Titin visualization in real time reveals an unexpected level of mobility within and between sarcomeres

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The giant muscle protein titin is an essential structural component of the sarcomere. It forms a continuous periodic backbone along the myofiber that provides resistance to mechanical strain. Thus, the titin filament has been regarded as a blueprint for sarcomere assembly and a prerequisite for stability. Here, a novel titin-eGFP knockin mouse provided evidence that sarcomeric titin is more dynamic than previously suggested. To study the mobility of titin in embryonic and neonatal cardiomyocytes, we used fluorescence recovery after photobleaching and investigated the contribution of protein synthesis, contractility, and calcium load to titin motility. Overall, the kinetics of lateral and longitudinal movement of titin-eGFP were similar. Whereas protein synthesis and developmental stage did not alter titin dynamics, there was a strong, inhibitory effect of calcium on titin mobility. Our results suggest a model in which the largely unrestricted movement of titin within and between sarcomeres primarily depends on calcium, suggesting that fortification of the titin filament system is activity dependent.

Introduction

The sarcomeric protein titin alias connectin is, after actin and myosin, the third most abundant protein in vertebrate striated muscle and expressed from mid-gestation through adult life (Fürst et al., 1989; Schaart et al., 1989). Its functional domains are assembled into various titin isoforms to adjust its mechanical and structural properties depending on developmental stage, functional requirements, and underlying disease (Neagoe et al., 2002; Lahmers et al., 2004; Opitz et al., 2004; Warren et al., 2004). The large cardiac titin N2BA isoform (3.5–3.7 MDa) is rapidly replaced by the smaller N2B isoform (3.0 MDa) both after birth and with reexpression of the fetal gene program in cardiac pathology (Neagoe et al., 2002; Lahmers et al., 2004; Makarenko et al., 2004; Opitz et al., 2004; Warren et al., 2004). This change in titin isoform expression helps adapt the elastic properties of the myocardium to enable efficient filling of the cardiac ventricle in diastole and has been characterized in detail both on the molecular and functional level (Lahmers et al., 2004; Opitz et al., 2004). Nevertheless, there is a gap in knowledge on how the altered titin isoform makeup is translated into altered sarcomeric protein composition, i.e., how titin molecules are replaced and relocated in the working sarcomere to adapt cardiac function.

Although the maintenance and remodeling of preexisting sarcomeres and the balance of assembly and disassembly in the working myocardium are still poorly understood, there has been considerable progress toward elucidating de novo sarcomere assembly during embryonic development (Dabiri et al., 1997; Du et al., 2003; Wang et al., 2005a; Weinert et al., 2006; Stout et al., 2008; Sanger et al., 2009). According to the pre-myofibril model, the initial formation of regular sarcomeres involves the polymerization of actin, incorporation of myosin, as well as assembly and alignment of Z-bodies, which incorporate titin’s N terminus and form the future Z-disc (Rhee et al., 1994; Sanger et al., 2000; Du et al., 2003). Subsequently titin’s C terminus is integrated into the M-band and connected to the muscle myosin filament (Nave et al., 1989; Obermann et al., 1996). The resulting continuous filament system has been regarded as a molecular ruler and as a blueprint for sarcomere assembly because titin’s PEVK-region, immunoglobulin, fibronectin, and kinase domains are associated with specific sections of the half-sarcomere and thus sublocalize the various titin-binding proteins along the myofilament (Labiet and Kolmerer, 1995; Trinick, 1996; van der Loop et al., 1996; Obermann et al., 1997; Gregorio et al., 1998). Within the

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Abbreviations used in this paper: BDM, 2,3-butanedione monoxime; CX, cycloheximide; Flp, flippase recombination enzyme; FRT, flippase recognition target; Neo, neomycin; ROI, region of interest.

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Z-disc, titin binds to T-cap alias telethonin (Gregorio et al., 1998), which assembles titin’s N terminus into an antiparallel sandwich complex (Zou et al., 2006). Titin’s structural relations to the thin filament are mediated by α-actinin, which connects to titin in the Z-disc (Ohtsuka et al., 1997a,b; Sorimachi et al., 1997). The interaction between titin’s PEVK region and actin within the I-band is calcium dependent and has been related to the passive properties of the sarcomere and its relaxation kinetics (Kulke et al., 2001; Yamasaki et al., 2001). Within the A-band titin is tightly linked to the thick filament via its multiple binding sites for myosin-binding protein C (MyBP-C; Labeit et al., 1992; Houmeida et al., 1995; Freiburg and Gautel, 1996). The titin–myosin interaction is reinforced at the M-band where titin interacts with myomesin and M-protein—both relevant for the assembly and structural maintenance of thick filaments (Bähler et al., 1985; Nave et al., 1989; Vinkemeier et al., 1993; Obermann et al., 1996). Thus, titin’s integration into the sarcomeric lattice is mediated by its interaction with multiple structural proteins along the half-sarcomere and provides an elastic connection between the thick and thin filament systems, thereby centering the A-band in the sarcomere (Houmeida et al., 1995).

In addition to its structural functions, titin relates to signal transduction and metabolism through its kinase domain, phosphorylation sites, and interaction with adaptor and signaling proteins. Four-and-a-half LIM domain protein 2 (FHL2) recruits metabolic enzymes to sites of high energy consumption such as the M-band and the cardiac N2B region within the I-band (Lange et al., 2002). Titin’s N2A region binds muscle ankyrin repeat proteins, which link myofibrillar stretch-induced signaling pathways and muscle gene expression (Miller et al., 2003) and the protease calpain 3 (p94), which has been suggested to play a role in myofibrillogenesis and sarcomere remodeling (Sorimachi et al., 1995; Kramerova et al., 2004). Additional binding sites for proteins related to signal transduction and protein degradation, such as MuRF (muscle-specific RING finger protein) 1 and 2 and calpain 3 are located at titin’s M-band (Kinbara et al., 1997; Centner et al., 2001; Lange et al., 2002; Witt et al., 2005). Thus, multiple binding proteins integrate titin into the sarcomere and decorate the titin filament with links to basic cellular functions such as trophic signaling and energy balance.

Although the mature myofibril appears as a stable structure, it is crucial that its components can readily detach, not only to replace proteins but also to adapt in conditions that require atrophy or hypertrophy. Thus, cardiac proteins are actively exchanged between organized structures such as the sarcomere and cytoplasmic pools (McKenna et al., 1985a; Sanger et al., 1986; Mittal et al., 1987). Within a few hours after injection, fluorescently labeled proteins are properly incorporated into the sarcomere (McKenna et al., 1985b; Mittal et al., 1987; Dome et al., 1988). The dynamics of proteins within the sarcomere as determined by photobleaching (exchange of bleached by neighboring fluorescent molecules) was reported to be much faster than expected based on their protein half-lives in the order of several days (Zak et al., 1977; Suzuki et al., 1998; Hasebe-Kishi and Shimada, 2000; Wang et al., 2005a; Skwarek-Maruszewska et al., 2009). Although the mobility of various titin-binding proteins has been investigated and related to sarcomere stability, stress, and signal transduction (McElhinny et al., 2002; Pizon et al., 2002; Miller et al., 2003), it has so far not been possible to obtain similar data for titin itself based on technical challenges related to titin’s exceptional size.

Here, we have generated a titin-eGFP knockin mouse and used fluorescence recovery after photobleaching (FRAP) to study titin dynamics in living cells. We were able to dissect titin mobility from de novo protein synthesis and demonstrated that the migration of titin molecules depends on calcium. We show that titin moves equally efficient within and between adjacent sarcomeres and that this process does not depend on cardiomyocyte contraction or major rearrangements of the myofilament. Furthermore, we provide evidence that titin dynamics are independent from the developmental stage as embryonic and neonatal cardiomyocytes display similar titin mobility. Our data suggest that titin molecules are rapidly exchanged between a pool of sarcomeric (bound) and nonsarcomeric (free) titin and that its mobility is inversely correlated with calcium levels but maintained from embryonic to postnatal development.

**Results**

**Generation and validation of the animal model**

Due to its size of up to 3.7 MDa, titin is not readily available for overexpression, gain-of-function studies, or the generation of a tagged full-length protein. To circumvent this problem, we have used gene targeting of the titin locus and generated a fluorescently labeled titin protein for expression analysis and real-time imaging. The targeting vector (Fig. 1 A) was designed to integrate eGFP into titin’s M-band exon 6 with a Neo resistance gene inserted into the 3′-untranslated region. After electroporation, selection, and verification of the targeted allele, positive embryonic stem cells were injected into blastocysts as described previously (Gotthardt et al., 2003). After germline transmission and excision of the Neo resistance cassette, subsequent generations were bred to hetero- or homozygosity as experiments warranted. Knockin, heterozygous, and wild-type animals were born at the expected Mendelian ratios. Both hetero- and homozygotes were viable and fertile, without any obvious phenotypic defects or changes in heart to body weight ratio (Fig. 1, B and C). Expression of titin-eGFP was confirmed by qPCR and Western blot, which both resulted in a strong signal in homozygote and an intermediate expression in heterozygote animals (Fig. 1, D and E; Fig. S1). The robust expression of titin-eGFP protein allowed for the detection of the native eGFP-fusion protein even in heterozygotes (Fig. 1 F). The periodic distribution of the fluorescent signal as detected by confocal imaging suggested proper integration of titin-eGFP into the sarcomere, which was confirmed by costaining with Titin-eGFP-tag at the M-band in heart, quadriceps, and soleus muscle.
was used to assess the quality of the culture. We used FRAP as a parameter of titin mobility with a minimal region of interest (ROI) that comprises one sarcomere length to reduce phototoxicity. Our preliminary analysis indicated stable recovery within 14 h. Calculated half-fluorescence mobility and mobile fractions did not change with an additional incubation through 24 h. Although myocytes contract and shift position in culture over time, the area of photobleaching was stable throughout the experiment. Based on these experiments we used homozygous titin-eGFP knockin cardiomyocytes to increase the signal-to-noise ratio and followed the exchange of bleached by unbleached titin-eGFP molecules for 14 h to reach a steady state of recovery (Fig. 3 A) and reduce the possibility for changes in the cellular phenotype in culture. To distinguish changes in the eGFP signal...
that result from protein synthesis versus protein mobility, we used cycloheximide (CX) as an inhibitor of protein synthesis that blocks translation elongation and has been applied to embryonic as well as to neonatal cardiomyocytes (Zaal et al., 1999; Wang et al., 2005a; Stahlhut et al., 2006). CX was added to the media 1 h before photobleaching and continued throughout the experiment. Fluorescence recovery rates were obtained by plotting the green fluorescence signal as a function of time after photobleaching. FRAP analysis of control cells (without treatment) as compared with CX-treated cells resulted in similar kinetics with complete fluorescence recovery after 14 h. Analysis of the recovery profile indicated no significant change in mobile fractions (49 ± 4% vs. 53 ± 9%) or half-lives of fluorescence recovery (2.2 vs. 2.6 h) in control compared with CX-treated cells (Fig. 3, B and C; Table I).

To dissect the contribution of sarcomere rearrangement versus molecular movement of titin-eGFP, we increased the ROI as indicated in Fig. 4. The mobile fraction was unchanged

Figure 2. Integration of titin-eGFP into the sarcomere. Expression and localization of titin-eGFP were documented in the heart, quadriceps, and soleus of homozygous, adult knockin mice. Coimmunofluorescence staining with antibodies directed against the Z-disc protein α-actinin (A), titin’s Z1/Z2 epitope (B), and titin’s M-band epitope MB/M9 (C) confirmed that titin-eGFP was properly integrated into the Z-disc and M-band of the sarcomere where it colocalized with the MB/M9 epitope in all striated muscle tissues analyzed. Bar, 10 µm.

Figure 3. Titin-eGFP dynamics are independent from protein synthesis. Cells were imaged for 14 h after photobleaching to follow recovery and treated with cycloheximide (CX) to distinguish titin mobility and de novo synthesis. (A) Images of control and CX-treated embryonic cardiomyocytes (E13.5) from homozygous titin-eGFP knockin mice were obtained before and immediately after bleaching (white arrows). Titin-eGFP was homogeneously distributed in embryonic cardiomyocytes and fluorescence had significantly recovered after 14 h. Bar, 10 µm. (B) Plot of fluorescence intensity vs. time after photobleaching to compare titin-eGFP protein recovery of control and CX-treated cells. Inhibition of protein synthesis did not influence the mobility of titin-eGFP (control, n = 4; CX, n = 3). (C) Quantification of the titin-eGFP mobile fractions indicated a level of ~50% in both control and CX-treated cardiomyocytes (control, n = 4; CX, n = 3). Error bars indicate SEM.
indicate that fluorescence recovery cannot solely be explained by rearrangement of myofibers but relates to the movement of individual titin-eGFP molecules.

Taken together, these data suggest that the titin filament system is not a rigid structure but contains a pool of titin molecules that migrate—albeit at a slower pace than other sarcomeric proteins—within the sarcomeric structure. Importantly, inhibition of protein synthesis revealed the same size of the dynamic titin pool and did not influence the mobility so that the changes in fluorescence are attributable to changes in titin mobility and not de novo synthesis of titin protein.

**Longitudinal and lateral dynamics of titin-eGFP**

Titin molecules are integrated into the M-band and Z-disc (Fürst et al., 1988; Wang et al., 1991; Labeit et al., 1997) and longitudinal movement along the sarcomere would require titin to disconnect, whereas lateral movement would retain titin within the half-sarcomere and merely require neighboring titin molecules to exchange positions. Conceivably, this could result in different kinetics for lateral and longitudinal diffusion of titin. To compare the mobility of titin within the myofibril (lateral movement) and between adjacent myofibrils (longitudinal movement), we followed titin movement in the photobleached area of 8 × 8 sarcomere lengths (Fig. S2; Video 1) and did not detect major differences in repopulation from lateral versus longitudinal. Furthermore, we altered the shape of the bleached area as indicated between small, medium, and large bleached areas, suggesting that the relative amount of titin-eGFP that can move into the bleached ROI is independent from the size of the area. The calculated exchange half-life is increased with the area of interest (Table II)—possibly reflecting the larger distance adjacent fluorescent molecules have to travel to completely fill the central photobleached area. Fluorescence recovery is indeed slightly faster at the edges of the bleached area (Fig. S2 and Video 1). Importantly, filament direction and spacing were remarkably conserved over the 14-h imaging period (Fig. 4; Fig. S2). This would indicate that fluorescence recovery cannot solely be explained by rearrangement of myofibers but relates to the movement of individual titin-eGFP molecules.

| Treatment or cell type | Mf [%] | t1/2 [h] |
|-----------------------|--------|----------|
| Control               | 49 ± 4 | 2.1 ± 0.4|
| CX                   | 53 ± 9 | 2.6 ± 0.4|
| LCa                  | 72 ± 4**| 3.6 ± 0.6|
| HCa                  | 28 ± 5*| 2.9 ± 0.9|
| BDM                  | 38 ± 1 | 2.3 ± 0.3|
| BDM + HCa            | 24 ± 1*| 1.7 ± 0.8|
| NCM                  | 56 ± 3 | 3.7 ± 0.4|

Control, embryonic cardiomyocytes without treatment at normal calcium (NCa); CX, cycloheximide treatment; LCa, low calcium concentration; HCa, high calcium concentration; BDM, 2,3-butanedione monoxime; NCM, neonatal cardiomyocytes (n = 24).

*, P < 0.05.

**, P < 0.01; one-way ANOVA, posttest Dunnett (comparison vs. control). Data are indicated as means ± SEM.
in Fig. 5 A with bleaching of 3 or 8 consecutive sarcomeres along or across the myofiber. Lateral and longitudinal movement were equally efficient as indicated in Fig. 5, B and C. Comparison of the mobile fractions longitudinal versus lateral for 3 sarcomeres (62 ± 5 and 58 ± 8, respectively) and longitudinal versus lateral for 8 sarcomeres (59 ± 1 and 47 ± 8, respectively) demonstrate that titin protein exchange occurs within one myofibril as well as between adjacent myofibrils at similar rates (Fig. 5, D and E). Furthermore, we found similar kinetics comparing lateral and longitudinal titin movement as indicated by the exchange half-lives of protein mobility (Fig. 5, D and E). Thus, titin mobility has more than one degree of freedom and is equally efficient along and across the sarcomere.

Calcium levels affect titin mobility
It has previously been shown that calcium does not only affect the proliferation of cardiomyocytes and protein expression, but also changes the mobility of sarcomeric proteins (Harayama et al., 1998). To investigate the effect of calcium on the mobility of titin we exposed embryonic cardiomyocytes to three different calcium concentrations (low, 0.9 mM; normal, 1.8 mM; high, 2.8 mM). Recovery was facilitated at reduced calcium levels, whereas treatment with high calcium resulted in a depressed recovery curve (Fig. 6, A and B). Differences in calcium-dependent recovery after photobleaching were largely attributable to differences in the mobile fractions, with 72 ± 4% at low calcium vs. 49 ± 4% at normal and 28 ± 5% at high calcium levels (Fig. 6 C). Calculated half-lives of titin-eGFP recovery after photobleaching were not significantly different (Table I). These data suggest that calcium levels determine the stability of titin’s integration in the sarcomere, rather than the movement of titin within the myofilament. To differentiate effects of calcium mediated by myosin or contraction versus signal transduction, we used 2,3-butanedione monoxime (BDM) as a cell-permeable inhibitor of myosin II. As indicated in Fig. 7, there was no effect of BDM on titin mobility comparing beating and BDM-treated cardiomyocytes (Fig. 7, B and C). Furthermore, the effect of high calcium was not overcome by BDM treatment (Fig. 7, D and E), both arguing for a myosin- and contraction-independent effect.

Comparison of titin-eGFP mobility in embryonic and neonatal cardiomyocytes
Titin protein undergoes a perinatal isoform switch leading to the expression of shorter titin isoforms starting at the day of birth (Greaser et al., 2005). We confirmed this difference in titin isoform expression by qPCR and found a significantly higher expression of N2B in neonatal compared with embryonic cultured cardiomyocytes (Fig. S3). Together with the altered isoform expression of sarcomeric titin-binding proteins such as myomesin (Grove et al., 1985), this might have implications for retention of titin in the M-band and altered motility in embryonic versus neonatal cardiomyocytes. To investigate whether the dynamic properties of titin protein change postnatally, we performed FRAP analysis on neonatal cardiomyocytes (NCM, P2). The comparison to embryonic cardiomyocytes did not indicate any major change in fluorescence recovery of titin (Fig. 8, A and B); mobile fractions of 56 ± 3% and exchange half-lives of titin mobility (3.7 ± 0.4) were not significantly different from those calculated for neonatal cardiomyocytes (Fig. 8 C; Table I). Thus, changes in perinatal isoform expression do not exert a discernable effect on titin mobility.

Discussion
Due to titin’s size and its expression in multiple isoforms it has been notoriously difficult to study the holoprotein both in vitro and in cultured cells. Circumventing the problems associated with the analysis of individual titin domains outside their native environment such as dominant-negative effects and mislocalization, researchers have used tissue- and species-specific titin isoform expression and subsequently knockout technology to gain insight into titin’s roles in sarcomere assembly and stability and mechanotransduction, as well as in cardiac and skeletal muscle physiology (Weinert et al., 2006; Radke et al., 2007; Gramlich et al., 2009; Granzier et al., 2009). Based on these studies it has been proposed that titin serves as an elastic scaffold to provide both a backbone for proper assembly and localization of sarcomeric proteins and a mechanical basis for the passive functions of striated muscle (Tskhovrebova and Trinick, 2003). Earlier work has shown that the accumulation of transfected titin fragments, which consist of the Z-disc region of titin fused to GFP, leads to disassembly of the sarcomere (Turnacioglu et al., 1997). In this study, we have used the titin-eGFP knockin mouse to prevent this toxic side effect and to constitutively express titin-eGFP in cardiomyocytes at physiological levels. We asked how stable the titin scaffold is over time using the titin-eGFP mouse as a novel animal model to investigate the mobility of the titin protein. The knockin allows us to study the dynamics of titin mobility and to follow titin-eGFP’s replacement after photobleaching in living cardiomyocytes.

Minimally invasive labeling of titin
Although the addition of the eGFP-tag only results in a minor addition to titin’s molecular weight (<1%), we considered the possibility of steric hindrance of protein interactions and avoided manipulating titin’s N terminus, which binds T-cap and is a crucial component of the Z-disc structure (Gregorio et al., 1998). Integration of titin in the M-band involves myomesin, which interacts with the Ig domain M4 (Obermann et al., 1997). There are no known interacting proteins at the very C terminus, which is thus amenable to addition of a protein tag. Indeed, the C-terminal fusion of eGFP did not affect the
proper integration of titin into the M-band or sarcomere assembly and maintenance. Attaching eGFP to the C terminus only labels full-length titin, but not the shorter (up to 700 kD) novex isoforms (Bang et al., 2001). Accordingly, we specifically visualize the pool of titin isoforms that contribute to the continuous titin filament system.

**Figure 5. Longitudinal and lateral movement of titin-eGFP within the sarcomere.** (A) Bleaching of 3 or 8 consecutive sarcomeres in embryonic cardiomyocytes was used to study longitudinal (white arrows) and lateral (open arrows) mobility of titin-eGFP. Titin-eGFP dynamics was monitored for 14 h. Bar, 10 µm. (B and C) Lateral and longitudinal movement of titin-eGFP were equally efficient as demonstrated by the plot of fluorescence intensity vs. time after photo-bleaching of 3 or 8 sarcomeres (3 sarcomeres, n = 4; 8 sarcomeres, n = 3). (D and E) The titin-eGFP mobile fractions were independent from the shape and area of photobleaching, whereas the half-fluorescence recovery increases with the size of the bleached area (3 sarcomeres, n = 4; 8 sarcomeres, n = 3). Error bars indicate SEM.

**Titin—more than a static scaffold**
Sarcomeres are highly ordered and therefore appear as a stable structure. Nevertheless, there is a continuous movement of structural, adaptor, signaling, and metabolic proteins to and from the sarcomere. Here we examine how titin, as a protein that builds the sarcomeric backbone, is replaced and travels...
Nevertheless, we did not see a drift of the bleached area within the cell or major changes in its shape, which we would expect if fluorescence recovery was driven by rearrangement only. Based on the directionality of the sarcomere, we would expect differences in rearrangements or titin mobility along and across the myofiber, unless titin molecules are detached, relocate passively, and get reattached as outlined below.

Titin spans the half-sarcomere integrating with its N terminus into the Z-disc and with its C terminus into the M-band overlapping head-to-head and tail-to-tail (Fürst et al., 1988; Wang et al., 1991; Labeit et al., 1997). The longitudinal movement of titin along the sarcomere would therefore require titin to disconnect and reconnect in an antiparallel orientation at the succeeding next half-sarcomere. For lateral movement of titin the protein remains within the half-sarcomere with neighboring titin molecules trading places as N termini and C termini move along the Z-disc and M-band, respectively. This should be reflected in different kinetics comparing lateral and longitudinal mobility of titin-eGFP.

Sarcomere dynamics

Mobility of titin could either reflect relocalization of individual titin molecules or rearrangement of myofibrils. Although we did not detect major structural changes of the filament system in the 14-h time-lapse (compare Fig. S2 and Video 1), minor changes in location of the myofibril might occur—in part because the cells contract and also as cells shift position in culture over time. Nevertheless, we did not see a drift of the bleached area within the cell or major changes in its shape, which we would expect if fluorescence recovery was driven by rearrangement only. Based on the directionality of the sarcomere, we would expect differences in rearrangements or titin mobility along and across the myofiber, unless titin molecules are detached, relocate passively, and get reattached as outlined below.

**Calcium interferes with titin-eGFP dynamics.** [A] Images of embryonic cardiomyocytes (E13.5) maintained at low vs. high calcium levels (0.9 mM LCa vs. 2.8 mM HCa) were obtained before and immediately after bleaching (white arrows). 14 h after bleaching the titin-eGFP signal was recovered only partially in cells treated with high as compared with low calcium. Bar, 10 µm. [B] Recovery profile of the bleached region over time. The titin-eGFP mobility was significantly increased at low calcium concentration and decreased at high calcium concentration as compared with control cells (normal, NCa, 1.8 mM; n = 4). [C] Mobile fractions decreased with increasing levels of calcium (n = 4 per group), *, P < 0.05; **, P < 0.01; ***, P < 0.001. Error bars indicate SEM.
moves in either direction before it reintegrates. Independently, we followed recovery after photobleaching of the 8 × 8 square and found that titin-eGFP movement into the bleached area was equally efficient from either side, supporting our findings from the directional photobleaching (Fig. S2; Video 1). Thus, we would suggest that a soluble or unattached pool of titin (de novo synthesized or under migration) is distinct from a sarcomeric or attached pool of titin that is tightly integrated into the myofila-
ment and that the unattached titin would freely move along and within the myofiber.

Calcium-dependent titin mobility
Calcium is a universal intracellular second messenger. Its multiple functions in the cardiomyocyte relate to electrophysiology, excitation–contraction coupling, contraction and relaxation, energy consumption, and cell growth and death, as well as transcriptional regulation (Bers, 2008). Toward understanding the effect of calcium on titin dynamics, it has been shown that low calcium concentrations in the culture media enhance the proliferation of cardiomyocytes and affect expression of sarcomeric proteins (Harayama et al., 1998). Within the sarcomere calcium activates contraction (Allen and Blinks, 1978) and regulates not only the interaction of actin and myosin, but also of titin and actin. High calcium levels increase the titin–actin interaction, which has been speculated to introduce a viscous force component opposing actin–myosin filament sliding (Kellermayer and Granzier, 1996; Linke et al., 2002). Furthermore, calcium binding to titin’s glutamate-rich PEVK exons leads to stiffening of the PEVK element (Labeit et al., 2003; Fujita et al., 2004). These data suggest that calcium levels determine the stability of titin’s integration in the sarcomere, rather than the transport of titin within the myofilament. Both the remodeling of the sarcomere with increased proliferation and the increased interaction with actin could affect titin’s mobility in the sarcomere. The former would be expected to increase titin mobility as sarcomeres disassemble, the calcium-dependent increase in titin–actin interaction would conversely lead to lower motility with increased calcium levels. Our data indeed show decreased titin mobility.
with increased calcium levels that would be consistent with a stronger retention of titin at the actin filament system. Nevertheless, the calcium-dependent interaction between titin and actin is relatively weak and may suggest that the titin–actin interaction is insufficient to explain the improved FRAP at reduced calcium levels we describe here (Linke et al., 2002). Alternatively, the effect of calcium on titin motility might be mediated by altered calcium signaling and transcriptional regulation. As suitable tools become available to dissect the effects of calcium on signal transduction and protein–protein interaction, it should be possible to determine their relative contribution to stabilizing titin in the sarcomere.

Titin movement in the developing sarcomere

The pattern of cardiac protein expression changes during development and several sarcomeric proteins switch their isoform type perinatally. Thus, in rat ventricle the predominant fetal isoform β-myosin heavy chain (β-MHC) is replaced by α-MHC after birth (Lompré et al., 1984). Similarly, the α-skeletal actin isoform, which is coexpressed with α-cardiac actin before birth, is gradually replaced with the α-cardiac actin isoform in postnatal development (Carrier et al., 1992). A similar isoform switching of sarcomeric proteins has been described for troponin-I, troponin-T, tropomyosin, and the titin M-band–binding protein myomesin (Grove et al., 1985; Saggin et al., 1988, 1989; Agarkova et al., 2000; Metzger et al., 2003). Cardiac titin is intensively alternatively spliced during development and postnatally. In the embryonic mouse heart the major titin isoform is N2BA—opposed to the adult heart with mainly N2B titin and neonatal hearts, which express both isoforms (Lahmers et al., 2004; Opitz et al., 2004; Warren et al., 2004). We used FRAP of titin-eGFP knockin cardiomyocytes to evaluate if these changes in isoform expression and protein makeup of the embryonic as compared with the neonatal heart lead to altered titin dynamics. The comparison of cardiomyocytes at different stages of development did not indicate a difference in FRAP, suggesting that titin’s motility is largely independent from its domain composition. Isolated adult cardiomyocytes were very sensitive to photobleaching and did not survive the FRAP procedure. Because titin displays a similar mobility in embryonic and neonatal cardiomyocytes at a time where the main changes in titin isoform expression and functional adaptation of the cardiomyocyte take place (Lahmers et al., 2004; Opitz et al., 2004; Warren et al., 2004), an additional change in the dynamics of titin in adult cardiomyocytes was not expected.

Comparative sarcomere dynamics

In FRAP experiments, the exchange of bleached with unbleached proteins occurs much faster than protein turnover would suggest, which reflects the contribution of protein movement versus de novo synthesis. Previous studies of sarcomere dynamics have shown a considerable difference in protein mobility with half-fluorescence recovery ranging from <1 to 12 min (Wang et al., 2005a), possibly related to different binding affinities or numbers of binding partners. Comparing FRAP of sarcomeric proteins allows speculation on structural functions versus a role in more dynamic processes such as signal transduction or metabolism. Within the Z-disc, the quick recovery after photobleaching of myotilin and α-actinin as compared with T-cap suggests a structural role for the latter (Wang et al., 2005a). Unlike various sarcomeric proteins studied previously, the half-fluorescence recovery of titin is in the range of hours (titin 2 h vs. T-cap ~5 min), which further substantiates the role of titin as the sarcomeric backbone.

Actin mobility has been studied extensively with half-fluorescence recovery ranging from 1 to 60 min between β-actin in neonatal rat cardiomyocytes (Skwarek-Maruszewska et al., 2009) versus G-actin in skeletal muscle (Suzuki et al., 1998; Hasebe-Kishi and Shimada, 2000). Skeletal muscle α-actinin (rabbit) microinjected into embryonic chicken cardiomyocytes does hardly recover after photobleaching, but almost completely recovers in fibroblasts (Hasebe-Kishi and Shimada, 2000). The available literature on sarcomeric protein mobility suggests that both mobile fractions and exchange half-lives depend on the
SDS-agarose electrophoresis and Western blot

Vertical SDS-agarose gel electrophoresis (VAGE) was used to detect titin-eGFP in cardiac and skeletal muscle (quadriceps and soleus) of adult knockin mice (Warren et al., 2003). For Western blot analysis the proteins were transferred to a PVDF membrane (GE Healthcare) with the PerfectBlue Semi-Dry Electroblotter SEDEC M (PEQLAB) at 250 mA for 1.5 h using anode buffer I (500 mM Tris-base, 0.05% SDS, and 10% methanol, pH 10.4), anode buffer II (25 mM Tris-base, 0.05% SDS, and 10% methanol, pH 10.4), and cathode buffer (25 mM Tris-base, 0.05% SDS, 10% methanol, 40 mM capric acid, and 10 mM β-mercaptoethanol, pH 10.4). The membrane was blocked with blocking solution (10 mM Tris/HCl, pH 8, 150 mM NaCl, 0.1% Tween 20, 0.5% BSA, and 2.5% skim milk) followed by incubation with the polyclonal primary antibody anti-titin IgG (1:1,000), or anti-titin M8/M9 (1:500, Trombitas et al., 2000) at 4°C overnight. Both titin antibodies were gifts from S. Labeit (Universitätsklinikum Mannheim, Mannheim, Germany). HRP-conjugated goat anti-rabbit IgG (1:2,500 in blocking solution; Sigma-Aldrich) was used as secondary antibody. Blots were washed twice with PBS-Tween 20 followed by a PBS wash and developed with chemiluminescence staining using ECL (SuperSignal West Femto Chemiluminescent Substrate; Thermo Fisher Scientific). Images were taken using the Stela 8300 imaging system (Raytest) and processed using the Aida Image Analyzer v.2.24 software (Raytest).

Real-time PCR

Heart and skeletal muscle from adult titin-eGFP wildtype, heterozygous, and homozygous mice were used for isolation of total RNA. Primary embryonic (E13.5) and neonatal cardiomyocytes (P2) from homozygous titin-eGFP mice were cultured for 3 d and used for RNA isolation. Snap-frozen tissue was homogenized two times for 2 min at a frequency of 30/s using the Tissuelyser II (QIAGEN). RNA was isolated using the peqGOLD TriFast kit (peqlab) and the RNeasy Mini kit (QIAGEN), followed by digestion of contaminating genomic DNA (RNase-Free DNase Set; QIAGEN). RNA concentration and purity were determined by spectrophotometry. 3 μg of total RNA was used for cDNA synthesis (Thermoscript First-Strand Synthesis System; Invitrogen). Quantitative real-time RTPCR was performed using the TaqMan probe-based chemistry (Applied Biosystems). Real-time PCR amplification reaction was performed on a Sequence Detection System (7900 HT; Applied Biosystems) using the qPCR MasterMix Plus (Eurogentec) according to the manufacturer’s instructions with 1x TaqMan Universal PCR master mix, 900 nM primers (EFGF-primer 5’-CTGCTGGCAGCAACAACTC3’; EFGF-primer 5’-ACCATGGATGGGCTGCCTCTC3’; Mex3-for 5’-CGGTCCTGATGGTCCTGGTCAGTA3’; Mex3-rev 5’-CTGGCCCTTCTTGATGGGA3’; N2BA-for 5’-TCAATACTTGCCCTTACG3’; N2BA-rev 5’-TTCTGACACTGTGATCGCGCTTC3’; endogenous control 18S RNA-for 5’-GCAAGGCCTGACATGGAATG3’; N2BA-primer 5’-AGGTGCCCGACGACGTC3’; endogenous control 18S RNA-rev 5’-GCAAGGCCTGACATGGAATG3’; N2BA-primer 5’-AGGTGCCCGACGACGTC3’; endogenous control 18S RNA-rev 5’-GCAAGGCCTGACATGGAATG3’; N2BA-primer 5’-AGGTGCCCGACGACGTC3’; endogenous control 18S RNA-rev 5’-GCAAGGCCTGACATGGAATG3’; N2BA-primer 5’-AGGTGCCCGACGACGTC3’; endogenous control 18S RNA-rev 5’-GCAAGGCCTGACATGGAATG3’; N2BA-primer 5’-AGGTGCCCGACGACGTC3’; endogenous control 18S RNA-rev 5’-GCAAGGCCTGACATGGAATG3’; N2BA-primer 5’-AGGTGCCCGACGACGTC3’; endogenous control 18S RNA-rev 5’-GCAAGGCCTGACATGGAATG3’; N2BA-primer 5’-AGGTGCCCGACGACGTC3’; endogenous control 18S RNA-rev 5’-GCAAGGCCTGACATGGAATG3’; N2BA-primer 5’-AGGTGCCCGACGACGTC3’; endogenous control 18S RNA-rev 5’-GCAAGGCCTGACATGGAATG3’; N2BA-primer 5’-AGGTGCCCGACGACGTC3’; endogenous control 18S RNA-rev 5’-GCAAGGCCTGACATGGAATG3’).

Immunofluorescence staining

Adult tissues from wild-type, homozygous, and heterozygous titin-eGFP mice were dissected and used for immunofluorescence staining as described previously [Weinert et al., 2006] with the following changes. Tissues were fixed in 4% PFA for 6 h, equilibrated with 30% sucrose in PBS overnight, and embedded in tissue Tek (O.C.T. compound; Sakura). Cryo-sections (5 μm) were blocked and permeabilized in blocking solution (10% goat serum, 0.3% Triton X-100, and 0.2% BSA in PBS) for 1 h. The sections were incubated with primary antibodies at 4°C overnight followed by the incubation with a biotin-conjugated antibody (1:500; BD) at room temperature for 1 h. Primary antibodies were used at the following dilutions: 1:200 monoclonal...
anti-sarcomeric α-actinin (Sigma-Aldrich), 1:50 anti-titin Z1/Z2, and 1:200 anti-titin M8/M9 (Trombitás et al., 2000). Fluorescence images were acquired with a laser-scanning confocal microscope (LSM 510 with software 3.2 SP2; Carl Zeiss) with a Plan-Apochromat 100x/1.4 oil DIC objective (Carl Zeiss). Images were assembled using Adobe Photoshop and Adobe Illustrator CS5.

Isolation of embryonic and neonatal murine cardiomyocytes
Timed matings were set up between homozygous animals (G/G). The morning of detection of the vaginal plug was regarded as day 0.5 after conception. Embryos were harvested at E13.5. The hearts were dissected and digested with 0.05% trypsin/EDTA (Invitrogen) for 2–4 h at 4°C followed by incubation at 37°C for 1.5 min. After centrifugation at 800 g for 3 min, the cells were resuspended in DME (Cambrex) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 1% penicillin/streptomycin (Invitrogen), and 1% nonessential amino acids (Invitrogen), and plated onto 0.1% gelatin-coated plastic dishes (Ibij). The culture was maintained at 37°C and 5% CO2 until transfer for FRAP experiments.

Neonatal (P2) hearts from homozygous titin-eGFP mice were dissected and pooled. Hearts were cut into small pieces, washed with PBS, and digested with 2.5 ml of 0.25% trypsin/EDTA (Invitrogen) at 34°C for 3 min. Trypsin solution was replaced and the tissue was incubated in 2.5 ml 0.25% trypsin/EDTA at 37°C for an additional 10 min. The supernatant was collected and the digestion was stopped with 1.25 ml of cold PBS. After centrifugation at 1,000 rpm for 8 min the pellet was resuspended in 20 µl DME and 750 µl PBS and stored on ice. The trypsination was repeated until full digestion of the hearts. All fractions were pooled and centrifuged at 1,000 rpm for 10 min. The pellet was resuspended in DME (Cambrex) supplemented with 10% FBS, 1% penicillin/streptomycin (Invitrogen), and 1% nonessential amino acids (Invitrogen) and plated onto 0.1% gelatin-coated plastic dishes (Ibij). The culture was maintained at 37°C and 5% CO2.

Fluorescence recovery after photobleaching and analysis of FRAP data
FRAP experiments were performed with a laser-scanning confocal microscope (LSM 510 Meta; Carl Zeiss). Titin-eGFP homozygous primary cardiomyocytes were kept at 37°C and 5% CO2 using a CO2 microscope incubation system (Okolab). For eGFP imaging the excitation wavelength and emission filters were 488 nm/band-pass 500–550 nm. Image processing was performed using LSM 510 Image Browser version 4.2 (Carl Zeiss). For FRAP experiments a Plan-Apochromat 63x/1.4 oil Ph3 objective was used and the confocal pinhole was set to 1.5 µm. 3–4 experiments (n = 3–4) were performed and each time 4–10 cells were selected. For every cell 1 or 2 different regions of interest (ROI) were chosen for bleaching to keep phototoxicity low and areas of interest were limited to squares of 1, 3, or 8 sarcomere lengths or areas of 1 sarcomere length × 3 or 8 sarcomere lengths along or across the myofiber. Images were taken before and immediately after bleaching. Photobleaching was done with 100% intensity of the 488-nm laser for 20 iterations. The fluorescence recovery was monitored 14 times every hour and measured using the LSM Image Examiner (Carl Zeiss). Images were assembled using Adobe Photoshop and Adobe Illustrator CS5.

The fluorescence intensity at zero after bleaching is defined as y0. K is normalized to the prebleach intensities in the corresponding regions generated from raw data as described in Al Tanoury et al. (2010). Back-the LSM Image Examiner (Carl Zeiss). Normalized FRAP curves were generated from raw data as described in Al Tanoury et al. (2010). Back-ground intensity (I(0)) was measured in a region outside of the cell and then subtracted from the intensity of the bleached area (I(0)) and the whole cell (I(0)) at each time point. The intensities were then normalized by rescaling to the prebleach intensities in the corresponding regions (I(0) and I(0)). The resulting equation for the normalized FRAP curve is:

\[
\frac{I(0) - I(0)}{I(0) - I(0)} = \frac{I(0) - I(0)}{I(0) - I(0)} = \frac{I(0)}{I(0)} \cdot \frac{I(0)}{I(0)}.
\]

To obtain the exchange half-life (t1/2) data were displayed as normalized fluorescence intensity versus time using Prism 5.0 (GraphPad Software). The model of one phase association was applied to fit the data:

\[
y(t) = y_0 + Mf \cdot (1 - e^{-t/K_{sep}}).
\]

The fluorescence intensity at zero time after bleaching is defined as y0. K is the rate constant. The exchange half-life (t1/2) is computed as follows:

\[
t_{1/2} = \frac{\ln(2)}{K_{sep}}.
\]
