Tyrosine phosphorylation of nuclear-membrane protein emerin by Src, Abl and other kinases

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Summary

X-linked recessive Emery-Dreifuss muscular dystrophy (EDMD) is caused by loss of emerin, a nuclear-membrane protein with roles in nuclear architecture, gene regulation and signaling. Phosphoproteomic studies have identified 13 sites of tyrosine phosphorylation in emerin. We validated one study, confirming that emerin is hyper-tyrosine-phosphorylated in Her2-overexpressing cells. We discovered that non-receptor tyrosine kinases Src and Abl each phosphorylate emerin and a related protein, LAP2β, directly. Src phosphorylated emerin specifically at Y59, Y74 and Y95; the corresponding triple Y-to-F ('FFF') mutation reduced tyrosine phosphorylation by ~70% in vitro and in vivo. Substitutions that removed a single hydroxyl moiety either decreased (Y19F, Y34, Y161F) or increased (Y4F) emerin binding to BAF in cells. Y19F, Y34F, Y161F and the FFF mutant also reduced recombinant emerin binding to BAF from HeLa lysates, demonstrating the involvement of both LEM-domain and distal phosphorylatable tyrosines in binding BAF. We conclude that emerin function is regulated by multiple tyrosine kinases, including Her2, Src and Abl, two of which (Her2, Src) regulate striated muscle. These findings suggest roles for emerin as a downstream effector and 'signal integrator' for tyrosine kinase signaling pathway(s) at the nuclear envelope.

Key words: Emerin, LEM domain, Nuclear envelope, Emery-Dreifuss muscular dystrophy, Src, Her2, Abl, Barrier-to-autointegration factor (BAF), Cardiomyopathy, Breast cancer, Laminopathy, Neuromuscular junction

Introduction

Mutations in the gene encoding emerin, a nuclear inner membrane protein, cause recessive X-linked Emery-Dreifuss muscular dystrophy (X-EDMD) (Bione et al., 1994), characterized by weakening of specific skeletal muscles, contractures, cardiomyopathy and cardiac conduction system defects that can cause sudden death (Muchir and Worman, 2007). Autosomal dominant EDMD (AD-EDMD) is caused by mutations in three other genes: *LMNA* (which encodes A-type nuclear lamin intermediate filaments) (Gruenbaum et al., 2005), *SYNE1* or *SYNE2* (which encode multiple isoforms of the spectrin-repeat, KASH-domain proteins nesprin-1 and nesprin-2, respectively) (Zhang, Q. et al., 2007). Emerin binds directly to products of all three genes, and its localization at the nuclear inner membrane in somatic cells requires A-type lamins (D'Angelo and Hetzer, 2006; Mislow et al., 2002; Zhang et al., 2005). Thus, EDMD disease might arise from the disruption of nuclear envelope structures that include lamin A, emerin and nesprins (Zhang, Q. et al., 2007). However, all four proteins have additional partners and roles that challenge our understanding of EDMD disease mechanisms.

Emerin is involved in tissue-specific gene regulation, mechanosensitive gene regulation, signaling and nuclear architecture (Bengtsson and Wilson, 2004; Lammerding et al., 2005; Muchir et al., 2009). The ~40-residue LEM (LAP2, emerin, MAN1) domain found in emerin and related proteins confers binding to BAF, a conserved chromatin regulator that also binds lamins (Margalit et al., 2007). Emerin also binds many other proteins directly, including transcription and splicing factors (GCL, Lmo7, β-catenin, Btf, YT521-B) (Haraguchi et al., 2004; Holaska et al., 2003; Holaska et al., 2006; Lee et al., 2001; Markiewicz et al., 2006; Wilkinson et al., 2003) and architectural components (lamin A, nesprins, nuclear myosin 1c, actin, tubulin) (Clements et al., 2000; Fairley et al., 1999; Holaska et al., 2004; Holaska and Wilson, 2007; Mislow et al., 2002; Salpingidou et al., 2007; Zhang et al., 2005). The purification of six distinct emerin-containing ternary complexes from HeLa cell nuclei (Holaska and Wilson, 2007) suggests that its interactions are differentially regulated. Indeed, emerin can be phosphorylated on Ser or Thr residues, including S49, S66, T67, S120, S163 and S175 (Brill et al., 2004; Hirano et al., 2005; Roberts et al., 2006; Shu et al., 2004). Mitotic phosphorylation of emerin S175 inhibits binding to BAF (Hirano et al., 2005), implicating regions outside the LEM domain in this interaction. During infection by Herpes simplex virus type 1, emerin is phosphorylated by several kinases (Leach et al., 2007; Morris et al., 2007).

Emerin is homologous to another LEM domain protein LAP2β, and they share several partners besides BAF (Furukawa, 1999; Lee et al., 2001), including transcription factor GCL (Holaska et al., 2003; Nili et al., 2001) and lamin B (Fairley et al., 1999; Foisner and Gerace, 1993). LAP2β is tyrosine phosphorylated (Otto et al., 2001), but specific sites or kinases were previously unidentified.

Phosphoproteomic studies suggested potential regulation of emerin by tyrosine kinases: at least 13 of the 18 tyrosine residues in emerin can be phosphorylated in vivo (see below), and tyrosine phosphorylation of emerin was reported to increase 9.9-fold in NIH-3T3 cells that overexpress Her2 (ErbB2), a member of the epidermal growth factor (EGF) family of receptor tyrosine kinases (Bose et al., 2006). Potential Her2-dependent tyrosine phosphorylation of emerin was not independently validated, nor were specific sites identified. Because Her2 activity is important for many tissues, including muscle and heart (Andrechek et al., 2002; Leu et al., 2003; Negro et al., 2004), we tested potential regulation of emerin by Her2. We also tested Src and Abl, non-

receptor tyrosine kinases that can be activated by Her2 or other signaling pathways (Roskoski, 2005; Srinivasan and Plattner, 2006). We confirm Her2-stimulated emerin tyrosine phosphorylation and report that emerin and LAP2β are phosphorylated directly by Src and Abl. We also show that tyrosine phosphorylation of emerin regulates binding to BAF.

Results

We compiled data from 13 independent proteomic studies (Amanchy et al., 2005; Brill et al., 2004; Cantin et al., 2008; Daub et al., 2008; Guo et al., 2008; Olsen et al., 2006; Pan et al., 2008; Rikova et al., 2007; Rush et al., 2005; Schlosser et al., 2006; Sui et al., 2008; Tao et al., 2005; Tsai et al., 2008), which identified 13 phosphorylated tyrosines in emerin (Fig. 1A). All sites except Y19 and Y41 were identified more than once. All 13 identified phosphorylated Tyr (Tyr-*P*) residues in human emerin are conserved in mouse, and six (corresponding to Y4, Y41, Y94, Y95, Y105, Y161) are conserved in *Xenopus* (Fig. 1B). These studies did not reveal responsible pathways or kinases.

We validated tyrosine phosphorylation of emerin by immunoprecipitating endogenous emerin from HeLa cells treated with 1 μM pervanadate (PV, a tyrosine phosphatase inhibitor) (Huyer et al., 1997) or buffer (PBS) as control, for 30 minutes before lysing cells. Emerin immunoprecipitated from PV-treated cells was specifically recognized by Tyr-*P* antibodies (not shown; see Fig. 2B). No other tyrosine-phosphorylated bands were detected (data not shown). Pervanadate enhances tyrosine phosphorylation by blocking dephosphorylation, and by activating tyrosine kinases (e.g. insulin receptor, EGFR, Src family kinases) regulated by tyrosine phosphorylation (Boulven et al., 2002; Fantus et al., 1989; Kim et al., 2003). Thus, PV-induced tyrosine phosphorylation probably reflects both ongoing physiological regulation and additional sites targeted by PV-activated kinases.

Emerin is tyrosine phosphorylated by Her2-mediated signaling

To test potential Her2-dependent phosphorylation (Bose et al., 2006), we immunoprecipitated endogenous emerin from NIH-3T3 cells that stably overexpressed either control vector or wild-type human Her2. Immunoprecipitates were resolved by SDS-PAGE and western blotted using antibodies specific for emerin or Tyr-*P* (Fig. 2A). Emerin was recovered from both Her2-expressing and control cells (Fig. 2A, lanes 2 and 4) and emerin precipitation was antibody dependent (Fig. 2A, lanes 1 and 3). Emerin tyrosine phosphorylation was detected in Her2-expressing cells (Fig. 2A, lane 4), independently confirming the proteomic result (Bose et al., 2006). Exogenous GFP-emerin, but not GFP, was also tyrosine phosphorylated in Her2-overexpressing cells (data not shown). Again, no other bands were detectably tyrosine phosphorylated under these conditions. We conclude that emerin is a target of Her2-mediated signaling in vivo. Her2, a plasma membrane protein, might also localize in the nucleus (Chen et al., 2005; Wang et al., 2004). However, Her2 more conventionally acts indirectly, by activating downstream effector tyrosine kinases (Yarden and Sliwkowski, 2001).

Emerin is directly phosphorylated by Src

We tested the model that Her2 acts indirectly, and focused on Src family kinases (SFKs), which can mediate Her2 signaling (Kim et al., 2005; Roskoski, 2005; Tan et al., 2005; Tan et al., 2006) and enter the nucleus (David-Pfeuty et al., 1993; David-Pfeuty and Nouvian-Dooghe, 1995; Ikeda et al., 2008; Radha et al., 1996; Rongish and Kinsey, 2000; Zhao et al., 1992). SFKs are physiologically relevant in heart and skeletal muscle, which are affected in EDMD patients. To determine whether SFKs might influence emerin, we compared PV-induced emerin tyrosine phosphorylation levels in control cells and cells pretreated with an SFK inhibitor, either PP2 or SU6656 (Fig. 2B). HeLa cells

A

Reference	Cell line, Tissue (species)	Phosphotyrosine Residues Identified											
		4	19	41	59	74	85	90	94	95	99	105	161	167
Cantin et al., 2008	HeLa, Cervix (human)				X									
Olsen et al., 2006	HeLa, Cervix (human)				X	X							X	X
Schlosser et al., 2006	HeLa, Cervix (human)				X	X	X						X	X
Amanchy et al., 2005	HeLa, Cervix (human)		X	X		X	X						X	X
Daub et al., 2008	HeLa, Cervix (human)											X		
Rush et al., 2005	Jurkat, T lymphocytes (human)					X	X	X	X	X	X			
Tao et al., 2005	Jurkat, T lymphocytes (human)											X		
Brill et al., 2004	Jurkat, T lymphocytes (human)													X
Rikova et al., 2007	NSCLC tumors, Lung (human)								X	X		X		
Guo et al., 2008	NSCLC tumors, Lung (human)								X				X	
Tsai et al., 2008	H1299, Lung (human)					X							X	X
Schlosser et al., 2006	Neuroblastoma N2a, Nervous (mouse)					X			X			X		
Pan et al., 2008	Hepa1-6, Liver (mouse)	X					X	X	X	X	X	X	X	X
Sui et al., 2008	Chang, Liver (human)	X												

B

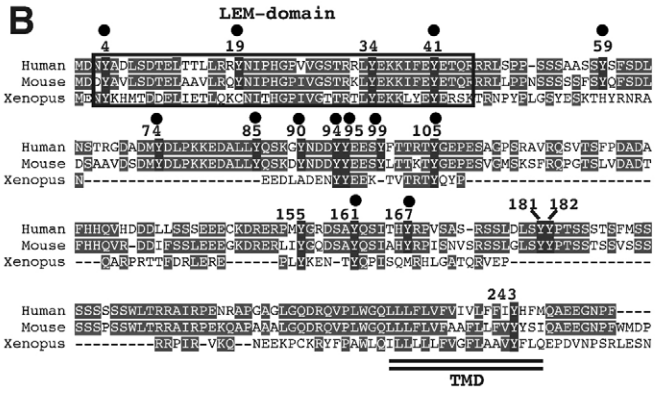

Fig. 1. Emerin is tyrosine phosphorylated. (A) Emerin Tyr-*P* sites identified in published proteomic mass spectrometry studies. (B) Amino acid sequences of emerin from human (Acc. no. NP_000108), mouse (NP_031953) and *Xenopus laevis* (NP_001089081). Conserved residues are light gray. All tyrosines are dark gray and numbered; black dots indicate those reported to be phosphorylated in A. The LEM domain is boxed and transmembrane domain (TMD) is double underlined.

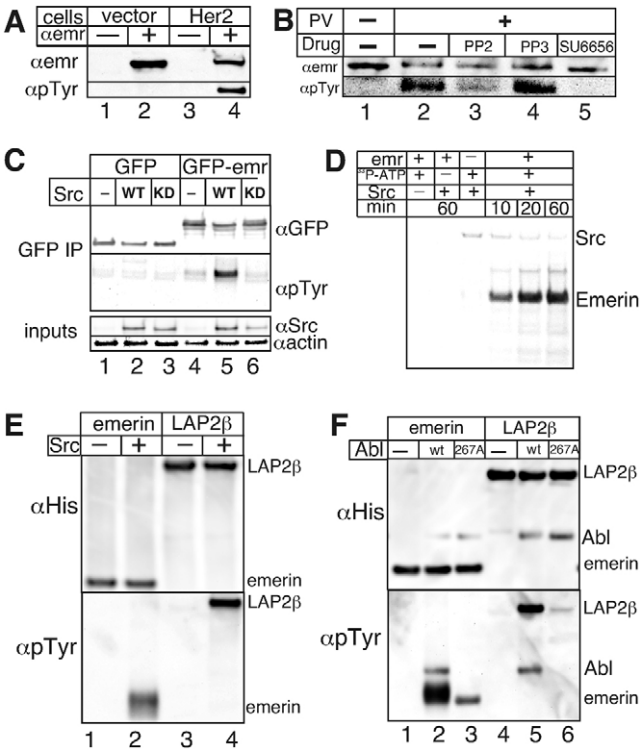

Fig. 2. Emerin tyrosine phosphorylation by Her2, Src and Abl. (A) Her2 overexpression induces emerin tyrosine phosphorylation. Endogenous emerin was immunoprecipitated (NCL-emerin antibody) from NIH-3T3 cells that stably expressed either control vector or a Her2 overexpression vector (Bose et al., 2006). Antibody was omitted from controls. Immunoprecipitates were analyzed by western blotting with antibodies against emerin (αemr; sc-15378) and Tyr-*P* (αpTyr). A representative blot is shown (*n*=3). (B) PV-induced emerin tyrosine phosphorylation is reduced by Src-specific inhibitors. HeLa cells were treated with either DMSO, DMSO and 10 μM PP2 (SFK inhibitor), DMSO and 1 nM SU6656 (SFK inhibitor) or DMSO and 10 μM PP3 (inactive analog of PP2) for 30 minutes before treatment with PBS or 1 μM PV for 30 minutes. Endogenous emerin was then immunoprecipitated (serum 2999), resolved by SDS-PAGE and western blotted for emerin (NCL-emerin) and Tyr-*P*. Representative blot is shown (*n*=3). (C) HeLa cells were transiently transfected to express GFP or GFP-emerin (GFP-emr), plus either wild-type Src (WT) or kinase-dead Src (KD). Lysates (1%) were western blotted for Src and actin as controls (inputs). GFP proteins were immunoprecipitated with GFP antibody (GFP IP), resolved by SDS-PAGE and western blotted for GFP and Tyr-*P*. A representative autoradiograph is shown (*n*=3). (D) In vitro Src phosphorylation using purified recombinant Src, purified recombinant untagged emerin (residues 1-222) and [^{33}P]ATP. Samples were resolved by SDS-PAGE and analyzed by autoradiography. A representative blot is shown (*n*=3). (E) Same in vitro Src phosphorylation assay as D, but incubated for 10 minutes with recombinant His-emerin (residues 1-176) or His-LAP2β (residues 1-408) as substrates, either with (+) or without (–) Src. Samples were resolved by SDS-PAGE and western blotted with antibodies against the His-tag and Tyr-*P*. A representative blot is shown (*n*=3). (F) Recombinant emerin (residues 1-176) and LAP2β (residues 1-408) were incubated in kinase buffer alone or with purified recombinant Abl kinase domain (wild type or mutant R367A). Reactions were resolved by SDS-PAGE and western blotted for His and Tyr-*P* (*n*=3); representative blots are shown.

were incubated for 30 minutes, with either DMSO (solvent control), 1 nM SU6656 (SFK inhibitor), 10 μM PP2 (SFK inhibitor) or 10 μM PP3 (negative control for PP2), then supplemented with PV (or PBS) for 30 minutes before lysing cells. Endogenous emerin was then immunoprecipitated from cell lysates, resolved by SDS-PAGE, and western blotted using antibodies against emerin or Tyr-*P* (Fig. 2B). As expected, emerin was detectably tyrosine phosphorylated in PV-treated, but not control (PBS-treated) cells (Fig. 2B, compare lane 2 with lane 1). The emerin Tyr-*P* signal, relative to total emerin in each sample, was reduced by both inhibitors (Fig. 2B, lanes 3 and 5; *n*=3) and unaffected by control PP3 (Fig. 2B, lane 4). Although each inhibitor can affect other kinases (Bain et al., 2007), these results supported potential SFK regulation of emerin.

To test potential Src regulation, we transiently cotransfected HeLa cells to overexpress either wild-type or kinase-dead Src, plus GFP or GFP-emerin. After 24 hours, whole-cell lysates were resolved by SDS-PAGE and western blotted to reveal Src levels relative to actin (Fig. 2C, inputs). GFP and GFP-emerin were immunoprecipitated from the same cell lysates using GFP antibodies, resolved and western blotted with antibodies against GFP or Tyr-*P*. GFP and GFP-emerin were recovered at similar levels from all samples (Fig. 2C, top). GFP was not detectably tyrosine phosphorylated (Fig. 2C, lanes 2 and 3). GFP-emerin was tyrosine phosphorylated in cells that expressed wild-type, but not kinase-dead, Src (Fig. 2C, compare lane 5 with lane 6). No additional Tyr-*P* bands were detected (data not shown). We concluded that Src or tyrosine kinases activated by Src (or both) phosphorylated emerin in HeLa cells.

Might Src phosphorylate emerin directly? Recombinant purified wild-type human emerin (untagged residues 1-222) was incubated for 10, 20 or 60 minutes with purified recombinant Src plus [^{33}P]ATP. Reactions were quenched with SDS sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose. Autoradiography confirmed Src autophosphorylation and showed increased emerin phosphorylation over time (Fig. 2D). Similar results were obtained using a His-tagged shorter emerin polypeptide (residues 1-176) incubated with Src for 10 minutes in vitro (Fig. 2E, lane 2). Another LEM protein, LAP2β (residues 1-408), was also phosphorylated by Src in vitro (Fig. 2E, lane 4).

Emerin is directly phosphorylated by Abl

Abl, an SFK-related kinase, is activatable by Her2 signaling (Srinivasan and Plattner, 2006) and localizes in the nucleus (Shaul, 2000). We tested emerin as a potential substrate for the purified recombinant His-tagged kinase domain (residues 229-511) of human Abl (Seeliger et al., 2005). As the control, we used Abl mutant R367A, which has 5000 times lower catalytic efficiency (Muratore et al., 2009). Recombinant His-tagged emerin (residues 1-176) was incubated 30 minutes alone or with each Abl kinase domain. We also tested recombinant LAP2β 1-408 as substrate. Reactions were resolved by SDS-PAGE and western blotted with Tyr-*P* or His antibodies (Fig. 2F). Emerin and LAP2β were tyrosine phosphorylated by the wild-type Abl domain, and weakly by the mutant domain (Fig. 2F, compare lanes 2 and 5 with 3 and 6). Thus, emerin and LAP2β were each phosphorylated directly by Abl in vitro.

MS identification of Abl and Src phosphorylation sites in emerin

Specific emerin residues phosphorylated in vitro by Src and Abl were identified by LC-MS/MS analysis. Tryptic digestion of recombinant GST-emerin residues 1-222, used to identify Src sites, yielded 12 predicted Tyr-containing peptides, nine of which were identified by LC-MS/MS (Fig. 3A). The Y4-containing GST-fusion peptide was not recovered. Small (e.g. Y34- or Y155-containing) tryptic peptides are typically not recovered by mass spectrometry. No phosphorylated tyrosines were detected in controls lacking Src (data not shown). However, three distinct emerin phosphopeptides

A

Emerin Tyr-containing Tryptic Peptides	Tyr	Identified by MS/MS		pTyr sites	
		Src	Abl	Src	Abl
MDNYADLSDTELTTLL	4	No	No		
YNIPHGPVVGSTR	19	Yes	Yes		
LYEK	34	No	No		
IFEYETQR	41	Yes	Yes		
LSPPSSSAASSYSFSDLNSTR	59	Yes	Yes	59	
GDADMYDLPK	74	Yes	Yes	74	
KEDALLYQSK	85	Yes	Yes		
GYNDDYYEESYFTTR	90,94,95,99	Yes	Yes	95	
TYGEPESAGPSR	105	Yes	Yes		
ERPMYGR	155	No	Yes		
DSAYQSITHYRPVSASR	161,167	Yes	Yes		167
SSLDLSYYPTSSSTSFMSSSSSSSSWLTR	181,182	Yes	–		

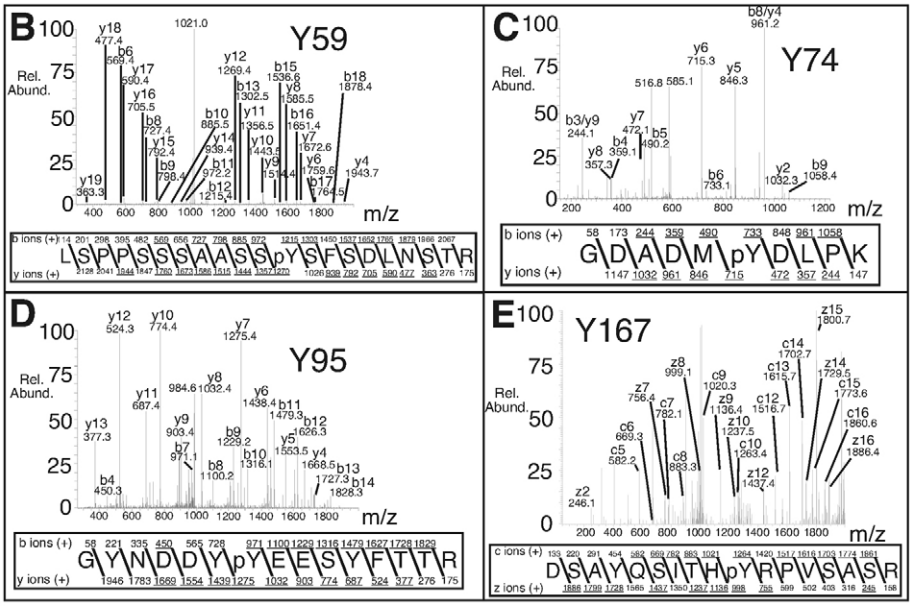

Fig. 3. LC-MS/MS identification of emerin tyrosine residues phosphorylated by Src and Abl. (A) Summary of emerin peptides identified by mass spectrometry. (B-D) Mass spectra of emerin peptides that demonstrated Src phosphorylation shown for Y59 (B; ion score=113; expect value=1.3×10^{8}), Y74 (C; ion score=53; expect value=0.0096) and Y95 (D; ion score=79; expect value=3.2×10^{-5}). Inserts in B-E show the predicted c-ion and z-ion masses of each identified sequence. Ions used for identification are underlined and corresponding peaks are labeled. (E) Mass spectrum demonstrating Abl phosphorylation of emerin Y167 (ion score=47; expect value=2.1).

were recovered that unambiguously identified phosphorylated emerin residues Y59, Y74 and Y95 (Fig. 3A-D). Thus, Src selectively recognized at least three of 14 available Tyr residues in the emerin 1-222 polypeptide. Tryptic digestion of the recombinant His-emerin 1-176 polypeptide, used to identify Abl sites, is predicted to yield 11 Tyr-containing peptides; nine were identified by LC-MS/MS (Fig. 3A). One Abl-phosphorylated peptide was recovered and identified as Y167 (Fig. 3E). These findings did not rule out additional sites.

Emerin residues Y59, Y74 and Y95 are major Src targets in vitro and in vivo

To test potential relevance of these sites, we repeated the in vitro Src phosphorylation assay using as substrates either wild-type His-tagged emerin (residues 1-176) or a corresponding polypeptide bearing Y-to-F substitutions at all three sites (‘FFF’). Controls showed no detectable emerin phosphorylation in reactions lacking Src (Fig. 4A, lanes 1 and 3) and Src-dependent phosphorylation of wild-type His-emerin (Fig. 4A, lane 2), as expected. In vitro tyrosine phosphorylation of the FFF mutant was reduced by 74% (Fig. 4B) (n=3; P<0.05). Residual (average 26%) phosphorylation of the emerin FFF mutant might be nonspecific, or due to unidentified specific site(s). We repeated the experiment with each double mutant combination; all showed significantly reduced (>50%) phosphorylation compared with the wild type (Fig. 4C,D) (n=3; FFF P<0.005; 59F/74F, P<0.005; 59F/95F, P=0.01; 74F/95F, P<0.05). We concluded that Y59, Y74 and Y95 are major in vitro sites of Src phosphorylation in emerin.

To assess any biological relevance, we first transiently expressed GFP-emerin (full-length wild-type or FFF) in HeLa cells; both localized predominantly at the nuclear envelope (Fig. 4E), suggesting that the FFF mutation did not grossly perturb exogenous emerin expression or localization. We deduced that the FFF mutation did not disrupt emerin binding to lamin A. Emerin binding to lamin A in vitro was unaffected by single Y-to-F mutations at these sites (data not shown). We next studied emerin tyrosine phosphorylation in HeLa cells 24 hours after transiently coexpressing Src (wild type or kinase-dead) plus either GFP or GFP-emerin (full-length wild type or FFF mutant). Whole-cell lysates were immunoprecipitated using GFP antibodies, resolved by SDS-PAGE and western blotted for GFP or Tyr-*P* (Fig. 4F). Control blots verified similar levels of Src expression, relative to actin (Fig. 4F, inputs). As expected, GFP-emerin was tyrosine phosphorylated in cells that coexpressed wild-type, but not kinase-dead, Src (Fig. 4F, lanes 3 and 2, respectively). Tyrosine phosphorylation of FFF-emerin was reduced by 71% in vivo (Fig. 4G) (n=3; P<0.01). We concluded emerin residues Y59, Y74 and Y95 are major targets of Src regulation in HeLa cells.

In HeLa cells, Src inhibitors reduced PV-induced emerin tyrosine phosphorylation (Fig. 2B), suggesting that SFKs contributed to this signal. We independently tested this contribution using the FFF mutant. HeLa cells were transfected to express GFP or GFP-emerin (wild type or FFF) for 24 hours, then treated with PV or PBS, immunoprecipitated using GFP antibodies, resolved by SDS-PAGE and western blotted for GFP or Tyr-*P* (Fig. 4H). Neither GFP nor GFP-emerin were detectably tyrosine phosphorylated in PBS-

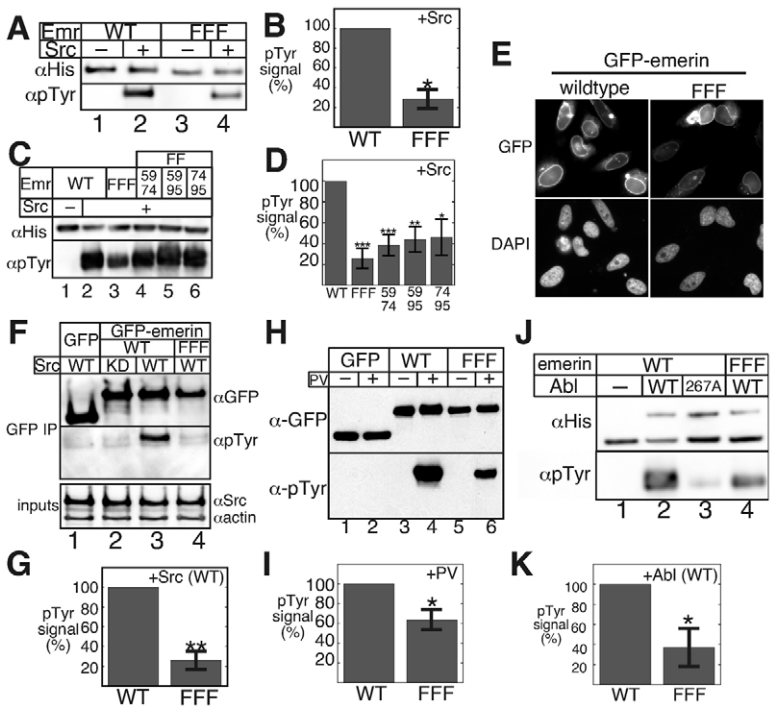

Fig. 4. Characterization of emerin Y-to-F missense mutations at identified Src phosphorylation sites. (A,B) In vitro phosphorylation assay with recombinant purified Src, recombinant purified emerin (residues 1-176; wild type or triple mutant Y59F, Y74F, Y95F; 'FFF') and incubated with ATP for 10 minutes, then resolved by SDS-PAGE and western blotted with antibodies against His tag or Tyr-*P* (A), and quantified (B) as the Tyr-*P*-to-His signal expressed as a percentage normalized to the wild type. Reduced tyrosine phosphorylation of FFF was significant; error bars show s.e.m.; **P*<0.05 (*n*=3). (C,D) Same in vitro Src phosphorylation assay as A but using as substrates either wild-type emerin residues 1-176, the corresponding FFF mutant, or each of three double mutant (FF) combinations: Y59F/Y74F, Y59F/Y95F or Y74F/Y95F. Reactions were resolved by SDS-PAGE and western blotted with antibodies to the His-tag or Tyr-*P* (C), then quantified (D) as described for B. Error bars show s.e.m.; **P*<0.05, ***P*<0.01, ****P*<0.005 (*n*=3). (E) Fluorescence micrographs of HeLa cells that transiently expressed GFP-emerin (wild type or FFF) for 24 hours, fixed, permeabilized, stained with DAPI and visualized by direct GFP fluorescence. (F) GFP-antibody immunoprecipitation of GFP-emerin (wild type or FFF mutant) after 24 hours co-expression in HeLa cells with either wild-type Src (WT) or kinase-dead Src (KD), resolved by SDS-PAGE and western blotted with antibodies against GFP or Tyr-*P*. As controls, input lysates (1%) were western blotted for Src and actin. (G) Results from F for wild-type Src were quantified as described in B. The reduced Tyr-*P* signal in the FFF mutant emerin was significant; ***P*<0.01; error bars show s.e.m. (*n*=4). (H) HeLa cells that transiently expressed GFP or GFP-emerin (wild type or FFF mutant) for 24 hours were treated 30 minutes with PBS or 1 μM PV, then lysed, immunoprecipitated with GFP antibodies, resolved by 16% Tris-Tricine SDS-PAGE and western blotted for GFP or Tyr-*P*. (I) Results from H were quantified as described in B. The reduced Tyr-*P* signal in the FFF mutant emerin was significant; **P*<0.05; error bars show s.e.m. (*n*=3). (J) Reduced Abl phosphorylation of FFF-mutant emerin. Recombinant emerin 1-176 (wild type or FFF mutant) polypeptides were incubated alone or with the Abl kinase domain (wild type or R367A). (K) Results from J for wild-type Abl were quantified as described in B. Error bars show s.e.m.; **P*<0.05 (*n*=3).

treated controls (Fig. 4H, lanes 1 and 3). In PV-treated cells, GFP-emerin was highly phosphorylated (Fig. 4H, lane 4) and this signal decreased by ~40% for the FFF mutant (Fig. 4H, lane 6; Fig. 4I, *n*=3, *P*<0.05). Thus, a significant fraction of PV-induced emerin phosphorylation in vivo involves residues Y59, Y74 and/or Y95.

These sites were also relevant to Abl, a Src-related kinase; in vitro Abl phosphorylation of FFF-mutated emerin residues 1-176 was reduced by 60% (Fig. 4J,K, *n*=3, *P*=0.028), suggesting these substitutions perturb substrate recognition by Abl, or remove additional Abl target site(s) in emerin.

Effects of emerin Y-to-F substitutions on binding to BAF

Tyrosine phosphorylation might, we hypothesized, regulate emerin interactions with specific partners (Fig. 5A), such as BAF. The LEM domain of emerin is sufficient (Cai et al., 2007) and necessary (Lee et al., 2001; Shumaker et al., 2001) to bind BAF. The human emerin LEM domain has four tyrosines, at least three of which (Y4, Y19, Y41) can be phosphorylated in vivo by unidentified kinases (see Fig. 1A). Y19 is conserved in mouse emerin, whereas Y4, Y34 and Y41 are also conserved in *Xenopus* emerin (Fig. 1B). Interestingly, emerin residue Y19 is conserved in LAP2 (all isoforms) and MAN1, but not other human LEM proteins (Fig. 5B). Residues Y4 and Y41 are unique to the LEM domain of emerin, suggesting that there is potential emerin-specific regulation (Fig. 5B). Residue Y34, highly conserved among emerin orthologs (and nearly all human LEM-domain proteins) (Fig. 5B), is in the hydrophobic groove that directly contacts BAF (Fig. 5C); the other three tyrosines, which are also surface exposed, are not predicted to contact BAF (Fig. 5C) (Cai et al., 2007).

To determine whether specific tyrosine hydroxyl moieties in emerin were required to bind BAF, we tested the effects of single Y-to-F substitutions in either the LEM domain (Y4F, Y19F, Y34F, Y41F) or the distal region (Y161F, Y167F) that was previously implicated in binding to BAF (Hirano et al., 2005). For this experiment, we used bacterially expressed recombinant GST or GST-emerin residues 1-176 (wild type, or single Y-to-F substitutions), which were assumed to lack eukaryotic post-translational modifications. Each recombinant polypeptide was incubated for 4 hours at 4°C with glutathione beads and HeLa cell protein lysates prepared by ~200-fold dilution in buffer lacking ATP and also lacking phosphatase inhibitors (see the Materials and Methods), as a source of endogenous BAF. Beads were then washed

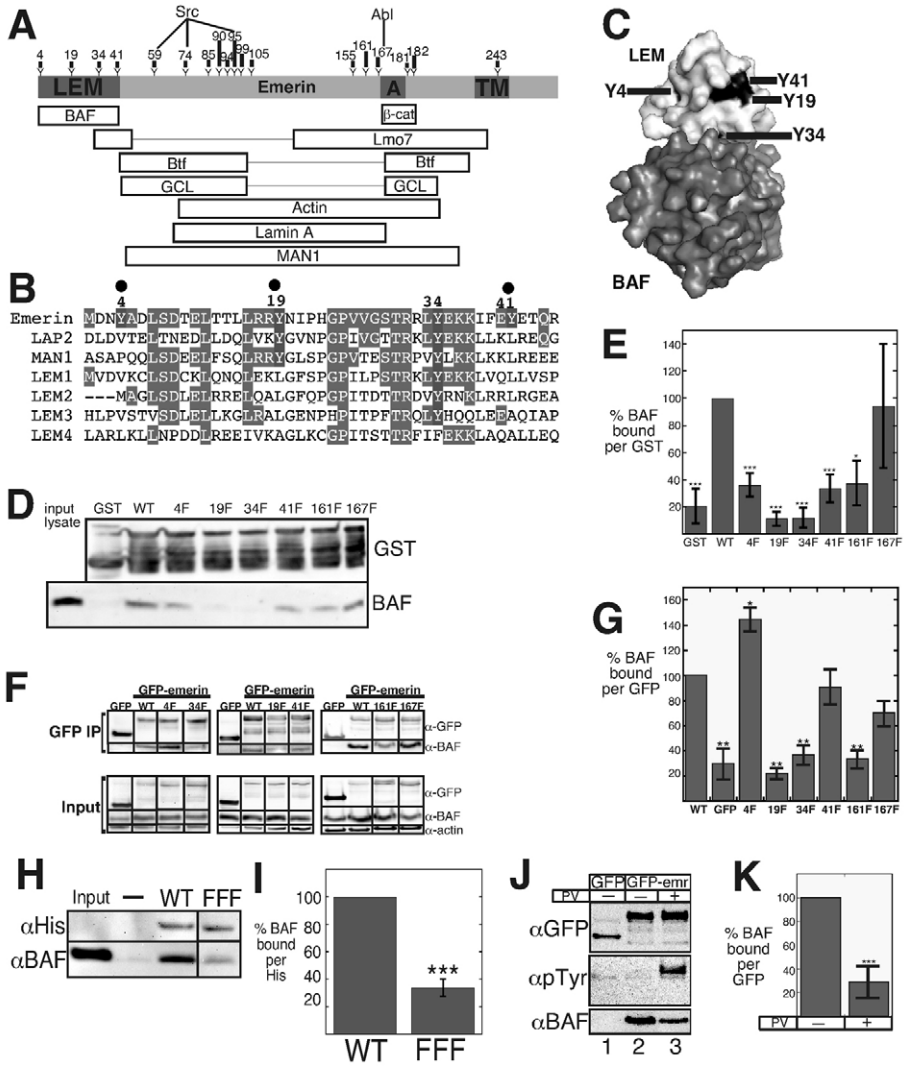

Fig. 5. Effects of tyrosine mutations and tyrosine phosphorylation on emerin binding to BAF. (A) Functional map based on mutations in emerin that disrupt binding to each indicated partner (Holaska et al., 2004; Holaska et al., 2006; Mansharamani and Wilson, 2005) and evidence that the APC-like ('A') region (residues 169-180) is sufficient to bind β-catenin (β-cat) (Markiewicz et al., 2006). The nesprin-binding region (residues 140-176) is not shown (Wheeler et al., 2007). LEM, LEM domain; TM, transmembrane. All Tyr residues are labeled 'Y' and numbered; identified Src and Abl targets are indicated. (B) LEM-domain amino acid sequences of all known human LEM-domain proteins (Wagner and Krohne, 2007). Conserved residues are light gray. All tyrosines are dark gray and numbered; black dots indicate known phosphorylation sites (Fig. 1A). (C) Surface representation of the NMR co-structure of BAF (gray) and the emerin LEM domain (white) (Cai et al., 2007); emerin residues Y4, Y19, Y34 and Y41 are shaded black. (D,E) Effects of Y-to-F mutations on emerin binding to BAF in vitro. Recombinant GST or GST-emerin (wild-type residues 1-176 or Y-to-F mutants) was incubated with HeLa cell lysates and pelleted using glutathione beads and western blotted for GST and BAF. Blots shown are representative of $n>3$ experiments and quantified in E as the BAF-to-His signal expressed as a percentage and normalized to wild-type GST-emerin. Error bars show s.e.m.; $*P<0.05$, $***P<0.005$ ($n=3$). (F,G) Effects of Y-to-F mutations on emerin binding to endogenous BAF in HeLa cells. HeLa cells that transiently expressed GFP or GFP-emerin (wild type, or each Y-to-F missense mutation) for 24 hours were immunoprecipitated with GFP antibodies (GFP IP); pellets were resolved by SDS-PAGE and western blotted for GFP and BAF. As loading controls (Input), 1% of each input lysate was directly probed with antibodies against GFP, BAF or actin. Blots shown in F are representative of $n>3$ experiments and quantified in G as the BAF-to-GFP signal expressed as a percentage and normalized to wild-type GFP-emerin. Error bars represent the s.e.m.; $*P<0.05$, $**P<0.01$ ($n>3$). (H,I) FFF mutation reduces binding of endogenous BAF from HeLa cell lysates to recombinant His-emerin residues 1-176. Blots shown in H are representative of $n=4$ experiments and were quantified in I as the BAF-to-His signal expressed as a percentage and normalized to wild-type His-emerin. Error bars indicate s.e.m.; $***P<0.005$ ($n=4$). (J,K) HeLa cells that transiently expressed GFP or GFP-emerin for 24 hours were treated with PBS or 1 μM PV for 30 minutes, then lysed, immunoprecipitated with GFP antibodies, resolved by 16% Tris-Tricine SDS-PAGE and western blotted for GFP, BAF or Tyr-*P*. Results from J were quantified in K as the BAF-to-GFP signal expressed as a percentage and normalized to PBS-treated cells. Error bars show s.e.m.; $***P<0.005$ ($n=3$).

and bead-associated proteins were western blotted for GST and BAF (Fig. 5D). Endogenous BAF bound wild-type GST-emerin, not GST (Fig. 5D), as expected. Binding to BAF in vitro was significantly reduced by the Y34F mutation, as predicted from the BAF-LEM domain co-crystal (Cai et al., 2007). However, binding was unexpectedly also reduced by Y4F, Y19F, Y41F and Y161F (Fig. 5D,E). We concluded that all five hydroxyl moieties each either contact BAF directly, or are required for emerin to adapt a BAF-binding conformation in vitro. This unexpected finding showed that the emerin molecule, including a membrane-proximal region, is more extensively engaged in binding to BAF than was previously thought. This requirement for specific tyrosine hydroxyl groups in

vitro predicted that BAF binding to emerin would be reduced by phosphorylation of these sites.

Tyrosine hydroxyl groups within, and outside, the LEM domain were also required to bind endogenous BAF in cells. We transiently expressed GFP alone or GFP-fused emerin (wild type, or single Y-to-F mutants) in HeLa cells for 24 hours, then immunoprecipitated with GFP antibodies, western blotted with antibodies against GFP or BAF, and quantified BAF binding to emerin. As loading controls, input samples were western blotted for GFP, BAF or actin (Fig. 5F, Input). Endogenous BAF co-immunoprecipitated with wild-type GFP-emerin (Fig. 5F,G, wt), not GFP alone (Fig. 5F,G, GFP), as expected. To quantify endogenous BAF binding to each GFP-fused emerin, we plotted the BAF-to-GFP signal ratio for each sample normalized to wild-type GFP-emerin (Fig. 5F,G) (n>3). BAF binding was reduced to similarly low levels by Y19F, Y34F and Y161F (Fig. 5F,G), confirming that each hydroxyl moiety is required (directly or indirectly) to bind BAF in cells. Phosphorylated Y19 and Y161 are detected in vivo (Fig. 1A), suggesting that cells might use Y19 and Y161 to regulate binding to BAF. Endogenous BAF co-immunoprecipitated with GFP-emerin mutants Y41F and Y167F at normal or near-normal levels, respectively (Fig. 5F,G), suggesting that these hydroxyl moieties were not essential for emerin to bind BAF in vivo, but leaving open the possibility that phosphorylation at Y41 or Y167 (our identified Abl site) might regulate binding to BAF. Why did BAF associate with emerin mutants Y4F and Y41F in cells (Fig. 5F,G), but not in vitro (Fig. 5D,E)? One possibility is that the C-terminal 78 residues (not present in recombinant emerin 1-176) normally contribute to BAF binding, and compensate in vivo for loss of the Y4 or Y41 hydroxyl group. Also interesting is why the Y4F mutation bound BAF better than wild-type GFP-emerin in cells (Fig. 5F,G). Enhanced BAF binding to the unphosphorylatable Y4F mutant is positive evidence that Y4, which is unique to the LEM domain of emerin, is subject to ongoing inhibitory phosphorylation in HeLa cells by unknown kinase(s).

The above findings showed that single tyrosine hydroxyl moieties either within or (surprisingly) outside the LEM domain are required to bind BAF. Might Src-regulated tyrosines also influence this important partner? We incubated recombinant wild-type or FFF-mutant His-tagged emerin residues 1-176 with HeLa cell lysates, pelleted nickel-bead-associated proteins and western blotted for the His-tag and endogenous BAF (Fig. 5H). The FFF mutant bound 70% less BAF in this assay (Fig. 5I). We concluded that one or more hydroxyl group(s) at Y59, Y74 and/or Y95 are required to bind BAF in vitro. This finding suggested, but did not prove, that emerin binding to BAF might be inhibited by phosphorylation of Y59, Y74 and/or Y95 in the context of full-length emerin.

To determine whether phosphorylation at Src-targeted sites influenced binding to BAF in cells, HeLa cells that transiently expressed GFP or GFP-emerin for 24 hours were treated for 30 minutes with either PV or PBS, then immunoprecipitated using GFP antibodies. Recall that SFKs are major, but probably not the only, contributors to emerin tyrosine phosphorylation in PV-treated cells (see Fig. 2B). Pellets were resolved by SDS-PAGE and western blotted for GFP, BAF or Tyr-P (Fig. 5J). BAF did not co-immunoprecipitate with GFP alone (Fig. 5J, lane 1), but did associate with GFP-emerin in both control and PV-treated lysates (Fig. 5J, lanes 2 and 3), as expected. Interestingly in PV-treated cells, the binding of wild-type GFP-emerin to endogenous BAF was reduced (Fig. 5J, lane 3) by 70% (Fig. 5K) (n=3; P=0.001). This result supports the hypothesis that tyrosine phosphorylation of emerin regulates at least one physiological function – binding to BAF – in cells.

Discussion

These findings suggest that emerin is regulated by at least three tyrosine kinases (Her2, Src, Abl). Our demonstration that emerin is tyrosine phosphorylated in Her2-overexpressing cells independently validates its identification as a major target of Her2 signaling (Bose et al., 2006). We discovered that Src, a potential downstream effector of Her2, directly and selectively phosphorylates at least three specific Tyr residues in human emerin: Y59, Y74 and Y95. All three residues as well as Y4, Y34, Y41, Y105 and Y155 are predicted Src sites (Prasad et al., 2008). Our results for the FFF mutant suggest that Y59, Y74 and Y95 comprise the majority of Src-regulated sites in emerin, both in vitro and in HeLa cells, but do not exclude other sites. Our results for Abl suggest, but do not prove, that Abl targets a distinct emerin residue, Y167, mutations in which had no significant effect on binding to BAF either in vitro or in cells. Interestingly Y-to-F substitutions at identified Src sites reduced recombinant emerin binding to endogenous BAF, revealing an unexpected contribution of tyrosine hydroxyl moieties in the ‘center’ of the emerin molecule. Further supporting functional regulation of emerin by tyrosine phosphorylation, BAF binding to emerin in HeLa cells was reduced significantly by PV treatment, which increases emerin tyrosine phosphorylation at sites that include Y59, Y74 and Y95 (the three identified Src sites). We also discovered that human LAP2β is directly phosphorylated in vitro by Src and Abl, but further work is needed to identify sites. These findings suggest two nonexclusive models for emerin: as a downstream effector of signaling by Her2, Src and/or Abl, and as a new signaling player that integrates information from different pathways, including those regulated by FGF7 and FGFR2IIIb (Luo et al., 2009), at the nuclear envelope. As discussed below, our findings predict that Her2 and Src signaling is disrupted in emerin-deficient heart and skeletal muscle cells.

LEM-domain tyrosines in emerin

Our analysis of LEM-domain tyrosines yielded unexpected insight: cells have the potential to regulate BAF binding to emerin uniquely, or concertedly with other LEM-domain proteins. The Y4F and Y41F substitutions in emerin residues 1-176 each reduced binding to BAF in vitro, but conferred either enhanced (Y4F) or normal (Y41F) binding to BAF when expressed in cells as full-length GFP-emerin. For Y4F, both results support the hypothesis that the Y4 hydroxyl group is important to bind BAF and that cells actively phosphorylate Y4 (via unidentified kinases) as a mechanism to release BAF. Kinase(s) that target emerin Y4 will be important to identify in the future. For Y41F, we speculate that the requirement for this hydroxyl moiety by BAF is somehow compensated in vivo, for example, by other partners or by additional residues present in full-length emerin. Since Y4 and Y41 are both unique to emerin and can be phosphorylated in vivo (Amanchy et al., 2005; Pan et al., 2008; Sui et al., 2008), we propose that they might be used to differentially regulate BAF binding to emerin, compared with other LEM-domain proteins. Conversely, phosphorylation of conserved LEM-domain tyrosines Y19 and Y34 has the potential to coordinately inhibit BAF binding to multiple LEM-domain proteins.

Tyrosines outside the LEM domain reveal extended involvement in binding to BAF

Our results directly implicate both central (e.g. residues 59-95) and membrane-proximal (e.g. Y161) regions of emerin in binding to BAF, which is consistent with previous evidence that BAF binding is inhibited by S175 phosphorylation (Hirano et al., 2005). We found

that loss of one hydroxyl group at Y161 (Y161F mutation) significantly reduced binding to BAF in all assays. We speculate that Y161 either contacts BAF directly or is required for the BAF-binding conformation of emerin. The hydroxyl group on nearby residue Y167, our identified Abl target site, is also frequently identified as a Tyr-*P* site in emerin (Fig. 1A), but was not required to bind BAF in cells. To account for these findings, we propose that BAF interacts concertedly with both the LEM domain and at least one other region of emerin in vivo.

Implications for heart, muscle, breast and cancer

Whether emerin is phosphorylated by Her2 directly remains unknown. Nevertheless our validation that emerin is regulated by Her2 suggests new mechanisms for EDMD disease. Mice in which Her2 function is specifically knocked out in cardiomyocytes are viable, but develop severe dilated cardiomyopathy with contractile defects that can lead to sudden death (Crone et al., 2002; Ozcelik et al., 2002). Might emerin be required for Her2-dependent signaling in cardiomyocytes? Mice that lack Her2 function in skeletal muscle experience poor motor coordination, altered gait and muscle wasting (Andrechek et al., 2002; Leu et al., 2003). In skeletal muscle, nuclei located under the neuromuscular junction (NMJ) are uniquely regulated by neuregulin-stimulated Her2 signaling, which controls genes encoding proteins (e.g. acetylcholine receptor) that are required for NMJ integrity (Leu et al., 2003; Lin et al., 2000; Ponomareva et al., 2006; Vock et al., 2008; Yang et al., 2005). Both mice and patients with *LMNA* mutations have defective recruitment or retention of nuclei at the NMJ and misregulation of NMJ-specific genes (Mejat et al., 2009). Nesprins, mutations in which also cause EDMD, are required to physically cluster nuclei at the NMJ in mice (Apel et al., 2000; Grady et al., 2005; Zhang, X. et al., 2007). Might loss of emerin perturb NMJ-specific gene regulation by Her2 in EDMD patients? Finally, amplification of Her2 is associated with ~25% of human breast tumors (Smith et al., 2006). Might susceptibility, or resistance, to Her2-overexpressing breast tumors be influenced by the emerin gene?

Our discovery that emerin is targeted by Src raises additional questions. In skeletal muscle, specific SFKs regulate myoblast proliferation or myofiber survival (Falcone et al., 2003; Laprise et al., 2002; Rosoff and Swope, 2002). In cardiomyocytes, SFKs regulate Ca^{2+}, Na^{+} and Cl^{-} conduction (Ahern et al., 2005; Browe and Baumgarten, 2003; Schroder et al., 2004) and attenuate ischemic injury (Stein et al., 2004). Might SFK signaling be disrupted in emerin-null EDMD patients? Activating mutations in Src cause many cancers (Frame, 2002); might emerin gene status influence susceptibility or resistance to such cancers?

'Disrupted signaling' hypothesis for EDMD disease

Our findings support an emerging disease model for X-linked EDMD: disrupted signaling. Through mechanisms that remain unknown, emerin can attenuate the activity of β-catenin and Lmo7, which transmit signals from the cell surface (Holaska et al., 2006; Markiewicz et al., 2006). Furthermore, genes regulated by the JNK, MAPK, NF-κB, integrin, Wnt or TGFβ signaling pathways are perturbed in emerin-null mouse hearts (Muchir et al., 2007).

Most EDMD patients with a mutated EMD gene lack detectable emerin protein (Morris and Manilal, 1999). However, in a few patients, the mutant emerin protein is expressed at normal or near-normal levels and localizes correctly at the nuclear envelope. These so-called 'special' disease-causing mutations include missense mutations (S54F, Q133H, P183T/H) and a five-residue deletion (Δ95-99) (Morris and Manilal, 1999). Biochemical studies of these mutations revealed normal binding to most partners, and no commonly affected partners were found that might explain why these alleles act as 'null' alleles. We now propose that these disease-causing mutations disrupt the post-translational regulation of emerin. For example Δ95-99 deletes two known phosphorylation sites: Y95 (a Src target) and Y99. We speculate that Δ95-99 might also disrupt phosphorylation of emerin Y94, which is detected 1-5 minutes after FGF7-activated FGFR2IIIb signaling in HEK293 cells (Luo et al., 2009). Cells from emerin Δ95-99 patients have altered levels of phosphorylation, determined by immunoprecipitating emerin from ^{32}P-labeled patient cells (Ellis et al., 1998). Interestingly, Src can also directly phosphorylate two emerin partners, splicing factor YT521B (Rafalska et al., 2004) and β-catenin (Lilien and Balsamo, 2005), suggesting that emerin might 'scaffold' Src targets. How emerin contributes to tyrosine kinase signaling or functions as a proposed signal integrator at the nuclear envelope are now important new questions for cell biology and human physiology.

Materials and Methods

Antibodies

Antibodies used in this work were rabbit anti-emerin serum 2999 (Lee et al., 2001), mouse NCL-emerin (Novacastra, Newcastle, UK), rabbit anti-GFP (cat. no. A6455, Invitrogen, Carlsbad, CA), rabbit anti-Src (36D10; cat. no. 2109, Cell Signaling Technology, Danvers, MA), rabbit anti-BAF serum 3273 (Haraguchi et al., 2001) and rabbit anti-actin (20-33; cat. no. A5060, Sigma), and five from Santa Cruz Biotechnology (Santa Cruz, CA): rabbit anti-emerin (FL-254; cat. no. sc-15378), mouse anti-GFP (B-2; sc-9996), mouse anti- Tyr-*P* (PY99; sc-7020), rabbit anti-His (H-15; sc-803) and anti-GST (B-14; sc-138).

Protein expression plasmids

Plasmids encoding the following polypeptides were described previously: pET23b human LAP2β residues 1-408 (Gant et al., 1999), pET11c human emerin residues 1-222 (Lee et al., 2001), and pEGFP-emerin (human, full-length) (Tsuchiya et al., 1999). pET29b-emerin 1-176 and pGEX-emerin 1-176 were generously provided by J. Ellis (Wheeler et al., 2007). Two plasmids were used to transiently express Src in mammalian cells: pUSE-amp Src (wild-type Src) and pUSE-amp Src K298M (kinase-dead Src), both from Upstate Biotechnology. (Charlottesville, VA). Control plasmid EGFPC1 was from Clontech (Mountain View, CA). To generate N-terminally GST-tagged emerin 1-222 (pGST-emerin 1-222), we inserted human emerin residues 1-222 into pGST-Parallel1 (Sheffield et al., 1999) using restriction sites *Bam*H1 and *Xho*I. Missense mutations were generated independently in pET29b-emerin 1-176 (for bacterial expression) and pEGFP-emerin (for mammalian expression) by QuikChange site-directed mutagenesis as per manufacturer's instructions (Stratagene, La Jolla, CA).

Recombinant proteins

Emerin residues 1-222 (in pET11c), C-terminally His-tagged emerin residues 1-176 (in pET29b), N-terminally GST-tagged emerin residues 1-176 (in pGEX 4T3), and C-terminally His-tagged LAP2β residues 1-408 (in pET23b) were purified from *E. coli* as described (Gant et al., 1999; Holaska et al., 2003; Wheeler et al., 2007). The pGST-emerin 1-222 construct was transformed into *E. coli* (BL21) cells and protein expression was induced at OD_{600} 0.5-0.8 using 40 μM IPTG for 4 hours. Bacteria were pelleted, resuspended in suspension buffer (PBS, 0.05% Tween, 2 mM EDTA, 0.1% BME, 1 mM PMSF), sonicated and centrifuged (30 minutes, 4°C, 20,000 ***g***). The insoluble pellet was resuspended in 8 M urea, diluted 1:10 in suspension buffer, incubated with glutathione agarose (Sigma) and washed three times before use as substrate for in vitro phosphorylation (described below). Purified recombinant wild-type Abl kinase domain (residues 229-511) (Seeliger et al., 2005) and corresponding mutant R367A (Muratore et al., 2009) were gifts from Philip Cole (Johns Hopkins School of Medicine, Baltimore, MD).

In vitro phosphorylation by purified kinases

Substrate proteins (emerin and LAP2β) were purified as described above. Emerin 1-222 or His-emerin 1-176 (in some experiments) was added directly to kinase reactions, whereas GST-emerin 1-222, His-emerin 1-176 (in other experiments) and His-LAP2β 1-408 were first conjugated to glutathione-Sepharose (GE Healthcare, Piscataway, NJ) or Ni-NTA agarose (Qiagen, Valencia, CA). Substrates were incubated for 10-60 minutes (30°C) as indicated in kinase reaction buffer [40 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 10% glycerol, 100 μM ATP, 2 mM DTT, protease inhibitor cocktail (Roche, Indianapolis, IN), plus either PhosSTOP (Roche) or 1 μM sodium orthovanadate] with either 16.7 μM Src (purified recombinant enzyme from

Invitrogen) or 30 nM purified recombinant Abl kinase domain (wild type or R367A mutant). Where indicated, the kinase reaction buffer also included 300 μCi/ml [γ-^{33}P]ATP (Perkin Elmer, Waltham, MA). Reaction products were resolved by SDS-PAGE (16% Tris-tricine or 4-12% Bis-Tris Nupage gels; Invitrogen), transferred to nitrocellulose (Schleicher and Schuell Bioscience, Keene, NH) and either immunoblotted with antibodies to Tyr-*P* (PY99; 1:1000-1:5000 dilution) or the His tag (1:5000-1:10,000 dilution), or phosphoimaged to detect incorporated ^{33}P. Western blots were visualized by horseradish peroxidase chemiluminescence (VersaDoc 5000; Bio-Rad, Hercules, CA) and quantified by densitometry using Quantity One Software (Bio-Rad).

Mass spectrometry identification of Src or Abl phosphorylation sites in emerin

GST-emerin 1-222 polypeptide bound to glutathione beads was incubated for 1 hour in vitro with or without purified Src. His-emerin 1-176 was incubated for 2 hours in vitro with or without purified Abl kinase domain, as described above. Src reactions were quenched with SDS-sample buffer and resolved by SDS-PAGE (4-12% Bis-Tris Nupage gel; Invitrogen). Proteins in gels were stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL), excised, and in-gel digested with trypsin (Promega, Madison, WI) as described (Shevchenko et al., 1996). Emerin proteins in Abl reactions were reduced and alkylated with iodacetamide and proteolyzed in solution with sequencing grade modified trypsin (Promega, Madison, WI). Peptides were identified by liquid chromatography/tandem mass spectrometry (LC-MS/MS) using an LTQ XL (Thermo Fisher Scientific, San Jose, CA) ion-trap MS equipped with an ETD (Electron Transfer Dissociation) fragmentation source. The peptides were desalted on a 75 μm × 2.5 cm YMC (Kyoto, Japan) AA12S11 5-15 μm C18 phase trap column at 5 μl/minute for 6 minutes in 1% acetonitrile and 0.1% formic acid and separated on a 75 μm × 100 mm YMC ODS-AQ C18 phase (5 μm, 120A) column in a 9-90% acetonitrile and 0.1% formic acid gradient over 18 minutes at 300 nl/minute using an Agilent 1100 (Palo Alto, CA) autosampler interfaced with a 2D nanoLC system (Eksigent, Dublin, CA). Eluting peptides were sprayed directly into the mass spectrometer and fragmented by collision-induced dissociation (CID) or electron transfer dissociation (ETD) in data-dependent mode with the MS1 scanning from 350-1800 m/z and selecting the top eight most abundant ions for MS2 fragmentation. In CID experiments, multistage activation was used to target the neutral loss from phosphorylated peptides losing 49 for the +2 charged peptides and 32.67 for the +3 charge state. For ETD experiments, peptides were reacted with fluoranthene for 125 milliseconds to dissociate peptides and all precursors and fragment ions were run in enhanced resolution mode owing to the complexity of ETD spectra. Peptide sequences were identified using Mascot (Matrix Science, Boston, MA) software to search the NCBI non-redundant database with the acquired fragmentation data. Peptide sequences with ion scores >95% confidence were considered significant. For the Abl experiment, Scaffold (Proteome Software, Portland, OR) was used to remove redundancies and validate confidence scores. Identified sequences and phosphosites were confirmed by manual inspection of fragmentation spectra.

Cell culture, inhibitors, transfections and microscopy

HeLa cells were cultured in Dulbecco's modified eagle's medium containing 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. NIH-3T3 stable cell lines expressing empty vector (Neo76 cells) or Her2 (8-13 cells) were obtained from Philip Cole (Johns Hopkins School of Medicine) and cultured as described (Bose et al., 2006). For pervanadate (PV) experiments, we added 1 μM PV or an equal volume of PBS for 30 minutes before lysing cells. Pervanadate was prepared fresh by mixing equimolar solutions of sodium orthovanadate (Sigma) and hydrogen peroxide (in PBS). For Src inhibitor experiments, cells were incubated for 30 minutes with either Src inhibitor PP2 (10 μM in DMSO; Biomol, Plymouth Meeting, PA), inactive analog PP3 (10 μM in DMSO; Calbiochem, Gibbstown, NJ), Src inhibitor SU6656 (1 nM in DMSO; Sigma-Aldrich, St Louis, MS) or solvent (DMSO) alone before PV treatment.

HeLa cells were transiently transfected using LT1 (Mirus, Madison, WI) per manufacturer protocol. Cells in 25, 10 or 3.5 cm plates were transfected with 10, 5 or 1 μg (respectively) of each plasmid plus 50, 25 or 5 μl (respectively) of LT1 in Opti-Mem (Invitrogen). For lysis, cells were harvested by scraping 24 hours after transfection (see below). For microscopy, cells were grown on coverslips in six-well plates, fixed for 15 minutes (3.7% formaldehyde), permeabilized for 20 minutes (PBS/0.2% Triton X-100), incubated briefly with 25 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) to visualize DNA and mounted using Vectashield (Vector Laboratories, Burlingame, CA). GFP fluorescence was directly visualized using an Eclipse E600 microscope (Nikon, Melville, NY).

Immunoprecipitation from cell lysates

Cells were lysed by scraping into 50 mM Tris-HCl pH 7.4, 0.3 M NaCl, 0.3% Triton X-100, 2 mM EDTA, 2 mM DTT, 1 μM sodium orthovanadate and protease inhibitor cocktail (Roche, Indianapolis, IN). Lysates were incubated for 4 hours (4°C) with each indicated antibody (0.25 μl GFP antibody A6455, 5 μl serum 2999 against emerin or 5 μl NCL-emerin) plus either 25 μl GammaBind G, protein-A-Sepharose or protein-G-Sepharose (all from Amersham Biosciences, Piscataway, NJ). Bound proteins were pelleted, washed three times in lysis buffer, resuspended in SDS sample buffer, resolved by SDS-PAGE using either 16% Tris-tricine gels or 4-12% Bis-Tris Nupage gels (Invitrogen), transferred to nitrocellulose (Schleicher and Schuell Bioscience), and western blotted using primary antibodies against GFP (sc-9996; 1:2000-1:5000 dilution), emerin (NCL-emerin at 1:5000 dilution or sc-15378 at 1:5000 dilution), BAF (serum 3273 at 1:1000 dilution), or Tyr-*P* (1:1000-1:5000 dilution). Western blots were visualized by horseradish peroxidase chemiluminescence on a VersaDoc 5000 (Bio-Rad) and quantified by densitometry using Quantity One Software (Bio-Rad).

Recombinant protein pull-downs

GST, GST-emerin 1-176 or His-emerin 1-176 proteins (10 μg) were each incubated 4 hours (4°C) with glutathione agarose (Sigma) or Ni^{2+} agarose (Qiagen), respectively, plus lysate from ~5×10^6 HeLa cells lysed in 1 ml lysis buffer [50 mM Tris-HCl pH 7.4, 0.3 M NaCl, 0.3% Triton X-100, 2 mM EDTA, 2 mM DTT, protease inhibitor cocktail (Roche, Indianapolis, IN)]. Pellets were washed thee times in lysis buffer, resuspended (SDS sample buffer), resolved by SDS-PAGE (16% Tris-tricine gels), transferred to nitrocellulose and western blotted using primary antibodies against GST (1:10,000 dilution), His (1:5000 dilution) and BAF (1:1000 dilution).

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