6 Summary

Titin M-line deficiency causes impaired myofibril maturation and cardiac dysfunction

The basic contractile unit of the striated muscle myofibril in vertebrates is the sarcomere. The filaments Myosin and Actin form a continuous system in the sarcomere. The third filament system is formed by Titin, the largest protein known so far. It contains different functional units that enable Titin to integrate into the Z-disc and M-band, provide elasticity, and support sarcomere assembly. Additionally, the presence of phosphorylation sites, a kinase domain, and binding sites for structural and signaling proteins suggest a role for Titin in signal transduction. Multiple Titin deficient animal models and cell lines have been investigated but so far there are no *in vivo* data on the function of Titin's M-line in early cardiac development.

To distinguish the role for M-line Titin in sarcomere assembly from a role in stabilizing preexisting sarcomeres and to address potential non-muscle functions, the conditional Titin M-line knockout was converted into a constitutive knockout. Heterozygous mice were viable and fertile. In contrast, homozygous Titin M-line deficient mice were not viable. Morphological studies revealed that knockout embryos developed normally until E9.0 apparent through a contracting heart and a regular number of somites. From E9.5 development of knockout embryos was delayed. They showed a reduced body size and number of somites, decreased trabeculation of the heart ventricle, and a thin myocardium. Knockouts failed to thrive and cardiac dysfunction caused lethality by E11.5. Ultrastructural analysis confirmed initial sarcomere assembly at E9.0 but impairment of lateral growth and destabilization of the sarcomere. From E10.5 sarcomeres disassembled and only few filaments in disarray remained at E11.0. Integration of Titin into the sarcomere was monitored by immunofluorescence studies. In knockout cardiac muscle cells Titin's N-terminus was incorporated into the Z-disc whereas Titin's M-line region did not integrate into the M-band. This suggested that the assembly of the I-band is an early event that is independent of Titin's M-line.

Titin's M-line provides binding sites for several structural and signaling proteins. Deletion of Titin's M-line might change expression or localization of these proteins and cause

disassembly. The disassembly of the knockout sarcomeres could likely be mediated by the structural protein Myomesin-EH. It contributes to the M-band lattice by interacting with Myosin and Titin. In this study Myomesin-EH was highly expressed in wildtype and knockout embryos. Immunostainings showed integration of Myomesin into the M-band independent of Titin's M-line. Localization was more diffuse most likely due to the absence of its binding site to Titin. The failure of Myomesin to cross-link Titin in the M-band could result in increased mobility of the sarcomeric filaments and thus enhance mechanical strain on the growing sarcomere leading to its disassembly.

Titin's kinase domain has been implied in sarcomere assembly and the control of muscle gene expression through the *in vitro* substrates Nbr1, Sqstm1, and T-cap. So far, no animal or tissue culture model was available to proof this hypothesis. This study showed that the phosphorylation of T-cap by Titin's kinase can not be the regulatory principle for initial cardiac sarcomere assembly. Not only did sarcomeres form in the absence of Titin's kinase, but furthermore T-cap was not detectable in early sarcomere development. Moreover, the substrates Nbr1 and Sqstm1 as well as the adaptor protein FHL2 were not expressed in significant amounts at the time when the knockout phenotype developed suggesting a role in maintenance of preexisting sarcomeres rather than assembly of sarcomeres. Titin's M-line region also contains binding sites for the ubiquitin ligases MuRF-1 and MuRF-2. These proteins act as cytoskeletal adaptors and signaling molecules. MuRF-1 is involved in protein degradation in the atrophic skeletal muscle and MuRF-2 regulates gene expression by interacting with Sqstm1. In this study both proteins were highly expressed at the time when the knockout phenotype developed. But neither the absence of Titin's M-line nor the lack of Sqstm1 nor the resulting atrophy altered the expression of MuRF-1 and MuRF-2. However, differences in the expression of sarcomeric proteins in the embryonic versus the adult heart refer to different molecular mechanisms of sarcomere assembly and maintenance of preexisting structures.

In the adult Titin M-line knockout mice, changes in the mitogen-activated protein (MAP) kinase signal transduction were observed. This is part of the hypertrophic signaling response secondary to the cardiomyopathy phenotype. However, differential regulation of hypertrophy markers, including genes in the MAP kinase pathway could not be detected in the embryonic heart of knockouts. Thus, the mechanism underlying impaired growth of the myofibril was independent of MAPK signal transduction.

In addition to striated muscle cells, Titin has been detected in non-muscle tissue such as in intestinal epithelial cells. Non-muscle functions of Titin were proposed through its localization to the mitotic spindle machinery and its interaction with the nuclear filament protein Lamin. Neither the presented Titin M-line knockout nor the published fish and mouse models did display any non-muscle phenotype apart from defects secondary to impaired cardiac and skeletal muscle function. Furthermore, cell culture data showed that mitosis did not require the Titin kinase since isolated cells divided and differentiated into various cell types such as cardiomyocytes.

This study provided *in vivo* data indicating that Titin's M-line is dispensable for initial sarcomere assembly but required for lateral growth and stabilization of the sarcomere. Furthermore it was shown that structural, mechanical, and signaling functions of Titin and its interacting proteins in the embryonic sarcomere are different from those observed in adult. The mouse model helps to understand how Titin and its binding protein shape and regulate sarcomere assembly in early cardiac development. The insights into the pathophysiology and molecular mechanism of cardiomyopathy can be used to develop new therapeutic strategies for cardiovascular diseases.