Regulation of Wnt Signaling and Embryo Patterning by an Extracellular Sulfatase

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The developmental signaling functions of cell surface heparan sulfate proteoglycans (HSPGs) are dependent on their sulfation states. Here, we report the identification of QSulf1, the avian ortholog of an evolutionarily conserved protein family related to heparan-specific *N*-acetyl glucosamine sulfatases. QSulf1 expression is induced by Sonic hedgehog in myogenic somite progenitors in quail embryos and is required for the activation of *MyoD*, a Wnt-induced regulator of muscle specification. QSulf1 is localized on the cell surface and regulates heparan-dependent Wnt signaling in C2C12 myogenic progenitor cells through a mechanism that requires its catalytic activity, providing evidence that QSulf1 regulates Wnt signaling through desulfation of cell surface HSPGs.

The developmental signaling molecules that control embryo patterning for body plan specification are now well known, but less understood are the mechanisms that generate spatially localized responses to these signals within developing embryos. HSPGs are candidate regulators of embryo patterning, as these molecules are localized to the cell surface where they influence diverse developmental signals (*1*, *2*). Furthermore, the sulfation states of *N*-acetyl glucosamine residues in heparan sulfate moieties of HSPGs influence their activities in FGF (*3*–*5*) and Wnt signaling (*6*, *7*), suggesting that HSPG sulfation has a regulatory function in developmental signaling.

In this report, we identify QSulf1 as a member of a family of evolutionarily conserved sulfatases related to the lysosomal *N*-acetyl glucosamine sulfatases (G6-sulfatases) (*8*, *9*) that catalyze the hydrolysis of 6-O sulfates from *N*-acetyl glucosamines of heparan during the degradation of HSPGs (*10*). QSulf1 is homologous with G6-sulfatase throughout its NH_2-terminal region, which includes the structural domains required for the formation of the active site (*8*, *11*). QSulf1 also contains a conserved cysteine residue at position 89, a site in sulfatase enzymes that undergoes posttranslational modification to *N*-formylglycine, forming a catalytically active enzyme for sulfate hydrolysis (Fig. 1A) (*9*). In addition, QSulf1 has a distinctive hydrophylic domain that characterizes human and *Caenorhabditis elegans* Sulf orthologs (*11*), as well as a predicted NH_2-terminal secretory signal peptide (*12*), indicating that QSulf1 is an extracellular HSPG-specific sulfatase.

QSulf1 was identified in a molecular clon-

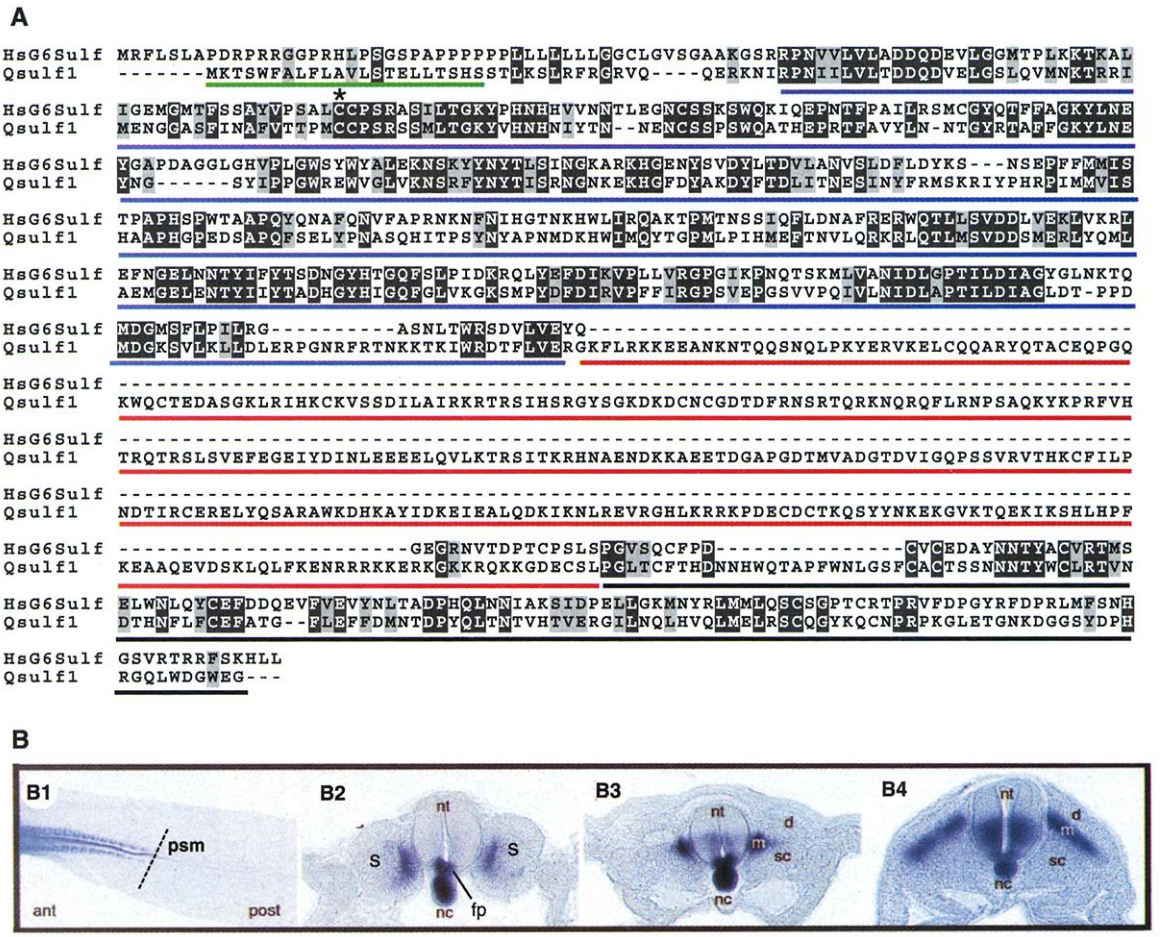

Fig. 1. QSulf1 sequence and embryonic expression. **(A)** QSulf1 (GenBank accession number AF410802) shares extensive sequence homology with human lysosomal *N*-acetylglucosamine 6-sulfatase (HuG6Sulf) (*10*). Dark and light shaded residues identify conserved amino acid residues (*33*). Bars show QSulf1 protein domains: green, signal peptide; blue, catalytic domain; red, hydrophilic domain; and black, conserved COOH-terminal domain. The asterisk indicates that *N*-formylglycine–modified cysteine is required for catalytic activity. **(B)** *QSulf1* expression in stage 12 quail embryos. (B1) *QSulf1* expression in an embryo whole mount. Dotted line shows boundary between the presegmental mesoderm (psm) and newly formed somites. (B2, B3, and B4) Transverse sections of whole mount embryos at somites II, VI, and XII. Abbreviations: ant, anterior; post, posterior; s, somite; d, dermotome; m, myotome; nt, neural tube; nc, notochord; fp, floor plate; and sc, sclerotome.

ing screen for Sonic hedgehog (Shh) response genes activated during somite formation in quail embryos (*13*, *14*). In situ hybridization studies show that QSulf1 is coexpressed with the muscle specification genes, *Myf5* and *MyoD*, in Shh-responsive epaxial muscle progenitors of newly formed somites (Fig. 1B) (*15*, *16*). *QSulf1* is also expressed in the notochord and floor plate, which produce Shh (*17*), and in the Shh-responsive ventral neural tube in the region of motor neuron specification (*18*). The role of Shh in *QSulf1* regulation was examined with Shh bead implantation and antisense inhibition studies (*14*). Disruption of notochord and floor plate *Shh* signaling through control bead implantation blocked *QSulf1* activation in epaxial somite progenitors on the displaced side of the embryo, whereas implantation of N-Shh–impregnated beads induced high-level *QSulf1* expression in somites on both sides (Fig. 2, A1 to A4). Antisense inhibition of *Shh* expression blocked *QSulf1* expression in epaxial somite muscle progenitors and neural tube progenitors, but not in the floor plate or notochord (Fig. 2, A5 to A8). Thus, *QSulf1* is a Shh response gene in the somite and neural tube.

QSulf1 function was investigated with antisense phosphorothiolated oligonucleotides developed to disrupt *QSulf1* transcript accumulation in embryos (Fig. 2, B1 to B4) (*19*). *QSulf1* antisense specifically inhibited activation of *MyoD* (Fig. 2, B5 to B8), but not *Myf5* (Fig. 2, B9 to B12), in the epaxial somite muscle progenitors and did not disrupt expression of *Pax3* or *Pax1* in the ventral somite (*11*). As *Myf5*, *Pax3*, and *Pax1* are Shh response genes (*14*, *20*), QSulf1 does not function in Shh signaling. However, *MyoD* is Wnt-inducible (*21*–*23*), implicating QSulf1 in Wnt signaling, which is controlled by HSPGs (*7*, *24*, *25*), the likely substrate of QSulf1 activity.

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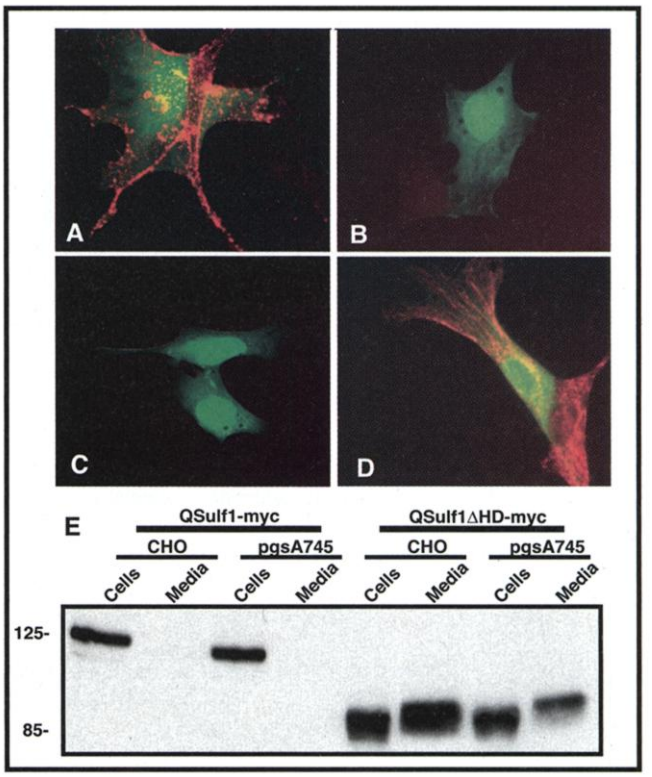

Fig. 3. Cell surface localization of QSulf1. **(A)** 10T1/2 cells transfected with QSulf1-myc and GFP expression plasmid and reacted as unpermeablized live cells with antibody to myc. **(B)** 10T1/2 cells transfected with pAG-myc and GFP expression plasmid (green) and reacted as unpermeablized cells with antibody to myc. **(C)** 10T1/2 cells transfected with QSulf1-myc and control GFP expression plasmid (green) and reacted as unpermeabilized cells with antibodies to β-tubulin. **(D)** 10T1/2 cells transfected with QSulf1-myc and control GFP expression plasmids and reacted with fixed and permeablilized cells before staining with antibodies to β-tubulin. Green, GFP; red, antibody to myc and antibody to β-tubulin. **(E)** Western blot assays of cell extracts and medium from CHO and *pgs*A745 mutant CHO cell cultures transfected with QSulf1-myc and hydrophilic domain mutant pAG-QSulf1(ΔHD)-myc expression plasmids.

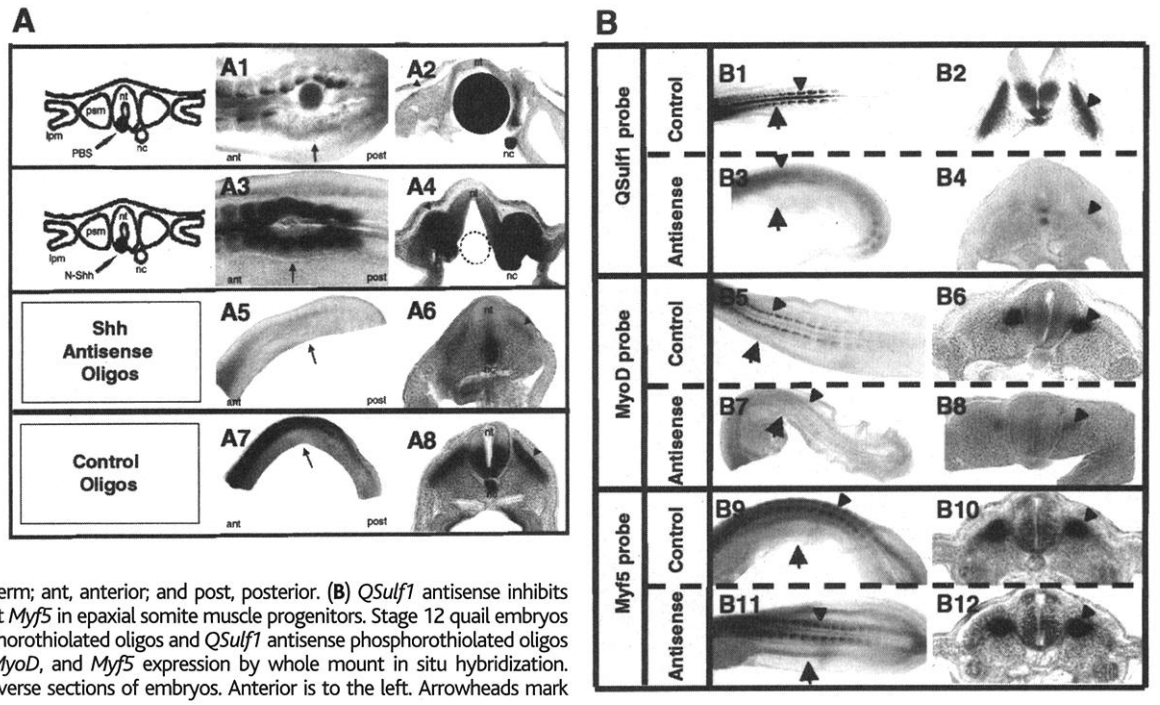

Fig. 2. *QSulf1* regulation and embryonic function. **(A)** *QSulf1* is a *Shh* response gene. In situ hybridization analysis of *QSulf1* expression in stage 12 quail embryos implanted with control beads impregnated with phosphate-buffered saline (PBS) (A1 and A2) and N-Shh–impregnated beads (A2 and A4) and embryos treated with *Shh* antisense oligos (A5 and A6) and with control oligos (A7 and A8). Arrows mark site of transverse embryo sections. Arrowheads mark somites. Abbreviations: nt, neural tube; nc, notochord; lpm, lateral plate mesoderm; psm, presegmental mesoderm; ant, anterior; and post, posterior. **(B)** *QSulf1* antisense inhibits activation of *MyoD*, but not *Myf5* in epaxial somite muscle progenitors. Stage 12 quail embryos treated with control phosphorothiolated oligos and *QSulf1* antisense phosphorothiolated oligos and assayed for *QSulf1*, *MyoD*, and *Myf5* expression by whole mount in situ hybridization. Arrows mark sites of transverse sections of embryos. Anterior is to the left. Arrowheads mark somites.

To assess whether QSulf1 is secreted, we cotransfected 10T1/2 cells with QSulf1-myc along with a green fluorescent protein (GFP) expression vector to identify transfected cells (*26*). Unpermeabilized cells were reacted with antibodies to myc to detect cell surface–localized QSulf1-myc or with antibody to β-tubulin, as a control for cell permeability. QSulf1-myc was detected on the cell surface (Fig. 3, A to D), whereas β-tubulin staining was undetected. Western blot assays reveal that QSulf1-myc is not freely released into the culture medium of transfected Chinese hamster ovary (CHO) cells, consistent with its cell surface localization (Fig. 3E). Cell surface localization of QSulf1 has been demonstrated in C2C12, CHO, 293T, and 10T1/2 cells with immunostaining and Western blot assays (*27*), suggesting that QSulf1 is docked to widely expressed cell surface components. QSulf1-myc was not released from the cell surface of CHO *pgsA745* mutant cells that lack xylosyltransferase and are defective for the synthesis of glycosaminoglycans (*28*), establishing that QSulf1 is not docked to heparan on HSPGs (Fig. 3E). QSulf1 is also not released from the cell surface by heparin or heparitinase (*27*), further demonstrating that QSulf1 is not docked by heparan. However, a hydrophylic domain mutant of QSulf1 with a deletion of amino acids 418 to 736 [QSulf1 (ΔHD)-myc] is freely released into the medium (Fig. 3E), indicating that QSulf1 is docked through interactions of the hydrophilic domain with cell surface components.

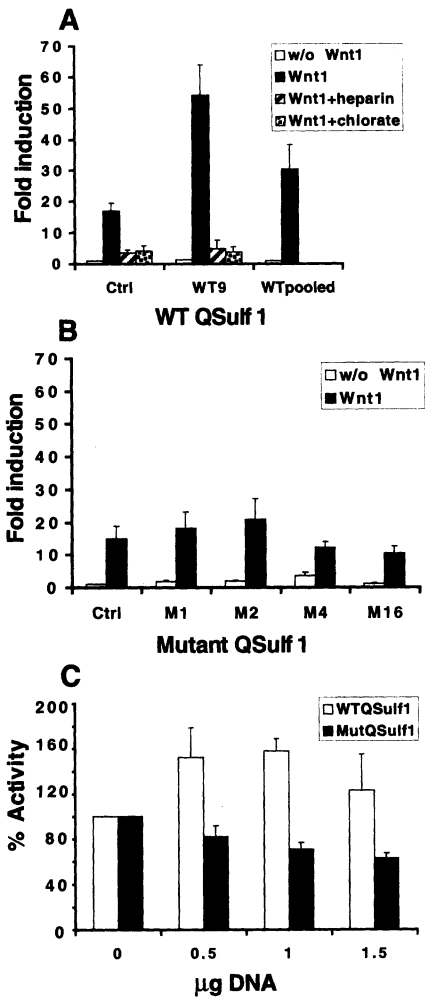

Fig. 4. Catalytically active QSulf1 promotes Wnt signal transduction in C2C12 muscle progenitors. (**A**) TCF luciferase reporter activities in control (Ctrl) C2C12 cells transfected with pAG-myc vector, a clonal C2C12 line (WT9) stably transfected with QSulf1-myc, and a pool of 200 C2C12 clones (WTpooled) transfected with QSulf1-myc and cocultured with Wnt1-expressing and control cells, in the presence or absence of heparin (10 μg/ml; Wnt1 + heparin) or 1 mM chlorate (Wnt1 + chlorate). (**B**) TCF reporter luciferase activities in C2C12 cells stably transfected with pAG-myc control vector (Ctrl) and clonal C2C12 cell lines (M1, M2, M4, and M16) expressing mutant QSulf1-myc (CC89,90AA). (**C**) TCF luciferase reporter activity in WT9 C2C12 cells expressing QSulf1-myc transfected with increasing concentrations of QSulf1-myc (WTQSulf1) and mutant QSulf1-myc (CC89,90AA) (MutQSulf1). Fold induction represents TCF luciferase activity normalized to the activity of the Renilla control plasmid. Results are averages of three independent experiments, and bars show SE.

QSulf1 function in Wnt signaling was assayed in C2C12 muscle progenitors with a quantitative TCF luciferase reporter gene (*29*, *30*). Wnt signaling was activated 17-fold in C2C12 cells cocultured with Wnt1-expressing cells relative to control uninduced cells (Fig. 4A). Wnt1 induction was inhibited both by heparin, which potentially binds Wnt1 and competes for its interaction with HSPGs, and by chlorate at concentrations that selectively inhibit the 6-O sulfation of *N*-acetyl glucosamine residues on HSPGs (*31*). Wnt1 induces pooled clones of QSulf1-expressing cells by 30-fold, which is twofold above the basal response of C2C12 cells to Wnt1. Clonal lines such as WT9 are induced more than 50-fold, which is a four- to fivefold increase above basal Wnt1 induction (Fig. 4A). Wnt1 induction of QSulf1-expressing C2C12 cells was also abolished both by heparin and by chlorate, establishing that QSulf1 functions to mediate HSPG-dependent Wnt signaling.

To assess whether sulfatase enzymatic activity is required for QSulf1 function, we mutated cysteines 89 and 90 to alanine (CC89,90AA) to block the *N*-formylglycine modification that is required for catalytic activity but not for substrate binding (*8*). Mutant QSulf1-myc (CC89,90AA) did not elevate Wnt signaling in C2C12, providing evidence that QSulf1 function is mediated through its enzymatic activity (Fig. 4B). Furthermore, expression of mutant QSulf1-myc (CC89,90AA) in QSulf1-expressing WT9 line (see Fig. 4C) progressively inhibited WT9 high-level Wnt1 signaling activity, whereas wild-type QSulf-1 further stimulated its Wnt signaling activity. This catalytically inactive QSulf1 (CC89,90AA) apparently acts as a dominant negative inhibitor of Wnt signaling, consistent with the expectation that mutant protein retains substrate binding activity to compete with the activity of wild-type QSulf1 in Wnt signaling.

On the basis of the high-affinity binding and sulfate-dependent activity of Wnts for heparan sulfate (*24*), QSulf1 could function in a two-step mechanism to regulate HSPG-dependent Wnt signaling. Wnts in the extracellular matrix would bind widely to heparan sulfate moieties on cell surface HSPGs, but only cells expressing QSulf1 on their cell surface would desulfate heparan sulfate to locally release HSPG-bound Wnts to its Frizzled receptor to initiate Wnt signaling. It is also possible that QSulf1 regulates FGF signaling, which is controlled by 6-O sulfation of *N*-acetyl glucosamine in HSPGs (*4*, *32*). As *QSulf1* expression is highly patterned in the embryo, QSulf1 provides a mechanism to regulate localized responses to widely distributed developmental signals for embryo patterning.

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30. Full-length QSulf1 cDNA was cloned into the pAG-myc vector (QSulf1-myc). C2C12 myoblasts were transfected with expression plasmids with a calcium phosphate protocol. Stably transfected clones were selected with hygromycin. Individual and pooled (200) clones were assayed for QSulf1 expression by immunostaining and Western blotting and for Wnt1 signaling activity by cotransfection with TCF luciferase reporter genes and with Renilla control vector (Promega Dual Luciferase Reporter Assay System). After 6 hours, transfected cells were cocultured for 24 hours with Wnt1-expressing or control rat B1a cells, in the presence or absence of heparin (Sigma; porcine intestinal mucosal) or sodium chlorate (Sigma). For some studies, WT9 QSulf1-expressing C2C12 cells were retransfected with QSulf1-myc or mutant QSulf1-myc (CC89,90AA) plasmids, as a mixture pAG empty vector, in a total of 1.5 μg of DNA per transfection.
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A Role for Thrombin Receptor Signaling in Endothelial Cells During Embryonic Development

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The coagulation protease thrombin triggers fibrin formation, platelet activation, and other cellular responses at sites of tissue injury. We report a role for PAR1, a protease-activated G protein–coupled receptor for thrombin, in embryonic development. Approximately half of *Par1*$^{-/-}$ mouse embryos died at midgestation with bleeding from multiple sites. PAR1 is expressed in endothelial cells, and a PAR1 transgene driven by an endothelial-specific promoter prevented death of *Par1*$^{-/-}$ embryos. Our results suggest that the coagulation cascade and PAR1 modulate endothelial cell function in developing blood vessels and that thrombin's actions on endothelial cells—rather than on platelets, mesenchymal cells, or fibrinogen—contribute to vascular development and hemostasis in the mouse embryo.

The serine protease thrombin is the product of a highly regulated cascade of zymogen activation that is triggered when plasma coagulation factors meet tissue factor. Tissue factor is expressed by cells that do not directly contact blood and by cytokine-activated leukocytes and endothelial cells. In the adult, thrombin is generated in the setting of tissue injury or inflammation. Thrombin cleaves fibrinogen to fibrin monomer, which polymerizes to form fibrin matrices. Thrombin also activates platelets and endothelial cells. These observations and the phenotypes of gain-of-function and partial loss-of-function mutations in coagulation factor genes in humans cast the coagulation cascade and its effector protease thrombin in the role of orchestrating hemostatic and inflammatory responses to tissue injury (*1*– *3*).

Cellular responses to thrombin are mediated, at least in part, by protease-activated G protein–coupled receptors (PARs) (*3*). Mouse embryos lacking PAR1 or coagulation factors die with varying frequency at midgestation, often with signs of bleeding (*4*–*10*). We sought to define the cellular basis for death of *Par1*$^{-/-}$ embryos and the relation between the phenotypes caused by deficiencies in coagulation factors and PAR1.

Matings between *Par1*$^{+/-}$ and *Par1*$^{-/-}$ mice in a C57BL/6J background (≥97%) yielded 135 *Par1*$^{+/-}$ and 73 *Par1*$^{-/-}$ progeny (number of *Par1*$^{-/-}$ offspring was 54% of that expected by Mendelian inheritance; $P < 0.001$ by chi-square test). Of the *Par1*$^{-/-}$ offspring, 36 were females, and 37 were males. These results confirmed the partial embryonic lethality initially reported in mixed 50%C57BL/6J-50%129/Sv and in pure 129/Sv backgrounds (*4*, *11*). *Par1*$^{-/-}$ × *Par1*$^{-/-}$ matings continued to yield approximately 50% embryonic loss over ~15 generations. This observation and the similar frequencies of embryonic death in the C57BL/6J and 129/Sv inbred backgrounds suggest that the partial penetrance of the *Par1*$^{-/-}$ phenotype is not due to a modifier gene.

Characterization of *Par1*$^{-/-}$ embryos in the C57BL/6J (≥97%) background revealed hemorrhage and cardiovascular failure at midgestation (*12*) (Fig. 1). At embryonic day 8.75 (E8.75), *Par1*$^{-/-}$ and wild-type embryos were indistinguishable by gross appearance, and somite counts were present in equal numbers in *Par1*$^{+/-}$ × *Par1*$^{+/-}$ litters. At E9.5, gross examination revealed blood in the exocoelomic and/or pericardial cavities in 22% of *Par1*$^{-/-}$ embryos. These embryos were pale, and their yolk sacs lacked blood-filled vessels. Embryos with gross bleeding usually had a dilated pericardial sac, a sign of cardiovascular failure. By E10.5, bleeding was seen in 35% of *Par1*$^{-/-}$ embryos; pericardial bleeding was especially prominent (Fig. 1D). By E12.5, 52% of *Par1*$^{-/-}$ embryos were dead with evident bleeding. The remaining E12.5 *Par1*$^{-/-}$ embryos were alive and appeared normal. Analysis of histological sections of gravid uterine segments containing *Par1*$^{-/-}$ embryos revealed that bleeding occurred earlier and more frequently than was apparent by gross examination (Fig. 1A). Collections of extravasated blood cells were seen as early as E9.0, before gross bleeding was detected, and by E9.5, blood cells were detected in the exocoelomic, amniotic, and/or pericardial and peritoneal cavities in 19 of 29 *Par1*$^{-/-}$ embryos (66%). No bleeding was detected in 16 wild-type E9.5 embryos exam-

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