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Emerin interacts *in vitro* with the splicing-associated factor, YT521-B

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Emerin is a nuclear membrane protein that interacts with lamin A/C at the nuclear envelope. Mutations in either emerin or lamin A/C cause Emery–Dreifuss muscular dystrophy (EDMD). The functions of emerin are poorly understood, but EDMD affects mainly skeletal and cardiac muscle. We used a high-stringency yeast two-hybrid method to screen a human heart cDNA library, with full-length emerin as bait. Four out of five candidate interactors identified were nuclear proteins: lamin A, splicing factor YT521-B, proteasome subunit PA28γ and transcription factor vav-1. Specific binding between emerin and the functional C-terminal domain of YT521-B was confirmed by pull-down assays and biomolecular interaction analysis (BIAcore). Inhibition by emerin of YT521-B-dependent splice site selection *in vivo* suggests that the interaction is physiologically significant. A 'bipartite' binding site for YT521-B in emerin was identified using alanine substitution or disease-associated mutations in emerin. The transcription factor GCL (germ cell-less) has previously been shown to bind to the same site. The results are consistent with an emerging view that lamins and lamina-associated proteins, like emerin, have a regulatory role, as well as a structural role in the nucleus. YT521-B joins a growing list of candidates for a role in a gene expression model of the pathogenesis of EDMD.

Keywords: Emery–Dreifuss muscular dystrophy; gene regulation; lamin; RNA splicing; yeast two-hybrid.

The *STA* gene (Xq28) encodes a nuclear protein, emerin, and mutations in *STA* cause the X-linked recessive form of Emery–Dreifuss muscular dystrophy (X-EDMD) [1]. An autosomal dominant form of EDMD (AD-EDMD) is caused by mutations in the lamin A/C gene [2] and displays a phenotype similar to X-linked EDMD [3,4]. Emerin and lamins colocalize at the nuclear rim [5,6]. Emerin and lamins also coimmunoprecipitate [7] and a direct interaction between emerin and lamin A has been demonstrated [8,9]. The LEM domain at the emerin N-terminus (amino acids 6–44) is shared by other nuclear membrane proteins, such as LAP2β [10] and MAN1 [11], and interacts with the DNA-binding protein, BAF [9]. Yeast two-hybrid studies have shown that the tail region of lamins A and C is responsible for binding emerin [12,13]. The lamin A/C knockout mouse displays muscular dystrophy [14], suggesting that EDMD is caused by functional defects in the lamin A/C–emerin complex (reviewed in [4]).

Most mutations in the emerin gene lead to early stop codons or cause complete deletion of the gene [15,16]. Missense mutations S54F and P183H/T, and a small deletion, ΔYEESY, also cause EDMD, though emerin is produced and is still able to localize to the nuclear membrane [7,9,17]. Understanding the molecular effects of these missense mutations is clearly important for understanding emerin function and the pathogenesis of EDMD. Recently, Lee *et al.* [9] located the lamin A binding region of emerin to amino acids 70–178 and showed that ΔYEESY within this region reduces lamin A binding. The Tsuchiya–Östlund sequence (amino acids 107–175 [18,19]), necessary for targeting emerin to the nuclear rim, lies within this lamin-binding region, consistent with evidence from the knockout mouse that lamin A/C is at least partially responsible for targeting emerin to the nuclear envelope [14]. Two missense mutations, S54F and P183H, did not affect binding to either lamin A or BAF [9], suggesting that there may be additional emerin interactions, yet to be identified, involving these residues. A third missense mutation, Q133H, lies within the lamin-binding region but does not affect binding to lamin A [20]. This mutation also causes aberrant splicing of emerin mRNA, which may contribute to the phenotype [21].

EDMD is predominantly a disease of cardiac and skeletal muscle and tendons, other tissues being unaffected [4]. The EDMD phenotype suggests that complexes between lamin A/C and emerin might have functions specific for muscle, potentially involving muscle-specific binding partners. Nesprin-1α, an integral membrane protein that localizes to the skeletal muscle nuclear membrane [22], binds to lamin A and is a high-affinity binding partner for emerin [23]. We therefore used the yeast two-hybrid method to screen a human heart cDNA library for novel emerin binding partners. Our identification of an RNA-splicing protein named YT521-B [24,25], reported here, adds significantly to the functional range of proteins that interact with emerin.

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Abbreviations: EDMD, Emery–Dreifuss muscular dystrophy; NAIP, neuronal apoptosis inhibitory protein; BIA, biomolecular interaction analysis; RU, resonance units.

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Small disruptions of the normal balance of gene expression may lead to the slow degeneration of cardiac and skeletal muscle tissues associated with EDMD.

Materials and methods

Yeast two-hybrid screening

A yeast two-hybrid human heart cDNA library in plasmid pPC86 (Proquest; Invitrogen), containing the activating domain, was screened using the full-length coding sequence of emerin cDNA as 'bait', in the binding domain plasmid, pDBLeu. The screen was performed in the MaV203 yeast strain according to the supplier's instructions. Emerin-pDBLeu was introduced into the yeast strain, followed by the library plasmid DNA (sequential transformation). Approximately 10^6 yeast transformants were screened. The transformation mixture was plated out onto dropout plates lacking leucine, tryptophan and histidine (SC-L/–T/–H) that had been supplemented with 25 mM 3-aminotriazole. *HIS*3-positive colonies were recloned and streaked onto dropout plates lacking leucine and tryptophan (SC-L/–T). The yeast were replica-plated onto filter paper on nutrient-rich YPAD medium and tested for activation of the *lac*Z gene using the X-gal assay.

Plasmid DNA was isolated from *lacZ*-positive clones and used to transform *Escherichia coli* DH5α for plasmid propagation and sequencing (Cytomyx, Cambridge, UK). BLAST (www.ncbi.nlm.nih.gov/BLAST) was used to search nucleotide databases for sequence similarities. The size of each interactor cDNA in pPC86 was determined by digestion with *Sal*I and *Not*I. The isolated plasmid (interactor in pPC86) was reintroduced into yeast carrying emerin-pDBLeu and retested for activation of the *lacZ*, *HIS3* and *URA3* reporter genes. HIS3 activation was assessed by growth on SC-L/–T/–H + 25 mM 3-aminotriazole dropout plates and URA3 by growth or lack of growth on SC-L/–T/–U or SC-L/–T + 5-fluoro-orotic acid dropout plates, respectively. Candidate partners were also tested in pDBLeu alone to control for auto-activation.

Protein expression and immunoprecipitation

Candidate interactor cDNAs in pPC86 were excised with *Sal*I and *Not*I, filled and cloned blunt ended into *Eco*RV-digested pET32a (Novagen). This vector adds an N-terminal thioredoxin sequence to improve recombinant protein solubility, as well as a His-tag. ^{35}S-methionine-labelled interactor proteins were expressed from the T7 promoter using the TNT Quick Coupled Transcription–Translation System (Promega) according to the manufacturer's protocol. The fragments corresponding to PA28γ and YT521-B included 966 and 568 bp, respectively, up to the natural stop codon. The predicted sizes of the recombinant proteins were 57 kDa for PA28γ and 42 kDa for YT521-B, including amino acids encoded by the vector. In addition to protein fragments from yeast two-hybrid cDNAs, ^{35}S-methionine-labelled full-length lamin A was produced from pcDNA4 [26] and PA28γ from pET16b (a generous gift from S. Wilk, Mount Sinai School of Medicine, New York). Full-length PA28γ (31 kDa) served as an essential control because the recombinant protein recovered in our two-hybrid screen included N-terminal residues encoded by the 5′-untranslated region, which are not normally expressed and might be responsible for nonphysiological binding to emerin.

The nucleoplasmic domain (residues 1–222) of wild-type and mutant emerins were expressed in *E. coli* BL21 (DE3)pLysS. The C-terminal 32 residues, which include the hydrophobic transmembrane span, were removed to avoid nonspecific binding to a region of emerin that is inaccessible to soluble proteins. This shorter form of emerin in pET11c was also used to generate mutant proteins S54F and P183H [9]. Log-phase cells were induced with isopropyl thio-β-D-galactoside at 37 °C for 3–4 h and washed once with TNE buffer (50 mM Tris/HCl, 150 mM NaCl, 10 mM EDTA pH 7). Emerin-containing inclusion bodies were washed twice with TNE and extracted by sonication with 8 M urea in NaCl/P_i. Two control recombinant proteins, full-length fukutin and a fragment of neuronal apoptosis inhibitory protein (NAIP; residues 112–476), were expressed and extracted under the same conditions. The amounts of emerin and control proteins were balanced, based on densitometry of Coomassie blue stained SDS/PAGE gels (data not shown).

^{35}S-Labelled interactor protein (8 μL reaction mix) and unlabelled emerin, diluted to < 0.2 M urea, were incubated for 1 h at 4 °C in IP buffer [20 mM Hepes pH 7.9, 150 mM NaCl, 10 mM EDTA, 0.1% NP-40 (v/v), 10% glycerol (v/v), 1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 20 $\mu g \cdot mL^{-1}$ leupeptin]. Rat anti-mouse Dynabeads (40 μL; Dynal) were incubated with MANEM1 mAb (30 μL [5]) for 15 min at room temperature then at 4 °C for 45 min. For nonspecific binding controls, NAIP and fukutin were captured in the same way using anti-NAIP and antifukutin mAbs, respectively (T. A. Lynch, Nguyen thi Man, T. Toda and G. E. Morris, unpublished data). The beads were washed thoroughly three times with 300 μL NaCl/P_i containing 0.1% Triton X-100. The protein mixture was added to the beads, made up to 80 μL in IP buffer and incubated for 2 h at 4 °C. The beads were washed with IP buffer and eluted with SDS sample buffer [62.5 mM Tris/HCl pH 6.8, 2% SDS (w/v), 5% 2-mercaptoethanol, 2% glycerol, 0.05 $mg \cdot mL^{-1}$ Bromophenol blue; 30 μL). An aliquot (10 μL) was analysed by SDS/PAGE (12.5% acrylamide), followed by fixing and drying, and exposed to X-ray film for 24–48 h.

Microtiter binding assays

Microtiter well-binding assays were carried out essentially as described [27,28]. To test for YT521-B binding to our collection of emerin mutants, ^{35}S-labelled YT521-B protein was synthesized in coupled transcription/translation extracts, and a subsaturating amount (40 nM) of ^{35}S-YT521-B was added to each well; wells contained 10 pmol emerin plus BSA to block nonspecific binding sites. Wells were not allowed to dry at any time during these assays. Negative control wells contained BSA only. Wells were washed, and bound ^{35}S-labelled proteins were extracted with 5% SDS and counted in a scintillation counter. Emerin mutants were described elsewhere [9,28], but are summarized here; each mutant comprised a small cluster of alanine substitutions and was numbered according to its most N-terminal mutated residue: m24 (24-GPVV to AAAA), m34

(34-YEKK to AAAA), m45A (45-RRR to AAA), m45E (45-RRR to EEE), m70 (70-DADMYD to AAAMAD), m76 (76-LPKKEDAL to APAKADAA), m112 (112-GPS RAVRQSVT to AASRAVAAAVA), m122 (122-TS to AA), m133 (133-Q to H), m145 (145-EE to AA), m151 (151-ER to AA), m161 (161-YQS to AAA), m164 (164-ITHYRPV to AAHARPA), m175 (175-SSL to AAA), m179 (179-LS to AA), m192 (192-SSSSS to ASAAA), m196 (196-SS to AA), m198 (198-SSWLTR to AAAAAA), m206 (206-IRPE to AAPA), m207 (207-RP to AA), m214 (214-GAGL to AAGA). Mutant m40 (40-E to K) is described here for the first time.

Biomolecular interaction analysis (BIA)

The BIAcore biosensor measures changes in surface plasmon resonance angle produced by changes in total mass at the surface of a sensor chip. Resonance units (RU) are proportional to the mass on the chip, so that real-time binding can be followed and quantified. Rabbit antimouse IgG was covalently bound to a dextran sensor chip by amino-coupling according to the manufacturer's instructions (BIAcore AB, Stevenage, UK). The operating flow-rate of the BIAcore-X apparatus was 20 $\mu L \cdot min^{-1}$ using HBS (20 mM Hepes pH 7.5, 150 mM NaCl, 3.4 mM EDTA, 0.005% P20 surfactant) as the running buffer. This buffer was also used to dilute all proteins to 20 $\mu g \cdot mL^{-1}$ just before use. Recombinant YT521-B (the two-hybrid fragment) and PA28γ proteins were extracted from *E. coli* with TNE buffer, as described above.

In vivo splicing assay

This was performed as described previously [29]. Briefly, 1 μg of the CD44 v5 minigene [29] was transfected into 3×10^5 HEK293 cells in the presence of cDNAs encoding EGFP-YT521-B [25] or emerin. RNA was isolated using RNeasy columns (Qiagen). 1 μg total RNA was reverse transcribed and amplified by PCR as described [29].

Results

Candidate binding partners for emerin from a yeast two-hybrid library

The Proquest yeast two-hybrid system minimizes nonspecific interactions by using low copy number plasmids and by having four different phenotype requirements for a positive score. In general, fewer interactors are pulled out of Proquest libraries than systems using high-copy-number plasmids (e.g. Clontech Matchmaker). We used full-length emerin as bait, including the hydrophobic transmembrane sequence. To control for the presence of the transmembrane domain, we subsequently checked all interactors biochemically for binding to the N-terminal nucleoplasmic domain only (residues 1–222). Five potential binding partners were identified in the initial two-hybrid screen: lamin A, PA28γ, YT521-B, lipoprotein lipase and the *Vav1* oncogene (Fig. 1). The mRNA map for each putative emerin-binding protein is shown in Fig. 2. Control experiments showed that these interactors did not auto-activate reporter genes when re-introduced into yeast without emerin, but did again activate reporter genes when re-cotransformed with the emerin bait (Fig. 1).

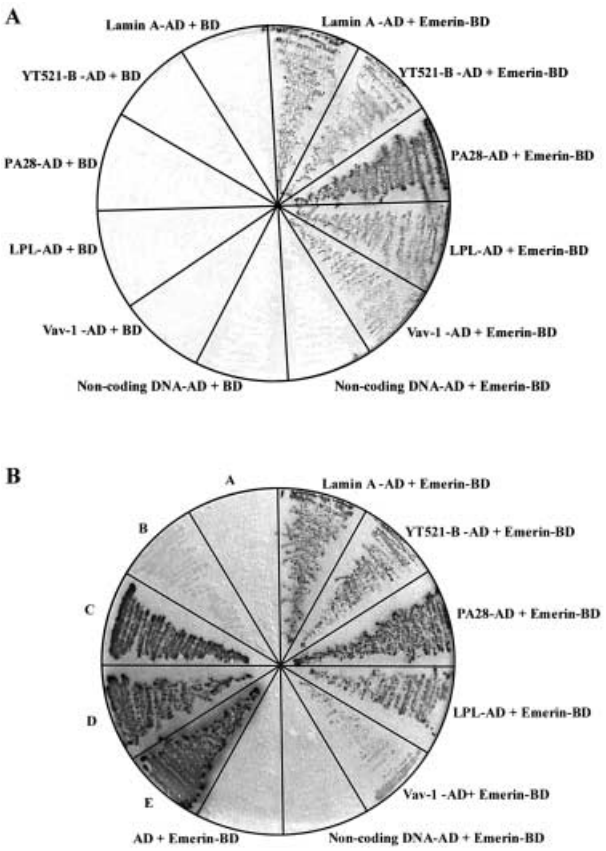

Fig. 1. Activation of the *lacZ* reporter gene by proteins interacting with emerin in a yeast two-hybrid assay. When the binding and activation domains of GAL4 interact, β-galactosidase is expressed yielding a blue colour with X-gal. (A) Colonies on the right half of this plate express both emerin (DNA-binding domain; BD) plus the indicated prey construct or noncoding DNA fused to the Activation Domain (AD). No blue signals were detected in cells that lacked emerin (left half of plate). (B) To compare interaction strengths, the same six interactors (right) are compared to five strains (left) that express pairs of proteins with known interaction affinities (provided with Proquest system). Anticlockwise, these controls were (A) negative control strain, (B) human RB and E2F1 [43], (C) *Drosophila melanogaster* DP and E2F [44], (D) rat c-fos and mouse c-jun [45] and (E) positive control (intact GAL4). Yeast marked 'AD + Emerin-BD' show that emerin alone does not autoactivate the *lacZ* reporter gene.

Finding lamin A, a known emerin-binding protein, showed that the two-hybrid screen worked well. Furthermore, the lamin cDNA recovered in our two-hybrid screen included the C-terminal globular 'tail' region of lamin A previously suggested to bind emerin [12,13]. Three of our novel interactors, Vav1, PA28γ and YT521-B, are known to be nuclear proteins, consistent with potential binding to emerin. Vav1, the nuclear proto-oncogene was not pursued further in this study because it is expressed mainly in haematopoietic cells [30], which are not affected by EDMD. The last interactor, lipoprotein lipase, was not studied further, as it is secreted by several cell types and has no known nuclear function [31]. For this report, we

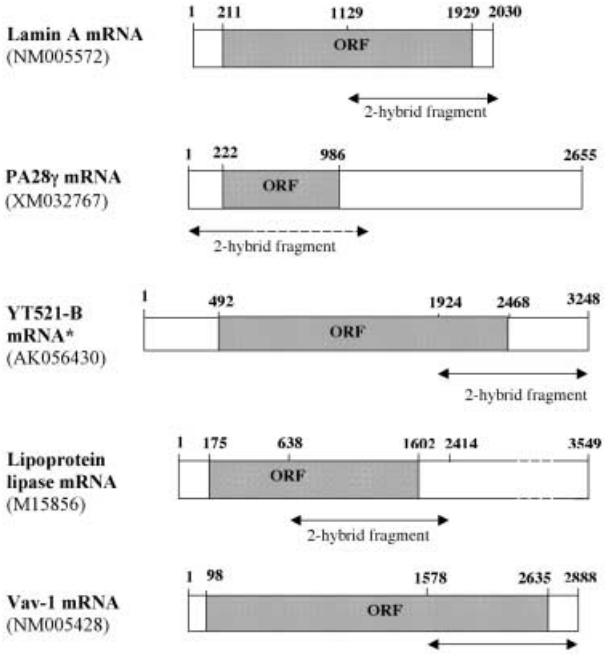

Fig. 2. Diagramatic representation of the cDNA sequences of the potential emerin interactors and their relationship to full-length mRNAs. The arrows denote the insert sequences of the clones from the human heart library. The PA28γ cDNA was not sequenced in its entirety and the dotted arrow shown denotes the estimated length of the insert. The lengths of the inserts were estimated by restriction digestion with *Sal*I and *Not*I.

focussed on two novel partner candidates, PA28γ and YT521-B.

The PA28γ fragment included 74 extra residues at the N terminus that derived from the 5′-untranslated region, and are presumably never synthesized *in vivo*. To determine if its interaction with emerin was due to these 'non-PA28γ' residues, all subsequent studies were performed using both the longer two-hybrid polypeptide and wild-type PA28γ. Full-length YT521-B was not available due to problems expressing this protein in bacteria, so we studied the function of the C-terminal fragment of YT521-B, which was sufficient to bind emerin in the yeast two-hybrid assay.

Biochemical evidence for emerin interaction with YT521-B

For immunoprecipitation studies, each purified recombinant protein (wild-type emerin residues 1–222, mutant emerins or control proteins) was preincubated with the ^{35}S-labelled 'interactor' protein, and then captured onto magnetic beads using the appropriate monoclonal antibodies against emerin or the control proteins. Approximately equal concentrations of each protein (± 10%) were present in each reaction, as estimated from densitometry of Coomassie-stained gels. Our negative control proteins were full-length fukutin and a recombinant fragment of NAIP expressed and extracted from bacteria in the same way as the emerins.

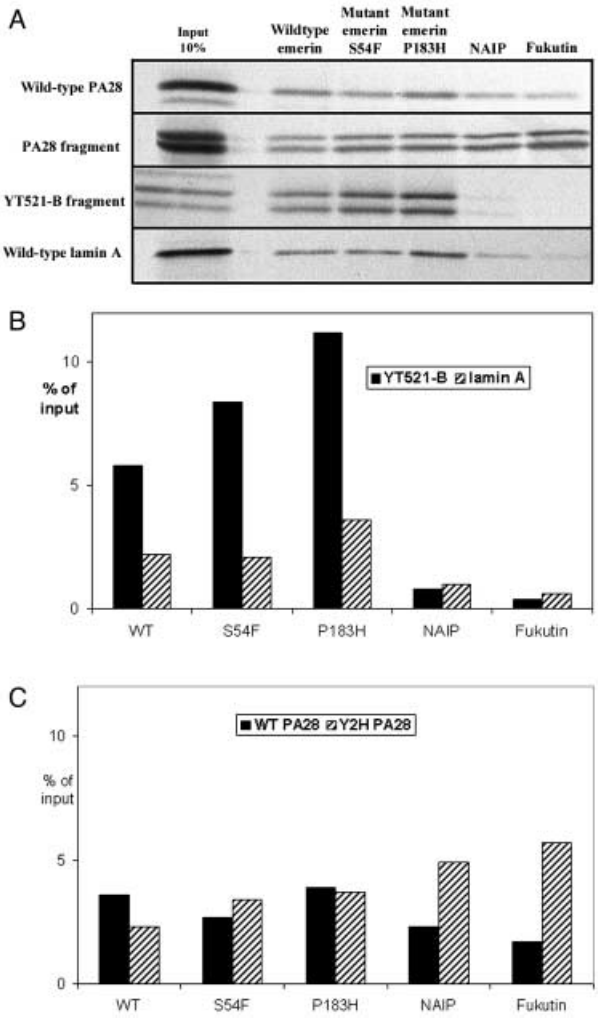

Fig. 3. Coimmunoprecipitation of *in vitro* translated yeast two-hybrid interactors by emerin and two emerin mutants. (A) Wild-type emerin and the EDMD mutants, S54F and P183H, were preincubated with the *in vitro* translated proteins shown on the left hand side of the figure and pulled down with anti-emerin mAb bound to Dynal magnetic beads. Anti-NAIP and anti-fukutin mAbs bound to Dynal magnetic beads were used as controls to detect any nonspecific binding of the *in vitro* translated proteins to fukutin or NAIP. Complexes were eluted, separated by SDS/PAGE on a 12.5% gel and *in vitro* translated proteins were detected by exposure to X-ray film. The lower (by about 3 kDa) M_r band in the two expressed fragments may result from cleavage of the N-terminal tag added by the pET32 plasmid or from a downstream initiation *in vitro*. (B) Binding of YT521-B (black columns) or lamin A (hatched columns) is expressed as a percentage of input ^{35}S-labelled protein. Densitometry of scanned film in (A) was performed using Laserpix software (Bio-Rad Laboratories). The Coomassie blue-stained gel showed almost equal input of all emerins and control proteins (± 10%) and a small correction was applied for any deviation from the mean. (C) Data for binding of wild-type PA28γ (WT PA28: black columns) and the PA28γ fragment from yeast two-hybrid (Y2H PA28: hatched columns) was analysed as in (B).

These pull-down assays showed that lamin A, PA28γ and the YT521-B protein all bound to wild-type emerin (Fig. 3A). The efficiency of capture was plotted as a

percentage of the input ^{35}S-probe (Fig. 3B,C). Binding of YT521-B to emerin was most efficient, with 5.8% of the input probe captured by emerin, compared to 2.2% of the input lamin A (Fig. 3B). Binding to YT521-B was specific, because an average of 8.5% of YT521-B input was captured by emerins compared with an average 0.6% captured by negative control proteins fukutin or NAIP (Fig. 3B). The corresponding numbers for lamin A were an average 2.6% of input captured by emerins compared with 0.8% by control proteins. In contrast, wild-type PA28γ and two-hybrid encoded PA28γ bound significantly to the negative control proteins, with an average 3.7% of input probes captured by negative control proteins, compared to 3.3% captured by emerins (Fig. 3C). We concluded that the two-hybrid interaction between emerin and PA28γ was non-specific. In summary, these biochemical results eliminated PA28γ as a candidate, and independently verified YT521-B as a *bona fide* emerin-binding protein.

We also tested two emerin missense mutations, S54F and P183H, for possible disruption of emerin binding to YT521-B (Fig. 3). Neither mutation disrupted emerin binding to lamin A, but the P183H mutation enhanced emerin binding to lamin A, consistent with earlier reports [9]. Neither disease-causing mutation reduced emerin binding to YT521-B. However, similar to its effects on lamin A [9], the P183H mutation increased emerin binding to YT521-B (Fig. 3). This enhanced binding of emerin mutant P183H was reproduced in multiple immunoprecipitation experiments and with different mutant emerin preparations, but its significance remains unclear (see Discussion).

The BIAcore biosensor detects protein–protein interactions in real time using surface plasmon resonance. Molecules binding or dissociating from the sensor chip are monitored by a change in the surface plasmon resonance response (RU) proportional to the mass on the chip [8]. An emerin mAb (MANEM1) was captured onto a rabbit antimouse sensor chip. YT521-B or PA28γ recombinant proteins were injected over the sensor chip to control for nonspecific binding of these proteins; the baseline was unchanged and thus no binding was detected (Fig. 4). Emerin was then injected and captured onto the same chip (Fig. 4; + 504 RU), and subsequent addition of YT521-B gave a positive binding signal (Fig. 4; + 180 RU). This result provided the third line of evidence supporting an interaction between emerin and YT521-B. No signal was seen for PA28γ (−20 RU), confirming its lack of binding to emerin in solution (data not shown).

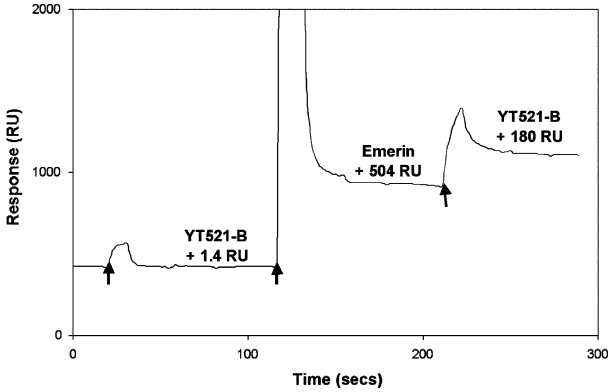

Fig. 4. BIAcore analysis of the interaction between emerin and YT521-B. The nucleoplasmic domain of emerin (residues 1–222) and the yeast two-hybrid fragment of YT521-B were expressed in *E. coli* BL21(DE3) from pET vectors. Emerin was captured onto a rabbit anti-mouse Ig chip via MANEM1 mAb (see Methods). YT521-B binds to emerin (+180 RU) but was unable to bind to the chip before emerin was added (+1.4 RU). The arrows show the injection points and the brief sharp peaks are due to refractive index changes as the buffer containing protein passes over the chip. Protein binding is quantified from the change in baseline after buffer and unbound proteins are washed away.

Mapping residues in emerin required to bind YT521-B

YT521-B was tested for binding to a collection of emerin mutants bearing clusters of alanine-substitution mutations, which were used previously to map the binding sites for BAF, lamin A and GCL [9,28]. Figure 5A shows that some mutants (namely mutants 45, 76, 122, 175, 179, 192 and 207) reduced YT521-B binding significantly, to levels at or near the BSA control. A few mutations had milder effects (mutants 40, 151, 161, 196 and 214), whereas the remaining mutants bound YT521-B at least as well as wild-type emerin. These results are mapped schematically in Fig. 5B. Two regions of emerin were important for binding to YT521-B; these regions flank the lamin-binding region and overlap partially with both the BAF- and lamin-binding regions. Interestingly, the regions important for binding to YT521-B overlap substantially with two transcriptional repressors that bind emerin ([28]; T. Haraguchi, J. M. Holaska, M. Yamame, K. L. Wilson & Y. Hiraoka, unpublished data; see Discussion]. This binding site is bipartite, which may imply some folding of emerin around its lamin-binding domain. Antibody studies have also suggested folding within the lamin-binding domain of emerin [32].

Emerin influences YT521-B-dependent splice sites *in vivo*

YT521-B has been shown to influence splice site selection in cotransfection experiments [25]. To determine whether emerin has a similar effect on splice site selection, we cotransfected HEK293 cells with expression constructs encoding emerin and a CD44 reporter gene. Fig. 6A shows that transfecting emerin alone had no effect on exon 5 inclusion, in contrast with transfection with YT521-B alone, which reduced exon 5 inclusion (Fig. 6B) as expected [25]. Cells transfected with 1 μg YT521-B had inclusion of exon 5 reduced significantly ($P = 0.006$; *t*-test) from 34% to 17% (Fig. 6B). To determine if emerin influenced YT521-B, we cotransfected cells with both proteins and tested their influence on exon 5 of the CD44 reporter gene [33]. If emerin was also present, the effect of YT521-B on splicing was partially reversed from 17% to 24% (Fig. 6B). Both emerin expression (Fig. 6C) and its effect on splicing (Fig. 6B) were dose-dependent and there was a significant difference in splicing ($P = 0.008$; *t*-test) between experiments with no detectable emerin expression (Fig. 6B, lanes 2 and 3) and those in which emerin was present (Fig. 6B, lanes 4–6). This is the first indication that full length YT521-B and emerin interact *in vivo*. Since emerin itself

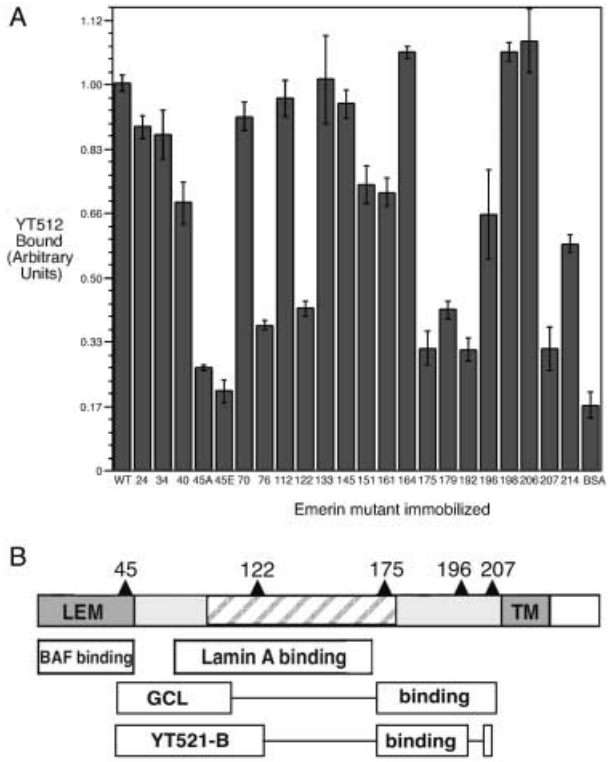

Fig. 5. Emerin residues required for binding to YT521-B. (A) Quantitative microtiter binding assays. Purified recombinant emerin protein (residues 1–222; either wild-type or each indicated mutant) was immobilized in microtiter wells, incubated with ^{35}S-YT521-B, washed and counted (see Materials and methods). Emerin proteins were extracted from parallel wells and immunoblotted to verify similar amounts of emerin per microtiter well (not shown). (B) Diagram of emerin, showing the positions of mutations that disrupt binding to YT521-B, relative to those that disrupt binding to BAF or lamin A (15) or transcription repressor GCL [28]. Black triangles indicate specific mutations that define the proposed binding domain for YT521-B in emerin. TM, Transmembrane domain; LEM, LEM-domain.

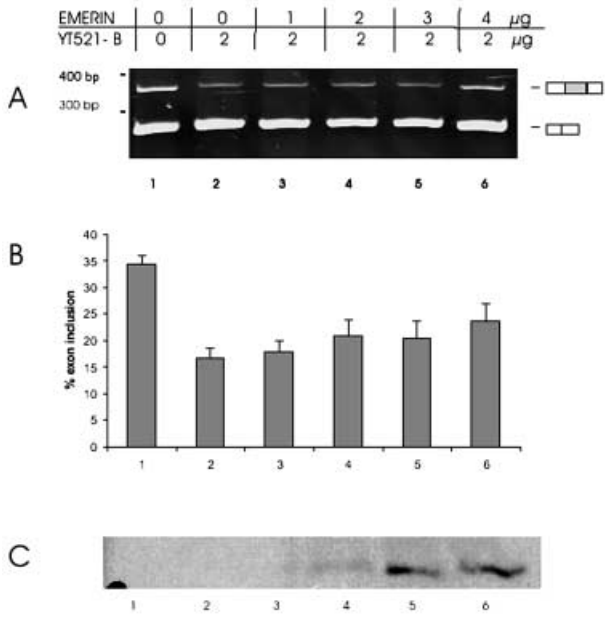

Fig. 6. Emerin reverses the influence of YT521-B on splice site selection. (A) Cotransfection assay demonstrating the influence of emerin and YT521-B on a CD44 exon v5 minigene. Two µg of a YT521-B expression clone were cotransfected with an increasing amount (indicated on top) of an emerin expression clone. The transfected DNA was adjusted to 6 µg with empty parental vector. One µg of a CD44 exon v5 reporter gene was present in all experiments. The gene products were detected by RT-PCR using minigene specific primers. One representative ethidium bromide stained gel is shown. (B) Quantitative analysis of at least three experiments. The standard deviations of at least three independent experiments are indicated as error bars. (C) The expression level of emerin in the experiment was determined by Western blot using MANEM5 mAb.

does not affect splice site selection, this effect is most likely mediated by its binding to YT521-B.

Discussion

This work has identified a novel emerin-binding protein, the ubiquitously expressed nuclear splicing-associated factor YT521-B [25]. The binding of emerin to YT521-B was confirmed by three independent biochemical methods (pull-downs, BIAcore and microtiter binding assays). The interacting region of YT521-B comprises part of a proline-rich domain that lacks secondary structure, plus the intact C-terminal 'Glu-Arg' domain ('R-D/E' domain), which is required for binding to several known RNA splicing factors including Sam68 and htra2-β1. Binding between Glu-Arg domains and splicing factors is regulated by the Src family kinase, p59^{fyn} [25]. Our results suggest that full-length YT521-B and emerin interact *in vivo*, and that this interaction is physiologically significant as emerin influenced YT521-B-dependent splicing *in vivo*. The binding regions on emerin for YT521-B also interact with a transcription factor, GCL, and binding of BAF to the adjacent LEM domain of emerin interferes with this GCL interaction [28]. This raises the possibility that interactions of this particular binding region of emerin are dynamic *in vivo* and occur only under specific circumstances. The fact that emerin and BAF only colocalize at specific stages of the cell cycle [34] supports this concept of dynamic interactions.

In our immunoprecipitation assays, emerin pulled down a higher proportion of input YT521-B than any other protein tested, including lamin A, suggesting that its binding is relatively tight *in vitro*. Binding to YT521-B was not disrupted by either of two emerin missense mutations that cause EDMD. However we are intrigued by the possibility that the P183H mutation might alter emerin dynamics by binding too tightly to both lamin A [9] and splicing factor YT521-B (Fig. 3B). It is interesting to note that myotonic dystrophies (DM1 and DM2) are now thought to be caused, at least in part, by defects in RNA splicing that result from the accumulation in the nucleus of expanded CUG repeats in nuclear RNA [35]. Myotonic dystrophies are multisystem disorders that share two major clinical features with EDMD: cardiac conduction system defects and skeletal muscle

wasting. Two additional features of myotonic dystrophy, myotonia and insulin resistance, have been directly linked to defective alternative splicing of mRNAs encoding a muscle chloride channel [36,37] and insulin receptor [38], respectively. In the heart, splicing of the mRNA encoding cardiac troponin-T is also altered in DM1 [39]. Thus, it is possible that many, if not all, clinical features of myotonic dystrophy are caused by defects in mRNA splicing.

YT521-B has been shown to modulate alternative splice site selection *in vivo* [25]; this activity requires the C-terminal Glu-Arg domain that our evidence suggests can also interact with emerin. If the normal functions of YT521-B are somehow regulated by interactions with emerin or the emerin–lamin A/C complex, some of the cardiac and skeletal muscle symptoms of EDMD may result from inappropriate splicing of tissue-specific mRNAs. This could explain how changes in widely expressed proteins like YT521-B and emerin might produce the tissue-specific effects in EDMD [4]. Although the functional effects of emerin on YT521-B may appear modest, it must be remembered that effects of complete absence of emerin in X-EDMD are also quite modest, with clinical features appearing many years after birth, and we would not expect lack of emerin to have any drastic effect on 'housekeeping' functions [28]. Splicing defects are often associated with late-onset disease or predisposition to disease [40].

Emerin binding to YT521-B might sequester or inactivate YT521-B, as suggested by our *in vivo* splicing results. Alternatively, the emerin–lamin A/C complex might help to organize transcription factors, splicing factors and chromatin to ensure that specific RNA transcripts are correctly spliced. Alternative splicing can regulate gene expression directly by changing the polypeptides encoded by primary RNA transcripts. However, transcription factors are themselves the products of alternative splicing, and defects in splice site selection may have downstream indirect effects on gene expression due to inappropriately expressed transcription factors (reviewed in [25]). The adipocyte differentiation and transcription factor known as 'sterol response element binding protein 1' (SREBP1), binds to lamin A and this binding is affected by a lamin A/C mutation that causes lipodystrophy [41]. The retinoblastoma transcription factor, Rb, binds to the emerin-related LEM domain protein, LAP2α [42]. Haraguchi *et al.* (personal communication) have shown that Btf, a transcriptional repressor that is highly expressed in muscle, interacts with emerin. It is impossible to say at present which of the currently known emerin-binding factors are involved in the pathogenesis of EDMD. However, the hypothesis that absence or disruption of the emerin–lamin A/C complex has downstream effects on gene expression that lead to specific degeneration of skeletal and cardiac muscle now deserves serious consideration.

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