Published in final edited form as:

Cytoskeleton (Hoboken). 2018 August ; 75(8): 339–351. doi:10.1002/cm.21454.

Nonmuscle myosin II in cardiac and skeletal muscle cells

Jushuo Wang, Yingli Fan, Jean M. Sanger, and Joseph W. Sanger*

Department of Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY 13210

Abstract

De novo assembly of contractile myofibrils begins with the formation of premyofibrils where filaments of non-muscle myosin (NM II), and actin organize in sarcomeric patterns with Z-Bodies containing muscle-specific alpha-actinin. Interactions of muscle specific myosin (MM II) with NM II occur in a nascent myofibril stage that precedes the assembly of mature myofibrils. By the final stage of myofibrillogenesis, the only myosin II present in the mature myofibrils is MM II. In this current study of myofibril assembly, the three vertebrate isoforms of NM II (A, B, and C) and sarcomeric alpha-actinin, ligated to GFP family proteins, were coexpressed in avian embryonic skeletal and cardiac muscle cells. Each isoform of NM II localized only in the mini-A-Bands of premyofibrils and nascent myofibrils. There was no evidence of localization of NM II in Z-Bodies of premyofibrils and nascent myofibrils or in Z-Bands of mature myofibrils. Fluorescence Recovery After Photobleaching (FRAP) experiments indicated similar exchange rates in premyofibrils for NM II isoforms A and B, whereas the IIC isoform was significantly less dynamic. Fluorescence Resonance Energy Transfer (FRET) measurements of colocalized fluorescent pairs of different NM II isoforms yielded signals similar to identical pairs, indicating copolymerization of the different NM II pairs. The role of NM II may reside in establishing the future sarcomere pattern in mature myofibrils by binding to the oppositely polarized actin filaments that extend between pairs of Z-Bodies along premyofibrils prior to their transformation into mature myofibrils.

Keywords

Myofibrillogenesis; sarcomere; premyofibril; nascent myofibril; mature myofibril; non-muscle myosin II isoforms; FRAP; FRET

Introduction

The *de novo* formation of avian embryonic skeletal and cardiac muscle results in assembly of linear arrays of repeating subunits designed to produce contractile force by muscle specific myosin II (MM II). Before MM II is organized in mature myofibrils to carry out that role, however, non-muscle isoforms of myosin II (NM II) are required for the assembly of premyofibrils and nascent myofibrils that lead to mature myofibril formation (Rhee et al., 1994; Du et al. 2003; 2008; White et al., 2014; Sanger et al. 2017). The same NM II

*Corresponding Author: Dr. Joseph W. Sanger, Department of Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY 13210., Tel.: 1-315-464-8538; sangerjo@upstate.edu.

isoforms of myosin are found in most eukaryotic cells in structures such as stress fibers and cleavage furrows (Sellers et al., 1999; Du et al. 2003; 2008; Vicente-Manzanares et al., 2009; Ma et al., 2010; Billington et al., 2013; Ebrahim et al. 2013; Ma and Adelstein, 2014). During *de novo* formation of myofibrils, small bands of NM II filaments that interact with actin filaments, align in an alternating pattern with muscle alpha-actinin in premyofibrils (Du et al., 2003; 2008; Sanger et al., 2017).

The study of living muscle cells that contain fluorescently tagged sarcomeric proteins has revealed a novel pathway of myofibrillogenesis: premyofibrils to nascent myofibrils to mature myofibrils (Sanger et al., 2017). Early studies using fluorescent dye coupled muscle alpha-actinin in avian embryonic cardiomyocytes suggested that the minisarcomeres, whose boundaries were marked by alpha-actinin containing Z-Bodies, increased in length to form the 2.5 micron long sarcomeres of mature myofibrils (Sanger et al., 1984). Studies of living skeletal muscle cells microinjected with fluorescent dye coupled alpha-actinin (Sanger et al., 1986), and living embryonic chick cardiomyocytes expressing muscle alpha-actinin-GFP (Dabiri et al., 1997), demonstrated decisively that the linear arrays of the minisarcomeres premyofibrils did increase in length to form the full sarcomeric spacings marked by the Z-Bands of mature myofibrils (Sanger et al., 1989; 2002, 2017).

This study is focused on examining NM II isoforms expressed in live embryonic avian muscle cells transfected fluorescent protein-linked nonmuscle myosin II heavy chains, muscle myosin II and alpha-actinin during myofibrillogenesis. NM IIB (Myosin 10) is the predominant NM II in cardiomyocyte premyofibrils (Rhee et al., 1994, Du et al., 2003), and NM IIA (Myosin 9) is the main isoform in premyofibrils in skeletal muscle (White et al., 2016; Sanger et al., 2017), whereas NM IIC (Myosin 14) is expressed primarily in adult organs with low levels in human fetal organs (Golomb et al., 2004). Each of the three nonmuscle isoforms, IIA, IIB, and IIC, localized in mini-A-bands in both types of muscle when expressed in cultures of transfected embryonic quail myotubes and avian cardiomyocytes. NM II alternated with alpha-actinin in premyofibrils in living skeletal and cardiac muscle cells reflecting antibody localizations seen in fixed muscle cells in precardiac explants (Du et al., 2003), intact embryonic hearts (Du et al., 2008), and cultured cardiac (Rhee et al., 1994) and skeletal (White et al., 2014) muscle cells. NM II isoforms were not localized in A-Bands of mature sarcomeres, and were not detected in Z-Bodies, or Z-Bands (Sanger et al., 2017). It has been reported by one group of investigators, however, that NM IIs localize in the Z-Bands of cardiac and skeletal muscle cells in antibody-stained tissue sections (Takeda et al, 2000; 2003). Our results reported here in living, transfected cardiac and skeletal muscle cells, as well as our previously reported antibody staining results do not support the localization of NM II isoforms in Z-Bands in either embryonic cardiac and skeletal muscle cells (Rhee et al., 1994; Du et al. 2003, 2008, White et al., 2014), or in freshly isolated cultured adult cat and rat cardiomyocytes (LoRusso et al., 1997).

Assessment of the dynamics of the three NM II isoforms in cultured embryonic skeletal and cardiac muscle cells with measurements of Fluorescence Recovery After Photobleaching (FRAP) showed similar exchange rates of IIA and IIB in premyofibrils, whereas the IIC isoform was markedly less dynamic. Fluorescence Resonance Energy Transfer (FRET) analyses of premyofibrils in skeletal muscle myotubes expressing combinations of cerulean

fluorescent protein (CeFP) and yellow fluorescent protein (YFP) pairs of NM IIA, IIB, or IIC showed strong signals between different NM II pairs, (e.g., IIA & IIB), comparable to identical NM II pairs (e.g., IIA & IIA), indicating the NM II isoforms may have copolymerized. Other FRET measurements in this report suggest that whereas NM II isoforms and MM II may copolymerize with each other in nascent myofibrils, proteins unique to the A-Bands may prevent copolymerization of NM II and MM II in mature myofibrils. Prior to the transformation of premyofibrils into nascent myofibrils, and then into mature myofibrils, the role of NM II may reside in establishing the future sarcomere pattern by binding to the oppositely polarized actin filaments that extend between pairs of Z-Bodies along premyofibrils.

Results

Localization of three isoforms of non-muscle II expressed in skeletal and cardiac muscle cells

Myotubes cotransfected with plasmids encoding sarcomeric alpha-actinin-CeFP and single isoforms of non-muscle myosin II coupled to GFP display colinear incorporation of NM II groups of filaments (Figure 1 a, d, e cyan in c, f, i), alternating with small bands of alpha-actinin (Z-Bodies) (Figure 1 b, e, h; magenta in c, f, i) in premyofibrils at the ends of myotubes. Each of the three NM II isoforms have similar patterns of NM II alternating with alpha-actinin: NM IIA (a, cyan in c); NM IIB (d, cyan in f); NM IIC (g, cyan in i). Toward the center of the myotube, distal to the area of premyofibrils in the myotube tip, Z-bands expressing sarcomeric alpha-actinin-CeFP span the width of mature myofibrils that have formed (Figure 2 b, e, h; magenta in c, f, i). Each of the expressed isoforms of NM II is restricted to thin fibrils in a subsarcolemmal localization along the middle shaft of the elongated myotube (Figure 2 IIA a, cyan in c; IIB d, cyan in f; IIC g, cyan in i). This GFP tagged localization matches the localization of endogenous NMII isoform IIA and IIB by specific antibody staining in myotubes published by our laboratory in White et al. (2014). The nonmuscle myosin IIC is not native to myotubes (Golomb et al., 2004). Our results demonstrate that NM IIC can localize to premyofibrils that contain normally NM IIA and NM IIB (White et al., 2014). There is no evidence that any of the NM II isoforms colocalized with the Z-Bands of the mature myofibrils (Figure 2 c, f, i).

A 3-D view of the disposition of nonmuscle myosin II and Z-Bands in mature myofibrils is shown along three axes (XY, XZ, and YZ) in another myotube doubly transfected with GFP-NM IIA (Figure 3 a, cyan in c) and CeFP-alpha-actinin (Figure 3 b, magenta in c). Nonmuscle myosin IIA is localized exclusively beneath the cell membrane that encircles the myotube, i.e., subsarcolemmal localizations (Figure 3 a, cyan in c). Z-bands fill the volume of cytoplasm within the membrane (Figure 3 b, magenta in c). The merged images of NM IIA and Z-Bands in three axes in Figure 3 c clearly show the separation of NM IIA and Z-Bands in the mature myofibrils. Identical results were obtained for NM II B, and NM IIC.

In some myotubes, NM II A was over expressed (Figure 4), allowing us to determine whether high levels of NM II resulted in its localization in Z-Bands or A-Bands of mature myofibrils. Contrary to its usual localization along the peripheral membranes of the mature myotubes (Figures 2 and 3), NM IIA, when over-expressed, assembled in stripes in the

middle of A-Bands (Figure 4 b, e, green in h) and also in aggregates with no specific sarcomeric localization (Figure 4 b). Post-fixation and staining with muscle-specific myosin antibody, F59, that shows the double bands of MM II bisected by a central bare zone characteristic of A-Bands (Figure 4 c, f, red in h and i) where NM IIA assembled when overexpressed. The arrowheads in Figure 4 (a, d blue in g, i), point to alpha-actinin expression in Z-Bands. These sites border the A-Bands, and are devoid of both NM IIA (Figure 4 b, e, h) and MM II (Figure 4 c, f, i). Thus, even in myotubes overexpressing GFP-NM IIA, NM II was not localized in Z-bands, the same result observed in myotubes expressing lower amounts of GFP-NM IIA isoforms (Figures 2 and 3).

In cultured cardiomyocytes (Figure 5), as shown in skeletal muscle, expression of NM II isoforms linked to GFP leads to its localization in small bands in premyofibrils (Figure 5a IIA, cyan in c; Figure 5d IIB, cyan in f; Figure 5g IIC, cyan in i). Alpha-actinin-CeFP was coexpressed with each fluorescently tagged NM II isoform in cardiomyocytes. Alpha-actinin-CeFP localized both in mature Z-Bands (Figure 5 b, e, h, magenta in c, f, i) and in small Z-Bodies, the precursors of Z-Bands, (Figure 5 b, e, h; magenta in c, f, i). The NM II isoforms marked by cyan (Figure 5 c, f, i) alternated with Z-Bodies along premyofibrils, and were not colocalized with Z-bands of the mature myofibrils.

Dynamics of the three different GFP-NM II isoforms in premyofibrils of skeletal and cardiac muscle cells

The dynamics of the three isoforms of NM II were measured with Fluorescence Recovery After Photobleaching (FRAP) to determine whether similar localization predicted similar dynamics in muscle cells. NM IIA and IIB have similar recovery curves and mobile fractions when compared to each other whether in skeletal or in cardiac muscle cells (Figure 6, Table 1). However, there is a marked decrease in the dynamics of NM IIC by comparison with NM IIA and NM IIB in the premyofibrils in skeletal (Figure 6 a, b) and cardiac (Figure 6 c, d) muscle cells (Table 1). A result of the slow dynamics of NM IIC can be seen in muscle cells expressing NM IIC-GFP that initially assemble into premyofibrils (Figure 7 a), but after several days in culture the premyofibrils disassemble in the ends of the myotubes, and myofibril assembly ceases (Figure 7 b).

Fluorescence Resonance Energy Transfer (FRET) experiments

To determine if pairs of isoforms of NM II that colocalized after expression in premyofibrils formed copolymers, we cotransfected skeletal muscle cells with combinations of plasmids encoding CeFP or YFP linked to NM II isoforms (Figure 8), and analyzed the proximity of the isoforms with FRET. Each coexpressed pair of NM II had identical localizations in the premyofibrils (Figure 8). Myotubes expressing seven different dual combinations of two differently labeled NM IIs (Table 2) were measured to derive values of Efficiency percentile (E%) to judge the proximity of the selected protein pairs using methods we previously described (Stout et al, 2008). All of the NM II colocalized pairs had E% in the range of 11–18% (Table 2), indicating close proximity or copolymerization of the different combinations of the three different NM II isoforms in mini A-Band filaments in premyofibrils in living transfected myotubes.

Muscle specific myosin (MM II), absent in premyofibrils (Figure 9 a to c), first appears during myofibrillogenesis with the formation of nascent myofibrils that exist as groups of thin overlapping fibrils of NM II and MM II (Figure 9 a to c) before only MM II assembles into A-Bands in mature myofibrils (Figure 9 d to f). Fibrils composed of NM II are concentrated at the ends (Figure 9 a, c) and sides (Figure 9 d, f) of myotubes (Figure 9 a small arrow, cyan in c), and fibrils of MM II are concentrated in the distal to the tip (Figure 9 b and magenta in c). FRET analysis was used to determine how closely aligned the two types of fibrils, NM II and MM II, were in nascent myofibrils (Figure 10). FRET values (E %) in fibrils of NM II isoform and fibrils of MM II isoform were indicative of colocalization and copolymerization (Figure 10). FRET analyses of A-Bands expressing muscle myosin II heavy chains (CeFP-MM II and YFP-MM II) revealed E% values (about 14%, Figure 10) similar to those E% values of nascent myofibrils (Figure 10, 13 to 18%). These FRET values are consistent with copolymerization of nonmuscle myosin II (A, B, or C) and muscle myosin II in the nascent myofibrils.

Discussion

How contractile units assemble *de novo* to form myofibrils in cardiac and skeletal muscle cells is a fundamental problem in muscle research (Piccirillo et al., 2013; Sanger et al., 2017). Early ultrastructural studies of embryonic striated muscle, fixed *in situ*, or cultured from embryonic muscle tissue, revealed bundles of actin and myosin filaments at the edges of the cells before myofibrils formed (Kelly, 1969; reviewed by Sanger et al., 2004). A key question then and now is how the filaments become aligned in sarcomeric structures capable of producing contractile force in myofibrils. In vertebrate embryonic muscle, the proteins of assembling sarcomeres, with one exception, are muscle-specific isoforms. Non-muscle isoforms of myosin IIs are present in premyofibrils and nascent myofibrils, two early stages in myofibrillogenesis (Rhee et al., 1994; Sanger et al., 2005; Du et al., 2003, 2008; White et al., 2014; Sanger et al., 2017).

There are three isoforms of non-muscle myosin II: (IIA, IIB, and IIC), in cells where they function in multiple contractile events ranging from cytokinesis to membrane ruffling and cell locomotion (Leal et al., 2003; Golomb et al., 2004; Vicente-Manzanares et al., 2009; Aguilar-Cuenca et al., 2013; Billington et al., 2013). Avian cardiomyocytes express only NM IIB both *in situ* and in culture (Rhee et al., 1994; Du et al., 2003, 2008). Mouse cardiomyocytes were reported by Tullio et al. (1997) to express only NM IIB in hearts *in situ*. But in both normal mouse cardiomyocyte cultures (Takeda et al., 2003), and in *in situ* hearts of knock out mice lacking NM IIB, the cardiomyocytes can upregulate the expression of NM IIA (Tullio et al., 1997). In contrast to Tullio et al. (1997), Lu et al. (2008) reported that both NM IIA and NM IIB, but not IIC, were expressed in early mouse heart development. Freshly isolated adult cat and rat cardiomyocytes do not express either NMII A or NM IIB in cultures by three to six hours in culture (LoRusso et al., 1997). However, after one to two days in tissue culture, NM IIB, but not NM IIA, is detected only in the spreading ends of the cat and rat cardiomyocytes. There are two reports that NM IIA and NM IIB antibodies stain Z-Bands in mouse cardiac and skeletal muscle cells (Takeda et al., 2000; 2003). In contrast, we did not detect antibody staining of NM IIA and NM IIB in Z-Bands of skeletal muscle cells, nor in Z-Bands of cardiac muscle cells (Rhee et al., 1994;

LoRusso et al., 1997; Du et al., 2003, 2008; Sanger et al., 2005; Sanger et al., 2010; White et al., 2014). These antibody staining differences may reflect differences in species, fixation artefacts, antibody specificities, or an artefact of antibody staining. Lu and Horowitz (2008) published images of cultured embryonic mouse cardiomyocytes stained with both muscle alpha-actinin and nonmuscle myosin IIB antibodies. These authors did not comment on the absence of nonmuscle myosin IIB in the mature myofibrils of the stage E 17.5 mouse cardiomyocytes. We observed in their published Figures that nonmuscle myosin IIB antibody staining was absent in the Z-Bands or any other part of the mature myofibrils in their cultured embryonic mouse cardiomyocytes. Thus, the analyses of living cardiac and skeletal muscle cells expressing fluorescently tagged proteins appeared an excellent alternative way of studying myofibrillogenesis. When NM IIs coupled to GFP, CeFP or YFP were expressed in muscle cells, none of the three isoforms localized in the Z-Bands of either skeletal or cardiac muscle cells. Thus, our results in both fixed (Sanger et al., 2017) and living muscle cells indicate that NM II is not a component of Z-Bands.

Colocalization studies of the three NM IIs lead to the question whether the NM IIs could copolymerize (Golumb et al., 2004; Beach et al., 2014). NM IIA and IIB are known to colocalize in filaments in nonmuscle cells (Marini et al., 2006). Two-color total internal reflection fluorescence structured illumination microscopy (TIRF-SIM) of nonmuscle cells transfected with NM II A-mApple and EGFP-NM IIB revealed that the two isoforms coassemble in individual bipolar filaments in the cells (Beach et al., 2014). During myofibril formation in avian skeletal and cardiac muscle cells, cotransfection of pairs of NM II isoforms results in their colocalization in premyofibrils (Figure 8). FRET efficiency measurements of the colocalized pairs suggest that the NM II pairs coassemble in premyofibrils (Table 2). In addition, FRET experiments demonstrate that the three NM IIs can coassemble with muscle specific heavy chains in nascent myofibrils in living skeletal muscle cells (Figure 10). This is similar to early biochemical experiments that purified platelet nonmuscle myosin II, now known to be an NM IIA isoform, can copolymerize with skeletal muscle myosin II (Pollard, 1975). Moreover, there is no indication that the three NM IIs normally coassemble with muscle myosin heavy chains in mature myofibrils. Our previous antibody results indicate that non-muscle myosin II is not present in the A-Bands of mature myofibrils in either cardiac (Rhee et al., 1994; Du et al., 2003, 2008) or skeletal (Sanger et al., 2005; White et al., 2014) muscle cells. We assume that normally during myofibrillogenesis, muscle myosin-binding proteins prevent the coassembly of nonmuscle myosin II isoforms in A-Bands of mature myofibrils. It is of interest that overexpression of nonmuscle myosin II heavy chains leads to their localization in the middle of the A-Bands. This aberrant localization suggests that putative A-Band proteins via mass action normally block the copolymerization of the C-termini of the heavy chains of NM II and MM II in the A-Bands. Future work should lead to discovery and properties of these putative blocking proteins. Even under these conditions of overexpression, nonmuscle myosin II was not detected in the Z-bands of the mature myofibrils.

These experimental approaches have the advantages that the experiments were accomplished in living cells, thereby avoiding the pitfalls of preparing extracting cells for pull down experiments, or false positives in yeast two hybrid assays. Embryonic cardiomyocytes are able to assemble and disassemble cleavage furrows during their normal cell divisions, and

then to assemble premyofibrils, nascent myofibrils and mature myofibrils using GFP-alpha-actinin (Dabiri et al., 1997; Sanger and Sanger, 2001). The embryonic skeletal muscle cells in this study are able to assemble premyofibrils, nascent myofibrils and mature myofibrils using GFP family probes ligated to nonmuscle myosin II isoforms and alpha-actinin.

Each NM II isoform has several distinct properties that reside in their cross-bridge ends at the N-terminal region of the myosin heavy complex (Vicente-Manzanares et al., 2009). The duty ratio, or time that the myosin is bound to F-actin producing force, is highest in the IIB isoform. The hydrolysis of ATP and the movement along actin filaments in cell motility assays is highest in the IIA isoform. The C-terminal tail ends of the myosin heavy chains are responsible for the assembly of myosin complexes into the non-muscle myosin II filaments (Sandquist and Means, 2008; Vicente-Manzanares et al., 2009). Billington et al. (2013) reported that nonmuscle myosin IIA and IIB formed monofilaments with similar binding properties to single and multiple actin filaments. These properties were in sharp contrast to the actin binding properties of minifilaments composed of NM IIC. Our FRAP results of the replacement of photobleached myosin complexes in the mini-A-Bands of premyofibrils are reflections of the C-terminal regions of the three different NM II isoforms. The NM IIA and IIB isoforms have similar exchange rates after FRAP after a 20-minute recovery period (Figure 6; Table 1). It is quite striking that the IIC isoform has a markedly lower exchange rate than IIA and IIB (Figure 6, Table 1). The slow exchange of the IIC isoform in embryonic myotubes is accompanied by the disappearance of premyofibrils and nascent myofibrils (Figure 7). The mature myofibrils in these myotubes are unaffected. Based on these results, it is not surprising that the isoform IIC is not expressed in embryonic muscle cells (Golomb et al., 2004; Swailes et al., 2006; Lu et al., 2008). The slow dynamics detected in FRAP experiments of the IIC isoform and the loss of the premyofibrils and nascent myofibrils indicate that certain levels of dynamics of the NM II isoforms as well as their ability to bind actin filaments are necessary for the transformation of premyofibrils into mature myofibrils. Two truncated Z-Band proteins i.e., 27 kD alpha-actinin fragment and the C-terminus of myotilin (Wang et al., 2005; 2011) also have slower FRAP dynamics than the full-length molecules, leading to the inhibition of myofibrillogenesis. In these two cases, the mature myofibrils were almost completely disassembled. The authors suggested that normal dynamics of these two full length proteins were necessary for the formation and maintenance of mature myofibrils (Wang et al. (2005; 2011).

In summary, NM II isoforms (IIA, IIB, and IIC) are neither Z-Body nor Z-Band proteins. FRAP experiments demonstrate that whereas the dynamics of NM IIA and IIB are similar, IIC isoform is markedly less dynamic. The dynamics of the three different NM IIs are consistent with the need for remodeling as premyofibrils are transformed to nascent myofibrils and then to mature myofibrils. The three NM II heavy chains yield a strong FRET signal (E%) indicating that the different non-muscle myosin IIs can coassemble with each other in premyofibrils in muscle cells. The three NM II isoforms can coassemble with MM II in nascent myofibrils but not in mature myofibrils. Together these results support a three-step model of *de novo* myofibrillogenesis: premyofibrils to nascent myofibrils to mature myofibrils (Rhee et al., 1994; Sanger et al., 2017).

Materials and Methods

Preparation, culture, constructs, and transfections of quail myoblasts

Myoblasts were isolated from the breast muscles of nine-day-old quail embryos, and cardiomyocytes were isolated from hearts of ten-day-old chick embryos as previously described (Dabiri et al., 1999, Wang et al. 2011). The muscle cells were transfected after two days of culture with GFP family of plasmids (GFP, CeFP and YFP) using Eugene HD transfection reagent (Roche Diagnostics, Mannheim, Germany) (Wang et al. 2005, 2011). Cells intended for immunostaining were fixed with buffered 3% paraformaldehyde, permeabilized, and stained with antibodies directed against muscle myosin II (F-59 antibody purchased from the Developmental Studies Hybridoma Bank, Iowa City, Iowa), and secondary antibodies labeled with Alexa-Fluor-594 (Invitrogen; Carlsbad, CA) as previously described (Dabiri et al., 1999).

Plasmids encoding Green Fluorescent Protein (GFP) linked to each isoform of nonmuscle myosin II were purchased from AddGene (Cambridge, MA) (plasmid IIA, # 11347; plasmid IIB, # 11348; and plasmid IIC # 10843). The DNA of the NMII plasmids (IIA, IIB, or IIC) was amplified by PCR and cloned into Yellow Fluorescent Protein (YFP) expression vectors, pEYFP-N1 and pEYFP-C3 (Clontech, Mountain View, CA) and Cerulean Fluorescent Protein (CeFP) expression vectors, CeFP-N1 and CeFP-C3 vectors using techniques previously reported (Wang et al., 2005, 2011). In these vectors, YFP or CeFP is linked either at the N-terminus of NM myosin II isoforms (C3 vectors) or at the C-terminus of NM myosin II isoforms (N1 vectors). The cloning sites for NM myosin II isoforms are the same as in their GFP vectors from Addgene (HindIII and SpeI for IIA, EcoRI and SacII for IIB and SalI and BamHI for IIC). Plasmids encoding embryonic chick MM II heavy chain were generously supplied by Professor Donald A. Winkelmann (Wang et al., 2003). MM II heavy chain DNA was cloned into the KpnI and BamHI sites of pEYFP-C3 and CeFP-C3 vectors for FRET studies. Table 2 lists the probes we have assembled for our studies, as well as the N- and C-terminal positions of the YFP and CeFP on the three different NM II isoforms. Plasmids encoding chicken sarcomeric alpha-actinin were amplified by PCR and cloned into HindIII site of CeFP-N1 vectors, as reported earlier (Dabiri et al., 1997, Wang et al. 2011). FRET studies were carried out with NM II isoforms and MM II linked to CeFP or YFP (Table 2). For co-localization studies, plasmids encoding GFP and the three different NM II isoforms, along with plasmids encoding alpha-actinin-CeFP were co-transfected into cardiac and skeletal muscle cells (Wang et al. 2011).

Microscopy

All images were acquired with a Leica SP5 Confocal Microscope with a 63× (N. A. 1.4) objective lens. The 3D images were acquired from stacks through 50 planes in the Z axis from the top to the bottom of quail myotubes cotransfected with GFP-NM II isoforms and CeFP-alpha-actinin. To evaluate the dynamics of the different isoforms of non-muscle myosin II heavy chains in living myotubes and cardiomyocytes, measurements of fluorescence recovery after photobleaching (FRAP) were collected at 30-second intervals over 20 minutes. Post-acquisition analyses are described in detail in previously published papers (Wang et al. 2005; 2007; 2008; Sanger et al., 2009).

Fluorescence Resonance Energy Transfer (FRET) experiments were conducted on the Leica SP5 Confocal Microscope using techniques previously published by Stout et al. (2008). Sensitized Emission FRET (SE-FRET) imaging was performed using the standard “3-cube method” (Gordon et al. 1998, Zal and Gascoigne, 2004). Images were acquired in three channels: the donor channel (D) (excitation laser: 458nm; emission detection: 460-500nm), the acceptor channel (A) (excitation laser: 514nm; emission detection: 550-580nm); and the FRET channel (DA) (excitation laser is 458nm, emission detect window at 550-580nm). FRET fluorescence was corrected by removing the acceptor and donor spectral bleed through and FRET efficiency was calculated as previously described (Gordon et al. 1998, Zal and Gascoigne, 2004, Stout et al. 2008). Empty YFP and CeFP were used as a negative control and FRET efficiencies of different non-muscle myosin II isoform pairs are shown in Table 2. Efficiency percentile provides an expression to determine the proximity of the two proteins, each labeled with a different member of the GFP family of plasmids (Stout et al., 2008). The donor NM IIs were coupled to CeFP, while the acceptor NMI Is was coupled to YFP. The control was to measure the base line proximities of free donor CeFP interacting with free acceptor YFP inside cotransfected myotubes. These cotransfected myotubes exhibited nonlocalized, and uniform fluorescent distributions for CeFP and YFP. The E% for these two soluble fluorescent probes was less than 4%, a base line indication or control for the random collisions of two soluble molecules (Table 2).

Acknowledgments

This work was supported by a NIAMS grant (R01 AR-57063), and an HLBI grant (HL-080426) awarded to JMS and JWS. JWS was also supported by the Hendricks Fund.

Grant Sponsor: NIH

References

- Aguilar-Cuenca R, Juanes-Garcia A, Vincente-Manzanares M. Myosin II in mechanotransduction: master and commander of cell migration, morphogenesis and cancer. *Cell Mol Life Sci.* 2014; 71:479–492. [PubMed: 23934154]
- Beach JR, Shao L, Remmert K, Li D, Betzig E, Hammer JA. Nonmuscle myosin II isoforms coassemble in living cells. *Curr Biol.* 2014; 24:1160–1166. [PubMed: 24814144]
- Billington N, Wang A, Mao J, Adelstein RS, Sellers JR. 2013. Characterization of three full-length human nonmuscle myosin II paralogs. *J Biol Chem.* 2013; 288:33398–33410. [PubMed: 24072716]
- Dabiri GA, Turnacioglu KK, Sanger JM, Sanger JW. Myofibrillogenesis visualized in living embryonic cardiomyocytes. *Proc. Natl. Acad. Sci. USA.* 1997; 94:9493–9498.
- Dabiri GA, Ayoob JC, Turnacioglu KK, Sanger JM, Sanger JW. Use of green fluorescent proteins linked to cytoskeletal proteins to analyze myofibrillogenesis in living cells. *Methods Enzymol.* 1999; 302:171–186. [PubMed: 12876770]
- Dhume A, Lu S, Horowitz R. Targeted disruption of N-RAP gene function by RNA interference: a role for N-RAP in myofibril organization. *Cell Motil Cytoskeleton.* 2006; 63:493–511. [PubMed: 16767749]
- Du A, Sanger JM, Linask KK, Sanger JW. Myofibrillogenesis in the first cardiomyocytes formed from isolated quail precardiac mesoderm. *Dev Biol.* 2003; 257:382–394. [PubMed: 12729566]
- Du A, Sanger JM, Sanger JM. 2008. Cardiac myofibrillogenesis inside intact embryonic hearts. *Developmental Biology.* 2008; 318:236–246. [PubMed: 18455713]
- Ebrahim S, Fujita T, Millis BA, Kozin E, Ma X, Kawamoto S, Baird MA, Davidson M, Yonemura S, Hisa Y, Conti MA, Adelstein RS, Sakaguchi H, Kachar B. NMII forms a contractile transcellular

- sarcomeric network to regulate apical cell junctions and tissue geometry. *Curr Biol.* 2013; 23:731–736. [PubMed: 23562268]
- Golomb E, Ma X, Jana SS, Preston YA, Kawamoto S, Shoha NG, Goldin E, Conti MA, Sellers JR, Adelstein RS. Identification and characterization of nonmuscle myosin IIC, a new member of the myosin II family. *J Biol Chem.* 2004; 279:2800–2808. [PubMed: 14594953]
- Gordon GW, Berry G, Liang XH, Levine B, Herman B. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys J.* 1998; 74:2702–2713. [PubMed: 9591694]
- Kelly DE. Myofibrillogenesis and Z-band differentiation. *Ana Rec.* 1969; 163:403–426.
- Leal A, Ende S, Stengel C, Huehne K, Loetterle J, Barrantes R, Winterpacht A, Rautenstrauss B. A novel myosin heavy chain gene in human chromosome 19q13.3. *Gene.* 2003; 312:165–171. [PubMed: 12909352]
- LoRusso SM, Rhee D, Sanger JM, Sanger JW. Premyofibrils in spreading adult cardiomyocytes in tissue culture: evidence for reexpression of the embryonic program for myofibrillogenesis in adult cells. *Cell Motil Cytoskeleton.* 1997; 37:183–198. [PubMed: 9227849]
- Lu S, Horowitz R. Role of nonmuscle myosin IIB and N-RAP in cell spreading and myofibril assembly in primary mouse cardiomyocytes. *Cell Motil Cytoskeleton.* 2008; 65:747–761. [PubMed: 18615632]
- Lu W, Seeholzer SH, Han M, Arnold AS, Serrano M, Garita B, Philp NJ, Farthing C, Steele P, Chen J, Linask KK. Cellular nonmuscle myosins NMHC-IIA and NMHC-IIB and vertebrate heart looping. *Dev Dyn.* 2008; 237:3577–3590. [PubMed: 18697221]
- Ma X, Jana SS, Conti MA, Kawamoto S, Claycomb WC, Adelstein RS. Ablation of nonmuscle myosin II-B and II-C reveals a role for nonmuscle myosin II in cardiac myocyte karyokinesis. *Mol Biol Cell.* 2010; 21:3952–62. [PubMed: 20861308]
- Ma X, Adelstein RS. The role of vertebrate nonmuscle Myosin II in development and human disease. *Bioarchitecture.* 2014; 4:88–102. [PubMed: 25098841]
- Marini M, Bruschi M, Pecci A, Romagnoli R, Musante L, Candiano G, Ghiggeri GM, Balduini C, Seri M, Ravazzolo R. Non-muscle myosin heavy chain IIA and IIB interact and co-localize in living cells: relevance for MYH9-related disease. *Int J Mol Med.* 2006; 17:729–36. [PubMed: 16596254]
- Piccirillo R, Demontis F, Perrimon N, Goldberg AL. Mechanisms of muscle growth and atrophy in mammals and *Drosophila*. *Dev Dyn.* 2014; 243:201–215. [PubMed: 24038488]
- Pollard TD. Electron microscopy of synthetic myosin filaments. Evidence for cross-bridge. Flexibility and copolymer formation. *J Cell Biol.* 1975; 67:93–104. [PubMed: 1236853]
- Rhee D, Sanger JM, Sanger JW. The premyofibrils: evidence for its role in myofibrillogenesis. *Cell Motil Cytoskeleton.* 1994; 28:1–24. [PubMed: 8044846]
- Sandquist JC, Means AR. The C-terminal tail region of nonmuscle myosin II directs isoform-specific distribution in migrating cells. *Mol Biol Cell.* 2008; 19:5156–67. [PubMed: 18843042]
- Sanger JW, Mittal B, Sanger JM. Formation of myofibrils in spreading chick cardiac myocytes. *Cell Motil.* 1984; 4:405–416. [PubMed: 6391683]
- Sanger JM, Mittal B, Pochapin BM, Sanger JW. Myofibrillogenesis in living cells microinjected with fluorescently labeled alpha-actinin. *J Cell Biol.* 1986; 102:2053–2066. [PubMed: 2423530]
- Sanger JM, Mittal B, Meyer TW, Sanger JW. 1989. Use of fluorescent probes to study myofibrillogenesis. In: Stockdale F, Kedes L, editors *Cellular and Molecular Biology of Muscle Development*. Alan R. Liss, Inc; New York: 1989. 221–235.
- Sanger JM, Danowski BA, Sanger JW. Microinjection of fluorescently labeled alpha-actinin into living cells. In: Tuan RS, Lo CW, editors *Methods in Molecular Biology: Developmental Biology Protocols*. Vol. III. Humana Press; 2000. 449–456.
- Sanger JW, Chowrashi P, Shaner NC, Spalthoff S, Wang J, Freeman N, Sanger JM. Myofibrillogenesis in skeletal muscle cells. *Clin Orthop Related Res.* 2002; 403S:S153–S162.
- Sanger JW, Sanger JM, Franzini-Armstrong C. Assembly of the skeletal muscle cell. In: Engel AG, Franzini-Armstrong C, editors *Myology*. 3rd. McGraw-Hill; New York: 2004. 35–65.
- Sanger JW, Kang S, Siebrands CC, Freeman N, Du A, Wang J, Stout AL, Sanger JM. 2005. How to build a myofibril. *J Muscle Res Cell Motility.* 2005; 26:343–354.

- Sanger JW, Wang J, Holloway JB, Du A, Sanger JM. 2009. Myofibrillogenesis in skeletal muscle cells in zebrafish. *Cell Motil Cytoskeleton*. 2009; 66:556–566. [PubMed: 19382198]
- Sanger JW, Wang J, Fan Y, White J, Sanger JM. Assembly and dynamics of myofibrils. *Journal of Biomedicine and Biotechnology*. 2010; 2010:8. Article ID 858606, www.hindawi.com/journals/jbb/2010/858606.html.
- Sanger JW, Sanger JM. Green fluorescent proteins improve myofibril research. *Biophotonics Internat*. 2001; 8:44–46.
- Sanger JW, Wang J, Fan Y, White J, Mi-Mi Lei, Dube DK, Sanger JM, Pruyne D. Assembly and maintenance of myofibrils in living muscle cells. *Handbook of Experimental Pharmacology*. 2017; 235:39–75. *The Actin Cytoskeleton*. [PubMed: 27832381]
- Sellers JR. *Myosins*. 2nd. Oxford University Press; New York: 1999.
- Stout AL, Wang J, Sanger JM, Sanger JW. Tracking changes in Z-band organization during myofibrillogenesis with FRET imaging. *Cell Motil Cytoskeleton*. 2008; 65:353–367. [PubMed: 18330906]
- Swales NT, Colegrave M, Knight PJ, Peckham M. Non-muscle myosins 2A and 2B drive changes in cell morphology that occur as myoblasts align and fuse. *J Cell Sci*. 2006; 119:3561–3570. [PubMed: 16895968]
- Takeda K, Yu ZX, Qian S, Chin TX, Adelstein RS, Ferrans VJ. Nonmuscle myosin II localizes to the Z-lines and intercalated discs of cardiac muscle and the Z-lines of skeletal muscle. *Cell Motil Cytoskel*. 2000; 46:59–68.
- Takeda K, Kishi H, Ma X, Yu Z, Adelstein RS. Ablation and mutation of nonmuscle myosin heavy chain II-B results in a defect in cardiac myocyte cytokinesis. *Circ Res*. 2003; 93:330–337. [PubMed: 12893741]
- Tullio AN, Accili D, Ferrans VJ, Yu ZX, Takeda K, Grinberg A, Westphal H, Preston YA, Adelstein RS. Nonmuscle myosin II-B is required for normal development of the mouse heart. *Proc Natl Acad Sci USA*. 1997; 94:12407–12412. [PubMed: 9356462]
- Vicente-Manzanares M, Ma X, Adelstein RS, Horwitz AR. Non-muscle myosin II takes centre stage in cell adhesion and migration. *Nature Rev Mol Cell Biol*. 2009; 10:778–790. [PubMed: 19851336]
- Wang J, Shaner N, Mittal B, Zhou Q, Chen J, Sanger JM, Sanger JW. Dynamics of Z-band based proteins in developing skeletal muscle cells. *Cell Motil Cytoskeleton*. 2005; 61:34–48. [PubMed: 15810059]
- Wang J, Sanger JM, Kang S, Thurston H, Abbott LZ, Dube DK, Sanger JW. 2007. Ectopic expression and dynamics of TPM1 α and TPM1 κ in myofibrils of avian myotubes. *Cell Motil. Cytoskeleton*. 2007; 64:767–776.
- Wang J, Thurston H, Essandoh E, Otoo M, Han M, Rajan A, Rajan A, Dube S, Zajdel RW, Sanger JM, Linask KK, Dube DK, Sanger JW. Tropomyosin expression and dynamics in developing avian embryonic muscles. *Cell Motil. Cytoskeleton*. 2008; 65:379–392.
- Wang J, Dube DK, Mittal B, Sanger JM, Sanger JW. Myotilin dynamics in cardiac and skeletal muscle cells. *Cytoskeleton*. 2011; 68:661–670. [PubMed: 22021208]
- Wang Q, Moncman CL, Winkelmann DA. Mutations in the motor domain modulate myosin activity and myofibril organization. *J Cell Sci*. 2003; 116:4227–4238. [PubMed: 12953063]
- White J, Barro MV, Makarenkova HP, Sanger JW, Sanger JM. Localization of sarcomeric proteins during myofibril assembly in cultured mouse primary skeletal myotubes. *Anat Record*. 2014; 297:1571–1584.
- Zal T, Gascoigne NRJ. Photobleaching-corrected FRET imaging of live cells. *Biophys J*. 2004; 86:3923–3939. [PubMed: 15189889]

Figure 1. Expression of each of the three isoforms of nonmuscle myosin II (NM II) in live myotubes leads to similar patterns of incorporation in premyofibrils
 Premyofibrils in the growing ends of live quail myotubes were recorded two days after cotransfections with plasmids encoding GFP and non-muscle myosin II isoforms (a, GFP NM IIA; d, GFP-NM IIB; g, NM IIC-GFP; cyan in c, f, i), together with sarcomeric alpha-actinin-CeFP (b, e, h, magenta in c, f, i). Each isoform of non-muscle myosin: IIA (a), IIB (d), and IIC (g) localized in premyofibrils in small bands that alternated with bands of alpha-actinin: merged images (c, f, i). Boxed areas are shown enlarged in the merged column. Bar = 10 microns (a-i). Bar = 2 microns (inset in c, f, i).

Figure 2. Expression of each isoform of NM II in older myotubes leads to their localization in thin premyofibrils along the myotube membrane adjacent to mature myofibrils
 Each isoform of non-muscle myosin II coupled to GFP incorporated in a few thin premyofibrils adjacent to the membrane surrounding mature myofibrils in the middle of myotubes: (a) IIA; (d) IIB; (g) IIC (cyan in c, f, i). alpha-actinin-CeFP localization in Z-bands marks mature myofibrils in the myotube center (b, e, h; magenta in c, f, i). Note that none of the three fluorescently labeled isoforms of NM II is localized in Z-Bands (c, f, i). Bar = 10 microns.

Figure 3. Spatially distinct patterns of localization of NM IIA and sarcomeric alpha-actinin are illustrated in axial views, XY, XZ, and YZ, of a myotube cotransfected with GFP-NM IIA and sarcomeric alpha-actinin-CeFP

Three views of a confocal image of the same live myotube show NM IIA (a, cyan in c) localized around the periphery of the myotube, and absent from the center of the myotube where alpha-actinin is concentrated in Z-Bands (b, magenta in c). Merged view (c) indicates the lack of any overlap between Z-Bands (magenta) and NM II (cyan) in mature myofibrils. Bar = 10 microns.

Figure 4. Overexpression of non-muscle myosin IIA in a myotube results in its localization in A-Bands and in aggregates

Four days after myotubes were cotransfected with (a) alpha-actinin-CeFP and (b) GFP-NM IIA, they were fixed and stained with muscle-specific myosin II (MM II) antibody, F-59 (c). Arrowheads (a, b, c; images enlarged in d to i) mark the positions of three Z-Bands (blue in g and i). (b, e, green in g) Single bands of GFP-NM IIA localize between the Z-bands (arrowheads). (c, f, i) F-59 antibody staining shows muscle myosin heavy chain (MM II) localized in a doublet pattern in A-Bands (red in h and i) between the Z-Bands (arrowheads). Note that even when overexpressed, NM IIA did not colocalize with alpha-actinin in Z-Bands in mature myofibrils (b, e, g). The magnified views of NM IIA (e, green in g and h) and MM II (f, red in h and i) indicate that the bands of NM IIA formed in the center of the MM II doublet in the A-Bands. Arrows in (b) point out nonsarcomeric aggregates of nonmuscle myosin IIA. Bar = 10 microns (a-c); 2 microns (d-i).

Figure 5. Expression of each of the three isoforms of nonmuscle myosin II (NM II) in living embryonic chick cardiomyocytes leads to similar patterns of incorporation in premyofibrils Premyofibrils are detected two days after cotransfections by their fluorescent non-muscle myosin II isoforms. (a) GFP-non-muscle myosin IIA; cyan in (c). (d) GFP-non-muscle myosin IIB; cyan in (f). (g) non-muscle myosin IIC-GFP; cyan in (i). (b, e, h) Mature myofibrils are detected in these same cells by the localization of sarcomeric alpha-actinin-CeFP in their Z-Bands (b, e, h; magenta in c, f, i). Note that none of the three different isoforms of non-muscle myosin II are localized in the Z-Bands (c, f, i). Bar = 10 microns.

Figure 6. Fluorescence Recovery after Photobleaching (FRAP) compares the dynamic exchange and mobile fractions of NM II isoforms in premyofibrils in skeletal and cardiac myocytes

In each muscle type, recovery curves and mobile fractions calculated after photobleaching show similar values for NM IIA and IIB in skeletal muscle (a, b) and in cardiomyocytes (c, d). Whereas in both types of muscle, IIC recovery occurred much more slowly (a, b and c, d).

Figure 7. Expression of NM IIC-GFP in myotubes leads initially to its incorporation in a banded pattern in premyofibrils. Several days after transfection, however, premyofibrils disassemble (a) NM IIC-GFP assembled in premyofibrils in the flat, spreading end of a myotube two days after transfection. (b) Four days after transfection of NM IIC-GFP, premyofibrils had disassembled. Bar = 10 microns.

Figure 8. SE-FRET analysis suggests that isoforms of NM II copolymerize in premyofibrils in live quail myotubes

Pairs of plasmids encoding CeFP or YFP and different isoforms of NM II were cotransfected in quail myotubes: (a, b) CeFP-NM IIA and YFP-IIB pair; (d, e) NM IIC-CeFP and YFP-IIA pair; and (g, h) NM IIC-CeFP and YFP-IIB pair. FRET signals were acquired in three channels: donor channel (a, d, g for CeFP), acceptor channel (b, e, h for YFP) and the resultant FRET channel (c, f, i). FRET efficiencies were calculated from these three-channel images and included in Table 2. Bar = 5 μ m.

Figure 9. NMII and MM II colocalize in nascent myofibrils early in myofibril assembly in living myotubes

Cultures of quail myotubes were cotransfected with CeFP-NM IIA (a, cyan in c, f), and YFP-MM II (b, magenta in c, f). NM IIA (a) localized in fibers at the spreading end of the living myotube (e.g. small arrow in a), on the periphery of the middle of the myotube (arrowheads in d, cyan in f). MM II localized in some of the fibers near the leading edge of the myotube (e.g. large arrow in b, c), and was organized in A-Bands of mature myofibrils in the central shaft of the myotube (e, magenta in f). The small arrow in (a) marks a fibril that lacks MM II (small arrow in b) and is termed a premyofibril. The larger arrow in (a) marks a fibril that has both NM IIA, and MM II (large arrow in b); this fiber is termed a nascent myofibril. The myofibrils that contain just MMII in their A-Bands are the mature myofibrils (e, magenta in c). Fibers that contain only NMIIA, i.e., premyofibrils, are also found on the periphery of the central shafts of myotubes (arrowheads in d, e, cyan in f). Bar = 5 microns.

Figure 10. Graph of values of E% determined from SE-FRET measurements in living myotubes cotransfected with different pairs of plasmids encoding MM II and isoforms of NM II
 The control FRET value was obtained from cotransfected myotubes expressing free CeFP and free YFP. The nascent myofibrils exhibited a strong FRET signal between MM II and NM IIA, IIB or IIC, suggesting close proximities of these different myosin IIs. The low FRET values of premyofibrils and mature myofibrils are consistent with the presence of just one type of myosin in these fibrils, i.e. NM II in premyofibrils and MM II in mature myofibrils. Quail myotubes expressing two differently labeled MM II (CeFP-MM IIs and YFP-MM II) in the A-Bands of mature myofibrils have a FRET E% value of about 14%, comparable to the FRET E% values obtained from the colocalization of fluorescently tagged NM II (IIA or IIB or IIC) and MM II in nascent myofibrils.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1
Summary of FRAP data for non-muscle myosin II isoforms in premyofibrils of skeletal (S) and cardiac muscle cells (C)

	$M_{(fast)} (\%)$	$K_{(fast)} (s^{-1})$	$T_{1/2 (fast)} (s)$	$M_{(slow)} (\%)$	$K_{(slow)} (s^{-1})$	$T_{1/2 (slow)} (s)$	$M_{(total)} (\%)$
NM IIA (S)	44 ± 12	0.035 ± 0.014	23 ± 11	49 ± 12	0.0056 ± 0.0015	174 ± 46	93 ± 12
NM IIB (S)	33 ± 19	0.027 ± 0.010	34 ± 15	61 ± 18	0.0037 ± 0.0014	234 ± 67	94 ± 9
NM IIC (S)	3.3 ± 1.7	0.050 ± 0.016	17 ± 5	24 ± 10	0.0017 ± 0.0004	406 ± 89	27 ± 10
NM IIA (C)	27 ± 13	0.010 ± 0.002	70 ± 5	67 ± 8	0.0018 ± 0.0001	391 ± 47	94 ± 6
NM IIB (C)	19 ± 6	0.012 ± 0.003	61 ± 15	76 ± 5	0.0015 ± 0.0001	485 ± 67	95 ± 10
NM IIC (C)	3.5 ± 1.8	0.021 ± 0.007	33 ± 10	26 ± 3	0.0010 ± 0.0003	735 ± 149	29 ± 3

Table 2
Evidence for the copolymerization of the three different isoforms with each other in premyofibrils

FRET-Pair		Efficiency(%)	N
IIA-IIA	<i>CeFP</i> -NM IIA+ <i>YFP</i> -NMIIA	13.18±1.8	10
IIA-IIB	<i>CeFP</i> -NM IIA+NM IIB- <i>YFP</i>	11.52±1.3	9
	NM IIA- <i>CeFP</i> +NM IIB- <i>YFP</i>	15.71±1.05	4
IIA-IIC	NM IIC- <i>CeFP</i> + <i>YFP</i> -NM IIA	13.92±1.9	10
	NM IIC- <i>CeFP</i> +NM IIA- <i>YFP</i>	17.57±2.07	7
IIB-IIC	NM IIC- <i>CeFP</i> +NM IIB- <i>YFP</i>	13.88±1.77	7
IIC-IIC	NM IIC- <i>CeFP</i> +NM IIC- <i>YFP</i>	12.88±1.13	25
Control	CeFP+YFP	3.27±0.49	10