JBC Papers in Press. Published on February 21, 2006 as Manuscript M509420200
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M509420200

FTDP-17 MUTATIONS COMPROMISE THE ABILITY OF TAU TO REGULATE MICROTUBULE DYNAMICS IN CELLS

Janis M. Bunker, Kathy Kamath, Leslie Wilson, Mary Ann Jordan, and Stuart C. Feinstein

Neuroscience Research Institute and Department of Molecular, Cellular and Developmental Biology, University of California, Santa Barbara, California 93106

Running Title: FTDP-17 Mutations Compromise Tau Function

Address correspondence to Dr. Stuart Feinstein, Neuroscience Research Institute, Bldg 571, Rm 6129, University of California, Santa Barbara, CA 93106; Tel. (805) 893-2659; Fax. (805) 893-2659, Email: feinstei@lifesci.ucsb.edu

The neural microtubule-associated protein tau binds directly to microtubules and regulates their dynamic behavior. In addition to being required for normal development, maintenance and function of the nervous system, tau is associated with several neurodegenerative diseases, including Alzheimer's disease. One group of neurodegenerative dementias known as FTDP-17 (fronto-temporal dementia with Parkinsonism linked to chromosome 17) are directly linked genetically to mutations in the tau gene, demonstrating that tau misfunction can cause neuronal cell death and dementia. These mutations result either in amino-acid substitutions in tau, or in altered tau mRNA splicing that skews the expression ratio of wild-type 3-repeat and 4-repeat tau isoforms. Since wild-type tau regulates microtubule dynamics, one possible mechanism underlying tau-mediated neurodegeneration is aberrant regulation of microtubule behavior. In this study, we microinjected normal and mutated tau proteins into GFP-tubulin-expressing cultured cells and measured the effects on the dynamic instability of individual microtubules. We found that the FTDP-17 amino-acid substitutions G272V (in both 3-repeat and 4-repeat tau contexts), ΔK280, and P301L all exhibited markedly reduced abilities to regulate dynamic instability relative to wild-type tau. In contrast, the FTDP-17 R406W mutation (which maps in a regulatory region outside the microtubule binding domain of tau) did not significantly alter the ability of 3-repeat or 4-repeat tau to regulate microtubule dynamics. Overall, these data are consistent with a loss-of-function model in which both amino-acid substitutions and altered mRNA-splicing in tau lead to neurodegeneration by diminishing the ability of tau to properly regulate microtubule dynamics.

Microtubules are dynamic polymers with growing and shortening behaviors that are exquisitely regulated. The dynamic behaviors of microtubules are both temporally and spatially regulated, even within individual cells (1). For example, microtubule dynamics in migrating cells differ at the leading and trailing edges (2).

The role of microtubule dynamics is best understood in the processes of mitosis and cell division, where microtubules play central roles in spindle assembly, the metaphase/anaphase checkpoint and chromosome segregation (3-5). Microtubule dynamics are also essential in post-mitotic neurons, serving critical roles in establishing cell polarity, axon outgrowth, cell signaling, adhesion, the organization of cellular organelles, and metabolic output (6).

The regulation of microtubule dynamics is known to be mediated by a large array of proteins that associate with tubulin and/or microtubules. Neurons possess an especially broad range of these regulators, including MAP2, stathmin, SCG10, APC, MAP1b, EB1, CLIPs, CRMP-2, and tau (7-12). For many of these regulators, we have only a relatively primitive understanding of their structure-function relationships and mechanisms of action. The presence of many neuronal proteins all serving to control various aspects of microtubule dynamics suggests that precise regulation of microtubule dynamics is crucially important to the development, maintenance and function of neurons.

The microtubule associated protein tau binds directly to microtubules and is among the major regulators of neuronal microtubule dynamics (13-15). The ability of tau to regulate microtubule dynamics is modulated both by alternative mRNA splicing, leading to the expression of either "3-repeat" or "4-repeat" tau (Fig. 1A, 16,17), and by complex combinatorial patterns of phosphorylation (reviewed in 18,19,20).

Whereas normal tau function is important for proper development and maintenance of the nervous system, tau dysfunction has long been correlated with neurodegenerative diseases, including Alzheimer's, fronto-temporal dementias, Pick's, and Progressive Supranuclear Palsy (21). In 1998, numerous independent dominant mutations in the tau gene were linked to a specific group of rare, familial neurodegenerative disorders collectively termed fronto-temporal dementia with Parkinsonism linked to chromosome 17 (also known as FTDP-17), which are characterized by neuronal cell death and dementia accompanied by abnormal tau fiber pathology (22-26).

Mutations in tau which cause FTDP-17 fall into two classes. One class consists of single amino-acid substitutions or deletions that generally decrease the ability of tau to bind microtubules and to promote microtubule assembly in vitro (23,27). The second class affects the regulation of tau mRNA splicing, altering the ratio of 4-repeat to 3-repeat tau expression (22,28-30). Thus, both tau dysfunction and mis-regulation can cause neurodegenerative disease.

The molecular mechanism(s) underlying tau-mediated neuronal cell death in FTDP-17 and other tauopathies are poorly understood. Consistent with the dominance of the FTDP-17 mutations, a widely held "gain-of-toxic-function" model suggests that mutations in tau increase its propensity to form abnormal fibers, which are posited to be cytotoxic (23,31,32). Alternatively, dominant phenotypes could also be achieved by a loss-of-function mechanism in which mutations in just one tau allele are sufficient to compromise the ability of the cell to properly regulate microtubule dynamics, leading to cell death.

Here, we sought to test a key prediction of the loss-of-function hypothesis, i.e., that FTDP-17 mutant tau isoforms possess compromised abilities to regulate microtubule dynamics relative to wild-type tau. To test this prediction, we injected identical amounts of either wild-type or FTDP-17 mutant tau into living MCF-7 cells, and analyzed the effects upon the dynamic instability behavior of individual microtubules in the thin lamellar peripheral region of the cells.

Materials and Methods

Tau protein purification - pRK expression vectors containing the human cDNA sequences for the shortest 4-repeat and 3-repeat tau isoforms (encoding 383 and 352 amino acids, respectively) were kind gifts from Dr. Kenneth Kosik (University of California, Santa Barbara) and Dr. Gloria Lee (University of Iowa). FTDP-17 mutations were introduced into both 4-repeat and 3-repeat tau constructs using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA; Figure 1A). The sequence of all constructs was verified by direct sequence analysis prior to use. Tau protein was expressed and purified as previously described (33,34). Briefly, tau expression was induced in BL21 (DE3) cells (Novagen, Madison, WI). Bacteria were lysed by sonication and the lysate was clarified by centrifugation (12,000 x g, 15 min, 4°C). Supernatants were boiled to precipitate heat-labile proteins, and re-centrifuged. The heat-stable proteins were adsorbed to a phosphocellulose column and eluted with a 0.2M to 1.0M NaCl gradient. Fractions containing tau protein were pooled and further purified using reverse-phase liquid chromatography (DeltaPak-C_{18}; Millipore, Billerica, MA). HPLC fractions containing tau were pooled, lyophilized, and re-suspended in PBS (Figure 1B). The concentration of each tau sample was determined by SDS-PAGE comparison with a "tau mass standard", the concentration of which was established by amino acid analysis (35).

Cell culture - MCF7 human breast cancer cells (ATCC, Manassas, VA) stably expressing the GFP-tubulin plasmid pEGFP-Tub (Clontech, Palo Alto, CA; (33) were maintained in DMEM (Gibco BRL, Grand Island, NY) supplemented with non-essential amino acids, 10% bovine serum, antibiotic-antimycotic (Gibco BRL) and Geneticin (400 μg/ml; Gibco BRL) at 37°C and 5.5% CO_2. Cells were seeded 36-48 hr before injection on 12 mm CellLocate coverslips (Eppendorf, Hamburg, Germany) coated with poly-D-lysine (100 μg/mL;

Sigma, St. Louis, MO) followed by human fibronectin (20 μg/mL; Gibco BRL) and laminin (10 μg/mL; Sigma). In order to induce a more flattened morphology, cells were serum-starved in media containing 2% bovine serum 12 hr before injection.

Microinjection - Seeded cells were transferred to serum-free DMEM lacking bicarbonate and containing 25 mM HEPES and 4.5 g/l glucose (recording media) (Gibco BRL). All wild-type and mutant tau proteins were diluted to a concentration of 13.3 μM in PBS plus 1.4 mM β-mercaptoethanol. Immediately prior to microinjection, the solution was centrifuged (50,000 x g, 15 min, 4°C) to remove any aggregates or debris. Pressure microinjection was performed using an Eppendorf Transjector 5246 and Injectman. The injection volume was approximately 10% of the cell volume (36), resulting in a ~ 1.3 μM final tau concentration in the cells. Injected cells were returned to normal media and incubated 2-3 hr at 37°C to allow equilibration of tau within the cells.

The quantity of tau injected into cells was based on the following rationale. Based upon the work of Dhamodharan and Wadsworth (37), we estimated that the total tubulin concentration in the cell to be approximately 20 μM (38) with 65% in polymer during interphase (39), resulting in 13 μM polymerized tubulin. With respect to tau, Drubin *et al*. (40) determined that the tau:polymeric tubulin molar ratio in neuronally differentiated rat PC12 cells is ~1:5 whereas it is ~1:34 in undifferentiated PC12 cells. Thus, we concluded that a 1:10 tau:polymeric tubulin molar ratio represents a reasonable approximation of in vivo neuronal conditions.

Immunocytochemistry - Cells were rinsed once with PBS and fixed by the rapid addition of 100% methanol (4°C). Fixed cells were incubated overnight in blocking buffer (3% BSA, 0.1% Triton-X-100 and 1% horse serum in PBS). Cells were incubated first with mouse monoclonal Tau 5 antibody (1:100; BioSource International, Camarillo, CA), then Cy3-conjugated donkey anti-mouse secondary antibody (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA), followed by FITC-conjugated mouse monoclonal tubulin antibody DM1α (1:50; Sigma). All incubations were for 1 hr at room temperature, followed by four 15 min washes in blocking buffer. Coverslips were mounted on glass slides using Prolong (Molecular Probes, Eugene, OR). Images were obtained using a laser scanning confocal microscope (MRC 1024; Bio-Rad, Hercules, CA). Images are 4 Kalman averages of the same plane in the z-axis. Images were processed using Adobe Photoshop 6.0.

Time-Lapse Microscopy and Image Acquisition - Microinjected cells were mounted in a Rose chamber (41) in recording media supplemented with Oxyrase (1:25 dilution; Oxyrase, Mansfield, OH) to reduce photobleaching. Images were captured with an inverted fluorescence microscope (Nikon Eclipse E800) with a Nikon plan apochromat 1.4 N.A., 100x objective lens, maintained at 36.5 ± 1°C. Thirty images per cell were taken at 4 s intervals using a Hamamatsu Orca II (Middlesex, NJ) digital camera driven by MetaMorph software (Universal Imaging, Media, PA).

Analysis of Microtubule Dynamic Instability – In order to determine the percentage of microtubules that displayed visually detectable growing and shortening dynamics, we analyzed the total change in length (growing plus shortening) of at least 50 randomly selected microtubules from 4 independent cells microinjected with a given tau isoform. A region of the cell periphery was randomly boxed and 10-20 microtubules within 1-2 boxes per cell were analyzed. A microtubule was considered to be not detectably dynamic if the total change in length during the 2 minute time-lapse sequence was less than or equal to 1.0 μm.

Analysis of microtubule dynamic instability behavior was performed as described previously (33,42). The positions of the plus-ends of individual microtubules over time were recorded using MetaMorph software (Universal Imaging), exported to Microsoft Excel and analyzed using RTM software (43). The lengths of individual microtubules were plotted as a function of time. Changes in length greater than 0.5 μm were designated as growth or shortening events. Periods in which length changes were less than 0.5 μm were designated as phases of attenuated microtubule dynamics (or pause). A catastrophe was defined as a transition from either growth or attenuation to shortening. The catastrophe frequency was calculated either as the total

number of catastrophes divided by the total time spent growing and attenuated, or as the total number of catastrophes divided by the total length grown. A rescue was defined as a transition from shortening to either growth or attenuation. Frequencies of rescue were calculated either as the total number of rescues divided by the total amount of time spent shortening, or as the total number of rescues divided by the total length shortened. Dynamicity was calculated as the total length grown and shortened divided by the total time measured.

Online Supplementary Material – Time-lapse movies of EGFP-microtubules undergoing dynamic instability in cells are available as supplemental material. Each movie consists of 30 frames taken at 4 sec intervals and is played 30 times faster than real time. Movie 1 shows microtubule dynamics in a control cell injected with buffer; Movie 2 shows microtubule dynamics in a cell injected with wild-type 4-repeat tau; Movie 3 shows microtubule dynamics in a cell injected with 4-repeat tau containing the ΔK280 mutation.

Results

Localization of microinjected wild-type and FTDP-17 mutated tau in cells

Prior to assessing the effects of FTDP-17 mutations on dynamic instability, we first sought to assess the sub-cellular distribution of injected wild-type and FTDP-17 mutated tau. We injected MCF7 cells, which do not express any endogenous tau, with either wild-type or FTDP-17 mutant tau and then returned the cells to the 37°C incubator for 2 hr to allow sufficient time for the tau to equilibrate throughout the cells. We then fixed the cells and double-stained for tau and tubulin. We found that wild-type and the various FTDP-17 mutated tau proteins associated with microtubules in injected cells, whereas, as expected, no tau was detected in uninjected cells (Figure 2). Tau staining was evenly distributed along the microtubules, with little or no tau staining elsewhere in the cells. Additionally, there was no detectable difference in the intensity of tau staining on microtubules between wild-type tau and tau containing the various FTDP-17 mutations. Thus, differences in the abilities of various tau molecules to regulate microtubule dynamics most likely reflect intrinsic mechanistic differences in the tau molecules being tested rather than differential binding of particular tau molecules to the microtubules.

Microinjection of exogenous wild-type or FTDP-17 mutant tau does not markedly affect the fraction of microtubules that display dynamic instability

In order to visualize dynamic instability behavior, we captured time-lapse images of microtubules in the flat peripheral region of living cells stably transfected with GFP-tubulin. As shown in Figure 3, and the online supplemental videos, the microtubules were well resolved and their ends were clearly visible. Initial analyses revealed that all cells possess two populations of microtubules, a stable population and a population which exhibits visually detectable dynamic behavior. To determine if the different tau isoforms used in this study differentially affected the percentage of dynamic versus non-dynamic microtubules in cells, we operationally defined dynamic microtubules as those exhibiting total length changes (growth plus shortening lengths) of 1.0 μm or more during two minutes of observation (dynamicity $\geq$ 0.5 μm/min); stable microtubules were defined as those which grew and/or shortened less than 1.0 μm during the same time period. Using these criteria, there were no significant differences in the percentage of dynamic microtubules in cells injected with control buffer versus any of the tau injected cells (Table 1). Most cells had ~80% dynamic microtubules. Cells injected with either 4-repeat wild-type tau or ΔK280 had slightly fewer dynamic microtubules than the control cells, but these differences were not statistically significant ($p = 0.20$ and $p = 0.39$, respectively using χ^2 test). These data are in contrast to our earlier assertion, based on non-quantitative assessments, suggesting that tau might increase the percentage of non-dynamic microtubules in the cells (33). In retrospect, it is likely that our earlier subjective observation was based upon the fact that tau markedly increases the percentage of time microtubules spend in the attenuated state (33).

Most FTDP-17 missense mutations reduce the ability of tau to regulate the percentage of time dynamic microtubules spend growing, shortening or attenuated

The FTDP-17 mutations examined here all map to regions of tau known to be important for normal tau function (see Figure 1). G272V is present in both 3-repeat and 4-repeat tau, residing in the first repeat. ΔK280 and P301L are both encoded by the alternatively spliced exon 10 and therefore are present only in 4-repeat tau; these mutations reside in the R1-R2 inter-repeat and at the end of repeat 2, respectively. Each of these mutations map to the region of tau believed to interact directly with microtubules (44-47). An additional mutation, R406W, is present in both 3-repeat and 4-repeat tau and resides in the flanking sequence on the carboxyl side of the repeat-inter-repeat region; these flanking sequences are believed to influence tau action indirectly via protein folding and/or phosphorylation effects (47,48).

To assess the effects of the various tau molecules on the regulation of microtubule dynamic instability, we injected cells either with buffer, wild type tau or FTDP-17 mutated tau and then measured the changes in length of individual microtubules over time by tracking the positions of microtubule ends (Materials and Methods). Typical microtubule life history plots for buffer-injected, wild-type 4-repeat tau-injected and ΔK280-injected cells are presented in Figure 4. From these plots, we determined the dynamic instability parameters (as described in Materials and Methods).

Dynamic microtubules transition among three phases: growth, shortening and attenuation (Figure 4). To begin our analyses, we first assessed the ability of each wild-type and FTDP-17 mutant tau isoform to influence the fraction of time that dynamic microtubules spent in each phase relative to the total time tracked. Wild-type 3-repeat and 4-repeat tau increased the fraction of time microtubules spent attenuated while reducing the fraction of time spent growing, relative to microtubules in buffer-injected control cells (33, see also Table 2). In contrast, four of the FTDP-17 tau mutations (3-repeat G272V, 4-repeat G272V, ΔK280 and P301L) were significantly compromised in their ability to influence the phase distribution relative to their wild-type counterparts (Table 2). For example, whereas wild-type 4-repeat tau reduced the fraction of time microtubules spent growing by 37% (from 0.39 to 0.24; Table 2), 4-repeat G272V, ΔK280 and P301L only caused 2-12% reductions. Similarly, whereas 4-repeat wild-type tau strongly increased the fraction of time microtubules spent attenuated (from 0.37 to 0.58, an increase of 58%), 4-repeat G272V, ΔK280 and P301L tau exhibited markedly reduced effects (10-29%)**.** Thus, these FTDP-17 tau isoforms have a decreased ability to regulate the dynamic behavior of cellular microtubules. In contrast to these large effects, the C-terminal tail mutation R406W, in both 4-repeat and 3-repeat contexts, exhibited no loss of effect relative to the respective wild-type tau isoforms. In fact, the R406W mutation slightly enhanced the ability of tau to decrease the time microtubules spent shortening in both 4-repeat and 3-repeat tau.

When the data for each mutant is plotted as percentage of the wild-type tau effect (for 4-repeat and 3-repeat wild type tau, as appropriate), it is clear that 4-repeat G272V, 3-repeat G272V, ΔK280 and P301L all exhibit marked loss-of-function effects upon tau's ability to regulate the time microtubules spend in each phase (Figure 5). In contrast, both the 4-repeat and 3-repeat R406W mutants exhibit near wild-type effects.

The amount of time a microtubule spends growing and shortening is in part determined by transition frequencies. In other words, tau might affect how long a microtubule grows or remains attenuated before it begins to shorten (defined as the catastrophe frequency) or conversely, tau might affect how long a microtubule shortens before it becomes attenuated or begins to grow (defined as the rescue frequency). Wild-type tau has a small effect on the catastrophe and rescue frequencies, which is most likely due to its regulatory effects on the growth and shortening rates (33). We calculated the catastrophe and rescue frequencies of microtubules in cells injected with FTDP-17 mutant tau proteins (calculated both per unit of time and per unit of length grown or shortened) and found no significant difference between wild-type tau and any of the mutants tau proteins tested in this study (data not shown).

FTDP-17 tau missense mutations exhibit loss-of-function effects on microtubule shortening events

As previously shown, 4-repeat wild type tau significantly reduced the average length a microtubule shortened during a shortening event in cells; in contrast, 3-repeat tau had a minimal effect on shortening events (33); see also Table 3). When we examined the effects of the various 4-repeat FTDP-17 mutated tau isoforms on the average length that a microtubule shortened, we found that most of the mutated tau proteins were less effective than wild-type tau, though to varying extents (Table 3; plotted as percentage of wild type tau effect in Figure 5). For instance, the P301L mutation caused a 57% loss of effect compared to wild-type 4R tau, while the ΔK280 mutation had no effect on the ability of tau to decrease the average length shortened.

The length that a microtubule shortens is determined by both the duration of the shortening event and the rate of shortening. When we compared the effects of wild-type and FTDP-17 mutant tau isoforms on the average duration of shortening, we found that the mutations had little or no effect, which might be expected since wild-type tau has little effect on this parameter (data not shown).

On the other hand, one of the most potent effects of wild-type 4-repeat tau on dynamic instability in vitro and in cells is to reduce the shortening rate (14,33,35). When we examined the effect of the FTDP-17 tau mutations on the microtubule shortening rate, we found that most of them (4-repeat G272V, P301L, and R406W) weakened tau's ability to reduce the shortening rate (Table 3; Figure 5). Interestingly, ΔK280 did not alter the effect of tau on the shortening rate. In the 3-repeat tau constructs, the FTDP-17 mutations had insignificant effects on tau's ability to modify the shortening rate. However, it would be difficult to detect a loss of effect on the shortening rate in these isoforms since 3-repeat wild-type tau itself has little effect on this parameter either in vitro or in cells (33,35).

FTDP-17 missense mutations decrease the ability of tau to regulate microtubule growth events

Both 4-repeat and 3-repeat wild type tau significantly reduced the average length that microtubules grew during a growth event (33, and Table 3). In order to determine whether FTDP-17 mutations in tau affect its ability to reduce the length grown during a growth event, we compared the average length grown per event in cells injected with buffer, wild-type tau or FTDP-17 mutated tau. We found that all FTDP-17 mutations tested decreased the ability of tau to regulate the length of a growth event (Table 3; Figure 5). Whereas growth events in control cells averaged 2.7 +/-0.2 μm, growth events in wild type 4-repeat tau-injected cells averaged 1.2 +/- 0.1 μm; the average growth event for the different 4-repeat FTDP-17 mutant tau isoforms ranged between 1.8 +/- 0.2 and 2.4 +/- 0.3 μm. Similar magnitudes of changes occurred with FTDP-17 mutant 3-repeat tau isoforms.

The length a microtubule grows during a growth event is a function of the duration of the growth event and the growth rate. We found that the FTDP-17 mutations affected both of these parameters. Whereas wild-type 4-repeat tau decreased the average growth duration by 32%, 4-repeat tau containing FTDP-17 mutations decreased the average growth duration by no more than 12% (data not shown). In addition, some FTDP-17 mutant tau proteins tested were also compromised in their ability to inhibit the growth rate (Table 3; Figure 5). The strongest loss-of-function occurred with ΔK280, which caused a 50% reduction in the ability of tau to regulate the growth rate when compared to 4-repeat wild-type tau. Two mutations, P301L and R406W (in both 4-repeat and 3-repeat isoforms), had almost no effect on this parameter, as they affected growth rates similarly to wild-type tau.

Taken together, the data demonstrate that every mutant tau isoform tested has a compromised ability to regulate some aspect(s) of microtubule growth events relative to their respective wild type tau isoform. The consequence of this loss of tau's regulatory ability is that microtubule growth proceeds more rapidly and/or for longer durations in cells with FTDP-17 mutant tau when compared to wild-type cells.

FTDP-17 missense mutations decreased the effect of tau on overall microtubule dynamicity

Dynamicity is a measure of the visually detectable amount of dynamic instability occurring

in a microtubule population. It is calculated as the total length grown and shortened divided by the time period observed through the process of dynamic instability. Both 4-repeat and 3-repeat wild-type tau greatly decreased the dynamicity of microtubules compared to microtubules in buffer-injected cells (Table 2 and 33). Most FTDP-17 tau mutant isoforms decreased microtubule dynamicity to a lesser extent than their respective wild-type isoforms, i.e., they were compromised in their abilities to regulate microtubule dynamics (Table 3; Figure 5). For example, the 4-repeat G272V, ΔK280, and P301L isoforms reduced dynamicity by only 32% to 39%, compared to the 56% reduction that occurred with wild-type 4-repeat tau. A similar loss of effect for the G272V mutation occurred in 3-repeat tau. However, in both 4-repeat and 3-repeat tau, the R406W mutation decreased microtubule dynamicity to a similar extent as the corresponding wild-type isoform. These results indicate that overall, with the exception of the R406W mutations, microtubules were 37% to 54% more dynamic in cells injected with FTDP-17 tau than in cells injected with wild-type tau.

Discussion

Given that the growing and shortening behaviors of microtubules are critical for proper cell function and viability (3-5) and that FTDP-17 mutations in tau can cause neuronal cell death and dementia, we sought to compare the effects of wild type and FTDP-17 mutant tau upon the dynamic instability behavior of microtubules. The hypothesis we tested was that FTDP-17 mutant tau isoforms are compromised in their ability to regulate the dynamic instability behavior of microtubules, which could in turn impact upon neuronal cell function and viability.

Analysis of microtubule dynamic instability in human neuronal cells is technically exceedingly difficult to accomplish. Thus, we used MCF-7 cells for this work. Attributes of these cells are that the dynamic instability behaviors of the microtubules in the thin peripheral regions of the cells are readily analyzed and that they do not express endogenous tau. Thus, the mechanistic effects of the FTDP-17 mutations on the regulation of dynamic instability could be analyzed in the absence of potentially complicating effects on dynamics contributed by wild-type tau isoforms. However, there are possible caveats to consider when extrapolating our data in MCF7 cells to human neuronal cells. Important differences between MCF-7 cells and neuronal cells are that they express different tubulin isotypes and different kinds of microtubule-associated proteins, both of which could influence the ability of tau or its mutated forms to regulate dynamic instability. Specifically, MCF7 cells express primarily the β1 tubulin isoform (49), whereas brain cells express primarily the βII isoform along with significant amounts of βIII (50). At the same time, our recent work shows that differences in the abilities of wild-type 3-repeat and 4-repeat tau to regulate microtubule dynamics first observed in *in vitro* assays is fully mirrored when these isoforms are expressed and analyzed in MCF7 cells (33,35). Furthermore, analysis of the same mutant tau isoforms tested here in MCF7 cells demonstrated almost identical effects in in vitro reactions (S. Levy and S.C. Feinstein, unpublished observations). Thus, while differences may indeed be found between the mechanistic effects of FTDP-17 tau mutations when present in MCF7 cells versus brain cells, it is likely that the data obtained in MCF7 cells provide a reasonable prediction of how the mutations will affect microtubule dynamics in neurons.

An additional point worthy of note is that the only tau present in our experimental system is the wild type or mutated tau that we microinject. In contrast, consistent with the dominance of the FTDP-17 mutations, cells in afflicted patients possess both wild type and mutant tau. However, the key point is that we are testing for the possible existence of mutation-induced alterations in the ability of tau to regulate microtubule dynamics in a cellular environment. The simultaneous presence of both wild type and mutant tau could blur our ability to detect such alterations. Indeed, recent in vitro work in our laboratories indicates that the level of microtubule dynamics regulatory activity exerted by a mixture of two different tau isoforms falls in between the levels of each isoform acting alone (A. LeBoeuf and S.C. Feinstein, unpublished observations). Taken together with the fact that subtle alterations in microtubule dynamics can have powerful cellular effects (51), we have sought to maximize the sensitivity of our assays by introducing only mutant tau into the cells. Indeed,

this strategy has been used by many investigators studying the cellular effects of various tau isoforms (for example see 52,53).

FTDP-17 missense mutations exhibit loss-of-function phenotypes with respect to the ability of tau to regulate microtubule dynamic instability

We found that all of the FTDP-17 tau mutations we examined caused loss-of-function effects (Tables 2 and 3; summarized graphically in Figure 5). For example, all of the mutations except R406W greatly reduced tau's ability to increase the amount of time microtubules spent attenuated, and all of the mutations reduced tau's ability to regulate the length of microtubule growth during individual growth events. The most dramatic loss-of-function effects were exhibited by the mutations that map to regions of tau believed to interact directly with microtubules (3-repeat G272V, 4-repeat G272V, P301L and ΔK280), while less marked effects occurred with 4-repeat R406W and 3-repeat R406W, both of which map outside the microtubule binding domain.

The differences we observed could possibly be explained by differences in the binding affinity of the mutated tau isoforms for the microtubule surface. Indeed, it has been demonstrated that many of the FTDP-17 mutations used in this study decrease the binding affinity of tau for microtubules (23,54). Therefore, the FTDP-17 tau isoforms could have a reduced ability to regulate microtubule dynamics simply by virtue of there being fewer molecules of mutated tau bound to the microtubules. We do not believe this to be the case for several reasons. First, when we calculated the percentage of tau that should be bound to microtubules using previously published microtubule binding constants (47) and our estimates of the tau and tubulin concentrations in the cell (see Materials and Methods), we determined that > 95% of both the wild-type and the FTDP-17 tau molecules should be bound to the microtubules. Consistent with this conclusion, not all dynamic parameters were affected equally by the presence of mutations in tau, as would be expected if the mutated tau were not binding to microtubules as well as wild-type tau. Furthermore, the R406W mutation has been shown to have a reduced affinity for microtubules (23), yet in our assays, it affects most dynamic instability parameters comparably to wild-type tau. Thus, we conclude that the loss-of-function effects of FTDP-17 mutant tau are manifested once the mutated tau binds to microtubules.

FTDP-17 missense mutations may act through multiple mechanisms

The different FTDP-17 mutations we examined had varying effects on dynamic instability. As noted above, the four mutations in the microtubule-binding region (3-repeat G272V, 4-repeat G272V, ΔK280 and P301L) can be grouped together relatively easily. These mutations all reduced the regulatory effects of wild type tau on growth length, on the fraction of time the microtubules spent growing and attenuated, and on dynamicity. There are likely to be subtle mechanistic differences even within this group, given that each mutation exhibits its own unique features. For instance, recalling that 4-repeat tau has a much stronger ability to repress the rate and extent of shortening than does 3-repeat tau, it is notable that the 4-repeat tau specific mutant P301L is especially weak with respect to regulating the rate and extent of shortening. This raises the possibility that this proline may play an important role in the ability of 4-repeat tau to suppress these parameters.

The R406W mutation behaved very differently than the other mutations, having only modest effects upon the ability of both 4-repeat and 3-repeat tau to regulate dynamic instability. Notably, this mutation does not map to the microtubule-binding region of tau; rather, it resides in the regulatory C-terminal tail. Rather than affecting the tau-microtubule interaction directly, this mutation may act primarily by affecting the phosphorylation of tau at nearby sites. Indeed, other studies have found that phosphorylation of tau was significantly altered by the presence of the R406W substitutions (2,48,52,55).

We propose that the 3-repeat G272V, 4-repeat G272V, ΔK280, and P301L mutations directly affect the tau-microtubule interaction. In contrast, we suggest that the direct effect of the R406W mutation is on the regulation of tau itself (for example, phosphorylation), which in turn impacts upon the tau-microtubule interaction and the regulation of microtubule dynamics. This

conclusion agrees with that of others using completely different bioassays (48).

Are these losses-of-function in the ability of tau to regulate microtubule dynamic instability meaningful to a cell?

It is well established that inappropriate alterations in microtubule dynamics can have major consequences during mitosis. Specifically, previous work has shown that low concentrations of taxol that suppress microtubule dynamicity by only ~31% are sufficient to impair mitotic progression, which may in turn lead to apoptosis (51,56). At the other extreme, increases in microtubule dynamicity of ~ 50-60 % inhibit mitotic progression in taxol-dependent cells unless microtubule dynamics are suppressed by treatment with taxol (42). Thus, both over-dynamic and under-dynamic microtubules can have severe consequences for cells.

Proper regulation of microtubule dynamics is critical in neurons as well. Disruption of the regulation of microtubule dynamics has been shown to interfere with axonal stability and the formation of synaptic boutons (57,58). Furthermore, increasing microtubule stability with microtubule-binding drugs ameliorates the axonal transport defects observed in a mouse model of tauopathy (54).

Based on these considerations and our recent in vitro and cellular work with wild-type 3-repeat and 4-repeat tau (33,35), we have proposed that microtubule dynamics in neuronal cells must be maintained within an acceptable range of activity levels. The model further suggests that, outside of this range, microtubules can not function normally, resulting in cumulative damage and eventual cell death. The work presented here demonstrates that the FTDP-17 missense mutations mapping to the microtubule binding region increase microtubule dynamicity by 37 – 54% when present at physiologically relevant levels, i.e., they significantly compromise the ability of tau to regulate microtubule dynamicity. Neurons harboring these mutations, and thus containing a mixture of wild-type and mutant tau, are to likely possess overly dynamic microtubules. This decrease in microtubule stability may not have an immediate effect on neuronal development and function, but over time, cumulative damage could lead to neurodegeneration.

These data complement our recent cellular work relevant to the FTDP-17 tau mRNA splicing mutations (33). In this work, we demonstrated that wild-type 4-repeat tau exhibits quantitatively and qualitatively different mechanistic capabilities than does wild-type 3-repeat tau, leading to the proposal that neurons harboring tau mRNA splicing mutations should also exhibit markedly different patterns of microtubule dynamics relative to normal neurons. Taken together, our data suggest that both classes of FTDP-17 mutations, amino-acid substitutions and altered tau mRNA splicing, compromise tau's ability to properly regulate the dynamic behavior of microtubules. These data are consistent with the model in which defects in tau or its regulation lead to microtubule dynamics which are outside of a tolerable range, resulting in disruption of microtubule function and consequent neuronal cell death.

REFERENCES

1. Akhmanova, A., and Hoogenraad, C. C. (2005) *Curr Opin Cell Biol* **17**, 47-54
2. Small, J. V., and Kaverina, I. (2003) *Curr Opin Cell Biol* **15**, 40-47
3. Maiato, H., Sampaio, P., and Sunkel, C. E. (2004) *Int Rev Cytol* **241**, 53-153
4. Zhou, J., and Giannakakou, P. (2005) *Curr Med Chem Anti-Canc Agents* **5**, 65-71
5. Jordan, M. A., and Wilson, L. (2005) in *Microtubules in Health and Disease* (Fojo, T., ed) Vol. (in press), Humana Press
6. Machesky, L. M., and Bornens, M. (2003) *Curr Opin Cell Biol* **15**, 2-5
7. Zhou, F. Q., and Snider, W. D. (2005) *Science* **308**, 211-214
8. Riederer, B. M., Pellier, V., Antonsson, B., Di Paolo, G., Stimpson, S. A., Lutjens, R., Catsicas, S., and Grenningloh, G. (1997) *Proc Natl Acad Sci U S A* **94**, 741-745
9. Nakao, C., Itoh, T. J., Hotani, H., and Mori, N. (2004) *J Biol Chem* **279**, 23014-23021
10. Cassimeris, L. (2002) *Curr Opin Cell Biol* **14**, 18-24
11. Tirnauer, J. S., Grego, S., Salmon, E. D., and Mitchison, T. J. (2002) *Mol Biol Cell* **13**, 3614-3626
12. Popov, A. V., and Karsenti, E. (2003) *Trends Cell Biol* **13**, 547-550
13. Drechsel, D. N., Hyman, A. A., Cobb, M. H., and Kirschner, M. W. (1992) *Mol Biol Cell* **3**, 1141-1154
14. Panda, D., Goode, B. L., Feinstein, S. C., and Wilson, L. (1995) *Biochemistry* **34**, 11117-11127
15. Trinczek, B., Biernat, J., Baumann, K., Mandelkow, E. M., and Mandelkow, E. (1995) *Mol Biol Cell* **6**, 1887-1902
16. Himmler, A. (1989) *Mol Cell Biol* **9**, 1389-1396
17. Lee, G., Cowan, N., and Kirschner, M. (1988) *Science* **239**, 285-288
18. Billingsley, M. L., and Kincaid, R. L. (1997) *Biochem J* **323 (Pt 3)**, 577-591
19. Stoothoff, W. H., and Johnson, G. V. (2005) *Biochim Biophys Acta* **1739**, 280-297
20. Buee, L., Bussiere, T., Buee-Scherrer, V., Delacourte, A., and Hof, P. R. (2000) *Brain Res Brain Res Rev* **33**, 95-130
21. Lee, V. M., Goedert, M., and Trojanowski, J. Q. (2001) *Annu Rev Neurosci* **24**, 1121-1159
22. Hutton, M., Lendon, C. L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A., Hackett, J., Adamson, J., Lincoln, S., Dickson, D., Davies, P., Petersen, R. C., Stevens, M., de Graaff, E., Wauters, E., van Baren, J., Hillebrand, M., Joosse, M., Kwon, J. M., Nowotny, P., Heutink, P., and et al. (1998) *Nature* **393**, 702-705
23. Hong, M., Zhukareva, V., Vogelsberg-Ragaglia, V., Wszolek, Z., Reed, L., Miller, B. I., Geschwind, D. H., Bird, T. D., McKeel, D., Goate, A., Morris, J. C., Wilhelmsen, K. C., Schellenberg, G. D., Trojanowski, J. Q., and Lee, V. M. (1998) *Science* **282**, 1914-1917
24. Spillantini, M. G., Crowther, R. A., Kamphorst, W., Heutink, P., and van Swieten, J. C. (1998) *Am J Pathol* **153**, 1359-1363
25. Clark, L. N., Poorkaj, P., Wszolek, Z., Geschwind, D. H., Nasreddine, Z. S., Miller, B., Li, D., Payami, H., Awert, F., Markopoulou, K., Andreadis, A., D'Souza, I., Lee, V. M., Reed, L., Trojanowski, J. Q., Zhukareva, V., Bird, T., Schellenberg, G., and Wilhelmsen, K. C. (1998) *Proc Natl Acad Sci U S A* **95**, 13103-13107
26. Goedert, M., Spillantini, M. G., Crowther, R. A., Chen, S. G., Parchi, P., Tabaton, M., Lanska, D. J., Markesbery, W. R., Wilhelmsen, K. C., Dickson, D. W., Petersen, R. B., and Gambetti, P. (1999) *Nat Med* **5**, 454-457

27. Hasegawa, M., Smith, M. J., and Goedert, M. (1998) *FEBS Lett* **437**, 207-210
28. Spillantini, M. G., Murrell, J. R., Goedert, M., Farlow, M. R., Klug, A., and Ghetti, B. (1998) *Proc Natl Acad Sci U S A* **95**, 7737-7741
29. Varani, L., Hasegawa, M., Spillantini, M. G., Smith, M. J., Murrell, J. R., Ghetti, B., Klug, A., Goedert, M., and Varani, G. (1999) *Proc Natl Acad Sci U S A* **96**, 8229-8234
30. Grover, A., Houlden, H., Baker, M., Adamson, J., Lewis, J., Prihar, G., Pickering-Brown, S., Duff, K., and Hutton, M. (1999) *J Biol Chem* **274**, 15134-15143
31. Barghorn, S., and Mandelkow, E. (2002) *Biochemistry* **41**, 14885-14896
32. Gamblin, T. C., King, M. E., Dawson, H., Vitek, M. P., Kuret, J., Berry, R. W., and Binder, L. I. (2000) *Biochemistry* **39**, 6136-6144
33. Bunker, J. M., Wilson, L., Jordan, M. A., and Feinstein, S. C. (2004) *Mol Biol Cell* **15**, 2720-2728
34. Goode, B. L., Denis, P. E., Panda, D., Radeke, M. J., Miller, H. P., Wilson, L., and Feinstein, S. C. (1997) *Mol Biol Cell* **8**, 353-365
35. Panda, D., Samuel, J. C., Massie, M., Feinstein, S. C., and Wilson, L. (2003) *Proc Natl Acad Sci U S A* **100**, 9548-9553
36. Lamb, N. J., and Fernandez, A. (1997) *Methods Enzymol* **283**, 72-83
37. Dhamodharan, R., and Wadsworth, P. (1995) *J Cell Sci* **108 (Pt 4)**, 1679-1689
38. Hiller, G., and Weber, K. (1978) *Cell* **14**, 795-804
39. Zhai, Y., and Borisy, G. G. (1994) *J Cell Sci* **107 (Pt 4)**, 881-890
40. Drubin, D. G., Feinstein, S. C., Shooter, E. M., and Kirschner, M. W. (1985) *J Cell Biol* **101**, 1799-1807
41. Rose, G. G., Pomerat, C. M., Shindler, T., and Trunnell, J. (1958) *J Biophys Biochem Cytol* **4**, 761-764
42. Goncalves, A., Braguer, D., Kamath, K., Martello, L., Briand, C., Horwitz, S., Wilson, L., and Jordan, M. A. (2001) *Proc Natl Acad Sci U S A* **98**, 11737-11742
43. Walker, R. A., O'Brien, E. T., Pryer, N. K., Soboeiro, M. F., Voter, W. A., Erickson, H. P., and Salmon, E. D. (1988) *J Cell Biol* **107**, 1437-1448
44. Brandt, R., and Lee, G. (1993) *J Biol Chem* **268**, 3414-3419
45. Butner, K. A., and Kirschner, M. W. (1991) *J Cell Biol* **115**, 717-730
46. Goode, B. L., and Feinstein, S. C. (1994) *J Cell Biol* **124**, 769-782
47. Goode, B. L., Chau, M., Denis, P. E., and Feinstein, S. C. (2000) *J Biol Chem* **275**, 38182-38189
48. Delobel, P., Flament, S., Hamdane, M., Jakes, R., Rousseau, A., Delacourte, A., Vilain, J. P., Goedert, M., and Buee, L. (2002) *J Biol Chem* **277**, 9199-9205
49. Nicoletti, M. I., Valoti, G., Giannakakou, P., Zhan, Z., Kim, J. H., Lucchini, V., Landoni, F., Mayo, J. G., Giavazzi, R., and Fojo, T. (2001) *Clin Cancer Res* **7**, 2912-2922
50. Luduena, R. F. (1998) *Int Rev Cytol* **178**, 207-275
51. Yvon, A. M., Wadsworth, P., and Jordan, M. A. (1999) *Mol Biol Cell* **10**, 947-959
52. Krishnamurthy, P. K., and Johnson, G. V. (2004) *J Biol Chem* **279**, 7893-7900
53. Vogelsberg-Ragaglia, V., Bruce, J., Richter-Landsberg, C., Zhang, B., Hong, M., Trojanowski, J. Q., and Lee, V. M. (2000) *Mol Biol Cell* **11**, 4093-4104
54. DeTure, M., Ko, L. W., Yen, S., Nacharaju, P., Easson, C., Lewis, J., van Slegtenhorst, M., Hutton, M., and Yen, S. H. (2000) *Brain Res* **853**, 5-14
55. del C. Alonso, A., Mederlyova, A., Novak, M., Grundke-Iqbal, I., and Iqbal, K. (2004) *J Biol Chem* **279**, 34873-34881

56. Jordan, M. A., Wendell, K., Gardiner, S., Derry, W. B., Copp, H., and Wilson, L. (1996) *Cancer Res* **56**, 816-825
57. Ruiz-Canada, C., Ashley, J., Moeckel-Cole, S., Drier, E., Yin, J., and Budnik, V. (2004) *Neuron* **42**, 567-580
58. Chang, L., Jones, Y., Ellisman, M. H., Goldstein, L. S., and Karin, M. (2003) *Dev Cell* **4**, 521-533

ACKNOWLEDGEMENTS

We are extremely grateful to Herb Miller for technical assistance and data analysis, to Herb Waite for performing the mass spectrometry and to Michelle Massie for assistance with figures. We also thank Allen Stewart-Oaten and Carol Vandenberg for valuable discussions, as well as Dmitri Leonoudakis for comments on the manuscript. This work was supported by National Institutes of Health grants NS35010 (SCF), NS13560 (LW) and CA57291 (MAJ) as well as Information Technology Research grant 0331697 from the National Science Foundation (P.I.: B.S. Manjunath, co-P.I.s SCF and LW).

FIGURE LEGENDS

Figure 1. Production of Wild-Type and FTDP-17 Tau Proteins. (A) Schematic of FTDP-17 mutations produced in 4-repeat and 3-repeat tau. Shaded boxes represent microtubule-binding repeats. (B) Electrophoretic fractionation of tau protein preparations using 8% SDS-PAGE gels stained with Coomassie Blue. 0.5 μg of protein was loaded in each lane.

Figure 2. Localization of Injected Wild-Type and FTDP-17 Tau by Immunofluorescence Microscopy. MCF7 cells were injected with 4-repeat or 3-repeat isoforms of either wild-type tau or tau containing the G272V mutation. Cells were fixed and stained for tau and tubulin as described in the Methods section. In the merged column the tau staining is pseudo-colored red and the tubulin staining is pseudo-colored green; yellow indicates co-localization. Tau co-localized with microtubules in the injected cells, while uninjected cells (seen next to the injected cell in some frames) did not stain positively for tau. Cells injected with other FTDP-17 mutated tau proteins exhibited staining that was indistinguishable from that shown for G272V (data not shown). Scale bar = 10 μm.

Figure 3. Microtubule Dynamic Instability in an MCF7 Cell Expressing GFP-tubulin. Time-lapse images of fluorescent (GFP) microtubules in a buffer-injected cell allow tracking of microtubule growth and shortening over time. One growing microtubule (closed arrowheads) and one shortening microtubule (open arrowheads) are highlighted. While microtubule ends are visible in these still images, it is much easier to discern changes in microtubule length in a time-lapse movie (online supplemental material). Scale bar = 5 μm.

Figure 4. Life history plots of the growth and shortening dynamics of individual microtubules. The positions of the ends of individual microtubule were tracked in cells injected with buffer, 4-repeat wild-type tau, or 4-repeat ΔK280 FTDP-17 tau. Changes in the length of the microtubules were plotted versus time. Each line represents a single microtubule. From these life history plots, individual growth, shortening, and attenuation events were identified and the rate and duration of each event were determined.

Figure 5. Abilities of FTDP-17 Tau Mutant Proteins to Regulate Individual Parameters of Microtubule Dynamic Instability, Relative to Wild-Type Tau. The effect of FTDP-17 tau proteins on various microtubule dynamic parameters is graphed as a percentage of the effect of the corresponding wild-type tau isoform, where the effect of wild-type tau is set to 100 and the effect of the buffer control injections is set to 0. %G = relative ability of mutant tau to reduce the percentage of time spent growing; %S = relative ability of mutant tau to reduce the percentage of time spent shortening; %A = relative ability of mutant tau to increase the percentage of time spent attenuated; GR = relative ability of mutant of tau to reduce the growth rate; GL = relative ability of mutant of tau to reduce the length grown per event; SR = relative ability of mutant of tau to reduce the shortening rate; SL = relative ability of mutant of tau to reduce the length shortened per event; Dyn = relative ability of mutant of tau to reduce microtubule dynamicity.

Table 1: Percentage of Dynamic Microtubules in Tau -Injected Cells

	N (# MTs)	% Dynamic
buffer control	60	80
4R WT	63	68
G272V	70	80
K280Δ	60	72
P301L	78	78
R406W	50	84
3R WT	60	83
G272V	65	77
R406W	78	82

Table 2: Effects of FTDP-17 mutations on the fraction of time microtubules spent growing, shortening and attenuated

		Fraction of time spent:		
		growing	shortening	attenuated
buffer control		0.39	0.24	0.37
4R WT	**	0.24	0.18	0.58
G272V	‡	0.34	0.18	0.48
K280Δ	‡	0.38	0.21	0.41
P301L	‡	0.36	0.18	0.46
R406W		0.26	0.15	0.59
3R WT	**	0.31	0.16	0.52
G272V	†	0.35	0.21	0.44
R406W		0.34	0.14	0.53

Note: Statistical significance determined using $\chi 2$ test:
** $p<0.001$ versus buffer control
† $p<0.01$ versus wild-type tau
‡ $p<0.001$ versus wild type tau

Table 3: Dynamic instability parameters of MTs in cells injected with wild type or FTDP-17 mutated tau

	N # MTs / # cells	Growth Rate (μm/min)	Growth Length (μm)	Shortening Rate (μm/min)	Shortening Length (μm)	Dynamicity (μm/min)
buffer control	56 / 18	13.3±0.4	2.7±0.2	27.1±1.5	5.2±0.4	11.7
4R WT	27 / 7	9.40±0.3**	1.2±0.1**	16.5±0.9**	2.9±0.4**	5.2
G272V	27 / 9	10.1±0.3	1.9±0.1‡	20.0±1.4†	3.2±0.4	7.1
K280del	27 / 8	11.3±0.5‡	2.4±0.3‡	17.4±1.0	2.8±0.3	8.0
P301L	27 / 11	9.6±0.5	1.9±0.2‡	21.0±1.5†	4.2±0.6	7.2
R406W	27 / 10	9.5±0.4	1.8±0.2†	20.5±1.9	3.6±0.6	5.5
3R WT	27 / 8	11.6±0.5*	1.8±0.2**	22.3±1.5*	3.6±0.4*	7.3
G272V	25 / 9	11.7±0.5	2.2±0.2	23.8±1.5	4.3±0.6	9.0
R406W	25 / 8	12.2±0.6	2.5±0.3 †	23.6±1.6	3.8±0.5	7.3

Note: Rate and length values are given as mean ± SEM

Note: Statistical significance determined using t-test with unequal variances:

* p<0.01 versus buffer control

** p<0.001 versus buffer control

† p<0.01 versus wild-type tau

‡ p<0.001 versus wild type tau

Figure 1

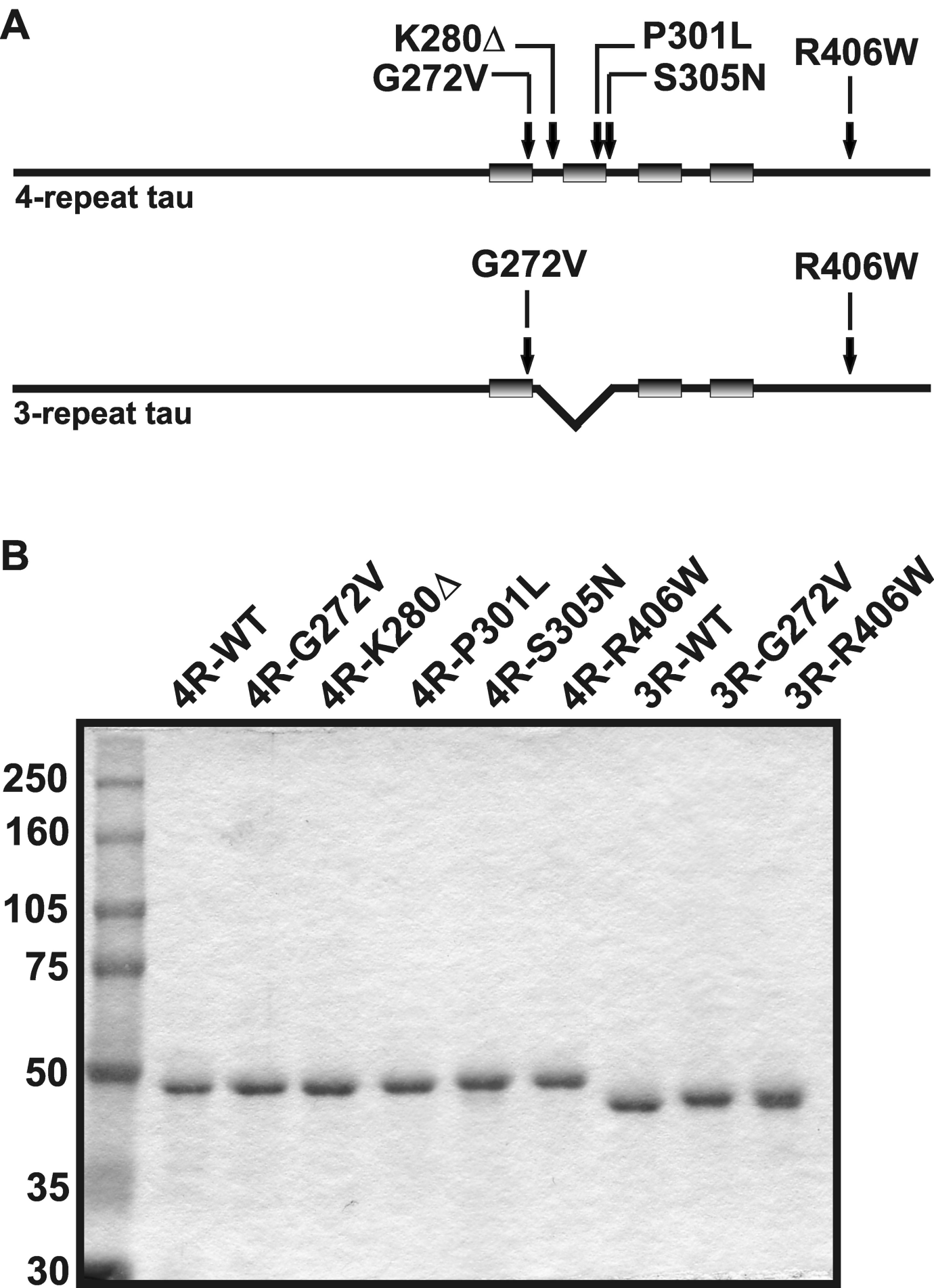

A
K280Δ
G272V
P301L
S305N
R406W
4-repeat tau
G272V
R406W
3-repeat tau
B
4R-WT
4R-G272V
4R-K280Δ
4R-P301L
4R-S305N
4R-R406W
3R-WT
3R-G272V
3R-R406W
250
160
105
75
50
35
30

Figure 2

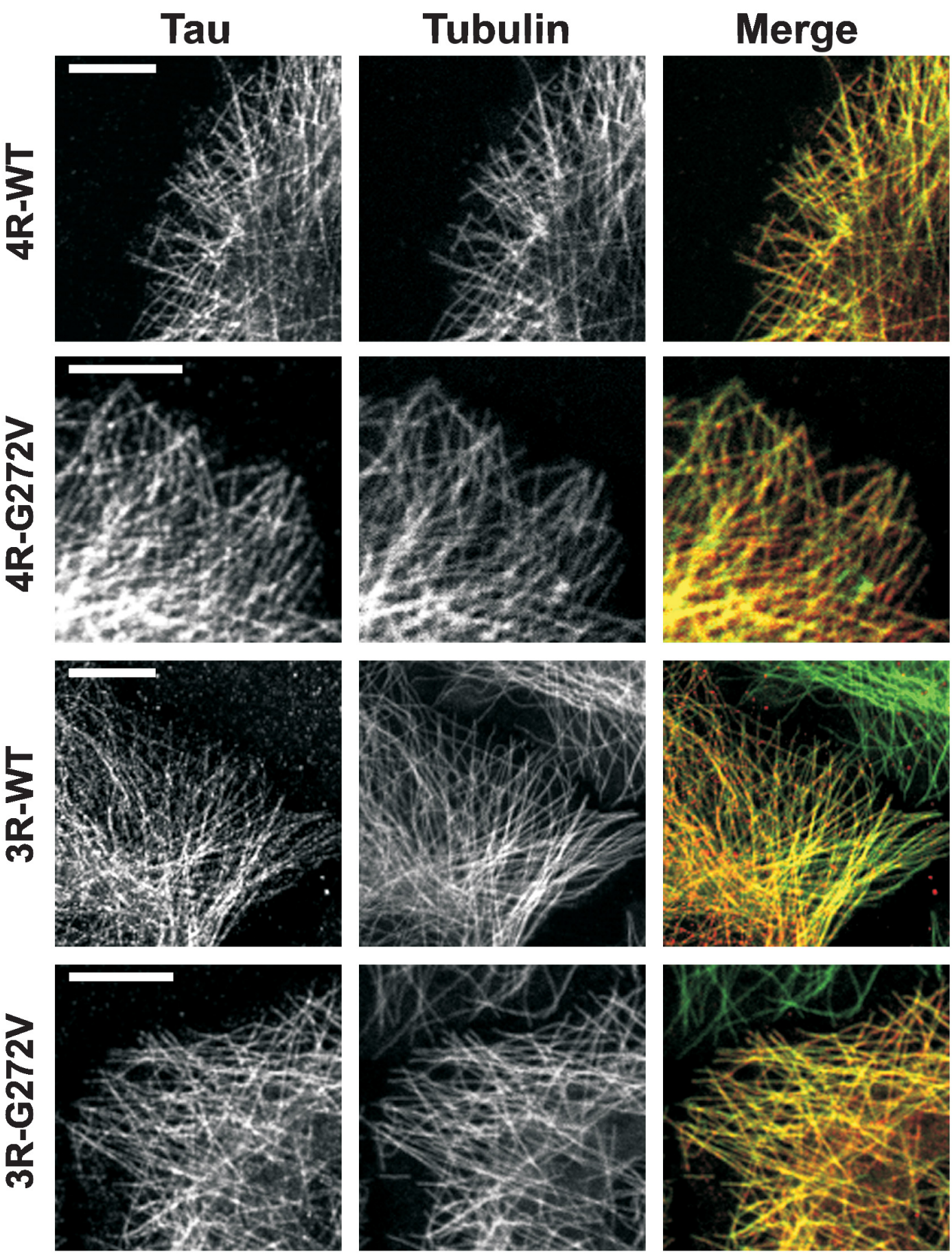


Figure 3

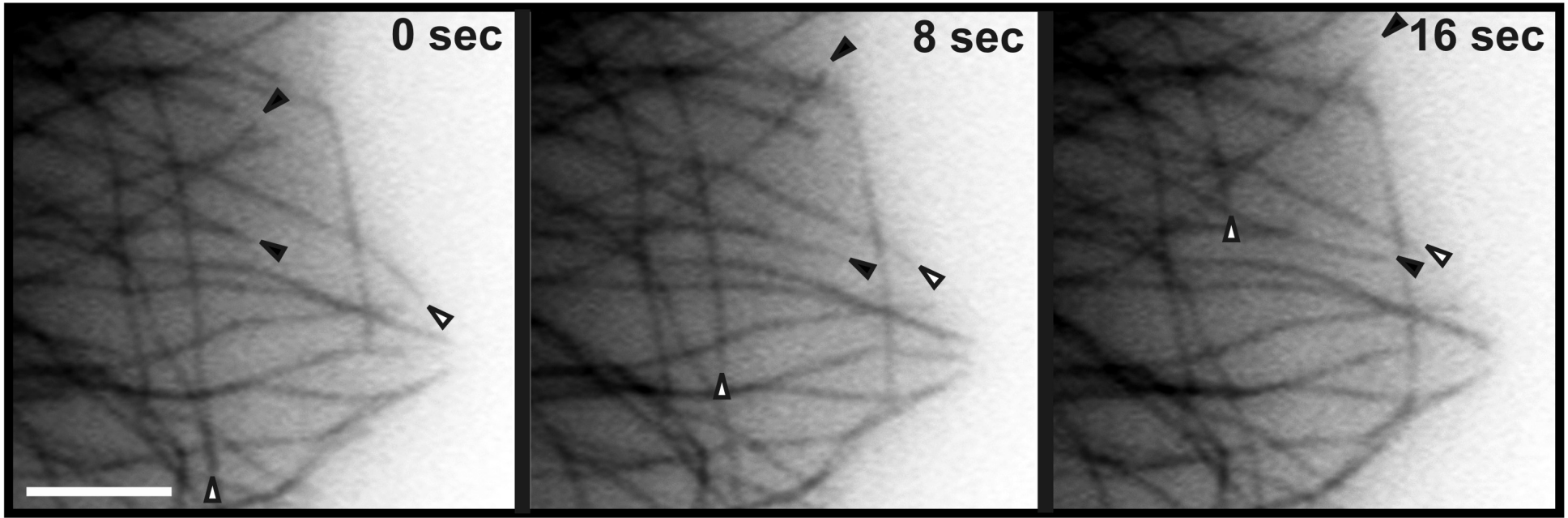


Figure 4

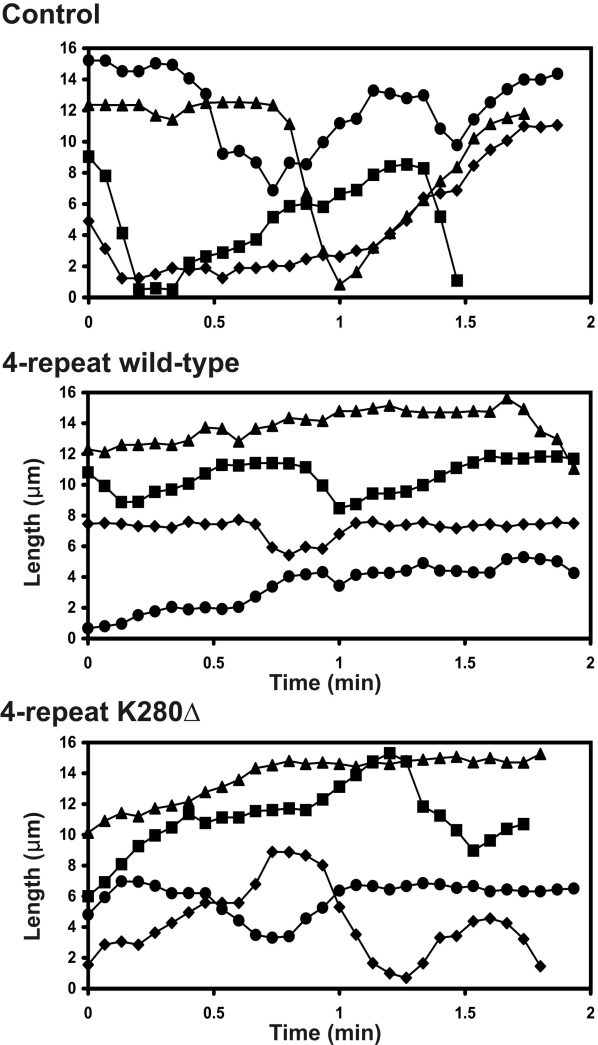
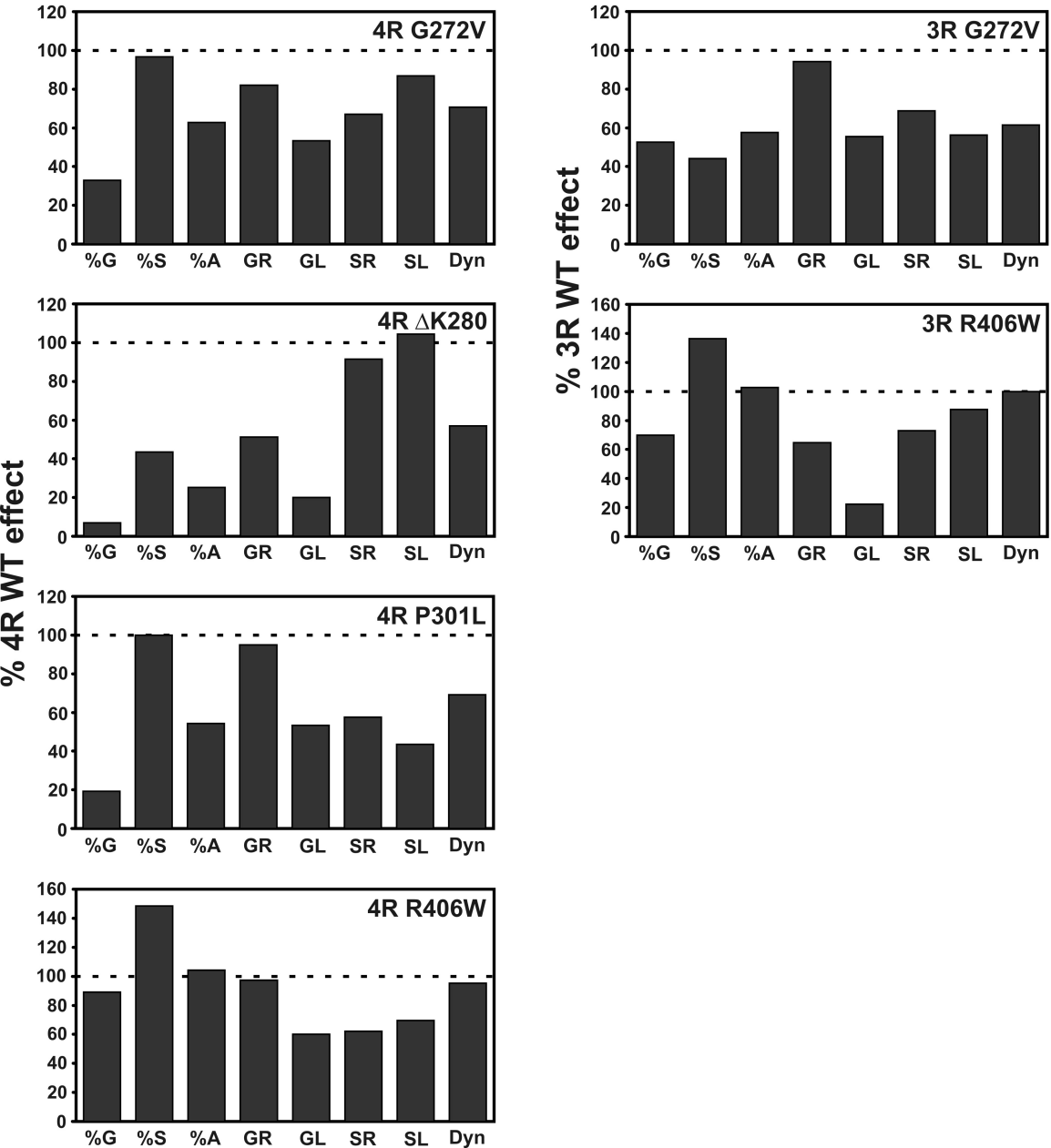

Control
4-repeat wild-type
Length (μm)
Time (min)
4-repeat K280Δ
Length (μm)
Time (min)

Figure 5


4R G272V
3R G272V
4R ΔK280
3R R406W
4R P301L
4R R406W
% 4R WT effect
% 3R WT effect
%G %S %A GR GL SR SL Dyn