POMK Regulates Dystroglycan Function via LARGE-mediated Elongation of Matriglycan

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ABSTRACT:

Protein O-Mannose Kinase (POMK) is a novel muscular dystrophy gene that phosphorylates mannose of core M3 (GalNac-β1,3-GlcNac-β1,4-Man) on α-dystroglycan (α-DG). Like-acetylglucosaminyltransferase-1 (LARGE) synthesizes matriglycan, a heteropolysaccharide [-GlcA-β1,3-Xyl-α1,3-]$_n$, that enables α-DG to function as an extracellular matrix (ECM) receptor. Extension of matriglycan enhances its binding capacity for ECM ligands and is required for skeletal muscle function. However, the mechanisms which regulate LARGE-mediated extension of matriglycan during muscle differentiation are unknown. Here we show that LARGE initiates matriglycan synthesis on α-DG without POMK but requires POMK to extend matriglycan to its mature length. Furthermore, mouse and human skeletal muscle lacking POMK express a short, non-extended form of matriglycan and exhibit muscular dystrophy. Our results demonstrate a POMK-dependent mechanism that regulates matriglycan synthesis and α-DG receptor function.

ONE SENTENCE SUMMARY:

POMK Mediates Matriglycan Extension

MAIN TEXT:

POMK is a glycosylation-specific kinase *(1-3)* that phosphorylates an *O*-mannosyl trisaccharide (GalNac-β1,3-GlcNac-β1,4-Man), termed core M3, on the high-affinity ECM receptor α-DG (Fig. 1A). The core M3 glycan structure terminates with matriglycan, a heteropolysaccharide [-GlcA-β1,3-Xyl-α1,3-]$_n$ that is synthesized by LARGE *(3-5)* (Fig. 1A). A single glucuronic acid-xylose (GlcA-Xyl) disaccharide repeat binds with high affinity to ECM ligands bearing laminin G (LG) domains *(4,8)*, and there is a direct correlation between the number of GlcA-Xyl repeats on α-DG and its binding capacity for ECM ligands *(10).* During skeletal muscle differentiation, LARGE elongates matriglycan, thereby enhancing the binding capacity of matriglycan for ECM ligands *(3, 4, 10)*. Defects in matriglycan synthesis lead to dystroglycanopathies, congenital and limb-girdle muscular dystrophies that can be accompanied by structural brain abnormalities *(7-15)*. Indeed, the clinical severity of dystroglycanopathy correlates with the degree of non-extension of matriglycan *(10)*. Despite the importance of mature, full-length matriglycan for proper muscle structure and function, little is known about how matriglycan elongation is regulated.

To understand the mechanisms regulating matriglycan elongation, we first studied skeletal muscle from a patient (NH13-284) with a POMK mutation and congenital muscular dystrophy (CMD) accompanied by structural brain malformations. Whole exome sequencing of genomic DNA demonstrated a homozygous missense mutation causing Aspartate to Asparagine substitution at residue 204 (D204N) of POMK (Fig. 1B). D204 is located in the catalytic loop of POMK and serves as the catalytic base in the phosphorylation reaction catalyzed by the kinase (Fig. 1B, S1). D204 is highly conserved (Fig. 1B), and mutation of D204 to Asparagine (D204N) or Alanine (D204A) is predicted to eliminate POMK activity (Fig. S1) (*1*). POMK activity from

skeletal muscle and skin fibroblasts of patient NH13-284 (POMK D204N) was undetectable when compared to control muscle and fibroblasts, respectively (Fig. 1C). B4GAT1 and LARGE activity was present in skeletal muscle and fibroblasts, respectively, of a control patient and patient NH13-284 (Fig. S2A, S3). Immunofluorescence analyses of POMK D204N muscle demonstrated partial IIH6 immunoreactivity, while the transmembrane subunit of DG, β-DG, was expressed normally in both POMK D204N and control muscle (Fig. 1D). Flow cytometry using IIH6 also demonstrated partial immunoreactivity in POMK D204N fibroblasts (Fig. S2B). To test the effect of the POMK mutation on ligand binding using a more sensitive method than IIH6 immunofluorescence, we performed a laminin overlay using laminin-111 on glycoproteins enriched from skeletal muscle cryosections with Wheat Germ Agglutinin (WGA)-agarose. We were surprised to find laminin binding (at ~90 to 100 kDa range) by a glycoprotein in POMK D204N skeletal muscle (Fig. 1E).

To understand the biochemical basis of the laminin binding at ~90 to 100 kDa, we knocked out POMK using LoxP sites and Cre driven by the Muscle Creatine Kinase (MCK) promoter, which is active in murine skeletal muscle and heart, or both the MCK promoter and the Paired Box 7 (Pax7) promoter, which is expressed by satellite cells and embryonic muscle (Fig. S4) (*16, 17*). Quadriceps muscle extracts of MCK-Cre; POMK$^{LoxP/LoxP}$ mice show reduced POMK activity compared to POMK$^{LoxP/LoxP}$ muscle but have similar levels of LARGE activity (Fig. 2B, S5). Histologic analyses of MCK-Cre; Pax7-Cre; POMK$^{LoxP/LoxP}$ (M-POMK KO) quadriceps muscles reveal hallmarks of a mild muscular dystrophy (Fig. 2A). M-POMK KO mice also showed reductions in 2-limb grip strength and body weight and elevations in post-exercise creatine kinase (CK) levels compared to littermate control POMK$^{LoxP/LoxP}$ mice (Fig. S6). Immunofluorescence analysis of M-POMK KO muscle shows that β-DG is expressed at the

skeletal-muscle sarcolemma (Fig. 2A, 2C); however, like patient NH13-284 IIH6 immunoreactivity persisted in M-POMK KO muscle, but at a reduced intensity. Biochemical analysis of control and M-POMK KO muscle showed a typical, lower molecular weight (MW) α-DG with anti-core DG antibody (Fig. 2C) like that has been previously reported for dystroglycanopathy patients (7,10); however, on laminin overlay, we observed laminin binding at 90-100 kDa (Fig 2D), similar to POMK D204N skeletal muscle (Fig. 1E). Solid-phase binding analyses of M-POMK KO and MCK-Cre; $POMK^{LoxP/LoxP}$ skeletal muscle demonstrated a reduced binding capacity (relative B_{max}) for laminin-111 compared to control muscle (Fig. S8A), but higher than that of $LARGE^{Myd}$ muscle, which lacks matriglycan due to a deletion in the *LARGE* gene.

To determine if a GlcA-Xyl disaccharide polymer, and, therefore, a form of matriglycan, is responsible for the laminin binding at 90-100 kDa in POMK-null muscle, we treated glycoproteins enriched from skeletal muscles of M-POMK KO and MCK-Cre; $POMK^{LoxP/LoxP}$ mice with two exoglycosidases, α-Xylosidase and β-Glucuronidase, which in combination digest matriglycan (*8*). Laminin overlay and solid phase analysis showed a reduction in laminin binding from muscles of M-POMK KO and MCK-Cre; $POMK^{LoxP/LoxP}$ mice (Fig. 2E, 2F, S8B) after dual exoglycosidase treatment. This demonstrates that matriglycan is responsible for the laminin binding at 90-100 kDa in POMK-deficient muscle.

We used a *POMK* KO HAP1 cell line to determine the identity of the above laminin-binding glycoprotein. The *POMK* KO HAP1 cells had undetectable levels of POMK activity and expression (Fig. 3A, S10) (*1*). A mass spectrometry (MS)-based glycomic analysis of O-glycans carried by recombinantly-expressed DG mucin-like domain protein further indicates the absence of an MS peak at *m/z* 873.5 corresponding to phosphorylated core M3 O-glycan (Fig. 3D, 3E),

consistent with an undetectable level of POMK activity in *POMK* KO HAP1 cells. Compared to WT HAP1 cells, immunoblots of *POMK* KO HAP1 cells showed a reduction in IIH6 immunoreactivity, a decrease in MW of core α-DG, and the presence of laminin binding at ~90 kDa on laminin overlay (Fig. 3C, S10). Laminin binding on overlay was rescued only after adenoviral transduction with wild-type (WT)-POMK, and not with POMK containing D204N (POMK D204N) or D204A (POMK D204A) mutations (Fig. 3C, S10). POMK D204N also lacked POMK activity *in vitro* but showed normal B4GAT1, B3GALNT2, and LARGE activity, thus confirming the pathogenicity of the D204N mutation (Fig. 3A, 3B, S11).

The bifunctional glycosyltransferase, LARGE, synthesizes matriglycan (*3-5*). To directly test if LARGE is required for synthesis of the 90 kDa laminin-binding glycoprotein in *POMK* KO HAP1 cells, we studied *POMK/LARGE* double knockout (DKO) HAP1 cells, which bear a CRISPR/Cas9-mediated deletion in *LARGE*. *POMK/LARGE* DKO HAP1 cells demonstrated the absence of the laminin binding at 90-100 kDa (Fig. 3F, S12), indicating that LARGE is required for the synthesis of the matriglycan responsible for laminin binding at 90-100 kDa. Moreover, *POMK/DG* DKO HAP1 cells demonstrated a complete absence of laminin binding (Fig. 3F) and IIH6 immunoreactivity at 90 kDa (Fig. S13), demonstrating that α-DG is the glycoprotein that binds laminin in the absence of POMK. We, therefore, refer to this glycoprotein as POMK-null α-DG (α-DG(POMK)). The length of matriglycan correlates with its binding capacity for ECM ligands (*4, 8, 10*). We hypothesized that, given the MW of α-DG(POMK) at 90-100 kDa, the glycan must be shorter than full-length matriglycan, and therefore, have a lower B_{max} for laminin. We measured the binding capacity of HAP1 α-DG using solid-phase binding assays. B_{max} of α-DG(POMK) for laminin-111 was reduced compared to wild-type α-DG (α-DG(WT)) but was greater than that of α-DG from *LARGE* KO HAP1 cells (Fig. 3G). *POMK/DG* DKO HAP1 cells

showed a reduction in B_{max} compared to *POMK* KO HAP1 cells, similar to the low levels observed in *LARGE* KO HAP1 cells (Fig. 3G). These data indicate that a short, non-extended form of matriglycan is synthesized on α-DG(POMK). This non-extended form of matriglycan has a lower binding capacity for laminin-111 and, thus, a reduced level of α-DG receptor function.

We next carried out a series of experiments to characterize the enzymatic requirements for synthesis of matriglycan on α-DG(POMK). After POMK phosphorylates core M3, Fukutin (FKTN) modifies GalNac with ribitol-phosphate for synthesis of full-length matriglycan (Fig.1A) *(4, 18)*. Overexpression of Isoprenoid Synthase Domain-Containing (ISPD), which synthesizes the substrate (CDP-ribitol) of FKTN (Fig.1A), in *POMK* KO HAP1 cells increases the matriglycan (without changing its MW) responsible for laminin binding at 90-100 kDa (Fig. 4A, S14) *(19-21)*. HAP1 cells lacking both POMK and ISPD do not express matriglycan, and adenoviral transduction of these cells with *ISPD* restores the 90-100 kDa laminin binding, (Fig. 4B, S15). FKTN overexpression in *POMK* KO HAP1 cells also increased the 90-100 kDa laminin binding (Fig. 4C, S16). These experiments collectively support a requirement for CDP-ribitol for synthesis of the non-extended form of matriglycan. The DG N-terminal domain (DGN) is required for interaction of α-DG with LARGE *(22-24)*. Synthesis of the non-extended form of matriglycan also requires this interaction, as a DG mutant lacking the DGN (DGE) expressed in *POMK/DG* DKO HAP1 cells did not show laminin binding at 90-100 kDa (Fig. S17). Similar experiments also indicated that synthesis of the non-extended matriglycan in HAP1 cells requires Threonine 317 of the mucin-like domain of α-DG (Fig. S17). Thus, these experiments show that the same pathway is used for the synthesis of the non-extended form of matriglycan.

Overexpression of LARGE has been shown to produce very high molecular weight matriglycan and rescues the defect in matriglycan synthesis in distinct forms of CMD and in *LARGE* KO HAP1 cells (Fig. 4D) *(25)*; however, overexpression of LARGE in *POMK* or *POMK/LARGE* KO HAP1 cells did not lead to synthesis of very high molecular weight forms of matriglycan (Fig. 4E, F, S18A). Only the rescue of *POMK/LARGE* KO HAP1 cells with POMK enabled LARGE to synthesize high molecular weight matriglycan (Fig. 4F, S18B). Thus, our results demonstrate that LARGE requires POMK in order to extend matriglycan on α-DG for the synthesis of mature matriglycan and very high molecular weight matriglycan.

Our study demonstrates that POMK is required for the LARGE-mediated synthesis of mature, full length forms of matriglycan (Fig 4G). In the absence of POMK, LARGE generates a short, non-extended form of matriglycan (Fig 4F), as observed by the laminin binding at 90-100 kDa. Mice and humans lacking POMK in skeletal muscle express this short, non-extended form of matriglycan, which has a reduced binding capacity of ECM ligands, however, both humans and mice develop muscular dystrophy due to a reduction in normal α-DG-ligand interactions. Studies in HAP1 cells indicated that the short, non-extended form of matriglycan is generated because LARGE can initiate matriglycan synthesis but is unable to elongate the polysaccharide due to absence of phosphorylation of core M3. Further studies demonstrated that the short, non-extended form of matriglycan requires DGN, CDP-ribitol and Threonine 317 of the mucin-like domain of α-DG. Our study, therefore, shows that LARGE requires POMK activity in order to extend matriglycan to its mature, full-length form and thereby, enable normal α-DG function. Collectively, these results indicate that phosphorylation of core M3 by POMK is a regulatory step in the synthesis of matriglycan, thus enabling full ECM binding capacity and preventing muscular dystrophy.

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Authors' Contributions

ASW and KPC designed experiments and wrote the manuscript. ASW, TY, TY, DV, MC, ST, MEA, LY, MD, SOL, and SJP performed experiments. AM, ST, and FM provided critical information and materials regarding the patient. FM was involved in meaningful discussions regarding the manuscript. All authors contributed to and gave feedback on the manuscript.

Competing interests

The authors declare that they have no competing interests.

Data and materials availability

All data generated or analyzed during this study are included in this published article.

Supplementary Materials

Materials and Methods

Supplementary Text

Figures S1–S24

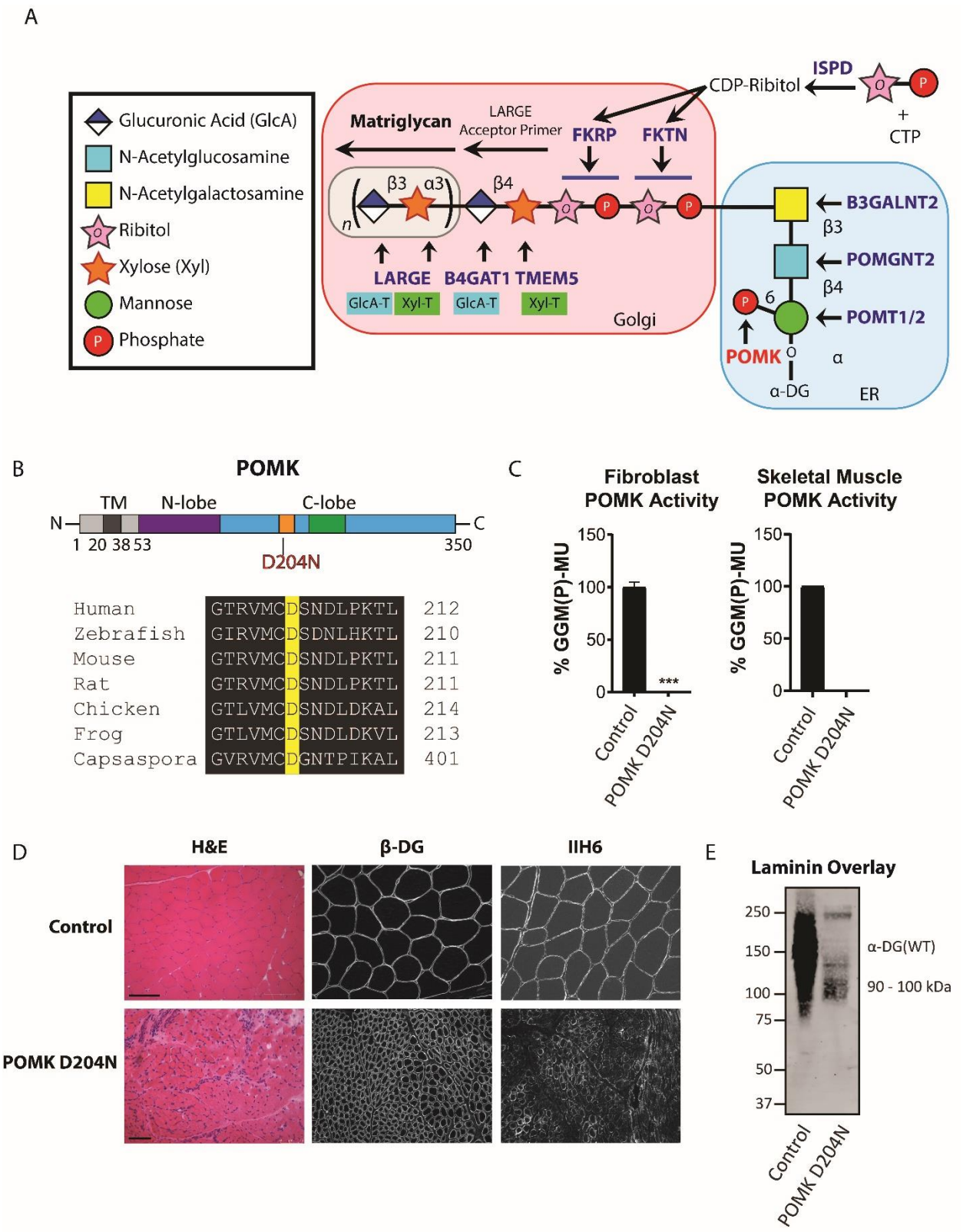
A
Glucuronic Acid (GlcA)
N-Acetylglucosamine
N-Acetylgalactosamine
Ribitol
Xylose (Xyl)
Mannose
Phosphate
Matriglycan
LARGE
Acceptor Primer
FKRP
FKTN
CDP-Ribitol
ISPD
+
CTP
β3
α3
β4
n
LARGE
B4GAT1
TMEM5
GlcA-T
Xyl-T
GlcA-T
Xyl-T
Golgi
B3GALNT2
β3
POMGNT2
β4
6
POMT1/2
POMK
α
α-DG
ER
B
POMK
TM
N-lobe
C-lobe
N
C
1 20 38 53
350
D204N
Human GTRVMCDSNDLPKTL 212
Zebrafish GIRVMCDSDNLHKTL 210
Mouse GTRVMCDSNDLPKTL 211
Rat GTRVMCDSNDLPKTL 211
Chicken GTLVMCDSNDLDKAL 214
Frog GTLVMCDSNDLDKVL 213
Capsaspora GVRVMCDGNTPIKAL 401
C
Fibroblast POMK Activity
Skeletal Muscle POMK Activity
% GGM(P)-MU
150
100
50
0
***
Control
POMK D204N
D
H&E
β-DG
IIH6
Control
POMK D204N
E
Laminin Overlay
250
150
100
75
50
37
α-DG(WT)
90 - 100 kDa
Control
POMK D204N

Fig. 1. Model of Matriglycan Synthesis and Characterization of a Patient with a POMK Mutation. A) Model depicting synthesis of the laminin-binding modification on α-DG and the enzymes involved. Core M3 (GalNac-β3-GlcNac-β4-Man) is synthesized by POMT1/2, POMGNT2, and B3GALNT2. POMK phosphorylates the C6 hydroxyl of mannose. The phosphorylated core M3 is elongated by FKTN, FKRP, TMEM5, and B4GAT1 in the Golgi. ISPD produces CDP-Ribitol in the cytosol, which is a substrate for FKTN and FKRP. LARGE synthesizes matriglycan, which is the ligand-binding moiety. **B)** Domain structure of POMK and location of D204N mutation. (above) Human POMK is composed of a signal peptide, transmembrane domain (TM), and a kinase domain (N-lobe and C-lobe). The kinase domain contains the catalytic loop (orange) and activation segment (green). The patient's missense mutation (D204N) is present within a conserved MCD motif. (below) Alignment of protein sequences flanking the D204N mutation. The mutation alters a highly conserved aspartate that serves as the catalytic base in the phosphorylation reaction catalyzed by POMK. **C)** POMK activity in control and patient NH13-284 (POMK D204N) fibroblasts (left) and skeletal muscle (right). For fibroblast POMK activity, n=3 experiments were performed. Triple asterisks indicate p-value<0.001. Due to the limited amount of skeletal muscle, n=1 experiment was performed. **D)** Histology and immunofluorescence staining of cryosections from control and POMK D204N skeletal muscle using IIH6, a monoclonal antibody against matriglycan, and an antibody against β-DG. (Scale bars: Control H&E- 200 µM, POMK D204N H&E 75 µM). **E)** Laminin overlay of control and POMK D204N skeletal muscle.

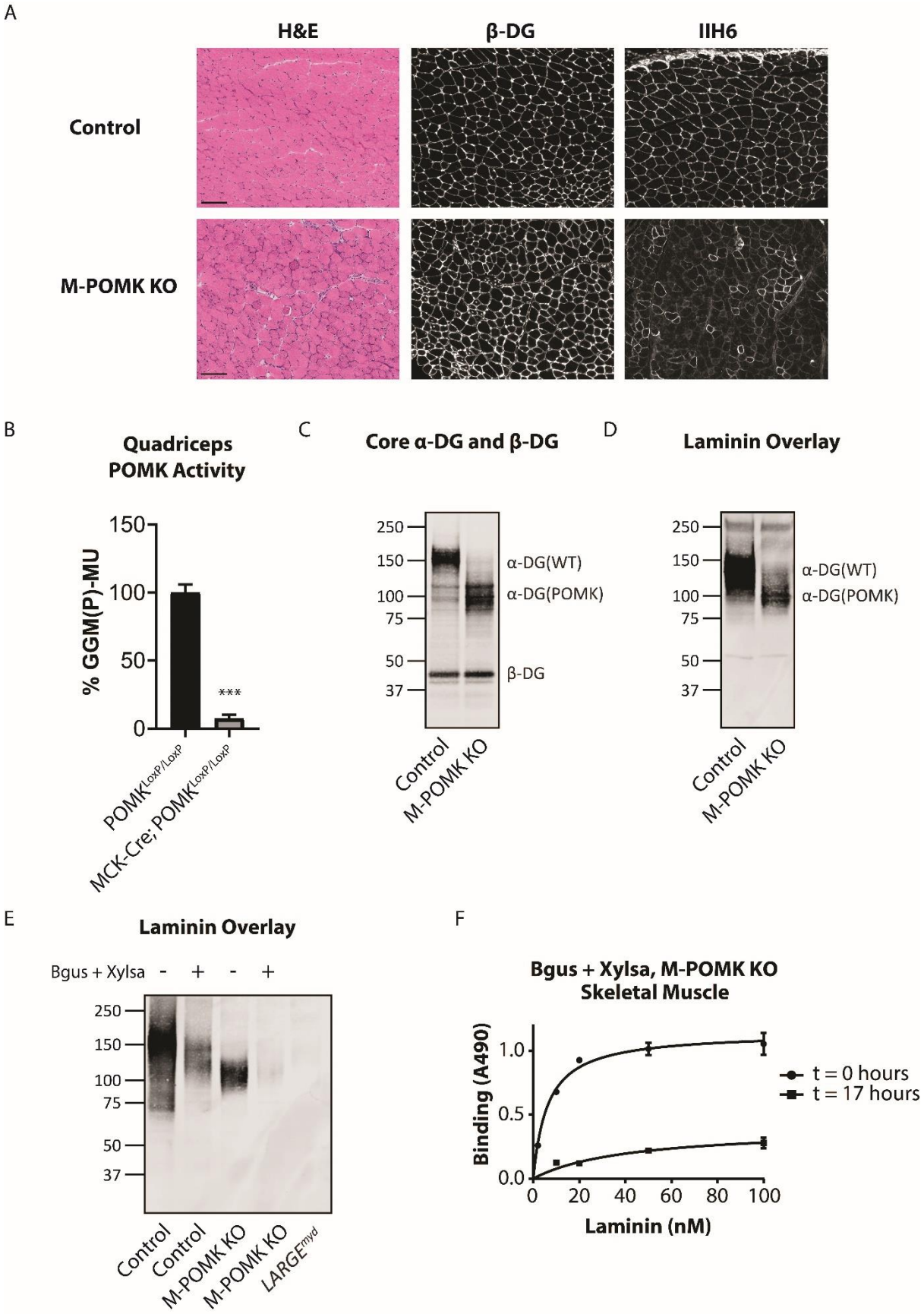
A
H&E
β-DG
IIH6
Control
M-POMK KO
B
Quadriceps
POMK Activity
% GGM(P)-MU
150
100
50
0
***
POMK LoxP/LoxP
MCK-Cre; POMK LoxP/LoxP
C
Core α-DG and β-DG
250
150
100
75
50
37
α-DG(WT)
α-DG(POMK)
β-DG
Control
M-POMK KO
D
Laminin Overlay
250
150
100
75
50
37
α-DG(WT)
α-DG(POMK)
Control
M-POMK KO
E
Laminin Overlay
Bgus + Xylsa - + - +
250
150
100
75
50
37
Control
Control
M-POMK KO
M-POMK KO
LARGE myd
F
Bgus + Xylsa, M-POMK KO
Skeletal Muscle
Binding (A490)
1.0
0.5
0.0
0
20
40
60
80
100
Laminin (nM)
t = 0 hours
t = 17 hours

Fig. 2. Mice with a Muscle-Specific Loss of POMK Express Matriglycan. A) Immunofluorescence and histologic analyses of MCK-Cre; Pax7-Cre; POMK$^{LoxP/LoxP}$ (M-POMK KO) and POMK$^{LoxP/LoxP}$ (Control) quadriceps skeletal muscle. Sections from 4 to 6 week-old mice were stained with antibodies against matriglycan (IIH6) and β-DG. Histology was evaluated by hematoxylin and eosin (H&E) staining (three replicates). Scale bars: 100 µM. **B)** POMK activity in extracts of MCK-Cre; POMK$^{LoxP/LoxP}$ and POMK$^{LoxP/LoxP}$ quadriceps skeletal muscles. Triple asterisks indicate statistical significance using a Student's unpaired t-test with a p-value<0.0001. **C)** Biochemical analysis of Control and M-POMK KO skeletal muscle. Glycoproteins were enriched from quadriceps skeletal muscles of mice using wheat-germ agglutinin (WGA)-agarose. Immunoblotting was performed with antibody AF6868, which recognizes core α-DG and β-DG (three replicates). **D)** Laminin overlay of quadriceps muscles of Control and M-POMK KO mice (three replicates). **E) and F)** Laminin overlay (E) and Solid phase analysis (F) of skeletal muscles of M-POMK KO mice treated with two exoglycosidases, α-xylosidase (Xylsa) and β-glucuronidase (Bgus) for 17 hours (three replicates).

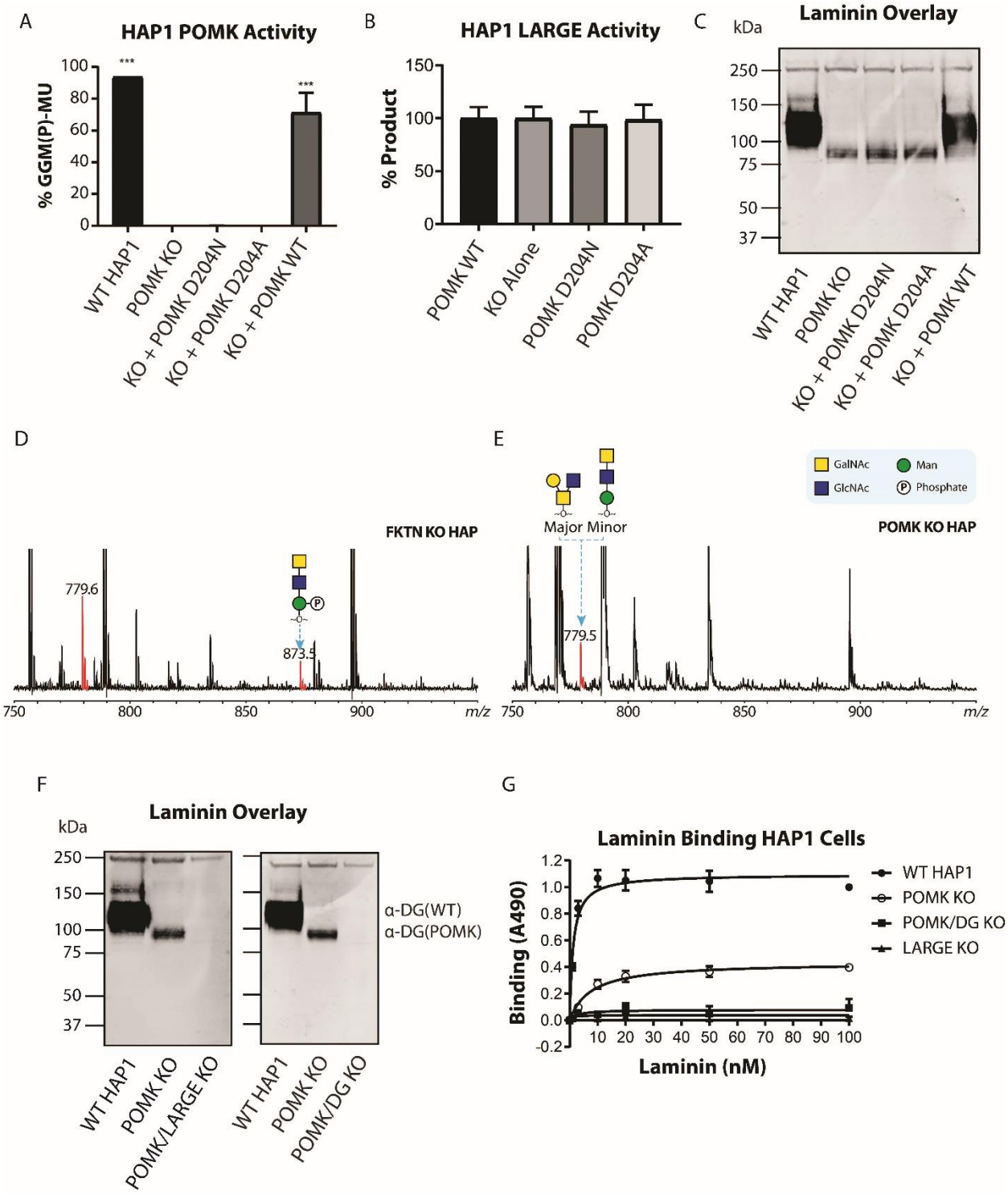
A
HAP1 POMK Activity
% GGM(P)-MU
100
80
60
40
20
0
***
***
WT HAP1
POMK KO
KO + POMK D204N
KO + POMK D204A
KO + POMK WT
B
HAP1 LARGE Activity
% Product
150
100
50
0
POMK WT
KO Alone
POMK D204N
POMK D204A
C
Laminin Overlay
kDa
250
150
100
75
50
37
WT HAP1
POMK KO
KO + POMK D204N
KO + POMK D204A
KO + POMK WT
D
FKTN KO HAP
779.6
873.5
750
800
850
900
m/z
E
GalNAc
Man
GlcNAc
Phosphate
Major Minor
POMK KO HAP
779.5
750
800
850
900
m/z
F
Laminin Overlay
kDa
250
150
100
75
50
37
α-DG(WT)
α-DG(POMK)
WT HAP1
POMK KO
POMK/LARGE KO
WT HAP1
POMK KO
POMK/DG KO
G
Laminin Binding HAP1 Cells
Binding (A490)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
10 20 30 40 50 60 70 80 90 100
Laminin (nM)
WT HAP1
POMK KO
POMK/DG KO
LARGE KO

Fig. 3. α-DG is Modified with Matriglycan in the Absence of POMK. A) POMK activity in wild-type (WT) HAP1 and *POMK* knockout (KO) HAP1 cells expressing POMK D204N, D204A, or POMK WT. Asterisks indicate P-value<0.001 compared to *POMK* KO alone using One-Way ANOVA with Dunnett's Test for Multiple Comparisons (three replicates, errors bars represent standard deviation). **B)** LARGE activity in *POMK* KO HAP1 cells with the indicated POMK mutants. **C)** Laminin overlay of *POMK* KO HAP1 cells expressing the indicated mutants was performed (three replicates). **D) and E)** Mass Spectrometry (MS)-based O-glycomic analyses of DG mucin-like domain protein (DG390TevHis) expressed in *Fukutin (FKTN)* KO HAP1 cells (D) or *POMK* KO HAP1 cells (E). The O-glycans were reductively released from the protein backbone and permethylated prior to Matrix-Assisted Laser Desorption/Ionization time-of-flight (MALDI-TOF) analyses. MS peaks at *m/z* 779.5 (779.6) correspond to a mixture of core 2 and core M3 O-glycan, and at 873.5, phosphorylated core M3 O-glycan (red). **F)** Laminin overlays of WT, *POMK* KO, and *POMK/LARGE* KO HAP1 cells (left) or WT, *POMK* KO and *POMK/DG* KO HAP1 cells (right). **G)** Solid phase analysis of WT, *POMK* KO, *POMK/DG* double KO, and *LARGE* KO HAP1 cells (three replicates).

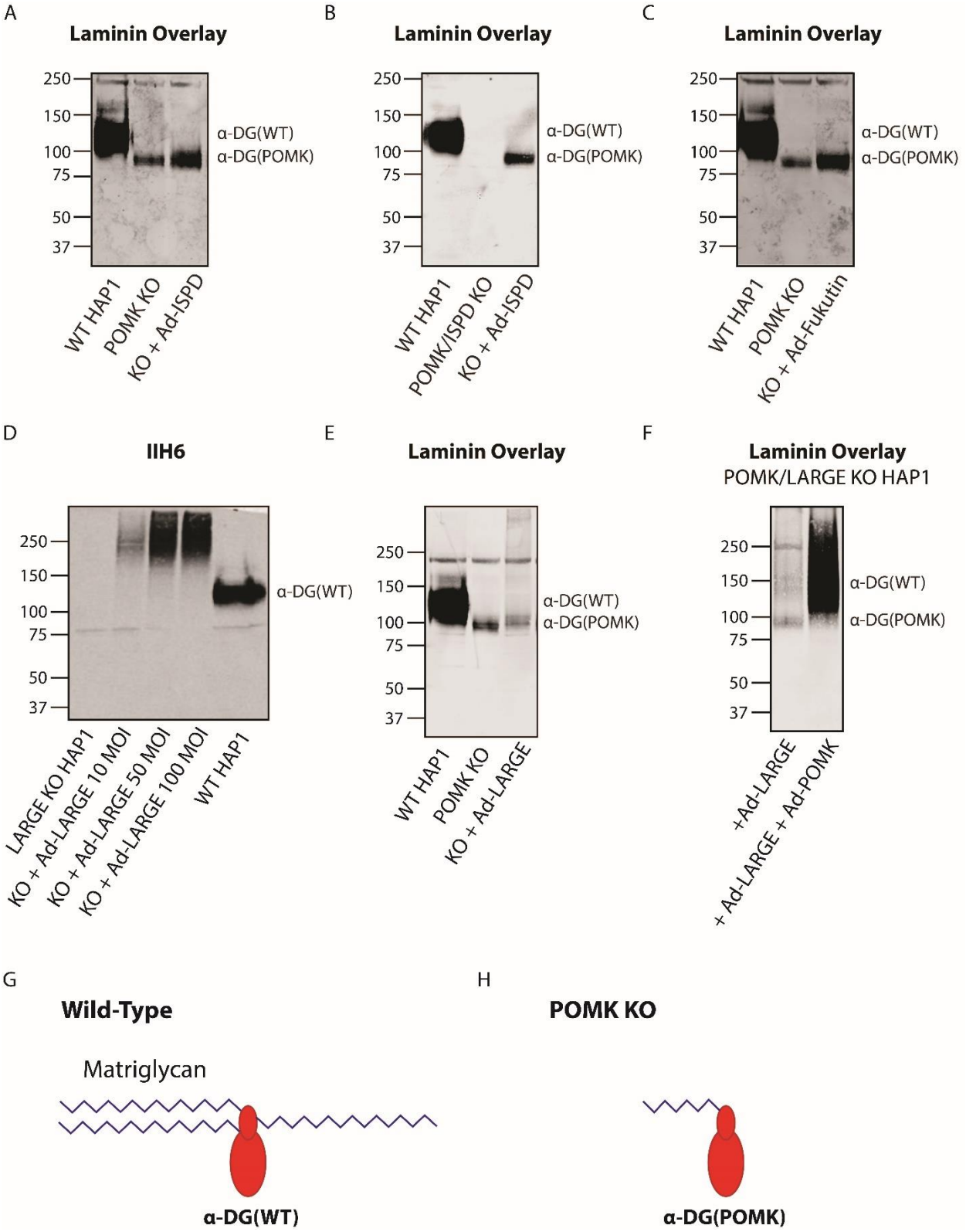
A
Laminin Overlay
250
150
100
75
50
37
α-DG(WT)
α-DG(POMK)
WT HAP1
POMK KO
KO + Ad-ISPD
B
Laminin Overlay
250
150
100
75
50
37
α-DG(WT)
α-DG(POMK)
WT HAP1
POMK/ISPD KO
KO + Ad-ISPD
C
Laminin Overlay
250
150
100
75
50
37
α-DG(WT)
α-DG(POMK)
WT HAP1
POMK KO
KO + Ad-Fukutin
D
IIH6
250
150
100
75
50
37
α-DG(WT)
LARGE KO HAP1
KO + Ad-LARGE 10 MOI
KO + Ad-LARGE 50 MOI
KO + Ad-LARGE 100 MOI
WT HAP1
E
Laminin Overlay
250
150
100
75
50
37
α-DG(WT)
α-DG(POMK)
WT HAP1
POMK KO
KO + Ad-LARGE
F
Laminin Overlay
POMK/LARGE KO HAP1
250
150
100
75
50
37
α-DG(WT)
α-DG(POMK)
+Ad-LARGE
+ Ad-LARGE + Ad-POMK
G
Wild-Type
Matriglycan
α-DG(WT)
H
POMK KO
α-DG(POMK)

Fig. 4. Characterization of the Synthesis of Non-Extended Matriglycan and Requirements for LARGE-Mediated Elongation. A), B), and C) Laminin overlays of *POMK* KO HAP1 cells overexpressing adenoviral ISPD (Ad-*ISPD*) (A), HAP1 cells lacking both POMK and ISPD overexpressing Ad-*ISPD* (B), and *POMK* KO HAP1 cells overexpressing adenoviral Fukutin (C). **D)** IIH6 immunoblotting of *LARGE* KO HAP1 cells expressing adenoviral LARGE (Ad-*LARGE*). **E) and F)** Laminin overlays of *POMK* KO HAP1 cells overexpressing Ad-*LARGE* (E), *POMK/LARGE* double KO HAP1 cells expressing Ad-*LARGE* or Ad-*LARGE* and Ad-*POMK* (F). All experiments were performed in triplicate. **G) and H)** Model depicting matriglycan on α-DG with POMK (G) and without POMK (H). Mature matriglycan is a long polysaccharide; however, a shorter, non-extended form of matriglycan is present in the absence of POMK.