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From myofibril to membrane; the transitional junction at the intercalated disc

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1. ABSTRACT

Cardiomyocytes are coordinated by linking together at their ends through the intercalated disc. The intercalated disc with its complex folded membrane, encompasses many structural and signalling functions and is thought to play a role in cell growth and sarcomere addition. Its relationship to the contractile myofibrils is central to myocyte function. The myofibrils continue their ordered sarcomeric structure up to the edge of the intercalated disc where there is no terminal Z-disc but, instead a transitional junction. Thin actin-containing filaments from the final half sarcomere extend beyond their normal length through the transitional junction to the folded intercalated disc membrane where tension is transmitted.

The peaks of the membrane folds also occur at the transitional level. They are spectrin rich and associated with sarcoplasmic reticulum vesicles. A subset of Z-disc proteins including titin, alpha-actinin and ZASP/cypher/oracle are found in the transitional region while others such as telethonin and FATZ/calsarcin/myozenin are absent. The presence of titin enables ordered sarcomeres to be maintained independently of changes in the amplitude of the membrane folds. The transitional junction is therefore poised to act as a site for a new Z-disc/SR/T-tubule complex and sarcomere addition. The evidence for this is reviewed.

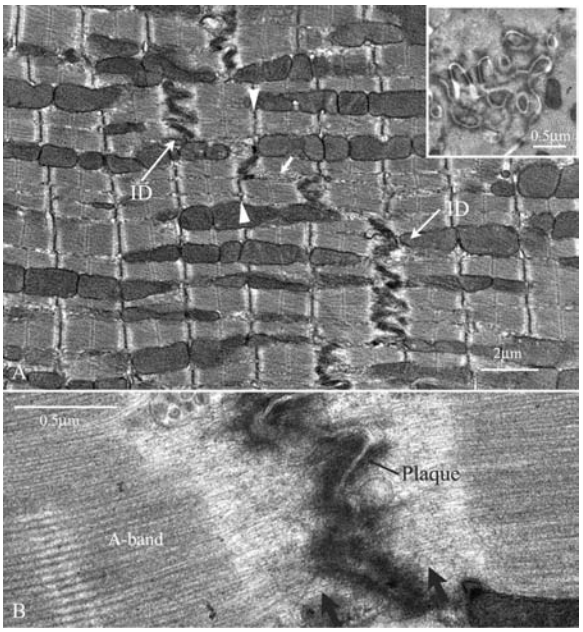

Figure 1. Electron micrographs of sections of mouse left ventricle. A. Longitudinal view of an ID showing stepped structure, well ordered sarcomeres and the relationship between neighbouring cells. Small arrow indicates the position of membrane on an axial 'riser' where gap junctions are usually found. Longer arrows indicate regions of folded ID membrane. Arrowheads indicate two Z-discs in neighbouring cells separated axially by the amplitude of the ID folds. *Inset.* Transverse section through an ID showing the circular shapes of the ID membrane peaks. B. High magnification longitudinal view of an inter-myofibrillar region of an ID showing the good order of the proximal A-bands. Large arrows indicate the position of the TJ and absence of a terminal Z-disc. Mouse heart tissue was fixed and processed for electron microscopy as described in (1).

2. INTRODUCTION TO THE TRANSITIONAL JUNCTION

2.1. General characteristics of the transitional junction at the intercalated disc

The muscle cells of the heart are linked at their ends through the intercalated disc (ID) in a way that allows both structural and functional coordination. The ID membrane is a complex folded structure which encompasses many domains that facilitate structural and signalling roles. Within the cardiomyocyte, myofibrils run from one end to the other between IDs (Figures 1, 2). The relationship of the contractile myofibrils to the ID is clearly central to the function of the myocyte but it is not well understood. A striking feature of the transition from myofibril to ID is that the sarcomere structure is maintained in an orderly way all the way to the edge of the ID. However, the Z-disc that normally delineates sarcomeres is absent at the end of the last sarcomere and the thin actin-containing filaments continue to the ID membrane. In addition, the ID membrane folds peak at the axial level where the ordered myofibrils end, that is, where the final Z-disc would have been if it were there. We have called the intersection between the orderly sarcomere structure and the ID together with the associated membrane peaks the 'transitional junction' (TJ) (1) (Figure 2). There we identified at the TJ the Z-disc components, titin, alpha-actinin and ZASP/Cypher and the membrane-associated protein, alphaII spectrin. Their presence suggested an important functional role for this region in maintaining sarcomere order up to this point.

This review is concerned with this relationship between fibril and ID, the properties of the transition and, also, its implications for growth and disease. Some new evidence from immunofluorescence and electron microscopy in support of these ideas is also presented.

These observations may be of wider interest since there are similarities between the intercalated disc in the heart and the myotendinous junction of skeletal muscle where the ordered sarcomeric structure is maintained up to where the myofibrils merge into the terminal actin bundles that insert into the tendon (see for example (2, 3)). Again there is no intervening Z-disc suggesting that a transitional-type junction is also present here.

2.2. General structure of the intercalated disc

When describing the ID it is often the membrane and its associated structures that are referred to. However, the presence of a transitional junction very strongly argues for all the material, including the thin filaments within the ID membrane folds to be part of the ID. It is in this sense that the term ID is used in this manuscript.

As befitting such a multifunctional organelle the structure of the intercalated disc is complex. See for example Fawcett and McNutt (4) and Forbes and Sperelakis (5) who have described the organisation in the left ventricle of the cat heart. They show a stepped organisation with regions of folded membrane transverse to the long axis of the cell (Figure 1A). The folded membrane in the ID is peaked and, in transverse views, rings of membrane can be seen resembling a section through an egg box (Figure 1A inset; (4)). The separation of the peaks laterally is about 500nm, that is, the order of the width of a 'myofibril' (personal observation). The amplitude of the folds can vary significantly from one ID to the next, but, generally speaking in healthy heart tissue, the amplitude of the ID folds between any two cells is the same across the whole area of interaction and independent of steps (Figure 1A). Further, as can be seen at steps in the ID, sarcomeres in neighbouring cells are displaced axially by the amplitude of the folds of the ID (arrowheads Figure 1A).

There are three prominent intercellular membrane junctions. The gap junctions which carry the electrical stimulus from cell to cell, are usually associated with the risers, the longitudinal, axial stretches of ID membrane. Here, the membranes are closely apposed and tightly and regularly packed with connexin proteins (6, 7). The ID membranes are mechanically riveted together by desmosome junctions (see review (8)). These dense well ordered structures often occur in the transverse regions of the ID particularly where mitochondria abut the membrane.

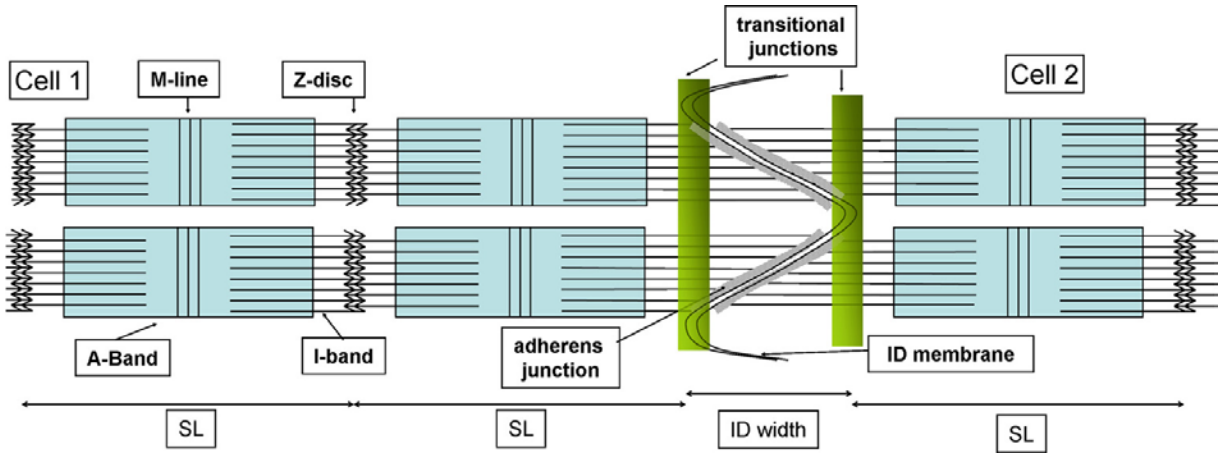

Figure 2. Diagram to show relationship between myofibrils, the TJ and the adherens junctions at the folded membrane of the ID. Sarcomeres are shown with their A-bands, thin filaments and limiting Z-discs to the left and right of an ID. The sarcomeres adjacent to the ID have no limiting Z-disc on the ID side, but the thin filaments continue into the fold of the ID and insert into the density of the AJ plaque. The transitional junctions (green boxes) are indicated at the positions equivalent to the Z-disc in the other half of the sarcomeres such that the distance between the TJ and the next Z-disc is a sarcomere length. The tops of the folds of the ID membrane are located axially at the position of the TJ.

The most prominent structures in the ID are the adherens or composite junctions seen in the interfibrillar regions. These are associated with complex folds where a dense rather amorphous plaque is seen on the slopes of the folds (Figure 1B). The proteins associated with the dense material are those normally associated with adherens junctions, e.g. N-cadherin, vinculin, beta-catenin (see (9)). However, in addition, Franke and his colleagues have shown that a number of desmosomal proteins are also found here, e.g. desmoplakin, plakoglobin. They have called these regions *areae compositae* or composite junctions (see a recent review (10)). The more traditional name, adherens junction (AJ) will be used for this review. Thin filaments from the sarcomere proximal to the ID continue and extend through the transitional junction into the ID membrane folds and invest into the AJ plaque so that the contractile tension generated is transmitted directly across the membrane from one cell to the next (5, 11) (Figure 1B and 2) .

In addition to these well characterised membrane domains, there are, between them, unspecialised regions of the ID membrane of unknown function. One such region is at the tops of the folds which are part of the transitional junction and will be described in more detail later (Figure 2).

3. EVIDENCE FOR THE TRANSITIONAL JUNCTION

3.1. Z-disc functions are separated at the intercalated disc

The ID has often been described as a functional Z-disc, operating between the fibrils of neighbouring cells. Indeed the plaque material of the AJ at the membrane could be considered to be equivalent to the Z-disc and certainly it is here that the contractile tension is transduced from one cell to the next. However, the question arises as to how the order of the last sarcomere is maintained when the thin filaments of its proximal half have such variable length. In the normal sarcomere the giant protein titin, stretching from M-band to Z-disk, is thought to maintain the thick filaments at the centre of the sarcomere through its elastic properties in the I-band (12-15). If titin were attached to the end of the thin filaments at the ID membrane, then, because of the variable length of these filaments, the tension exerted on the thick filaments would also be very variable and the A-band in the final sarcomere would be very disordered, contrary to observation. We showed by immunofluorescence that titin is indeed present in the ID region but the N-terminal epitopes, normally associated with the Z-disc, are not found at the ID membrane at the end of the thin filaments, rather as two bands, one either side of the ID, appearing as a doublet of intensity ((1); see Figure 3). The position of this doublet, later defined as the position of the transitional junctions, is shown in Figure 2. The separation of the doublet varies depending on the amplitude of the ID folds (the ID width). However, each of the doublet lines bears the same relationship to its neighbouring Z-disc as do two successive Z-discs, i.e. a separation equivalent to the sarcomere length. The observation suggested that titin functions normally in the end sarcomere and explains why the sarcomeres are so well ordered up to the ID. Hence, the Z-disc function of anchoring titin is separated from tension transmission at the ID membrane. How this can occur is another matter. One approach is to determine what other proteins and structures are associated with this region.

3.2. Myofibrillar proteins at the transitional junction

3.2.1. I-band proteins

We can ask what other thin filament proteins characterise the TJ. Further immunofluorescence evidence reinforced the idea that the last half sarcomere has a typical myofibrillar thin filament protein distribution. Half sarcomeres of striated muscle I-band proteins, tropomyosin

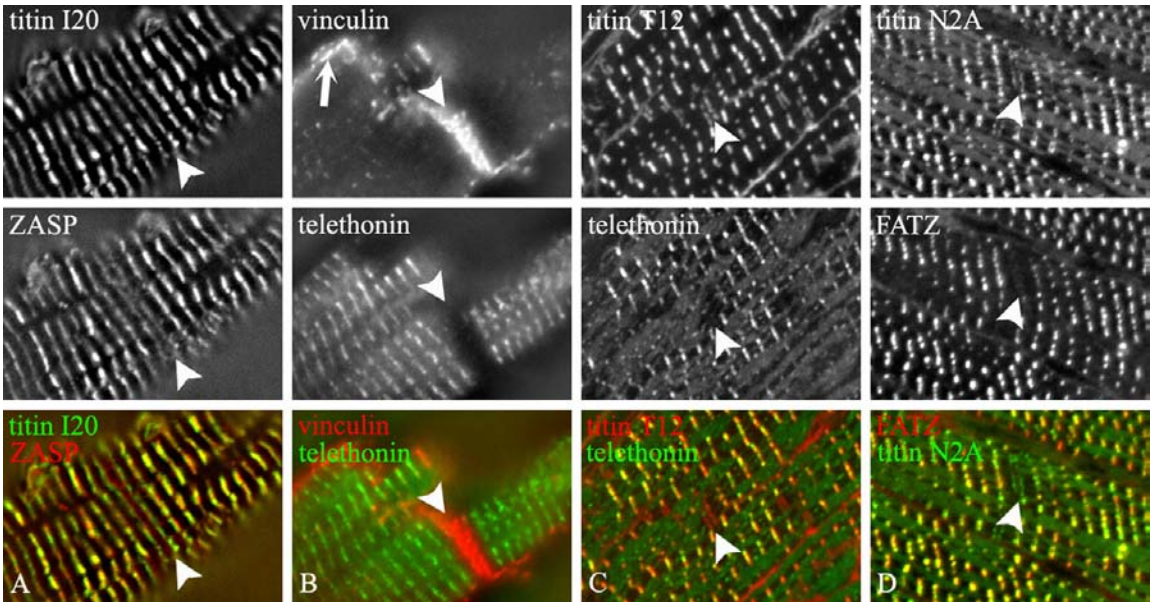

Figure 3.. Immunofluorescence of mouse heart tissue labelled with indicated antibodies. A and B; myofibril preparation. C and D; thin cryosections. Arrowheads indicate IDs. A. Antibodies to I-band titin (I20 epitope close to the C-terminal region of the elastic PEVK region (102)) and ZASP both label near the Z-disc and as a doublet about the ID at the TJ. ZASP (red) being at the Z-disc, its doublet separation at the ID is seen to be slightly smaller than that from the titin (green). B. Antibody to vinculin (red) strongly labels the ID and the costameres on the plasma membrane (arrow in top frame). Telethonin (green) has no presence at the ID but is clear at the Z-disc. C. Antibodies to Z-disc titin (red) (epitope T12 is located approximately 100 nm from the edge of the Z-disc (103)) and telethonin (green) colocalise at the Z-disc but telethonin is not present in the ID at the TJ. D. Antibodies to I-band titin (green) (N2A epitope is close to the Z-disc at the N-terminal end of the elastic PEVK region (104)) and FATZ (red) both localise near the Z-disc but FATZ is not present in the ID at the TJ. Immunofluorescence of thin cryosections was as described in (1). Antibodies not described there were mouse polyclonal anti-ZASP1 and anti-FATZ2 from Dr Georgine Faulkner, rabbit anti-telethonin from Professor Mathias Gautel and rabbit anti-titin N2A from Dr Wolfgang Linke.

and troponin, are seen either side of the ID (1). It was also suggested that alpha-cardiac actin, present in normal sarcomeres, was constrained to this region and not in the ID.

Phalloidin staining confirms the presence of actin within the ID folds although the precise isoform has not been established (1). Neither has any other thin filament ancillary proteins such as non-striated muscle tropomyosin, been identified in the ID folds.

3.2.2. Z-disc proteins

In the absence of Z-disc density at the TJ, which, if any, of the normal complement of Z-disc proteins are present? Many proteins have been associated with the Z-disc and several recent reviews have described the properties and interactions of these components (16-20). Figure 4A shows diagrammatically a selected group of proteins found at the Z-disc which are present in sufficient amounts that they may contribute to its structural integrity. These comprise the core proteins actin, alpha-actinin and titin together with the actin binding proteins capZ and nebulette, the titin associated protein, telethonin, and the alpha-actinin associated proteins, ZASP, myotilin, filamin and FATZ. Ringed in the figure are the proteins that will be considered here. Figure 4B shows which of those proteins are found to be present at the TJ and which are absent. Below, the salient features of these proteins and how they may relate to the TJ are described.

3.2.2.1. Actin

In the Z-disc it is the barbed ends of the thin filaments that overlap and are cross linked by alpha-actinin. The amount of overlap, that is, the width of the Z-disc depends on the muscle and is about 140nm in the heart. At the TJ there is no antiparallel array of thin filaments. Forbes and Speriakis (5) showed by stereo microscopy that the filaments were continuous from within the last sarcomere to the ID membrane. In addition, the thin filaments labelled with myosin subfragment-1 all had the same orientation with their barbed ends at the ID membrane (11). The immunofluorescence results seem to show that the actin isoform in the proximal half sarcomere is at least partly alpha-cardiac actin, the same as in the rest of the fibril, and different from the isoform in the ID folds (1). The ID fold isoform has not been identified and it may be a non-muscle isoform. Non-muscle myosin II is found in the ID (see X. Ma & R.S. Adelstein: *In vivo* studies on non-muscle myosin II expression and function in heart development. Front Biosci. In press – this volume) and it normally interacts with non-muscle actin. Of the non-muscle isoforms, gamma-actin is expressed in muscle cells only at a low level and is found near the plasma membrane where myofibrils are attached through their Z-discs at

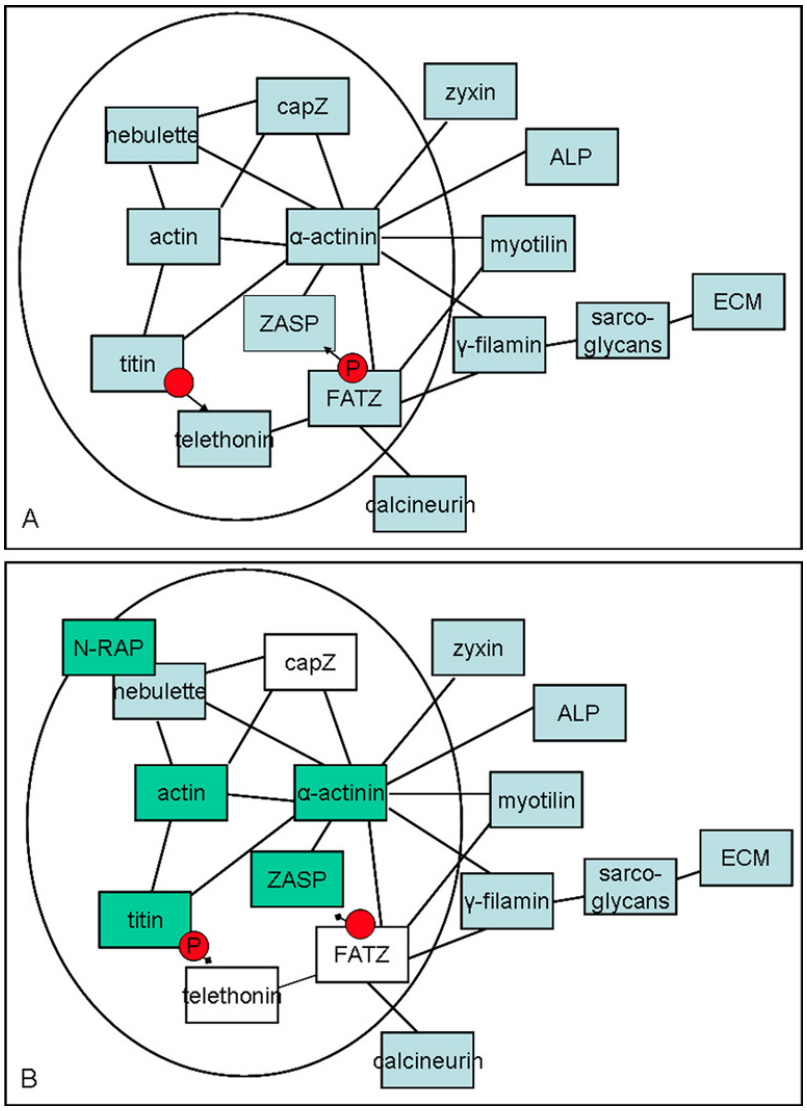

Figure 4. A. Z-disc proteins at the ID. A selection of Z-disc proteins with a structural function are shown with lines indicating their interactions. The large circle surrounds the proteins investigated at the TJ and discussed in the text. The small red circles indicate a phosphorylation dependent interaction. Putative phosphorylated proteins are indicated with a P within the circle. B. The same group of proteins but colour coded to show whether they have been shown to be present at the TJ (dark blue), are not present (white) or have not been tested (pale blue). The protein N-RAP is added since it has been shown to have a strong split presence at the ID.

costameres (21). Its location at the ID is not known. beta-actin has recently been found at the ID in feline cardiomyocytes (22).

3.2.2.2. CapZ

CapZ is an alpha/beta heterodimer that acts as a barbed end capping protein for F-actin and is found at the end of the thin filaments in the Z-disc (23, 24). Its structure has been determined and relationship to the thin filament modelled (25). CapZ is present at the intercalated disc but since the thin filaments are continuous from the myofibril to the ID membrane there should be no binding site for capZ at the TJ. This is supported by the evidence that it is the non muscle subunit beta2 and not the sarcomeric beta1 that is located at the ID suggesting that capZ binds at the end of the thin filaments near the membrane (26).

3.2.2.3. Nebulette

Nebulette is thought to replace the skeletal muscle thin filament protein, nebulin, in the heart generally (27). Like nebulin it has a C-terminal SH3 domain which locates it at the Z-disc end of the thin filament where it interacts with actin, alpha-actinin 2 and tropomyosin (28). Nebulin also binds to capZ, so it is likely that nebulette does too (29). The main body of nebulette comprises a number of tandem nebulin repeats which bind sequentially to actin monomers for about 150nm running along the filament through the Z-disc towards the pointed end. It is not clear

whether nebulette is present in the region close to the ID. Some evidence that it may be can be deduced from the presence of nebulin at the myotendinous junction in skeletal muscles. This junction, as mentioned above, has significant similarities to the ID. Ordered sarcomeric structure is maintained up to where the myofibrils merge into the terminal actin bundles. Furthermore, here, as in the ID there is another nebulin related protein, N-RAP, associated with these bundles (see below). Immunoelectron microscopy has shown that nebulin is present in the final half sarcomere in its normal position (3). The similarities between nebulin and nebulette in their C-terminal sequences and their association with the Z-disc imply that nebulette will be found at the TJ.

3.2.2.4. Alpha-actinin

alpha-actinin is an ubiquitous molecule and its structure and function has been recently reviewed by Sjoblom et al. (30). It is an antiparallel rod shaped homodimer some 40nm in length. Its N-terminal actin binding domains (ABD) is linked to C-terminal calmodulin (CaM-like) domain by a central rod of four spectrin repeats, R1-R4, each a triple alpha-helical coiled coil bundle. All parts of the molecule act as a binding partner for other proteins; so much so that Hampton *et al* (31) have suggested that alpha-actinin essentially acts as a hook to hang other proteins on.

The cardiac sarcomeric isoform, alpha-actinin 2, cross-links antiparallel actin filaments at the cardiac Z-disc and forms part of the angled Z-bridges. It is also present at the TJ. It has the same doublet appearance across the ID as titin ((1)). Like titin, the fluorescence is weaker than that at the Z-discs consistent with there being fewer alpha-actinin molecules concentrated at any position. Also, the doublet is quite diffuse suggesting that molecules may be spread over a greater length of the filaments. The alpha-actinin link between actin filaments is very flexible as has been demonstrated by rotating an actin filament attached to another by an alpha-actinin link by 180^{o} using optical tweezers (32). Taylor and his co-workers ((31, 33) have shown that alpha-actinin can link both parallel and anti-parallel actin filaments *in vitro*, the characteristic spacing between parallel filaments being bigger (up to 38nm compared to 22nm). This is consistent with the bigger spacing expected at the TJ between parallel filaments compared to that between twice the number of antiparallel ones at the Z-disc. The flexibility of the alpha-actinin - actin link suggests that additional components may be required to stabilise the actin/alpha-actinin structure in its parallel or anti-parallel conformation (31).

3.2.2.5. Titin

The sequence of the giant protein titin has been described as a blueprint for filament organisation in the sarcomere (14, 34, 35). The N-terminal sequence associated with the Z-disc is no exception (36-40). The sequence starts with 3 Ig domains, Z1-Z3, followed by several short 45aa Z repeats. The number of Z repeats depends on the muscle since they are differentially spliced, but in the heart the maximum 7 are present. Further Ig domains and linker sequences follow.

Z-disc titin interacts with a number of proteins. At the end of the titin molecule, the Z1Z2 Ig domains are the binding site for telethonin/Tcap (see below). There are significant interactions of titin with alpha-actinin. Two types of alpha-actinin binding sites have been identified. One is in the zq –Z4 sequence, C-terminal to the Z repeats, which associates with the alpha-actinin rod repeats R2R3 ((36)). The other is in the alpha-helical Z repeats which bind to the C-terminal EF34 hands of the CaM domain (36, 38, 41). The NMR structure of this complex shows that the way in which the alpha-helical Z repeat relates to the EF hands is similar to a number of other structures such as the troponinC/troponinI interface and the binding of myosin light chain to the IQ domain of the myosin heavy chain (42, 43).

Some parts of I band titin can bind to actin filaments. The near Z-disc Ig domains Z10 –I1 form a complex with actin (44). In addition, the PEVK region which constitutes the principle elastic region in the I-band under normal sarcomere lengths, associates with the thin filament. It has been found to inhibit the movement of actin filaments over myosin in the sliding filament assay (45-47). This inhibition is calcium sensitive in the presence of the calcium binding protein S100A1 (46).

These multiple interactions suggest that titin, actin and alpha-actinin may, under the influence of thermal vibrations, be able to zip up to form the basic structure of the thin filament in the Z-disc and at the TJ.

3.2.2.6. ZASP/Cypher/Oracle

ZASP/cypher/oracle is a member of the enigma family of proteins (48) and is found at the Z-disc. We previously reported that it was present at the TJ and evidence for this is shown in Figure 3A (1). The protein is characterised by an N-terminal PDZ domain and C-terminal LIM domains (49-51). There are at least 6 differentially spliced isoforms, three specific to cardiac muscle and three to skeletal muscle (52). In heart there are two principle isoforms, the longer with 3 LIM domains and the shorter, which lacks the LIM domains. The longer is present throughout development and the shorter is upregulated after birth and is the predominant isoform in adult heart. We have investigated the location of the short isoform, ZASP1, in relation to N-terminal titin by immunofluorescence. Figure 3A shows that, as well as at the Z-disc, it is clearly present at the TJ as demonstrated by the characteristic doublet about the ID.

ZASP has been found to interact with several Z-disc proteins. The PDZ domain interacts with C-terminal PDZ canonical binding motif of FATZ (see below), myotilin and alpha-actinin (53). Near the centre of the ZASP sequence is a special Z-disc binding motif, ZM, which targets the rod domain of alpha-actinin (54, 55). This domain is also found in other enigma proteins such as ALP which are also found at the Z-disc. Thus ZASP has two alpha-actinin binding sites.

The role of ZASP at the Z-disk is not very well defined but its importance is illustrated by the number of

muscle diseases associated with ZASP mutations, so-called 'ZASPologies' (56). Its presence at the TJ also indicates its essential role. The discovery of two alpha-actinin binding sites has led to the suggestion that it acts as a strut and stabilises alpha-actinin binding to the thin filament (57). In addition, the LIM domains of ZASP are a binding partner for many protein kinase C (PKC) isoforms alpha, beta1, zeta, gamma, delta and epsilon and they can be phosphorylated by PKCbeta1 (50). It is suggested that in this way PKC could be targeted to the Z-disc and hence could be involved in a regulatory role in Z-disc and, therefore, TJ formation.

3.2.2.7. Telethonin/T-cap

The titin binding protein telethonin (T-cap) is a small 2 domain protein that interacts very tightly with the Z1Z2 Ig domains of two titin molecules (58-60). The crystal structure of the complex shows that the titin molecules are antiparallel and sandwich the N-terminal 90 residues of telethonin (61). The interaction is very strong as demonstated by atomic force microscopy and very tight (62). In support of this, FRAP experiments on quail breast muscle and zebrafish skeletal muscle expressing GFP tagged proteins show that telethonin in Z-discs exchanges only very slowly in comparison to the other Z-disc proteins tested including alpha-actinin (63, 64).

In view of the strength of interaction between titin and telethonin it was somewhat surprising to find that telethonin was absent from the TJ. Figure 3B shows its location in relation to the ID protein, vinculin. There is a clear separation of the vinculin (red) signal in the ID membrane folds from the telethonin (green) signal. This is reinforced in Figure 3C where telethonin is compared to a Z-disc titin epitope. The signals colocalise at the Z-disc but only titin (green) is present in a doublet at the ID. It has been shown that telethonin is a late arrival at the scene during sarcomerogenesis (63, 65). Even though it is important for sarcomere formation (66) it is only found at the Z-disc after mature sarcomeres are formed suggesting it is involved in stabilising the final structure. The binding of titin to telethonin is apparently dependent on the conformation of the N-terminal titin, possibly, it has been suggested, on phosphorylation of titin on the linker between the Z2 and Z3 Ig domains (60). Since the expressed fragments of titin longer than Z1Z2 do not bind telethonin and are likely to be unphosphorylated the implication is that the linker must be phosphorylated for binding. The absence of telethonin at the TJ indicates that the structure is in some way transient or flexible or perhaps in a state that can be easily modified.

3.2.2.8. FATZ/Calsarcin/Myozenin

FATZ/calsarcin/myozenin is a small 32KDa Z-disc associated protein with many binding partners (67-70). It is probably globular but the sequence reveals no known canonical domains. Of the three isoforms, FATZ2/calsarcin1/myozenin2 is predominant in the heart. FATZ binds through its C-terminal to the spectrin repeat 3 and 4 of alpha-actinin (67, 69, 70). It also binds gamma-filamin, calcineurin and telethonin. The C-terminus of FATZ is a class III PDZ binding motif and interacts with ZASP-PDZ in a phosphorylation dependent manner (53).

FATZ is not found at the TJ as shown in comparison to titin in Figure 3D. Possibly this is due to the absence of telethonin as a binding partner but in developing cardiomyocytes FATZ is seen colocalised with alpha-actinin in the premyofibrils and Z-bodies where there is no telethonin (63). However, the premyofibrils are thought to have an antiparallel actin arrangement in their Z-bodies. Possibly, the TJ with its parallel filaments is a special structure so that even the presence of two of its binding partners, alpha-actinin and ZASP, is not sufficient for FATZ binding. In addition, lack of phosphorylation of its PDZ binding motif may play a part (Figures 3D and 4B).

3.2.2.9. Other Z-disc proteins

Other proteins are shown in Fig 4 whose distribution has not been characterised in the TJ. Many of these proteins have been found in the ID but not in sufficient detail to localise precisely in relation to the TJ or the ID membrane. These include proteins such as myotilin and gamma-filamin which are binding partners for Z-disc proteins, particularly alpha-actinin and ZASP as described above. However, with the absence of a significant extra protein mass at the TJ, it is clear that the array of proteins normally associated with the Z-disc cannot be accommodated at the TJ.

3.3. NON-MYOFIBRILLAR PROTEINS

3.3.1. N-RAP

N-RAP was identified by Horowits and his colleagues as a nebulin like protein at the ID and the myotendinous junction (71, 72). It has 45 nebulin actin-binding repeats (nr) and most of these are grouped into 5 nebulin super repeats (equal to 7nr) which in nebulin associate with the actin/tropomyosin/troponin repeats of the thin filament (73, 74). The molecule is therefore some 240nm long. N-RAP differs from nebulin/nebulette in that it does not have the C-terminal SH3 domain that locates it to the Z-disc (28, 29). Rather, it has an N-terminal LIM domain. The linker between the LIM domain and the first nebulin repeat has been shown to bind alpha-actinin (73, 75) and muscle LIM protein (MLP) (73). In addition to actin the super repeats bind gamma-filamin (75).

It is reported that N-RAP has a split distribution across the ID in adult cardiac muscle where it colocalised with alpha-actinin presumably at the TJ (76). The antibody used for these studies targeted the alpha-actinin binding domain. However, in the actin bundles in the myotendinous junction its position is remote from the end of the final sarcomere close to the tendon (3).

A further puzzle is the function of the super repeats. Their presence suggests that N-RAP should be oriented along the thin filament in the same way as nebulin and that the repeats should be associated with the tropomyosin/troponin containing sarcomeric part of the thin filaments. However, if the LIM domain is near the TJ then

the main body of the molecule will be involved with thin filaments in the ID fold.

3.3.2. Spectrin associated membrane cytoskeleton

The presence of the ID membrane peaks at the axial level of the myofibrillar TJ suggests that there is a relationship between the two. Given the similarity of the fibrillar TJ with the Z-disc we can ask if the ID membrane peaks have any similarity with the lateral plasma membrane at the level of the Z-disc. Here, there are costameres where the membrane skeleton in conjunction with focal adhesion proteins like vinculin supports an array of intermediate filaments (desmin) which anchor the fibrils at the Z-level within the cell (see review (77)). Associated with the costameres and the lateral plasma membrane, in general, are two classes of membrane cytoskeleton. One is the dystrophin system anchored to the membrane through dystroglycans. It has been shown that dystrophin is only present at the lateral membrane and not at the ID (78)

The other membrane system is the spectrin associated cytoskeleton including ankyrin, protein 4.1 and adducin (79). Several of these proteins are found in the heart and are differentially localised, some at the ID (see (80)). Spectrin is an antiparallel heterodimer of alpha and beta subunits which link end to end to form a tetramer. The tetramers link to short actin filaments via the actin binding domain of beta-spectrin to form sub membrane networks (review (79)). There are several isoforms of both alpha and beta spectrin which are also differentially spliced. In the heart the erythrocyte isoforms of spectrin, alphaI/betaI, are found on the lateral plasma membrane (81). alphaII and betaII spectrin has been located on the plasma membrane, at the level of the Z-discs in T-tubules and at the ID (1, 80-84)). Recently, betaIV spectrin was identified at the ID and in the T-tubules but not on the lateral plasma membrane (85). The organisation of spectrin alphaII at the ID has been investigated most closely. It has a split distribution across the ID by immunofluorescence and using immunogold EM we found that it is present at the tops of the ID folds (1). A cardiac specific spliced isoform of alphaII spectrin which has a weaker association with bets spectrin has been recently identified but it is associated with early growth and is not seen at the plasma membrane or the ID in mature cardiomyocytes (86). The specific distribution of the other spectrins and their associated components remains to be established

Protein 4.1 bonds the actin-spectrin complex through its ABD. There are 4 isoforms of which three are found in human heart (87). Two of these, Protein 4.1R and 4.1N, are found at the ID as well as elsewhere in the cell (80). Ankyrin associates with repeats 14 and 15 on β spectrin in the middle of the spectrin arms (88, 89). One isoform, ankyrin G, is known to be present at the ID as well as at the Z-disc level in the myocyte (90).

In the erythrocyte, proteins 4.1R and ankyrinR each coordinate the assembly of macro complexes of transmembrane proteins (91). While the two complexes (the "junctional complex" and the "ankyrin based carbon dioxide metabolon") are separate, they contain an overlapping spectrum of membrane proteins. For example, the erythrocyte anion exchanger band 3 and the channel protein Rh, each bind 4.1R and ankyrinR.

In heart, electrophysiological analysis of mutant mice reveals functional interactions between 4.1R and ankyrinG and membrane proteins required in the regulation of heart beating, including the voltage gated sodium channel NaV1.5 and the sodium/calcium exchanger NCX1 (90, 92). In particular, a human disease correlation with this is found in Brugada syndrome, in which mutations in NaV1.5 that inhibit interaction with ankyrinG give rise to abnormal heart beating (90). A portion of both NaV1.5 and NCX1, as well as another ankyrin-interactive protein, the sodium/potassium-ATPase, which creates the sodium gradient required for NCX1, are present in the intercalated disc.

Given that there seem to be multiple transmembrane signalling proteins that interact with either or both of ankyrin and protein 4.1, it seems likely that they coordinate the formation of macro complexes of transmembrane proteins at the intercalated disc, in a manner analogous to those found in red blood cells (93).

3.3.3. Membrane myofibril connection

How the connection between the membrane and myofibrillar TJ is maintained is not clear. Pinder and Baines (94) have introduced the idea of a protein accumulator whereby the spectrin complex brings together interdependent components. In this way membrane proteins and extra cellular molecules can be connected up with, for example, the costameric complex. Ervasti (77) has detailed the interactions delineating the costameric network and shows several pathways by which spectrin could be linked to actin and alpha-actinin, on one hand, through desmin or filamin to integrins. Details of such interactions at the TJ remain to be elucidated.

4. MORPHOLOGICAL OBSERVATIONS

4.1. Thin filaments

Given the evidence so far presented, what are the structural observations? Early electron microscopic observations while showing beautiful pictures of the ID and its environs did not reveal a structure that would correspond to the TJ (see also Figure 1B). We have carried out some electron tomography to investigate this area (Bennett and Wilson unpublished observations). Figure 5A shows a frame of a tomogram of a longitudinal section showing the region between the end of the A-band and the ID membrane. The approximate level of the TJ is indicated by white arrows. In a copy of the same picture (Figure 5B) two thin filaments are highlighted in red and can be followed from their ordered position in the thick filament lattice all the way to the ID membrane confirming the observation of Forbes and Sperelakis (5) that the filaments are continuous throughout this region.

There is no structural density running across the myofibril which obviously corresponds to the TJ. Hence, the specific location of an alpha-actinin, ZASP and N

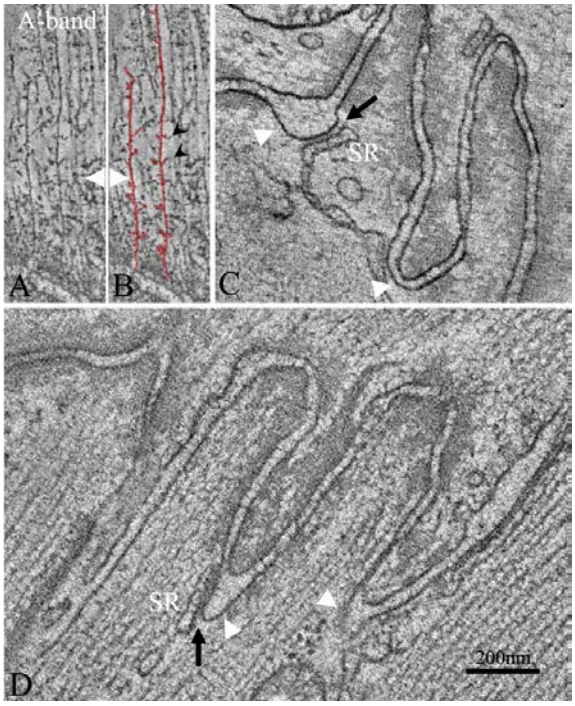

Figure 5. Images from tomograms from electron micrographs of mouse heart IDs. 300nm thick sections of mouse papillary muscle were stained with 2% uranyl acetate in 30% ethanol at 37^{o}C for 20m and 5 nm gold particles were used for fiducial markers. Tilted images at 1^{o} intervals over a range of +/- 70^{o} in two orthogonal directions were obtained in an FEI T20 electron microscope. Tomographic reconstructions were calculated using IMOD (Boulder Laboratory, University of Colorado). A shows thin filaments between the proximal A-band and the ID membrane White arrows indicate the approximate position of the TJ. B. The same image as in A where two of the filaments have been coloured red to show the continuity of the filaments from myofibril through the TJ to the ID membrane. Attached proteins (black arrowheads) are seen to hang off the filaments at regular intervals of ~40nm. C and D. The two frames from different tomograms show slices through peaks of the ID membrane. The tops of the peaks are bare of plaque (white arrowheads). Black arrows indicate where SR vesicles form peripheral couplings apposed to the ID membrane in the region of the peak tops.

terminal titin complex cannot be recognised at this resolution. However, it is possible to see material hanging off the filaments at relatively regular intervals (small arrows in Figure 5B). Some of these at least are the right size to correspond to alpha-actinin. This suggests that there are additional links between the actin filaments along their length all the way to the ID membrane. However, the sharp focus of titin and ZASP make it unlikely that they have the same distributed location.

4.2. Membrane peaks

The views shown in Figure 5C and D are also frames from tomograms and are chosen to pass through the tops of some of the ID membrane peaks. Such views confirm that here the membrane is bare of plaque. The AJ plaque is located on the slope of the folds where the thin filaments invest. Tidball has argued that mechanically this is the optimal arrangement for transmitting the tension across the membrane (95, 96). Regions of membrane bare of structural and functional domains such as AJs and desmosomes would be expected to have a membrane cytoskeleton. It is therefore not surprising that the spectrin complex is present at the tops of the folds. An additional observation is that there are no T-tubules seen in the TJ but there are often sarcoplasmic reticulum (SR) vesicles which form peripheral couplings with the ID membrane. This would allow calcium to be released into the terminal half sarcomere during activation. This might be one reason why the tops of the folds are so closely associated with the TJ since the fold/SR dyad will have the same relationship with the TJ as the T-tubule/SR does at the normal Z-disc; an additional reason for spectrin to be here since it has been associated with T-tubules. Often at the tops of the peaks the membranes separate and one of them tends to balloon (Figure 5C) suggesting that the membrane may bleb off, possibly to become part of a T-tubule.

5. ROLE OF THE TRANSITIONAL JUNCTION

5.1. The model structure of the transitional junction and its function

Figure 6 shows a diagram for the TJ which attempts to draw together the information discussed above. Parallel actin filaments are cross-linked by alpha-actinin. The alpha-actinin links are strengthened by ZASP struts from the rod to C-terminal PDZ binding motif. N-terminal titin is attached through two forms of association with alpha-actinin; the zq-Z4 sequence and at least one Z repeat of titin, with the rod and the C-terminal EF hands of alpha-actinin, respectively. Titin and ZASP are not known to interact with one another but the connection of both with two similar locations on alpha-actinin suggests that they may intimately affect each others binding ability. One circumstance that is likely to influence the arrangement is the level of phosphorylation of the components. In particular, phosphorylation of the linker region of titin between Z2 and Z3 may influence the structure enough to inhibit binding to telethonin. Another unknown is the function of N-RAP. It seems likely that its N-terminal LIM domain is associated with alpha-actinin near the TJ. These proteins, alpha-actinin, titin, ZASP and N-RAP and possibly nebulette may be the minimal needed to locate the titin molecule at the correct position to maintain the axial order of the last sarcomere. It is, however, in no way clear how the position of titin on one filament is communicated laterally to neighbouring filaments. Nonetheless, the sharpness of the titin and ZASP doublets in immunofluorescence point to this precision. Possibly a combination of lateral forces between the filaments and longitudinal elastic forces on titin is instrumental in maintaining this order.

The most obvious difference between the myofibrillar region of the TJ and the Z-disc is the lack of Z-disc-like density. This is supported by the observed absence of telethonin and FATZ. It remains to be seen what other

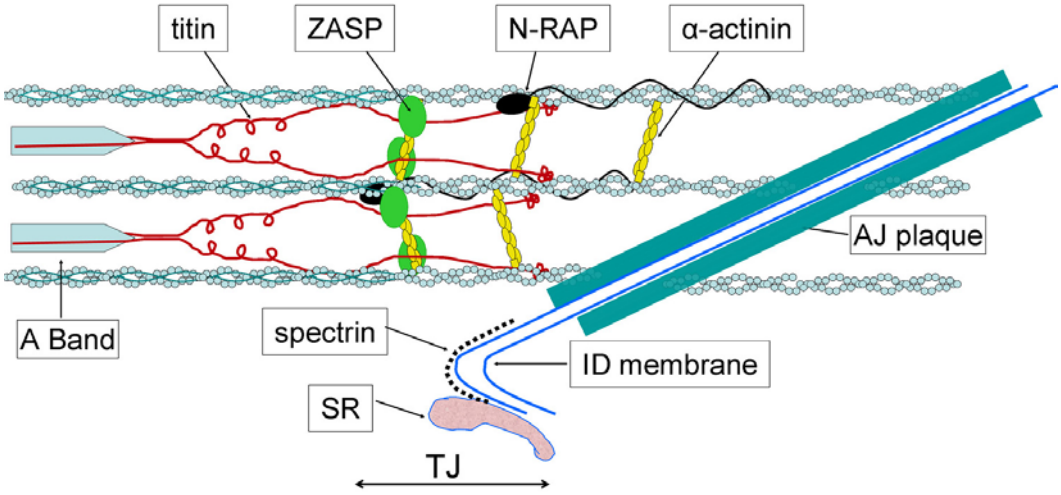

Figure 6. Model for the transitional junction in cardiac muscle. The diagram shows the curve of the ID membrane and the relationship of the thin filaments coming from the proximal A-band crossing the transitional junction, whose approximate position is indicated (TJ), and inserting into the AJ plaque on the membrane. The membrane fold extends axially to peak at the axial level of the myofibrillar TJ. At the TJ, alpha-actinin cross-links parallel actin filaments. ZASP and titin and N-RAP are also present. Two types of interaction of titin with alpha-actinin are shown. Other alpha-actinin molecules may cross-link actin elsewhere in the folds. The top of the membrane fold is associated with spectrin. SR vesicles form a dyad with the ID membrane in the same region.

proteins, Z-disc or otherwise, are necessary to maintain the TJ structure. On the other hand there is significant density in the plaque at the AJ where the thin filaments insert. This implies that much of the density seen at the Z-disc is needed to organise and maintain the thin filaments in their antiparallel conformation and to supply enough strength to counteract the contractile forces that they experience.

At the TJ level the ID membrane sweeps down to form a peak that is associated with a spectrin network, a network that is geared to maintain complex shapes such as those found in the red blood cell. The connection between this and the myofibrillar TJ is not known but may include filamin and other costameric proteins. Associated in a peripheral coupling with the ID membrane a junctional SR vesicle is shown.

The components identified suggest that the TJ is an incipient or nascent T-tubule/SR/Z-disc structure.

5.2. The role of the transitional junction in sarcomere addition and cell growth

The Z-disc has at least two roles. One is to act as a scaffold for the attachment of the elastic filament titin so that the sarcomere order is maintained. The other is to transmit the contractile tension from one cell to the next through the strong attachment of antiparallel thin filaments to one another. We have seen that in the ID these two functions are separated. The tension transduction is carried out through the AJs at the ID membrane while the titin tethering function occurs at the TJ.

The arrangement of separated functions at the ID allows the amplitude of the ID folds to change without affecting contractile function. The range of amplitude observed in mouse heart is from 0.2 μm to greater than 1μm with an average of ~ 0.5μm (personal observations). At a sufficient amplitude a new sarcomere could be inserted into the fold of the ID (1, 97) (see Figure 7). Yoshida et al have presented some evidence for this (97). They showed that in overloaded rabbit heart, myocytes grow in length by increasing the number of sarcomeres and there are concomitant periodic changes in the structure of the ID. The observed amplitude of the folds increases to a size that would accommodate a new sarcomere. The resulting long tongue of ID membrane then breaks down leaving an ID with a small width.

It was noted above (section 2.2) that the ID width remains constant across the interface between two myocytes. It is then clear that if a muscle was stretched uniformly, two cells could pull apart uniformly and the increase in ID width would increase uniformly until it was wide enough to insert a sarcomere from one or the other side of the ID.

The TJ is in a position to be able to respond to this scenario. There is already essentially a half Z-disc equipped with actin, alpha-actinin, ZASP and titin together with a connection to the membrane; A template from which to grow a new sarcomere with SR and T-tubule when the resources become available.

5.3. The role of the transitional junction in disease

In dilated cardiomyopathy (DCM) the ID is clearly affected and the precise order of the myofibrils is lost (98). The heart of the muscle LIM protein (MLP) knock out mouse illustrates these changes (Figure 7). Frequently, the amplitude of the ID membrane folds is significantly greater than in normal hearts. Furthermore the

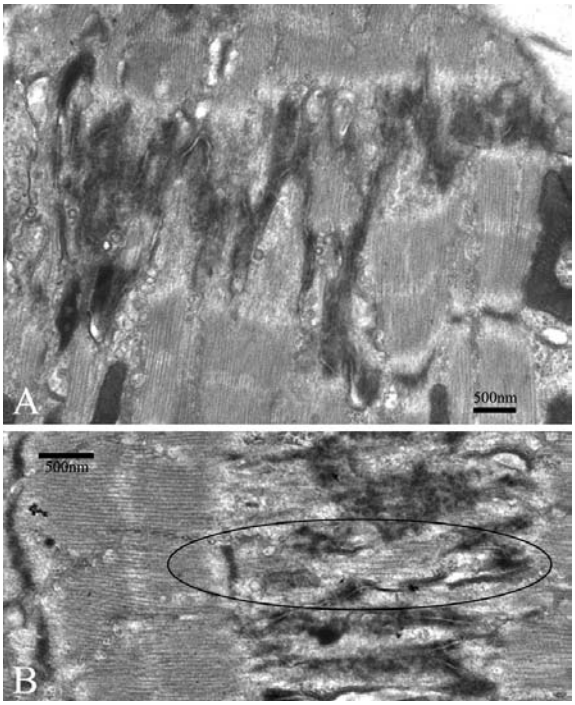

Figure 7. Electron micrographs of MLP knock out mouse heart illustrating changes typically seen at the ID in dilated cardiomyopathy. (A) Variations in ID width across a myocyte. The width is normal to the right and becomes abnormally large, greater than one sarcomere length, to the left. (B) Insertion of a sarcomere in a long fold of an ID (ringed). A new Z-disc and thick filaments can be seen encompassed by the folded membrane. Electron microscopy was as in Figure 1.

amplitude is not constant for any one ID but varies across the cell (Figure 7A). When the amplitude is very large then new sarcomere like structures are often seen within the folds (Figure 7B). The large but variable amplitude therefore allows precocious accumulation of new sarcomeres at the ID that result in unregulated growth. This would explain the greater variation in observed cell length and width as well as the brush like ends to the cells (99, 100). What exactly is the cause of this structural breakdown is not clear but the lack of controlled growth may result from a greater sensitivity to slight variations in stretch leading to an overproduction of ID fold membrane and other components, or a loss in communication laterally across the cell. Such communication could come from the TJ. It is interesting that MLP, as well as being located at the Z-disc has also been shown to bind to TJ components, N-RAP and beta spectrin (100, 101). Furthermore N-RAP is upregulated in the heart of the MLP knock out mouse, as well as in the TOT (transgenic overexpression of tropomodulin) mouse which also exhibits a DCM phenotype (100).

6. PERSPECTIVE

The intercalated disc is an important organising region of the heart, integrating myocytes and coordinating function. Although it appears to be structurally rather disorganised, on closer inspection it is clear that the precise order of the sarcomeres is maintained while allowing the freedom for the cells to grow and accommodate other forces. An important part of this is the separation of the myofibrillar function from the cell-cell junctions by a transitional zone. Further, this transitional region incorporates membrane elements and appears to be needed for controlled cell growth. Some of the proteins involved have been identified but the mechanism by which the myofibrillar TJ is maintained and connected to the membrane–associated TJ remains to be clarified.

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Abbreviations: ID, intercalated disc; AJ, adherens junction; TJ, transitional junction; SR, sarcoplasmic reticulum

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