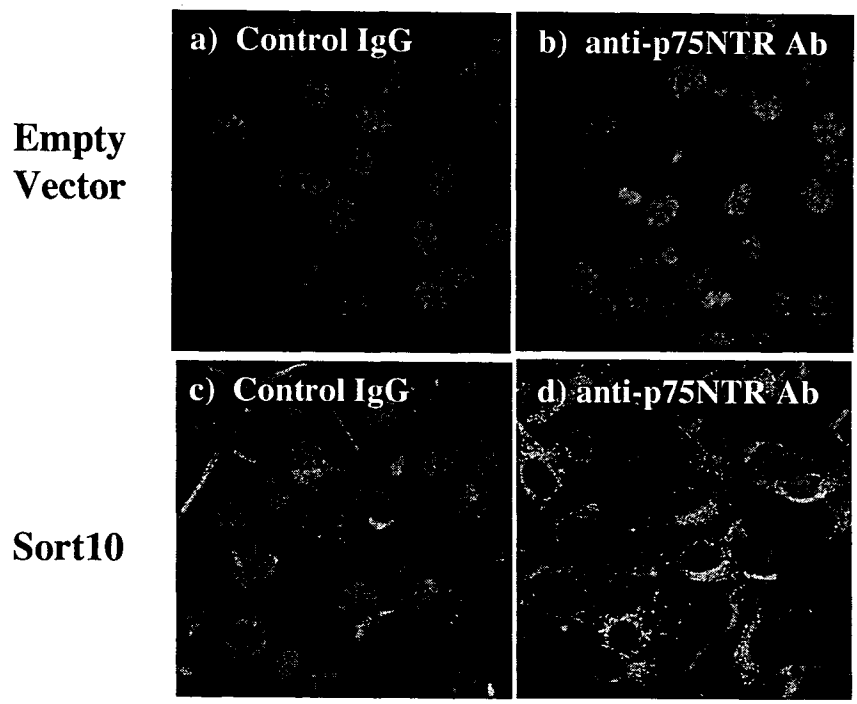


Ariga, M. *et al.* Figure 2C

A)

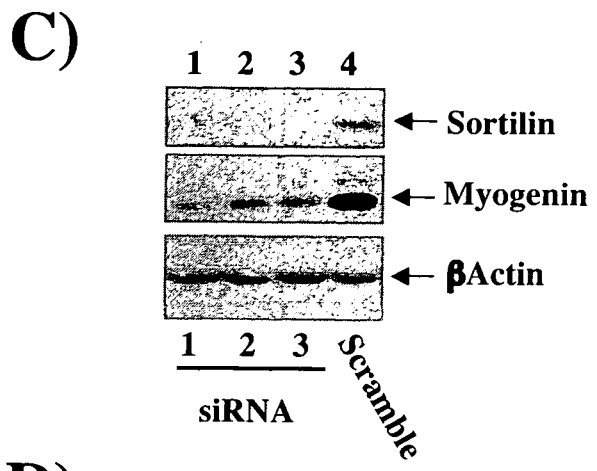


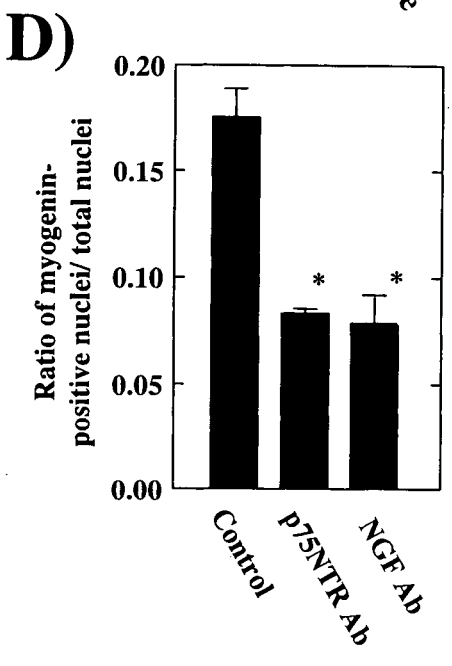
B)

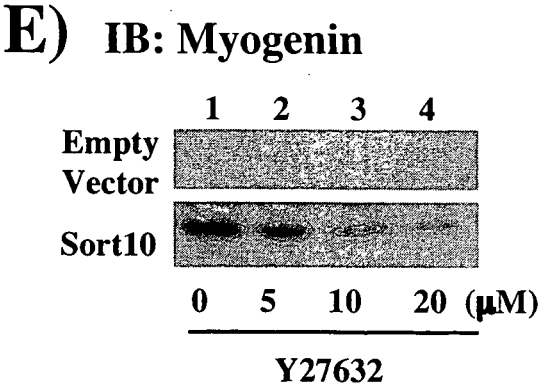


Sortilin / Endocytosed IgG / DAPI

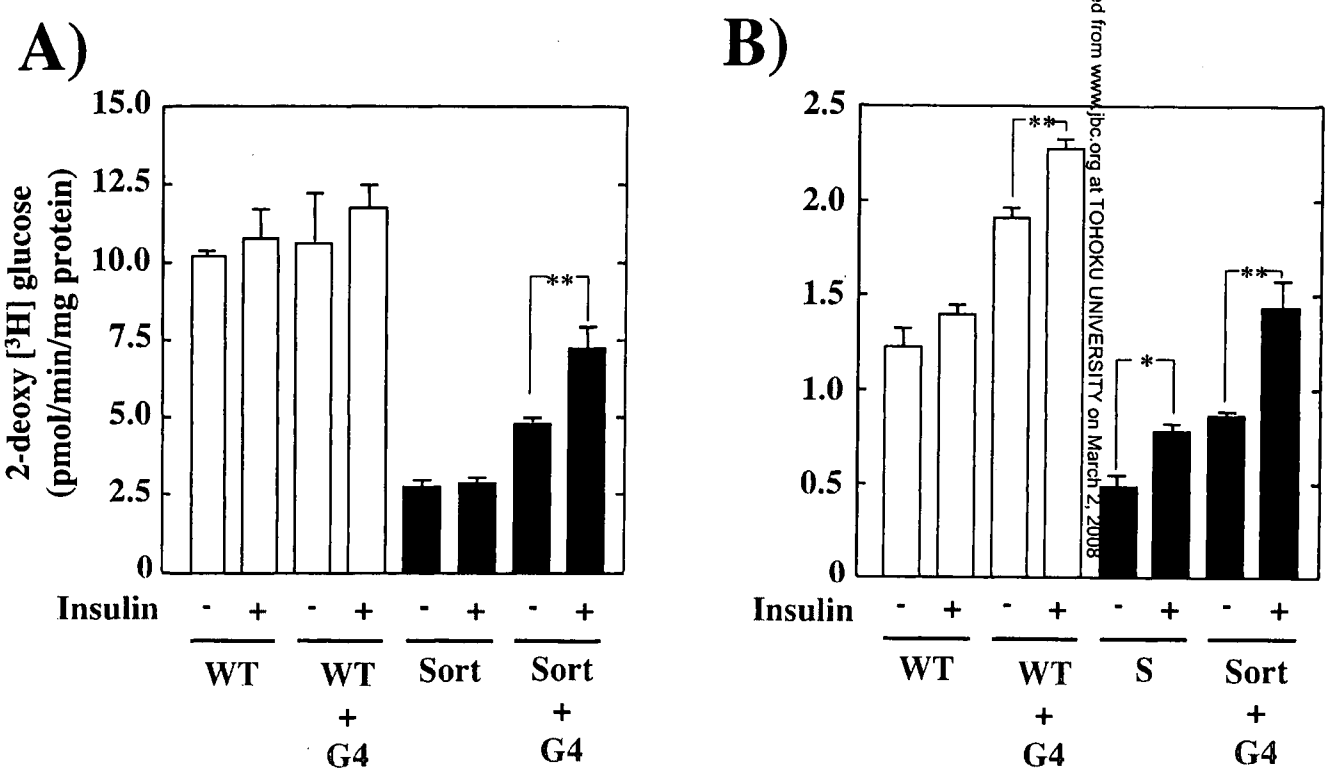
Ariga, M. *et al.* Figure 3AB



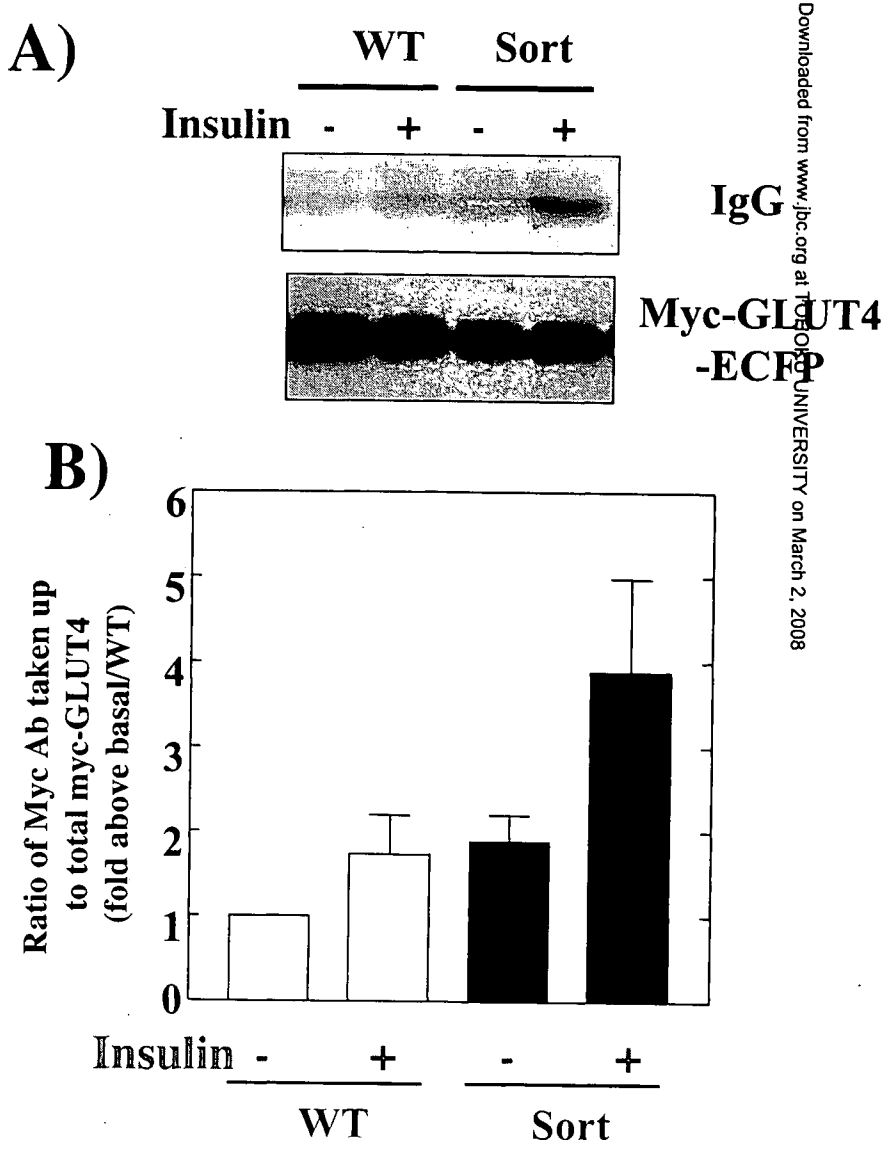




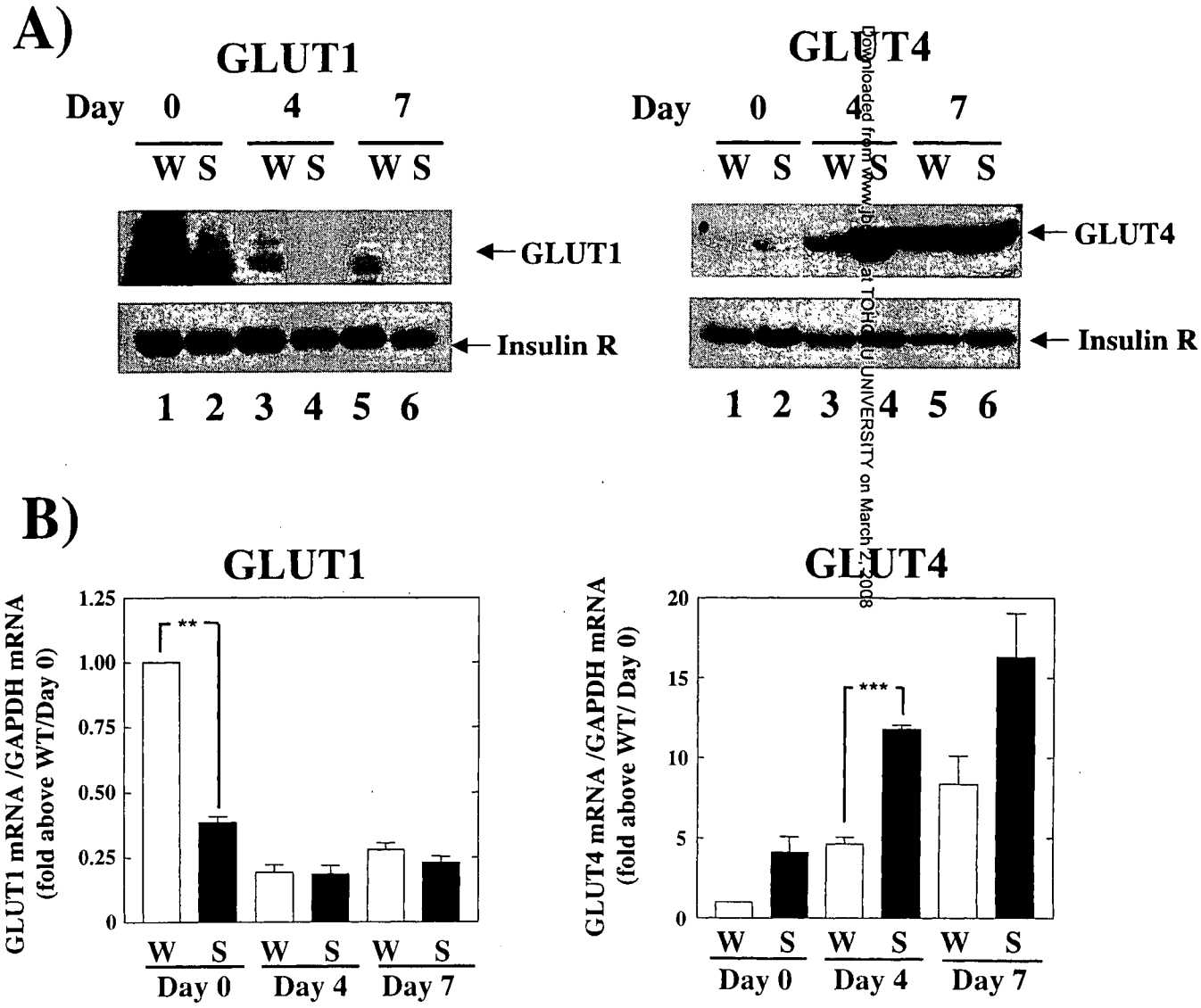
Ariga, M. *et al.* Figure 3CDE



Ariga, M. *et al.* Figure 4

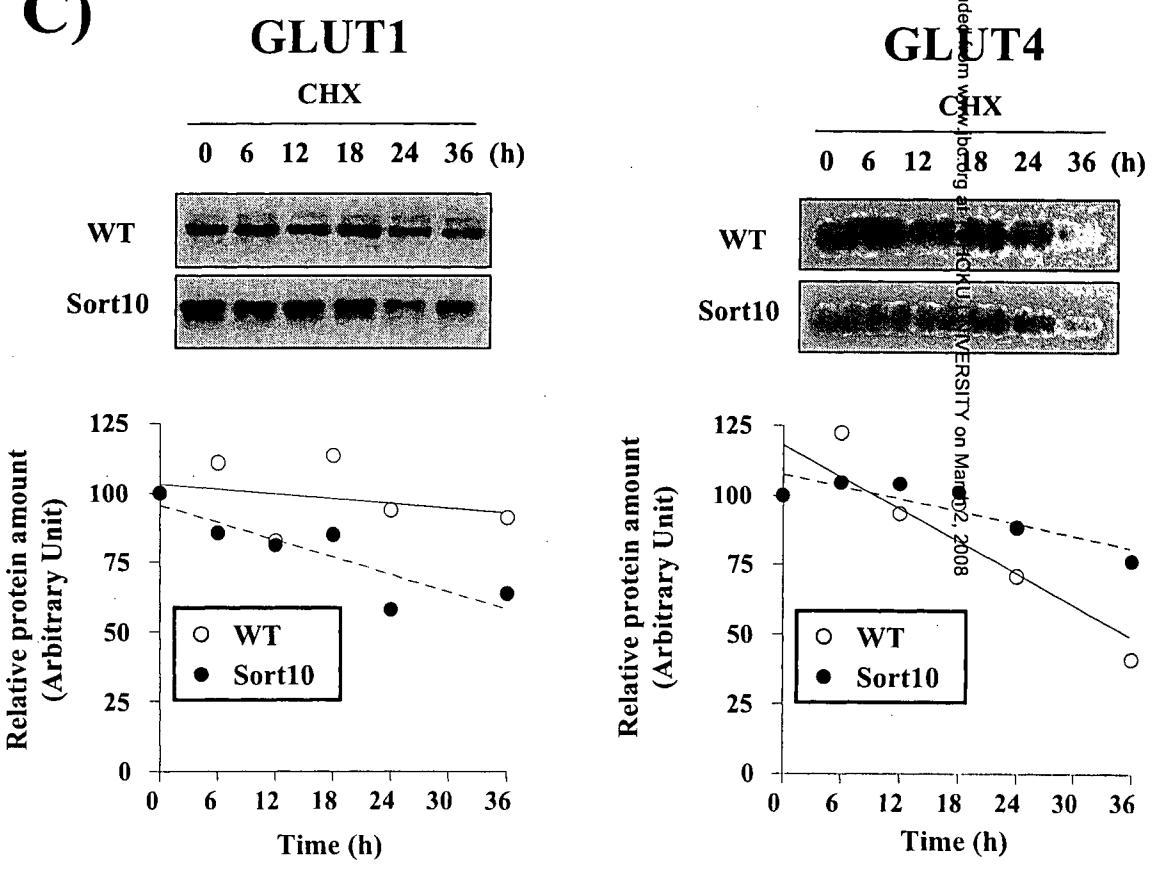


Ariga, M. *et al.* Figure 5 AB

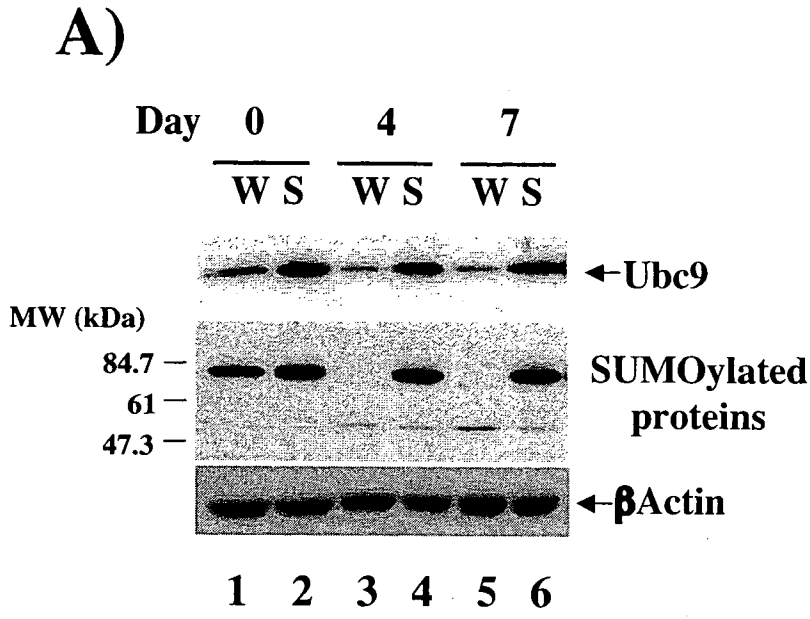


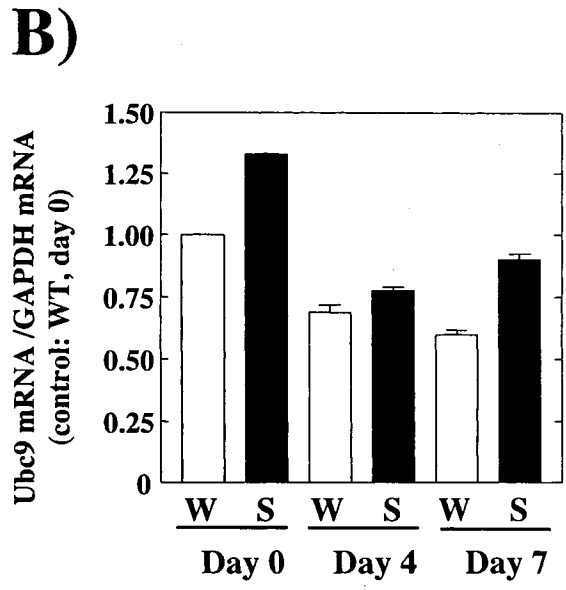
Ariga, M. *et al.* Figure 6AB

C)



Ariga, M. *et al.* Figure 6C





Ariga, M. *et al.* Figure 7 AB

The Wolfram syndrome 1 (*Wfs1*) gene expression in the normal mouse visual system

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ABSTRACT

Wolfram syndrome (OMIM 222300) is a neurodegenerative disorder defined by insulin-dependent diabetes mellitus and progressive optic atrophy. This syndrome has been attributed to mutations in the *WFS1* gene, which codes for a putative multi-spanning membrane glycoprotein of the endoplasmic reticulum. The function of WFS1 (wolframin), the distribution of this protein in the mammalian visual system, and the pathogenesis of optic atrophy in Wolfram syndrome are unclear. In this study, we made a detailed analysis of the distribution of *Wfs1* mRNA and protein in the normal mouse visual system by using in situ hybridization and immunohistochemistry. The mRNA and protein were observed in the retina, optic nerve, and brain. In the retina, Wfs1 expression was strong in amacrine and Müller cells, and moderate in photoreceptors and horizontal cells. In addition, it was detectable in bipolar and retinal ganglion cells. Interestingly, moderate Wfs1 expression was seen in the optic nerve, particularly in astrocytes, while little Wfs1 was expressed in the optic chiasm or optic tract. In the brain, moderate Wfs1 expression was observed in the zonal, superficial gray, and intermediate gray layers of the superior colliculus, in the dorsomedial part of the suprachiasmatic nucleus, and in layer II of the primary and secondary visual cortices. Thus, *Wfs1* mRNA and protein were widely distributed in the normal mouse visual system. This evidence may provide clues as to the physiological role of Wfs1 protein in the biology of vision, and help to explain the selective vulnerability of the optic nerve to WFS1 loss-of-function.

(249 words)