Cardiac regeneration and remodelling of the cardiomyocyte cytoarchitecture

Hashim Ali[1,2], Luca Braga[1,2] and Mauro Giacca[1,2,3]

1 British Heart Foundation Centre of Research Excellence, School of Cardiovascular Medicine & Sciences, King's College London, UK
2 Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy
3 Department of Medical, Surgical and Health Sciences, University of Trieste, Italy

Keywords
actin; cardiomyocyte; centrosome; cytoskeleton; intercalated disc; mechanosensing; regeneration; sarcomere; tubulin; YAP

Correspondence
M. Giacca, King's College London, School of Cardiovascular Medicine & Sciences, The James Black Centre, 125 Coldharbour Lane, London SE5 9NU, UK
Tel: +44 207 848 1309
E-mail: mauro.giacca@kcl.ac.uk

Hashim Ali and Luca Braga are contributed equally.

Accepted 18 November 2019)

Adult mammals are unable to regenerate their hearts after cardiac injury, largely due to the incapacity of cardiomyocytes (CMs) to undergo cell division. However, mammalian embryonic and fetal CMs, similar to CMs from fish and amphibians during their entire life, exhibit robust replicative activity, which stops abruptly after birth and never significantly resumes. Converging evidence indicates that formation of the highly ordered and stable cytoarchitecture of mammalian mature CMs is coupled with loss of their proliferative potential. Here, we review the available information on the role of the cardiac cytoskeleton and sarcomere in the regulation of CM proliferation. The actin cytoskeleton, the intercalated disc, the microtubular network and the dystrophin–glycoprotein complex each sense mechanical cues from the surrounding environment. Furthermore, they participate in the regulation of CM proliferation by impinging on the yes-associated protein/transcriptional co-activator with PDZ-binding motif, β-catenin and myocardin-related transcription factor transcriptional co-activators. Mastering the molecular mechanisms regulating CM proliferation would permit the development of innovative strategies to stimulate cardiac regeneration in adult individuals, a hitherto unachieved yet fundamental therapeutic goal.

Introduction

Despite remarkable progress in terms of early diagnosis and prevention, the prevalence of cardiovascular disease is on the rise globally [1]. In particular, heart failure remains a prominent cause of deaths, disability and financial costs, estimated to affect approximately 38 million people worldwide [2]. In a vast majority of cases, this condition ensues as the consequence of an acute (myocardial infarction) or chronic ischaemic injury to the myocardium, followed by irreversible loss of cardiomyocytes (CMs), since these cells are

Abbreviations
ADF, actin-depolymerizing factor; ALMS1, Alstrom syndrome 1; AMOT, angiomotin; CK1, casein kinase 1; CM, cardiomyocyte; Dag1, dystroglycan 1; DDR, DNA damage response; DGC, dystrophin–glycoprotein complex; ECM, extracellular matrix; GSK-3β, glycogen synthase kinase 3β; GTP, guanosine-5′-triphosphate; ICD, intercalated disc; LINC, linker of nucleoskeleton and cytoskeleton; MAPs, microtubule-associated proteins; MMPs, matrix metalloproteinases; MRTF, myocardin-related transcription factor; MTOC, microtubule organizing centre; MTs, microtubules; MuRF, muscle ring finger; Mybpc3, myosin-binding protein C; NRG1, neuregulin 1; OSM, oncostatin M; PCM, pericentriolar material; RhoA, Ras homolog gene family member A; ROCK, rho-associated, coiled-coil protein kinase; RUNX1, runt-related transcription factor 1; SRF, serum response factor; SUN, Sad1/UNC-84; TAZ, transcriptional co-activator with PDZ-binding motif; TEAD, transcriptional enhancer factor domain family member; Wnt, wingless-int; YAP, yes-associated protein.

incapable of clinically significant replication in adult life. Carbon dating of CM DNA in humans indeed indicates that the renewal capacity of the heart is < 50% in a 70-year lifetime [3], an estimate that is consistent with information obtained in mice by imaging mass spectrometry, showing that CM renewal is to the order of < 1% every year [4]. As a consequence of the incapacity of CMs to replicate, scarring and fibrosis represent a general hallmark of cardiac repair after damage. Similar considerations also apply to other causes of CM loss, including untreated hypertension, viral infection and the side effects of anticancer chemotherapy.

In sharp contrast with adult mammals, some vertebrates, including urodeles and fish, completely regenerate significant portions of lost cardiac tissue after myocardial injury [5,6]. Genetic fate mapping experiments have indicated that the regenerative capacity of the zebrafish heart is due to the intrinsic capacity of resident CMs to partially dedifferentiate after damage and to re-enter the cell cycle [7,8]. In mammals, myocardial infarction also triggers an attempt at cardiac repair through CM replication, as testified by an increase in the rate of CM renewal [4] and the presence of mitotic CMs in the infarct border zone [9]. This attempt, however, is limited or abortive, and certainly below clinical significance.

At difference with postnatal life, mammalian CMs exhibit robust replicative activity during embryonic and fetal development, with subsequent waves of proliferation [10]. Replication stops abruptly immediately at birth to never resume again significantly. Further cardiac enlargement occurs by cell hypertrophy, characterized by marked increased in cytoplasmic volume and the accumulation of organized sarcomeric structures. Stop of cell division at birth is often accompanied by the uncoupling of DNA synthesis and mitosis from cytokinesis, by which over 90% of cells become bi-nucleated between P4 and P14 in the mouse [11,12]; binucleation is less frequent but still present in humans (~ 25% of adult ventricular CMs) [13,14]. In addition, ~ 45% and 66% of ventricular CM nuclei are polyploid in mice and humans, respectively [13].

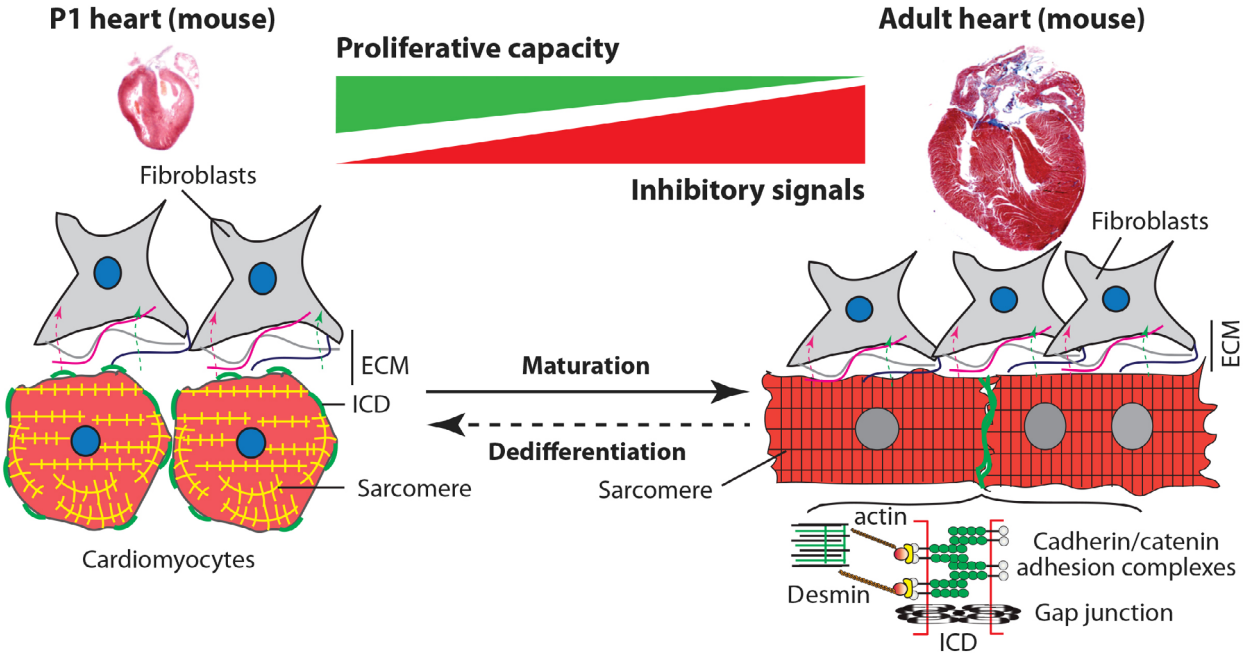
The withdrawal of CMs from the cell cycle after birth impacts profoundly on the capacity of the mammalian heart to undergo repair after damage: in the mouse, loss of myocardial tissue in the fetal or early neonatal life is healed through the generation of new contractile tissue [15], while fibrosis and scarring predominate later. A recent case report in a newborn child indicates complete recovery of cardiac function after an ischaemic insult can also occur early in life in humans [16].

Figure 1 schematically shows the complex molecular features that accompany CM withdrawal from the cell cycle. In particular, replicating CMs need to cope with the highly complex and ordered structure of their cytoplasmic molecular structures. During heart development from fetal to adult stages, the cardiac cytoskeleton undergoes gradual maturation, which leads to increase in size, number and complexity of myofibrils organization [17–20]. For CM proliferation to occur, many cytoskeletal complexes in mature CMs need transient disentanglement or disengagement. A successful mitotic event requires the formation of mitotic spindles and the contractile ring, which is unlikely to occur without preceding myofibril disassembly and cytoskeleton rearrangement. In addition to steric hindrance to cell division, and probably most important, the cytoskeletal structures most likely regulate the CM cell cycle by specific molecular signals.

Our understanding of the molecular pathways controlling assembly and disassembly of the CM cytoskeleton and its interconnection with the regulation of cell replication is still very patchy. In addition, it is largely unclear to which extent the vast information concerning the regulation of the cytoskeleton during the cell cycle in other cell types (most notably, cancer cells and fibroblasts) also applies to highly specialized cells such as CMs. Here, we specifically focus on the peculiarities of the CM cytoskeleton and its passive and active roles during replication of these cells.

The cardiac cell cytoskeleton

In all cell types, the cytoskeleton is a complex and highly dynamic structure, which not only defines the cell shape and structural integrity but also acts as an essential component of various aspects of cell physiology, including determination of cell polarity, provision of physical and mechanical cues to cell division and migration and definition of extracellular matrix (ECM) patterning. In a broader sense, the cell cytoskeleton acts as a signalling hub that senses, integrates and transmits both intracellular and extracellular signals [21]. This is of particular significance in CMs, since connection between the myofibrils and the ECM is essential to permit exertion of force during cell contraction in systole. At the same time, specialized cytoskeletal structures interconnect with the cell membrane at intercalated discs (ICDs) to permit proper



Immature cardiomyocyte
Smaller size with fewer sarcomeres
Less organized cytoskeleton
Positive cell cycle regulators expressed
Cell cycle inhibitors downregulated
Glycolytic metabolism
Rigid matrix
Mononucleated
Immature ICDs
Intact centrosomes

Mature cardiomyocyte
Larger size with multiple sarcomeres
Highly organized cytoskeleton
Positive cell cycle regulators downregulated
Cell cycle inhibitors expressed
Oxidative metabolism
Compliant matrix
Mono- and bi-nucleated
Mature ICDs
No apparent centrosomes

Fig. 1. Properties of developing and mature CMs. Schematic diagram showing immature CMs and their associated physiological and morphological changes towards maturation. These include modifications in myofibril density, cytoskeleton organization, switch of metabolism from glycolysis to oxidative phosphorylation, centrosome remodelling, ECM maturation, binucleation, ICD maturation and modification in the levels of positive and negative cell cycle regulators.

interconnection between orderly spaced neighbouring CMs.

The CM cytoskeleton components can schematically be divided into three different categories based on structural and functional properties, as schematically shown in Fig. 2.

The sarcomeric cytoskeleton

The sarcomere is the contractile unit of the cardiac muscle fibre. Thick and thin filaments, composed of myosin heavy- and light-chain molecules and by polymerized actin filaments, respectively, are integrated in para-crystalline manner together with a large number of regulatory and scaffolding proteins (Fig. 2) [22,23].

The nonsarcomeric cytoskeleton

The nonsarcomeric cytoskeleton is composed of actin, tubulin and desmin, each of which assemble and generate their respective polymeric forms [F-actin, microtubules (MTs) and intermediate filaments, respectively]. MT filaments are noncovalent polymers of α- and β-tubulin forming highly dynamic structures with continuous elongation and shortening during all stages of the cell cycle [24]. In CMs, the majority of tubulin is found in its monomeric form (~ 70%), while the rest (~ 30%) in the form of MTs [25]. These interact with the desmin cytoskeleton and regulate the assembly of myofibrils during cardiac muscle development [26]. The desmin cytoskeleton in CMs is transversely dispersed along the

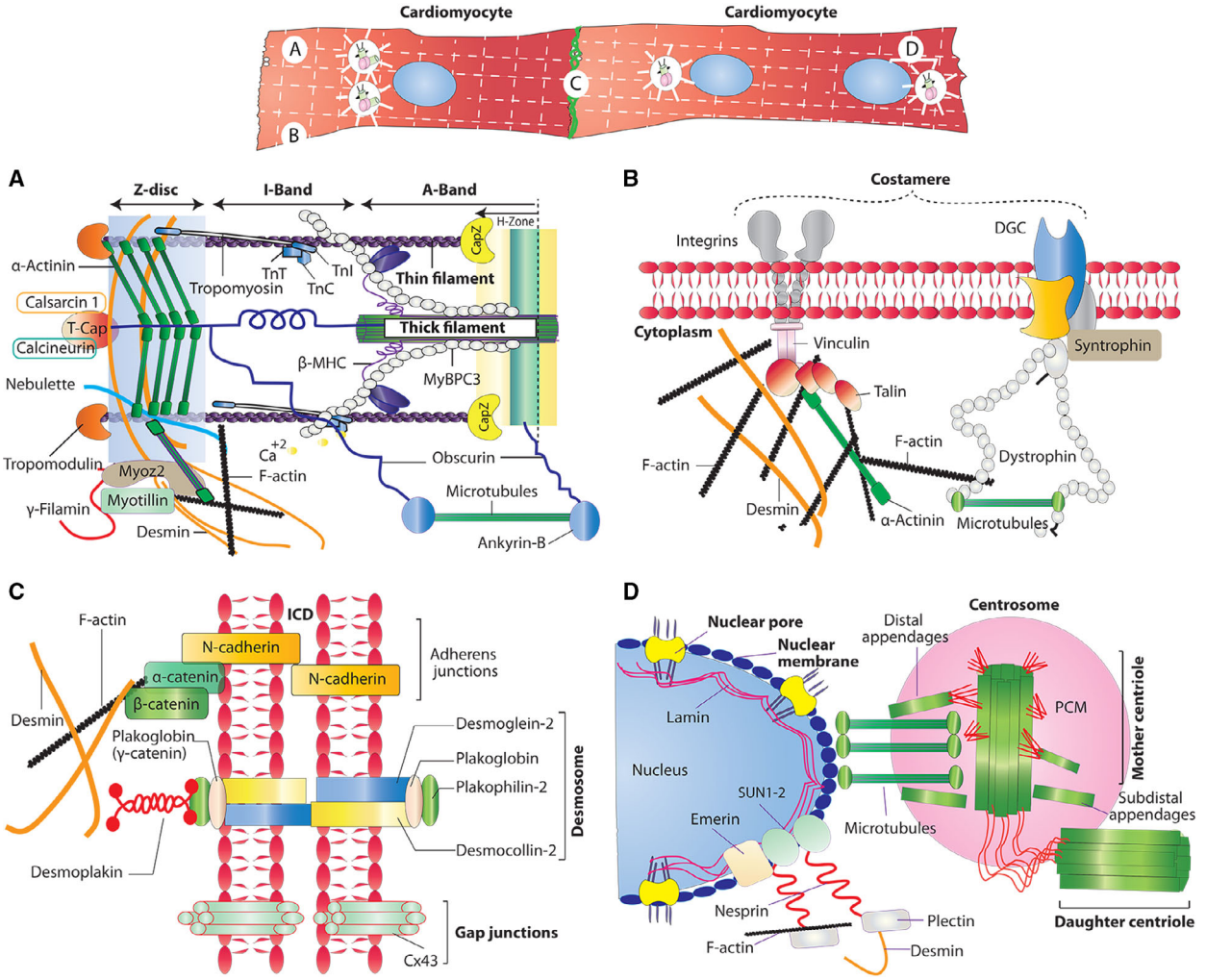


Fig. 2. Molecular anatomy of the cardiac muscle cell and of its functional complexes. The cartoon on top shows two connected CMs with the localization of the four structures shown in panels A, B, C and D (sarcomere, costamere, ICD and nucleoskeleton/centrosome, respectively). (A) Sarcomere. The cartoon shows half of the sarcomere structure. The thick filament of the sarcomere is composed of myosin heavy and light chain molecules, while the thin filament contains repeated actin molecules. Actin filaments are closely associated with the regulatory troponin complex (T, I, C) and tropomyosin, which plays a key role in regulating sarcomere contraction. Additional essential components of the sarcomere that provide mechanical resistance and structural integrity to CMs are titin, Mybpc3, α-actinin and myomesin. At the electron microscope, the sarcomere shows different topographical regions: M-band in the middle of the sarcomere, A-band in the centre, H-zone that lacks cross-bridges and only consists of myosin tails, I-band, in which there are only aligned actin filaments with no overlapping myosin filaments and, finally, Z-disc, which comprises the lateral border of the sarcomere. (B) Costamere. The costamere is composed of the DGC and the integrin–vinculin–talin complex, which connects the ECM to the myofibrils and sarcolemma via the actin and MT cytoskeletons. (C) ICDs connect neighbouring CMs and are essential for electrochemical coupling and tissue integrity. An ICD contains adherens junctions, desmosomes and gap junctions. Desmosomes and adherens junctions connect with the actin and desmin cytoskeleton via catenins. Catenins (alpha and beta) are known to regulate CM proliferation. (D) Nucleoskeleton and centrosome. The nucleoskeleton components (lamin, SUN1-2 and emerin) are tightly connected to the actin, MT and desmin cytoskeleton via nesprins. Mutations in lamin and nesprins are associated with cardiac disease. The centrosome contains two centrioles (mother and daughter), which are surrounded by a matrix of proteins known as the PCM. Centrosome integrity plays a key role in CM division and in organizing the cytoskeleton network.

myofibril area, where it links with the border zone of the Z-disc and ICD, and plays a vital role in maintaining sarcomere integrity [27]. Besides their function throughout the cell cycle, MTs form the filamentous structure of the mitotic spindle to segregate the newly formed sister chromatids [28].

Specialized structures associated with the cardiomyocyte cytoskeleton

At least three specialized structures play essential roles in CM biology and appear to be involved, to various extents, in the regulation of CM proliferation and maturation. These are the membrane-associated cytoskeleton, the ICD and the centrosome. In CMs, the main structural–functional component of the membrane-associated cytoskeleton is the costamere (Fig. 2B), which includes two types of membrane-associated protein complexes: the dystrophin–glycoprotein complex (DGC) and the integrin–vinculin–talin complex, both of which play a key role in regulating a biomechanical signalling between the sarcomere, sarcolemma and the ECM [29]. Adjacent CMs are connected through the ICD, consisting of multifunctional/structural connections that are essential for electrochemical and mechanical signalling (Fig. 2C) [30]. Finally, multiple, but still scattered, evidence indicates that an important determinant of CM proliferation lies within the components of the centrosome, a specialized MT-nucleating organelle that duplicates once per cell cycle forming the pole of the mitotic spindle (Fig. 2D) [31].

The actin cytoskeleton and the regulation of CM proliferation

During interphase, nonsarcomeric actin exists as an extensive network that rapidly dissolves as soon as the cells enter mitosis and round their shape. Consolidated evidence indicates that, in most cell types, the cell cycle machinery controls the actin cytoskeleton while, in turn, the state of organization of the cytoskeleton regulates cell cycle progression [32–34]. Perturbation of proper cytoskeleton reorganization leads to cell cycle arrest due to the activation of checkpoints in both the G1 [35] and G2/M [36] phases of the cell cycle. For example, overexpression of Cofilin 1, an actin-depolymerizing factor (ADF), arrests the cells in G1 [37], while increased F-actin polymerization due to genetic mutations or cell treatment with Jasplakinolide leads to delayed mitosis and defective cytokinesis, which eventually determines cell multinucleation [38]. Should a connection between altered nonsarcomeric actin cytoskeleton dynamics and defects of cell division also hold true in CMs, it could explain both nuclear division of CMs in the absence of cytokinesis and block of the cell cycle in G2 when these cells stop proliferating.

Over 150 proteins have been identified to contain an actin-binding domain and thus influence nonsarcomeric actin dynamics or mediate signalling from it [39,40]. In particular, the dynamic formation of the actin cytoskeleton and its rapid response to cellular cues – such as those related to sensing of the extracellular environment, as it will be discussed later – is the consequence of the ratio between polymerized, filamentous actin and monomeric, globular actin (F-actin and G-actin, respectively). This ratio also regulates CM proliferation. In particular, our own past work has shown that several microRNA can stimulate cell proliferation once overexpressed in neonatal rat and mouse CMs [41]. A common characteristic of most of these microRNA is to downregulate one or more members of the families of factors that prevent actin polymerization by directly interacting with G-actin; these include Cofilins, Twinfilins, Thymosin β4 and Profilins. Among the proteins that are downregulated by these pro-proliferative microRNA are also cysteine and glycine rich protein 3, MT-associated monooxygenase, calponin and LIM domain containing 3 and Aurora A kinase, all of which are directly or indirectly involved in the regulation of actin polymerization [42]. A specific, direct target of four miRNA that are very effective in stimulating CM proliferation (miR-199a-3p, miR-1825, miR-302d and miR-373 [41]) is Cofilin 2, a muscle-specific member of the ADF/Cofilins family of proteins, which modulate actin filament nonequilibrium assembly and disassembly by preventing assembly of actin monomers and causing depolymerization of acting filaments [43,44]. Downregulation of Cofilin 2 by RNAi, which markedly promotes actin polymerization and formation cortical stress fibres besides leading to disorganization of the sarcomeric architecture, is sufficient to stimulate neonatal CM entry into the cell cycle [42].

The mechanism by which actin polymerization leads to CM proliferation is still poorly understood. Of interest, the members of the myocardin-related transcription factors (MRTF-A and MRTF-B) are sequestered in the cytoplasm through their specific interaction with G-actin monomers. When actin becomes polymerized, typically as a response to increased substrate stiffness via Ras homolog gene family member A (RhoA) and rho-associated, coiled-coil protein kinase (ROCK) [45], these factors are released from actin binding and translocate into the nucleus. Here, they act as co-activators of the serum response factor (SRF), controlling expression of a set of genes carrying CArG boxes in their promoters and determining myogenic specification, cytoskeletal organization and cell proliferation [46,47]. A testable hypothesis, therefore, is that, also in CMs, MRTF could link actin polymerization with activation of proliferation.

Another essential molecular component connecting sensing of mechanical cues, actin cytoskeleton and cell

proliferation is the Hippo pathway [48], which acts as a key regulator of CM proliferation during heart development. In CMs, the positive effectors of this pathway are the yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) transcriptional co-activators, which, in their dephosphorylated form, localize to the nucleus and associate with the transcriptional enhancer factor domain family member 1–4 (TEAD1–4) transcription factors to drive transcription of genes involved in cell proliferation [49]. Cardiac-specific YAP knockout causes embryonic lethality at E10.5 due to myocardial hypoplasia and contractile dysfunction [50,51], while YAP overfunction induces heart regeneration in both neonatal and adult hearts [50]. Consistent with this information, transgenic mice overexpressing activated YAP or lacking the inhibitory mammalian sterile 20 kinase 1 kinase (Hippo in *Drosophila*, in which the pathway was originally discovered) both repair myocardial injury through regeneration instead of fibrosis [52,53].

Multiple evidence links YAP-mediated CM proliferation to the F-actin cytoskeleton. Chromatin immunoprecipitation-sequencing analysis of YAP target genes showed interaction with several genes involved in cell cycle progression, but also genes regulating F-actin polymerization and connecting the ECM to the actin cytoskeleton [54]. YAP/TAZ also regulates expression Cofilin 1/2, Capzb and Gelsolin, all of which are well-known organizers of actin filament dynamics [55] and in turn inhibit YAP/TAZ function [56]. Finally, our own work has shown that a series of microRNA that are capable to induce CM proliferation act through the activation of YAP [42]. In particular, miR-199a-3p, which is one of the most effective in driving cardiac regeneration in both mice [41] and pigs [57], directly targets the 3′UTR of TAOK1 and β-TrCP; the former is an inhibitory kinase in the Hippo pathway [58,59], while the latter acts as the E3 ubiquitin ligase responsible of YAP degradation through the proteasome [60]. Convergence of CM pro-proliferative miRNA onto the Hippo pathway was also shown by the Mercola laboratory after high-throughput screening for human miRNA stimulating iPS cell-derived CM proliferation [61].

Thus, at least two different transcriptional programmes connect cytoskeletal reorganization and cell replication, the RhoA–actin–MRTF pathway and the YAP/TAZ pathway. A crosstalk actually exists between these pathways. First, they both respond to actin polymerization. While MRTF binds and senses the levels of G-actin directly, YAP/TAZ instead interact with angiomotin (AMOT), which in turn binds actin filaments [62]. Second, emerging evidence indicates that, in different cell types, the MRTF/SRF and YAP/TEAD transcriptional pathways are also interconnected at the target gene levels, since several promoters require both sets of transcriptional factors/cofactors for their proper expression [63–65]. Thus, it appears likely that both pathways might functionally interact to also control CM proliferation, especially in response to mechanical cues.

The microtubular network at the interface between mechanosensing and the regulation of cardiomyocyte replication

Microtubules are highly dynamic cytoskeletal filaments composed of α-/β-tubulin heterodimers, which are essential for a broad range of cellular processes, including intracellular vesicle transport, division, definition of cell polarity and morphology, and cell motility [66]. MT polymerization is regulated by the hydrolysis of β-tubulin-bound guanosine-5′-triphosphate (GTP), which only occurs after a GTP–tubulin dimer becomes successfully incorporated into the growing MT tips. In this manner, growing MT ends always maintain a stabilizing GTP cap, the loss of which leads to fast depolymerization. The MT end that exposes β-tubulin is defined as the plus end, while the MT minus end is the one that exposes α-tubulin [67].

An extensive range of post-translational modifications is known to decorate both α- and β-tubulin subunits; the most common of these are tyrosination/detyrosination, acetylation, polyglutamylation, polyglycylation and phosphorylation. Notably, these modifications are nonrandomly distributed along MTs [68] and their nature and pattern finely regulate binding of several MT-associated proteins (MAPs), which contribute to define function of the microtubular network [69]. Among these MAPs, MT polymerases (XMAP125 family, +tracking proteins) bind MTs and significantly increase MT elongation by recruiting tubulin dimers at the MT-plus ends. On the contrary, MT depolymerases, such as nonmotile kinesin-13, destabilize MT-plus ends by removing terminal tubulin subunits and disrupting lateral tubulin interaction in an ATP hydrolysis-dependent manner [70].

The idea to target mitotic MT dynamics to block cell cycle progression has been one of the most successful strategies to develop anticancer therapies [24]. The class of MT targeting agents we have available today can broadly be classified into two groups based on their specific mechanisms of action: MT-stabilizing agents, which include taxanes (paclitraxel, docetaxel),

and MT-destabilizing agents, which contain structurally diverse chemicals such as vinblastine, vincristine, nocodazole and colchicine. The taxanes are tubulin-polymerizing inducers, which stabilize MTs and disturb MT depolymerization during cell cycle progression. Nocodazole, vincristine and colchicine instead disrupt MT function by binding various sites on β-tubulin and inducing MT depolymerization [24].

In cardiac cells, the microtubular network exerts at least two essential roles: first, as in all cell types, it is essential for mitosis, since perturbation of MT assembly or disassembly results in inhibition of spindle attachment to the kinetochore, causing delay or arrest of cells in G2/M phase of the cell cycle. Second, it specifically senses tension transmitted to the cells by the extracellular environment and responds dynamically to it by adapting cell size and shape [71]. The dynamic role of MTs in postnatal cardiac development has been shown already in 1985 by Cartwright *et al.* [72], who reported that, by measuring the number of MTs per squared micron in the cross-sectional area of rat cardiac papillary muscle, the MT network constantly increases in density during the early postnatal period, to reach its maximum at postnatal day 5. From this time point, microtubular density constantly decreases and reaches a steady-state level typical of the adult heart at postnatal day 14. This was one of the first indications that the microtubular network adapts to the modified conditions of ventricular load occurring immediately after birth and persisting thereafter during the adult life. Consistent with this function, a number of investigators have later reported that progressive increase in density of the CM MT network sustains pathological cardiac hypertrophic remodelling and accompanies subsequent development of heart failure [25,73–75].

The molecular mechanism by which increased MT polymerization impacts on contractile dysfunction has recently been studied by B. Prosser and coworkers. These investigators found that the MT network regulates CM passive stiffness by binding the contractile apparatus and forming load-bearing spring elements that span alongside sarcomeres [76]. In particular, desmin, a pivotal regulator of sarcomere architecture, binds detyrosinated residues of α-tubulin and, through this interaction, MT directly experience contractile force and themselves deform under load. Proteomics and single-cell mechanical study have revealed that MTs of adult CMs from human failing hearts are more abundant and heavily detyrosinated compared to nonfailing CMs [77]. Accordingly, both pharmacological suppression of detyrosination with parthenolide (an inhibitor of the detyrosinase tubulin carboxypeptidase) and overexpression of tubulin tyrosine ligase (which rapidly tyrosinates detyrosinated MTs) lower the viscoelasticity of failing myocytes and restore 40–50% of lost contractile function [77].

To what extent these modifications in MT structure and density might also correlate with the regulation of CM proliferation remains an open question. To date, for obvious reasons, the effect of MT targeting agents has been extensively studied in highly proliferative cells for their anti-mitotic properties, while very few investigations have addressed their function in resting cells. A study published in cell almost 40 years ago reported that, quite contra-intuitively, MT-depolymerizing drugs were able to induce DNA synthesis in serum-free cultures of nonproliferating fibroblasts; upon MT repolymerization, the treated cells effectively re-entered the cell cycle. In the same experimental conditions, taxols exerted an opposite effect [78]. These observations indicate that integrity of the MT network might be inhibitory to cell proliferation, possibly by inhibiting factors that are essential for cell entry into the S phase. This is of potential interest in the light of understanding the molecular mechanisms that determine CM exit from the cell cycle immediately after birth: the same mechanism by which these cells sense increased ventricular load and build a more stable MT network might also be responsible for the arrest of their proliferation.

The factors by which the MT network might control CM proliferation are still unexplored. Of potential interest, two unrelated screenings performed in other cell types have revealed an unexpected link between tubulin depolymerization and wingless-int (Wnt) pathway activation. Using a human radiation hybrid cell panel, Khan and coworkers found that the E3 ubiquitin ligase zinc and ring finger 3, which negative controls Wnt signalling, rescues toxicity of the tubulin-depolymerizing drug colchicine [79]. Fukuda *et al.* [80] instead identified tubulin as the target proteins for a small molecule identified in a screening for Wnt agonists; inhibition of tubulin polymerization was thus associated with Wnt activation. This is also in agreement with the notion that Wnt signalling proteins, such as Dishevelled and β-catenin, bind components of the MT system [81]. On the other hand, Wnt signalling is also known to regulate the tubulin network, in particular through Wnt- mediated inhibition of glycogen synthase kinase 3β (GSK-3β), which leads to MT stabilization [82]. To what extent these findings, which were obtained in different cell types, also extend to CMs remains an open matter. Wnt signalling is known to exert a crucial function in heart development and CM specification [83]. Yet, the roles of this pathway

on heart regeneration are still poorly understood [84], and current evidence appears to indicate that inhibition of this pathway promotes CM proliferation during zebrafish regeneration [85] and in infarcted mice [86].

A deeper understanding of the molecular events linking MT dynamics with Wnt signalling specifically in CMs is definitely needed.

Extracellular mechanical cues and cardiomyocyte proliferation

The ability of CMs to sense extracellular mechanical stimuli is of pivotal importance in the heart adaptive response to hemodynamic changes. At least three major sets of molecular interactions mediate anchorage of the CM cytoskeleton to the ECM, namely the DGC, the MT network and transmembrane integrins (Fig. 2B).

The DGC binds the ECM to the dystrophin protein, while dystrophin anchors the DGC to the F-actin cytoskeleton [87]. The DCG complex is also connected to MTs via ankyrin B [88,89], a member of the ankyrin family known to participate in multiple processes involved in physiological cardiac homeostasis. MTs tend to bud from Z-discs and then grow parallel to the long axis of sarcomeres, thus wrapping the contractile apparatus. On the one hand, this allows providing mechanical resistance to the myocyte, while, on the other hand, it connects sarcomeres with the extracellular membrane via the DGC [90]. In addition, detyrosinated MTs directly bind desmin, which is a pivotal regulator of CM architecture and dynamics, as also discussed later. Finally, transmembrane integrins bind vinculin and talin, which bridge the ECM to actin, thus mediating biomechanical signalling to the cytoskeleton [91,92].

Structural and functional integrity of the DGC also plays a specific role in the regulation of CM proliferation. A component of this complex, dystroglycan 1 (Dag1), was reported to directly bind YAP and inhibit its pro-proliferative function [93]. Of note, Dag1 and the DGC are also the targets of the ECM proteoglycan agrin, which promotes cardiac regeneration in neonatal mouse hearts [94]. This protein is known to induce actin polymerization and cytoskeletal remodelling in cooperation with the Hippo pathway [95,96]. Agrin-mediated YAP activation is inhibited by RhoA and ROCK inhibitors and by Latrunculin A, which affects F-actin polymerization [97]. Agrin-stimulated CM proliferation following interaction with Dag1 induces subsequent disassembly of the DGC and release of YAP into the nucleus [94]. Together, these results again highlight the relevance of ECM-mediated regulation of cardiac regeneration by mechanisms mediated through the interaction of the DGC with the cellular actin cytoskeleton and converting onto YAP activation.

Intermediate filaments and propagation of mechanosensing to the nucleus

The cytoplasmic network is tightly linked to nuclear lamins and DNA chromatin via linker of nucleoskeleton and cytoskeleton (LINC), a protein complex composed of Sad1/UNC-84 (SUN)-domain proteins and integrins (reviewed in ref. [98]). Lamins (LMNA, LMNC and LMNB), which are the most abundant nuclear intermediate filament, directly bind SUN proteins that span through the inner nuclear membrane and to nesprins in the perinuclear space. Nesprins in turn cross the outer nuclear membrane to interact with multiple classes of cytoskeletal proteins (Fig. 2D). This ensures that a direct link exists between extracellular mechanical stimuli and the nuclear cytoskeleton.

In particular, nesprin-1/2 directly connects cytoplasmic actin to nuclear lamin and emerin [99]; nesprin-3 bridges nuclear and desmin cytoplasmic intermediate filaments via plectins [100]; nesprin-4 interacts with MTs by directly binding kinesin proteins [101]. Desmin, which belongs to the class of cytoplasmic intermediate filaments, is the major load-bearing structure in CMs, thus actin as a pivotal mechanosensors and mechanotransducers in the heart [102]. In addition, thanks to its interaction with the LINC complex, desmin acts as a hub in transducing stimuli deriving from the ECM into the nucleus. Mutations in desmin, lamin or nesprins are associated with severe cardiac disease [103–106]. Mice heterozygous for a lamin A mutation show decreased response to left-ventricular pressure overload, due to impaired activation of the mechanosensitive gene Egr-1 [107]. Similarly, the hearts of nesprin 1/2 double knockout mice fail in raising a protective gene expression programme to face changes in biomechanical load [108]. Finally, recent evidence indicates that embryonic hearts progressively stiffen during development, while lamin A accumulates to oppose stress-driven nuclear rupture, increased DNA damage, and cell cycle arrest [109].

Taken together, this information is concordant in indicating that both cytoplasmic and nuclear intermediate filaments are essential transducers of mechanical cues from the extracellular environment into the nucleus. To what extent the proteins forming these filaments are also involved in the CM cell cycle arrest

concomitant with increased cardiac load after birth remains an exciting topic for future investigation.

Remodelling of the sarcomeric cytoskeleton during cardiomyocyte replication

The contractile nature of CMs poses additional problems to cell replication compared to other cell types. CM myofibrils have long been thought to impede nuclear division and cell cleavage, blocking the onset of replication or leading to cell bi- or multinucleation [19,20]. Thus, historically, the terminally differentiated phenotype of CMs has been considered as an irreversible state. Over the past two decades, however, it has become progressively evident that adult CMs are plastic and can remodel their cytoskeleton and sarcomere profoundly both *in vivo* and *in vitro* and thus cope with the issue of duplicating their genetic material and divide despite their nature of contractile cells.

The heart is the first functional organ that develops intracellular myofibrils and starts contracting at embryonic day 8.5 in mice [110]. The relative volume of myofibrils in the CM cytoplasm increases from 10% to 20% in the early and midgestational stages to ~ 30% soon after birth [111,112]. Neonatal mouse CMs have been reported to have a progressively extended S/G2/M phase compared to embryonic stages. The reason for this delay prior to cytokinesis might be attributed to the increased number and complex organization of neonatal sarcomeres [113].

Despite these functional and structural changes, embryonic, fetal and early neonatal CMs continue to divide. Early reports had already indicated that intact myofibrils are found next to condensed chromosomes [114,115] or that the cross-striated pattern of CMs is completely lost in cytokinesis [19,116]. Later advances in microscopy proved that dedifferentiated CMs can indeed progress through complete cell division, which requires disassembly of the myofibrils [117]. During this process, the primary event is the dissociation of Z-disc- and thin filament-associated proteins, followed by M-band and thick filament disassembly. After complete cell division, cardiac sarcomeric proteins reassemble into their cross-striated pattern. This process is also known to occur after exogenous stimulation of CM proliferation [42,118,119].

Disassembly of sarcomeric structures also occurs during adult heart regeneration in zebrafish [7]. In the mouse, apical resection within the first week of life is healed by regeneration rather than scarring; this process is again accompanied by disassembly of sarcomeric structures, followed by cell division [15]. Transcriptome analysis in regenerating fish hearts showed that this process is accompanied by downregulation of several sarcomeric genes, suggesting a transcriptional component in the control of dedifferentiation [120]. Concordant findings were also reported in newts [121]. Of note, structural changes of a similar type are also present in human myocardium during hibernation after cardiac injury [122]. Hibernating CMs typically show depletion of sarcomeric structures and an expression pattern of structural proteins closely resembling that of fetal heart CMs [123].

Loss of the mammalian heart proliferative potential shortly after birth is concomitant with the onset of CM binucleation and hypertrophy [11]. In 1-day-old mice, ~90% CMs are mononucleated and have a high proliferative potential. However, just a few weeks after birth, more than 90% of CMs become binucleated and lose their proliferative capacity [11,124]. The advent of binucleated CMs is considered indicative of lack of coupling of karyokinesis to cytokinesis owing to deficient myofibril disassembly in the last stage of cell division [12,118]; other reasons, however, might be responsible for the lack of cell division, in particular related to the biology of CM centrosome (cf. later). The physiological role of binucleated CMs remains largely elusive; the increased number of mRNA transcripts from the two nuclei was reported to be essential to maintain the high metabolic demands of differentiated CMs [125].

The majority of CM nuclei in adult human hearts are those already present at birth, as concluded from ^{14}C dating studies [3]. However, CMs undergo regular turnover of their sarcomeric proteins, with an average half-life for these proteins that ranges from a few days to 2 weeks [126–128]. Understanding the molecular correlates of this continuous turnover not only has importance to counteract the mechanisms leading to cardiac hypertrophy but could also shed light on the dynamics of the sarcomeric cytoskeleton during CM division. Inhibition of myosin-binding protein C (Mybpc3), a structural protein of the sarcomere that regulates myofilament stiffness and rigidity [129], reduces sarcomere density [130], leading to cardiac hypertrophy. Of interest, in heterozygous Mybpc3$^{+/-}$ mice, the hypertrophic phenotype is mainly due to increase in CM cell size, however in homozygous Mybpc3$^{-/-}$ animals this occurs mainly due to increased CM proliferation [131,132]. The cardiac cytoskeleton in homozygous Mybpc3 knockout hearts contains less rigid sarcomeres and has decreased myofibrillar density. These animals also show more mononucleated CMs compared to wild-type mice. The transition from early induction of CM proliferation to

later stimulation of hypertrophy in homozygous Mybpc3 knockout hearts raises the question as to how the proliferative and hypertrophic signalling are interconnected.

A main controller of disassembly and degradation of CM sarcomeric structures is the ubiquitin/proteasome system. Several E3 ubiquitin ligases have been shown to stimulate sarcomeric protein degradation. Among these are the muscle ring finger (MuRF) members of a family of muscle-specific ubiquitin E3 ligases, of which MuRF1 (TRIM63), MuRF2 (TRIM55) and MuRF3 (TRIM54) are the most studied in cardiac and skeletal muscle cells [133–135]; the Fbxo28 protein (also called atrogin-1), which plays as essential role during muscle atrophy [136,137]; TRIM32, which is involved in the degradation of the thin filament (actin, tropomyosin, troponins) and Z-band (alpha-actinin) components as well as of the desmin cytoskeleton [134,138]; the cardiac-specific F-box protein Fbxl22, which promotes the ubiquitin-proteasome mediated degradation of sarcomeric proteins-α-actinin and filamin C [139]; and, finally, the E2 enzymes Ube2i and Ube2g1, which disrupt the cardiac sarcomere structure in a STAT3-dependent manner [140].

Cellular proteases might also play a role in sarcomere disassembly by acting upstream of the proteasome. This is the case of myofibril disassembly occurring during myopathies, which is mainly regulated by the activity of muscle-specific calpain proteases. These enzymes, which associate with the I-band region of titin [141,142], induce release of myofilaments and Z-band disintegration, followed by activation of the ubiquitin–proteasome pathway. Matrix metalloproteinases (MMPs), specially MMP-2, are also abundantly expressed in CMs [143] and, besides being secreted, also localize to different intracellular compartments, including the sarcomere Z-disc region [144]. MMP-2 was reported to specifically cleave a number of intracellular substrates, including α-actinin [145], troponin I [143], myosin light chain-1 [146], titin [144] and GSK-3β [147].

A few factors recognized for their property to induce CM cell division are known to induce sarcomere dis- and re-assembly (Fig. 3). Oncostatin M (OSM), a macrophage-derived cytokine, determines a de-differentiate phenotype in CMs [148]. OSM also induces expression of MMP-2, which might mediates sarcomere degradation [149]. Neuregulin 1 (NRG1), which plays important roles in zebrafish and mice cardiac development and homeostasis, induces proliferation of both neonatal and adult CMs involving dedifferentiation followed by re-differentiation [150]. Constitutively active expression of ERBB2, the NRG1 receptor, increases CM mitotic activity paralleled by disassembly and rarefaction of sarcomeres together with re-expression of progenitor cell markers (among which, runt-related transcription factor 1 and disabled 2) [151]. Agrin, an ECM heparan sulphate proteoglycan that binds the Dag1 component of the DGC to induce CM proliferation (cf. before) also induces sarcomere disassembly [94]. Hippo-deficient mouse hearts, in which YAP activation drives a CM hyperproliferative phenotype, show increase in the disassembly of sarcomeric structures [54]. Finally, delivering a cocktail of three CM reprogramming genes (FoxM1, Id1 and Jnk3-shRNA) that induce CM proliferation *in vivo*, also results in low levels of sarcomeric alpha-myosin heavy chain gene and less organized sarcomeres [152].

Taken together, this information is consistent in showing that cardiac regeneration is an evolutionary conserved mechanism from teleost fish to mammals that involves a ‘dedifferentiation’ step including the disassembly and rarefaction of cardiac contractile units. The actual molecular mechanisms for sarcomere disassembly and downregulation of sarcomeric protein levels, however, remain still largely uncharacterized. Additionally, despite the wealth of information concerning sarcomere disassembly, little is known on how this process coordinates with, or regulates, CM proliferation specifically.

Dynamics of the centrosome during cardiomyocyte mitosis and cytokinesis

Multiple, but still scattered, evidence indicates that an important determinant of CM proliferation lies within the components of the centrosome. In metazoans, the centrosome is a single, MT-nucleating organelle in juxtanuclear position, comprising two centrioles (named mother and daughter) and a complex assembly of proteins collectively termed pericentriolar material (PCM; Fig. 2D). Centrioles are cylindrical structures composed of nine triplet MTs that duplicate once per cell cycle, starting at the G1/S transition, with the two resulting centrosomes forming the pole of the mitotic spindle [31] (Fig. 4A). In interphase, the centrosome anchors a radial array of MTs forming the routes for intracellular transport, named the MT organising centre (MTOC). In most quiescent, terminally differentiated cells, the mother centrosome assembles at the cell membrane to form a single, nonmotile, primary cilium (termed a basal body when ciliated), which, in several cell types, is responsible for mechano- and chemo-sensing, as well as for motility. In addition, the centrosome also nucleates actin filaments [153] and serves as a hub

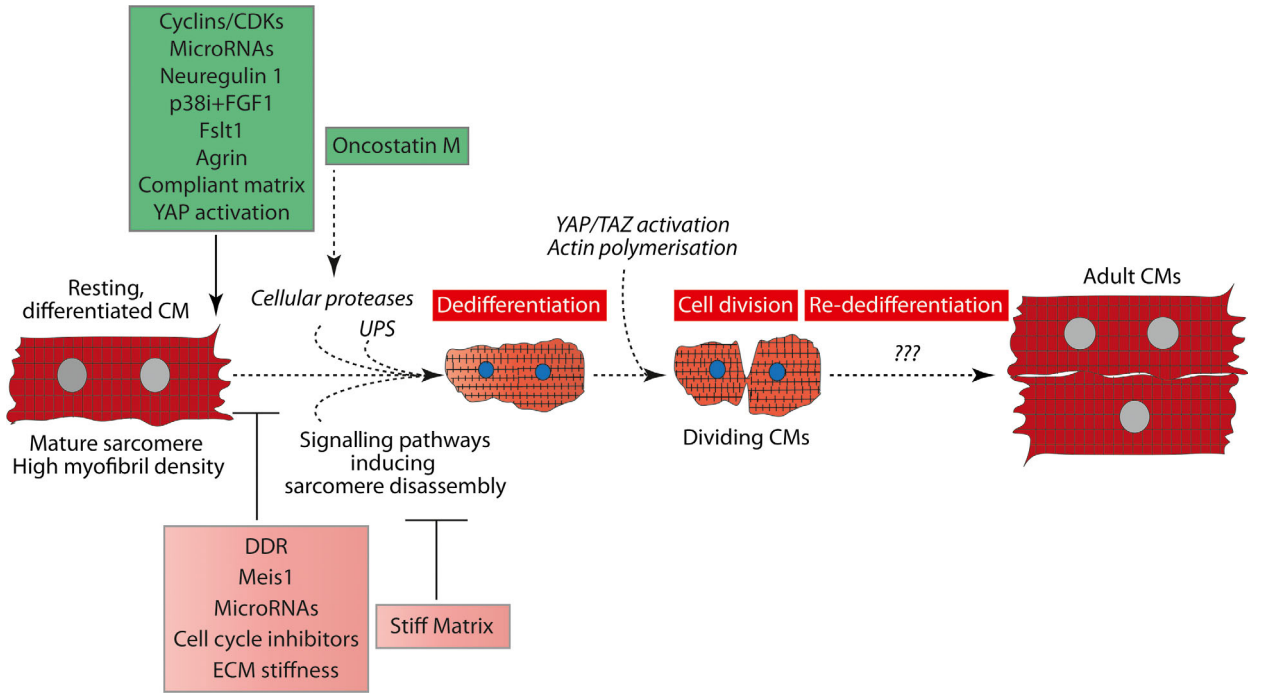


Fig. 3. Known mechanisms modulating CM proliferation and dedifferentiation via cytoskeleton remodelling. A few soluble factors were discovered that stimulate CM proliferation during development and in models of heart regeneration. These include NRG1, agrin, fibroblast growth factor-1 and insulin-like growth factor-1. OSM is a secreted cytokine involved in proliferation by promoting CM dedifferentiation. In the mammalian heart, CMs exit the cell cycle soon after birth and are incompetent to re-enter the cell cycle because of the accumulation of negative cell cycle regulators such as Meis1, some miRNA (e.g. miR-15), activation of the DDR and of the p38 mitogen-activated protein kinase. Different factors including other miRNA (e.g. miR-302, miR-199a-3p and miR-590-3p) can promote re-entry of mature CMs into the cell cycle and induce cardiac regeneration by targeting genes of various signalling pathways. YAP/TAZ acts as a transcriptional co-activator and is essential for CM proliferation; its import into the nucleus is regulated by both structural and functional components of CMs. Functional and structural properties of the ECM also regulate CM proliferation. Factors inducing cytokinesis after binucleation and cytoskeletal components impeding cytokinesis are still poorly characterized.

for components of the cell cycle regulatory and DNA damage response machineries [154–156]. Over 100 proteins participate in centrosome structure and dynamics [157].

Several observations link the biological events occurring at the centrosome and the primary cilium to the regulation of CM proliferation. First, biallelic mutations in the giant protein Alstrom syndrome 1 (ALMS1), which is broadly expressed in most cell types and localizes to the proximal end of the centrioles [158], cause a form of rare cardiomyopathy characterized by delayed cell cycle arrest of neonatal CMs [159,160]. Patients with this ciliopathy show persisting postnatal CM proliferation, a phenotype that can be reproduced in homozygous mutant mice and anti-ALMS1 siRNA-treated cultured CMs; in normal conditions, expression of the protein increases during terminal differentiation of neonatal CMs [159]. Thus, ALMS1 appears to act as a suppressor of CM proliferation [158].

Second, centrioles/primary cilia can act as sensory organelles that respond to stimuli in the micro-environment by orchestrating a cell response through different signal transduction mechanisms, involving the Hippo, Wnt and Notch pathways [161,162], all of which are known to regulate CM replication. In particular, in basal conditions, in various cell types the levels of cytoplasmic β-catenin are low since this protein is targeted by the β-catenin destruction complex, which is formed of Axin, adenomatous polyposis coli, casein kinase 1 (CK1) and GSK-3β; CK1 and GSK-3β continuously phosphorylate β-catenin and drive its degradation through the SCF-β TrCP-dependent ubiquitin–proteasome pathway. When canonical Wnt signalling is activated, the destruction complex becomes inhibited and the levels of β-catenin in the cytoplasm increase [163]. In mouse embryos, primary fibroblasts and embryonic stem cells, this process occurs at the cilium [164]. Then, β-catenin is free to translocate to the nucleus, where it acts as a transcriptional

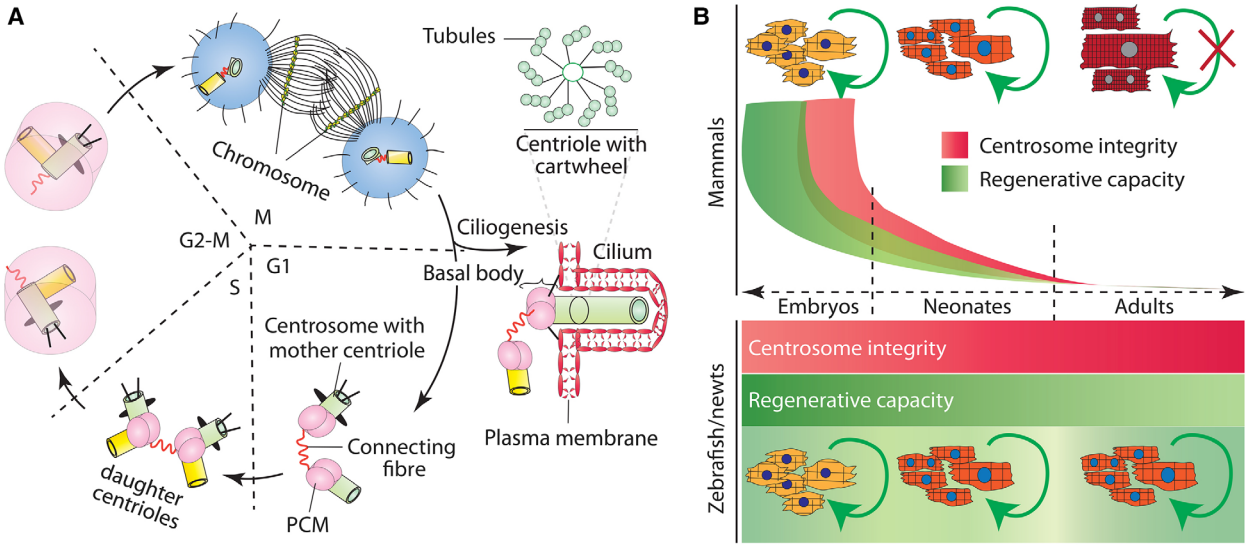


Fig. 4. Centrosome dynamics during the eukaryotic cell cycle and CM proliferation. (A) In most of the newly formed cells, the G1 phase centrosome contains two centrioles, which are connected together with a flexible linker and surrounded by a matrix of proteins known as PCM. Each centriole duplicates in the S phase, forming daughter centrioles. Once the cells enter into G2/M, the centrosome starts separating and split into two, while the mother centrioles enlarge their PCM. An enlarged PCM permits centrosome nucleation and organization of MTs, which is important to bring together and position the mitotic spindle. As the cells exit mitosis, the chromosomes segregate on the mitotic spindle and the mother and daughter centrioles detach from one another, although they often remain loosely associated through a flexible connecting fibre. In many cells that have exited, the cell cycle centrioles migrate to the cell periphery, where the mother centriole builds a basal body from which the cilium extends. (B) Graphical representation of centrosome integrity and rate of CM proliferation in mammals and lower vertebrates (zebrafish and newts). Mammalian CMs maintain high replicative capacity and centrosome integrity during the embryonic and fetal life. Concomitant with the sudden decline in proliferative potential at birth, CMs loose centrosome integrity, whereas zebrafish and newts maintain both replicative potential and centrosome integrity throughout life.

co-activator of members of the T-cell factor/lymphoid enhancer factor family of proteins. In CMs, inhibition of GSK3-β, through genetic means [165] or by treatment with the inhibitory small molecule 6-bromoindirubin-3′-oxime [166], results in β-catenin stabilization and CM mitosis and proliferation. A major mechanism controlling CM proliferation is the regulation of β-catenin through the YAP and TAZ transcriptional cofactors in the Hippo pathway [167,168]. In addition, ALMS1 itself was identified as an interactor or substrate of the E3 ubiquitin ligase SCF-β-TrCP [169], which controls degradation of both YAP and β-catenin, and of the YAP-inhibitory proteins large tumour suppressor kinase 2 and AMOT [170]. The functional relevance of these interactions still remains largely unexplored in CMs. They might provide a novel angle to connect mechanosensing and proliferation in the heart.

Third, experimental evidence suggests that centrosomal dynamics correlates with the incapacity of CMs to progress through mitosis and cytokinesis (Fig. 4B). Work from the Engel laboratory has shown that, in postnatal mouse CMs, centrioles lose their cohesion during the G0/G1 phase, in contrast to neonatal mouse and zebrafish CMs, which retain regenerative capacity [171]. This split-centriole division would result in relocalization of several centrosomal components to the nuclear envelope and be a block to further cell division. An interpretation of these findings is that centrosome disassembly might lead to cytoskeletal reorganization to better handle postnatal increased hemodynamic load, thanks to a more effective organization of the MTOC in correspondence to the nuclear envelope, as it also occurs in skeletal muscle [172]. Failure to complete cytokinesis might also then ensue due to mislocalization of proteins required to form an effective actomyosin ring for cleavage furrow formation [173]. Further studies will certainly reveal whether these are the cause or effects of mammalian CM withdrawal from the cell cycle, and to what extent these effects might be reversible.

Finally, a recently identified protein interacting with both tubulin and actin filaments and being essential for proper termination of cell division is GAS2L3. This a member of the GAS2 family of proteins, which crosslinks actin and MTs in interphase and growth-arrested cells [174,175]. GAS2L3, which is highly expressed throughout the cell cycle, plays an essential

role in mitotic spindle assembly, cytokinesis and during abscission, being localized to the midbodies and contractile ring during the final stage of cytokinesis [176,177]. In cells in which GAS2L3 is downregulated, the key players of cleavage furrow ingression, RhoA and Myosin IIA, are mislocalized, resulting in failure to stably position the contractile ring ultimately leading to defects in cytokinesis [178]. Mice knockout for GAS2L3 die prematurely of heart failure due to anticipated termination of CM proliferation during embryonic development [177]. The proliferation arrest of CMs is accompanied by binucleation through incomplete cytokinesis. Whether this protein might be involved in the physiological propensity of CMs to undergo bi- and multinucleation after birth remains a matter for interesting future investigation [179].

Cardiomyocyte proliferation and intercalated disc junctions

The ICD is a CM-specific structure that includes three different types of cell–cell connections: desmosomes, adherens junctions and gap junctions. Adherens junctions and desmosomes (which together form the *area composita*) are major cell–cell plaque-bearing contacts in the ICD, which anchor myofibrils and are connected to the CM cytoskeleton. Desmosomes includes proteins that link the intercellular network with the desmin cytoskeleton in the CM cytoplasm (Fig. 2C). At least 200 different proteins are known to be associated with the ICD; mutation of at least 40% of these proteins are associated with different forms of cardiomyopathy [180,181]. Intercellular proteins include desmosomal cadherins, desmoglein and desmocollin, while cytoplasmic proteins comprise desmoplakin, plakophilin and plakoglobin. Adherens junctions, which anchor the sarcomeric actin filaments, are composed of N-cadherin as a transmembrane component that provides a link to the neighbouring cell and a cytoplasmic plaque that contains, among other proteins, α-catenin and β-catenin and connects to actin filaments [182]. In CMs, gap junctions act as ion channels that are mainly composed of connexin family members and are involved the in rapid and coordinated electrochemical communication, which is essential for rhythmic heart function. In particular, Connexin-43 is mainly expressed in ICDs of adult heart ventricles [183].

During early cardiac development, the ICD components are distributed along the membrane of immature, round-shaped CMs [18]. However, soon after birth the sarcomeric cytoskeleton starts to elongate and CMs acquire a rod-shaped structure, which is concomitant with ICD junctional complexes being relocated to the longitudinal ends of neighbouring CMs [18,184]. Intriguingly, these changes in the distribution of ICD components concur with exit of CMs from the cell cycle soon after birth [11,12], suggesting a possible function of ICD components in the regulation of CM proliferation. Conditional knockout studies have revealed that αE-catenin, an ICD component, plays a key role in the proliferation of different cell types [185,186], apart from anchoring the cadherin–catenin complex to the actin cytoskeleton. Similarly, inhibition of α-catenin expression in mice after MI induces CM proliferation by releasing YAP from cadherin-catenin complexes [187]. Consistent with a role of the ICD in controlling CM proliferation, it was also shown that perturbation of ICD maturation (by inhibiting expression of αE-catenin and αT-catenin) induces CM proliferation in the adult heart [188].

It is of interest to observe that the *area composita* is not found in lower vertebrates, such as amphibians and fish [189], which suggests that this structure might have evolved to sustain the augmented mechanical load of the mammalian ventricles. Since increased load is considered to be a main reason for withdrawal of CMs from the cell cycle immediately after birth [190], the molecular connection between the ICD and the regulation of CM proliferation definitely needs more systematic, molecular analysis.

Conclusions and translational perspectives

Understanding the molecular correlates regulating assembly of the CM cytoskeleton and sarcomere and their disassembly during replication holds paramount importance in the light of developing innovative therapeutic strategies for myocardial infarction and heart failure. Direct administration of stem cells or various derivation to the heart have failed to provide significant regeneration over the last 15 years [191]. Embryonic stem cells or iPS cells can be expanded in the laboratory to generate large number of CMs, to be directly injected into the heart [192] or used for the formation of large patches of myocardial tissue *ex vivo* [193,194]. However, a possibly much simpler and translatable possibility is to re-awaken the endogenous potential of CMs to proliferate and thus mimic what spontaneously occurs in fish, amphibians and neonatal mammals to repair cardiac damage. MicroRNA endowed with pro-proliferative activity [41,57], cyclin/CDKs reactivating cell cycle entry [195] or exogenously administered proteins [94,150,196] all hold great potential for cardiac regeneration (Fig. 3).

It remains clear that the formation of reparative cardiac tissue is a complex biological event, certainly centred on CM replication but also involving all cells in the heart, including endothelial cells, vascular smooth muscle cells, pericytes and cardiac fibroblasts. Newly formed CMs need to communicate electrically and mechanically through specialized junctions; force needs to be appropriately transmitted by maturation of their sarcomeric structures; newly formed tissue demands proper vascularization through connection with pre-existing vessels; ECM requires proper structure and compaction. While this complexity adds further levels of regulation to the regeneration process, the existing information from neonates and adults in which the heart is able to regenerate are concordant in indicating that the regulation of the capacity of CMs to proliferate remains the primary event that controls cardiac regeneration.

Thus, whatever the mechanism through which CM replication will operate for regenerative purposes, it will need to overcome the molecular brake to replication imposed by the cardiac cytoskeleton and sarcomere. Finding molecular means that help promoting disassembly of these structures and inactivate the negative signals imposed to mammalian cardiac cells immediately after birth are essential goals in the light of developing effective regenerative therapies in the near future.

Acknowledgements

This work was supported by European Research Council (ERC) Advanced Grant 787971 'CuRE', grant 825670 'CardioReGenix' from the European Commission Horizon 2020 programme, the Leducq Foundation Transatlantic Network of Excellence grant 14CVD04 and the continuous support of Fondazione CRTrieste, Trieste, Italy.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

HA, LB and MG conceived the manuscript and wrote the text.

References

1 Roth GA, Johnson C, Abajobir A, Abd-Allah F, Abera SF, Abyu G, Ahmed M, Aksut B, Alam T, Alam K *et al.* (2017) Global, regional, and national burden of cardiovascular diseases for 10 causes, 1990 to 2015. *J Am Coll Cardiol* **70**, 1–25.

2 Moran AE, Forouzanfar MH, Roth GA, Mensah GA, Ezzati M, Murray CJ & Naghavi M (2014) Temporal trends in ischemic heart disease mortality in 21 world regions, 1980 to 2010: the Global Burden of Disease 2010 study. *Circulation* **129**, 1483–1492.

3 Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H *et al.* (2009) Evidence for cardiomyocyte renewal in humans. *Science* **324**, 98–102.

4 Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu TD, Guerquin-Kern JL, Lechene CP & Lee RT (2013) Mammalian heart renewal by pre-existing cardiomyocytes. *Nature* **493**, 433–436.

5 Poss KD, Wilson LG & Keating MT (2002) Heart regeneration in zebrafish. *Science* **298**, 2188–2190.

6 Oberpriller JO & Oberpriller JC (1974) Response of the adult newt ventricle to injury. *J Exp Zool* **187**, 249–253.

7 Jopling C, Sleep E, Raya M, Marti M, Raya A & Izpisua Belmonte JC (2010) Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464**, 606–609.

8 Kikuchi K, Holdway JE, Werdich AA, Anderson RM, Fang Y, Egnaczyk GF, Evans T, Macrae CA, Stainier DY & Poss KD (2010) Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. *Nature* **464**, 601–605.

9 Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA *et al.* (2001) Evidence that human cardiac myocytes divide after myocardial infarction. *N Engl J Med* **344**, 1750–1757.

10 Sedmera D, Reckova M, DeAlmeida A, Coppen SR, Kubalak SW, Gourdie RG & Thompson RP (2003) Spatiotemporal pattern of commitment to slowed proliferation in the embryonic mouse heart indicates progressive differentiation of the cardiac conduction system. *Anat Rec A Discov Mol Cell Evol Biol* **274**, 773–777.

11 Soonpaa MH, Kim KK, Pajak L, Franklin M & Field LJ (1996) Cardiomyocyte DNA synthesis and binucleation during murine development. *Am J Physiol* **271**, H2183–H2189.

12 Li F, Wang X, Capasso JM & Gerdes AM (1996) Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. *J Mol Cell Cardiol* **28**, 1737–1746.

13 Mollova M, Bersell K, Walsh S, Savla J, Das LT, Park SY, Silberstein LE, Dos Remedios CG, Graham D, Colan S *et al.* (2013) Cardiomyocyte proliferation contributes to heart growth in young humans. *Proc Natl Acad Sci USA* **110**, 1446–1451.

14 Bergmann O, Zdunek S, Felker A, Salehpour M, Alkass K, Bernard S, Sjostrom SL, Szewczykowska M, Jackowska T, Dos Remedios C *et al*. (2015) Dynamics of cell generation and turnover in the human Heart. *Cell* **161**, 1566–1575.
15 Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN & Sadek HA (2011) Transient regenerative potential of the neonatal mouse heart. *Science* **331**, 1078–1080.
16 Haubner BJ, Schneider J, Schweigmann U, Schuetz T, Dichtl W, Velik-Salchner C, Stein JI & Penninger JM (2016) Functional recovery of a human neonatal heart after severe myocardial infarction. *Circ Res* **118**, 216–221.
17 Ehler E, Rothen BM, Hammerle SP, Komiyama M & Perriard JC (1999) Myofibrillogenesis in the developing chicken heart: assembly of Z-disk, M-line and the thick filaments. *J Cell Sci* **112**(Pt 10), 1529–1539.
18 Hirschy A, Schatzmann F, Ehler E & Perriard JC (2006) Establishment of cardiac cytoarchitecture in the developing mouse heart. *Dev Biol* **289**, 430–441.
19 Rumyantsev PP (1977) Interrelations of the proliferation and differentiation processes during cardiact myogenesis and regeneration. *Int Rev Cytol* **51**, 186–273.
20 Zak R (1974) Development and proliferative capacity of cardiac muscle cells. *Circ Res* **35**(suppl II), 17–26.
21 Bezanilla M, Gladfelter AS, Kovar DR & Lee WL (2015) Cytoskeletal dynamics: a view from the membrane. *J Cell Biol* **209**, 329–337.
22 Sequeira V, Nijenkamp LL, Regan JA & van der Velden J (2014) The physiological role of cardiac cytoskeleton and its alterations in heart failure. *Biochim Biophys Acta* **1838**, 700–722.
23 Gautel M & Djinovic-Carugo K (2016) The sarcomeric cytoskeleton: from molecules to motion. *J Exp Biol* **219**, 135–145.
24 Jordan MA & Wilson L (2004) Microtubules as a target for anticancer drugs. *Nat Rev Cancer* **4**, 253–265.
25 Tsutsui H, Ishihara K & Cooper GT (1993) Cytoskeletal role in the contractile dysfunction of hypertrophied myocardium. *Science* **260**, 682–687.
26 Hein S, Kostin S, Heling A, Maeno Y & Schaper J (2000) The role of the cytoskeleton in heart failure. *Cardiovasc Res* **45**, 273–278.
27 Milner DJ, Weitzer G, Tran D, Bradley A & Capetanaki Y (1996) Disruption of muscle architecture and myocardial degeneration in mice lacking desmin. *J Cell Biol* **134**, 1255–1270.
28 Nogales E (2000) Structural insights into microtubule function. *Annu Rev Biochem* **69**, 277–302.
29 Peter AK, Cheng H, Ross RS, Knowlton KU & Chen J (2011) The costamere bridges sarcomeres to the sarcolemma in striated muscle. *Prog Pediatr Cardiol* **31**, 83–88.
30 Ehler E (2016) Cardiac cytoarchitecture - why the "hardware" is important for heart function! *Biochim Biophys Acta* **1863**, 1857–1863.
31 Nigg EA & Holland AJ (2018) Once and only once: mechanisms of centriole duplication and their deregulation in disease. *Nat Rev Mol Cell Biol* **19**, 297–312.
32 Assoian RK & Zhu X (1997) Cell anchorage and the cytoskeleton as partners in growth factor dependent cell cycle progression. *Curr Opin Cell Biol* **9**, 93–98.
33 Thery M & Bornens M (2006) Cell shape and cell division. *Curr Opin Cell Biol* **18**, 648–657.
34 Spector I, Shochet NR, Kashman Y & Groweiss A (1983) Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. *Science* **219**, 493–495.
35 Reshetnikova G, Barkan R, Popov B, Nikolsky N & Chang LS (2000) Disruption of the actin cytoskeleton leads to inhibition of mitogen-induced cyclin E expression, Cdk2 phosphorylation, and nuclear accumulation of the retinoblastoma protein-related p107 protein. *Exp Cell Res* **259**, 35–53.
36 Gachet Y, Tournier S, Millar JB & Hyams JS (2001) A MAP kinase-dependent actin checkpoint ensures proper spindle orientation in fission yeast. *Nature* **412**, 352–355.
37 Lee YJ & Keng PC (2005) Studying the effects of actin cytoskeletal destabilization on cell cycle by cofilin overexpression. *Mol Biotechnol* **31**, 1–10.
38 Moulding DA, Blundell MP, Spiller DG, White MR, Cory GO, Calle Y, Kempski H, Sinclair J, Ancliff PJ, Kinnon C *et al*. (2007) Unregulated actin polymerization by WASp causes defects of mitosis and cytokinesis in X-linked neutropenia. *J Exp Med* **204**, 2213–2224.
39 Pollard TD (2016) Actin and actin-binding proteins. *Cold Spring Harb Perspect Biol* **8**, a018226.
40 Lappalainen P (2016) Actin-binding proteins: the long road to understanding the dynamic landscape of cellular actin networks. *Mol Biol Cell* **27**, 2519–2522.
41 Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S & Giacca M (2012) Functional screening identifies miRNAs inducing cardiac regeneration. *Nature* **492**, 376–381.
42 Torrini C, Cubero RJ, Dirkx E, Braga L, Ali H, Prosdocimo G, Gutierrez MI, Collesi C, Licastro D, Zentilin L *et al*. (2019) Common regulatory pathways mediate activity of microRNAs inducing cardiomyocyte proliferation. *Cell Rep* **27**, 2759–2771.e5.
43 Bernstein BW & Bamburg JR (2010) ADF/cofilin: a functional node in cell biology. *Trends Cell Biol* **20**, 187–195.

44 Bravo-Cordero JJ, Magalhaes MA, Eddy RJ, Hodgson L & Condeelis J (2013) Functions of cofilin in cell locomotion and invasion. *Nat Rev Mol Cell Biol* **14**, 405–415.
45 Miralles F, Posern G, Zaromytidou AI & Treisman R (2003) Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* **113**, 329–342.
46 Parmacek MS (2007) Myocardin-related transcription factors: critical coactivators regulating cardiovascular development and adaptation. *Circ Res* **100**, 633–644.
47 Olson EN & Nordheim A (2010) Linking actin dynamics and gene transcription to drive cellular motile functions. *Nat Rev Mol Cell Biol* **11**, 353–365.
48 Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, Zanconato F, Le Digabel J, Forcato M, Bicciato S *et al*. (2011) Role of YAP/TAZ in mechanotransduction. *Nature* **474**, 179–183.
49 Panciera T, Azzolin L, Cordenonsi M & Piccolo S (2017) Mechanobiology of YAP and TAZ in physiology and disease. *Nat Rev Mol Cell Biol* **18**, 758–770.
50 Xin M, Kim Y, Sutherland LB, Qi X, McAnally J, Schwartz RJ, Richardson JA, Bassel-Duby R & Olson EN (2011) Regulation of insulin-like growth factor signaling by Yap governs cardiomyocyte proliferation and embryonic heart size. *Sci Signal* **4**, ra70.
51 von Gise A, Lin Z, Schlegelmilch K, Honor LB, Pan GM, Buck JN, Ma Q, Ishiwata T, Zhou B, Camargo FD *et al*. (2012) YAP1, the nuclear target of Hippo signaling, stimulates heart growth through cardiomyocyte proliferation but not hypertrophy. *Proc Natl Acad Sci USA* **109**, 2394–2399.
52 Xin M, Kim Y, Sutherland LB, Murakami M, Qi X, McAnally J, Porrello ER, Mahmoud AI, Tan W, Shelton JM *et al*. (2013) Hippo pathway effector Yap promotes cardiac regeneration. *Proc Natl Acad Sci USA* **110**, 13839–13844.
53 Heallen T, Morikawa Y, Leach J, Tao G, Willerson JT, Johnson RL & Martin JF (2013) Hippo signaling impedes adult heart regeneration. *Development* **140**, 4683–4690.
54 Morikawa Y, Zhang M, Heallen T, Leach J, Tao G, Xiao Y, Bai Y, Li W, Willerson JT & Martin JF (2015) Actin cytoskeletal remodeling with protrusion formation is essential for heart regeneration in Hippo-deficient mice. *Sci Signal* **8**, ra41.
55 Pollard TD & Cooper JA (2009) Actin, a central player in cell shape and movement. *Science* **326**, 1208–1212.
56 Aragona M, Panciera T, Manfrin A, Giulitti S, Michielin F, Elvassore N, Dupont S & Piccolo S (2013) A mechanical checkpoint controls multicellular growth through YAP/TAZ regulation by actin-processing factors. *Cell* **154**, 1047–1059.
57 Gabisonia K, Prosdocimo G, Aquaro GD, Carlucci L, Zentilin L, Secco I, Ali H, Braga L, Gorgodze N, Bernini F *et al*. (2019) MicroRNA therapy stimulates uncontrolled cardiac repair after myocardial infarction in pigs. *Nature* **569**, 418–422.
58 Boggiano JC, Vanderzalm PJ & Fehon RG (2011) Tao-1 phosphorylates Hippo/MST kinases to regulate the Hippo-Salvador-Warts tumor suppressor pathway. *Dev Cell* **21**, 888–895.
59 Poon CL, Lin JI, Zhang X & Harvey KF (2011) The sterile 20-like kinase Tao-1 controls tissue growth by regulating the Salvador-Warts-Hippo pathway. *Dev Cell* **21**, 896–906.
60 Zhao B, Li L, Tumaneng K, Wang CY & Guan KL (2010) A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). *Genes Dev* **24**, 72–85.
61 Diez-Cunado M, Wei K, Bushway PJ, Maurya MR, Perera R, Subramaniam S, Ruiz-Lozano P & Mercola M (2018) miRNAs that induce human cardiomyocyte proliferation converge on the hippo Pathway. *Cell Rep* **23**, 2168–2174.
62 Mana-Capelli S, Paramasivam M, Dutta S & McCollum D (2014) Angiomotins link F-actin architecture to Hippo pathway signaling. *Mol Biol Cell* **25**, 1676–1685.
63 Foster CT, Gualdrini F & Treisman R (2017) Mutual dependence of the MRTF-SRF and YAP-TEAD pathways in cancer-associated fibroblasts is indirect and mediated by cytoskeletal dynamics. *Genes Dev* **31**, 2361–2375.
64 Katschnig AM, Kauer MO, Schwentner R, Tomazou EM, Mutz CN, Linder M, Sibilia M, Alonso J, Aryee DNT & Kovar H (2017) EWS-FLI1 perturbs MRTFB/YAP-1/TEAD target gene regulation inhibiting cytoskeletal autoregulatory feedback in Ewing sarcoma. *Oncogene* **36**, 5995–6005.
65 Yu OM, Miyamoto S & Brown JH (2016) Myocardin-related transcription factor A and yes-associated protein exert dual control in g protein-coupled receptor- and RhoA-mediated transcriptional regulation and cell proliferation. *Mol Cell Biol* **36**, 39–49.
66 Akhmanova A & Steinmetz MO (2015) Control of microtubule organization and dynamics: two ends in the limelight. *Nat Rev Mol Cell Biol* **16**, 711–726.
67 Tran PT, Walker RA & Salmon ED (1997) A metastable intermediate state of microtubule dynamic instability that differs significantly between plus and minus ends. *J Cell Biol* **138**, 105–117.
68 Wloga D & Gaertig J (2010) Post-translational modifications of microtubules. *J Cell Sci* **123**, 3447–3455.

69 Janke C (2014) The tubulin code: molecular components, readout mechanisms, and functions. *J Cell Biol* **206**, 461–472.
70 Desai A, Verma S, Mitchison TJ & Walczak CE (1999) Kin I kinesins are microtubule-destabilizing enzymes. *Cell* **96**, 69–78.
71 Lara-Gonzalez P, Westhorpe FG & Taylor SS (2012) The spindle assembly checkpoint. *Curr Biol* **22**, R966–R980.
72 Cartwright J Jr & Goldstein MA (1985) Microtubules in the heart muscle of the postnatal and adult rat. *J Mol Cell Cardiol* **17**, 1–7.
73 Wang X, Li F, Campbell SE & Gerdes AM (1999) Chronic pressure overload cardiac hypertrophy and failure in guinea pigs: II. Cytoskeletal remodeling. *J Mol Cell Cardiol* **31**, 319–331.
74 Zile MR, Green GR, Schuyler GT, Aurigemma GP, Miller DC, & Cooper GT (2001) Cardiocyte cytoskeleton in patients with left ventricular pressure overload hypertrophy. *J Am Coll Cardiol* **37**, 1080–1084.
75 Tagawa H, Koide M, Sato H, Zile MR, Carabello BA & Cooper GT (1998) Cytoskeletal role in the transition from compensated to decompensated hypertrophy during adult canine left ventricular pressure overloading. *Circ Res* **82**, 751–761.
76 Robison P, Caporizzo MA, Ahmadzadeh H, Bogush AI, Chen CY, Margulies KB, Shenoy VB & Prosser BL (2016) Detyrosinated microtubules buckle and bear load in contracting cardiomyocytes. *Science* **352**, aaf0659.
77 Chen CY, Caporizzo MA, Bedi K, Vite A, Bogush AI, Robison P, Heffler JG, Salomon AK, Kelly NA, Babu A *et al*. (2018) Suppression of detyrosinated microtubules improves cardiomyocyte function in human heart failure. *Nat Med* **24**, 1225–1233.
78 Crossin KL & Carney DH (1981) Evidence that microtubule depolymerization early in the cell cycle is sufficient to initiate DNA synthesis. *Cell* **23**, 61–71.
79 Khan AH, Bloom JS, Faridmoayer E & Smith DJ (2016) Genetic screening reveals a link between Wnt signaling and antitubulin drugs. *Pharmacogenomics J* **16**, 164–172.
80 Fukuda Y, Sano O, Kazetani K, Yamamoto K, Iwata H & Matsui J (2016) Tubulin is a molecular target of the Wnt-activating chemical probe. *BMC Biochem* **17**, 9.
81 Stolz A, Neufeld K, Ertych N & Bastians H (2015) Wnt-mediated protein stabilization ensures proper mitotic microtubule assembly and chromosome segregation. *EMBO Rep*. **16**, 490–499.
82 Anastas JN & Moon RT (2013) WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer* **13**, 11–26.
83 Rao TP & Kuhl M (2010) An updated overview on Wnt signaling pathways: a prelude for more. *Circ Res* **106**, 1798–1806.
84 Ozhan G & Weidinger G (2015) Wnt/beta-catenin signaling in heart regeneration. *Cell Regen (Lond)* **4**, 3.
85 Zhao L, Ben-Yair R, Burns CE & Burns CG (2019) Endocardial notch signaling promotes cardiomyocyte proliferation in the regenerating Zebrafish heart through Wnt pathway antagonism. *Cell Rep* **26**, 546–554.e5.
86 Yang D, Fu W, Li L, Xia X, Liao Q, Yue R, Chen H, Chen X, An S, Zeng C *et al*. (2017) Therapeutic effect of a novel Wnt pathway inhibitor on cardiac regeneration after myocardial infarction. *Clin Sci (Lond)* **131**, 2919–2932.
87 Rybakova IN, Amann KJ & Ervasti JM (1996) A new model for the interaction of dystrophin with F-actin. *J Cell Biol* **135**, 661–672.
88 Ayalon G, Davis JQ, Scotland PB & Bennett V (2008) An ankyrin-based mechanism for functional organization of dystrophin and dystroglycan. *Cell* **135**, 1189–1200.
89 Prins KW, Humston JL, Mehta A, Tate V, Ralston E & Ervasti JM (2009) Dystrophin is a microtubule-associated protein. *J Cell Biol* **186**, 363–369.
90 Drum BM, Yuan C, Li L, Liu Q, Wordeman L & Santana LF (2016) Oxidative stress decreases microtubule growth and stability in ventricular myocytes. *J Mol Cell Cardiol* **93**, 32–43.
91 Harston RK & Kuppuswamy D (2011) Integrins are the necessary links to hypertrophic growth in cardiomyocytes. *J Signal Transduct* **2011**, 1–8.
92 Hemmings L, Rees DJ, Ohanian V, Bolton SJ, Gilmore AP, Patel B, Priddle H, Trevithick JE, Hynes RO & Critchley DR (1996) Talin contains three actin-binding sites each of which is adjacent to a vinculin-binding site. *J Cell Sci* **109**(Pt 11), 2715–2726.
93 Morikawa Y, Heallen T, Leach J, Xiao Y & Martin JF (2017) Dystrophin-glycoprotein complex sequesters Yap to inhibit cardiomyocyte proliferation. *Nature* **547**, 227–231.
94 Bassat E, Mutlak YE, Genzelinakh A, Shadrin IY, Baruch Umansky K, Yifa O, Kain D, Rajchman D, Leach J, Riabov Bassat D *et al*. (2017) The extracellular matrix protein agrin promotes heart regeneration in mice. *Nature* **547**, 179–184.
95 Cartaud A, Stetzkowski-Marden F, Maoui A & Cartaud J (2011) Agrin triggers the clustering of raft-associated acetylcholine receptors through actin cytoskeleton reorganization. *Biol Cell* **103**, 287–301.
96 Chakraborty S, Lakshmanan M, Swa HL, Chen J, Zhang X, Ong YS, Loo LS, Akincilar SC, Gunaratne J, Tergaonkar V *et al*. (2015) An oncogenic role of

Agrin in regulating focal adhesion integrity in hepatocellular carcinoma. *Nat Commun* **6**, 6184.
97 Chakraborty S, Njah K, Pobbati AV, Lim YB, Raju A, Lakshmanan M, Tergaonkar V, Lim CT & Hong W (2017) Agrin as a mechanotransduction signal regulating YAP through the hippo pathway. *Cell Rep* **18**, 2464–2479.
98 Martino F, Perestrelo AR, Vinarsky V, Pagliari S & Forte G (2018) Cellular mechanotransduction: from tension to function. *Front Physiol* **9**, 824.
99 Zhang Q, Ragnauth C, Greener MJ, Shanahan CM & Roberts RG (2002) The nesprins are giant actin-binding proteins, orthologous to *Drosophila melanogaster* muscle protein MSP-300. *Genomics* **80**, 473–481.
100 Wilhelmsen K, Litjens SH, Kuikman I, Tshimbalanga N, Janssen H, van den Bout I, Raymond K & Sonnenberg A (2005) Nesprin-3, a novel outer nuclear membrane protein, associates with the cytoskeletal linker protein plectin. *J Cell Biol* **171**, 799–810.
101 Roux KJ, Crisp ML, Liu Q, Kim D, Kozlov S, Stewart CL & Burke B (2009) Nesprin 4 is an outer nuclear membrane protein that can induce kinesin-mediated cell polarization. *Proc Natl Acad Sci USA* **106**, 2194–2199.
102 Lyon RC, Zanella F, Omens JH & Sheikh F (2015) Mechanotransduction in cardiac hypertrophy and failure. *Circ Res* **116**, 1462–1476.
103 Goldfarb LG & Dalakas MC (2009) Tragedy in a heartbeat: malfunctioning desmin causes skeletal and cardiac muscle disease. *J Clin Invest* **119**, 1806–1813.
104 Zhou C, Li C, Zhou B, Sun H, Koullourou V, Holt I, Puckelwartz MJ, Warren DT, Hayward R, Lin Z *et al.* (2017) Novel nesprin-1 mutations associated with dilated cardiomyopathy cause nuclear envelope disruption and defects in myogenesis. *Hum Mol Genet* **26**, 2258–2276.
105 Salvarani N, Crasto S, Miragoli M, Bertero A, Paulis M, Kunderfranco P, Serio S, Forni A, Lucarelli C, Dal Ferro M *et al.* (2019) The K219T-Lamin mutation induces conduction defects through epigenetic inhibition of SCN5A in human cardiac laminopathy. *Nat Commun* **10**, 2267.
106 van Berlo JH, de Voogt WG, van der Kooi AJ, van Tintelen JP, Bonne G, Yaou RB, Duboc D, Rossenbacker T, Heidbuchel H, de Visser M *et al.* (2005) Meta-analysis of clinical characteristics of 299 carriers of LMNA gene mutations: do lamin A/C mutations portend a high risk of sudden death? *J Mol Med (Berl)* **83**, 79–83.
107 Cupesi M, Yoshioka J, Gannon J, Kudinova A, Stewart CL & Lammerding J (2010) Attenuated hypertrophic response to pressure overload in a lamin A/C haploinsufficiency mouse. *J Mol Cell Cardiol* **48**, 1290–1297.
108 Banerjee I, Zhang J, Moore-Morris T, Pfeiffer E, Buchholz KS, Liu A, Ouyang K, Stroud MJ, Gerace L, Evans SM *et al.* (2014) Targeted ablation of nesprin 1 and nesprin 2 from murine myocardium results in cardiomyopathy, altered nuclear morphology and inhibition of the biomechanical gene response. *PLoS Genet* **10**, e1004114.
109 Cho S, Vashisth M, Abbas A, Majkut S, Vogel K, Xia Y, Ivanovska IL, Irianto J, Tewari M, Zhu K *et al.* (2019) Mechanosensing by the lamina protects against nuclear rupture, DNA damage, and cell-cycle arrest. *Dev Cell* **49**, 920–935.e5.
110 Schoenwolf GC & Garcia-Martinez V (1995) Primitive-streak origin and state of commitment of cells of the cardiovascular system in avian and mammalian embryos. *Cell Mol Biol Res* **41**, 233–240.
111 Brodsky V, Sarkisov DS, Arefyeva AM, Panova NW & Gvasava IG (1994) Polyploidy in cardiac myocytes of normal and hypertrophic human hearts; range of values. *Virchows Arch* **424**, 429–435.
112 Pilny J (1975) The amount of contractile matter per myocardial cell nucleus in the rat during postnatal ontogenesis. II. *Folia Morphol* **23**, 342–346.
113 Hashimoto H, Yuasa S, Tabata H, Tohyama S, Hayashiji N, Hattori F, Muraoka N, Egashira T, Okata S, Yae K *et al.* (2014) Time-lapse imaging of cell cycle dynamics during development in living cardiomyocyte. *J Mol Cell Cardiol* **72**, 241–249.
114 Kelly AM & Chacko S (1976) Myofibril organisation and mitosis in cultured cardiac muscle cells. *Dev Biol* **48**, 421–430.
115 Manasek FJ (1968) Mitosis in developing cardiac muscle. *J Cell Biol* **37**, 191–196.
116 Kaneko H, Okamoto M & Goshima K (1984) Structural change of myofibrils during mitosis of newt embryonic myocardial cells in culture. *Exp Cell Res* **153**, 483–498.
117 Ahuja P, Perriard E, Perriard JC & Ehler E (2004) Sequential myofibrillar breakdown accompanies mitotic division of mammalian cardiomyocytes. *J Cell Sci* **117**, 3295–3306.
118 Engel FB, Schebesta M, Duong MT, Lu G, Ren S, Madwed JB, Jiang H, Wang Y & Keating MT (2005) p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. *Genes Dev* **19**, 1175–1187.
119 Engel FB, Schebesta M & Keating MT (2006) Anillin localization defect in cardiomyocyte binucleation. *J Mol Cell Cardiol* **41**, 601–612.
120 Sleep E, Boue S, Jopling C, Raya M, Raya A & Izpisua Belmonte JC (2010) Transcriptomics approach to investigate zebrafish heart regeneration. *J Cardiovasc Med* **11**, 369–380.
121 Laube F, Heister M, Scholz C, Borchardt T & Braun T (2006) Re-programming of newt cardiomyocytes is

induced by tissue regeneration. *J Cell Sci* **119**, 4719–4729.
122 Wijns W, Vatner SF & Camici PG (1998) Hibernating myocardium. *N Engl J Med* **339**, 173–181.
123 Dispersyn GD, Geuens E, Ver Donck L, Ramaekers FC & Borgers M (2001) Adult rabbit cardiomyocytes undergo hibernation-like dedifferentiation when co-cultured with cardiac fibroblasts. *Cardiovasc Res* **51**, 230–240.
124 Walsh S, Ponten A, Fleischmann BK & Jovinge S (2010) Cardiomyocyte cell cycle control and growth estimation *in vivo*–an analysis based on cardiomyocyte nuclei. *Cardiovasc Res* **86**, 365–373.
125 Ahuja P, Sdek P & MacLellan WR (2007) Cardiac myocyte cell cycle control in development, disease, and regeneration. *Physiol Rev* **87**, 521–544.
126 Martin AF (1981) Turnover of cardiac troponin subunits. Kinetic evidence for a precursor pool of troponin-I. *J Biol Chem* **256**, 964–968.
127 Martin AF, Rabinowitz M, Blough R, Prior G & Zak R (1977) Measurements of half-life of rat cardiac myosin heavy chain with leucyl-tRNA used as precursor pool. *J Biol Chem* **252**, 3422–3429.
128 Morkin E, Kimata S & Skillman JJ (1972) Myosin synthesis and degradation during development of cardiac hypertrophy in the rabbit. *Circ Res* **30**, 690–702.
129 Previs MJ, Beck Previs S, Gulick J, Robbins J & Warshaw DM (2012) Molecular mechanics of cardiac myosin-binding protein C in native thick filaments. *Science* **337**, 1215–1218.
130 Palmer BM, Noguchi T, Wang Y, Heim JR, Alpert NR, Burgon PG, Seidman CE, Seidman JG, Maughan DW & LeWinter MM (2004) Effect of cardiac myosin binding protein-C on mechanoenergetics in mouse myocardium. *Circ Res* **94**, 1615–1622.
131 Jiang J, Burgon PG, Wakimoto H, Onoue K, Gorham JM, O'Meara CC, Fomovsky G, McConnell BK, Lee RT, Seidman JG *et al*. (2015) Cardiac myosin binding protein C regulates postnatal myocyte cytokinesis. *Proc Natl Acad Sci USA* **112**, 9046–9051.
132 Nixon BR, Williams AF, Glennon MS, de Feria AE, Sebag SC, Baldwin HS & Becker JR (2017) Alterations in sarcomere function modify the hyperplastic to hypertrophic transition phase of mammalian cardiomyocyte development. *JCI Insight* **2**, e90656.
133 Centner T, Yano J, Kimura E, McElhinny AS, Pelin K, Witt CC, Bang ML, Trombitas K, Granzier H, Gregorio CC *et al*. (2001) Identification of muscle specific ring finger proteins as potential regulators of the titin kinase domain. *J Mol Biol* **306**, 717–726.
134 Cohen S, Zhai B, Gygi SP & Goldberg AL (2012) Ubiquitylation by Trim32 causes coupled loss of desmin, Z-bands, and thin filaments in muscle atrophy. *J Cell Biol* **198**, 575–589.
135 Fielitz J, van Rooij E, Spencer JA, Shelton JM, Latif S, van der Nagel R, Bezprozvannaya S, de Windt L, Richardson JA, Bassel-Duby R *et al*. (2007) Loss of muscle-specific RING-finger 3 predisposes the heart to cardiac rupture after myocardial infarction. *Proc Natl Acad Sci USA* **104**, 4377–4382.
136 Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K *et al*. (2001) Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* **294**, 1704–1708.
137 Gomes MD, Lecker SH, Jagoe RT, Navon A & Goldberg AL (2001) Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. *Proc Natl Acad Sci USA* **98**, 14440–14445.
138 Volodin A, Kosti I, Goldberg AL & Cohen S (2017) Myofibril breakdown during atrophy is a delayed response requiring the transcription factor PAX4 and desmin depolymerization. *Proc Natl Acad Sci USA* **114**, E1375–E1384.
139 Spaich S, Will RD, Just S, Spaich S, Kuhn C, Frank D, Berger IM, Wiemann S, Korn B, Koegl M *et al*. (2012) F-box and leucine-rich repeat protein 22 is a cardiac-enriched F-box protein that regulates sarcomeric protein turnover and is essential for maintenance of contractile function *in vivo*. *Circ Res* **111**, 1504–1516.
140 Haghikia A, Missol-Kolka E, Tsikas D, Venturini L, Brundiers S, Castoldi M, Muckenthaler MU, Eder M, Stapel B, Thum T *et al*. (2011) Signal transducer and activator of transcription 3-mediated regulation of miR-199a-5p links cardiomyocyte and endothelial cell function in the heart: a key role for ubiquitin-conjugating enzymes. *Eur Heart J* **32**, 1287–1297.
141 Kinbara K, Ishiura S, Tomioka S, Sorimachi H, Jeong SY, Amano S, Kawasaki H, Kolmerer B, Kimura S, Labeit S *et al*. (1998) Purification of native p94, a muscle-specific calpain, and characterization of its autolysis. *Biochem J* **335**(Pt 3), 589–596.
142 Taylor RG, Tassy C, Briand M, Robert N, Briand Y & Ouali A (1995) Proteolytic activity of proteasome on myofibrillar structures. *Mol Biol Rep* **21**, 71–73.
143 Wang W, Schulze CJ, Suarez-Pinzon WL, Dyck JR, Sawicki G & Schulz R (2002) Intracellular action of matrix metalloproteinase-2 accounts for acute myocardial ischemia and reperfusion injury. *Circulation* **106**, 1543–1549.
144 Ali MA, Cho WJ, Hudson B, Kassiri Z, Granzier H & Schulz R (2010) Titin is a target of matrix metalloproteinase-2: implications in myocardial ischemia/reperfusion injury. *Circulation* **122**, 2039–2047.

145 Sung MM, Schulz CG, Wang W, Sawicki G, Bautista-Lopez NL & Schulz R (2007) Matrix metalloproteinase-2 degrades the cytoskeletal protein alpha-actinin in peroxynitrite mediated myocardial injury. *J Mol Cell Cardiol* **43**, 429–436.
146 Sawicki G, Leon H, Sawicka J, Sariahmetoglu M, Schulze CJ, Scott PG, Szczesna-Cordary D & Schulz R (2005) Degradation of myosin light chain in isolated rat hearts subjected to ischemia-reperfusion injury: a new intracellular target for matrix metalloproteinase-2. *Circulation* **112**, 544–552.
147 Kandasamy AD & Schulz R (2009) Glycogen synthase kinase-3beta is activated by matrix metalloproteinase-2 mediated proteolysis in cardiomyoblasts. *Cardiovasc Res* **83**, 698–706.
148 Kubin T, Poling J, Kostin S, Gajawada P, Hein S, Rees W, Wietelmann A, Tanaka M, Lorchner H, Schimanski S *et al*. (2011) Oncostatin M is a major mediator of cardiomyocyte dedifferentiation and remodeling. *Cell Stem Cell* **9**, 420–432.
149 Fan X, Hughes BG, Ali MA, Chan BY, Launier K & Schulz R (2016) Matrix metalloproteinase-2 in oncostatin M-induced sarcomere degeneration in cardiomyocytes. *Am J Physiol Heart Circ Physiol* **311**, H183–H189.
150 Bersell K, Arab S, Haring B & Kuhn B (2009) Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* **138**, 257–270.
151 D'Uva G, Aharonov A, Lauriola M, Kain D, Yahalom-Ronen Y, Carvalho S, Weisinger K, Bassat E, Rajchman D, Yifa O *et al*. (2015) ERBB2 triggers mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation. *Nat Cell Biol* **17**, 627–638.
152 Cheng YY, Yan YT, Lundy DJ, Lo AH, Wang YP, Ruan SC, Lin PJ & Hsieh PC (2017) Reprogramming-derived gene cocktail increases cardiomyocyte proliferation for heart regeneration. *EMBO Mol Med* **9**, 251–264.
153 Farina F, Gaillard J, Guerin C, Coute Y, Sillibourne J, Blanchoin L & Thery M (2016) The centrosome is an actin-organizing centre. *Nat Cell Biol* **18**, 65–75.
154 Mullee LI & Morrison CG (2016) Centrosomes in the DNA damage response–the hub outside the centre. *Chromosome Res* **24**, 35–51.
155 Pascreau G, Churchill ME & Maller JL (2011) Centrosomal localization of cyclins E and A: structural similarities and functional differences. *Cell Cycle* **10**, 199–205.
156 Ma XS, Lin F, Wang ZW, Hu MW, Huang L, Meng TG, Jiang ZZ, Schatten H, Wang ZB & Sun QY (2016) Geminin deletion in mouse oocytes results in impaired embryo development and reduced fertility. *Mol Biol Cell* **27**, 768–775.
157 Bornens M (2012) The centrosome in cells and organisms. *Science* **335**, 422–426.
158 Hearn T (2019) ALMS1 and Alstrom syndrome: a recessive form of metabolic, neurosensory and cardiac deficits. *J Mol Med (Berl)* **97**, 1–17.
159 Shenje LT, Andersen P, Halushka MK, Lui C, Fernandez L, Collin GB, Amat-Alarcon N, Meschino W, Cutz E, Chang K *et al*. (2014) Mutations in Alstrom protein impair terminal differentiation of cardiomyocytes. *Nat Commun* **5**, 3416.
160 Louw JJ, Corveleyn A, Jia Y, Iqbal S, Boshoff D, Gewillig M, Peeters H, Moerman P & Devriendt K (2014) Homozygous loss-of-function mutation in ALMS1 causes the lethal disorder mitogenic cardiomyopathy in two siblings. *Eur J Med Genet* **57**, 532–535.
161 Hergovich A (2016) The roles of NDR protein kinases in hippo signalling. *Genes (Basel)* **7**, 1–16.
162 Pala R, Alomari N & Nauli SM (2017) Primary cilium-dependent signaling mechanisms. *Int J Mol Sci* **18**, 1–19.
163 Kim SE, Huang H, Zhao M, Zhang X, Zhang A, Semonov MV, MacDonald BT, Zhang X, Garcia Abreu J, Peng L *et al*. (2013) Wnt stabilization of beta-catenin reveals principles for morphogen receptor-scaffold assemblies. *Science* **340**, 867–870.
164 Corbit KC, Shyer AE, Dowdle WE, Gaulden J, Singla V, Chen MH, Chuang PT & Reiter JF (2008) Kif3a constrains beta-catenin-dependent Wnt signalling through dual ciliary and non-ciliary mechanisms. *Nat Cell Biol* **10**, 70–76.
165 Singh AP, Umbarkar P, Guo Y, Force T, Gupte M & Lal H (2019) Inhibition of GSK-3 to induce cardiomyocyte proliferation: a recipe for in situ cardiac regeneration. *Cardiovasc Res* **115**, 20–30.
166 Tseng AS, Engel FB & Keating MT (2006) The GSK-3 inhibitor BIO promotes proliferation in mammalian cardiomyocytes. *Chem Biol* **13**, 957–963.
167 Azzolin L, Zanconato F, Bresolin S, Forcato M, Basso G, Bicciato S, Cordenonsi M & Piccolo S (2012) Role of TAZ as mediator of Wnt signaling. *Cell* **151**, 1443–1456.
168 Varelas X, Miller BW, Sopko R, Song S, Gregorieff A, Fellouse FA, Sakuma R, Pawson T, Hunziker W, McNeill H *et al*. (2010) The Hippo pathway regulates Wnt/beta-catenin signaling. *Dev Cell* **18**, 579–591.
169 Coyaud E, Mis M, Laurent EM, Dunham WH, Couzens AL, Robitaille M, Gingras AC, Angers S & Raught B (2015) BioID-based identification of Skp cullin F-box (SCF)beta-TrCP1/2 E3 ligase substrates. *Mol Cell Proteomics* **14**, 1781–1795.
170 Couzens AL, Knight JD, Kean MJ, Teo G, Weiss A, Dunham WH, Lin ZY, Bagshaw RD, Sicheri F, Pawson T *et al*. (2013) Protein interaction network of

the mammalian Hippo pathway reveals mechanisms of kinase-phosphatase interactions. *Sci Signal* **6**, rs15.
171 Zebrowski DC, Vergarajauregui S, Wu CC, Piatkowski T, Becker R, Leone M, Hirth S, Ricciardi F, Falk N, Giessl A *et al*. (2015) Developmental alterations in centrosome integrity contribute to the post-mitotic state of mammalian cardiomyocytes. *eLife* **4**, 1–16.
172 Espigat-Georger A, Dyachuk V, Chemin C, Emorine L & Merdes A (2016) Nuclear alignment in myotubes requires centrosome proteins recruited by nesprin-1. *J Cell Sci* **129**, 4227–4237.
173 Leone M, Musa G & Engel FB (2018) Cardiomyocyte binucleation is associated with aberrant mitotic microtubule distribution, mislocalization of RhoA and IQGAP3, as well as defective actomyosin ring anchorage and cleavage furrow ingression. *Cardiovasc Res* **114**, 1115–1131.
174 Stroud MJ, Kammerer RA & Ballestrem C (2011) Characterization of G2L3 (GAS2-like 3), a new microtubule- and actin-binding protein related to spectraplakins. *J Biol Chem* **286**, 24987–24995.
175 Stroud MJ, Nazgiewicz A, McKenzie EA, Wang Y, Kammerer RA & Ballestrem C (2014) GAS2-like proteins mediate communication between microtubules and actin through interactions with end-binding proteins. *J Cell Sci* **127**, 2672–2682.
176 Pe'er T, Lahmi R, Sharaby Y, Chorni E, Noach M, Vecsler M, Zlotorynski E, Steen H, Steen JA & Tzur A (2013) Gas2l3, a novel constriction site-associated protein whose regulation is mediated by the APC/C Cdh1 complex. *PLoS ONE* **8**, e57532.
177 Stopp S, Grundl M, Fackler M, Malkmus J, Leone M, Naumann R, Frantz S, Wolf E, von Eyss B, Engel FB *et al*. (2017) Deletion of Gas2l3 in mice leads to specific defects in cardiomyocyte cytokinesis during development. *Proc Natl Acad Sci USA* **114**, 8029–8034.
178 Wolter P, Schmitt K, Fackler M, Kremling H, Probst L, Hauser S, Gruss OJ & Gaubatz S (2012) GAS2L3, a target gene of the DREAM complex, is required for proper cytokinesis and genomic stability. *J Cell Sci* **125**, 2393–2406.
179 Grundl M, Engel FB & Gaubatz S (2017) GAS2L3: coordinator of cardiomyocyte cytokinesis? *Cell Cycle* **16**, 1853–1854.
180 Zhao G, Qiu Y, Zhang HM & Yang D (2019) Intercalated discs: cellular adhesion and signaling in heart health and diseases. *Heart Fail Rev* **24**, 115–132.
181 Estigoy CB, Ponten F, Odeberg J, Herbert B, Guilhaus M, Charleston M, Ho JWK, Cameron D & Dos Remedios CG (2009) Intercalated discs: multiple proteins perform multiple functions in non-failing and failing human hearts. *Biophys Rev* **1**, 43–49.
182 Bass-Zubek AE, Godsel LM, Delmar M & Green KJ (2009) Plakophilins: multifunctional scaffolds for adhesion and signaling. *Curr Opin Cell Biol* **21**, 708–716.
183 van Kempen MJ, ten Velde I, Wessels A, Oosthoek PW, Gros D, Jongsma HJ, Moorman AF & Lamers WH (1995) Differential connexin distribution accommodates cardiac function in different species. *Microsc Res Tech* **31**, 420–436.
184 Angst BD, Khan LU, Severs NJ, Whitely K, Rothery S, Thompson RP, Magee AI & Gourdie RG (1997) Dissociated spatial patterning of gap junctions and cell adhesion junctions during postnatal differentiation of ventricular myocardium. *Circ Res* **80**, 88–94.
185 Schlegelmilch K, Mohseni M, Kirak O, Pruszak J, Rodriguez JR, Zhou D, Kreger BT, Vasioukhin V, Avruch J, Brummelkamp TR *et al*. (2011) Yap1 acts downstream of alpha-catenin to control epidermal proliferation. *Cell* **144**, 782–795.
186 Lien WH, Klezovitch O, Fernandez TE, Delrow J & Vasioukhin V (2006) alphaE-catenin controls cerebral cortical size by regulating the hedgehog signaling pathway. *Science* **311**, 1609–1612.
187 Lin Z, von Gise A, Zhou P, Gu F, Ma Q, Jiang J, Yau AL, Buck JN, Gouin KA, van Gorp PR *et al*. (2014) Cardiac-specific YAP activation improves cardiac function and survival in an experimental murine MI model. *Circ Res* **115**, 354–363.
188 Li J, Gao E, Vite A, Yi R, Gomez L, Goossens S, van Roy F & Radice GL (2015) Alpha-catenins control cardiomyocyte proliferation by regulating Yap activity. *Circ Res* **116**, 70–79.
189 Pieperhoff S & Franke WW (2008) The area composita of adhering junctions connecting heart muscle cells of vertebrates. VI. Different precursor structures in non-mammalian species. *Eur J Cell Biol* **87**, 413–430.
190 Canseco DC, Kimura W, Garg S, Mukherjee S, Bhattacharya S, Abdisalaam S, Das S, Asaithamby A, Mammen PP & Sadek HA (2015) Human ventricular unloading induces cardiomyocyte proliferation. *J Am Coll Cardiol* **65**, 892–900.
191 Eschenhagen T, Bolli R, Braun T, Field LJ, Fleischmann BK, Frisen J, Giacca M, Hare JM, Houser S, Lee RT *et al*. (2017) Cardiomyocyte regeneration: a consensus statement. *Circulation* **136**, 680–686.
192 Liu YW, Chen B, Yang X, Fugate JA, Kalucki FA, Futakuchi-Tsuchida A, Couture L, Vogel KW, Astley CA, Baldessari A *et al*. (2018) Human embryonic stem cell-derived cardiomyocytes restore function in infarcted hearts of non-human primates. *Nat Biotechnol* **36**, 597–605.
193 Tiburcy M, Hudson JE, Balfanz P, Schlick S, Meyer T, Chang Liao ML, Levent E, Raad F, Zeidler S,

Wingender E *et al*. (2017) Defined engineered human myocardium with advanced maturation for applications in heart failure modeling and repair. *Circulation* **135**, 1832–1847.

194 Weinberger F, Breckwoldt K, Pecha S, Kelly A, Geertz B, Starbatty J, Yorgan T, Cheng KH, Lessmann K, Stolen T *et al*. (2016) Cardiac repair in guinea pigs with human engineered heart tissue from induced pluripotent stem cells. *Sci Transl Med* **8**, 363ra148.

195 Mohamed TMA, Ang YS, Radzinsky E, Zhou P, Huang Y, Elfenbein A, Foley A, Magnitsky S & Srivastava D (2018) Regulation of cell cycle to stimulate adult cardiomyocyte proliferation and cardiac regeneration. *Cell* **173**, 104–116.e12.

196 Wei K, Serpooshan V, Hurtado C, Diez-Cunado M, Zhao M, Maruyama S, Zhu W, Fajardo G, Noseda M, Nakamura K *et al*. (2015) Epicardial FSTL1 reconstitution regenerates the adult mammalian heart. *Nature* **525**, 479–485.