

Titin: a stiff proposal

Professor Wolfgang A Linke is conducting groundbreaking research into titin, a large protein prevalent in the human body. Here, he talks about his 20 years' experience in the field

PROFESSOR WOLFGANG A LINKE



To begin, could you outline the core objectives of your research?

About two decades ago, my group started working on an obscure and poorly understood protein called titin – the largest in the human body – abundantly found in skeletal and cardiac muscle cells within contractile units called sarcomeres. We developed an approach to measure the mechanical properties of single sarcomeres, which established that titin is mainly responsible for the stiffness of muscle sarcomeres – including cardiac sarcomeres – and largely determines the stiffness of the cardiac muscle cell and cardiac walls.

Titin molecules are like tiny rubber bands, making the muscle extensible and springy, but the spring properties of titin can be pathologically deranged in heart disease. These discoveries set the path for our current objectives; we are studying how titin function is dynamically regulated in cardiac muscle cells. For example, the stiffness of titin, and thus of the cardiac wall, can be acutely altered by biochemical modification called phosphorylation; we are studying the molecular basis for these modifications and seeking novel treatments to rectify problems in failing hearts.

An atomic force microscope (AFM) was used by your team to study the mechanical properties of single cytoskeletal proteins from muscle. Were any interesting results uncovered during this process?

We did not develop this technique, but were among the first to adapt it to our specific needs, revealing that the mechanical properties of the single titin molecule explain the elastic properties of a cardiomyocyte. Using single-molecule AFM force spectroscopy, we were

the first to show that phosphorylation of titin alters its elasticity, demonstrating why this biochemical modification has such an impact on cardiac muscle cell and cardiac wall stiffness. Although these measurements represent a very reductive approach, they are extremely useful to understand the function of titin at an atomic level.

Diastolic heart failure (DHF) accounts for a large proportion of heart failure cases in the Western world. What more can be done to understand its pathophysiology?

The symptoms of DHF are less well defined than for systolic heart failure making it harder to diagnose, with treatment options not really currently available to the cardiologist. The main problem is a lack of mechanistic understanding. However, cardinal symptoms in DHF do include increased cardiac wall stiffness and subsequently reduced diastolic filling capacities of the heart ventricles. Because titin greatly contributes to cardiac wall stiffness, and because phosphorylation can reduce this, we are looking into therapeutic approaches which target titin, or enzymes that phosphorylate this protein. Reducing this stiffness would likely benefit many DHF patients.

How has research on biological changes in the giant polypeptide titin enabled you to progress with your work?

There is increasing evidence that titin is genetically altered in patients with heart or muscle conditions such as cardiomyopathy and muscular dystrophy. The most common inherited cardiomyopathy is dilated cardiomyopathy (DCM), associated with more than 30 known disease genes, where the heart becomes weakened and enlarged and cannot pump blood efficiently. We have learned very recently that by far the most frequently mutated gene in human DCM is TTN, which encodes titin. Titin has since gained much attention from physicians, but the mechanisms involved are not well established; we want to address this by studying the functional consequences of titin mutations in cardiac muscle cells.

Where are you hoping to focus your research efforts next? Do you currently have any unique plans for the study of vertebrate muscle cells?

The heart is an amazing organ which starts beating about three weeks after conception and can continue uninterrupted for 100 years. What causes many hearts to stop before this is still not fully understood. We are interested in why

a heart, which can compensate minor forms of pathological remodelling for years, suddenly becomes weak and thin-walled. From observing that mutations in many different cytoskeletal proteins can cause chronic heart disease, we know the sarcomeric cytoskeleton of the cardiac muscle cells likely plays an important role in the disease-related remodelling processes. We will continue our basic research on the sarcomere; learn more about the diverse functions of titin; and proceed with translational science – using knowledge of basic mechanisms to answer relevant research questions. We will also touch on the molecular mechanisms of mechanical signalling and mechanosensing in the heart and their role in heart disease.

A molecular approach to diastolic heart failure

Ongoing studies at the **Ruhr University**, Germany, are seeking new targets in the fight against diastolic heart failure. The researchers are shedding light on the elasticity of heart muscle cells

DIASTOLIC HEART FAILURE (DHF)

accounts for over 50 per cent of all heart failure cases in the Western world and, as such, presents a major challenge to public healthcare. Despite the high incidence of the disease, understandings of the mechanisms behind it, and clinical outcomes, remain poor. Current treatment recommendations are often reliant on disease-orientated evidence such as pathophysiology; extrapolations from similar cardiovascular conditions; results from relatively small studies; and expert opinion. In order to develop novel therapeutic approaches, knowledge of the basic mechanisms which alter diastolic function must first be improved.

THE ROLE OF TITIN

Studies at the Department of Cardiovascular Physiology, Ruhr University in Bochum, Germany, led by Professor Wolfgang Linke, have been looking into the function of the protein titin in relation to DHF. Despite the fact that the average adult human body contains up to half a kilogram in dry weight of titin, very little was known about the protein's function or structure when Linke and his fellow researchers began their work – and even less was understood about its role in heart disease.

Since Linke's work began 20 years ago, knowledge on the role of titin in DHF has started to unravel, although much research remains to be done. DHF is characterised by abnormal diastolic function of the left ventricle (LV), two prominent features of which are impaired relaxation and an increase in diastolic stiffness. The latter is evident from high cardiomyocyte passive stiffness

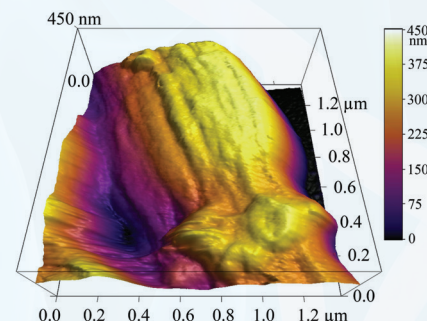
(Fpassive), which is associated with the elastic properties of titin. Thus, Linke and his colleagues are attempting to devise therapies which target titin and its role in abnormalities of myocardial function present in patients with DHF.

A NODAL POINT?

Linke believes that titin could be a crucial regulatory node in the sarcomeric cytoskeleton. The protein is involved in interactions with multiple molecules, some of which are known to participate in signalling pathways which cause increased cardiac growth, or hypertrophy, which can either be physiologic or pathologic, and includes an inherited condition called familial hypertrophic cardiomyopathy. This, and other forms of hypertrophy, can result from increased muscle stretch, which causes titin to extend and perhaps trigger hypertrophic gene activation. Linke explains: "This observation leads us to speculate that titin is a nodal point within the cardiac muscle cell, integrating and perhaps even coordinating some of these hypertrophic signalling pathways". In addition to this, stretch could be a factor in the phosphorylation of titin.

SILDENAFIL

Linke's laboratory has also been involved in a collaborative study with an experienced team of clinician-scientists at the Mayo Clinic in Rochester, USA, who have created a significant animal model which demonstrated similar diastolic abnormalities to those shown in cases of human DHF. Having identified titin as a substrate of phosphorylating enzymes called kinases, which act to reduce titin-based

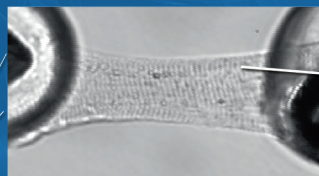
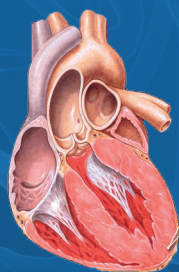


A MUSCLE SARCOMERE RECORDED BY ATOMIC FORCE MICROSCOPY

stiffness, the researchers attempted to boost the activity of one such kinase – protein kinase G – in the hope that it would increase cardiac titin phosphorylation and lower stiffness in the cardiac wall.

The investigation was successful; a drug called Sildenafil – better known as the active ingredient in Viagra – made it easier for the heart to fill with blood, partially restoring diastolic function. Whilst studies demonstrate that Sildenafil will not aid all sufferers of heart disease – although it can improve symptoms in some systolic heart failure patients – will continue to assess whether the results from these animal models are mirrored in human DHF patients. A clinical trial known as RELAX is currently underway, and the researchers remain optimistic that this approach could lead to a novel therapeutic strategy. In order to fine-tune such a strategy, Linke's team is also testing Sildenafil on genetically modified mice in which they have inactivated one of the components of the protein kinase G signalling

MECHANICAL STUDIES ON SINGLE CARDIAC MUSCLE CELLS AND MYOFIBRILS



ISOLATED MUSCLE CELL



SINGLE MYOFIBRIL

INTELLIGENCE

LINKE LAB

OBJECTIVES

To elucidate the molecular basis of elasticity of skeletal and heart muscle cells, especially the function of the giant protein titin; understand the dynamics of the specialised muscle cytoskeleton (focus: sarcomere) under physiological and disease conditions; assess the role of cardiomyocyte remodelling in heart failure, including human diastolic and systolic heart failure; detect novel signalling pathways important for muscle mechanosensing; and detect novel potential targets of pharmacological intervention within the cardiomyocytes, with the aim of reducing pathologically high diastolic myocardial stiffness in heart failure.

KEY COLLABORATORS

Dr Johannes Backs, University of Heidelberg, Germany • **Professor Cristobal dos Remedios**, University of Sydney, Australia • **Professor Julio M Fernandez**, Columbia University, USA • **Professor Gerd Hasenfuss**, University Medicine Center, Göttingen, Germany • **Professor Walter Paulus**; **Dr Jolanda van der Velden**, University of Amsterdam, The Netherlands • **Professor Margaret M Redfield**, Mayo Clinic Rochester, USA

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undertook his PhD at Halle-Wittenberg, Germany, before becoming a postdoc at the University of Washington in the USA. He received his Habilitation at the University of Heidelberg in Germany. Linke is currently Professor at the Ruhr University Bochum, Germany, and Adjunct Professor of Cardiac Mechanotransduction at the Heart Center, University Medicine Center, Göttingen, Germany. He sits on the editorial boards of a number of journals, including the *Journal of Molecular and Cellular Cardiology*, *Journal of Muscle Research and Cell Motility* and the *European Journal of Cardiovascular Medicine*.

pathway. This will enable the researchers to test how the stiffness of hearts in mice responds to Sildenafil treatment.

PROTEIN METHYLATION

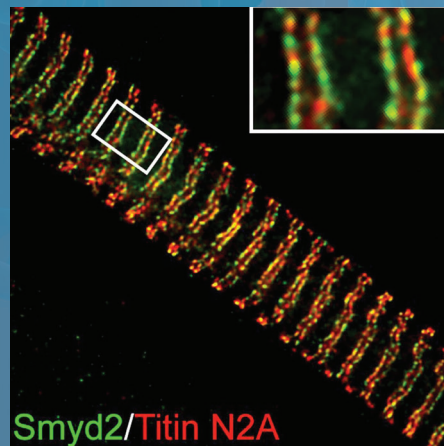
More recently, Linke and his fellow researchers have become fascinated by the role which protein methylation appears to play in the function of skeletal and cardiac muscle cells. "We discovered that a lysine methyltransferase – an enzyme which transfers methyl groups to a certain amino acid in proteins – is abundant in the muscle cytoplasm, where among others it binds to titin," he reveals. This discovery, in the context that protein lysine methylation is one of the most common biochemical modifications that occurs in the nuclei of eukaryotic cells, is potentially very exciting. Lysine methylation of nuclear proteins acts to increase the formation of protein complexes which are in control of gene expression and the replication and repair of DNA.

The effect of this process on the cytoplasm remains unclear, but Linke suggests that the cytoplasmic lysine methylation could perform a similar role as it does in the nucleus, promoting the formation of protein complexes. "In this instance, a complex between the lysine methyltransferase, titin and an important chaperone or heat shock protein called Hsp90 is formed in the cytoplasm of cardiac or skeletal muscle cells upon methylation of Hsp90," he elaborates. This modification seemed, in Linke's trials, to help control the quality of the titin molecule, aiding it to remain functional. The group is eager to continue these investigations, with much hope that they can learn more about protein quality control in the cardiac muscle cells under both normal and disease conditions.

SPlicing FACTORS

Despite the fact that there is only one titin gene in humans, the protein exists in multiple isoforms. These can vary hugely in size, and are a result of the alternative splicing of the titin messenger ribonucleic acid (mRNA) following gene transcription. This alternative splicing of mRNA, which occurs in the nucleus, can only come about

Muscle fibre with the elastic titin region and methyltransferase Smyd2 visualised by immunofluorescence microscopy.

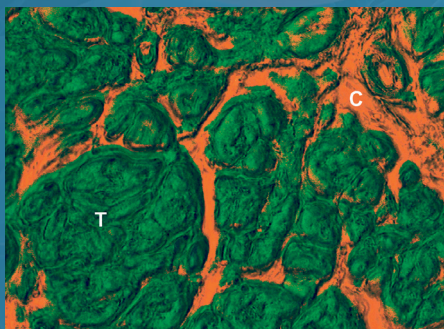


The study was successful; a drug called Sildenafil made it easier for the heart to fill with blood, partially restoring diastolic function

with the contribution of splicing factors, one of which, it has recently been discovered, can cause aberrant titin splicing and expression of abnormal titin isoforms if mutated or missing in the heart. Although the splicing of around 30 proteins is deregulated by such a mutated splicing factor, the changes it produces in titin are the most significant, creating discrepancies in the size of titin isoforms of hundreds of kilodaltons (proteins are normally no larger than around 200 kilodaltons). Because the composition of titin isoforms is pathologically altered in failing hearts, as Linke's group has previously shown, he and his collaborators are looking at the role played by these splicing factors in alterations to the heart's function.

LOOKING AHEAD

Given the complexity of the role and functions of titin, the road ahead for Linke's group remains a long one. Their studies, though, have already shed light on the molecular basis of the elasticity of skeletal and heart muscle cells; the mechanical mechanisms of the specialised muscle cytoskeleton; the role of cardiomyocyte remodeling in failing hearts; and, to some extent, the signalling pathways involved in mechanosensing of muscles. As their understanding of these grows, it will become increasingly likely that they can develop a novel pharmacological intervention that will reduce pathologically high diastolic myocardial stiffness in those with heart failure.



Cross-section of failing human myocardium with muscle cells (titin-T-green) and interstitial tissue (collagen-C-orange) highlighted.