Desmin intermediate filaments and tubulin detyrosination stabilize growing microtubules in the cardiomyocyte

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Abstract

The microtubule network of the cardiomyocyte exhibits specialized architecture, stability and mechanical behavior that accommodate the demands of working muscle cells. Stable, post-translationally detyrosinated microtubules are physical coupled to the sarcomere, the contractile apparatus of muscle, and resist sarcomere motion to regulate muscle mechanics and mechanosignaling. Control of microtubule growth and shrinkage dynamics represents a potential intermediate in the formation of a stable, physically coupled microtubule network, yet the molecular determinants that govern dynamics are unknown. Here we test the hypothesis that desmin intermediate filaments may stabilize growing microtubules at the sarcomere Z-disk in a detyrosination-dependent manner. Using a combination of biochemical assays and direct observation of microtubule plus-end dynamics in primary adult cardiomyocytes, we determine that: 1) tyrosination increases the frequency of microtubule depolymerization and reduces the pausing of microtubules at the Z-disk, leading to a more dynamic microtubule; and 2) desmin intermediate filaments stabilize both growing and shrinking microtubules specifically at the Z-

disk and protect them from depolymerization. This stabilizes iteratively growing, detyrosinated microtubules between adjacent sarcomeres, which promotes the formation of high-energy microtubules that buckle between sarcomeres and elevates myocyte viscoelasticity. Our findings inform on how the tubulin code and intermediate filaments regulate microtubule dynamics, and provide mechanism to the establishment of a spatially organized, physically coupled, and long-lived microtubule network in the cardiomyocyte.

Introduction

Microtubules are polymers of α - and β -tubulin that are characterized by cyclical transitions between polymerization and depolymerization, a behavior called dynamic instability¹. Tuning this dynamic behavior confers unique functionality to specific sub-populations of microtubules². Control of microtubule dynamics is cell type- and context-specific and can occur either by modulating polymer addition or subtraction at the ends, or through lateral interaction with the microtubule polymer³. The temporal and spatial control of dynamics can be tuned by post-translational modification of tubulin, which in turn affects the biophysical properties of the microtubule and interactions with microtubule associated proteins (MAPs)⁴. For example, detyrosination, the post translational removal of the C-terminal tyrosine residue on α -tubulin, has been shown to increase microtubule stability in mitotic cells by preventing their interaction with depolymerizing MAPs⁵.

In the cardiomyocyte, microtubules fulfill both canonical roles in intracellular trafficking and organelle positioning⁶, as well as non-canonical functions matched to the unique demands of working myocytes. In the interior of the myocyte, microtubules form a predominantly longitudinal network that runs perpendicular to the transverse Z-discs that define the sarcomere, the basic contractile unit of muscle (**Fig 1A**). Sub-populations of microtubules form physical connections with the Z-disc that serve as lateral reinforcements along the length of the microtubule⁷. Upon stimulation and sarcomere shortening, these physically coupled microtubules buckle at short stereotypical wavelengths between sarcomeres to resist the change in myocyte length⁷. The physical ramifications of these reinforced microtubules for the myocyte are significant, as reinforced microtubules can resist 1000x more force than uncoupled microtubules^{8,9}. This viscoelastic resistance becomes particularly problematic in heart failure, where proliferation of longitudinal, coupled microtubules stiffens the cardiomyocyte and impairs myocyte motion¹⁰.

Physical coupling of the microtubule to the sarcomere is tuned by detyrosination. Genetic reduction of detyrosination by overexpression of tubulin tyrosine ligase (TTL), the enzyme responsible for ligating the terminal tyrosine residue on detyrosinated tubulin, reduces sarcomeric buckling and the viscoelastic resistance provided by microtubules, increasing myocyte contractility¹⁰. Tyrosination status also governs microtubule-dependent mechanotransduction in muscle that regulates downstream second messengers and is implicated in myopathic states¹¹. Given its ability to lower stiffness and improve the function of myocardium from patients with heart failure¹⁰, tyrosination is under pursuit as a novel

therapeutic approach. Yet how detyrosination promotes the interaction of microtubules with the sarcomere to mediate their mechanical impact remains poorly understood.

Several observations suggest this interaction may be mediated at least in part through desmin intermediate filaments that wrap around the Z-disk. Detyrosination promotes microtubule interaction with intermediate filaments^{7,12}, and in the absence of desmin, microtubules are disorganized and detyrosination no longer alters myocyte mechanics⁷. Additionally, a recent pre-print indicates that intermediate filaments can directly stabilize dynamic microtubules in vitro¹³.

Despite this evidence, the effects of desmin or detyrosination on the dynamics of cardiomyocyte microtubules have not been investigated. We hypothesized that desmin may serve to stabilize growing microtubules at the Z-disk, which would represent an important intermediary in laterally reinforcing the network and coupling microtubules to the sarcomere. Here, using a combination of genetic manipulations, biochemical assays, and direct live-cell observation of dynamic microtubules, we directly interrogated the effect of tyrosination status and desmin depletion on microtubule dynamics. We find that desmin dictates the spatial organization of microtubule dynamics and that growing microtubules are stabilized at the Z-disc in a desmin-dependent fashion. Additionally, we find that tyrosinated microtubules are more dynamic, a characteristic that precludes their ability to efficiently grow between adjacent sarcomeres and form stabilizing interactions at the Z-disk. These findings provide insight into the organizing principles of myocyte cytoarchitecture and into the mechanism of action for therapeutic strategies that target detyrosinated microtubules.

Results

Tyrosination alters the dynamics of the microtubule network

We first sought to determine the effect of detyrosination on the dynamics of the cardiomyocyte microtubule network. To reduce detyrosination, we utilized adenoviral delivery of TTL into isolated adult rat cardiomyocytes⁷. TTL binds and tyrosinates tubulin in a 1:1 complex, and so we also utilized adenoviral delivery of TTL-E331Q (E331Q), a verified catalytically dead mutant of TTL¹⁴, to separate effects of tubulin tyrosination from tubulin sequestration. We have previously confirmed that TTL overexpression under identical conditions reduces detyrosination below 25% of initial levels, while TTL-E331Q does not significantly affect detyrosination levels with similar overexpression¹⁴. To specifically quantify the effects of reducing detyrosination on the dynamic microtubule population, we adapted a subcellular fractionation assay from Fasset et al., 2009¹⁵ that allowed us to separate free tubulin from polymerized tubulin in the dynamic (i.e. cold-sensitive) microtubule pool (**Fig 1B**).

Expression of TTL, but not E331Q, resulted in significantly less detyrosinated tubulin in the dynamic microtubule pool (**Fig 1C**). Further, only TTL expression shifted tubulin away from the polymerized fraction towards the free tubulin fraction, resulting in an increased ratio of free: polymerized tubulin (**Fig 1C, S. Fig 1A**). This suggests that tyrosination effects the cycling of tubulin within the dynamic microtubule pool. If indeed tyrosinated microtubules are more dynamic, then levels of acetylation, a canonical marker of long-lived microtubules¹⁶, should also be decreased by TTL. Consistent with this, TTL, but not E331Q, led to a robust reduction in levels of microtubule acetylation, suggesting that tyrosination reduces microtubule lifetime in the cardiomyocyte (**Fig 1D**).

As acetylation itself is linked to microtubule stability^{17,18}, the TTL-dependent change in the dynamic microtubule pool (**Fig 1C**) could be directly related to tyrosination or secondary to the associated reduction in acetylation (**Fig 1D**). To discriminate between these two hypotheses, we directly modulated acetylation. To this end, we developed adenoviral constructs encoding histone deacetylase 6 (HDAC6) and α tubulin acetyltransferase 1 (α TAT1). HDAC6 expression reduced total microtubule acetylation to 25% of initial levels (**Fig 1E**) and α TAT1 expression increased acetylation 12-fold (**Fig 1E**). Because α TAT1 has been shown to modulate microtubule dynamics independent of enzymatic activity¹⁹, we also used a pharmacological inhibitor of HDAC6, Tubastatin A (TubA) to increase acetylation through an orthogonal approach (**Fig 1E**). Having validated robust tools to modulate acetylation, we next determined the effect of acetylation on the dynamic microtubule pool utilizing the same fractionation assay. HDAC6 expression resulted in less acetylation in the cold-sensitive fraction while both α TAT1 expression and TubA treatment increased levels of acetylated tubulin. Neither genetic methods to increase or decrease acetylation altered the free:polymerized tubulin (**Fig 1G, S. Fig 1B**); however, TubA treatment modestly increased the free:polymerized tubulin ratio, suggesting that while microtubule dynamics are likely unaffected by changing levels of acetylation, the presence of HDAC6 protein may affect microtubule dynamics as observed previously²⁰. Given that modulating tyrosination altered levels of acetylation (**Fig 1D**), we also asked whether this relationship was reciprocal. However, whole cell levels of detyrosination were largely unaffected by modulating acetylation (**Fig 1F**), except for a modest increase with HDAC6 expression that may be related to HDAC6 association with microtubules increasing their stability and availability for detyrosination²⁰.

Figure 1. Tyrosination specifically alters microtubule stability. **A** Left, Schematic depicting de/tyrosinated microtubules (orange) buckling due to physical coupling with desmin at the Z-disc. Uncoupled tyrosinated microtubules (blue) do not buckle⁷. Right, De/tyrosination and Acetylation cycles. **B** Overview of the cell fractionation assay adapted from Fasset et al. ¹⁵ that allows for separation of free tubulin and polymerized microtubules within the dynamic tubulin pool. **C** Representative western blot (top) and quantification (bottom) of α-tubulin, de/tyrosinated (dTyr) tubulin, and GAPDH in free and cold-sensitive microtubule fractions from adult rat cardiomyocytes infected with either null, TTL, or TTL-E331Q adenovirus. Detyrosinated tubulin values normalized to α-tubulin in cold-sensitive fraction. **D** Representative western blot (top) and quantification (bottom) of α-tubulin, acetylated tubulin, and GAPDH in whole-cell lysate from null, TTL, or E331Q expressing myocytes. **E** Validation of HDAC6 and αTAT1 constructs and Tubastatin a (TubA) treatment. Representative western blot (top) and quantification (bottom) of β-tubulin, acetylated tubulin, and GAPDH in whole-cell lysate from adult rat cardiomyocytes infected with HDAC6, αTAT1, or null adenovirus or treated with 1 μM TubA or DMSO overnight. **F** Representative western blot (top) and quantification (bottom) of α-tubulin, dTyr, and GAPDH in whole cell lysate from adult rat cardiomyocytes infected with HDAC6, αTAT1, or null adenovirus or treated with 1 μM TubA or DMSO overnight. **G** Representative western blot (top) and quantification (bottom) of β-tubulin, acetylated tubulin, and GAPDH in free and polymerized dynamic fractions. Lysate from myocytes infected with HDAC6, αTAT1, or null adenovirus or treated with 1 μM TubA or DMSO overnight. Western blot data points represent technical replicates (2 from each biological replicate) normalized to their respective controls (N=4 in C+E; N=3 in D, E, G). Statistical significance determined via two-sample T test with alpha adjusted for multiple comparisons. Data are presented as mean±SEM unless otherwise specified.

Tyrosination increases catastrophes of growing microtubules

We next wanted to more precisely quantify the effects of tyrosination on the dynamics of individual microtubules. Microtubule dynamics (**Fig 2A**) are characterized by their rates of growth (polymerization) and shrinkage (depolymerization) as well as the frequency of transitions between these two states. Events are categorized as catastrophes (transitions from growth to shrinkage), rescues (transitions from shrinkage to growth), and pauses (neither growth nor shrinkage). We directly observed the microtubule plus-ends by time-lapse imaging of a fluorescent end-binding protein 3 (EB3-GFP) construct expressed via adenovirus in isolated rat cardiomyocytes (**S. Movie 1**). By creating kymographs from these videos, we were able to quantify dynamic events (rescue, catastrophe, and pause) in addition to microtubule growth and shrinkage kinetics (**Fig 2B**). We harnessed off-target EB3-GFP Z-disc localization to characterize dynamic events as occurring on or off the Z-disc, as in Drum et al.²¹ As we noted higher variability in measurements from one cell isolation to another than between cells from the same isolation, experimental data were normalized to control data that were acquired from the same animals.

We began with multiple control experiments. Although EB interaction is thought to be unaffected by microtubule detyrosination²², we wanted to validate that EB3 labeling of microtubules did not systematically differ with TTL expression. EB3 fluorescence intensity along the length and at the tip of the microtubule was unchanged in control, TTL or E331Q expressing cells (**S. Fig 2A**), indicating that EB3 expression or labeling of microtubules was not altered by our experimental interventions.

As adult ventricular cardiomyocytes are normally paced in vivo but quiescent in vitro, we sought to determine whether electrical pacing and evoked contractions would overtly alter microtubule dynamics. To this end, we timed the imaging of EB3 puncta to occur during the diastolic rest interval between each contraction of cardiomyocytes that were electrically paced at 0.5Hz. Internally controlled, pre- and post-comparisons of microtubule dynamics before and during electrical stimulation showed no significant effect of stimulation on microtubule dynamic events or growth kinetics in rat cardiomyocytes (**S. Fig 2B**). As such, for the remainder of this study we interrogated quiescent cardiomyocytes to minimize movement artifacts.

Under basal conditions, we observed a stark spatial bias in microtubule dynamic behavior, similar to that observed by Drum et al²¹. The initiation of microtubule growth, as well as pausing of growth, predominantly occurred on the Z-disc (**Fig. 2C**). Conversely, catastrophes predominantly occurred off the Z-disc, while rescue from catastrophe again occurred more frequently at the Z-disc. As exemplified in **S. Movies 1-2**, in control (AdV-null) expressing myocytes microtubules tend to grow iteratively from one Z-disk to another, often pausing at each Z-disk region. If a microtubule undergoes catastrophe before reaching a Z-disk, it tends to shrink to a previous Z-disk, where rescue is more likely to occur. These data suggest factors at the Z-disc region strongly bias microtubule behavior and support the initialization as well as stabilization of growing microtubules.

Figure 2 Tyrosinated microtubules are more dynamic **A** Schematic of microtubule dynamic events. **B** Representative kymographs from cardiomyocytes infected with EB3-GFP plus null, TTL or E331Q adenovirus. Arrowheads denote Z-discs. **C** Number of initiations, rescues, pauses, and catastrophes that occur on and off the Z-disc in isolated cardiomyocytes infected with null adenovirus. **D** Cell-wide catastrophe (left) and pause (right) frequency in myocytes infected with null, TTL, or E331Q adenovirus. **E** Number of catastrophes (left) and pauses (right) that occur on and off the Z-disc in myocytes infected with null (black), TTL (blue), or E331Q (yellow) adenovirus. **F** Gross measurements of microtubule dynamics. Tortuosity, the distance a microtubule grows divided by its displacement (left), and number of catastrophes in relation to number of successful Z-disk crossing (right), in myocytes infected with null, TTL, or E331Q adenovirus. **G** Frequency distribution of average microtubule growth displacement, duration, and velocity in myocytes infected with null, TTL, or E331Q adenovirus. Data are normalized to the average null value for the corresponding experiment. Statistical significance was determined with Kruskal-Wallis ANOVA with post-hoc test. Data are presented as mean $\pm$ SEM unless otherwise specified.

We next interrogated the role of tyrosination. As seen in **S. Movie 3**, microtubules in TTL treated cells also initiated growth at the Z-disk, but often had shorter runs and underwent catastrophe prior to reaching a subsequent Z-disk. Consistently, TTL overexpression significantly increased the frequency of catastrophes, while tending to reduce the frequency of pausing (**Fig 2B and D**). E331Q expression did not alter event frequency (**S. Movie 4**), suggesting a tyrosination-specific effect on microtubule dynamics. Further examination of spatial dynamics revealed that TTL similarly increased the number of catastrophes both on and off the Z-disc while reducing the number of pauses specifically on the Z-disc (**Fig 2E**). As a readout of inefficient growth, TTL increased microtubule tortuosity, the ratio of growth distance to growth displacement (**Fig 2F**). Combined, the lack of stabilization at the Z-disc and more frequent catastrophes resulted in tyrosinated microtubules breaking down ~4 fold as often before successfully crossing a Z-disk when compared to either null or E331Q expressing cells (**Fig 2F**). Consistent with an increased frequency of catastrophe, growth events in TTL myocytes were shorter in duration and traveled less distance, despite similar growth velocity (**Fig 2G**). Of note, cells expressing E331Q showed comparable growth metrics and event frequencies to control infected myocytes, indicating that TTL's effect on microtubule dynamics is due to tyrosination and not tubulin binding. Substantial overexpression of TTL or E331Q could reduce growth velocity by limiting tubulin available for polymerization; we calculated the predicted effect of our level of overexpression on tubulin growth velocity and found a negligible effect on growth kinetics (**S. Fig 2D**), consistent with our experimental observations (**Fig. 2G**).

Desmin structures the growing microtubule network

We hypothesized that desmin intermediate filaments could be the Z-disc stabilizing factor for growing microtubules, based in part on the gross microtubule misalignment observed in the absence of desmin²³, and from our observation that microtubules tend to traverse the Z-disc at desmin-enriched regions¹⁰. To determine if desmin regulates the dynamic population of microtubules, we utilized the previously described fractionation assay (**Fig 1B**) with lysate from rat cardiomyocytes infected with adenoviruses encoding shRNA against desmin (Des KD) or a scramble control (scram). This construct has been previously validated to reduce desmin protein levels ~70% under identical conditions²⁴. Desmin depletion resulted in an increased free to polymerized ratio in the dynamic microtubule pool (**Fig 3A, S. Fig 3A**), suggesting that desmin coordinates the stability of dynamic microtubules. We next quantified microtubule acetylation and detyrosination, markers of long-lived microtubules, and found that both were decreased in desmin depleted myocytes, without alterations in whole cell tubulin content (**Fig 3A**).

We next directly quantified plus-end microtubule dynamics by EB3-GFP upon desmin depletion. As seen in **S. Movies 5-6**, upon desmin depletion microtubule growth still initiated at the Z-disc, but the iterative, longitudinal growth from one Z-disc to another seen in control cells was lost. Instead, microtubules often grew past Z-disc regions without pausing (**Fig 3B**), and when breaking down they were less likely to be rescued at the previous Z-disc. Blind quantification of global event frequency revealed that desmin depletion modestly increased the frequency of catastrophes while more robustly reducing both the frequency of rescues and pauses (**Fig 3C**). Interrogation of where dynamic events occurred in relation to the Z-disc revealed that desmin depletion specifically increased the number of catastrophes that occurred only on the Z-disc (**Fig**

3D), while reducing the number of catastrophes that occurred off the Z-disk. More strikingly, desmin depletion markedly reduced the number of pauses and rescues that occur specifically on the Z-disk, while not affecting pause or rescue behavior elsewhere (**Fig 3D**). Growth events tended to travel longer distance with higher velocities in Des KD cells (**Fig 3E**). Together, these results indicate that desmin is spatially coordinates microtubule dynamics and stabilizes both the growing and shrinking microtubule at the Z-disk.

As the Z-disk region coordinates microtubule dynamics, we sought to summarize how our different interventions (TTL, Desmin KD) affected the spatial organization of microtubule behavior. By taking the ratio of events that occurred on vs. off the Z-disk and performing a log₂ transform, we calculated a Z-disk bias for each type of dynamic event (**Fig 3F**). Of note, this metric only reflects the spatial bias of events, not their frequencies. TTL reduced the preference for microtubule pausing at the Z-disk, but did not affect the spatial preference of rescues, catastrophes, or initiations (TTL increased catastrophe frequency in a spatially-agnostic fashion). Desmin depletion, on the other hand, virtually eliminated any spatial preference of pausing, rescue, or catastrophe behavior. Initiations had a strong Z-bias regardless of intervention, which likely reflects nucleating events from microtubule organizing centers at Golgi outposts proximal to the Z-disk that are not affected by these manipulations²⁵.

Figure 3 Desmin stabilizes the growing microtubule at the Z-disc **A** Representative western blot (left) and quantification (right) of α -tubulin, dTyr, acetylated tubulin, and GAPDH in free, cold-sensitive, or whole-cell lysate from isolated rat cardiomyocytes expressing either scramble (scrm) or desmin specific shRNA (Des KD). **B** Representative EB3-GFP kymograph from control or desmin KD myocytes. **C** Cell-wide catastrophe (left), rescue (middle), and pause (right) frequency with or without desmin KD. **D** Number of catastrophes (left), pauses (middle), and rescues (right) that occur on or off the Z-disc with or without desmin KD. **E** Frequency distribution of average microtubule growth displacement, duration, and velocity with or without desmin KD. **F** Z-disc bias score (log2 transform of the ratio of events that occurred on vs. off the Z-disc) for all experimental conditions. Data are normalized to the average control value for the corresponding experiment. Statistical significance determined with Two Sample Kolmogorov-Smirnov Test. Data are presented as mean $\pm$ SEM unless otherwise specified.

Cardiomyocytes from global, desmin germ-line knockout mice are characterized by misaligned and degenerated sarcomeres²⁶ with a disorganized microtubule network⁷. Gross restructuring of the myofilaments could affect microtubule dynamics due to a change in the physical environment that is permissive to microtubule growth, for example by increasing the spacing between Z-discs of adjacent myofilaments. To assess if our acute desmin depletion altered myofilament spacing or alignment, we performed quantitative measurements on electron micrographs from desmin depleted cardiomyocytes. Blind analysis indicated that acute desmin depletion did not change myofilament spacing or alignment at this level of resolution (**S Fig 3B**), consistent instead with a direct stabilizing effect of desmin intermediate filaments on the microtubule network.

We next wanted to visualize the effect of desmin depletion on whole-cell microtubule network organization. Airyscan imaging and blind image quantification indicated a reduction of the percent cell area occupied by both detyrosinated and tyrosinated microtubules (**Fig 4A-B**), consistent with reduced stability of microtubules upon desmin depletion. As a reduction in detyrosinated microtubules and their association with the Z-disc is associated with reduced cardiomyocyte viscoelasticity⁷, we hypothesized that desmin-depleted myocytes would be less stiff. To test this, we performed transverse nanoindentation of cardiomyocytes and quantified Young's modulus of the myocyte over a range of indentation rates. Desmin depletion specifically reduced the rate-dependent (viscoelastic) stiffness of the myocyte (**Fig 4C**), without significantly altering rate-independent (elastic) stiffness. Reduced viscoelasticity is consistent with reduced transient interactions between dynamic cytoskeletal filaments. To directly test if the

326 reduction in desmin alters microtubule buckling between sarcomeres, we performed a semi-
 327 automated, blind analysis of microtubule buckling, as in our previous work⁷. In control cells,
 328 most microtubules buckle in a clear sinusoidal pattern with a wavelength corresponding to the
 329 distance of a contracted sarcomere (~ 1.5 - $1.9 \mu\text{m}$) (**Fig 4D+G, S Movie 7**). Upon desmin
 330 depletion, fewer polymerized microtubules were observed in general, with more chaotic
 331 deformations and organization upon contraction (**S Movie 8**). For microtubules that did buckle,
 332 the amplitude of buckles was reduced (**Fig. 4E**), as well as the proportion of microtubules that
 333 buckled at wavelengths corresponding to the distance between 1 or 2 sarcomeres (1.5 - 1.9 or 3.0 -
 334 $3.8 \mu\text{m}$, respectively, **Fig 4F**). Combined, these results are consistent with desmin coordinating
 335 the physical tethering and lateral reinforcement of detyrosinated microtubules at the
 336 cardiomyocyte Z-disk to regulate myocyte viscoelasticity.

Figure 4 Desmin facilitates the microtubule contribution to viscoelastic resistance **A.** Representative Airyscan images of desmin (magenta), detyrosinated tubulin (orange), and tyrosinated tubulin (cyan) in isolated adult rat cardiomyocytes with or without desmin KD. **B** Quantification of percent cell area coverage of detyrosinated microtubules and tyrosinated microtubules with or without desmin KD. **C** Nanoindentation measurement of cardiomyocyte viscoelasticity. Left, Stiffness (elastic modulus) as a function of probe indentation velocity with or without desmin KD. Right, quantification of rate-dependent (viscoelastic) and independent (elastic) stiffness. **D** Representative images of microtubules in a cardiomyocyte at rest (top) and at the peak of contraction (bottom) with or without desmin KD. **E** Quantification of microtubule buckle amplitude. **F** Left, histogram of wavelength distribution of individual microtubule buckles. Right, distribution difference (scram – des KD from **G**). Statistical significance determined via 2-sided t test.

Discussion

In this paper we identify that 1) the dynamic, catastrophe-prone nature of tyrosinated microtubules precludes their ability to faithfully grow to subsequent Z-discs; and 2) desmin intermediate filaments structure and stabilize the growing microtubule network.

This study represents the first direct observation that tyrosination increases the dynamics of cardiac microtubules. Increased dynamicity seems unlikely to arise from the PTM itself,²⁷ as the C-terminal tyrosine is not believed to be critical involved in the formation of lateral contacts with other tubulin dimers in the microtubule lattice. Yet, removal of this large hydrophobic residue and subsequent exposure of acidic residues will alter hydrophobic and electrostatic interactions on the outer surface of the polymerized microtubule. Through such a mechanism, tyrosination can promote microtubule dynamics via increased interaction with destabilizing MAPs, or through decreased interaction with stabilizing MAPs^{27,28}.

For example, detyrosination reduces the affinity of the depolymerizing kinesin MCAK for the microtubule, increasing microtubule stability⁵. We hypothesized that increased MCAK affinity may contribute to the increased rate of catastrophe we observed in tyrosinated microtubules; yet due to its low abundance in the cardiomyocyte, our attempts to detect and knock down MCAK levels were unreliable, precluding the immediate test of this hypothesis. The low abundance of MCAK, while not ruling out a physiological rule, also motivates interrogation into alternative stabilizing or destabilizing effector proteins.

Tyrosination status is also known to impact the recruitment of plus-end tip proteins (+Tips), such as CLIP-170 and p150^{glued}²², which may tune microtubule dynamics through either direct or indirect effects. +Tip proteins also serve to couple the growing microtubule plus end with cellular effectors²⁹. In the search and capture mechanism, dynamic microtubules ‘search’ for sites on the plasma membrane, chromosomes, and organelles to ‘capture’ via +Tip proteins³⁰. The interaction of microtubules with cellular structures, such as chromosomes or the cell cortex, has been shown to promote rescue events through the formation of tension at the plus-end³¹. The expression and localization of these proteins has been unexplored in the cardiomyocyte and represents a potential mechanism for recognition of detyrosination- or intermediate filament-dependent capture sites and the formation of durable detyrosinated microtubules.

We also identified that the intermediate filament desmin provides structure to the growing microtubule network by stabilizing microtubules at the Z-disc. What is the mechanism of desmin-dependent stabilization? A recent elegant *in vitro* study using reconstituted vimentin intermediate filaments and microtubules indicates that intermediate filaments are sufficient to stabilize growing microtubules through electrostatic and hydrophobic interactions¹³. The interaction with intermediate filaments reduces catastrophes and promotes rescues, in strong accordance with our *in cellulo* findings here. While MAPs may also be involved in modulating microtubule-intermediate filament interactions, this direct effect is sufficient to explain the primary phenotypes observed upon desmin depletion (i.e. increased catastrophes and reduced pausing in the absence of desmin at the Z-disk, and a loss of Z-disk rescues).

The intermediate filament stabilization of growing microtubules would then provide a longer-lived microtubule substrate to facilitate reinforcing, detyrosination-dependent interactions, such as those previously documented between desmin and the microtubule through intermediates such as Kinesin-1³² or members of the plakin family of scaffolding proteins³³. Desmin-mediated interaction along the length of the microtubule may also lead to the loss of tubulin dimers at sites of contact; these lattice defects are replaced by GTP-tubulin, which upon microtubule catastrophe may function as a rescue sites³⁴. Lateral interactions between microtubules and intermediate filaments govern microtubule mechanical behavior upon compressive loading of microtubules⁹ allowing desmin to orchestrate microtubule buckling in the cardiomyocyte. ³⁴

Combined with past and current work, we propose a unifying model for microtubule-intermediate filament interactions in the cardiomyocyte and how they contribute to myocardial mechanics. Detyrosinated microtubules, with less frequent depolymerization, experience more chance interactions with intermediate filaments at the Z-disc. The altered surface chemistry of detyrosinated microtubules may also strengthen the electrostatic interactions with intermediate filaments and additional cross-linking proteins. The periodic, lateral reinforcement of microtubules increases their stability, leading to longer-lived microtubules and providing a dynamic cross-link with the sarcomere, increasing the viscoelastic resistance to myocyte motion and the ability of microtubules to transduce mechanical stress. Increased microtubule lifetimes also promote microtubule acetylation, which itself increases the ability of microtubules to withstand mechanical stress³⁵ and increases myocyte viscoelasticity³⁶. In the setting of heart disease, the increased abundance of both desmin intermediate filaments and detyrosinated

microtubules thus promotes a feed-forward substrate for enhanced mechanotransduction and myocardial stiffening.

Methods

Animals

Animal care and procedures were approved and performed in accordance with the standards set forth by the University of Pennsylvania Institutional Animal Care and Use Committee and the Guide for the Care and Use of Laboratory Animals published by the US National institutes of Health.

Rat cardiomyocyte isolation and culture

Primary adult ventricular myocytes were isolated from 6- to 8-week-old Sprague Dawley rats as previously described³⁷. Briefly, rats were anesthetized under isoflurane while the heart was removed and retrograde perfused on a Lutgendorf apparatus with a collagenase solution. The digested heart was then minced and triturated using a glass pipette. The resulting supernatant was separated and centrifuged at 300 revolutions per minute to isolate cardiomyocytes that were resuspended in rat cardiomyocyte media at a density that ensured adjacent cardiomyocytes did not touch. Cardiomyocytes were cultured at 37°C and 5% CO₂ with 25 µmol/L of cytochalasin D. The viability of rat cardiomyocytes upon isolation was typically on the order of 50-75% rod-shaped, electrically excitable cells, and the survivability for 48hrs of culture is >80% (See Heffler et al.²⁴ for our quantification of cardiomyocyte morphology in culture).

439

440 Rat cardiomyocyte media: medium 199 (Thermo Fisher 115090) supplemented with 1x Insulin-
441 transferrin-selenium-X (Gibco 51500056), 1 $\mu\text{g } \mu\text{l}^{-1}$ primocin (Invivogen ant-pm-1), 20 mmol/L
442 HEPES at pH 7.4 and 25 $\mu\text{mol/L}$ cytochalasin D.

443

444 *Fractionation assay of free tubulin and cold-sensitive microtubules*

445 Free tubulin was separated from cold-labile microtubules using a protocol adapted from Tsutusi
446 et al., 1993 and Ostlud et al., 1979. Isolated rat cardiomyocytes were washed once with PBS and
447 homogenized with 250 μl of microtubule stabilizing buffer using a tissue homogenizer. The
448 homogenate was centrifuged at 100,000 $\times g$ for 15 minutes at 25°C and the resulting supernatant
449 was stored at -80°C as the free tubulin fraction. The pellet was resuspended in ice-cold
450 microtubule destabilizing buffer and incubated at 0°C for 1 hour. After centrifugation at 100,000
451 $\times g$ for 15 minutes at 4°C the supernatant containing the cold-labile microtubule fraction was
452 stored at -80°C.

453

454 Microtubule stabilizing buffer: 0.5 mM MgCl_2 , 0.5 mM EGTA, 10mM Na_3PO_4 , 0.5 mM GTP,
455 and 1X protease and phosphatase inhibitor cocktail (Cell Signaling #5872S) at pH 6.95
456 Microtubule destabilizing buffer: 0.25 M sucrose, 0.5 mM MgCl_2 10 mM Na_3PO_4 , 0.5 mM GTP,
457 and 1X protease and phosphatase inhibitor cocktail (Cell Signaling #5872S) at pH 6.95

458

459 *Western blot*

460 For whole cell protein extraction, isolated rat cardiomyocytes were lysed in RIPA buffer
461 (Cayman #10010263) supplemented with protease and phosphatase Inhibitor cocktail (Cell

Signaling #5872S) on ice for 1 hour. The supernatant was collected and combined with 4X loading dye (Li-COR #928-40004), supplemented with 10% 2-mercaptoethanol, and boiled for 10 minutes. The resulting lysate was resolved on SDS-PAGE gel and protein was blotted to nitrocellulose membrane (Li-COR #926-31902) with mini Trans-Blot Cell (Bio-Rad). Membranes were blocked for an hour in Odyssey Blocking Buffer (TBS) (LI-COR #927-50000) and probed with corresponding primary antibodies overnight at 4 °C. Membranes were rinsed with TBS containing 0.5% Tween 20 (TBST) three times and incubated with secondary antibodies TBS supplemented with extra 0.2% Tween 20 for 1 hour at room temperature. Membranes were washed again with TBST (0.5% Tween 20) and imaged on an Odyssey Imager. Image analysis was performed using Image Studio Lite software (LI-COR). All samples were run in duplicates and analyzed in reference to GAPDH.

Antibodies and labels

Acetylated tubulin; mouse monoclonal (Sigma T6793-100UL); western blot: 1: 1,000
Detyrosinated tubulin; rabbit polyclonal (Abcam ab48389); western blot: 1: 1,000
Alpha tubulin; mouse monoclonal, clone DM1A (Cell Signaling #3873); western blot: 1:1,000
Beta tubulin; rabbit polyclonal (Abcam ab6046); western blot: 1,000
Tyrosinated tubulin; mouse monoclonal (Sigma T9028-.2ML); Immunofluorescence: 1:1000
Desmin; rabbit polyclonal (ThermoFisher PA5-16705); western blot and immunofluorescence: 1: 1,000
GAPDH; mouse monoclonal (VWR GenScript A01622-40); western blot: 1:1,000
Goat anti-mouse AF 488 (1:1000 Life Technologies A11001)
Goat anti-rabbit AF 565 (1:1000 Life Technologies A11011)

IRDye 680RD Donkey anti-Mouse IgG (H + L) (LI-COR 926-68072); western blot: 1:10,000.

IRDye 800CW Donkey anti-Rabbit IgG (H + L) (LI-COR 926-32213); western blot: 1:10,000.

Microtubule Dynamics by EB3

Isolated rat cardiomyocytes were infected with an adenovirus containing an EB3-GFP construct.

After 48 hours, cells were imaged on an LSM Zeiss 880 inverted confocal microscope using a

40X oil 1.4 numerical aperture objective. Cells expressing EB3-GFP only at the tip were imaged

for four minutes at a rate of 1fps. Files were blinded, Gaussian blurred, and Z-compressed using

Image J (National Institutes of Health) to generate kymographs. The number of catastrophes,

rescues, and pauses were recorded per kymograph in addition to manual tracing of microtubule

runs to quantify time, distance, and velocity of microtubule growth or shrinkage. We refer to the

entire kymograph as the microtubule ‘track’ that is made up of individual growth and shrinkage

events we call ‘runs’. Catastrophe and rescue frequency were calculated per cell by dividing the

number of catastrophes or rescues by total time spent in growth or shrinkage time, respectively.

Catastrophes and rescues occurring specifically on or off the Z-disc were normalized by the total

time of microtubule growth and shrinkage. Experimental values were normalized to their

respective control cells (Null for TTL and E331Q, or shScrm for shDes) acquired from the same

animals. A minimum of 3 separate cell isolations were performed for each group.

Immunofluorescence

Cells were fixed in pre-chilled 100% methanol for 8 minutes at -20°C. Cells were washed 4x

then blocked with Sea Block Blocking Buffer (abcam #166951) for at least 1 hour followed by

antibody incubation for 24-48 hours. Incubation was followed by washing 3x with Sea Block,

then incubated with secondary antibody for 1 hour. Fixed cells were mounted using Prolong Diamond (Thermo #P36961)

We used ImageJ to calculate the percent area fraction of both desmin and microtubules. An ROI was drawn to include the entire cell boundary minus the nucleus. For each image we identified the percent fractional coverage of a fluorescence signal over a manually identified threshold as described previously¹⁰.

Buckling analysis

Adult rat cardiomyocytes were isolated as previously described and infected with adenovirus carrying the microtubule-binding protein EMTB chimerically fused to 3 copies of GFP. The purpose of this construct was to label microtubules fluorescently for imaging. The cells were allowed 48 hours to express the construct. All cells chosen were those that contained sufficient brightness and contrast to observe microtubule elements and where the health of the myocyte was not compromised. To interrogate microtubule buckling amplitude and wavelength, cells were induced to contract at 1 Hz 25 V and imaged during the contraction. For analysis, images were blinded, and a microtubule was located that could be followed during the contraction. The backbone was manually traced at rest and during its peak of contraction and the ROI was saved. The ROI was then analyzed using a macro that rotated so that the ROI had the peak of contraction 90 degrees to the axis of contraction to protect from aliasing errors. The program then calculated the distance between the axis of the ROI and its peak and calculated the peak (amplitude) and the width (half wavelength).

Electron Microscopy

Transmission electron microscopy images were collected as previously described²⁴. Images at 7500x were rotated so the cells were parallel to the longitudinal axis. ROIs were generated between adjacent Z-discs to quantify sarcomere spacing and the angle relative to 90°.

Statistics

Statistical analysis was performed using OriginPro (Version 2018). Normality was determined by Shapiro-Wilk test. For normally distributed data, two sample t-test or one-way ANOVA was utilized as appropriate. For non-normally distributed data, two sample Kolmogorov-Smirnov test or Kruskal-Wallis ANOVA was utilized as appropriate. Specific statistical tests and information of biological and technical replicates can be found in the figure legends. Unless otherwise noted, ‘N’ indicates the number of cells analyzed and ‘n’ indicates number of microtubule runs.

S. Figure 1. Supplemental figures associated with figure 1. A Representative western blot (top) and quantification (bottom) for α-tubulin, dTyr tubulin, and GAPDH in free and cold-sensitive fractions from rat cardiomyocytes infected with null, TTL, or E331Q adenovirus. **B** Representative western blot (top) and quantification (bottom) for α-tubulin, dTyr tubulin, and GAPDH in free and cold-sensitive fractions from rat cardiomyocytes either infected with null, HDAC, or ATAT1 adenovirus or treated with DMSO or TubA. Statistical significance determined via ANOVA with post hoc Bonferroni test.

S. Figure 2. Supplemental figures associated with figure 2. **A** Background (left) EB3-GFP fluorescence intensity, EB3 tip fluorescence intensity (center) and the ratio of EB3 tip fluorescence intensity to microtubule EB3 intensity (right) in isolated rat cardiomyocytes co-infected with null, TTL, or E331Q adenovirus. Statistical significance determined via ANOVA with post hoc Bonferroni test. **B** Representative EB3-GFP kymograph from isolated rat cardiomyocyte before and after 0.5 Hz electrical stimulation. **C** Quantification of catastrophe frequency, rescue frequency, microtubule growth displacement and growth velocity before and after electrical stimulation. **D** To reduce levels of detyrosination in this study we relied on overexpression of TTL, which is known to associate with the free α/β tubulin heterodimer in a 1:1 complex with a K_d of $1 \mu M$ ³⁸. Due to the nature of this interaction, TTL overexpression can decrease the amount of “polymerization-competent” tubulin, leading to a decrease of *in vitro* microtubule polymerization³⁸. The relationship between free tubulin concentration and microtubule polymerization is described as a simple 1D model below, where v_g is the velocity of microtubule growth, d is the dimer length (assumed to be 8 nm), k_{on} is the tubulin dimer association constant per protofilament (assumed to be $4.8 \mu M^{-1}s^{-1}$)³⁰, k_{off} is the tubulin dissociation rate constant per protofilament (assumed to be $15s^{-1}$)³⁰, and $[Tb]$ is the concentration of free tubulin³⁹.

$$v_g = d(k_{on}[Tb] - k_{off}).$$

Solving for the equation of the line at a physiologic tubulin concentration of $5 \mu M$ provides a theoretical value of $4.32 \mu m s^{-1}$ for growth velocity. Using the K_d value for TTL-tubulin interaction at a tubulin concentration of $5 \mu M$ suggests that an 8-fold overexpression of TTL¹⁴ will produce $0.2 \mu M$ of TTL-tubulin complex. If we assume that tubulin bound to TTL cannot be polymerized, then only $4.8 \mu M$ of free tubulin is polymerization-competent at any time. This change in polymerization-competent tubulin would lead to $\sim 10\%$ decrease in growth velocity. We did not observe an effect of E331Q on microtubule growth kinetics or on event frequency suggesting that the local tubulin concentration at the microtubule plus-tip is unaffected by the sequestration activity of TTL. Statistical significance determined via 2-sided t test.

S. Figure 3. Supplemental figures associated with figure 3. A Representative western blot (**top**) and quantification (**bottom**) for α -tubulin and GAPDH in free and cold-sensitive fractions from rat cardiomyocytes infected with adenovirus containing scramble shRNA (scrm) or shRNA for desmin (Des KD). **B** Representative electron microscopy images of isolated rat cardiomyocytes with or without desmin knockdown. **C** Probability density plot of angle (**top**) and distance (**bottom**) between adjacent Z-disks with or without desmin knockdown.

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Competing interests

The authors declare no competing interests.

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