

# Matrix assembly, regulation, and survival functions of laminin and its receptors in embryonic stem cell differentiation

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Laminin-1 is essential for early embryonic basement membrane assembly and differentiation. Several steps can be distinguished, i.e., the expression of laminin and companion matrix components, their accumulation on the cell surface and assembly into basement membrane between endoderm and inner cell mass, and the ensuing differentiation of epiblast. In this study, we used differentiating embryoid bodies derived from mouse embryonic stem cells null for  $\gamma$ 1-laminin,  $\beta$ 1-integrin and  $\alpha$ / $\beta$ -dystroglycan to dissect the contributions of laminin domains and interacting receptors to this process. We found that (a) laminin enables  $\beta$ 1-integrin-null embryoid bodies to assemble basement

membrane and achieve epiblast with  $\beta$ 1-integrin enabling expression of the laminin  $\alpha$ 1 subunit; (b) basement membrane assembly and differentiation require laminin polymerization in conjunction with cell anchorage, the latter critically dependent upon a heparin-binding locus within LG module-4; (c) dystroglycan is not uniquely required for basement membrane assembly or initial differentiation; (d) dystroglycan and integrin cooperate to sustain survival of the epiblast and regulate laminin expression; and (e) laminin, acting via  $\beta$ 1-integrin through LG1–3 and requiring polymerization, can regulate dystroglycan expression.

## Introduction

Basement membranes are extracellular matrices (ECMs)\* that affect the survival and differentiation of adherent cells. Regulation of their assembly plays a crucial role during development, and laminins are essential for this process (Colognato and Yurchenco, 2000). Initiation of basement membrane in a tissue requires synthesis of  $\alpha$ ,  $\beta$ , and  $\gamma$  laminin subunits and heterotrimer formation (Yurchenco et al., 1997). Once secreted, laminin engages the cell surface through several receptors, notably cognate integrins,  $\alpha$ / $\beta$ -dystroglycan, and

syndecans (Oh et al., 1997; Edwards et al., 1998; James et al., 2000), and self-assembles into a matrix polymer (Yurchenco, 1994). Although  $\beta$ 1-integrin and dystroglycan have each been proposed to be key mediators of basement membrane formation (Henry and Campbell, 1998; Klass et al., 2000; Lohikangas et al., 2001), several exceptions suggest that such mediation is not tightly coupled to assembly (Cote et al., 1999; Feltri et al., 2002) and may be indirect. One possibility is that these receptors regulate the synthesis or turnover of basement membrane components rather than the assembly process itself. To distinguish these contributions and to dissect functions of laminin, its domains, and interacting receptors, we undertook an analysis of cellular differentiation in embryoid bodies (EBs) derived from mouse embryonic stem (ES) cells that recapitulate crucial events of basement membrane formation during early gastrulation.

The first basement membranes to form during mouse embryonic development are those located between visceral

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\*Abbreviations used in this paper: AEBSF, aminoethyl benzene sulfonyl fluoride; EB, embryoid body; ECM, extracellular matrix; ES, embryonic stem; ICM, inner cell mass; NFL, neurofilament; RT, reverse transcription.

Key words: basement membrane; gastrulation; integrin; dystroglycan; apoptosis

**Table I. Basement membrane formation and epiblast differentiation in wild-type, dystroglycan-null,  $\gamma$ 1-laminin-null, and  $\beta$ 1-integrin-null embryoid bodies**

EBs	Treatment	Basement membrane formation	Epiblast differentiation	Number counted
		%		
<b>Nulls</b>				
Wild-type		72 $\pm$ 9	64 $\pm$ 7	145
DG <sup>-/-</sup>		75 $\pm$ 4	51 $\pm$ 6	191
$\gamma$ 1-Lm <sup>-/-</sup>		0	0	253
$\gamma$ 1-Lm <sup>-/-</sup>	Laminin-1	57 $\pm$ 9	34 $\pm$ 5 <sup>a</sup>	150
$\beta$ 1-integrin <sup>-/-</sup>		0	0	173
$\beta$ 1-integrin <sup>-/-</sup>	Laminin-1	46 $\pm$ 2	18 $\pm$ 2 <sup>a</sup>	174
<b>Isoforms</b>				
$\gamma$ 1-Lm <sup>-/-</sup>	Laminin-1 ( $\alpha$ 1 $\beta$ 1 $\gamma$ 1)	59	36	150
$\gamma$ 1-Lm <sup>-/-</sup>	Laminin-2/4 ( $\alpha$ 2 $\beta$ 1 $\gamma$ 1/ $\alpha$ 2 $\beta$ 2 $\gamma$ 1)	37	9	35
$\gamma$ 1-Lm <sup>-/-</sup>	Laminin-4 ( $\alpha$ 2 $\beta$ 2 $\gamma$ 1)	40	20	40
$\gamma$ 1-Lm <sup>-/-</sup>	Laminin-5 ( $\alpha$ 3A $\beta$ 3 $\gamma$ 2)	0	0	44
$\gamma$ 1-Lm <sup>-/-</sup>	Laminin-8 ( $\alpha$ 4 $\beta$ 1 $\gamma$ 1)	0	0	42
<b>Modified laminin-1 and fragments</b>				
$\gamma$ 1-Lm <sup>-/-</sup>	AEBSF-laminin-1	0	0	39
$\gamma$ 1-Lm <sup>-/-</sup>	Laminin-1 + E1'	0	0	39
$\gamma$ 1-Lm <sup>-/-</sup>	Laminin-1 + AEBSF-E1'	53	11	66
$\gamma$ 1-Lm <sup>-/-</sup>	Laminin-1 + E4	2	0	43
$\gamma$ 1-Lm <sup>-/-</sup>	Laminin-1 + E3	0	0	58
$\gamma$ 1-Lm <sup>-/-</sup>	Laminin-1 + E8	39	16	44

EB, embryoid body.

<sup>a</sup>*P* < 0.001 versus wild type. Data were obtained from four to six separate experiments for each group and expressed as mean  $\pm$  SE.

endoderm and developing epiblast, and underneath the parietal endoderm (Reichert's membrane), which extends over the trophectoderm (Leivo et al., 1980). Although primitive endodermal cell differentiation precedes basement membrane assembly, epiblast differentiation and proamniotic cavitation require and follow it (Murray and Edgar, 2000; Murray and Edgar, 2001a,b). In this study, we examined wild-type,  $\gamma$ 1-laminin-null,  $\beta$ 1-integrin-null, and dystroglycan-null differentiating EBs. We report that the integrin- and laminin-deficient cells are unable to form basement membranes or undergo epiblast differentiation and cavitation because, in both states, they fail to express heterotrimeric laminin. Exogenous laminin bypasses the defect in each null embryoid body, restoring basement membrane along with epiblast differentiation and cavitation. This activity requires participation of long arm laminin LG modules that include a critical heparin-binding sequence as well as polymerization mediated by the three short arms. Strikingly, neither integrin nor dystroglycan is uniquely required for basement membrane assembly. Instead, they are necessary for regulation of their own expression, that of major basement membrane components, and cell differentiation. Finally, dystroglycan and integrin promote epiblast survival.

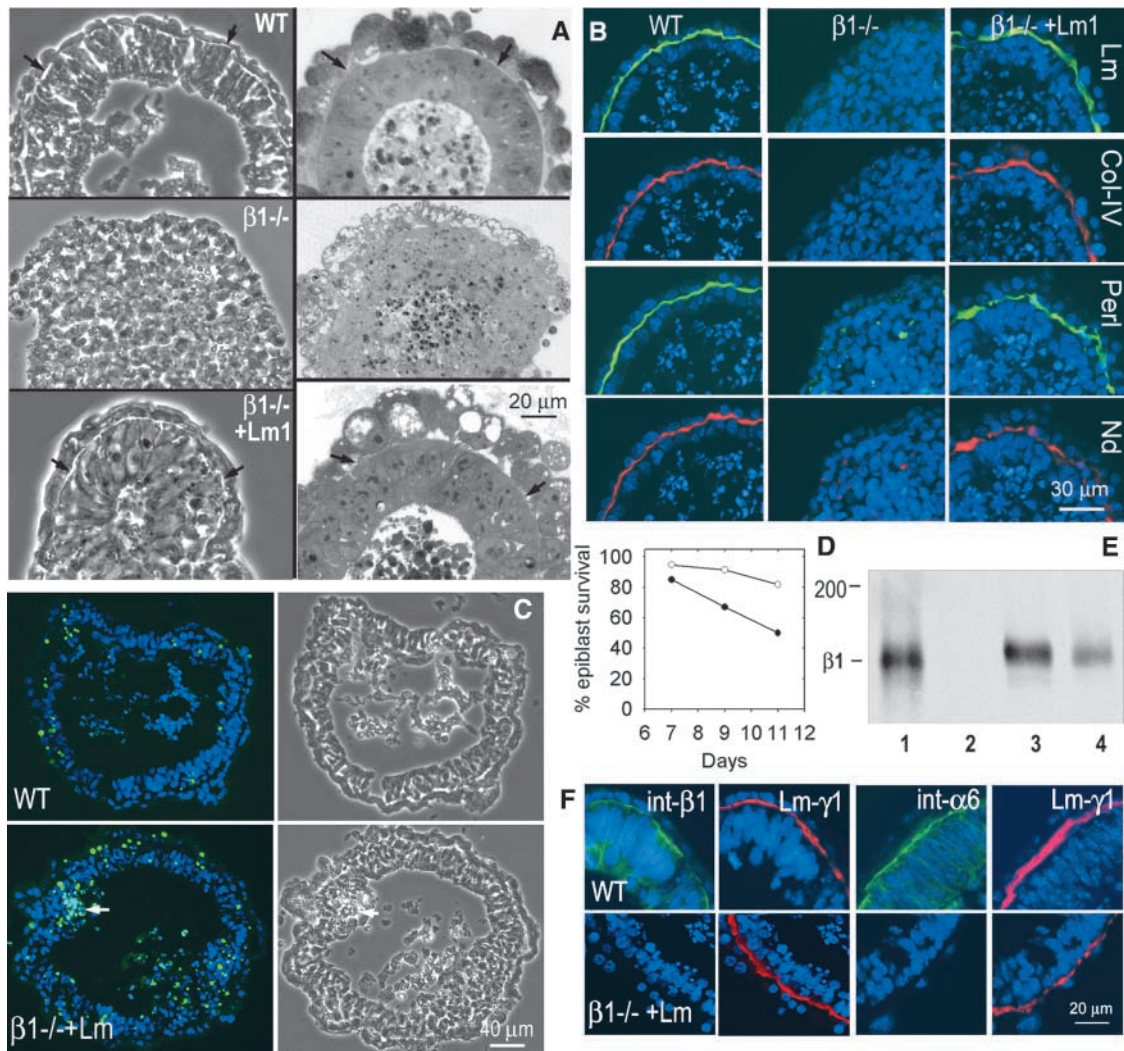
## Results

### Laminin-1 rescue of basement membrane and differentiation in $\beta$ 1-integrin-null EBs

It has previously been shown that  $\beta$ 1-integrin-null (-/-) EBs fail to develop basement membranes and that  $\alpha$ 1-laminin chain expression is decreased (Aumailley et al., 2000). It has also been proposed that  $\beta$ 1-integrins are required as receptors for basement membrane assembly (Raghavan et al., 2000; Lohikangas et al., 2001). However, given the  $\alpha$ 1-

laminin subunit is necessary to assemble a heterotrimeric laminin (Yurchenco et al., 1997), we considered the possibility that the failure to form a basement membrane and differentiate is due to the absence of laminin  $\alpha$ 1-chain expression rather than the receptor-mediated cell surface assembly process itself. To test this hypothesis, we compared the behavior of  $\beta$ 1-integrin-null EBs incubated in the presence of exogenous laminin-1 with untreated null and wild-type EBs (Fig. 1 and Table I).

When dispersed wild-type embryonic stem cells are suspended in LIF-free medium for several days, they form EBs that (a) develop an outer endodermal layer, (b) form a sub-endodermal basement membrane, and (c) differentiate to form epiblast and a central proamniotic-like cavity (Couchanis and Martin, 1995; Murray and Edgar, 2000). These progressions were observed in wild-type controls with endoderm appearing at 3–4 d, basement membrane at 4–5 d, and epiblast at 5–7 d. Although integrin  $\beta$ 1-integrin-null EBs developed morphological features of endodermal differentiation (a distinct outer layer with flattened cells and/or cells containing vacuoles), neither basement membrane, epiblast, nor central cavity formed. However, when  $\beta$ 1-integrin-null ES cells were incubated with 25  $\mu$ g/ml laminin-1, nearly half of the EBs underwent striking morphological and immuno-histochemical changes (Fig. 1). By phase-contrast microscopy, these EBs possessed a distinctive second cell layer consisting of polarized cells in a pseudo-stratified columnar arrangement and with the innermost aspect facing a sharply demarcated central cavity (Fig. 1 A). A thin bright line corresponding to an ECM circumscribed the outermost edge of this layer. By immunofluorescence microscopy, the EBs exhibited colocalization of laminin ( $\gamma$ 1 epitope), type IV collagen, nidogen, and perlecan in a linear and generally continuous pattern located between endoderm and epiblast



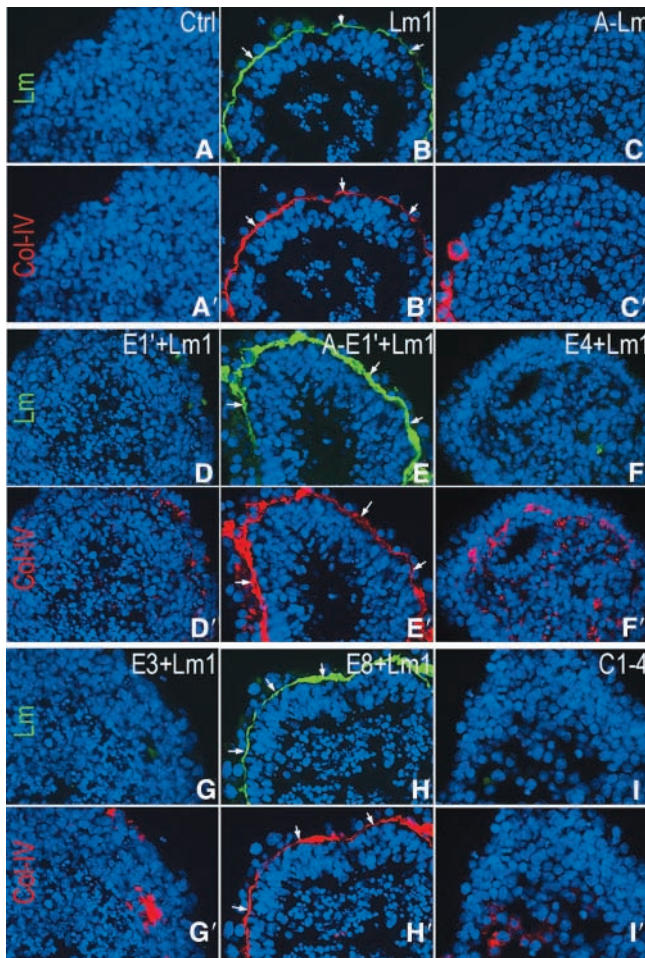
**Figure 1. Laminin induction of basement membrane and epiblast in  $\beta 1$ -integrin-null EBs.** Wild-type and  $\beta 1$ -integrin-null ES cells were grown in suspension for 7 d, the latter maintained alone or in the presence of laminin-1 (25  $\mu\text{g}/\text{ml}$ ). (A) Phase micrographs (left) and methylene blue-stained sections (right) of wild-type (top), untreated (middle) and laminin-1-treated (bottom)  $\beta 1$ -integrin-null EBs. (B) Immunofluorescence micrographs of consecutive sections of the above EBs stained with DAPI (nuclei, blue) and antibody to laminin- $\gamma 1$  (Lm), type IV collagen (Col-IV), perlecan (Perl) and nidogen (Nd). (C and D)  $\beta 1$ -integrin-null EBs were treated with 25  $\mu\text{g}/\text{ml}$  laminin-1 and cultured for 7–11 d. TUNEL (green) and DAPI (blue) costaining revealed developing segmental (arrow) apoptosis (plot was calculated after subtracting EBs with full-thickness segmental apoptosis from the total). (E) Immunoblot detection of the  $\beta 1$ -integrin subunit in wild-type (lane 1),  $\beta 1$ -integrin-null (lane 2), dystroglycan-null (lane 3), and  $\gamma 1$ -laminin-null (lane 4) EBs. (F) Immunofluorescence micrographs showing  $\beta 1$ -integrin (first frame),  $\alpha 6$ -integrin (third frame), and  $\gamma 1$ -laminin (second and fourth frames) of wild-type (top) and laminin-treated  $\beta 1$ -integrin-null EBs (bottom).

(Fig. 1 B). We examined the survivability of the laminin-induced EBs and found, using TUNEL staining, that an increase in segmental cell layer apoptosis occurred selectively in the epiblast layer (Fig. 1, C and D); however, the layer remained intact even after 11 d.

Wild-type and  $\beta 1$ -integrin-null EBs treated with exogenous laminin-1 were examined for  $\beta 1$ - and  $\alpha 6$ -integrin expression (Fig. 1, E and F). These integrin chains were localized in differentiated wild-type EBs in the epiblast and in the basement membrane zone. In contrast, the integrin-null EBs, regardless of treatment, failed to express and accumulate  $\alpha 6$ -integrin.  $\beta 1$ -integrin levels in dystroglycan-null EBs were similar to those detected by immunoblotting in the wild-type state (and were absent in the integrin-null state), suggesting that integrin does not compensate for loss of dystroglycan by up-regulation.

### Contributions of laminin polymerization and LG modules

Incubation of  $\gamma 1$ -laminin-null EBs with exogenous laminin-1 results in basement membrane formation, epiblast differentiation, and cavitation (Murray and Edgar, 2000), providing a basis to analyze laminin domain contributions. We evaluated (a) the three short arms that mediate laminin polymerization as well as  $\alpha 1\beta 1$ ,  $\alpha 2\beta 1$  integrin, and heparin binding and (b) G-domain that mediates  $\alpha 6\beta 1$ ,  $\alpha 7\beta 1$  integrin-, heparin/heparan sulfate-, and  $\alpha$ -dystroglycan binding (Colognato and Yurchenco, 2000). To assess polymerization and discriminate it from other short arm functions, we determined the ability of nonpolymerizing laminin-1, prepared by treatment with the selective polymer-inactivating agent aminoethyl benzene sulfonyl fluoride (AEBSF; Colognato et al., 1999), to assemble a basement membrane in  $\gamma 1$ -laminin-null EBs (Fig. 2 and Table I) and evaluated the ac-



**Figure 2. Laminin domains contributing to basement membrane assembly.**  $\gamma$ 1-Laminin-null EBs were cultured for 7 d without laminin as a control (Ctrl), or with either laminin-1 (Lm1, 25  $\mu$ g/ml), non-polymerizing laminin-1 (A-Lm1, AEBSF-treated, 25  $\mu$ g/ml), or fragment C1-4 (25  $\mu$ g/ml) as shown. In addition, EBs were treated with laminin-1 mixed with an  $\sim$ 50-fold molar excess of polymer-inhibiting fragment E1', noninhibiting AEBSF-E1' control (A-E1'), polymer-inhibiting fragment E4,  $\alpha$ 6 $\beta$ 1-integrin-binding fragment E8, or dystroglycan/heparin/sulfatide-binding fragment E3 (fragment map shown in Fig. 10).

tivity of laminin-1 maintained in the presence of fragments that selectively inhibit polymerization through either the  $\alpha$ 1/ $\gamma$ 1 (E1', AEBSF-treated E1' as noninhibiting control) or the  $\beta$ 1 (E4) short arms. Neither a basement membrane immunostaining pattern of laminin and type IV collagen nor epiblast formation was detected after inhibition of laminin polymerization, regardless of reagent used. This inhibition was considered specific because the only activity of E4 is polymerization inhibition, and the cell adhesion, heparin-binding, and nidogen-binding properties of laminin and E1' are not found affected by AEBSF treatment (Colognato et al., 1999; unpublished data).

To analyze laminin long arm contributions, we determined the ability of polymerizing-laminin that lacks the coiled-coil and G-domains (fragment C1-4) to induce matrix assembly, and evaluated the potential of fragments E3 (containing LG module 4, a ligand for heparin, sulfatide, and dystroglycan) and E8 (LG modules 1-3, ligand for  $\alpha$ 6

and  $\alpha$ 7 integrins) to inhibit laminin-1 induction of basement membrane. C1-4 treatment of  $\gamma$ 1-laminin-null EBs for 7 d did not result in the formation of a basement membrane or in epiblast differentiation. When fragment E3 was incubated in molar excess with intact laminin-1, neither basement membrane nor epiblast differentiation was detected (Fig. 2, rows 5 and 6). In contrast, fragment E8, when similarly incubated with laminin-1, did not prevent laminin rescue of assembly. We concluded that one or two of the LG modules contained within E3 (LG4-5) were required for basement membrane.

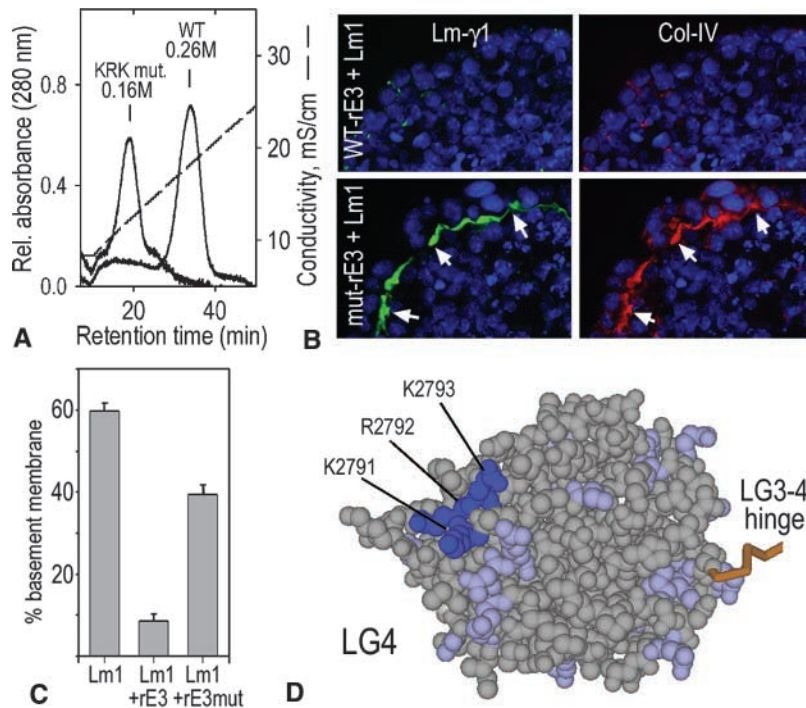
After treatment with either laminin-4 or laminin-2/4, laminin, type IV collagen, nidogen, and perlecan were detected in a subendodermal linear pattern accompanied by epiblast differentiation and cavitation (Table I). In contrast, laminin-8 ( $\alpha$ 4 $\beta$ 1 $\gamma$ 1) failed to induce basement membrane-type immunostaining, epiblast differentiation, or central cavitation. Laminin-5 ( $\alpha$ 3 $\beta$ 3 $\gamma$ 2) adhered to the outer surface of the EBs (image not depicted); however, laminin-5,  $\gamma$ 1-laminin,  $\gamma$ 3-laminin, and nidogen did not accumulate within the basement membrane zone. Although a weaker and discontinuous linear subendodermal pattern of type IV collagen and perlecan was noted, no ECM was detected by electron microscopy. Thus, of the laminins tested, only  $\alpha$ 1- and  $\alpha$ 2-laminins, both polymerizing laminins, were capable of inducing basement membrane.

#### Site of activity in an LG module

To determine whether heparin/dystroglycan binding within LG module 4 is required for basement membrane, we inactivated the KRK (residues 2791-2793) sequence common to both by alanine substitution (Andac et al., 1999) in recombinant LG4-5 and determined its ability to block laminin-1 rescue of Lm- $\gamma$ 1-null EBs (Fig. 3). The mutant LG4-5 showed substantially reduced binding by heparin affinity chromatography (Fig. 3 A), eluting at 0.16 M NaCl compared with 0.26 M for wild-type protein. In contrast to its recombinant control, the KRK mutant protein was largely unable to block laminin rescue of the Lm- $\gamma$ 1-null phenotype (Fig. 3, B and C). In keeping with this result, 0.1 mg/ml heparin completely prevented laminin-1 induction of basement membrane. We concluded that this particular surface-exposed triplet basic sequence (Fig. 3 D) plays a critical role in basement membrane assembly, likely contributing to anchorage.

#### Mesodermal differentiation

Because laminin treatment of  $\beta$ 1-integrin and  $\gamma$ 1-laminin-null EBs enabled differentiation of epiblast morphology, we asked whether the "rescue" also initiated mesodermal differentiation. To address this, we examined the transcriptional expression of low molecular weight neurofilament (NFL; expressed in ectodermal derivatives), BMP-4 (ES cells and mesoderm), brachyury (mesodermal T transcriptional factor), and  $\zeta$ -globulin (mesoderm) by semi-quantitative reverse transcription (RT)-PCR (Fig. 4). All markers were detected in wild-type EBs. By 7 d of culturing, NFL and BMP-4 were detected in both  $\beta$ 1-integrin-null and laminin- $\gamma$ 1-null EBs if treated with laminin-1, but not if untreated. Brachyury and  $\zeta$ -globulin were detected in  $\gamma$ 1-



**Figure 3. Heparin-binding site in LG4 is required by laminin mediation of basement membrane assembly in laminin  $\gamma$ 1-null EBs.** (A) Elution of recombinant wild-type and KRK $\rightarrow$ AAA mutant recombinant LG4-5 from a heparin 5PW column with a NaCl gradient. (B) Immunofluorescence (laminin  $\gamma$ 1, type IV collagen) micrographs of  $\gamma$ 1-laminin-null EBs treated with either laminin-1 in the presence of a 20-fold molar excess of wild-type or mutated LG4-5. (C) Quantitation of degree of basement membrane formation. (D) Space-filling model of Lm- $\alpha$ 1 LG4 KRK sequence (blue) superimposed upon the crystal structure of  $\alpha$ 2-LG4 determined from coordinates submitted to the Brookhaven protein database.

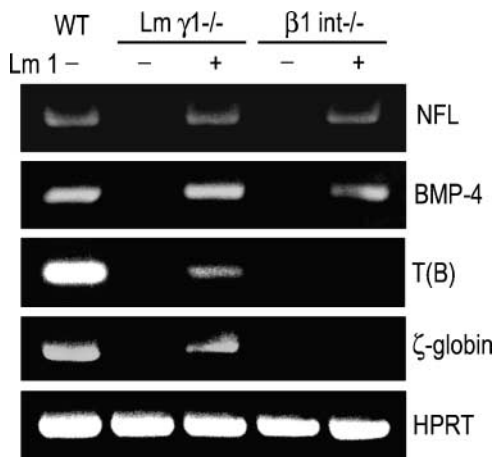
laminin-null EBs treated with laminin, but not  $\beta$ 1-integrin-null EBs treated with laminin. By week 2 of culturing, brachyury was detected in the  $\beta$ 1-integrin-null EBs treated with laminin as well (unpublished data). These data reflect a delay of mesodermal differentiation in the integrin-deficient EBs.

#### Contribution of dystroglycan to basement membrane assembly and epiblast survival

Given that a site in LG4 mediating both heparin- and dystroglycan binding is essential for basement membrane assembly, we asked whether dystroglycan was responsible for

this interaction. We found that by 5 d of culturing, most dystroglycan-null EBs possessed linear subendodermal colocalized distributions of laminin, type IV collagen, nidogen, and perlecan (Fig. 5 and Table I), i.e., characteristics of a basement membrane later confirmed by electron microscopy. The spontaneous formation of these ECMs (i.e., without exogenous laminin) was accompanied by epiblast differentiation and cavitation. These results were consistent with the mouse knockout phenotype in which epiblast-associated basement membranes were detected (Williamson et al., 1997), and the data suggest that a heparan sulfate proteoglycan such as a member of the syndecan family (and/or possibly sulfatide), rather than dystroglycan, mediates an essential laminin-LG-4 interaction.

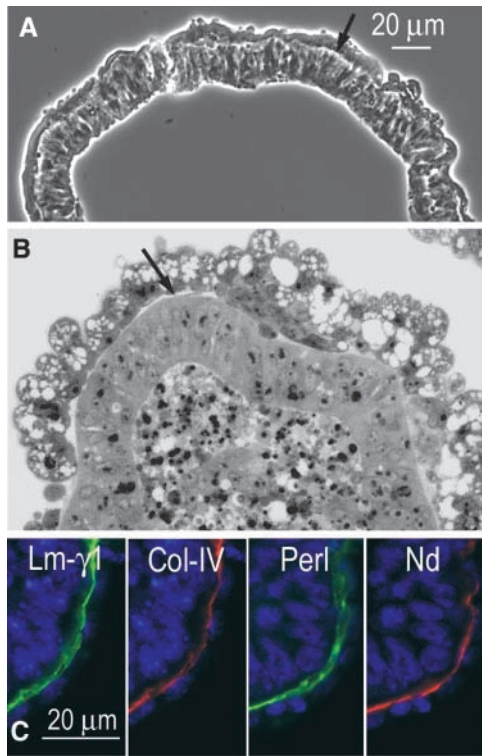
The epiblast cells that developed in dystroglycan-null EBs were clearly polarized but tended to be shorter in length. By 7 d, many EBs were noted to possess unusually thick basement membranes by light microscopy (Fig. 6). By 9 d, the epiblast layer was found to have partially or completely degenerated in most EBs, leaving behind EBs consisting only of an outer endoderm layer resting on an otherwise acellular basement membrane with a hollow cavity. A progression of epiblast loss could be followed from 7 to 9 d. This ectodermal degeneration, in which remaining cells had smaller and denser nuclei, suggested that the epiblast cells were undergoing apoptosis. TUNEL staining and coincident nuclear DAPI staining confirmed the development of apoptosis in dystroglycan-null EBs by 7 d, culminating in the loss of the layer in most EBs by 9 d. The increase in TUNEL staining could be seen in association with both thin and thick basement membranes.



**Figure 4. Expression of differentiation markers.**  $\beta$ 1-integrin-null ( $\beta$ 1 int $^{-/-}$ ) and  $\gamma$ 1-laminin-null (Lm  $\gamma$ 1 $^{-/-}$ ) ES cells untreated or treated with laminin-1 (Lm1, 25  $\mu$ g/ml) and wild-type ES cells were cultured in suspension for 7 d. Total RNA was isolated and subjected to semi-quantitative RT-PCR analysis for epiblast (low molecular weight NFL) and mesoderm (brachyury, T(B), and  $\zeta$ -globin) marker expression. HPRT was used as normalizing control.

#### Basement membrane zone ultrastructure

Thin sections of wild-type,  $\beta$ 1-integrin-null,  $\gamma$ 1-laminin-null, and dystroglycan-null EBs were examined by electron



**Figure 5. Basement membranes assemble in dystroglycan-null EBs.** Dystroglycan-null ES cells, suspended at the first passage from feeder cell layers, were allowed to form EBs for 5 d in the absence of any treatment. (A) Phase micrograph and (B) methylene blue-stained section show epiblast differentiation, cavitation, and thin basement membrane-like structures (arrows). (C) EBs, visualized by immunofluorescence, reveal a subendodermal basement membrane pattern costaining with antibodies for  $\gamma$ 1-laminin, type IV collagen, perlecan, and nidogen.

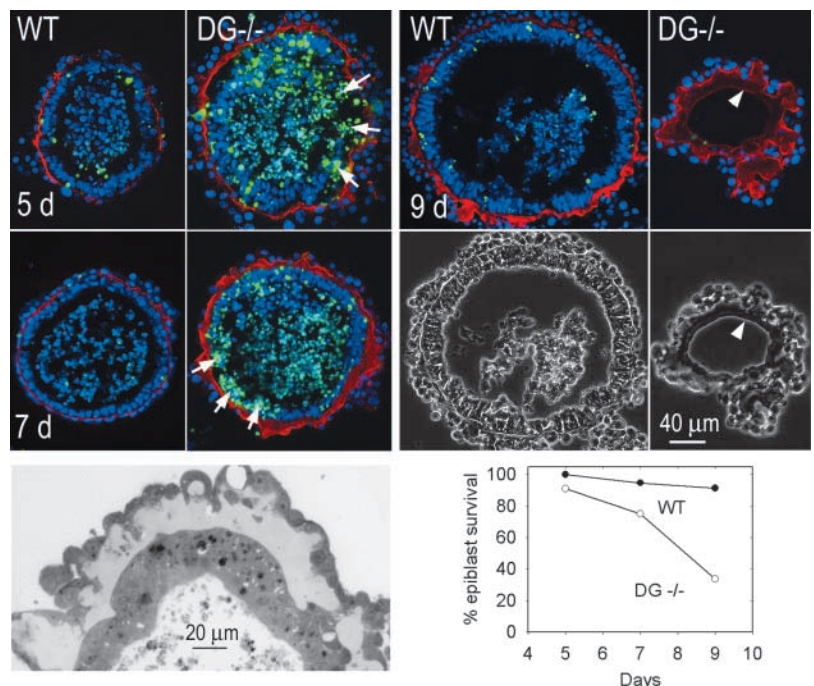
microscopy (Fig. 7). The overlying endoderm in all four types of EBs possessed an increase in prominence of RER and mitochondria compared with adjacent inner cell mass

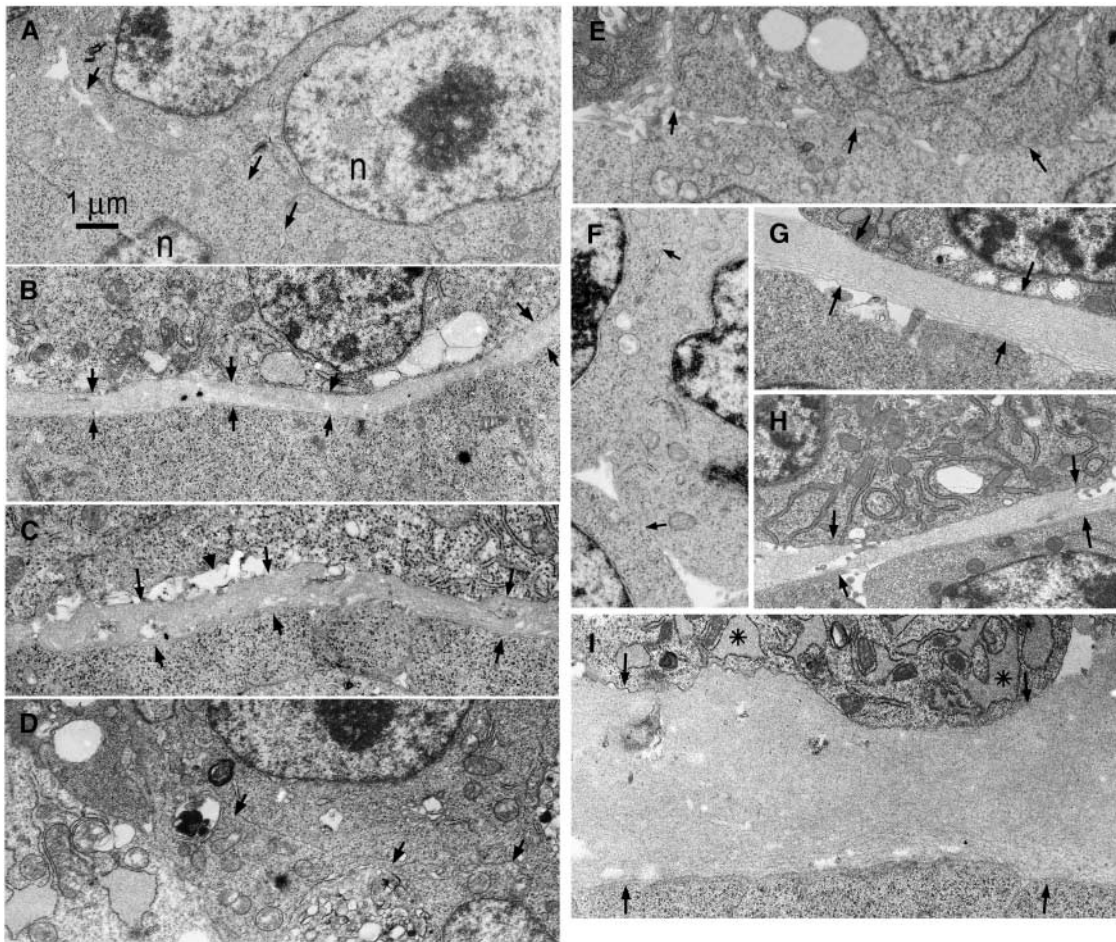
(ICM) or epiblast. Wild-type basement membranes located between endoderm and epiblast layer ranged in thickness from  $\sim 0.25$  to  $\sim 1.5$   $\mu$ m (Fig. 7 B) and often had a layered appearance, particularly in cell-adjacent zones. Untreated  $\beta$ 1-integrin-null and  $\gamma$ 1-laminin-null EBs lacked a basement membrane, even by 9 d of culturing (Fig. 7, A and D). The endoderm layer instead showed direct contact with underlying cells, occasionally interrupted by a small, apparently empty space. In contrast, a distinct basement membrane was observed after treatment of  $\beta$ 1-integrin-null (Fig. 7 C) or  $\gamma$ 1-laminin-null EBs (Fig. 7 G) with exogenous laminin. The morphology was similar to that observed in the wild-type state. The epiblast layer contained adjacent elongated cells with a cytoplasm containing minimal RER and a finely granular cytoplasm. Junctional complexes were noted between cells (images not shown). The epiblast features were the same as observed in differentiated wild-type EBs. Dystroglycan-null EBs also developed prominent basement membranes ( $>10$   $\mu$ m; Fig. 7, H and I) that substantially separated endodermal layers from epiblast layers. The fraction of the thick basement membranes was noted to increase with culturing extending out to 7 d. The RER of the dystroglycan-null EBs was noted to be more prominent than those of wild-type EBs, containing more RER cisternae that were especially dilated in dystroglycan-null EBs with thick basement membranes.

$\gamma$ 1-Laminin-null EBs treated with laminin-5 (Fig. 7 E), nonpolymerizing laminin-1 (Fig. 7 F), or laminin-1 plus E1' did not contain a recognizable basement membrane at the ultrastructural level. The endoderm was located either in close apposition to the ICM or separated by very narrow and largely empty clefts. Scattered amorphous deposits, possibly corresponding to ECM protein, were sometimes detected within the clefts. Thus, although type IV collagen immunostaining was elevated over null controls, after treatment with laminin-5 and (to a lesser extent) nonpolymerizing laminin, no organized ECM was detected (Fig. 7 E).

**Figure 6. Development of epiblast apoptosis and basement membrane thickening in dystroglycan-null EBs.**

Untreated dystroglycan-null EBs and wild-type controls were cultured for 5–9 d. Thick basement membranes developed in dystroglycan-null EBs as seen both in immunofluorescence micrographs (laminin- $\gamma$ 1 epitope in red) and the thick section. An epiblast layer is seen in both forms of EBs. By 9 d, epiblast layer degeneration is observed. TUNEL (green) and DAPI (blue) costaining reveals apoptosis in wild-type and dystroglycan-null cells over time. Epiblast apoptosis was prominent in the dystroglycan-null, but not the wild-type, EBs, and was augmented over time. The EBs with partial and complete degeneration and loss of the epiblast layer were subtracted from the total EBs counted. The plot shows the percentage of remaining EBs with surviving epiblast layers.





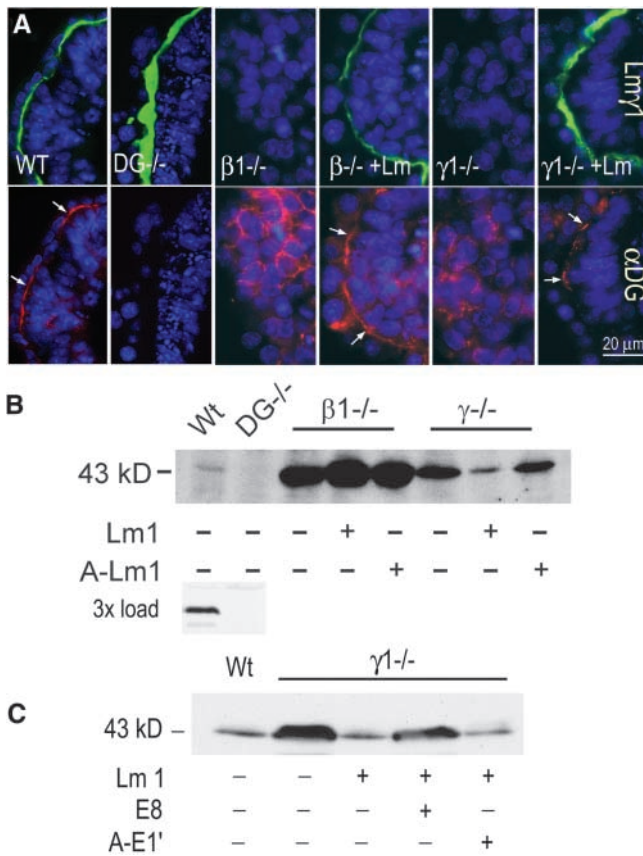
**Figure 7. Ultrastructure of EBs.** Wild-type (R1),  $\gamma$ 1-laminin-null,  $\beta$ 1-integrin-null, and dystroglycan-null EBs, untreated and laminin-treated, were examined by electron microscopy after incubation in suspension culture for 5–7 d. The regions containing junctions of the endodermal layer and ICM or epiblast layer are shown. (A)  $\beta$ 1-Integrin-null embryoid body, 7 d. Endoderm (above arrows) was present; however, neither basement membrane nor epiblast differentiation is present. Arrows indicate endodermal/ICM cell boundary and n indicates nucleus. (B) Wild-type embryoid body (7 d) reveals basement membrane between endoderm and epiblast (between arrows). (C)  $\beta$ 1-Integrin-null embryoid body treated with laminin-1 (25  $\mu$ g/ml, 7 d). Basement membrane (arrows) is located between endoderm and epiblast layers. Scattered small clefts (arrowhead) located between cell and matrix were present more frequently in these EBs compared with wild-type. (D)  $\gamma$ 1-Laminin-null EB, 7 d. No basement membrane was detected at the endoderm/ICM cell boundary (arrows). (E)  $\gamma$ 1-Laminin-null EB treated with laminin-5 (7 d). Endodermal differentiation in the absence of basement membrane was seen. Endodermal/ICM interface indicated by arrows. (F)  $\gamma$ 1-Laminin-null EBs treated with nonpolymerizing laminin-1 (25  $\mu$ g/ml, 7 d). No basement membrane or epiblast differentiation was detected. (G)  $\gamma$ 1-Laminin-null EBs treated with (polymerizing) laminin-1 (25  $\mu$ g/ml, 7 d). Note prominent basement membrane between endoderm and epiblast layers (arrows). (H and I) Dystroglycan-null EBs, 5 d. Note typical basement membrane (H) lying between endoderm and epiblast cell layers. The RER (asterisk) of the endoderm is dilated.

### Dystroglycan expression

Examination of  $\alpha$ -dystroglycan immunofluorescence of early and differentiated wild-type EBs revealed that  $\alpha$ -dystroglycan was initially diffusely distributed throughout the ICM in a pericellular pattern, but then redistributed to the basement membrane zone after basement membrane formation (Fig. 8). The latter pattern appeared to be largely confined to the epiblast aspect of the zone (this was particularly evident if the epiblast layer became detached from basement membrane during sectioning). No staining, as expected, was observed in dystroglycan-null EBs. The pericellular dystroglycan immunostaining intensity was greater in  $\beta$ 1-integrin and  $\gamma$ 1-laminin-null EBs compared with wild-type EBs (Fig. 8 A). After laminin-1 treatment of the integrin-null EBs, dystroglycan became redistributed to the

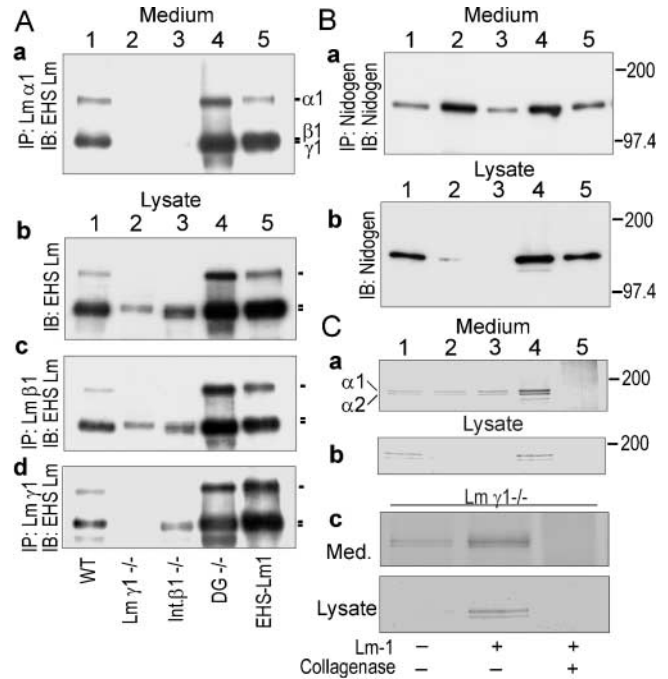
basement membrane zone, whereas the staining intensity remained high. Laminin-1 treatment of the  $\gamma$ 1-laminin-null EBs similarly caused redistribution of dystroglycan to the basement membrane zone; however, the staining intensity was now decreased.

The relative abundance of  $\beta$ -dystroglycan present in wild-type and null EBs was measured in immunoblots of detergent extracts (Fig. 8 B) in which equal amounts of total protein were loaded in each lane. A relatively small amount of dystroglycan was detected in wild-type and no dystroglycan was detected in the dystroglycan-null EBs as expected. The dystroglycan level was severalfold higher compared with wild-type EBs in both the untreated and laminin-treated  $\beta$ 1-integrin-null EBs, consistent with the immunofluorescence data. We concluded that  $\beta$ 1-integrin and laminin are



**Figure 8. Dystroglycan distribution and expression.** (A) Dystroglycan distribution of wild-type (first column), dystroglycan-null (second column), both laminin-untreated (third column) and treated (fourth column)  $\beta 1$ -integrin-null EBs, and laminin-untreated (fifth column) and treated (sixth column)  $\gamma 1$ -laminin-null EBs. (B) Wild-type EBs, dystroglycan-null (DG  $-/-$ ) EBs,  $\beta 1$ -integrin-null ( $\beta 1 -/-$ ) EBs untreated, laminin-1-treated (Lm1), or nonpolymerizing laminin treated (A-Lm1), and  $\gamma 1$ -laminin-null EBs, untreated, laminin-1-treated, or nonpolymerizing laminin treated EBs cultured for 7 d were detergent-extracted, normalized for total protein, analyzed by reducing SDS-PAGE, and transferred onto membranes that were incubated with  $\beta$ -dystroglycan-specific mAb with the bands detected with sheep anti-mouse IgG-HRP. Inset shows heavier sample load for wild-type and dystroglycan-null EBs. (C) Using the above conditions, E8 and AEBSE-E1' (each an integrin ligand) were incubated in 50-fold molar excess with laminin-1 followed by immunoblotting to detect  $\beta$ -dystroglycan subunit expression.

both required to maintain dystroglycan at a normal level. Therefore, we asked whether this protein expression was directly regulated by laminin-1, a potential ligand.  $\gamma 1$ -Laminin-null EBs were incubated with laminin-1 in the presence of excess E8 fragment that contains the  $\alpha 6\beta 1$  integrin-binding site (Fig. 8 C). E8 treatment reversed the effect of laminin-1 to down-regulate  $\beta$ -dystroglycan in  $\gamma 1$ -laminin-null EBs. In contrast, AEBSE-E1' that possesses the binding activities for  $\alpha 1\beta 1$  and  $\alpha 2\beta 1$  integrins, did not prevent the laminin-1-mediated decrease in dystroglycan expression. As shown earlier, neither of these two fragments inhibits basement membrane assembly (Fig. 2). The results support the hypothesis that the specific ligand-mediating dystroglycan regulation is a property of the E8 region of laminin and likely lies within laminin LG1-3.

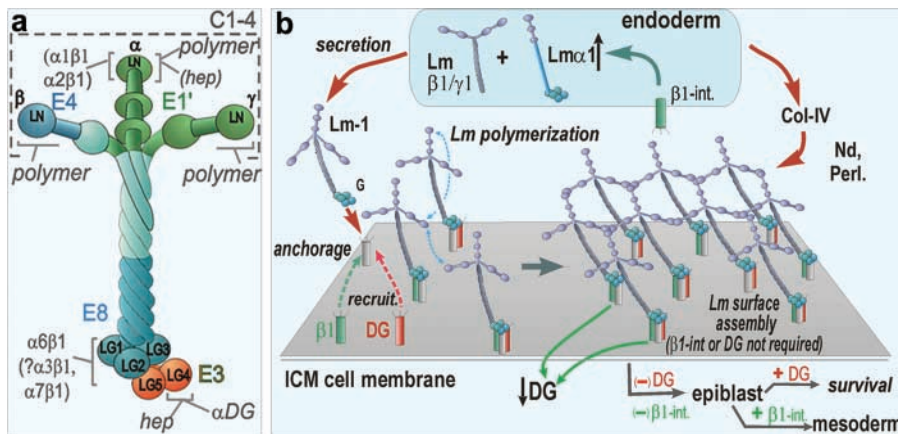


**Figure 9. Expression and accumulation of basement membrane components.** Conditioned media (10 ml from the last 2 d) and EBs were collected from cultures of wild-type,  $\beta 1$ -integrin-null,  $\gamma 1$ -laminin-null, and dystroglycan-null ES cells maintained for 7 d. The cell pellets were extracted with 0.5 ml of lysis buffer, 0.5 ml conditioned medium, or 0.15 ml EB lysates were incubated with antibody specific for the laminin- $\alpha 1$  (anti-RG50),  $\beta 1$  (anti-E4), or  $\gamma 1$  (rat anti-mouse  $\gamma 1$  chain mAb), and then pulled down with protein A or protein G coupled to agarose beads (immunoprecipitation [IP]). Alternatively, the extract or medium fraction was analyzed directly with EHS laminin-1-specific pAb in immunoblots (IB). (A) Laminin. Medium (IP/IB) and embryoid body cell pellet (IB or IP/IB). Samples correspond to EBs prepared from wild-type (lane 1),  $\gamma 1$ -laminin-null (lane 2),  $\beta 1$ -integrin-null (lane 3), and dystroglycan-null (lane 4) ES cells, shown in comparison to purified EHS laminin-1 (lane 5). (B) Nidogen. Media and extracted EB pellets were analyzed in immunoprecipitates/immunoblots with specific antibody for nidogen as follows: wild-type (lane 1),  $\gamma 1$ -laminin-null (lane 2),  $\beta 1$ -integrin-null (lane 3), and nidogen standard (lane 5). (C) Type IV collagen-specific antibody was used to immunoprecipitate the collagen from media and EB fractions followed by reducing SDS-PAGE and Coomassie blue staining. Type IV collagen immunoprecipitated from wild-type conditioned medium or EBs could be digested with bacterial collagenase (lane 5).

### Alterations of basement membrane component synthesis and accumulation

The expression and accumulation of basement membrane components were evaluated (Fig. 9). Loads of all fractions for analysis were normalized to total protein present in each EB extract. Conditioned medium protein contained the ECM components that accumulated into a final "pool" in transit from the EB, resulting either from turnover or cell death. The EB cell lysate, containing  $\sim 10\%$  of total endogenous basement membrane proteins present in each culture, represented the material that accumulated in basement membrane and cell (most epitopes were present within the basement membrane zone as determined by microscopy). When EB extracts or conditioned media were immunoprecipitated with laminin  $\alpha 1$ ,  $\beta 1$ , or  $\gamma 1$  chain-specific anti-





**Figure 10. Model of laminin interactions.** (a) Laminin-1 and its fragments. (b)  $\beta$ 1-Integrin initiates laminin- $\alpha$ 1 expression in endoderm, enabling heterotrimer formation and secretion. The laminin becomes anchored to and concentrated on the endodermal (not depicted) and (shown) outer ICM cell surfaces largely via LG4–5, accompanied by recruitment of  $\beta$ 1-integrins and  $\alpha$ / $\beta$ -dystroglycan (DG) that interact with LG1–3 and LG4, respectively. Laminin polymerizes through its short arms creating a multivalent network. The ICM, requiring this network, but not requiring integrin or dystroglycan, becomes polarized and converted to

epiblast.  $\alpha$ 6 $\beta$ 1-Integrin, interacting with polymerizing laminin through LG1–3, down-regulates dystroglycan (DG), and dystroglycan down-regulates basement membrane components. Type IV collagen forms a second network and nidogen and perlecan are incorporated into a more stable ECM. Mesodermal differentiation is delayed in the laminin-treated integrin-null EBs.

bodies, no heterotrimeric laminin-1 was detected in the  $\gamma$ 1-laminin-null state. In addition to the expected absent  $\gamma$ 1 chain, the  $\alpha$ 1 chain was absent, possibly a consequence of degradation. In  $\beta$ 1-integrin-null EBs, laminin chains were not detected with EHS-laminin-specific antibody after precipitation with  $\alpha$ 1 subunit-specific antibody from either medium or cell lysates. However, immunoprecipitation of  $\beta$ 1-integrin-null cell extracts with either laminin  $\beta$ 1- or laminin  $\gamma$ 1-specific antibody revealed an incompletely resolved  $\beta$ / $\gamma$  doublet. We concluded that these chains, present in low amount, are present within the endodermal cell cytoplasm because no basement membrane formed in these EBs, whereas weak diffuse intracellular endodermal staining could be detected. These data support and extend the conclusions of Aumailley et al. (2000) but do not support those of Lohikangas et al. (2001) i.e., laminin  $\alpha$ 1 expression is selectively absent in  $\beta$ 1-integrin-null EBs. It follows that the block to assembly is due to the laminin expression defect rather than to a role of  $\beta$ 1-integrin in the polymerization of laminin or in its cell surface receptor-mediated assembly.

In contrast to the wild-type state, both cell and media fractions obtained from dystroglycan-null EBs contained elevated levels of laminin-1, type IV collagen, and nidogen. This overexpression of components correlates with the appearance of dilated ER in the endodermal cells, the principle source of basement membrane proteins. Endodermal ECM overexpression may explain the unusual thickness that develops in the dystroglycan-null basement membranes. The absence of basement membrane formation in the  $\beta$ 1-integrin-null and  $\gamma$ 1-laminin-null EBs was accompanied by the presence of only trace amounts of nidogen and type IV collagen in the EB fractions. However, lack of a basement membrane was not accompanied by a substantial decrease of either nidogen or type IV collagen expression, as these proteins still accumulated in conditioned media. Thus, we concluded that the absence of laminin polymer accumulation between endoderm and ICM results in a failure to sequester nidogen and collagen within the basement membrane zone, even though these components continue to be synthesized and secreted.

## Discussion

Embryonic basement membrane assembly, a process in which soluble extracellular monomers form a supramolecular architecture in association with a specific cell surface, required that laminin polymerize and interact with the surface through its G-domain, critically depending upon the heparin-binding KRK sequence within LG4 (Fig. 10). On the other hand, in the presence of laminin-1, neither  $\beta$ 1-integrin nor dystroglycan was uniquely needed for this assembly or for the subsequent differentiation of the epiblast. Instead, we found that  $\beta$ 1-integrin and dystroglycan acted upstream and downstream of assembly, mediating laminin  $\alpha$ 1-chain expression and affecting the regulation of dystroglycan and other basement membrane components.

### $\beta$ 1-Integrin functions

The ability of exogenous laminin-1 to rescue the integrin-defect with restoration of basement membrane formation and epiblast development indicates that the early differentiation block in  $\beta$ 1-integrin-null EBs is due to the failure of laminin  $\alpha$ 1-chain expression (either transcriptional or post-transcriptional) and is not at the level of basement membrane anchorage and assembly. This contribution may be specific for laminin- $\alpha$ 1 because similar regulation has not been observed for Schwann cell basement membranes lacking this integrin subunit (Feltri et al., 2002).  $\beta$ 1-Integrin was not required for the integration of type IV collagen, nidogen, or perlecan into the basement membrane, suggesting that their incorporation into a laminin scaffold is mediated either directly through laminin interactions or through novel cell surface molecules. Furthermore, the data show that  $\beta$ 1-integrin, once the laminin synthesis block is bypassed, is not required for epiblast differentiation and cavitation, although it is essential for mesodermal differentiation. Although other  $\beta$ -integrins might compensate for the missing  $\beta$ 1 subunit, there is no obvious candidate. None of the known laminin-interacting integrins ( $\alpha$ 6 and therefore  $\beta$ 4) were detected in  $\beta$ 1-integrin-null ES cells and/or EBs.

Although integrin compensation was not detected, dystroglycan was substantially overexpressed in  $\beta$ 1-integrin-null EBs. Laminin induced a topographical redistribution of dys-

troglycan such that it localized to sites in the basement membrane zone; however, it did not restore normal dystroglycan levels. Dystroglycan was similarly overexpressed in the  $\gamma$ 1-laminin-null EBs, suggesting that laminin is required to maintain normal dystroglycan expression. The hypothesis was supported by the finding that exogenous laminin mediated both the correct basement membrane zone localization and normalization of dystroglycan expression. Furthermore, treatment of  $\gamma$ 1-laminin-null EBs with E8 (ligand for  $\alpha$ 6 $\beta$ 1 integrin) abrogated laminin-mediated dystroglycan down-regulation. These data not only show that dystroglycan expression and localization is regulated by laminin and  $\beta$ 1-integrin, but also suggest that this regulation requires the direct ligation of laminin G-domain within a polymer to the integrin.

### Role of a heparin-binding site in LG4

$\alpha$ 6 $\beta$ 1,  $\alpha$ 7 $\beta$ 1, and  $\alpha$ 6 $\beta$ 4 integrin-binding sites are located in LG modules 1–3, whereas heparin/heparan sulfate, sulfatide, and  $\alpha$ -dystroglycan-binding sites are located within LG module-4. A third cell-interactive domain, capable of binding to heparin and  $\alpha$ 1 $\beta$ 1 and  $\alpha$ 2 $\beta$ 1 integrins, and located within the LN domain of the  $\alpha$ 1 subunit (Cognato-Pyke et al., 1995; Cognato et al., 1997), was not found to participate in embryonic basement membrane assembly. In EBs, LG module 4 was found to be required for basement membrane assembly, which is consistent with the concept that it provides for the key anchorage to the cell surface. We examined this further by alanine mutagenesis of an important heparin/dystroglycan-binding sequence, EYIKRKAF, located between inter- $\beta$  strand loops H and I of LG4 (Tisi et al., 2000). We found that the mutation, which substantially decreased heparin-binding, inactivated LG4–5 inhibition of assembly. The possibility that dystroglycan was an essential receptor for assembly, mediated through this site, was ruled out because dystroglycan-null EBs spontaneously formed basement membrane. This in turn suggests that the critical LG4 interaction is mediated by a heparan sulfate proteoglycan, or possibly by a sulfatide. However, an important remaining question is whether laminin anchorage and basement membrane assembly can occur in the absence of both integrin and dystroglycan, requiring only LG4 heparin-type binding and polymerization. Because integrin and dystroglycan may provide some of the anchorage activity themselves, it is possible that a minimum of two of the three binding sites in G-domain are required. Alternatively, the heparin-site may provide sufficient anchorage in the absence of either receptor. Resolution of this question will require further experimentation.

### Role of dystroglycan

Our study has shown that dystroglycan is not required for formation of the developmentally critical basement membrane between endoderm and epiblast. This conclusion is seemingly in disagreement with the Henry and Campbell (1998) article. In that analysis, it was reported that basement membranes failed to form in dystroglycan-null EBs, and it was therefore suggested that dystroglycan is essential for basement membrane assembly. However, this could not represent a general receptor requirement as was implied be-

cause knockout of the dystroglycan gene in mice is characterized by a loss of Reichert's membrane, but not a loss of the embryonic basement membrane adjacent to epiblast (Williamson et al., 1997). Furthermore, the skeletal muscle of dystroglycan-deficient chimeric mice has been found to possess basement membrane (Cote et al., 1999). Of note, neither the wild-type nor dystroglycan-null EBs used in the study of Henry and Campbell developed epiblast and only 1% of EBs cavitated (both central attributes of embryonic differentiation), making unclear what step of differentiation was modeled. Together, we conclude that dystroglycan is not a fundamental requirement for basement membrane assembly in tissues.

A striking finding in our analysis was the loss of the epiblast layer through apoptosis. It has previously been observed that only those cells that adhere to basement membrane survive to differentiate with the nonadherent cells undergoing anoikis (Cocouvanis and Martin, 1995). However, continued survival of the epiblast was clearly dependent upon a dystroglycan interaction. This receptor dependency was significantly greater than that which we observed in the laminin-rescued integrin null, and a general survival role for dystroglycan is supported by *in vitro* studies conducted on muscle cells (Montanaro et al., 1999). The survival deficit seen in both receptor nulls raises the possibility that cell adhesion strength determines survival regardless of the specific receptor involved, and that the observed difference in survivability is due to asymmetric compensation in which only the integrin-null loss of receptor binding is largely replaced by high cell surface expression of dystroglycan.

EBs lacking  $\gamma$ 1-laminin,  $\beta$ 1-integrin, or functional FGF receptors fail to express essential laminin subunits, fail to form a basement membrane, and fail to differentiate (Li et al., 2001, and this study). In each case, assembly and differentiation could be rescued with exogenous laminin-1, strongly suggesting that lack of extracellular laminin, rather than a problem with cell surface ability to mediate assembly, caused the defect. During development, laminin expression became restricted to the zone underneath the endodermal layer, the major source of laminin synthesis and secretion (Murray and Edgar, 2001a). This step requires FGF signaling and  $\beta$ 1-integrin. Interestingly, the findings of Li et al. (2001) argue that laminin is both necessary and sufficient to mediate epiblast differentiation in the absence of endoderm. Our data provide evidence for a mechanism in which laminin must both polymerize through its LN domains (Yurchenco and Cheng, 1993) and interact with the cells of the ICM through a heparin-binding sequence in LG4 to initiate site-specific basement membrane assembly and to trigger differentiation. The new findings also argue that the laminin polymer creates the initial architectural scaffolding that must assemble before other components can accumulate into the ECM, and that is crucial for cellular differentiation.

## Materials and methods

### Culturing of embryonic stem cells and EBs

Wild-type R1 (Smyth et al., 1999) and D3 ES cells (Doetschman et al., 1985),  $\gamma$ 1-laminin-null (Smyth et al., 1999), and dystroglycan-null (Cote et al., 1999) ES cells were grown on feeder layers of mitomycin-treated (10

$\mu\text{g/ml}$ , 2 h) SNL STO cells in ES medium (MEM  $\alpha$ -medium; catalog No. 12463-014; Life Technologies) supplemented with 15% ES-grade FCS (Life Technologies), 0.1 mM nonessential amino acids, 0.1 mM  $\beta$ -mercaptoethanol, 1 mM sodium pyruvate, 100  $\mu\text{g/ml}$  penicillin, 100  $\mu\text{g/ml}$  streptomycin, and 1,000 U/ml leukemia inhibitory factor (LIF; Life Technologies).  $\beta$ 1-Integrin-null (clone G201) ES cells (Fässler et al., 1995) were cultured directly on Falcon tissue culture dishes (Becton Dickinson) in ES medium. ES cells were subcultured at semi-confluence, and the medium was changed every day to maintain the cells in an undifferentiated state. To culture EBs, subconfluent ES cells were dispersed with 0.25% trypsin-0.53 mM EDTA and plated onto gelatin-coated dishes for 3 h to allow feeder cells to selectively attach. Nonadherent ES cell aggregates were then dispersed and cultured on bacteriological petri dishes in ES medium without LIF.

### Proteins and antibodies

Laminin-1 (DEAE-unbound fraction) and laminin fragments E1' (short arm complex), E3 ( $\alpha$ 1-LG modules 4–5), E4 ( $\beta$ 1-domains VI and V), E8 (lower coiled-coil with LG1–3), and C1–4 (polymerizing  $\alpha$ 1 $\beta$ 1 $\gamma$ 1 short-arm complex) were prepared from the mouse EHS tumor as described previously (Yurchenco and Cheng, 1993; Yurchenco and O'Rear, 1994). Nonpolymerizing laminin-1 was prepared by treatment with 5 mM AEBSF in 50 mM Tris-HCl and 90 mM NaCl, pH 7.4, in the cold overnight (Colognato et al., 1999). AEBSF-E1' (nonpolymerization inhibition control) was prepared by incubation of E1' under the same conditions followed by dialysis to remove AEBSF. Laminin-2/4 and laminin-4 were prepared from collagenase-treated human placenta as described previously (Cheng et al., 1997). Recombinant laminin-5 ( $\alpha$ 3 $\beta$ 3 $\gamma$ 2), produced in transfected HEK-293 cells, was a gift of Dr. Ariel Boutaud (BioStratum Incorporated, Research Triangle Park, NC). Reducing SDS-PAGE revealed 150-kD ( $\alpha$ 3), 140-kD ( $\beta$ 2), and 105-kD ( $\gamma$ 2) bands. Recombinant laminin-8 ( $\alpha$ 4 $\beta$ 1 $\gamma$ 1) was prepared as described previously (Kortesmaa et al., 2000).

Rat monoclonal anti-laminin  $\gamma$ 1 (clone A5; Upstate Biotechnology), rabbit anti-mouse type IV collagen antibody (Rockland Immunochemicals), rat anti-mouse perlecan mAb, and rabbit anti-mouse type I collagen antibody (CHEMICON International, Inc.) were used for immunostaining at 1, 2.5, 2, and 2.5  $\mu\text{g/ml}$  respectively. Rabbit pAbs specific for laminin-1, E4 ( $\beta$ 1 subunit), mouse laminin-1 RG50 ( $\alpha$ 1 LG 4–5) fractionated from recombinant G-domain were prepared and characterized as described previously (Yurchenco and Ruben, 1987; Handler et al., 1997; Yurchenco et al., 1997). E4 and RG50 antibodies were used for immunoprecipitation at 10  $\mu\text{g/ml}$  and EHS-laminin-1 antibody was applied on immunoblots at 3  $\mu\text{g/ml}$ . Rabbit polyclonal nidogen-specific antibody was generated with purified EHS-nidogen, affinity-purified with immobilized nidogen and cross-absorbed with laminin, and used at 3 (immunoprecipitation) and 1  $\mu\text{g/ml}$  (immunoblotting, immunofluorescence). Mouse monoclonal IgM antibody IH6 hybridoma medium specific for  $\alpha$ -dystroglycan (Ervasti and Campbell, 1991), a gift of Kevin Campbell (Howard Hughes Medical Institute, University of Iowa, Iowa City, Iowa), was used as conditioned hybridoma medium at 1:2 dilution. Mouse mAb specific for  $\beta$ -dystroglycan (Novocastra Laboratories Ltd) was used at a dilution of 1:100. Rat anti-mouse integrin  $\beta$ 1-chain mAb (2  $\mu\text{g/ml}$  for immunoblotting and 5  $\mu\text{g/ml}$  for immunofluorescence), and hamster anti-mouse integrin  $\beta$ 3-chain mAb (5  $\mu\text{g/ml}$  for immunofluorescence) were obtained from BD Biosciences.

FITC- and Cy5-conjugated antibodies specific for mouse IgG, mouse IgM, and rabbit IgG (Jackson ImmunoResearch Laboratories) were used at 1:100 dilutions. HRP-linked antibodies specific for mouse IgG, rat IgG, and rabbit IgG (Amersham Pharmacia Biotech) were used as secondary antibodies for immunoblotting at a dilution of 1:3,000.

### Sample preparation

EBs were collected into 10-ml tubes and allowed to sediment by gravity. After washing in PBS with 0.5% BSA, the EBs were fixed with 3% paraformaldehyde in PBS and followed by incubation in 7.5% sucrose-PBS for 3 h at room temperature and then in 15% sucrose-PBS at 4°C overnight. The EBs were embedded in Tissue-Tek OCT (Miles, Inc.) and 4- $\mu\text{m}$ -thick frozen sections were prepared. Nonspecific binding sites were blocked with 5% goat serum. FITC- and/or Cy5-conjugated antibodies were used as secondary reagents and nuclei were counterstained with DAPI.

### Microscopy

Slides were viewed by indirect immunofluorescence using an inverted microscope (model IX70; Olympus) fitted with an IX-FLA fluorescence observation attachment and a MicroMax 5-mHz CCD camera (Princeton Instruments) controlled by IP Lab 3.0 (Scanalytics). EBs were allowed to settle in 15-ml conical tubes, and then washed with PBS by resuspension/settling. The cell pellet was fixed in 0.5% glutaraldehyde and 0.2% tannic acid in PBS for 1 h

(room temperature), washed with 0.1 M sodium cacodylate buffer, transferred to modified Karnovsky's fixative, post-fixed in 1% osmium tetroxide for 1 h, and then dehydrated and embedded in Epon/SPURR resin (EM Science). Thick (1  $\mu\text{m}$ ) and thin sections ( $\sim$ 90 nm) were cut with a diamond knife on an ultramicrotome. Thick sections were stained with 1% methylene blue in 1% sodium borate for light microscopy, and thin sections were stained with saturated uranyl acetate followed by 0.2% lead citrate. Images were photographed with an electron microscope (model JEM-1200EX; JEOL USA, Inc.).

**TUNEL staining.** Apoptosis was assessed by terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end-labeling (Promega). EB cryosections were washed in PBS, fixed with 3% paraformaldehyde in PBS for 30 min, and permeabilized with 0.2% Triton X-100 in PBS. DNA fragments were end-labeled with 0.5 U/ml terminal transferase and 5 mM fluorescein 12-dUTP for 1 h at 37°C. Slides were washed twice in 2 $\times$  SSC followed by three washes in PBS. EBs were immunostained for laminin and counterstained with DAPI.

### Protein assays

Protein in solution was determined either by absorbance at 280 nm or the Bradford assay (Bio-Rad Laboratories). SDS-PAGE was performed in 3.5–12% linear gradient gels and electrophoretic transfer of proteins onto PVDF membranes was performed as described previously (Yurchenco and Cheng, 1993; Cheng et al., 1997). Blots were blocked with 5% nonfat dried milk and 0.2% Tween 20 in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, and then incubated with primary antibody followed by antibody-HRP. Reacting bands were detected by ECL (Amersham Biosciences). Immunoprecipitation (IP) was performed at 4°C with the addition of protease inhibitor cocktails (Sigma-Aldrich) to all the protein samples and buffers. EB-conditioned medium or lysates were precleared with 20  $\mu\text{l}$  of 50% protein A-agarose (pAb IP) or protein G-Sepharose bead slurry (mAb IP). Samples were incubated with antibody overnight and precipitated with 40  $\mu\text{l}$  protein A-agarose or protein G-Sepharose beads for 2 h and followed by washing in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, and 0.1% SDS. After an additional wash, the supernatant was removed and the immunoprecipitates were analyzed by SDS-PAGE. Duplicates of type IV collagen antibody immunoprecipitates were incubated with 5 U bacterial collagenase (CLSPA; Worthington Biochemical Corporation) at 37°C for 1 h. After collagenase digestion, the immunoprecipitates were washed twice in PBS and analyzed.

### Semi-quantitative RT-PCR

Total RNA was isolated with TRIzol<sup>®</sup> reagent (Life Technologies) and reverse-transcribed to cDNA using SuperScript<sup>™</sup> II reverse transcriptase (Life Technologies). The primers and PCR annealing conditions for brachyury, BMP4, low molecular weight NFL,  $\zeta$ -globulin, and hypoxanthine guanine phosphoribosyl transferase (HPRT) were described previously (Levinson-Dushnik and Benvenisty, 1997; Rohwedel et al., 1998; Weinhold et al., 2000). PCR products were electrophoretically resolved on 2% agarose gels.

### Production of recombinant E3 and its mutant

Laminin- $\alpha$ 1LG4–5 was amplified by PCR from a mouse laminin  $\alpha$ 1 chain cDNA. BM40 signal sequence and a FLAG epitope were introduced into the 5'-end of the cDNA fragment. The heparin/dystroglycan-binding site KRK in LG4 was replaced with AAA via PCR-based mutagenesis as described previously (Andac et al., 1999). Both wild-type and KRK mutant were cloned into mammalian expression vector pcDNA3.1/Zeo+ (Invitrogen) and the sequence of the inserts was confirmed by automated sequencing. The constructs were expressed in HEK 293 cells and stable clones expressing wild-type or the mutant E3 were selected with Zeocin<sup>™</sup>. Recombinant LG4–5 proteins were purified to homogeneity by FLAG affinity chromatography (Yurchenco et al., 1997).

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