

Published in final edited form as:

*Development*. 2008 October ; 135(20): 3447–3458. doi:10.1242/dev.025916.

## NUMB Mediates the Interaction between Wnt and Notch to Modulate Primitive Erythropoietic Specification from the Hemangioblast

Xin Cheng<sup>1</sup>, Tara L. Huber<sup>2</sup>, Vincent C. Chen<sup>1</sup>, Paul Gadue<sup>1</sup>, and Gordon M. Keller<sup>3,\*</sup>

<sup>1</sup>Department of Gene and Cell Medicine; Mount Sinai School of Medicine; New York, NY 10029, USA

<sup>2</sup>Stem Cell and Developmental Biology Department; Genome Institute of Singapore 138672; Singapore

<sup>3</sup>McEwen Centre for Regenerative Medicine; University Health Network; Toronto, Ontario, Canada

### Summary

During embryonic development, the establishment of the primitive erythroid lineage in the yolk sac is a temporally and spatially restricted program that defines the onset of hematopoiesis. In this report we have used the embryonic stem cell differentiation system to investigate the regulation of primitive erythroid development at the level of the hemangioblast. We show that the combination of Wnt signaling together with inhibition of the Notch pathway is required for the development of this lineage. Inhibition of Notch signaling at this stage appears to be mediated by the transient expression of Numb in the hemangioblast-derived blast cell colonies. Activation of the Notch pathway was found to efficiently inhibit primitive erythropoiesis through the upregulation of inhibitors of the Wnt pathway. Together, these findings demonstrate that specification of the primitive erythroid lineage is controlled, in part, by the coordinated interaction of the Wnt and Notch pathways and position Numb as a key mediator of this process.

### Introduction

The development of blood islands in the yolk sac marks the onset of hematopoiesis in the mammalian embryo. These blood islands consist of a population of maturing primitive erythrocytes surrounded by developing endothelial cells that will form part of the yolk sac vasculature (reviewed by Ferkowicz and Yoder, 2005). Primitive erythrocytes represent the predominant population generated during the yolk sac stage of hematopoiesis and are characterized by their large size and their expression of embryonic hemoglobin (Barker, 1968). Production of this lineage, primitive erythropoiesis, represents a unique developmental program within the hematopoietic system in that it is transient and restricted to one site, the yolk sac (Palis et al., 1999). Along with the primitive erythrocytes, the yolk sac generates a subset of other hematopoietic cell types including macrophages and progenitors of the definitive erythroid, megakaryocyte and mast cell lineages (Palis et al., 1999). These populations are considered as the yolk sac definitive hematopoietic lineages and differ from the primitive erythroid lineage in that they are also generated in other hematopoietic sites including the fetal liver and bone marrow (reviewed by Keller et al., 1999).

\*corresponding authors Contact: gkeller@uhnresearch.ca, phone: 416-581-7694, fax: 416-595-5719 (G.K.).

The hematopoietic and vascular lineages of the yolk sac are derived from the first mesodermal population generated during gastrulation (Haar and Ackerman, 1971). Following induction, these mesodermal cells migrate proximally from the primitive streak to the extra-embryonic region where they rapidly differentiate and give rise to vascular and hematopoietic cells (Tam and Behringer, 1997). Insights into the developmental progression of mesoderm to these derivative lineages have come from studies using the embryonic stem (ES) cell differentiation model (reviewed in Keller, 2005). These studies demonstrated that one of the earliest steps in this process is the generation of a progenitor that displays both hematopoietic and vascular potential (Choi et al., 1998 and Nishikawa et al., 1998). This progenitor known as the blast colony-forming cell (BL-CFC) co-expresses the receptor tyrosine kinase Flk-1 and the T box transcription factor brachyury and is considered to represent the *in vitro* equivalent of the hemangioblast (Fehling et al., 2003). Analysis of the hematopoietic potential of the BL-CFC revealed that it displays the capacity to generate primitive erythroid cells as well as the spectrum of definitive lineages found in the yolk sac (Kennedy et al., 1997; Choi et al., 1998). As such, the hemangioblast can be considered to be the immediate progenitor of the primitive erythroid lineage. Following the discovery of the BL-CFC in mouse ES cell cultures, a progenitor with virtually identical characteristics was identified in the posterior primitive streak (PS) of the gastrulating embryo at a stage prior to the establishment of the yolk sac blood islands (Huber et al., 2004). The properties of this progenitor suggest that it represents the yolk sac hemangioblast, the progenitor of the yolk sac hematopoietic program.

The transient nature of primitive erythropoiesis indicates that the regulation of this lineage is tightly controlled, possibly by mechanisms that differ from those that control development of the other lineages in the yolk sac. Targeting studies have identified several key regulators of yolk sac hematopoiesis including VEGF/Flk-1 (Shalaby et al., 1995), TGF $\beta$ 1 (Dickson et al., 1995) and erythropoietin/EpoR (Wu et al., 1995; Lin et al., 1996) and have demonstrated that they function at specific stages, ranging from establishment of the hematopoietic and vascular lineages, to expansion of specific populations following their induction. To date, none of these signaling pathways has been shown to specifically regulate primitive erythropoiesis. Several recent studies have provided evidence suggesting that the Notch and Wnt pathways may play a role in the regulation of this early blood cell lineage. The Notch pathway appears to function in an inhibitory capacity as Notch1 null ES cells and RBP-J $\kappa$  mutant embryos display enhanced primitive erythroid potential compared to wild-type counterparts (Hadland et al., 2004; Robert-Moreno et al., 2007). Wnt signaling, on the other hand appears to positively regulate the development of this lineage, as blocking the pathway dramatically reduced primitive erythroid development from Flk-1+ progenitor cells in mouse ES cell differentiation cultures (Nostro et al., 2008). The opposite effects of the Notch and Wnt signaling on primitive erythroid development suggest that these two pathways comprise part of the regulatory network that controls this early blood cell program.

In the current study we have further evaluated the role of the Notch and Wnt pathways in the specification of the earliest hematopoietic lineages, focusing on the BL-CFC/hemangioblast stage of development. From these studies, we provide evidence that the combination of canonical Wnt signaling together with the inhibition of the Notch pathway by Numb is required for primitive erythroid specification from this progenitor. Notch signaling was found to inhibit primitive erythropoiesis through the upregulation of inhibitors of the Wnt pathway. Immunostaining analysis of early stage embryos demonstrated the presence of Numb and  $\beta$ -catenin in the region of the developing yolk sac, suggesting that these pathways also regulate this lineage *in vivo*. Together, these findings provide the first detailed insights into the signaling networks controlling specification of the primitive erythroid lineage from the hemangioblast.

## Materials and Methods

### ES cell lines

The ES line Bry-GFP (Fehling et al., 2003) was used for the gene expression analysis of developing hemangioblast-derived colonies. The ES line A2lox.sβcat (sβ-cat) that contains a stabilized β-catenin gene under control of a doxycyclin-regulated promoter (Lindsley et al., 2006) was a gift from Dr. Kenneth Murphy at Washington University School of Medicine. The AinV/GFP-Bry/CD4-Foxa2 ES cell line is a tet-on inducible ES line modified from the AinV18 ES cell line (Kyba et al., 2002) with EGFP and CD4 cDNAs targeted into the *Brachyury* and *Foxa2* loci, respectively. The tet-inducible Numb and Notch1-IC expressing ES lines were generated by targeting either the PRR<sup>S</sup> Numb (Petersen et al. 2006) (gift from Dr. Weimin Zhong,) or Notch1-IC (Pear et al. 1996) (gift from Dr Warren S. Pear) cDNA into the HPRT locus of the AinV/GFP-Bry/CD4-Foxa2 ES cell line as previously described (Kyba et al., 2002).

### Flk-1+ cell reaggregation assay

For reaggregation, Flk-1+ cells were isolated by cell sorting from 2.75 EBs generated from either the GFP-Bry, Notch1-IC, Numb or sβ-cat ES cell lines. The isolated Flk-1+ cells were cultured at densities of 150,000 -200,000 cells/ml in low cluster dishes (Costar) for one or two days in a serum-free media (IMDM/F12, 3:1; with 0.5% N2-supplement and 1% B27, Gibco) supplemented with 10 ng/ml hVEGF in the presence or absence of Dox, and/or agonists or antagonists of Wnt or Notch signaling pathways. Following this short culture period, the aggregates were harvested, the cells dissociated and plated in methylcellulose hematopoietic progenitor assays to evaluate hematopoietic potential.

### RT-PCR and quantitative real-time PCR

Gene expression analyses of colonies or colony-derived single cells were performed by a modified polyA<sup>+</sup> global amplification polymerase chain reaction (PCR) as previously described (Robertson et al., 2000). Briefly, 3' cDNA was diluted and subjected to gene-specific PCR using primers designed within the most 3' 600 bp, including the UTR. The primer sequences are listed in Supplemental Table 1. PCR conditions were: 94°C, 5 min followed by X cycles (94°C, 30 s; 55-65°C, 30 s; 72°C, 30 s); 72°C, 10 min.

Real-time quantitative PCR was performed as previously described (Nostro et al., 2008) on a MasterCycler<sup>®</sup> EP RealPlex (Eppendorf) or the ABI 7900HT (Applied Biosystems). All experiments were done in triplicate using Platinum SYBR Green qPCR SuperMix or SYBR GreenER qPCR SuperMix (Invitrogen). The oligonucleotide sequences and PCR cycle conditions are listed in the supporting text (Supplemental Table S2). A 10-fold dilution series of mouse genomic DNA standards ranging from 100 ng/ml to 10 pg/ml was used to evaluate the efficiency of the PCR and calculate the copy numbers of each gene relative to the house keeping gene *actb*. cDNA samples from three independent experiments were analyzed for the expression of each genes. To reveal the gene expression patterns of developing blast colonies, 3' cDNA samples of seven individual colonies of each time point were pooled and diluted and subject to qPCR.

### Blast colony assay

The serum-based blast colony assay was as previously described (Kennedy et al., 1997). In brief, sorted Flk-1+ cells were plated in 1% methylcellulose-containing media with 10% FCS (Summit), VEGF (5 ng/ml), interleukin-6 (IL-6; 5 ng/ml) and 25% D4T endothelial cell-conditioned medium. The serum-free blast colony condition M10 was established by substituting the FCS and D4T with StemPro-34 serum-free medium (Invitrogen), and supplementing with a combination of the following 10 cytokines: KL (50ng/ml); mIL3

(20ng/ml); hMBP4 (5ng/ml); hIL11 (5ng/ml); EPO (2U/ml); hVEGF (5ng/ml), mLIF (2ng/ml); mIL6 (10ng/ml); bFGF (5ng/ml); TGF $\beta$ 1 (2pg/ml). All cytokines were purchased from R&D Systems. To evaluate their hematopoietic and vascular potential, the resulting blast colonies were picked from the methylcellulose and cultured on a matrigel-coated surface in media containing both hematopoietic and endothelial cytokines, as previously described (Choi et al., 1998). For quantification of their hematopoietic potential, pools of blast colonies were picked, the cells disassociated by treatment with 1% collagenase at 37°C for 30 min followed by trypsin for 5 min and replated in methylcellulose progenitors assays. (Kennedy and Keller, 2003). For analyzing the role of Wnt signaling in blast colony development, Dox and hDkk1 were added at 0hr and 6hr of culture respectively for a period of 24 hours. Following this treatment, the colonies were washed with IMDM to remove Dox and hDkk1 and then replated in hemangioblast conditions for an additional 2-3 days.

### Immunofluorescence

Developing blast colonies (approximately 400) were pooled in 100  $\mu$ l serum, and spread on coverslips by centrifugation at 1000rpm for 5 min with a cyto centrifuge (Thermo Shandon Ltd.). The coverslips were fixed with 4% paraformaldehyde for 30 min at room temperature, and washed with PBS three times. Fixed colonies were blocked and the cell permeabilized by incubation in 3% donkey serum and 0.1% BSA in PBS containing Triton X-100 (blocking buffer) for 30 min. The cells were incubated overnight (4°C) with either rabbit anti-mNumb (anti-NMBR1, a gift from Dr Weimin Zhong, Yale University; 1:500 in blocking buffer) or rabbit anti-activated Notch1 (GeneTex Inc. GTX28925; 1:300 in blocking buffer) and monoclonal anti-active  $\beta$ -catenin (Upstate, clone 8E7, 1:500 in blocking buffer) Following this incubation, the cells were washed six times (10 min each) and then incubated with either Cy2-conjugated donkey anti-rabbit-IgG (Jackson ImmunoResearch, 1:300 in blocking buffer at room temperature for 2 h) to reveal Numb and Notch1-IC positive cells or with Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, 1:500 in blocking buffer for 2h) to detect active  $\beta$ -catenin. After an additional six washes the coverslips were mounted onto slides with Slowfade Gold antifade reagent with DAPI (Invitrogen, Molecular Probes). Images were captured with Leica SP5 confocal laser scanning microscope (Leica Microsystems; 63 $\times$ , oil lens) by single layer scanning.

### Transient transfections, luciferase assays, and reporter plasmids

The TOP/FOP Flash reporter assay was performed to evaluate the TCF/LEF transcriptional activity induced by activated  $\beta$ -catenin. In brief,  $1 \times 10^6$  day 2.75 sorted GFP-Bry<sup>+</sup>Flk-1<sup>+</sup> cells generated from the Numb or Notch1-IC ES cell lines were co-electroporated (Mouse ES Cell Nucleofactor kit and Nucleofactor device, Amaxa Inc., with the Nucleofactor program set to O17) with two sets of plasmids, either TOPflash reporter plasmids (10  $\mu$ g/100 $\mu$ l) plus Renila-tk plasmids (1  $\mu$ g/100 $\mu$ l) or FOPflash plasmids (10  $\mu$ g/100 $\mu$ l) plus Renila-tk plasmids (1  $\mu$ g/100 $\mu$ l). The plasmids used were obtained from Dr. Sergei Sokol, Mount Sinai School of Medicine. Electroporated cells were allowed to reaggregate in the presence or absence of Dox (2  $\mu$ g/ml), and/or hDkk1 (300 ng/ml) and/or Wnt3a (100 ng/ml). Following 24 hours of culture, both the firefly and the renila luciferase activities were measured using Dual Luciferase Reporter Assay System Kit (Promega). TOP/FOP activities were calculated following the formula: TOP/FOP = (TOP firefly luciferase activity / renila luciferase activity) / (FOP firefly luciferase activity / renila luciferase activity). This experiment was repeated three times.

## Whole mount immunohistochemistry of mouse embryos

Dissected embryos were washed with PBS three times and fixed with ice-cold 100% acetone for 10 min. Following three washes with PBS+0.5%BSA, embryos were blocked and permeabilized in PBS with 0.01% Triton X-100 and 3% nonfat milk (PBSMT/40), rotating at 4°C overnight. Fixed embryos were stained with rabbit anti-mNumb (anti-NMBR1; 1:300), rabbit anti-activated Notch1 (GeneTex Inc. 1:300), or monoclonal anti-active  $\beta$ -catenin (Upstate, clone 8E7, 1:300), and with control antibodies, rabbit IgG (for numb and activated Notch1) or Mouse IgG2a (for  $\beta$ -Catenin), rotating in 50  $\mu$ l PBSMT/40 for 4 h at room temperature. The embryos were washed eight times for 15 min each in PBSMT/40 solution, then incubated for 2 h at room temperature with rotation in the presence of Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Inc., 1:300) or Cy3-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Inc., 1:300) in PBSMT/40. Following eight washes for 15 min each in PBS+0.5% BSA, embryos were mounted onto slides with Slowfade Gold antifade reagent with DAPI (Invitrogen, Molecular Probes). Images were acquired with Leica SP5 confocal laser scanning microscope (Leica Microsystems; 10 $\times$  or 20 $\times$  lenses, optimized Z-stacking). In the z (depth) direction, approximately half of the embryo was processed. Software Autodeblur (Media Cybernetics) was used for resolution improvement and noise reduction of the confocal images. Leica confocal software was used to analyze the images.

## Statistical Analysis

Results for continuous variables are expressed as mean  $\pm$  standard deviation or mean  $\pm$  standard error. Treatment groups were compared with the independent samples' t-test. P values < 0.05 were considered statistically significant.

## Results

### Gene expression analysis of developing hemangioblast-derived colonies

As a first step in defining the signaling pathways that regulate commitment of the BL-CFC/hemangioblast to its derivative lineages, we analyzed the gene expression patterns of individual blast colonies in 12 hour intervals over a 48 hour time period. During the first 24 hours of growth, the blast colonies appear as compact structures that consist of tightly packed clusters of cells (Fig. 1A). Within the next 12 to 24 hours, round cells begin to “bud” from the periphery of these structures and over the next 2 days this outer “budding” population expands dramatically and forms a loose cluster of cells that completely surrounds the inner core, resulting in a colony with typical blast colony morphology. Previous studies have shown that the outer loose cells represent the hematopoietic component of the blast colonies whereas the inner core contains the progenitors with vascular potential.

Expression analyses revealed that the mesoderm/primitive streak gene *brachyury* (Kispert and Herrmann, 1994) was downregulated within the first 24 hours of colony growth (Fig. 1B). With the loss of *Brachyury*, we observed the upregulation of expression of *CD31/PECAM1* (Watt et al., 1995), *Tie2* (Takakura et al., 1998), *Scl* (Robb et al., 1995; Shivdasani et al., 1995) and *Runx1/AML1* (Okuda et al., 1996; Wang et al., 1996), genes associated with both hematopoietic and vascular development as well as of *CD41* (Ferkowicz et al., 2003) and *Gata-1* (Orkin, 1992), genes indicative of hematopoietic commitment. The genes defining the earliest stages of endothelial specification and development, *Flk-1* (Millauer et al., 1993; Yamaguchi et al., 1993; Shalaby et al., 1995), *Flt-1* (Fong et al., 1996), and *VE-cadherin/Cdh5* (Breier et al., 1996; Nishikawa et al., 1998) were expressed in colonies at all time points analyzed. These expression patterns indicate that the developing blast colonies rapidly progress from the hemangioblast stage, characterized by co-expression of *Brachyury*

and *Flk-1* and undergo commitment to the vascular and hematopoietic lineages within 24 hours of culture.

Methylcellulose colony assays confirmed the molecular analysis and demonstrated that the 24-hour-old blast colonies contained readily detectable numbers of hematopoietic progenitors, including those of the primitive erythroid (Ep) and macrophage (Mac) lineages as well as bipotential cells able to generate both the erythroid and macrophage lineages (E/Mac) (Fig. 1C). Primitive erythroid progenitors were predominant at this early stage and represented more than 50% of the total progenitor population. The hematopoietic potential of the colonies continued to increase over the following 24 hours at which time multipotential progenitors (Mix) able to generate mixed lineage colonies (3 or more lineages) were also detected. Most of this increase was due to an increase in the number of definitive hematopoietic progenitors which now account for greater than 70% of the total number of progenitors. These findings demonstrate that the hemangioblast undergoes hematopoietic specification within the first 24 hours of culture and suggest that the primitive erythroid lineage is generated in the blast colonies before the definitive populations.

Analysis of components of the notch signaling pathway revealed that *Notch1* and *Notch4* were expressed in the colonies at all time points (Fig. S1). In contrast, *Notch2* was not detected at any stage of colony growth. The negative modulator of Notch signaling, *Numb*, but not its homologue *Numblike* (Zhong et al., 1997), was also expressed in the blast colonies throughout the time course. Among the  $\beta$ -catenin-dependent Wnt family members analyzed, only *Wnt3* was expressed in the developing colonies and the levels appeared to be highest in the earliest colonies. Neither *Wnt3a* nor *Wnt8a* were detected at any stage of colony growth. *Wnt5a*, a  $\beta$ -catenin-independent/non-canonical Wnt was expressed in colonies at all time points analyzed.

Quantitative PCR analyses on pools of colonies revealed a rapid upregulation of expression of the Notch ligand *Jagged1*(*Jag1*) and of two transcriptional targets of the Notch pathway, *Hes5* and *Hey1*, suggesting the initiation of active Notch signaling early in the blast colonies (Fig. 1D). Consistent with this interpretation is the observation that expression of *Numb* declines over this same period of time. In contrast to Notch, the Wnt pathway appears to be active in the isolated Flk-1+ population as demonstrated by the expression of *Wnt3* and *Axin2*, the downstream target of Wnt signaling (Jho et al., 2002). With the onset of colony development, one observes a rapid downregulation of *Axin2* expression and an upregulation of inhibitors of the pathways including the soluble inhibitors *Sfrp1*, *Sfrp2*, *Sfrp5* (reviewed in Kawano and Kypta, 2003), non-canonical *Wnt5a* and nemo-like kinase (*Nlk*), a gene that encodes a component of MAPK pathway that antagonizes canonical Wnt signaling (Ishitani et al., 1999). These patterns suggest that Wnt signaling is inhibited early in the development of the blast colonies. The expression patterns of *gata-1* are consistent with specification of the hematopoietic lineage by 24 hours of growth. Together, these findings reveal dynamic changes in the signaling program within the developing blast colonies, and suggest a rapid transition from the Wnt to the Notch pathway.

Cells from different aged blast colonies were next stained with appropriate antibodies to demonstrate the presence and distribution of *Numb*, the cleaved intracellular active portion of Notch1 (Notch1-IC or NIC) and activated  $\beta$ -catenin protein. Cells from the 12 and 24-hour stage colonies revealed a striking pattern for *Numb*, as the protein was predominantly restricted to the boundaries of cell-cell contact (Fig. 2A). Beyond this stage the levels of *Numb* protein appeared to decline and undergo re-distribution to the cytoplasm (48 hour colony). These patterns are consistent with those of the qPCR analysis (Fig. 1D) and suggest a role of membrane-associated *Numb* protein at the onset of colony growth, possibly functioning to inhibit Notch signaling at this stage. Analysis of colonies stained with the

anti-Notch1-IC antibody demonstrated the presence of Notch1-IC in the colonies at all time points, indicating active Notch signaling at these stages (Fig. 2B). Activated  $\beta$ -catenin appeared to be predominantly localized to the cell membrane in the early 12 hour-old colonies, similar to the pattern observed for Numb. In most early colonies, staining was also detected in the nucleus (Fig. 2A and B). The level of  $\beta$ -catenin appears to decline beyond this time, indicating a rapid downregulation of this pathway in the developing blast colonies.

### Wnt and Notch signaling affect blast colony development from the hemangioblast

To dissect the signaling pathways involved in hematopoietic and vascular development from the hemangioblast, it was important to first establish serum-free conditions for the growth of blast colonies from these progenitors. Replacing serum with a combination of 10 different cytokines (M10) resulted in the development of 3-fold more blast colonies from 3.25 EB-derived GFP-Bry<sup>+</sup>Flk1<sup>+</sup> cells (Fig. S2A). Blast colonies grown under the serum free conditions gave rise to hematopoietic colonies when replated in methylcellulose assays (Fig. S2B). They also generated both vascular and hematopoietic progeny when plated on a thin layer of Matrigel in liquid media in the presence of hematopoietic and vascular cytokines (liquid expansion assay) (data not shown), indicating that growth in the absence of serum did not impact their developmental potential. Expression analysis of the blast colonies grown in serum-free cultures revealed patterns similar to those observed in the colonies grown in the presence of serum (Fig. S2C).

To determine if Wnt and Notch signaling play a role in the generation of the blast colonies from the hemangioblasts, we manipulated these pathways in the developing colonies either by the addition of agonists or antagonists to the methylcellulose cultures or through the inducible expression of components of the pathways in the Flk-1<sup>+</sup> hemangioblasts. For the latter approach, the A2lox ES cell line or the AinV/GFP-Bry/CD4-Foxa2 ES line, that allow doxycyclin (Dox)-inducible expression of a gene of interest, were engineered to express the stabilized form of  $\beta$ -catenin ( $\beta$ -cat) (Lindsley et al., 2006), the constitutively activated Notch1-IC or the PRR<sup>S</sup> isoform of Numb. The resulting cell lines were designated as  $\beta$ -cat, Notch1-IC and Numb ES cell lines, respectively. Expression of  $\beta$ -cat results in constitutive  $\beta$ -catenin-dependent signaling whereas expression of Notch1-IC activates the Notch pathway without the need for ligand stimulation. The PRR<sup>S</sup> isoform of Numb contains a truncated C-terminal region and was used for these studies, as it is more abundantly expressed in early blast colonies than the PRR<sup>L</sup> isoform (Fig. S3). The PRR<sup>S</sup> isoform was found to be functionally indistinguishable from other Numb isoforms when ectopically expressed in *Drosophila* (Petersen et al., 2006). Dox-induced expression of  $\beta$ -cat, Notch1-IC and Numb in the respective ES cell lines was confirmed by western blot analyses (Fig. S4). Activation of Notch signaling following Dox-induction was also demonstrated using a RBP-Jk reporter assay (Fig. S5).

As a first step in evaluating the role of Wnt signaling in blast colony development, the Wnt inhibitor DKK1 was added to the serum-free cultures of Flk-1<sup>+</sup> hemangioblasts generated from the  $\beta$ -cat ES cell line. Blocking Wnt signaling by the addition of DKK1 resulted in a dramatic suppression of blast colony formation (Fig. 3A). Induction of the Wnt pathway through  $\beta$ -cat completely reversed the inhibitory effects of DKK1 (+Dox+DKK1), indicating that the reduction of blast colonies was in fact due to inhibition of the Wnt/ $\beta$ -catenin pathway. While administration of DKK1 suppressed the formation of typical blast colonies, compact (or core) colonies with the morphology of immature blast colonies developed in these cultures (Figs. 3B, S6). The total number of blast and core colonies in the untreated and treated groups was the same suggesting that the core colonies are blast colonies without the outer population of hematopoietic cells. Pools of 3-day-old blast colonies from the untreated group and core colonies from the DKK1 treated cultures were picked, the cells dissociated and replated in the hematopoietic methylcellulose assay. While

both types of colonies gave rise to secondary definitive hematopoietic colonies, only the blast colonies generated secondary primitive erythroid colonies (Fig. 3C) When plated on a thin layer of Matrigel in liquid expansion assay, these compact core colonies generated both adherent (vascular) and non-adherent (hematopoietic) progeny comparable to those derived from typical blast colonies (Fig. S7). These findings indicate that Wnt signaling is required for the establishment of the primitive erythroid lineage from the hemangioblast. Development of the definitive hematopoietic and vascular lineages from this progenitor, in contrast, is not dependent on this signaling pathway. Inhibition of Notch signaling either by addition of  $\gamma$ -secretase inhibitor X (gSI) or through the induction of Numb with Dox led to a moderate but significant increase in blast colony number (Fig. 3D). These colonies were larger in size than those from the control cultures and did give rise to both vascular and hematopoietic cells in the liquid expansion cultures (data not shown). Conversely, the induction of activated Notch1 inhibited blast colony development, but did permit the formation of compact core colonies, comparable to those observed in the cultured treated with DKK1 (Figs. 3E, S8A) When replated in hematopoietic methylcellulose cultures, these compact colonies displayed definitive but not primitive hematopoietic potential (Fig. S8B). These observations demonstrate that active Notch signaling inhibits primitive erythroid development from the hemangioblast.

### **Interaction between Notch and Wnt signaling modulates primitive erythroid development from the hemangioblast**

To further dissect the roles of canonical Wnt and Notch signaling in hematopoietic specification, the Flk-1+ population that contains hemangioblasts was cultured in liquid rather than in methylcellulose to enable easy access to these progenitors for more precise stage-specific manipulation of the signaling pathways. When cultured under appropriate conditions in liquid culture, the cells form aggregates and undergo hematopoietic commitment in a time frame similar to that observed in the blast colonies growing in methylcellulose cultures (Nostro et al., 2008; Keller G. and Cheng X. unpublished observation). For the following set of experiments, Flk-1+ cells were induced from the different ES cell lines using a combination of Wnt3a, Activin and BMP4 in the absence of serum as previously described (Nostro et al., 2008). The Flk-1+ cells were isolated by cell sorting and allowed to reaggregate for different periods of time in serum-free media supplemented with 10 ng/ml VEGF in the presence or absence of Dox.

Expression of Wnt3 and Axin2 within the aggregates declined sharply within the first 24 hours of culture (Fig. 4A) suggesting that this pathway may be required only at the onset of hematopoietic commitment. To determine if this is the case, we delayed the addition of DKK1 to the Flk-1+ cells for either 6 or 12 hours and then analyzed the population for both primitive and definitive hematopoietic potential at 48 hours (Fig. 4B). Consistent with our previous study, the addition of DKK1 at the onset of the culture significantly reduced the development of primitive erythroid progenitors (Fig. 4B, DKK-0h). Definitive hematopoiesis was not affected by the addition of DKK1. A delay in the addition of DKK1 by 6 hours did not alter its inhibitory effect on primitive erythroid development (Fig. 4B, DKK-6h). However, a further delay of 6 hours significantly diminished this inhibition (Fig. 4B, DKK-12h), indicating that Wnt signaling does function early and transiently in the Flk-1 population to establish the primitive erythroid lineage.

Inhibition of Notch signaling by the Dox-induced expression of Numb or by the addition of gSI increased the number of primitive erythroid progenitors that developed from the Flk-1+ population (Fig. 4C). In contrast, activation of Notch1-IC resulted in a dramatic decrease in primitive erythroid development, 24 hours following induction (Fig. 4D). Manipulation of Notch signaling by Numb or by Notch1-IC did not significantly impact definitive hematopoietic development (Fig. 4C, D). Taken together, these findings demonstrate that

Notch signaling specifically inhibits primitive erythroid development from the Flk-1+ population in liquid culture, as observed in the hemangioblast methylcellulose assay.

The opposing effects of the Wnt and Notch signaling pathways on primitive erythroid development suggest that they may interact in the specification of this lineage and in the establishment of the embryonic hematopoietic system. To determine if this is the case and to define the relationship between them, epistasis analyses were carried out. The addition of Wnt to aggregates induced to express Numb or to those treated with gSI dramatically increased the development of primitive erythroid progenitors compared to those treated with Wnt, Dox or gSI alone (Fig. 5A, B). When added to Notch1-IC induced cells, Wnt3a was able to rescue the block in primitive erythroid development (Fig. 5C). In contrast, expression of Numb was unable to rescue the DKK1-induced inhibition in primitive erythroid development (Fig. 5D). Together, these findings demonstrate that Wnt signaling is essential for the establishment of the primitive erythroid lineage from the Flk-1+ hemangioblast population and that inhibition of residual Notch signaling enhances development of the lineage. They also suggest that the inhibitory effects of Notch are mediated through the inhibition of the Wnt pathway. The inability of Numb to overcome the DKK1 block in primitive erythroid development, supports the interpretation that it functions to inhibit Notch signaling rather than to directly activate the Wnt pathway.

To further investigate the interactions of these pathways, TOP/FOP Flash reporter assays were used to evaluate TCF/LEF transcriptional activity in Flk-1+ populations induced to express either Notch1-IC or Numb. For this assay, appropriate reporter plasmids were introduced into the Flk-1+ cells and the cells reaggregated in the conditions outlined in Figure 5E. Following 24 hours of culture, TOP or FOP reporter activities were quantified by measuring firefly luciferase activities, and normalized to the renilla luciferase activities. TOP/FOP values represent the specific  $\beta$ -catenin-dependent TCF/LEF activity. In the absence of exogenous Wnt3a,  $\beta$ -catenin activity was significantly suppressed by the Dox-induced Notch1-IC expression. As expected the addition of Wnt3a induced high levels of  $\beta$ -catenin activity. This level was reduced by the induction of Notch1-IC, but remained substantially higher than the levels in the Dox-induced cells in the absence of exogenous Wnt3a. Induction of Numb either in the absence or presence of exogenous Wnt3a resulted in a significant increase of  $\beta$ -catenin activity (Fig. 5E). Almost no activity was detected in the groups treated with DKK1. These results demonstrate that Notch signaling can modulate Wnt signaling in Flk-1-derived populations and reveal a strong correlation between the  $\beta$ -catenin activity in TOP/FOP reporter assay (Fig. 5E) and the number of primitive erythroid progenitors in hematopoietic progenitor assays (Fig. 5A, C, D). This suggests that Wnt signaling is dominant for primitive erythropoiesis from the hemangioblast, while Notch-Numb signaling acts on this program through its ability to influence Wnt signaling.

To reveal the mechanism by which Notch1-IC and Numb can modulate canonical Wnt signaling, the aggregates grown in the presence or absence of Dox were analyzed by quantitative PCR for the expression of genes known to encode inhibitors of the Wnt pathway. As expected, *Hey1*, a downstream target of Notch signaling (Jarriault et al., 1995) was up-regulated following induction of Notch1-IC. Induction of Numb reduced *Hey1* expression during the first 24 hours of culture (Fig. S9). Expression of Notch1-IC led to the rapid upregulation of expression of *Sfrp1*, *Sfrp5*, *Dkk1* (Fig. 6A, B and E), soluble inhibitors of Wnt signaling, as well as of *Wnt5a* (Fig. 6C) and *Nlk* (Fig. 6D). The induction of *Numb* had more modest effects but did lead to the reduction of expression of *Wnt5a*, *Nlk*, *Dkk1* (Fig. 6C through E) for the first 24 hours of culture. The levels of *Sfrp1* and *Sfrp5* were not significantly impacted by Numb expression (data not shown). Collectively, these qPCR data strongly suggest that Notch signaling negatively regulates Wnt/ $\beta$ -catenin signaling through the upregulation of antagonists of the pathway. Through its capacity to inhibit Notch, Numb

suppressed a subset of these Wnt inhibitory molecules. To determine if the Notch-induced canonical Wnt antagonists SFRP1 and Wnt5a can affect primitive erythropoiesis from the Flk-1+ population, they were added individually to the Flk-1+ aggregation cultures. As observed with DKK1, both SFRP1 and Wnt5a did specifically inhibit primitive erythroid development (Fig. S10).

### Expression of Numb, Notch1-IC and activated $\beta$ -catenin in the early embryo

To gain some insight into the potential role of these pathways in the regulation of primitive erythropoiesis in the early embryo, whole mount immunostaining was performed on E7.5 (neural plate), E8.25 (3-7 somite-pair) and E8.5 (10-12 somite-pair) stage mouse embryos to establish the spatial expression patterns of Numb, Notch1-IC and activated  $\beta$ -catenin. These stages span the primitive erythropoiesis phase (E7.5 to E8.5) of hematopoiesis (Palis et al., 1999). As shown in Figures 7A and S11B, whole membrane-tethered NUMB was preferentially localized to the posterior PS and developing YS of these early embryos, the sites of hemangioblast development and primitive erythropoiesis respectively. Expression of Numb in the yolk sac decreased substantially by E8.25 (3-7 somite-pair stage) and remained low at E8.5 (10-12 somite-pair stage). Numb was detected in the embryo proper at these stages (Fig. 7A and not shown). Notch1-IC was present in the distal and lateral region of the early stage embryo, but not in developing yolk sac (Figs. 7B, S11B). This distribution confirms a previous report showing the absence of Notch-1C in the E7.5 yolk sac blood islands (Hadland et al., 2004). By E8.25 (7 somite-pair stage), Notch-1C was detected in specific regions of the yolk sac, and by E8.5 it was broadly distributed in this tissue. Activated  $\beta$ -catenin showed a pattern similar to that of NUMB and appears to be preferentially enriched in the YS and the posterior primitive streak of the early embryo (Figs. 7C, S11B). The levels of  $\beta$ -catenin remained high in the yolk sac of the E8.25 embryo and then declined dramatically by E8.5. Taken together, these findings demonstrate that Numb and activated  $\beta$ -catenin are present in the yolk sac during the primitive erythroid stage of development, supporting the interpretation that the interaction of these pathways regulates the development of this lineage in vivo, as observed in vitro.

### Discussion

Cell fate is determined during embryonic development by multiple signaling pathways, including Wnt and Notch, which function both synergistically and antagonistically (Hayward et al., 2008). The primitive erythroid lineage is the first hematopoietic fate to be established in the mammalian embryo, in a region that will form the yolk sac. The development of this lineage is rapid, synchronous and restricted both in time and embryonic site. Detailed analyses of the developing mouse yolk sac indicate that primitive erythropoiesis is active for approximately 48 hours and then rapidly shut down (Palis et al., 1999), suggesting control by both positive and negative regulators. Primitive erythropoiesis in ES cell differentiation cultures follows a similar temporal pattern of development, suggesting that the regulation of the lineage is preserved in this model system (Keller et al., 1993). While long recognized as the first blood cell lineage to develop, little is known about the pathways that control primitive erythropoiesis. In this study we provide evidence that the Wnt pathway is a pivotal regulator of primitive erythropoiesis at the level of the hemangioblast and that it functions in concert with Numb, an inhibitor of the Notch pathway (Zhong et al., 1996; Petersen et al., 2006) to establish this lineage (Fig. 8).

Analyses of the gene expression patterns in the developing blast colonies provide a picture of dynamic change as the hemangioblast undergoes specification to the hematopoietic and vascular lineages (Fig. 1B, D). Of particular interest is the change in expression of components of the Wnt and Notch pathways (Fig. 1D), indicating a transition from Wnt to Notch signaling. The rapid downregulation in Wnt signaling, predicted from the gene

expression patterns (Figs. 1D, 4A) are in line with the inhibition studies (Fig. 4B) demonstrating that Wnt/ $\beta$ -catenin signaling is required for a remarkably short period of time to establish the primitive erythroid fate. This restricted period of Wnt signaling may, in part, determine the transient nature of primitive erythropoiesis. The rapid onset of Notch signaling during blast colony development (Fig. 1D), together with the observation that this pathway induces Wnt inhibitors (Fig. 6), suggests that Notch may play a role in restricting the window of Wnt signaling and thereby regulate the primitive erythroid phase of hematopoiesis. Attempts to prolong primitive erythropoiesis by sustaining Wnt signaling while inhibiting the Notch pathway with  $\gamma$ -secretase inhibitor were not successful (data not shown), suggesting that other regulators are also likely to be required to establish this temporal pattern.

The TOP/FOP reporter assays and PCR analyses provide strong evidence that activation of the Notch pathway leads to the inhibition of Wnt/ $\beta$ -catenin signaling, whereas enforced expression of Numb potentiates Wnt/ $\beta$ -catenin signaling (Fig. 5E, 6). The rapid induction of Wnt inhibitors by Notch activation strongly suggests that this is the mechanism by which Notch mediates this antagonistic effect. The observation that the addition of Wnt can overcome the Notch-induced inhibition of primitive erythropoiesis supports this interpretation (Fig. 5C). The presence of multiple CSL-binding sites in the vicinity of the genes coding region or within the coding region of these Wnt inhibitors suggests that they might be the direct targets of Notch signaling (data not shown). Previous studies have shown that Notch can function as an antagonist for Wnt signaling through a number of different mechanisms including the modulation of Armadillo/ $\beta$ -catenin activity (Hayward et al., 2005; Nicolas et al., 2003; Deregowski et al., 2006), the induction of NLK (Röttinger et al., 2006), the interaction with Axin (Hayward et al., 2006) and the regulation of GSK3 $\beta$  (Brack and Conboy et al., 2008). The induction of Wnt inhibitors by Notch demonstrated in this study provides another mechanism through which these pathways can interact. While the reciprocal interaction of Wnt regulating Notch signaling is less well established, observations that Numb is induced in the chick somites by the canonical Wnt signaling and that a series of Notch target genes including *Jag1* and *Hes1* are induced upon  $\beta$ -catenin activation in the mouse skin provide strong evidence for this level of control (Holowacz et al. 2006; Estrach et al., 2006; Ambler et al., 2007). This reciprocal regulation of these critical signaling pathways may function to provide the appropriate level of temporal control necessary for establishing specific cell fates, including the primitive erythroid lineage, during development.

Our immunostaining analyses demonstrating transient expression of activated  $\beta$ -catenin (Figs. 7C, S11B) and Numb (Fig. 7A, S11B) in the yolk sac during the primitive erythropoiesis stage of development are consistent with the interpretation that these pathways interact to regulate this lineage in vivo. Findings from recent targeting studies have reported an increase in primitive erythropoiesis in the yolk sac of embryos lacking RBP-J $\kappa$ /CBF1, the canonical effector of Notch signaling, suggesting that this pathway does inhibit the development of this lineage in vivo (Robert-Moreno et al., 2007). This difference is in contrast to an earlier study that failed to document enhanced primitive erythropoiesis in Notch1 $^{-/-}$  embryos (Hadland et al., 2004). In this case, it is possible that other Notch family members such as Notch4 known to be expressed in the yolk sac (Shirayoshi et al., 1997 and Chen V. unpublished data) provide some compensatory function for primitive erythroid development. Numb $^{-/-}$  embryos die around E11.5 from premature depletion of neural progenitors and other defects including abnormal vasculature (Zhong et al., 2000). As primitive erythropoiesis was not evaluated in this study, it is unclear if the development of this lineage was impacted in these embryos. The role of Wnt signaling in primitive erythropoiesis in vivo is currently not known as embryos deficient in key components of this

pathway, including Wnt3 or  $\beta$ -catenin die during gastrulation (Liu et al., 1999;Huelsenken et al., 2000).

In summary, the findings reported here have uncovered a novel role for Numb in mediating the interplay between canonical Wnt and Notch signaling in the establishment of the primitive erythroid lineage from the hemangioblast. The observed specificity of these pathways to the primitive erythroid lineage further highlights the unique properties of this blood cell population and lays the foundation for future studies aimed at identifying other regulators of the earliest stages of hematopoietic commitment. The approach used in this study also demonstrates the power of the ES differentiation model in dissecting signaling pathways that regulate early developmental programs that would be difficult, if not impossible, to study in the normal embryo.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank members of the Keller lab for critically reading this manuscript. We thank Dr Lei Yang and Dr Jun Liao for the embryo dissection. We also thank the Mount Sinai School of Medicine Flow Cytometry Shared Research Facility for assistance with cell sorting, and the Microscopy Shared Research Facility (supported with funding from NIH-NCI shared resources grant 5R24 CA095823-04) for assistance with confocal laser scanning. This work was supported by National Institutes of Health grants R01 HL 48834-09 and R01 HL 71800 to G.K. The authors declare that they have no competing financial interests.

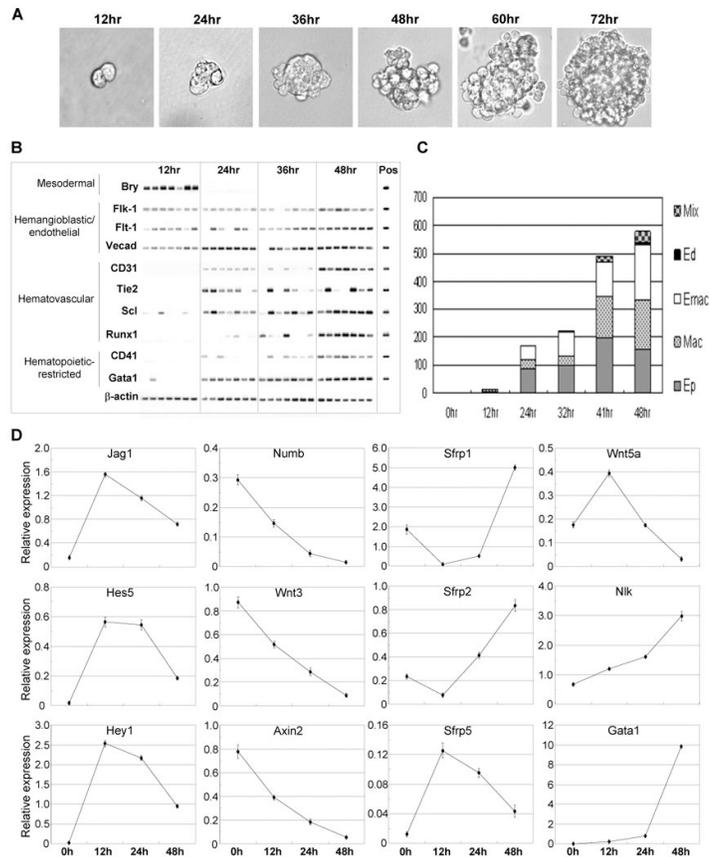
## References

- Ambler CA, Watt FM. Expression of Notch pathway genes in mammalian epidermis and modulation by beta-catenin. *Dev Dyn* 2007;236:1595–601. [PubMed: 17474126]
- Barker JE. Development of the mouse hematopoietic system. I. Types of hemoglobin produced in embryonic yolk sac and liver. *Dev Biol* 1968;18:14–29. [PubMed: 5669501]
- Brack AS, Conboy IM, Conboy MJ, Shen J, Rando TA. A Temporal Switch from Notch to Wnt Signaling in Muscle Stem Cells Is Necessary for Normal Adult Myogenesis. *Cell Stem Cell* 2008;2:50–59. [PubMed: 18371421]
- Breier G, Breviario F, Caveda L, Berthier R, Schnürch H, Gotsch U, Vestweber D, Risau W, Dejana E. Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. *Blood* 1996;87:630–641. [PubMed: 8555485]
- Burns CE, Traver D, Mayhall E, Shepard JL, Zon LI. Hematopoietic stem cell fate is established by the Notch-Runx pathway. *Genes Dev* 2005;19:2331–42. [PubMed: 16166372]
- Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. *Development* 1998;125:725–732. [PubMed: 9435292]
- Deregowski V, Gazzo E, Priest L, Rydzziel S, Canalis E. Notch 1 overexpression inhibits osteoblastogenesis by suppressing Wnt/beta-catenin but not bone morphogenetic protein signaling. *J Biol Chem* 2006;281:6203–6210. [PubMed: 16407293]
- Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ. Defective haematopoiesis and vasculogenesis in transforming growth factor- $\beta$ 1 knock out mice. *Development* 1995;121:1845–1854. [PubMed: 7600998]
- Estrach S, Ambler CA, Lo Celso C, Hozumi K, Watt FM. Jagged 1 is a beta-catenin target gene required for ectopic hair follicle formation in adult epidermis. *Development* 2006;133:4427–38. [PubMed: 17035290]
- Fehling HJ, Lacaud G, Kubo A, Kennedy M, Robertson S, Keller G, Kouskoff V. Tracking mesoderm induction and its specification to the hemangioblast during embryonic stem cell differentiation. *Development* 2003;130:4217–4227. [PubMed: 12874139]

- Ferkowicz MJ, Starr M, Xie X, Li W, Johnson SA, Shelley WC, Morrison PR, Yoder MC. CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development* 2003;130:4393–4403. [PubMed: 12900455]
- Ferkowicz MJ, Yoder MC. Blood island formation: longstanding observations and modern interpretations. *Exp Hematol* 2005;33:1041–1047. [PubMed: 16140152]
- Fong GH, Klingensmith J, Wood CR, Rossant J, Breitman ML. Regulation of flt-1 expression during mouse embryogenesis suggests a role in the establishment of vascular endothelium. *Dev Dyn* 1996;207:1–10. [PubMed: 8875071]
- Hadland BK, Huppert SS, Kanungo J, Xue Y, Jiang R, Gridley T, Conlon RA, Cheng AM, Kopan R, Longmore GD. A requirement for Notch1 distinguishes 2 phases of definitive hematopoiesis during development. *Blood* 2005;104:3097–105. [PubMed: 15251982]
- Haar JL, Ackerman GA. A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse. *Anat Rec* 1971;170:199–223. [PubMed: 4931266]
- Hayward P, Brennan K, Sanders P, Balayo T, DasGupta R, Perrimon N, Martinez Arias A. Notch modulates Wnt signalling by associating with Armadillo/beta-catenin and regulating its transcriptional activity. *Development* 2005;132:1819–1830. [PubMed: 15772135]
- Hayward P, Balayo T, Martinez Arias A. Notch synergizes with axin to regulate the activity of armadillo in *Drosophila*. *Dev Dyn* 2006;235:2656–66. [PubMed: 16881048]
- Hayward P, Kalmar T, Martinez Arias A. Wnt/Notch signalling and information processing during development. *Development* 2008;135:411–24. [PubMed: 18192283]
- Holowacz T, Zeng L, Lassar AB. Asymmetric localization of numb in the chick somite and the influence of myogenic signals. *Dev Dyn* 2006;235:633–45. [PubMed: 16425215]
- Huber TL, Kouskoff V, Fehling HJ, Palis J, Keller G. Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* 2004;432:625–630. [PubMed: 15577911]
- Huelsken J, Vogel R, Brinkmann V, Erdmann B, Birchmeier C, Birchmeier W. Requirement for beta-catenin in anterior-posterior axis formation in mice. *J Cell Biol* 2000;148:567–578. [PubMed: 10662781]
- Ishitani T, Ninomiya-Tsuji J, Nagai S, Nishita M, Meneghini M, Barker N, Waterman M, Bowerman B, Clevers H, Shibuya H, Matsumoto K. The TAK1-NLK-MAPK-related pathway antagonizes signaling between  $\beta$ -catenin and transcription factor TCF. *Nature* 1999;399:798–802. [PubMed: 10391247]
- Jarriault S, Brou C, Logeat F, Schroeter EH, Kopan R, Israel A. Signalling downstream of activated mammalian Notch. *Nature* 1995;377:355–358. [PubMed: 7566092]
- Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol Cell Biol* 2002;22:1172–83. [PubMed: 11809808]
- Kawano Y, Kypta R. Secreted antagonists of the Wnt signalling pathway. *J Cell Sci* 2003;116:2627–2634. [PubMed: 12775774]
- Keller G, Kennedy M, Papayannopoulou T, Wiles MV. Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol Cell Biol* 1993;13:473–86. [PubMed: 8417345]
- Keller GM. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev* 2005;19:1129–1155. [PubMed: 15905405]
- Kennedy M, Firpo M, Choi K, Wall C, Robertson S, Kabrun N, Keller G. A common precursor for primitive and definitive hematopoiesis. *Nature* 1997;386:488–493. [PubMed: 9087406]
- Kennedy M, Keller GM. Hematopoietic commitment of ES cells in culture. *Methods in enzymology* 2003;365:39–59. [PubMed: 14696336]
- Kispert A, Herrmann BG. Immunohistochemical analysis of the Brachyury protein in wild-type and mutant mouse embryos. *Dev Biol* 1994;161:179–193. [PubMed: 8293872]
- Kojika S, Griffin JD. Notch receptors and hematopoiesis. *Exp Hematol* 2001;29:1041–52. [PubMed: 11532344]
- Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP, Gallahan D, Closson V, Kitajewski J, Callahan R, Smith GH, Stark KL, Gridley T. Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev* 2000;14:1343–1352. [PubMed: 10837027]

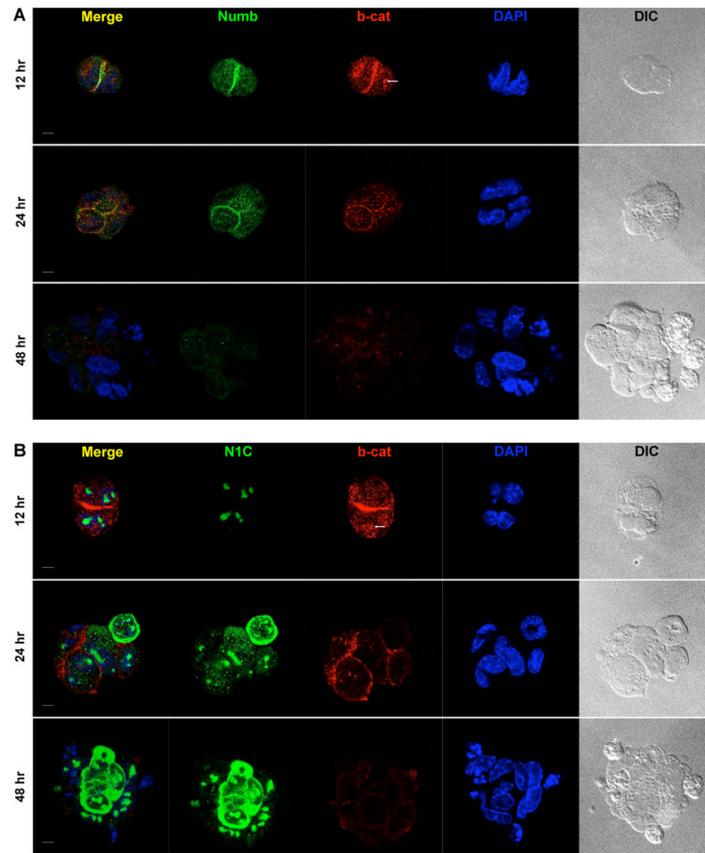
- Kumano K, Chiba S, Kunisato A, Sata M, Saito T, Nakagami-Yamaguchi E, Yamaguchi T, Masuda S, Shimizu K, Takahashi T, Ogawa S, Hamada Y, Hirai H. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* 2003;18:699–711. [PubMed: 12753746]
- Kyba M, Perlingeiro RC, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* 2002;109:29–37. [PubMed: 11955444]
- Lin CS, Lim SK, D'Agati V, Costantini F. Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. *Genes Dev* 1996;10:154–64. [PubMed: 8566749]
- Lindsley RC, Gill JG, Kyba M, Murphy TL, Murphy KM. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. *Development* 2006;133:3787–3796. [PubMed: 16943279]
- Liu P, Wakamiya M, Shea MJ, Albrecht U, Behringer RR, Bradley A. Requirement for Wnt3 in vertebrate axis formation. *Nat Genet* 1999;22:361–365. [PubMed: 10431240]
- Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W, Ullrich A. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 1993;72:835–846. [PubMed: 7681362]
- Miller JR. The Wnts. *Genome Biol* 2002;3:REVIEWS3001. [PubMed: 11806834]
- Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, Hui CC, Clevers H, Dotto GP, Radtke F. Notch1 functions as a tumor suppressor in mouse skin. *Nat Genet* 2003;33:416–421. [PubMed: 12590261]
- Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development* 1998;125:1747–1757. [PubMed: 9521912]
- Nostro MC, Cheng X, Keller GM, Gadue P. Wnt, Activin and BMP signaling regulate distinct stages in the developmental pathway from embryonic stem cell to blood. *Cell Stem Cell* 2008;2:60–71. [PubMed: 18371422]
- Okuda T, van Deursen J, Hiebert SW, Grosveld G, Downing JR. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* 1996;84:321–330. [PubMed: 8565077]
- Orkin S. GATA-binding transcription factors in hematopoietic cells. *Blood* 1992;80:575–581. [PubMed: 1638017]
- Palis J, Roberston S, Kennedy M, Wall C, Keller G. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* 1999;126:5073–5084. [PubMed: 10529424]
- Pear WS, Aster JC, Scott ML, Hasserjian RP, Soffer B, Sklar J, Baltimore D. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J Exp Med* 1996;183:2283–91. [PubMed: 8642337]
- Petersen PH, Tang H, Zou K, Zhong W. The enigma of the numb-Notch relationship during mammalian embryogenesis. *Dev Neurosci* 2006;28:156–168. [PubMed: 16508312]
- Pevny L, Simon MC, Robertson E, Klein WH, Tsai SF, D'Agati V, Orkin SH, Costantini F. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* 1991;349:257–260. [PubMed: 1987478]
- Robb L, Lyons I, Li R, Hartley L, Kontgen F, Harvey RP, Metcalf D, Begley CG. Absence of yolk sac hematopoiesis from mice with a targeted disruption of the *scl* gene. *Proc Natl Acad Sci* 1995;92:7075–7079. [PubMed: 7624372]
- Robert-Moreno A, Espinosa L, Sanchez MJ, de la Pompa JL, Bigas A. The notch pathway positively regulates programmed cell death during erythroid differentiation. *Leukemia* 2007;21:1496–503. [PubMed: 17476283]
- Röttinger E, Croce J, Lhomond G, Besnardeau L, Gache C, Lepage T. Nemo-like kinase (NLK) acts downstream of Notch/Delta signaling to downregulate TCF during mesoderm induction in the sea urchin embryo. *Development* 2006;133:4341–4353. [PubMed: 17038519]

- Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1 deficient mice. *Nature* 1995;376:62–66. [PubMed: 7596435]
- Shirayoshi Y, Yuasa Y, Suzuki T, Sugaya K, Kawase E, Ikemura T, Nakatsuji N. Proto-oncogene of int-3, a mouse Notch homologue, is expressed in endothelial cells during early embryogenesis. *Genes Cells* 1997;2:213–24. [PubMed: 9189758]
- Shivdasani R, Mayer E, Orkin SH. Absence of blood formation in mice lacking the T-cell leukemia oncoprotein tal-1/SCL. *Nature* 1995;373:432–434. [PubMed: 7830794]
- Tam PP, Behringer RR. Mouse gastrulation: the formation of a mammalian body plan. *Mechanisms of development* 1997;68:3–25. [PubMed: 9431800]
- Takakura N, Huang XL, Naruse T, Hamaguchi I, Dumont DJ, Yancopoulos GD, Suda T. Critical role of the TIE2 endothelial cell receptor in the development of definitive hematopoiesis. *Immunity* 1998;9:677–686. [PubMed: 9846489]
- Wang Q, Stacy T, Binder M, Marin-Padilla M, Sharpe AH, Speck NA. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc Natl Acad Sci* 1996;93:3444–3449. [PubMed: 8622955]
- Watt SM, Gschmeissner SE, Bates PA. PECAM-1: its expression and function as a cell adhesion molecule on hemopoietic and endothelial cells. *Leuk Lymphoma* 1995;17:229. [PubMed: 8580791]
- Witte ON. Steel locus defines new multipotent growth factor. *Cell* 1990;63:5–6. [PubMed: 2208282]
- Wu H, Liu X, Jaenisch R, Lodish HF. Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell* 1995;83:59–67. [PubMed: 7553874]
- Yamaguchi TP, Dumont DJ, Conlon RA, Breitman ML, Rossant J. flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development* 1993;118:489–98. [PubMed: 8223275]
- Zhong W, Feder JN, Jiang MM, Jan LY, Jan YN. Asymmetric localization of a mammalian Numb homolog during mouse cortical neurogenesis. *Neuron* 1996;17:43–53. [PubMed: 8755477]
- Zhong W, Jiang M, Weinmaster G, Jan LY, Jan YN. Differential expression of mammalian Numb, Numblike and Notch1 suggests distinct roles during mouse cortical neurogenesis. *Development* 1997;124:1887–1897. [PubMed: 9169836]
- Zhong W, Jiang MM, Schonemann MD, Meneses JJ, Pedersen RA, Jan LY, Jan YN. Mouse numb is an essential gene involved in cortical neurogenesis. *Proc Natl Acad Sci* 2000;97:6844–6849. [PubMed: 10841580]



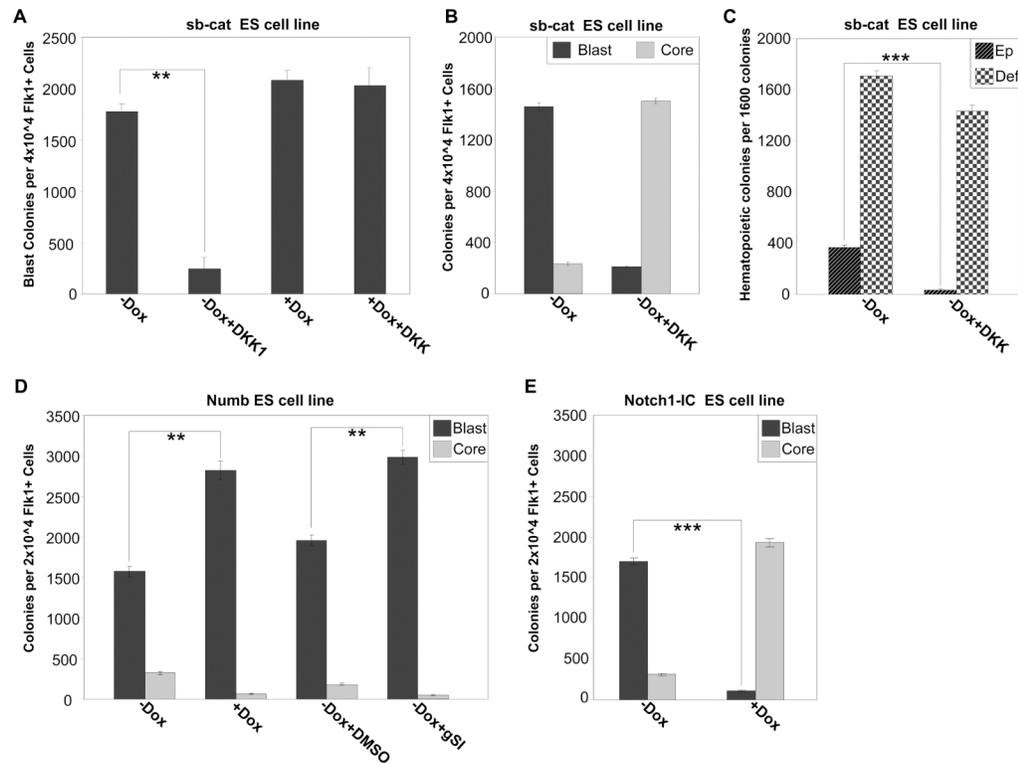
**Figure 1. Gene expression analysis of developing blast colonies**

Flk-1<sup>+</sup> cells isolated from Bry-GFP ES cell-derived day 3.25 EBs were cultured in serum-containing hemangioblast methylcellulose media and the developing blast colonies assayed at the indicated time intervals. (A) Images showing the morphology of representative developing blast colonies over a 72 hour culture period. (B) Expression profiles of individual developing blast colonies isolated at the indicated times. Each lane represents an individual colony. Pos: positive control. (C) Hematopoietic progenitor potential of different aged blast colonies. 700 colonies of each time points were collected, dissociated and the cells plated in methylcellulose media supplemented with hematopoietic cytokines. Primitive erythroid (Ep), macrophage (Mac), and bipotential macrophage/erythroid (E/Mac) colonies were scored following 4-5 days of culture, while definitive erythroid (Ed) and multipotential myeloid/erythroid (Mix) colonies were scored at day 9 of culture. (D) Expression of Notch and Wnt signaling components in developing blast colonies revealed by quantitative PCR. 3' cDNA samples of day 3.25 Flk-1<sup>+</sup> cells (0h) and 3' cDNAs of seven individual blast colonies used in (B) were pooled and analyzed at each time point (12-48h). Average expression normalized to *actb* is shown. Error bars represent the standard errors of mean from three independent experiments.

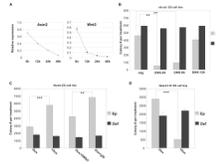


**Figure 2. Immunostaining of Numb, cleaved Notch1 intracellular domain and active  $\beta$ -catenin in developing blast colonies**

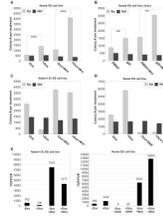
Immunostaining showing (A) Numb and activated  $\beta$ -catenin (b-cat), (B) cleaved Notch1 intracellular domain (NIC) and activated  $\beta$ -catenin (b-cat) in 12 hr, 24 hr and 48 hr blast colonies. Merged images show the overlay of three channels (DAPI, Cy2 and Cy3). Light transmission images (DIC) display the morphology of the cells. Arrows indicate the nuclear presence of activated  $\beta$ -catenin. Scale bars represent 5  $\mu$ m.



**Figure 3. Wnt and Notch signaling affects blast colony formation from the hemangioblast**  
 (A) Role of Wnt signaling in blast colony development.  $4 \times 10^4$  Flk1+ cells isolated from the day 2.75 EBs generated from either sb-cat or Notch1-IC ES cells were cultured in M10 serum-free hemangioblast media in the presence or absence of doxycyclin (Dox; 2  $\mu$ g/ml) and DKK1 (DKK1; 300ng/ml) as indicated. Dox was added at 0 hr of culture, while DKK1 was added at 6 hr of culture. Colonies were washed with media at 24 hr to deplete DKK1 and Dox, and replated back into fresh M10. Blast and core colonies were scored following 4 days of culture. (B) Effect of DKK1 treatment on blast and core colony development. (C) Hematopoietic potential of DKK1 treated colonies. 1600 colonies of each group were picked at day 3 of culture, pooled, dissociated and replated into hematopoietic methylcellulose cultures. Ep: primitive erythroid colonies; Def: combined macrophage, bipotential macrophage/erythroid, definitive erythroid and multipotential myeloid/erythroid colonies. (D) Role of Numb in blast colony development. gSI  $\gamma$ -secretase inhibitor X (2.5  $\mu$ M) (E) Role of Notch signaling in blast colony development. (A-E) Error bars represent standard deviations of the mean of number of colonies from n independent experiments (A, n=3; B, n=3; C, n=3; D, n=4; E, n=4). (\*\*P-value<0.01; \*\*\*P-value<0.001)

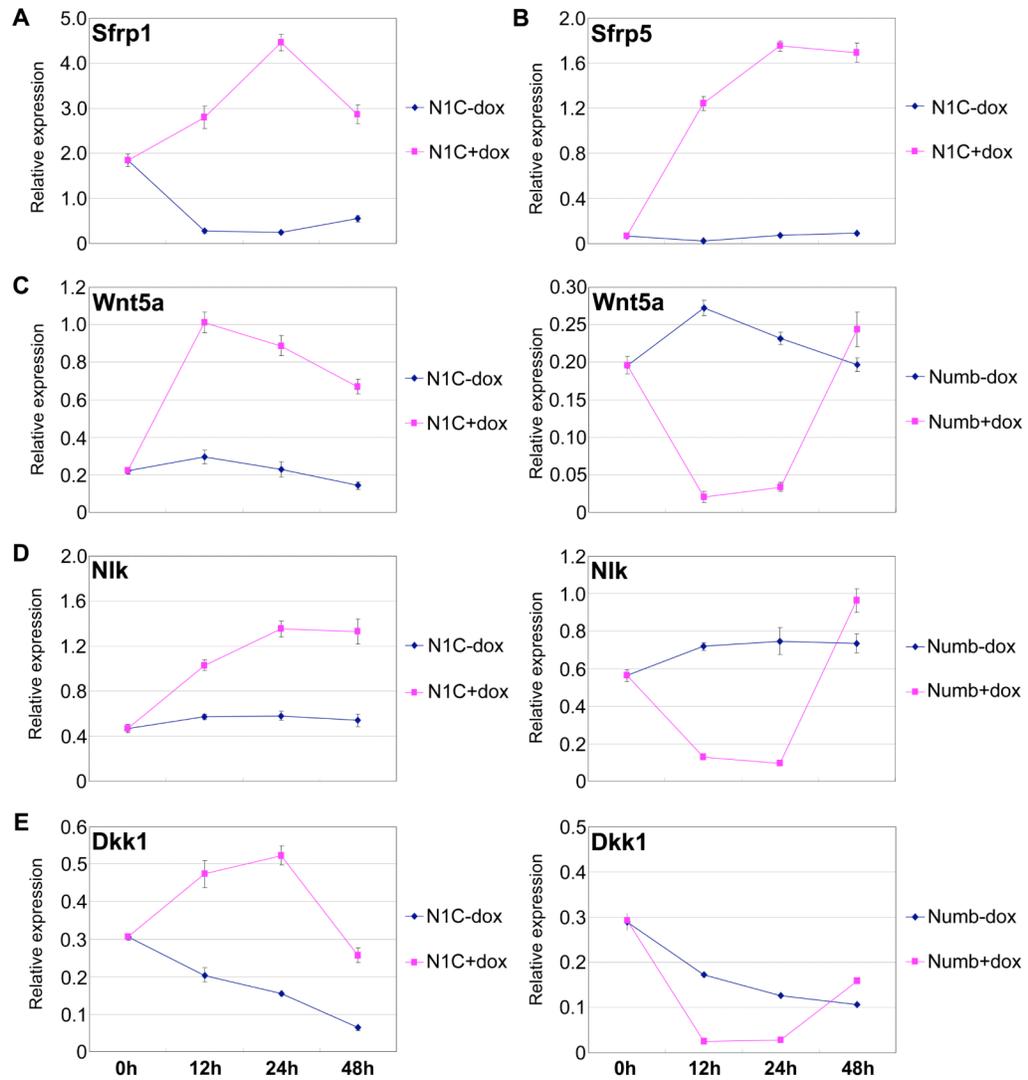


**Figure 4. Wnt and Notch signaling regulate primitive erythroid development from Flk-1+ cells**  
 Flk-1<sup>+</sup> cells isolated from day 2.75 EBs generated from the indicated ES cell lines were cultured as aggregates in serum-free media containing VEGF (10 ng/ml) and either DKK1 (300 ng/ml), Dox (2  $\mu$ g/ml),  $\gamma$ -secretase inhibitor (2.5  $\mu$ M) or DMSO (2.5  $\mu$ l/ml), as specified. (A) Expression of *Axin2* and *Wnt3* in aggregates at different times. Average expression normalized to *actb* is shown. (B) Transient requirement for Wnt signaling in primitive erythroid specification of Flk-1+ cells Ep: primitive erythroid colonies; Def: combined macrophage, bipotential macrophage/erythroid, definitive erythroid and multipotential myeloid/erythroid colonies. Total colony numbers are shown (C) Effect of Numb expression or addition of gSI on primitive erythroid development from Flk-1+ cells. -Dox: doxycyclin-untreated group; +Dox: doxycyclin was added for the first 24 hours, and then washed away. (D) Effect of Notch expression on primitive erythroid development from Flk-1+ cells. Dox was added as indicated above in (C). (B-D) Error bars represent standard deviation of the mean number of colonies from n independent experiments (B, n=3; C, for the first three groups n=6, and last two groups n=3; D, n=6). (\*\*P-value<0.01; \*\*\*P-value<0.001)



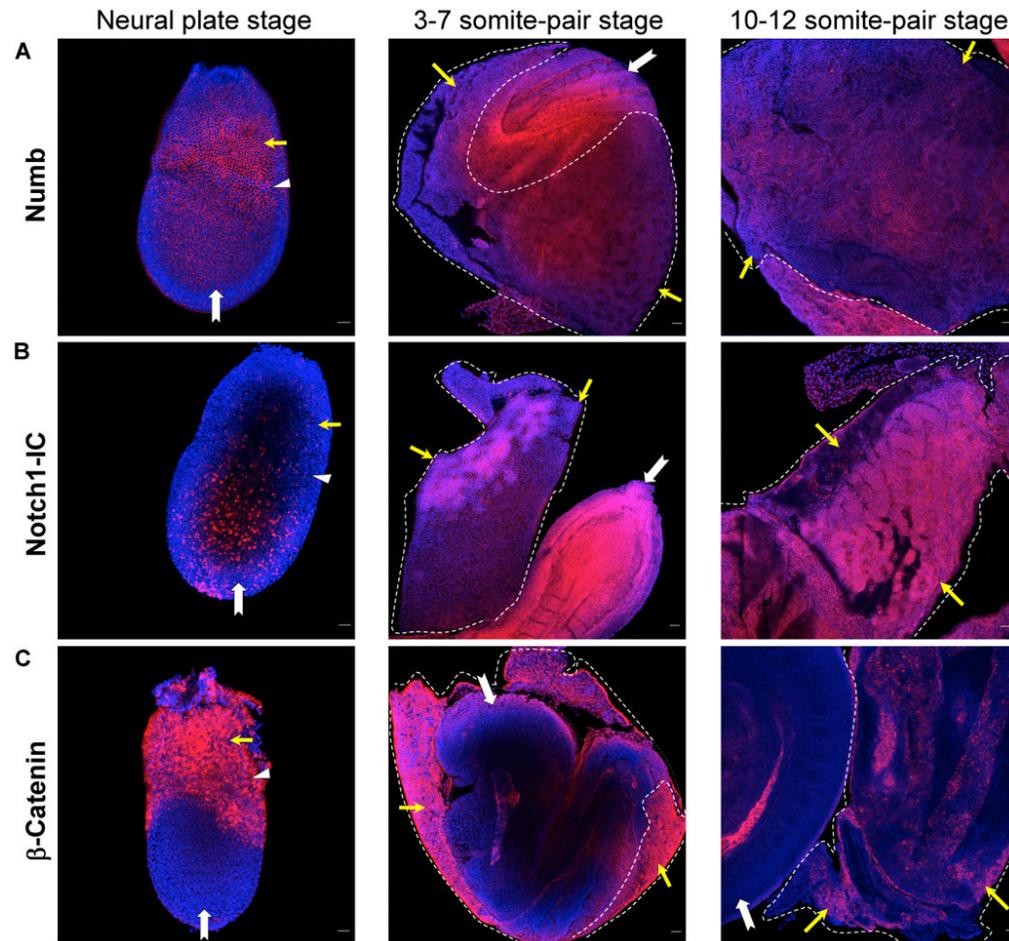
**Figure 5. Interaction of Wnt and Notch signaling in modulating primitive erythropoiesis from Flk-1+ cells**

Flk-1 cells isolated from day 2.75 EBs generated from the indicated ES cell lines were cultured as aggregates in serum free media containing hVEGF (10ng/ml) and either Wnt3a (100 ng/ml), DKK1 (300 ng/ml), Dox (2  $\mu$ g/ml),  $\gamma$ -secretase inhibitor (gSI; 2.5  $\mu$ M) or DMSO (2.5  $\mu$ l/ml), as specified. Hematopoietic potential of the aggregates was assayed as described in the Figure 4 legend. (A) Synergistic effects of Numb and Wnt3a on primitive erythroid development from Flk-1+ cells. (B) Synergistic effects of  $\gamma$ -secretase inhibitor and Wnt3a on primitive erythroid development from Flk-1+ cells. (C) Effect of Wnt3a on Notch1-IC-induced suppression of primitive erythroid development. (D) Effect of Numb expression on DKK1-induced block in primitive erythropoiesis. DKK1 was added to the reaggregation media after 6 hours of culture to allow time for Numb protein to accumulate. (A-D) Error bars represent the standard deviation of the mean of the number of colonies from n independent experiments (A, n=6; B, n=4; C, n=5; D, n=3) (\*\*P-value<0.01; \*\*\*P-value<0.001). (E) The TOP/FOPflash reporter assay demonstrates interaction between Wnt and Notch pathways. Error bars indicate standard deviation of TOP/FOP values of triplicate electroporations. Numbers above each bar represent actual TOP/FOP values.

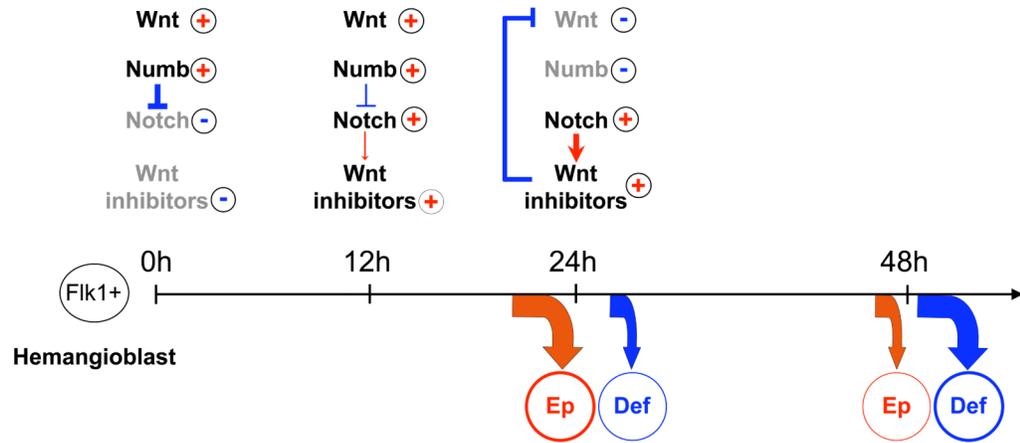


**Figure 6. Expression analyses of Flk-1+ cell-derived populations following induction of Notch1-IC or Numb**

Flk-1<sup>+</sup> cells isolated from day 2.75 EBs generated from either the Numb or Notch1-IC ES cell line were cultured as aggregates with VEGF (10 ng/ml) in the presence or absence of Dox (2  $\mu$ g/ml). Flk-1<sup>+</sup> cells prior to culture (0h) or aggregates following 12hr, 24hr, and 48hr of culture were harvested for qPCR. Average expression normalized to *actb* is shown. Error bars represent the standard errors of mean from three independent experiments. (A) *Sfrp1*; (B) *Sfrp5*; (C) *Wnt5a*; (D) *Nlk*; (E) *Dkk1*



**Figure 7. Expression of Numb, Notch1-IC, and activated  $\beta$ -Catenin in early embryos**  
 Confocal images (maximum projection by z-stacking, XY planes) of whole mount immunostaining for (A) NUMB, (B) cleaved Notch1 intracellular domain (Notch1-IC) and (C) activated  $\beta$ -catenin in different staged mouse embryos and yolk sacs. Yellow arrows indicate the yolk sac; white arrow-heads indicate the posterior primitive region in neural plate stage embryos, and white arrows indicate the embryo proper. Dashed lines delineate the YS region in somite-stage embryos. Scale bars represent 50 $\mu$ m. Red: Numb, or Notch1-IC or activated  $\beta$ -catenin; Blue: DAPI



**Figure 8. Model of Primitive Erythroid Development**

Shown are the changes in the signaling pathways during the first 24 hours of blast colony development. Both Wnt and Numb are indicated as active in the isolated Flk-1+ population (0h) as well as in the 12 hour colonies. The Notch pathway is not active in the starting Flk-1 population, but is activated within the first 12 hours of colony growth. The activation of Notch induces different Wnt inhibitors, which in turn inhibits the Wnt pathway by 24 hours. Arrows below the line indicate specification to the primitive erythroid and definitive hematopoietic lineages. The thicker red arrow at 24 hours represents a higher proportion of primitive erythroid progenitors at this stage, whereas the thicker blue arrow at 48 hours depicts an expansion of the definitive lineages.