Minireview

Cardiac microtubules in health and heart disease

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Impact statement

Advancements in cell biological and biophysical approaches and super-resolution imaging have greatly broadened our view of tubulin biology over the last decade. In the heart, microtubules and microtubule-based transport help to organize and maintain key structures within the cardiomyocyte, including the sarcomere, intercalated disc, protein clearance machinery and transverse-tubule and sarcoplasmic reticulum membranes. It has become increasingly clear that post translational regulation of microtubules is a key determinant of their sub-cellular functionality. Alterations in microtubule network density, stability, and post-translational modifications are hallmarks of pathological cardiac remodeling, and modified microtubules can directly impede cardiomyocyte contractile function in various forms of heart disease. This review summarizes the functional roles and multi-leveled regulation of the cardiac microtubule cytoskeleton and highlights how refined experimental techniques are shedding mechanistic clarity on the regionally specified roles of microtubules in cardiac physiology and pathophysiology.

Abstract

Cardiomyocytes are large ($\sim$40,000 μm^3), rod-shaped muscle cells that provide the working force behind each heartbeat. These highly structured cells are packed with dense cytoskeletal networks that can be divided into two groups—the contractile (i.e. sarcomeric) cytoskeleton that consists of filamentous actin-myosin arrays organized into myofibrils, and the non-sarcomeric cytoskeleton, which is composed of β- and γ-actin, microtubules, and intermediate filaments. Together, microtubules and intermediate filaments form a cross-linked scaffold, and these networks are responsible for the delivery of intracellular cargo, the transmission of mechanical signals, the shaping of membrane systems, and the organization of myofibrils and organelles. Microtubules are extensively altered as part of both adaptive and pathological cardiac remodeling, which has diverse ramifications for the structure and function of the cardiomyocyte. In heart failure, the proliferation and post-translational modification of the microtubule network is linked to a number of maladaptive processes, including the mechanical impediment of cardiomyocyte contraction and relaxation. This raises the possibility that reversing microtubule alterations could improve cardiac performance, yet therapeutic efforts will strongly benefit from a deeper understanding of basic microtubule biology in the heart. The aim of this review is to summarize the known physiological roles of the cardiomyocyte microtubule network, the consequences of its pathological remodeling, and to highlight the open and intriguing questions regarding cardiac microtubules.

Keywords: Microtubule, cytoskeleton, myocytes, heart, heart failure, post-translational modification

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Overview—The cardiomyocyte cytoskeleton

Cardiomyocytes are the cellular working units of the heart and utilize coordinated force generation to contract the ventricles and power blood-flow through the circulatory system. Within the myocyte, densely packed myofibrils consisting of actin and myosin (and regulatory and structural proteins like troponin and titin) comprise the sarcomeric cytoskeleton, which converts chemical energy into mechanical work by adenosine triphosphate (ATP) hydrolysis. Since sarcomeres drive contraction, the structural "health" of the sarcomere is integral for cardiac function; and as cardiac myocytes are extremely long-lived (decades[1,2]), maintaining sarcomere homeostasis presents a tall order to the cell. This order is carried out by a number of specialized support systems that are largely coordinated by the non-sarcomeric cytoskeleton.

Sarcomeric proteins must be readily recycled and replaced, which requires the effective delivery of mRNA and protein, as well as the clearance and degradation of aged constituents. Mitochondria, which provide the ATP

to fuel contraction, must be properly positioned and maintained. The transverse tubule (T-tubule) and sarcoplasmic reticulum (SR) membranes must be organized in close apposition to one another to support excitation–contraction (E-C) coupling. Inter-myocyte connections that mediate synchronous contraction must be closely coordinated via cell–cell junctions at the intercalated disc. The proper positioning, regulation, and turnover of each of these components is facilitated by the meshwork of microtubules and intermediate filaments making up the non-sarcomeric cytoskeleton. Microtubules deliver ion channels, translational machinery and facilitate the clearance of misfolded proteins by orchestrating their sorting and delivery to distinct subcellular locales. Scaffolding for the myofilaments and positioning of organelles within the myocyte is also mediated by the non-sarcomeric cytoskeleton. As it forms an interconnected lattice throughout the cell, the non-sarcomeric cytoskeleton is well positioned to sense and transmit mechanical signals[3–5] that enable the heart to regulate gene transcription and adapt to changing demands. Furthermore, microtubules are deformed during contraction and can directly regulate contractility by mechanically impeding myocyte shortening. Despite the many known functions of the microtubule cytoskeleton, there are even more unanswered questions regarding its role in myocyte biology. In this review we will focus on the microtubules as a major component of the non-sarcomeric cytoskeleton; for a recent review of other elements, please see Grimes *et al.*[6]

Microtubules—What they are, what they do

Microtubules are hollow, 25 nm diameter tubes formed by the polymerization of α/β-tubulin dimers. They can run tens of microns in length within the cell, and are the stiffest of the cytoskeletal filaments—microtubules have a persistence length ~2–3 orders of magnitude greater than actin filaments,[7] which are in turn an order of magnitude stiffer than intermediate filaments.[8] Microtubules can be "dynamic," undergoing rounds of growth (polymerization) and shrinkage (catastrophe), or "stable," meaning they present as fixed structures over a time scale of minutes to hours.[9] The canonical functions of microtubules are to provide cellular structural support, enable chromosome movement during cell division, and serve as the tracks by which cargo is transported throughout the cell.[10] Cargo transport is achieved via the microtubule motor proteins kinesin and dynein, with kinesin trafficking cargo towards the growing, plus end of a microtubule, and dynein moving in the opposite, retrograde direction.

Microtubule populations in the heart

The cardiomyocyte microtubule network can be spatially separated into three distinct (yet overlapping) populations. Early characterization of microtubules in cardiac muscle by electron microscopy identified interfibrillar microtubules running parallel to and in close association with myofibrils and mitochondria, as well as an additional population encircling the nucleus (Figure 1).[11] Later, cortical microtubules, which wrap around the cardiomyocyte perpendicular to myofibrils, were implicated in the mechanotransduction of external cues and in the regulation of transmembrane proteins and ion channels.[12,13] Interfibrillar microtubules are involved in organelle positioning, T-tubule and SR membrane regulation,[5,14,15] maintenance of the intercalated disc,[16] and regulation of myofilament mechanics.[17–19] Perinuclear microtubules encircle the nucleus (or nuclei) of the cardiomyocyte, organize perinuclear organelles, and directly interact with the LINC (Linker of Nucleoskeleton and Cytoskeleton) complex to mechanically couple the microtubule cytoskeleton with the nucleoskeleton.[20–22] A dense highway of microtubules is typically observed spanning between nuclei in cardiomyocytes (Figure 1) but little is known about any unique functionality of this structure. Cortical microtubules, interfibrillar microtubules, and perinuclear microtubules are spatially distinct, vary in stability, post-translational profile and function, but utilize much of the same microtubule-associated machinery to conduct their tasks.

Microtubules in the failing heart

Consistent with the diverse roles played by the non-sarcomeric cytoskeleton in the myocyte, numerous and impactful modifications to the non-sarcomeric cytoskeleton are observed in heart failure. The density of the microtubule and intermediate filament network increases significantly in end-stage heart failure of diverse etiology[23,24] with an accompanying decrease in sarcomeric protein density.[23,25,26] There is an accumulation of microtubule post-translational modifications (PTMs) in heart failure that alter the microtubule interactome to likely favor microtubule stabilization by facilitating interactions with microtubule associated proteins (MAPs) and intermediate filaments.[17,23,27,28] These cumulative alterations in network density, dynamics, and binding partners interfere with the physiological roles of the microtubule (through incompletely understood mechanisms), affecting E-C coupling, intercalated disc maintenance, myofilament contractility, and proteostasis. As such, treatments to restore the density or dynamics of cardiac microtubules could provide benefit to a number of the maladaptive processes observed in cardiac pathology, but the role of microtubules must be better defined in order to inform specific interventions. Below we will break down the role of microtubules within the cardiomyocyte, and highlight how they are altered in and may contribute to heart disease.

Microtubules and tubulin turnover

In the healthy myocyte, roughly 50% of tubulin is in its free, soluble form, while the remaining half is incorporated into polymerized microtubules.[29–31] The half-life of a microtubule can vary greatly based on its interactions with stabilizing factors, but is on the order of minutes to hours,[32] while the lifetime of free tubulin is on the order of 1–2 days.[33,34] Compared to other cell types, microtubules in mature cardiomyocytes appear particularly stable,[35] which can be assessed using nocodazole, a drug that

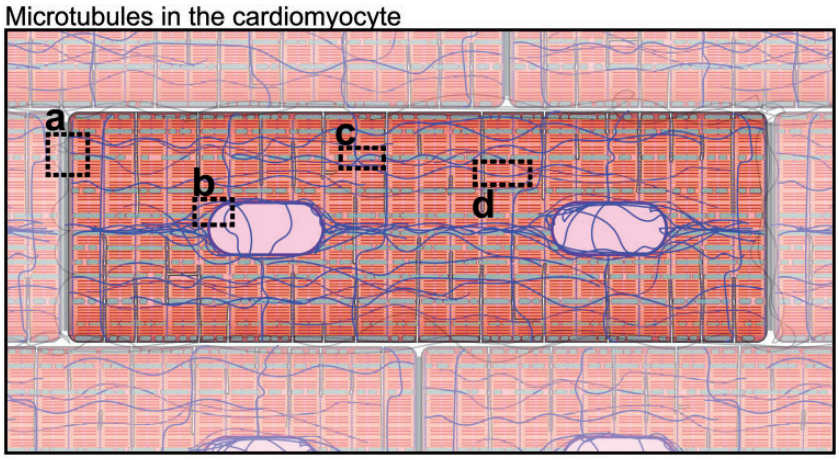

Figure 1. Microtubules in the cardiomyocyte. (a) Intercalated disc region. (b) Nuclear region. (c) Mitochondrial associated microtubules. (d) Microtubules at the dyad/Z-disc. Microtubules (blue), sarcomeric cytoskeleton (red/orange), mitochondria (green), nuclei (light purple). (A color version of this figure is available in the online journal.)

binds free tubulin and prevents it from incorporating into the microtubule lattice.[36] Thus, the rate at which the microtubule lattice disintegrates upon nocodazole treatment is an indirect assessment of microtubule stability.[37,38] Nanomolar concentrations of nocodazole reduce microtubule dynamicity in most cell types,[39] including immature cardiac cells, while higher concentrations are required to depolymerize microtubules in adult cardiomyocytes.[37,38] In adult ventricular myocytes the most stable microtubules are largely concentrated around the myocyte nuclei, and remain after nocodazole or colchicine treatment sufficient to eliminate most other microtubules (Belmadani *et al.*[38] and our unpublished observations).

Further stabilization of the microtubule network has been demonstrated in patient tissue and various animal models of cardiac hypertrophy and heart failure (cat pulmonary artery banding[19,37]; dog left ventricular pressure overload[40,41]; mouse transverse aortic constriction[30,42,43]; human patients[23,24]; see review[31]). Pressure-overload induced hypertrophy stabilizes cardiac microtubules, as inferred from increased resistance to nocodazole treatment[37] and increased tubulin content in insoluble fractions.[30,42,43] In patients with end-stage heart failure of ischemic, dilated, or hypertrophic origin, markers of stable microtubules are increased, including PTMs, stabilizing MAPs, and a greater increase in polymerized microtubule density than total tubulin content (Figure 2(b) to (f)).[23,24] Microtubule stabilization likely occurs early on in disease progression, as indicated by a rapid rise of tubulin levels and stabilizing MAPs in pressure overload.[27,28,37,44,45] Elevated tubulin levels also persists into disease progression, remaining six months following pulmonary artery banding.[44] Significant work is still needed to clarify the temporal progression of microtubule stabilization during the onset, progression, and reversion of discrete myopathic etiologies—particularly of those independent of significant pressure-overload—as well as to establish causality between microtubule stabilization and hypertrophy or decompensation.

Microtubule post-translational modifications

Microtubule turnover is highly affected by the stability, rigidity and dynamics of the network, which is regulated by a combination of the "tubulin code" and MAPs. The "tubulin code" encompasses the diversity of tubulin isoforms and PTMs that can combine in numerous ways to confer unique functionality to subsets of microtubules within the cell.[46–49]

Typically, the longer lived a microtubule, the more post-translationally modified it becomes. However, the increased PTM of microtubules observed in disease could both *arise from*, as well as *confer* increased stability. For example, stable, long-lasting microtubules provide a substrate for luminal acetylation, but acetylation itself also can prolong the lifetime of a microtubule.[50] Acetylation occurs within the microtubule lumen by αTAT1, while the tubulin deacetylase HDAC6 predominantly acts on free tubulin (Figure 2(a)).[51,52] Highly acetylated microtubules exhibit increased flexural compliance and decreased damage under repetitive bending cycles,[53] which can increase their lifetime in the face of mechanical stress. This may be important for maintaining network integrity in the heart, where microtubules are observed to buckle during each contraction.[17] If we assume a 60min lifetime for a stable microtubule, this would equate to about 3600 bending cycles per cardiac microtubule in a resting human, and 36,000 bending cycles/microtubule in a mouse. It remains to be shown whether cyclic buckling fatigues or "breaks" microtubules in a myocyte the way repetitive bending does *in vitro*,[54] and whether damaged microtubules repair themselves to maintain network integrity.

The most well studied PTM of the microtubule network in the heart is the C-terminal detyrosination of α-tubulin. The buckling, load-bearing behavior of microtubules is promoted by detyrosination,[17] and detyrosination directly increases myocyte viscoelastic stiffness (as expanded upon below) and contributes to contractile dysfunction in cardiomyocytes from patients with heart failure.[17,23] Regulation of the "tyrosination cycle" occurs on free and

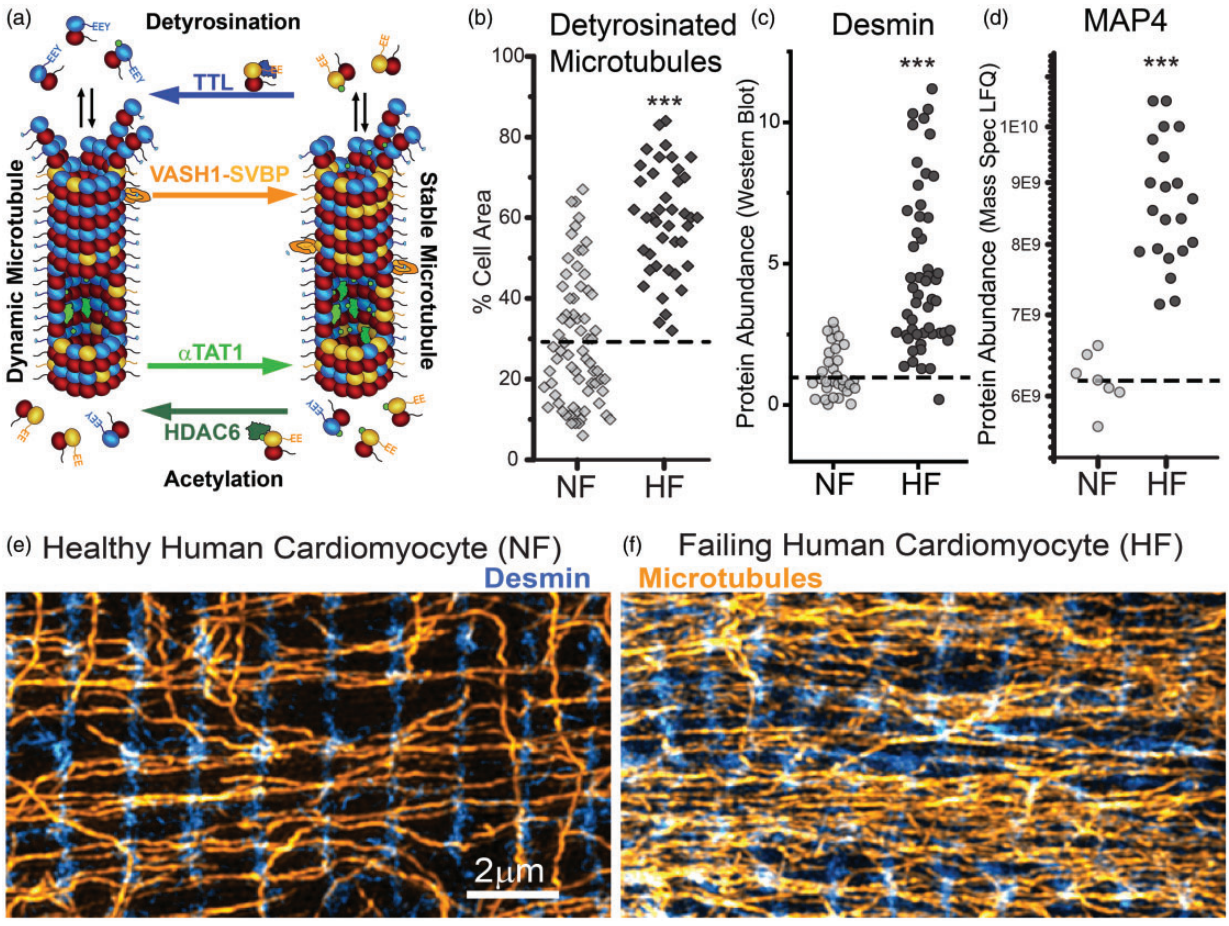

Figure 2. Microtubule network stabilization in heart failure. (a) Schematic of C-terminal detyrosination and luminal acetylation. (b to d) Microtubule detyrosination, desmin, and MAP4 are consistently increased in human heart failure (modified from Chen *et al.*[23]) (e and f) Immunofluorescence. Microtubules intersect with desmin intermediate filaments at the Z-disc of healthy and failing human cardiomyocytes. (A color version of this figure is available in the online journal.)

polymerized tubulin, with polymerized tubulin as the primary substrate for detyrosination by vasohibin enzymes or other carboxypeptidases,[55,56] and free tubulin as the primary target for tyrosination by tubulin tyrosine ligase (TTL) (Figure 2(a)).[57] TUBA4A is the only tubulin isotype translated in its detyrosinated form,[58] and interestingly is one of the most highly expressed isotypes in the heart.[23] Thus, total levels of detyrosination may reflect a combination of TUBA4A synthesis and PTM of other tubulin isoforms. Microtubule detyrosination is also associated with enhanced microtubule stability,[32,38] which may be conferred by promoting interactions with intermediate filaments[59,60] such as desmin.[17] Increased microtubule detyrosination is observed in end stage human heart failure and is sufficient to increase myocyte viscoelasticity, while reducing detyrosinated tubulin in cardiomyocytes from failing hearts is sufficient to reduce myocyte stiffness and improve contractility.[17,30]

Other common tubulin PTMs, such as glutamylation and glycylation, are receiving emerging attention for their important regulatory roles in neuronal biology. For example, glutamylation can promote microtubule severing by enzymes such as katanin and spastin to regulate network density,[61,62] and aberrant glutamylation perturbs neuronal transport and can cause neurodegeneration.[63,64] The role of these PTMs has not been examined in the heart to our knowledge, but as regulators of network stability, organization, and MAP association, they warrant further investigation, which will benefit from the generation of mouse models with cardiac-specific manipulation of tubulin modifying enzymes.

Microtubule associated proteins

There are two primary classes of MAPs: molecular motors and structural MAPs.[46,65–67] Two families of microtubule-based motors, kinesin and dynein, drive anterograde and retrograde cargo transport respectively. Structural MAPs (e.g. tau, MAP4, dynactin, end-binding protein 1 (EB1)) can influence the stability of microtubules either by blocking or recruiting severing proteins or by affecting the association of microtubules with other cytoskeletal elements including the sarcomeres.[27] MAPs interact with each other, either cooperatively or competitively, and the affinity of MAPs for microtubules is strongly impacted by PTMs.

The balance of intracellular transport can be shifted by PTM of the microtubule and the binding of structural MAPs. While the interactions between different kinesins and detyrosinated microtubules have not been exhaustively explored, detyrosination tends to enhance kinesin

recruitment to the microtubule or its processivity[68–70] while highly processive dynein complexes are recruited to tyrosinated microtubules.[71] Thus, the tyrosination state of the microtubule lattice influences the balance between retrograde (dynein-driven) and anterograde (kinesin-driven) transport, which can have important ramifications for the cell. For example, dynein-mediated retrograde transport is required for autophagy-mediated protein recycling,[72] while delivery of cytosolic cargo[70] and export of endocytic machinery to the cell surface[73] is primarily kinesin driven. Proper patterning of microtubule tyrosination is also necessary for long-range transport, as has been demonstrated in neurons where an intact tyrosination cycle is required for neuronal differentiation and organization.[56,74] Furthermore, structural MAPs along the microtubule can also influence the balance of motor-based transport. For example, MAP7 promotes kinesin-based transport while tau inhibits it, but neither impact dynein motility.[65] In sum, these examples demonstrate the multiplexed ability to tune microtubule transport via various combinations of PTMs, MAPs, and tubulin isotypes.

While the aforementioned phenomena have largely been characterized in neurons, many of the same MAPs are expressed in the heart. One of the most highly expressed structural MAPs in the heart, MAP4,[23,37] is involved in both pathological densification of the microtubule network[27,28,43] and inhibition of microtubule-based transport.[75] An increase in MAP4 expression is observed in heart disease,[37] and cardiac-specific overexpression of MAP4 is sufficient to increase tubulin expression and microtubule stability.[76] This increase in network density and stability may also come at the cost of jamming motor-based transport with excessive MAP4 decoration of the microtubules, leading to defective cargo delivery throughout the myocyte.[75,77]

Phosphorylation of MAPs is a common regulator of MAP-microtubule affinity, which can have a pronounced effect on microtubule dynamics and network architecture.[78] MAP phosphorylation is tuned by protein kinases and phosphatases[78] that respond to neurohormonal signals elicited by cardiac stress, and that are well-established mediators of cardiac hypertrophy.[79–81] For example, dephosphorylation of MAP4, which is demonstrated in pressure-overload hypertrophy, increases its association with and stabilization of cardiac microtubules.[28,43] By contrast, phosphorylation of MAP4 reduces its microtubule affinity and can destabilize the network, which may also lead to pathological remodeling, as phosphomimetic-MAP4 knock-in mice develop hypertrophy, fibrosis and systolic dysfunction.[82] Consequently, it appears a proper balance of de- and phosphorylated MAP4 (and by extension hyper- and hypo-stabilized microtubules) is optimal for the heart, and shifting of the equilibrium in either direction can lead to pathological remodeling.

Phosphorylation also regulates the microtubule associated protein tau. Famous for its role in neurodegenerative disease, tau is also expressed in the heart[23,83,84] (and see proteomic data set PXD008934). Tau can protect microtubules from severing,[85] and may provide additional stabilization of cardiac microtubules. A direct interaction between tau and bridging integrator-1 (BIN1) has been characterized *in vitro*, and phosphorylation of tau destabilizes its interaction with both BIN1[86] and microtubules,[87] but the specific role played by tau in the heart remains to be determined.

A family of structural MAPs and motors known as tip-tracking proteins are responsible for stabilization and steering of the microtubule growing-end (+TIP).[88] In the heart +TIP interactions are largely coordinated by EB1, which promotes microtubule stability and recruits a family of CAP-Gly domain-containing tip-tracking proteins including cytoplasmic linker proteins 170, and 115 (CLIP170 and CLIP115) and p150^{glued}.[89–91] Tip-tracking proteins are preferentially recruited to tyrosinated microtubules and phosphorylation of EB1 enhances its localization to the +TIP. Also favoring tyrosinated tubulin, tip-tracking kinesins can facilitate microtubule disassembly (kinesin-13 family members, MCAK and KIF2A),[92] which may contribute to the decreased stability of tyrosinated microtubules.[93] Thus, PTMs to either MAPs or the microtubules on which they bind regulate intrinsic microtubule properties and their interactions within the myocyte, highlighting the complex, multi-layered regulation of the microtubule cytoskeleton.

While not classically considered MAPs, cytoskeletal linker proteins also influence microtubule organization and stability in the cardiomyocyte. Plectins can directly cross-link microtubules and intermediate filaments,[94] while microtubule actin cross-linking factor 1 (MACF1) stabilizes and guides microtubule growth along actin filaments. Cardiac specific deletion of MACF1 leads to aberrant microtubule redistribution that is strongly correlated with ventricular hypertrophy and contractile dysfunction upon pressure overload,[29] while plectin deletion leads to the disruption of desmin intermediate filaments[95] that typically help maintain microtubule network organization.[17] These data highlight the interdependence of the cytoskeletal networks in the cardiomyocyte, which must be considered when attempting to perturb specific components.

Microtubules at the membrane and costamere

Beneath the myocyte membrane lies a cortical network of microtubules that wraps around the myocyte and is predominantly aligned with the short axis of the cell. While the functionality of this pool of microtubules is far from fully elucidated, cortical microtubules regulate sarcolemmal ion channel localization, turnover, and activity,[96] caveolae formation and signaling via G-protein coupled receptors (GPCRs),[97] and mechanosensation and signaling, at least partly via connections to transmembrane complexes such as the dystrophin/dystroglycan complex.[3]

Vesicular trafficking is dependent on the microtubule network and is essential for the delivery and recycling of proteins between the SR, Golgi, and plasma membrane. Cycling of β-adrenergic receptors[98,99] and insulin receptors[100] depends on microtubules, while microtubules and actin microfilaments restrict cyclic adenosine monophosphate (cAMP) formation by localizing adenylyl cyclase

signaling components in caveolae in adult myocytes.[97] Ion channel localization and recycling at the cortex requires intact microtubules. Microtubule depolymerization reduces both kinesin-mediated anterograde[101,102] and dynein-mediated retrograde[103,104] transport of potassium channels in cardiomyocytes, leading to either decreased or increased surface expression of these channels. Inhibiting dynamic microtubules with nocodazole increases the expression of the chloride channel ClC2,[105] while stabilization of the microtubule network with taxol reduces sarcolemmal sodium channel density.[106] Microtubules are also implicated in the regulation of channel function independent of surface expression, as is demonstrated for KCNQ1.[96] Thus, perturbations to either microtubule density or dynamics can disrupt ion flux in the cardiomyocyte.

Costameres are cortical hubs of mechanotransduction in striated muscle that connect the cell membrane to the sarcomeres at the Z-disc. At the costamere, the dystrophin/dystroglycan complex (DGC) bridges the extracellular matrix and the cytoskeleton.[107,108] Dystrophin, the protein compromised in Duchenne muscular dystrophy, facilitates costameric organization,[109,110] recruits glycoprotein complexes and associates with intermediate filaments,[111] actin[112] and microtubules.[3,113,114] For a cartoon schematic of the DGC and its interactions with the cytoskeleton please see review.[114] Proper localization of dystrophin, dystroglycan, and microtubules at the costamere protects muscle from exercise-induced injury. A functional hierarchy was proposed,[115,116] where β2 spectrin localizes ankyrin-B to costameres, and ankyrin-B in turn localizes dynactin-4 and dystrophin, the latter of which likely facilitates dynactin-4 mediated stabilization of the costamere-associated microtubule network. In a commonly used model of Duchenne muscular dystrophy (the *mdx* mouse), subsarcolemmal microtubule network derangement increases reactive oxygen species (ROS) production and leads to aberrant calcium signaling.[4,5,117] Transgenic overexpression of dystrophin or mini-dystrophin, but not utrophin, prevents cortical microtubule disorganization in *mdx* mice, protects skeletal muscle from eccentric contraction-induced force loss, and improves physical activity of the mice.[3] However, further fine-mapping studies of dystrophin domains showed that the microtubule binding domain of dystrophin is dispensable for its microtubule-organizing function *in vivo*, and that full-length dystrophin is not sufficient to maintain normal microtubule network organization in the absence of other proteins in the dystrophin-glycoprotein complex.[3] This suggests dystrophin plays an indirect role in microtubule organization. Loss of dystrophin is coincident with the transition from compensated hypertrophy to heart failure,[118] but it remains to be demonstrated if a loss of dystrophin localization at the costamere is related to microtubule disarray and microtubule-dependent dysfunction in the progression of heart disease.

Microtubules at the dyad

Similar to costameric association at the cortex, microtubules also associate with interfibrillar structures at the Z-disc regulating the transport and positioning of a number of key components involved in E-C coupling. The SR and T-tubule membranes are well distributed throughout the myocyte and make regular contact at the Z-line in structures known as dyads (see review[119]). Dyads are hubs of E-C coupling (see review[120]), where action potential propagation leads to calcium entry through L-type calcium channels on the T-tubule membrane, which is then amplified by ryanodine receptors (RyRs) via calcium-induced calcium release from SR stores (Figure 3, inset). Efficient E-C coupling requires an extensive and well-ordered network of T-tubules and tight proximal interaction between T-tubules and the SR, as well as normal distribution of L-type calcium channels.

Microtubules maintain the structural and functional integrity of dyads through multiple mechanisms, including targeted delivery of L-type calcium channels and T-tubule scaffolding proteins.[121,122] The T-tubule scaffolding protein BIN1 is important for both L-type calcium channel localization[121] and T-tubule structural regulation[123–125] (see review by Fu and Hong[126]), while junctophilin-2 (JPH2) directly couples the T-tubule and SR membranes.[122] The growing tip of microtubules links to the T-tubule network through an interaction between the tip tracking protein CLIP-170 and BIN1.[14] L-type calcium channels and SR membranes (containing RyRs) are moved on microtubules by kinesin motors.[127,128] Triadin, named for its role in maintaining the triad (the analogous structures to dyads in skeletal muscle), is involved in shaping and remodeling SR membranes, likely in part through its interaction with microtubules via 63 kDa cytoskeleton-linking membrane protein (CLIMP63).[129] In both right and left ventricular pressure overload, microtubule proliferation is associated with reduced expression and localization of JPH2, pathological T-tubule remodeling and RyR disorganization.[15,130] T-tubule disruption and calcium mishandling are secondary to microtubule-mediated misregulation of JPH2,[131] and reducing microtubule density can restore dyad structure and improve ventricular function, suggesting a causative role of microtubules in the misregulation of E-C coupling in disease.

Microtubules also regulate myocyte sensitivity to mechanical stretch through the E-C coupling machinery. During diastolic stretch, microtubules serve as mechanotransducers to activate the production of ROS by NADPH oxidase 2 (NOX2) in the T-tubule membranes, a process termed X-ROS signaling (Figure 3, inset).[5,117] Under normal conditions, ROS can rapidly sensitize nearby RyRs, which primes the E-C coupling machinery at an appropriate phase of the cardiac cycle (late diastole). However, overproduction of ROS can lead to excessive RyR calcium release and subsequent arrhythmia. In disease states such as myopathy arising from Duchenne muscular dystrophy, increased NOX2 activity, microtubule disorganization and detyrosination, and altered mechanical loads may all lead to excessive X-ROS signaling that disrupts calcium homeostasis and causes oxidative stress in both cardiac and skeletal muscle.[4,132] Upregulation of the β-tubulin isoform tubb6 has been shown to at least partly underlie microtubule disorganization in dystrophic

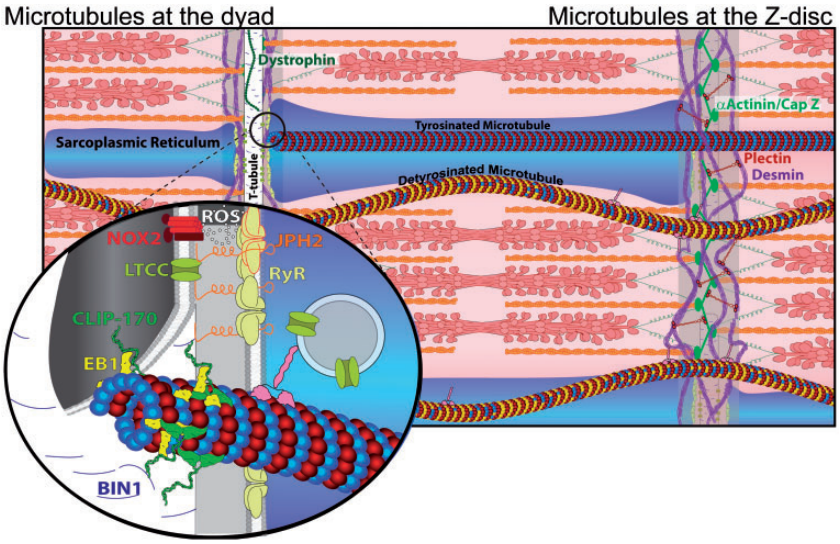

Figure 3. Role of microtubules at the dyad and Z-disc. Desmin (purple) wraps myofilaments at the Z-disc where it interacts with α-actinin (green) through plectin (red), and microtubules possibly through kinesin (pink) or plectin. Detyrosinated (yellow) microtubules link to the Z-disc and buckle during contraction while tyrosinated microtubules are not mechanically coupled and slide past myofilaments. Inset: A structural schematic of the dyad: L-type calcium channels and NOX2 on the T-tubule (white, cut open) are held in proximity to RyRs (pale yellow) at the SR (blue) by JPH2 (orange). Reactive oxygen species (ROS) emitted by NOX2 sensitizes RyRs. Microtubules link to the T-tubule via an interaction between CLIP-170 and BIN1. (A color version of this figure is available in the online journal.)

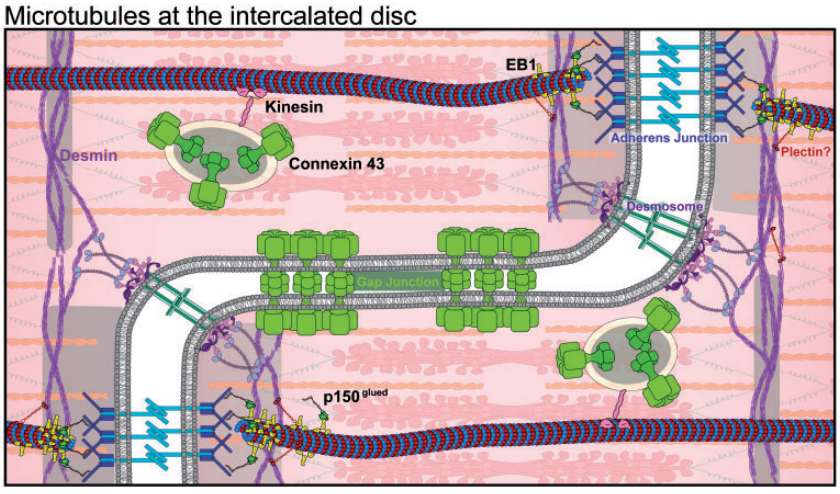

Figure 4. Microtubule interactions at the intercalated disc. Microtubule +TIPs anchoring to the adherens junctions (blue) via BIN1 and p150^{glued} directs the delivery of connexin 43 (light green) and other intercalated disc machineries. Desmosomes connect to desmin intermediate filaments via desmoplakin. Gap junctions (light green) provide cytoplasmic connectivity between cells. (A color version of this figure is available in the online journal.)

muscle.[133] While the complex interplay between microtubules, NOX2, and dystrophin requires further elucidation, efforts to target this axis—by restoration of microtubule network organization, tyrosination,[4,134,135] or NOX2 activity[4,136]—have each been shown to confer protection in dystrophic muscle.

Microtubules at the intercalated disc

Inter-myocyte coupling is achieved at the intercalated disc, specialized regions at myocyte ends that are enriched in desmosomes, adherens and gap junction complexes[137] (Figure 4). Myocytes are electrically coupled through gap junctions, which are large, non-specific transmembrane channels consisting of connexin hexamers that span adjacent myocyte membranes to provide electrical, metabolic, and immunological connectivity. Connexin protein is continuously made, transported, and degraded, having a half-life of only 1–5 h,[138,139] and its turnover is regulated by both intracellular and extracellular stresses. In heart disease, for example following myocardial infarction, connexin is readily downregulated and protein localization to the intercalated disc is compromised[140]. Connexin is also readily post-translationally modified, and its altered phosphorylation is linked to gap junction redistribution and arrhythmias.[141,142] Microtubules deliver connexons (oligomers of

connexin) to the intercalated disc by motor-based transport, with the help of EB1 and p150^{glued} anchoring of the growing end of microtubules to adherens junctions through N-cadherin.[16,139,143] Analogous to the loss of the dyadic structure in heart failure, alterations to the microtubule cytoskeleton are also associated with the disorganization of intercalated discs.[144] Perhaps consistently, administration of agents that disrupt microtubule dynamics are associated with ventricular arrhythmias.[145–147] Yet the mechanism by which this occurs is far from settled, motivating future studies to evaluate whether reversing microtubule alterations in disease may exert beneficial effects via restoration of cell-cell coupling.

Microtubules and the myofilaments

In contrast to the circumferential cortical network, a longitudinal network of interfibrillar microtubules interacts with the sarcomeres through transverse desmin intermediate filaments at the Z-disc to form a lattice-like scaffolding associated with myofilaments (Figure 3). Coupled to the sarcomeres, microtubules transmit force as they are pulled taut during diastole and compressed during systole.[17] Lateral reinforcement of microtubules by cross-links to cytoskeletal proteins enables microtubules to bear compressive loads and mechanically impede sarcomere shortening.[148] Microtubules buckling between sarcomeres during contraction of adult cardiomyocytes provides visual evidence for such mechanical coupling between sarcomeres and microtubules, and importantly this sarcomeric mode of buckling is promoted by detyrosination.[17] The interaction between microtubules and desmin intermediate filaments and the Z-disc[59] could be mediated through Kif5b[149] or plectin,[94] or may be non-specific and dictated largely by proximity within the cell. Regardless, the lateral reinforcement and thus mechanical behavior of microtubules within the myocyte is dependent on cross-linking between detyrosinated microtubules and other cytoskeletal elements.

Microtubule depolymerization or tyrosination increases myocyte contractility and accelerates relaxation without altering calcium signaling,[18,19,23,31] consistent with a mechanical uncoupling of microtubules from myofilaments. Measurements of myocyte and myocardial stiffness support a direct mechanical explanation for this altered contractility,[17,18,23,150–153] as myocyte viscoelasticity is reduced by microtubule tyrosination or depolymerization. A viscoelastic contribution of the network implies that the buckling of detyrosinated microtubules dissipates energy (resisting both contraction and relaxation). Due to its viscoelastic nature, this resistance will increase with increasing shortening or lengthening speeds, which has been demonstrated in both human and rat myocytes.[18,23]

As the microtubule contribution to myocyte mechanics strongly depends on the network density and interactome, it differs significantly in various cellular contexts and pathological states. This manifests as a considerably larger improvement in myocyte mechanical behavior when microtubules are targeted in myocytes from diseased vs. healthy hearts,[19,23] and may underlie the inverse correlation between detyrosinated microtubules and ejection fraction observed in human and murine heart disease.[17,24,42]

It should also be noted that common methodologies used to assess cardiac mechanics—including permeabilizing cells or tissues, isolating myofilaments, or maintaining a preparation in cold relaxing solution—compromises the integrity of the microtubule network, and thus confounds the mechanical assessment. In rat cardiomyocytes, the transverse and shear modulus were found to be decreased by colchicine and increased by taxol, while the tensile elastic modulus was not changed.[94] In a separate study, colchicine was found to have variable effects on the tensile stiffness of cardiomyocytes, but on average accounted for roughly 10% of the passive tension of the cell.[154] These findings are consistent with more recent work demonstrating that differences in cardiomyocyte tensile properties only arise at physiological strain rates[18] and are almost undetectable in healthy cells upon a slow application of stretch. Thus, the effective mechanical response depends on the pathological context, technical conditions of the preparation, and the orientation and speed of the mechanical test. These complexities have likely contributed to past confusion regarding the mechanical role of cardiac microtubules.

In tissue and organ level studies, a similar pathology-dependent mechanical contribution has been observed in some,[41,152,155] but not all[156,157] investigations. Colchicine treatment significantly improved myocardial performance after severe pressure overload in the right ventricle of cats[152,155] and the left ventricle of dogs,[41] with little impact on normal myocardium. Colchicine-dependent preservation of myocardial function *in vivo* has also been observed in a rat model of pulmonary hypertension[130] and upon left ventricular pressure overload in rats[158] and mice.[15]

Taken together, a picture emerges where the microtubule contribution to cardiac mechanics is highly context dependent; there are subtle, yet measurable phenotypes in healthy cardiomyocytes, and an increasing relative contribution in disease that appears most prominent upon severe pressure overload or advanced heart failure. While in our opinion the contribution to isolated myocyte mechanics has been reasonably well defined, more work is needed to delineate how this contribution scales to the tissue and organ level.

Microtubules and mitochondria

In non-muscle cells, variable energy demand is met by rapid remodeling and redistribution of the mitochondrial network,[159–161] in what is largely a microtubule driven process.[162,163] Perturbing microtubules is sufficient to remodel the mitochondrial network, likely by changing the balance of mitochondrial fission and fusion,[164,165] or by disrupting microtubule motor-dependent forces that shape mitochondrial morphology. Fusion is supported by mitochondrial motility,[160] which is driven by the microtubule motors kinesin for anterograde[166] and dynein for retrograde distribution of mitochondria and is regulated by the motor adaptor

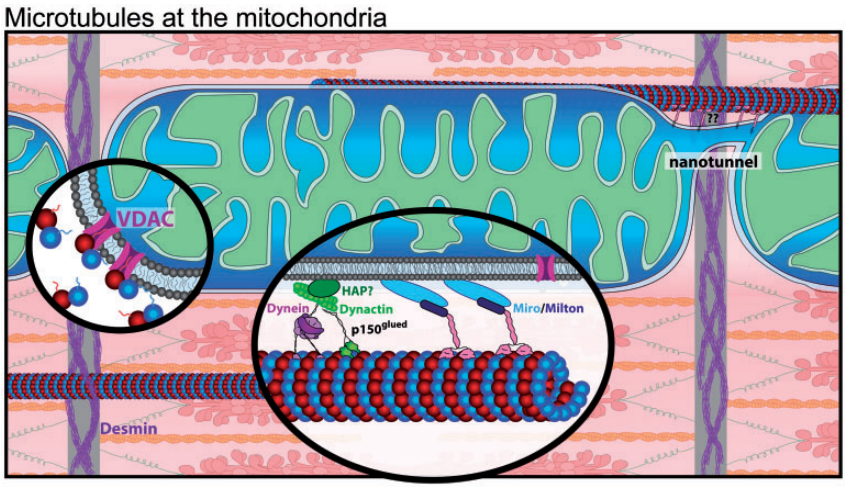

Figure 5. Microtubules are in close proximity to the mitochondria in the cardiomyocyte. Nanotunnels (top right) span adjacent mitochondria. Inset (middle): Mitochondria interact with both kinesin and dynein motors. Inset (left): VDAC is inhibited by free tubulin through binding of the β-tubulin tail. (A color version of this figure is available in the online journal.)

complex Miro/Milton in a calcium depending manner[167–169] (Figure 5, inset, middle).

In comparison to non-muscle cells, mitochondria in cardiomyocytes are less mobile. A constant high energy demand is met by a dense population of mitochondria that are packed between myofibrils, occupying 35–40% of the cell volume.[170,171] Dense packing of mitochondria restricts their mobility, fission and fusion,[172,173] and leads to spatially distinct populations of subsarcolemmal, inter-myofibrillar, and perinuclear mitochondria.[174–176] The subsarcolemmal mitochondria lie beneath membrane crests spanning between costameres, and loss of cortical microtubules may displace subsarcolemmal mitochondria.[177] Distinct from fusion, cardiac mitochondria do form direct connections via nanotunnel extensions that permit exchange of matrix content (Figure 5).[171,173,178] The association of nanotunnels with microtubules in electron micrographs suggests nanotunnels may be generated or stabilized by an interaction between the mitochondrial membrane and microtubules.[178] Further, recent evidence suggests mitochondria also undergo fusion and fission at higher rates than previously thought, and these dynamics are likely regulated by several relevant stimuli.[173] Thus, the interplay between mitochondrial and microtubule dynamics in various pathophysiological contexts warrants future investigation.

Beyond regulating mitochondrial positioning and exchange of matrix content, microtubule-based transport is important for the functional regulation of mitochondria. Microtubules mediate the delivery of purinosomes to the mitochondria,[179,180] multienzyme complexes that regulate metabolic flux and provide precursors for DNA synthesis. Additionally, free tubulin can directly regulate mitochondrial respiration through reversible blockage of the mitochondrial voltage-dependent anion channel (VDAC) by β-tubulin c-terminal tails (Figure 5, inset, left).[181–184] Thus, the interplay between microtubule dynamics and mitochondrial dynamics may elicit complex effects on metabolism and ATP production. Myocytes from failing hearts exhibit shifts in mitochondrial fusion and fission balance that affect myocyte bioenergetics,[185–187] which we speculate may at least partly arise due to altered microtubule dynamics, but this is not well understood.

Microtubules and the nucleus

Cardiomyocytes are often multi-nucleated, with nuclei aligned in the interior of the myofibrils and connected by a dense highway of microtubules (Figure 1). This differs from skeletal muscle, where nuclei are extruded to the cell periphery. The perinuclear microtubules in a cardiomyocyte are amongst the most stable in the cell resisting treatments with colchicine or nocodazole,[38] which may indicate that they are protected by heavy decoration with interacting partners.

In most eukaryotic cells, microtubules emanate from a microtubule organizing center (MTOC) at juxta-nuclear centrosomes, where the minus (−) ends of microtubules originate near the nucleus and plus (+) ends radiate toward the periphery.[188] Yet in cardiomyocytes, centrosomes disintegrate shortly after birth,[189] and non-centrosomal MTOCs decorate the nuclear envelope, associated Golgi and Golgi outposts in muscle cells.[190] While microtubules are still concentrated around the nucleus, the lack of a centrosomal MTOC creates a more widely distributed and less uniformly polarized microtubule network (Figure 1).

Regardless, centrosomal proteins such as PCM-1, AKAP450, pericentrin, and γ-tubulin localize to the nuclear periphery and can nucleate microtubule growth,[191–193] recruit motor proteins[194] and facilitate interactions between the "cage" of perinuclear microtubules and the nuclear mechanotransduction machinery known as the LINC complex. The LINC complex consists of the outer nuclear membrane nesprin proteins and inner nuclear membrane SUN proteins that connect the cytoskeleton to the nuclear lamina (Figure 6). These nuclear intermediate filaments (lamins) couple with regions of chromatin to form lamin-associated domains (LADs),[195] which are associated with silenced genes.[196] The LINC complex is thus capable

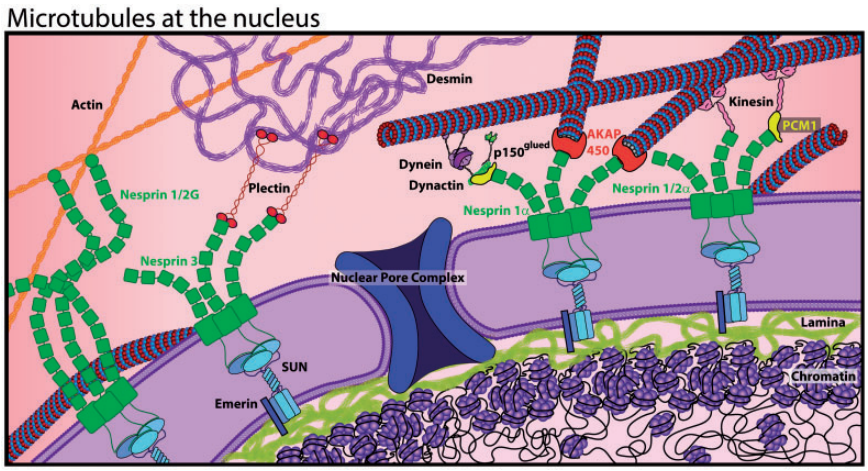

Figure 6. The LINC complex links the nucleoskeleton with the cytoskeleton. Cytoskeletal filaments (actin, desmin, and microtubules) are anchored to nesprins at the outer nuclear membrane, which couple to the SUN family of proteins spanning the inner nuclear membrane to connect with the nucleoskeleton (lamins). (A color version of this figure is available in the online journal.)

of transmitting mechanical signals from the cytoskeleton to LADs to regulate gene expression, an attractive mechanism for the myocyte to sense changes in environmental stress and respond accordingly. Microtubules interact with the LINC complex via nesprin 1α, nesprin 2α,[197] and nesprin 4[198] by binding the microtubule motors kinesin and dynein,[194,199] and considerable evidence supports a nesprin 1α interaction in the cardiomyocyte.[200,201] The importance of these LINC complex interactions is highlighted by mutations in LINC components causing diverse cardiomyopathies (see review[22,196]), yet much remains unknown about the mechanistic underpinnings of these connections and their role in nuclear homeostasis and cardiac mechanotransduction.

Microtubules and hypertrophy

Mechanotransduction in the myocyte facilitates a remarkable plasticity of the heart to adapt to chronic changes in load, as occurs with frequent exercise or elevated blood pressure. Thickening of the ventricular walls (hypertrophy) helps minimize wall stress under increased load, while atrophy of the heart occurs in response to unloading. Each requires individual myocytes to quickly and efficiently change their size, as myocyte proliferation is not sufficient to drive hypertrophy.[2,202,203]

Microtubule proliferation is closely associated with cardiac hypertrophy induced by pressure overload on the left and right ventricle in various small and large animal studies,[15,42,44,45,158] and seems to scale with the severity of wall stress.[40,204] Microtubules appear required for hypertrophy in response to increased external stress, as treatment with colchicine significantly blunts the hypertrophic response to pressure overload.[42,45,130,158] In a more subtle, clinically relevant setting of chronic hypertension, colchicine treatment has been demonstrated to arrest the progression of hypertrophy in spontaneously hypertensive rats.[205] Microtubules have also been shown to be required for phenylephrine-induced hypertrophy *in vitro*, and the anti-hypertrophic effects of adenosine have been attributed to a reduction in stable, detyrosinated microtubules.[30] In a clever approach exploiting transgenic mice harboring hyper- or hypo-stable forms of tubulin, Cheng *et al.*[206] showed that stabilization of tubulin is sufficient to induce modest cardiac hypertrophy, which is then further exacerbated upon pressure overload. These findings appear consistent with that of Fassett *et al.*,[30,42] where increased microtubule stability upon genetic manipulation of adenosine metabolism is associated with compensated hypertrophy at baseline and worsening cardiac function upon modest aortic constriction of murine hearts. Thus, there is evidence in the literature that microtubules are both necessary and sufficient for the cardiac hypertrophic response, although we consider the evidence stronger for the former.

Given their promiscuity in the cell, it is likely that microtubules could mediate the hypertrophic response via multiple independent pathways. For example, microtubules help transport sarcomeric mRNAs for local translation and incorporation into myofibrils, which may be important for building new sarcomeres during hypertrophic growth.[75,207] They can also serve as mechanotransducers, transmitting signals such as cell stretch to downstream effectors[79–81,208] that may regulate hypertrophic gene expression. Dissecting the molecular mechanisms by which microtubules mediate cardiomyocyte hypertrophy will require more nuanced investigation into the numerous components involved in prosecuting a hypertrophic stimulus, and the specific subsets of microtubules that may enable this response. While there is considerable evidence that destabilizing microtubules can blunt the hypertrophic response, it remains an open question as to whether destabilization could reverse established cardiac remodeling, and whether such a treatment may be tolerated.

Microtubules and protein turnover

Continuous elimination of misfolded or damaged proteins and organelles is of particular importance in the cardiomyocyte, given its long lifetime (see review[209]). If the

recycling machinery becomes overwhelmed, the accumulation of misfolded protein is associated with and can cause heart failure.[210] The degradation of proteins in cardiac muscle is predominately carried out by two proteolytic systems: the ubiquitin-proteasome system (UPS) and autophagy (see review[211]), both of which depend on microtubule-based transport for the collection and clearance of waste material.

Proteins are marked for elimination by the UPS system by post-translational ubiquitination, but if ubiquitinated proteins are not degraded by proteasomes they can accumulate and are transported into perinuclear structures known as aggresomes.[212] Aggresome formation is a microtubule-dependent process requiring dynein-mediated transport, and typically occurs in close proximity to MTOCs.[213–216] Mutations in UPS components are associated with hereditary hypertrophic and dilated cardiomyopathy, and can result in proteotoxicity, endoplasmic reticulum (ER) stress and autophagic activation (see review[217]).

The other primary method of waste clearance is autophagy, which is driven by the fusion of lysosomes with autophagosomes to form an autolysosome that degrades proteins and organelles. Microtubules regulate multiple stages in both basal and stress-induced autophagy (see review[218]), including the formation of autophagosomes,[219,220] dynein-mediated retrograde transport of the autophagosome,[221] and autophagosome-lysosome fusion.[222–224] Thus, an intact microtubule network is necessary at multiple levels to maintain autophagic flux.

As a regulator of microtubule-based transport, there is increasing evidence that microtubule PTMs can potently influence autophagic flux. Tubulin hyperacetylation was found to be protective against cardiac proteotoxicity by promoting autophagy in a mouse model of desminopathy.[210] Microtubule acetylation may activate autophagy by increasing the polymerized microtubule pool, enhancing the recruitment and movement of kinesin 1 and dynein,[225] and triggering phosphorylation of JNK which promotes the association of factors necessary for initiating autophagosome formation.[220,226] Microtubule detyrosination has also been implicated in autophagy during starvation.[224,227] A small subset of detyrosinated microtubules was found to be optimal to recruit and concentrate lysosomes via kinesin-1, which encouraged fusion with autophagosomes and protein degradation. In this model, an appropriate level of detyrosination allows for optimal autophagic flux, as too much or too little compromises the process by either dispersing and stalling lysosomes across the microtubule network, or by preventing their recruitment to microtubules, respectively.

While considered as largely independent systems targeting proteins for degradation in the proteasome and lysosome, the UPS and autophagy execute coordinated protein degradation with shared molecular machinery.[228] Methods to increase the efficiency of proteasomal and/or autophagic activity have demonstrated cardioprotective potential,[229–233] which should be considered when designing any microtubule-based approach to combat cardiac disease states, particularly given the regulation of autophagy by tubulin PTMs.

Microtubule-based therapies

Extrapolating from the literature reviewed above, tailored tuning of the microtubule network could provide a means to increase power output, blunt hypertrophy, enhance protein recycling, reduce oxidative stress, and prevent arrhythmia. Yet accomplishing all this in a single therapy seems a tall order (or perhaps pure fantasy), and at minimum will require a highly nuanced understanding of microtubules in the heart.

To date, therapeutic interventions targeting microtubules for the treatment of cardiovascular disease have been restricted to colchicine treatment. While this is a blunt tool, beneficial effects on the heart have been demonstrated in numerous animal studies, such as alleviating hypertrophy[44,126,153,199] and improving systolic function.[41,42,130] More granular manipulations, such as pharmaceutical inhibition of detyrosination with parthenolide, can acutely lower stiffness and improve contractile function of failing human cardiomyocytes, but still produces off-target alterations to E-C coupling,[23] and is associated with complex immunological[231] and oxidative responses[234] that may or may not be due to its inhibition of detyrosination. Taxanes (microtubule stabilizers) commonly used in chemotherapy are associated with cardiomyopathy, conduction abnormalities, and impaired contractility, particularly when combined with anthracyclines.[235,236] Longitudinal, in vivo imaging of non-failing patient hearts before and after this chemotherapeutic regiment indicate compromised diastolic function,[237] suggesting that stabilizing cardiac microtubules can be detrimental in humans.

In patients, meta-analysis suggests long-term, low-dose colchicine is associated with reduced risk of overall cardiovascular events, although establishing efficacy for modifying the course of coronary artery disease or heart failure requires further investigation.[238] Moreover, these benefits in patients likely arise largely due to colchicine's anti-inflammatory effects mediated through non-muscle cells. To achieve an appreciable depolymerization of cardiac microtubules (measured as an increase in the ratio of free: polymerized tubulin), a dosage of more than 0.4 mg/kg/day was required with either chronic or acute administration in animal models.[41,42,45,130,158,205] This is more than ~20 times the maximal recommended dosage of colchicine (1.2 mg/day) approved for humans for the treatment of gout,[239] which must be kept low to minimize gastrointestinal side-effects. With a half-life of less than 20 min, plasma colchicine peaks at ~2 μM within minutes and rapidly drops to 0.25 μM within an hour of treatment at maximal dosage.[240] Thus, plasma levels suggest it unlikely that the dosage of colchicine tolerated in humans will significantly depolymerize cardiac microtubules and explain the beneficial effects observed in patients. We should note, however, that intracellular concentrations may easily differ from plasma levels, and subtle destabilization of myocyte microtubules could still have significant consequences.

Regardless, grossly targeting the microtubule network could lead to numerous undesirable effects, and more specific pharmacologic and genetic therapies should be pursued. Cardiac decompensation is associated with the

stabilization of microtubules, a shift in their post-translational profile, and altered associations with MAPs, which has diverse implications for microtubule regulation of the dyad, myofilaments, mitochondria, and intercalated disc (to name a few). A clearer picture of how microtubules, their PTMs and MAPs impact these processes in the myocyte will likely inform more effective and specific therapies to treat patients with heart disease.

Closing thoughts

Not surprisingly, as cytoskeletal directors of cargo transport through the myocyte, microtubules are implicated in diverse cellular processes, and several areas appear particularly ripe for further investigation in cardiac microtubule biology. The role of microtubules in mechanotransduction and proteostasis appears critical for the acute and chronic adaptation of the heart to changing loads, and may be central to delineating between physiological and pathological remodeling. A more detailed understanding of how microtubules shape and maintain the unique membrane systems of the cardiomyocyte—and the effect this has on E-C coupling and arrhythmia—is required. Single cell studies and the cell-free reductionist assays that are the bulwark of the cytoskeletal field are instrumental in revealing molecular mechanisms and should be applied to the problems above. Yet it is also essential to test how targeting microtubules, MAPs and their modifying enzymes impact cardiac function at the organ level and *in vivo*, and to evaluate the long-term efficacy of more refined microtubule-based therapies.

A careful reader may note that much of our knowledge on cardiac microtubules has been inferred from studies using blunt pharmacological reagents to depolymerize or artificially stabilize the network. While these tools are practical and useful in identifying a contribution of the microtubule network to the process at hand, it is often difficult to glean mechanistic clarity, due largely to the fact that microtubules contribute to multiple, intermingling processes in the cell. Greater mechanistic insight requires that these approaches be combined (or replaced) with more refined techniques, such as live-cell monitoring of specific populations of dynamic and stable microtubules, genetically modulating the enzymes, MAPs and tubulin isotypes that confer unique functionality to subsets of microtubules, or perturbing specific microtubule motors and their ability to traffic particular cargo throughout the cell. An excellent toolkit for interrogating microtubule biology has been developed over the past 20 years, but its application to cardiomyocyte biology has somewhat lagged. We hope that beyond serving as a useful reference point, this review will help stimulate investigation into such a rich area of myocyte biology, with broad implications for cardiac health and disease.

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