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Novel Roles for Podocalyxin in Regulating Stress Myelopoiesis, Rap1a and Neutrophil Migration

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Abstract

Podocalyxin (Podxl) is a CD34 orthologue and cell surface sialomucin with reported roles in renal podocyte diaphragm slit development, vascular cell integrity, and the progression of blood, breast, and prostate cancers. Roles for Podxl during non-malignant hematopoiesis, however, are largely undefined. Presently we have developed a Vav-Cre *Podxl* knockout mouse model, and report on novel roles for Podxl in governing stress myelopoiesis. At steady-state, Podxl expression among hematopoietic progenitor cells was low-level but was induced by GCSF (granulocyte colony stimulating factor) in myeloid progenitors, and by TPO (thrombopoietin) in HSCs. In keeping with low level *Podxl* expression at steady-state, Vav-Cre deletion of Podxl did not markedly alter peripheral blood cell levels. G-CSF challenge in Podxl-KO mice, in contrast, hyper-elevated peripheral blood neutrophil and monocyte levels. Podxl-KO also substantially heightened

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Authorship contributions

All authors contributed in substantial ways to experimental designs, study execution, data acquisition plus analysis, and manuscript construction.

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neutrophil levels following 5-fluorouracil myeloablation. These LOF phenotypes were selective, and Podxl-KO did not alter lymphocyte, basophil or eosinophil levels. Within bone marrow (and following G-CSF challenge), Podxl deletion moderately decreased CFU-GEMM and CD16/32^{pos}CD11b^{pos} progenitors but did not affect Gr-1^{pos} cell populations. Notably, Podxl-KO did significantly heighten peripheral blood neutrophil migration capacities. To interrogate Podxl's action mechanisms, a co-immunoprecipitation plus LC-MS/MS (liquid chromatography – mass spectrometry) approach was applied using hematopoietic progenitors from G-CSF-challenged mice. Rap1a, a Ras-related small GTPase, was a predominant co-retrieved Podxl partner. In bone marrow HPC's, Podxl-KO led to heightened GCSF activation of Rap1a^{GTP}, and Rap1a^{GTP} inhibition attenuated Podxl-KO neutrophil migration. Studies reveal novel roles for Podxl as an important modulator of neutrophil and monocyte formation, and of Rap1a activation, during stress hematopoiesis.

Keywords

Podocalyxin; stress myelopoiesis; G-CSF; 5-fluorouracil; Rap1a

INTRODUCTION

Podocalyxin (Podxl) is a transmembrane sialomucin, and an orthologue of CD34 and Endoglycan [1] [2]. Investigations of Podxl's actions have predominantly focused on renal podocytes, in which Podxl is important for podocyte foot extension and glomerular diaphragm slit formation [3]. Podxl has also been implicated in supporting vascular integrity [4], and its co-expression with Flk1 distinguishes embryonic Flk1^{pos}Podxl^{neg} endothelial precursor mesodermal cells from definitive Flk1^{pos}Podxl^{pos} hematopoietic progenitors [5]. Podxl additionally can mark sub-populations of embryonic CD34^{pos} hematopoietic stem cells [6], and Podxl/Pclp1 has been defined as functional marker of hematopoiesis within AGM regions [7]. Recent studies have further linked elevated Podxl expression with B-ALL and AML leukemogenesis [8] [9, 10] as well as invasive breast carcinoma [11], and tumor angiogenesis [12].

Roles for Podxl during non-malignant adult hematopoiesis are less well understood. Presently, we have investigated actions of Podxl during blood cell development by generating Podxl$^{flox/flox}$ and conditional Vav-Cre Podxl-KO mice. In keeping with observed low-level expression of Podxl at steady-state, Podxl-KO did not substantially alter peripheral blood cell levels. However, under conditions of 5-fluorouracil (5-FU) myeloablation or GCSF challenge, Podxl-KO proved to substantially and selectively up-regulate the formation of peripheral blood neutrophils and monocytes. Mechanistically (and as studied in primary hematopoietic progenitor cells) Podxl further proved to associate with Rap1a, to regulate Rap1a^{GTP} formation, and to enhance the migration properties of peripheral blood neutrophils. Studies point to Podxl as a novel hematopoietic growth factor (HGF) target, and regulator of stress granulomyelopoiesis.

MATERIALS AND METHODS

For all approaches, method details are defined in Supplemental Text.

RESULTS AND DISCUSSION

As a basic but informative analysis, *Podxl* transcript expression profiles among hematopoietic cells first were assessed. In silico analyses defined generally low level expression of *Podxl* at steady-state (as contrasted with *CD34*) (Fig-S1). Previously, we described the induced expression of Podxl by EPO in primary bone marrow erythroid progenitors [13, 14]. Here, we extended these studies to assess possible Podxl regulation by three additional hematopoietic growth factors (HGFs). Based additionally on indicated roles for Podxl in myeloid leukemia [9, 15], we first investigated possible G-CSF modulation of *Podxl*. G-CSF challenge in vivo significantly heightened *Podxl* transcript levels in isolated bone marrow myeloid HPCs (2.85 +/− 0.47-fold). Such increases in Podxl also were observed at the protein level via flow cytometry (Fig-1A). For GM-CSF, effects on *Podxl* expression were assessed using isolated bone marrow GMPs, with possible Tpo effects investigated in bone marrow HSCs. In GMP, *Podxl* levels were unchanged due to GM-CSF challenge (Fig-1B). In HSCs, however, Tpo induced *Podxl* 7.18 +/− 2.09-fold (Fig-1C). Select HGFs therefore can significantly heighten Podxl levels in HPCs.

To investigate functional roles for Podxl in regulating hematopoietic cell formation, we generated *Podxl*$^{+/flox}$ ES cells, and *Podxl*$^{f/f}$ mice. Figs-1D,E outline Podxl's protein and gene structures, together with the constructs used to flox (and delete) *Podxl* gene exons 3–7. *Podxl*$^{+/flox}$ mice were generated via ES cell blastocyst fusion, and mice with high chimerism were interbred to yield *Podxl*$^{flox/flox}$ mice. Crosses with Vav-Cre mice were then performed to generate conditionally deleted *Podxl*$^{\Delta HC}$ (hematopoietic cell deleted) mice. Flow cytometry analysis using bone marrow cells from wild type vs knockout mice further demonstrated a clear loss of signal due to Podxl-KO (Fig-1G). Podxl protein levels additionally were assessed by western blotting (Supplemental Figure S2).

At steady-state, *Podxl*'s hematopoietic deletion (Podxl$^{\Delta HC}$ mice) did not significantly alter peripheral blood cell levels (with the exception of a limited ~2.8% increase in hematocrits) (Supplemental Table-1). Following 5-FU challenge, however, neutrophil levels in Podxl$^{\Delta HC}$ mice were heightened up to 2.5-fold over wild-type congenic control mice (Fig-2A). When Podxl$^{\Delta HC}$ mice were challenged with G-CSF, peripheral blood neutrophil levels similarly were hyper-elevated, with significant increases in monocyte production also observed (Fig-2B). These effects were selective, with no such effects of Podxl-KO observed for other blood cell types. When bone marrow progenitors were analyzed (following G-CSF challenge), CFU-GEMM progenitor levels were moderately decreased (Fig-2C). Podxl-KO therefore may compromise levels of select myeloid progenitor pools. For bone marrow CD11b cells and Gr-1^{pos} HPCs, however, levels were not significantly affected by Podxl-KO (absolute numbers for CD11b: wt=117250±8504/mL, Podxl-KO=137583±4666/mL; absolute numbers for Gr1: wt=298083±927/mL, Podxl-KO=301066±1760/mL) (Fig-2D).

To begin to understand how peripheral blood neutrophil levels become elevated due to Podxl-KO, we further analyzed bone marrow cells from GCSF- dosed wt vs. KO mice using three markers: CD16 (early myeloid/monocytic progenitor marker), CD11b/Mac1 (myeloid/granulocytic progenitor marker), and Ly6G (granulocytic progenitor/granulocyte marker). In Podxl-KO bone marrow, a modest decrease in CD16^{pos}CD11b^{pos} progenitors was observed

(Supplemental Figure S3A, B), and may relate to modest decreases observed for CFU-GEMM (Fig-2C). Otherwise, Podxl-KO effects on these progenitors were limited (i.e., not significant). These findings tend to discount hyper-expansion of pro-neutrophil progenitors within bone marrow as a possible explanation for elevated production of neutrophils due to Podxl-KO.

To initially seek candidate molecular mechanisms associated with Podxl's discovered effects on stress myelopoiesis, a co-immunoprecipitation plus LC-MS/MS approach was employed. Specifically, wild-type C57BL/6 mice were dosed with G-CSF (125μg/kg, d1–d5). Bone marrow HPCs were then isolated (d6), and cell lysates were prepared. Podxl together with associated partner proteins were then immunoprecipitated, reduced and alkylated. Tryptic peptides were generated and analyzed by LC-MS/MS, essentially as previously described [16]. One prime Podxl co-immunoprecipitated protein was the Ras family small GTPase, Rap1a (Fig-3A). Follow-up western blot experiments revealed interesting effects of Podxl deletion on activated $Rap1a^{GTP}$. As analyzed in primary bone marrow HPCs, levels of total Rap1a were unaffected by Podxl-KO (Figs 3B–D). In the absence of G-CSF challenge, Podxl-KO led to modestly lowered baseline levels of activated $Rap1a^{GTP}$. Upon G-CSF (but not GM-CSF) challenge, levels of activated $Rap1a^{GTP}$ became markedly elevated (Figs-3B,D). Analyses of activated p-MAPK indicated only limited consequences of Podxl-KO on this candidate target of $Rap1a^{GTP}$ (Figs-3C,D). Results similar to those observed for G-CSF also were exhibited when HPCs were challenged with IL3 (Fig-3D). To additionally assess possible pre-association of Podxl with Rap1a in the absence of GCSF challenge, co-immunoprecipitation experiments were performed using lineage negative bone marrow HPCs from wild-type mice, and detectable co-IP of Rap1a with Podxl was observed (AK and PS, data not shown). This latter observation may help to explain how, in the absence of GCSF challenge, Podxl-KO resets baseline activation of Rap1a-GTP. Podxl therefore may be prepositioned to regulate Rap1a.

In a final set of experiments, migration properties of Podxl-KO peripheral blood neutrophils were analyzed. Migration rates (as assayed using trans-well chambers) interestingly were significantly enhanced over $Podxl^{+/+}$ neutrophils (Fig-3E). To initially assess roles for Rap1a in Podxl-KO potentiated neutrophil migration, we utilized a specific Rap1a inhibitor, GGTI-2147. GGTI-2147 treatment attenuated Podxl KO induced neutrophil migration ≥3 fold (Fig-3E). It is notable, however GGTI-2147 did not significantly affect the migration of Podxl wild-type peripheral blood neutrophils. Heightened sensitivity of Podxl-KO neutrophils to GGTI-2147 therefore may relate to decreased levels of $Rap1a^{GTP}$ observed in bone marrow granulomonocytes (see Fig-3B). In Figure 3G, working models are outlined for HGF regulation of *Podxl*, and for Podxl governing of neutrophil migration from bone marrow and/or (e)migration potential.

In summary of the present findings, using a floxed allele plus Vav1-Cre approach, we have generated a blood cell conditional knockout mouse model to investigate Podocalyxin's roles during adult hematopoiesis (and prospectively, leukemogenesis). In keeping with low *Podxl* expression among HPCs at steady-state (see Fig-S1), Podxl-KO did not significantly alter peripheral blood populations (with the exception of mild polycythemia) (see Supplemental Table-S1). G-CSF dosing, however, proved to induce *Podxl* expression in bone marrow

myeloid progenitors, and this focused our investigations on stress hematopoiesis. Following G-CSF or 5-FU treatment, Podxl-KO substantially heightened peripheral blood neutrophil levels, with G-CSF additionally boosting Podxl-KO monocyte levels. In relating these phenotypes to bone marrow HPC pools, CFU-GEMM were diminished compared to *Podxl*$^{f/f}$ controls as were bone marrow CD16/32^{pos}CD11b^{pos} granulomyeloid progenitors. Gr1^{pos} cell levels, however, were not significantly affected. Podxl-KO effects on heightened blood granulocytes (and monocytes) therefore likely involve mechanisms beyond a simple over-production of myeloid progenitors. Our initial data further indicate roles for Podxl in neutrophil migration (Fig-3E). Notably, as a complex cell surface sialomucin, Podxl has been demonstrated to interact with select integrins and selectins [1, 2] and via intracellular domains can also interact with Ezrin and actin as cytoskeletal components [1, 2].

Co-immunoprecipitation LC-MS/MS experiments provided initial molecular insight into Podxl's effects on granulomyelocytic cells, with Rap1a identified as a novel Podxl partner. One major effect of Podxl-KO was to increase Rap1a^{GTP} levels in G-CSF challenged hematopoietic progenitors. As Ras-related GTPases, Raps are tightly regulated by select GEFs and GAPs, and by extracellular cues [17]. Their engagement in diverse tissues and cell types in addition can modulate cell migration, and adhesion [18]. This is consistent with our observed effects of Podxl-KO on enhancing neutrophil migration (see Fig-3E).

Taken together, the present investigations have uncovered novel roles for Podxl in governing stress myelopoiesis. We have also begun to define correlations between Podxl deletion, Rap1a^{GTP} dysregulation, and heightened neutrophil migration due to Podxl-KO. This points to a Podxl plus Rap1a circuit that may regulate peripheral blood neutrophil levels during stress hematopoiesis (Fig-3F). Consistent with our findings, the knockout of the positive Rap1a^{GTP} regulator, Radil, recently has been reported to compromise neutrophil chemotaxis [19]. Rap1 additionally has been characterized as a mediator of TLR4 effects on neutrophil beta-2 Integrin activation and leukocyte rolling [20]. To further understand Podxl plus Rap1 connections and actions during myelopoiesis, future studies involving the manipulation of GEF, GAP and Rap1 factors will be required. Such investigations may extend to hematopoietic malignancies, and dysregulation of Podxl, and of Rap1, recently has been associated with T-cell acute lymphoblastic leukemia [21].

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HIGHLIGHTS

- Podxl is induced in hematopoietic progenitors by GCSF and TPO
- Podxl-KO increases PB granulocyte and monocyte levels following GCSF or 5FU dosing
- Podxl-KO neutrophils exhibit heightened migration capacities
- Rap1a is a Podxl partner that modulates Podxl's effects on neutrophil migration

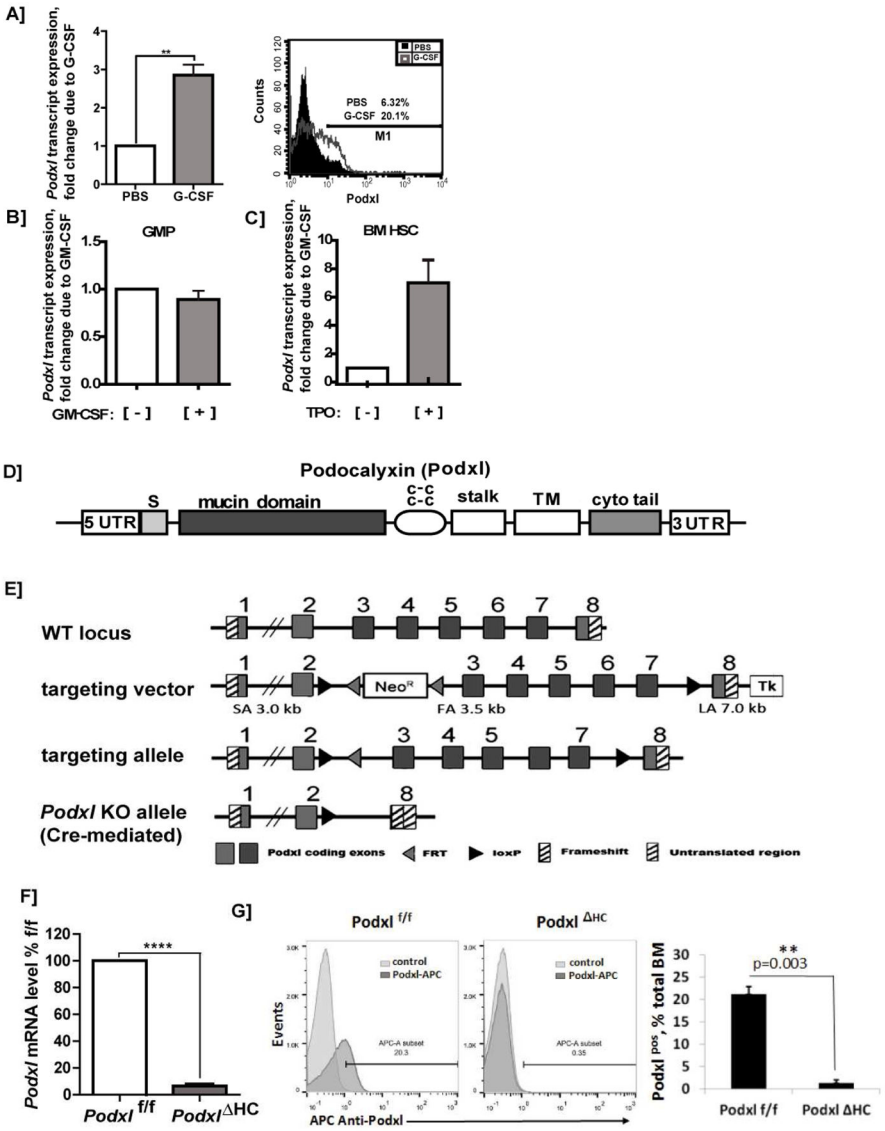
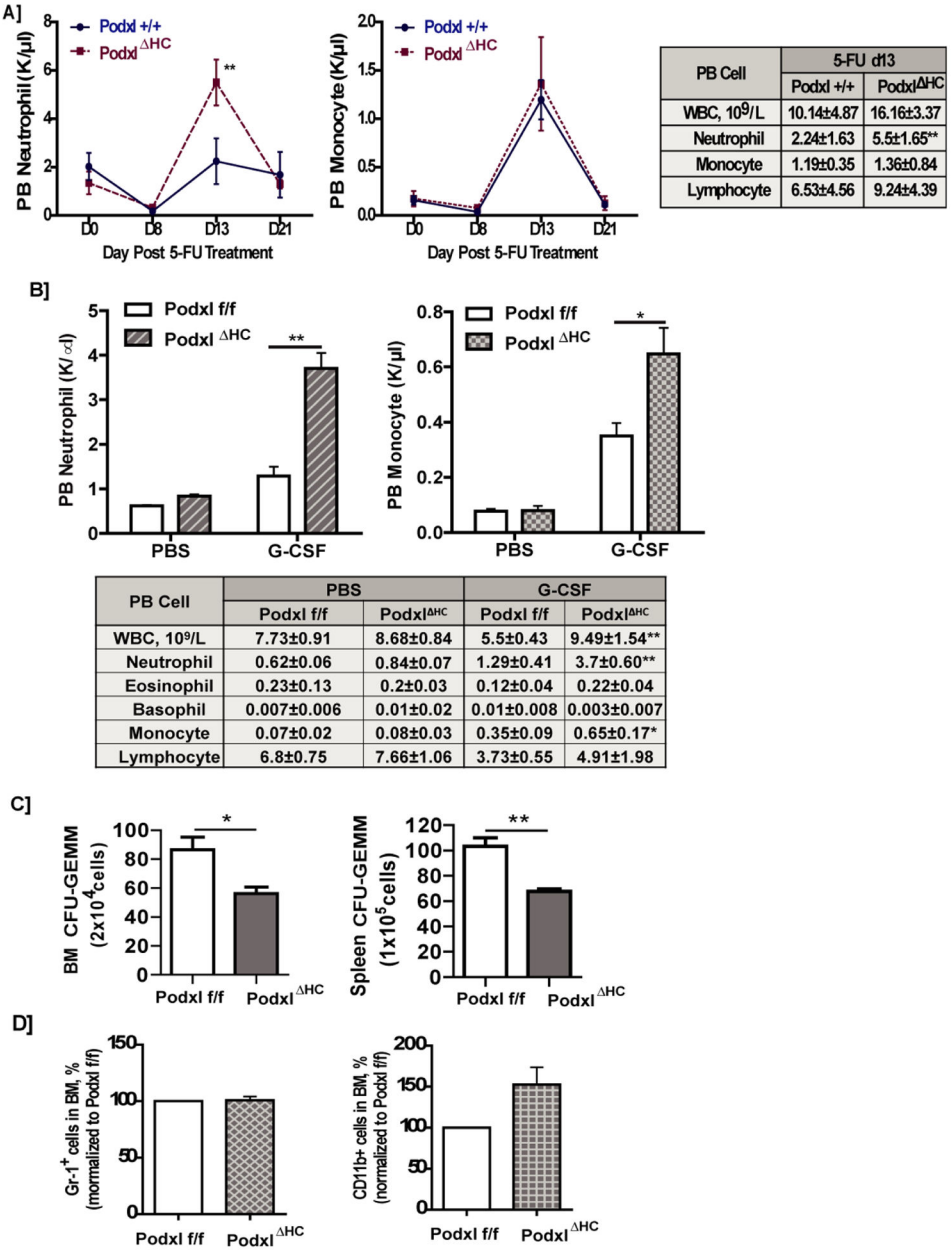


Figure 1. Hematopoietic growth factor induction of *Podxl*, locus targeting, and hematopoietic deletion of *Podxl*

A–C] GCSF, and TPO induction of Podxl expression: A] Up-modulation of *Podxl* transcript expression in bone marrow granulomyeloid progenitors in vivo: Wild-type C57Bl/6 mice were administered G-CSF (125μg/kg/IP) or PBS. On d6, granulomyeloid progenitor populations were isolated from bone marrow via the retrieval of CD11b, Gr-1, Ly6G positive cells. These hematopoietic progenitor cells (HPCs) were then analyzed for *Podxl* expression by RT-PCR (left sub-panel)(mean values +/− SE, n=3), and by flow cytometry (right sub-panel). **B]** Possible effects of GM-CSF on *Podxl* expression were assessed in isolated bone marrow GM progenitors (GMPs) as lineage negative, $CD117^{+}$, $Sca\text{-}1^{-}$, $CD34^{+}$, $CD16/32^{+}$ cells. These HPCs were challenged ex vivo [+/− GMCSF (granulocyte macrophage colony stimulating factor)], and isolated total RNA was used to determine *Podxl* levels. **C]** In HSC, possible effects of TPO on *Podxl* expression were assessed. HPCs were isolated from bone marrow as Lin^{neg} cells (using biotinylated

antibodies to CD5, CD11b, CD19, CD45R, Ly6G/C, Ter119). Lin^{neg} cells were then labeled with fluorescent antibodies to c-Kit and Sca1, and HSCs were purified via FACS. HSCs were then challenged [+] vs [−] TPO. Total RNA was purified, and used to determine *Podxl* expression levels. **D]** Podxl's structural subdomains are diagrammed including its signal peptide (S), mucin domain, cysteine-rich region (c-c), stalk plus transmembrane (TM) domains, and cytoplasmic tail (cyto tail). **E]** Targeting (floxing) of the Podxl gene: Details are diagrammed for exon floxing, and Cre-mediated deletion. **F]** RT-qPCR analysis of *Podxl* transcripts in hematopoietic progenitor cells from wild-type and $Podxl^{\Delta HC}$ bone marrow: Hematopoietic progenitor cells (HPCs) were prepared as Lin^{neg} populations. *Podxl* levels were then determined as normalized to *beta-Actin* (means +/− SE, n=3). **G]** Representative flow cytometric analysis of Podxl expression in bone marrow cell preparations from wild-type and $Podxl^{\Delta HC\text{-}KO}$ mice (left panel). In the right panel, results for triplicate samples are illustrated (mean % positive +/− SE).



PB Cell	5-FU d13	
	Podxl +/+	Podxl$^{\Delta HC}$
WBC, 10^9/L	10.14±4.87	16.16±3.37
Neutrophil	2.24±1.63	5.5±1.65**
Monocyte	1.19±0.35	1.36±0.84
Lymphocyte	6.53±4.56	9.24±4.39

PB Cell	PBS		G-CSF	
	Podxl f/f	Podxl$^{\Delta HC}$	Podxl f/f	Podxl$^{\Delta HC}$
WBC, 10^9/L	7.73±0.91	8.68±0.84	5.5±0.43	9.49±1.54**
Neutrophil	0.62±0.06	0.84±0.07	1.29±0.41	3.7±0.60**
Eosinophil	0.23±0.13	0.2±0.03	0.12±0.04	0.22±0.04
Basophil	0.007±0.006	0.01±0.02	0.01±0.008	0.003±0.007
Monocyte	0.07±0.02	0.08±0.03	0.35±0.09	0.65±0.17*
Lymphocyte	6.8±0.75	7.66±1.06	3.73±0.55	4.91±1.98

Figure 2. Governing of stress myelopoiesis by Podxl

A] *Podxl*$^{+/+}$ and *Podxl*$^{\Delta HC}$ mice were administered 5-FU (150 mg/kg). On the days indicated, peripheral blood cell populations were assayed. Left and center panels report on numbers of peripheral blood (PB) neutrophils and monocytes among *Podxl*$^{+/+}$ and *Podxl*$^{\Delta HC}$ mice (means +/− SE, n=4). The right panel reports on total white blood cell (WBC), neutrophil, monocyte and lymphocyte counts in PB from *Podxl*$^{+/+}$ and Podx*l*$^{\Delta HC}$ mice at d13 post 5-FU dosing (means +/− SE, n=4). **B]** Podxl deletion dysregulates G-CSF induced neutrophil and monocyte formation: Podxl$^{f/f}$ and Podxl$^{\Delta HC}$ mice (n=4) were dosed on d1–5 with G-CSF (125μg/kg) or PBS. Levels of peripheral blood cells then were determined. Upper panels define neutrophil and monocyte numbers at d6 (means +/− SE). In the lower

panel, overall levels of peripheral blood neutrophils, eosinophils, basophils, monocytes and lymphocytes are reported. **C]** Following G-CSF administration (125μg/kg, d1–5), bone marrow and splenic cells were prepared from *Podxl*$^{f/f}$ and *Podxl*$^{\Delta HC}$ mice. HPCs were then prepared as lineage negative bone marrow populations (depleted using antibodies to CD5, CD11b, CD19, CD45R, Ly6G/C and Ter119) and were used in CFU assays (means +/− SE< n=3). Findings for CFU-GEMM are illustrated. For CFU-GM no significant effects of Podxl-KO were observed (data not shown) **D]** For granulomyeloid progenitors prepared from *Podxl*$^{f/f}$ or *Podxl*$^{\Delta HC}$ mouse bone marrow (at steady-state) populations of FITC-anti-Gr-1 or FITC-anti-CD11b positive HPCs were assayed by flow cytometry. Here, granulomyeloid progenitors were prepared as CD11b, Gr-1, Ly6G positive populations. Data are mean frequencies of positive cells (means +/− SE, n=3).

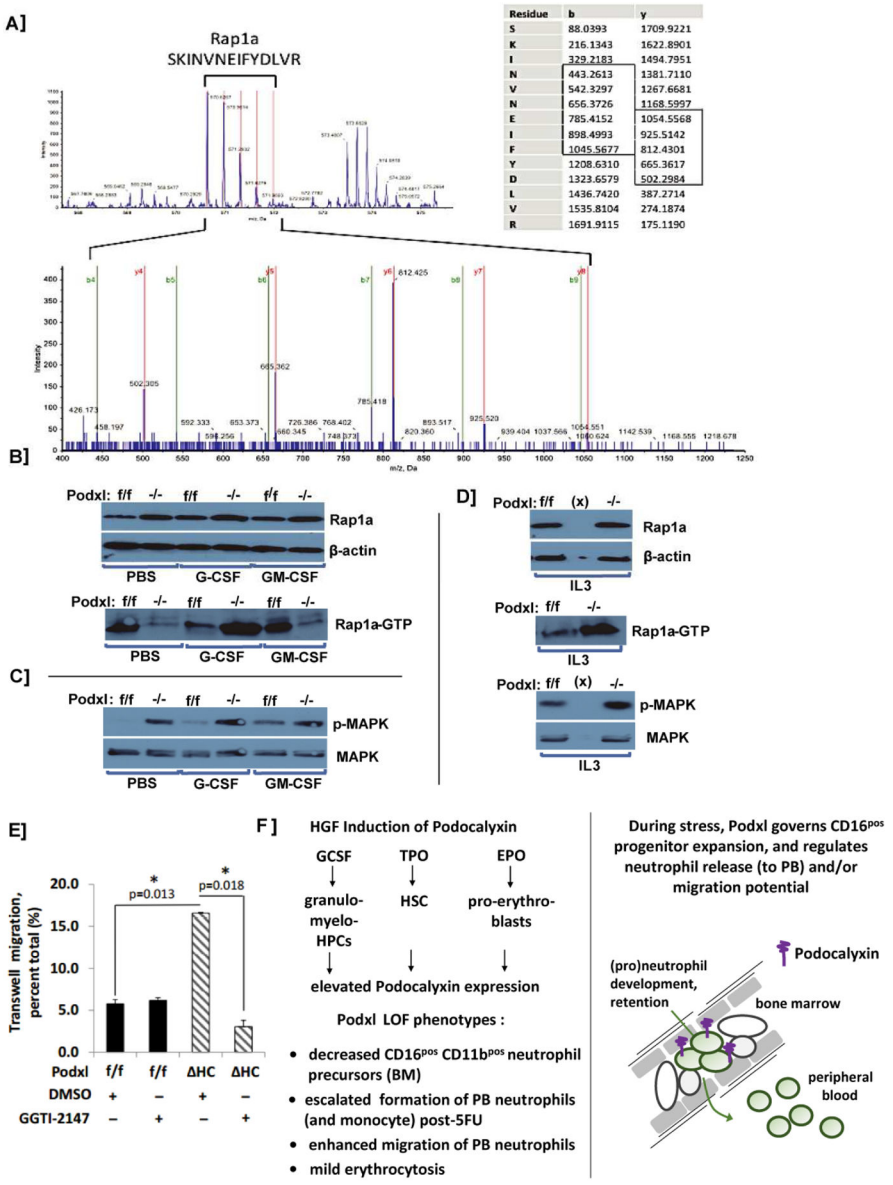


Figure 3. Podxl interacts with Rap-1A in HPCs, and regulates Rap1a^{GTP} levels

A] Following G-CSF dosing of wild-type mice, hematopoietic progenitor cells (HPCs) were isolated from bone marrow cell preparations as Lin^{neg} populations (depleted for CD5, CD11b, CD19, CD45R, Ly6G/C and Ter119). From cell lysates, Podxl (co-)immunoprecipitates were then prepared, reduced, alkylated and used to generate tryptic peptides. Peptides were analyzed by LC-MS/MS. For one predominant Podxl partner, Rap1a, representative time of flight mass spectra (MS) are shown (e.g., SKINVNEIFYDLVR). The left panel identifies precursor peptide ion mass 1708.9148, z=3 and delta mass= −0.0001 (99% confidence). The right panel summarizes collision-induced decay fragment spectra for sequence determination of SKINVNEIFYDLVR. **B]** Podxl regulates levels of activated Rap1a-GTP: Hematopoietic progenitor cells were prepared from the bone marrow of *Podxl*$^{f/f}$ and *Podxl*$^{\Delta HC}$ mice, and plated in IMDM (1x10^{6}cells/mL).

Here, Lin^{neg} bone marrow HPCs from wild type and Podxl-KO mice were used. Cells were then exposed to PBS, G-CSF, or GM-CSF. At 30 minutes, lysates were generated and analyzed by western blotting for total Rap-1A levels, and levels of GTP-bound Rap-1A (lower panel). **C]** For the above samples (4B), levels of p-MAPK and total MAPK also were assessed. **D]** Podxl-KO dysregulates IL3- induced $Rap1a^{GTP}$ formation: HPCs isolated from $Podxl^{+/+}$ and $Podxl^{\Delta HC}$ mouse bone marrow. Following HGF withdrawal, cells were stimulated with IL3 (10ng/mL, 30 minutes). Cell lysates were then prepared, and levels of Rap1a and $Rap1a^{GTP}$ were determined (as in 4B above). **E]** Podxl deletion enhances neutrophil migration: Peripheral blood neutrophils were prepared via density gradient centrifugation using Histopaque-1077 and Histopaque-1119. Trans-well migration of peripheral blood neutrophils from wild type and Podxl-KO mice was then assessed +/− the Rap1a inhibitor GGTI-2147. Graphed results are mean values +/− SE (n=3) for trans-membrane migrating cells. **F]** Initial model for Podxl governing of stress myelopoiesis: Left panel: HGF effects on *Podxl* expression are outlined (upper sub-panel), together with Vav1-conditional Podxl-KO phenotypes. Right panel: A working model is outlined at a cellular level for proposed Podocalyxin governing of peripheral blood neutrophil (e)migrations.