INVESTIGATING THE ROLE OF ANKYRIN REPEAT AND SOCS BOX PROTEIN 4 (ASB4) DURING VASCULAR DEVELOPMENT

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ABSTRACT

James E. Ferguson, III: Investigating the Role of Ankyrin Repeat and SOCS Box Protein 4 (ASB4) During Vascular Development

(Under the direction of Cam Patterson)

The molecular mechanisms of endothelial differentiation into a functional vascular network are incompletely understood. To identify novel factors in endothelial development we employed a microarray screen using differentiating embryonic stem (ES) cells that identified Ankyrin Repeat and SOCS Box Protein 4 (ASB4) as the most highly differentially expressed gene in the vascular lineage during early differentiation. Like other SOCS box-containing proteins, ASB4 acts as the substrate recognition molecule of an Elongin-B/Elongin-C/Cullin/Roc ubiquitin ligase complex that mediates the ubiquitination and degradation of substrate protein(s). High levels of ASB4 expression in the embryonic vasculature coincide with drastic increases in oxygen tension as placental blood flow is initiated. However, as vessels mature and oxygen levels stabilize, ASB4 expression is quickly downregulated, suggesting that ASB4 may function to modulate an endothelial-specific response to increasing oxygen tension. Consistent with the hypothesis that ASB4 function is regulated by oxygen concentration, ASB4 directly interacts with Factor Inhibiting HIF1α (FIH), and is a substrate for FIH-mediated hydroxylation via an oxygen-dependent mechanism. Additionally, overexpression of ASB4 in ES cells promotes differentiation into

the vascular lineage in an oxygen-dependent manner. We propose that hydroxylation of ASB4 in normoxia promotes binding to and degradation of substrate protein(s) to modulate vascular differentiation.

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LIST OF ABBREVIATIONS

ASB4	Ankyrin Repeat and SOCS box Containing Protein 4
SOCS	Suppressor of Cytokine Signaling
FIH	Factor Inhibiting HIF1α
HIF	Hypoxia Inducible Factor
ES cells	Embryonic Stem Cells
EB	Embryoid Body
TK	Thymidine Kinase
DT	Diphtheria Toxin

Chapter 1 GENERAL INTRODUCTION

A. ASB4 IS AN E3 UBIQUITIN LIGASE

Ankyrin repeat and SOCS box protein 4 (ASB4) is a member of the Suppressor of Cytokine Signaling (SOCS) superfamily of proteins whose members all share a constant C-terminal SOCS box domain, and variable N-terminal protein-protein interaction motifs (Fig. 1-1A). The SOCS box has been shown to bind to an Elongin-B/Elongin-C/Cullin/Roc complex of proteins whose function as an E3 ubiquitin ligase has been well characterized (Fig. 1-1B). The N-terminal protein interaction motif binds to substrate protein(s) to optimally position them for ubiquitination and subsequent degradation. Thus, SOCS proteins are the substrate-receptors for an E3 ubiquitin ligase complex that controls steady-state levels of substrate proteins. Because of this, SOCS proteins like ASB4 are carefully regulated both at the transcriptional and post-translational level to tightly control substrate protein levels.

Generally, polyubiquitin chain formation on proteins is a molecular signal for their proteasome-mediated degradation. Polyubiquitination is achieved through a cascade of biochemical reactions (Fig. 1-1B). First, E1 ubiquitin activating enzyme covalently binds to the 7 kDa ubiquitin protein via an ATP-dependent process. Next, the E1 enzyme transfers the ubiquitin to an E2 ubiquitin conjugating enzyme. Finally, in the case of cullin-containing complexes the E2 is closely approximated to the substrate by binding to the ring-finger domain of the E3 complex, which results in covalent attachment of ubiquitin to lysine residues of the substrate protein. This cycle is repeated and additional ubiquitin molecules are

added to the substrate-attached ubiquitin to produce a polyubiquitin chain. Ubiquitin chains longer than three molecules long are recognized by the proteasome and rapidly degraded.

Ankyrin repeat and SOCS box proteins (ASBs) constitute one subclass of the SOCS superfamily and are characterized by variable numbers of N-terminal ankyrin repeats as substrate-binding domains (reviewed in [1]). Over the past five years, at least 18 family members have been identified in mammals, and preliminary functional characterization is currently underway. So far, ASB proteins have been shown to mediate the ubiquitination of a broad range of target proteins including TNF receptor II (ASB3)[2], Creatine Kinase B (ASB9)[3], and Adaptor protein with PH and SH2 domains (APS) (ASB6)[4]. Since ankyrin repeats function as generic scaffolds creating modular binding sites that mediate interactions with an almost unlimited variety of binding motifs and domains,[5, 6] it is not surprising that ASBs interact with and promote the degradation of a wide diversity of target substrate proteins. Thus, while these family members share the common function of ubiquitination and target protein degradation, the biologic ramifications of their activities are likely to be as diverse as the wide variety of substrates which they degrade.

In order to identify novel and/or uncharacterized genes whose function is important to early vascular differentiation and development of the vascular lineage, we performed a microarray screen that identified genes that are highly differentially expressed in vascular cells (Flk1 positive cells) compared to non-vascular (Flk1 negative cells) during embryonic stem (ES) cell differentiation.[7] Out of 20,000 genes, ASB4 was the most highly differentially expressed gene at early time points during differentiation into the vascular lineage. This dissertation describes characterization of the involvement of the previously uncharacterized

gene ASB4 during vascular development, and more specifically, endothelial differentiation and maturation.

B. Blood Vessel Development

Endothelial Commitment (Vasculogenesis) is the first step in blood vessel development. Early in development, just after gastrulation, precursor cells of the vascular lineage differentiate from pluripotent mesoderm cells in response to inductive signals from neighboring endoderm.[8, 9] These cells colocalize with hematopoietic precursors within the yolk-sac in structures called blood islands, raising the possibility that both lineages arise from a common precursor, the hemangioblast. Studies have indicated that both lineages bear certain common molecular markers, and cells with the potential to produce either lineage (as well as vascular smooth muscle cells (VSMCs) and cardiomyocytes, under specific conditions) have been isolated from differentiating mouse embryoid bodies.[10, 11] However, it does not seem likely that hemangioblast identity is a required step for all endothelial development, and in fact direct differentiation of angioblasts from mesoderm is a well-supported phenomenon.[8, 12] In addition, it has been suggested that in some cases hematopoietic cells may arise directly from differentiated endothelium—so-called hemogenic endothelium.[13, 14] Clearly, differentiation into the vascular and hematopoietic lineages during development is closely intertwined.

Blood vessel formation is classically divided into two categories. Vasculogenesis refers to the *in situ* differentiation of endothelial cells to form blood vessels. In contrast, angiogenesis refers to the formation of new blood vessels via extension or remodeling of existing blood vessels. Angiogenesis occurs throughout development and in adulthood,

whereas vasculogenesis is generally thought to occur during a limited period early in embryonic development. Vasculogenesis is further subdivided based on whether it occurs within the extraembryonic or intraembryonic compartments. The best available evidence suggests that these two waves of vasculogenesis are temporally and spatially distinct, and molecular studies indicate that they are also partially distinct at the mechanistic level.

Extraembryonic vasculogenesis precedes intraembryonic vascular development, and in mammals is first apparent as blood islands assembling within the mesodermal layer of the yolk sac. Blood islands are foci of hemangioblasts that differentiate *in situ*, forming a loose inner mass of embryonic hematopoietic precursors and an outer luminal layer of angioblasts. Blood islands eventually coalesce into a functional vascular network that constitutes the vitelline circulation, which is adapted to transfer nutrients from the yolk sac to the embryo proper. Recent evidence indicates that extraembryonic blood vessels may also arise independently of blood islands via direct differentiation of angioblasts from mesoderm.[12] Vessels arising via yolk sac vasculogenesis communicate with the fetal circulation via the vitelline vein, but otherwise do not contribute to intraembryonic vasculature.

De novo extraembryonic vasculogenesis also occurs in the allantois, a structure responsible for the induction of placental development and for the formation of the umbilical vessels. Avascular allantoides can be excised from the developing embryo and cultured in isolation for one day, during which time they develop a definitive vascular plexus, indicating that vasculogenesis can proceed independently in the allantois during development.[15, 16] Because of this, primitive vessels are already in place in the allantois as it makes contact with the chorion to facilitate the formation of the maternal–fetal circulation. Whether these cells also contribute to hematopoiesis and whether they receive inductive signals from endoderm

or allantoic mesothelium is still undergoing debate, and may differ between species.[17, 18] Furthermore, given the close proximity of the developing allantois to the yolk sac mesoderm and posterior primitive streak,[8] structures with potent hemangioblastic potential, further studies have sought to address the exact temporal and anatomic origins of the vasculogenic cells resident in the allantois.[19]

Intraembryonic vasculogenesis. Recent evidence suggests that intraembryonic vasculogenesis can occur throughout most of the intraembryonic mesoderm, and much work has been done to characterize the different origins of particular vascular structures. As might be expected, the endocardium and great vessels are the first intraembryonic endothelial structures formed during development.[12] The endocardium originates in mammals from clusters of migrating angioblasts derived from presomitic cranial mesoderm that enter the pericardial area to form a vascular plexus adjacent to the developing myocardium. This plexus then undergoes remodeling to form the endocardial tube. Simultaneous to heart development, vasculogenesis is initiated within the aortic primordia, a collection of mesoderm just lateral to the midline, to give rise to the dorsal aortae and the cardinal veins.[20] During this development, differentiating angioblasts assemble into primary vascular networks, which are then remodeled in a bidirectional fashion to generate the bilateral embryonic aortae. This region, later termed the para-aortic splanchnopleure (PAS), and then the aorta-gonad-mesonephros (AGM) region, continues to be a "hot-spot" for hematovascular differentiation during development.[13]

As the heart enlarges, passive diffusion of nutrients and waste becomes limiting, and a coronary vasculature is formed to supply the metabolically active heart tissue. Vascular precursor cells (including endothelial and smooth muscle cell progenitors) reside in the pro-

epicardium (PE)—a mass of cells that appears to originate from splanchnic mesoderm in conjunction with the septum transversum just inferior to the heart.[21] This cell mass makes contact with the developing heart tube and quickly spreads over the entire heart. Once spread, these cells undergo epithelial-to-mesenchymal transformation and invade the underlying mesoderm to give rise to the capillaries, veins, and arteries of the coronary vasculature.[22] The development of the coronary circulation is clearly unique, and still requires investigation to determine the timing and molecular nature of the signals necessary to induce endothelial differentiation in the PE.[23, 24]

Capillary Plexi are Remodeled to Generate Mature Vasculature. Before blood flow can be established in the primitive vasculature, the capillary plexi of endothelial cells must be remodeled into a unidirectional circuit to allow proper blood circulation. To this end, endothelial cells are determined for either arterial or venous fate. Once this is accomplished, and simultaneous to blood flow initiation, VSMCs are recruited to surround the endothelial tubes and provide structural support and elasticity to the vessel wall. Once reinforced by this VSMC layer, the vasculature can develop in the midst of drastic increases in mechanical hemodynamic stresses during embryonic development.

Once blood flow is established, the vasculature can be considered "mature" since it is exposed to the relatively constant environmental forces that will exist for the rest of the life of the organism (i.e. blood flow/shear stress, blood pressure/mechanical stress, blood oxygenation/oxygen stress, etc). However, when these environmental forces are perturbed, the vasculature can undergo a programmed response to correct the perturbation. Collectively, this response is termed "angiogenesis" and is thought to be largely mechanistically distinct from the process of "vasculogenesis" described above. During angiogenesis, new vessels

sprout from pre-existing vessels in response to signals from the surrounding tissues. Hypoxia is often the initial cue that sets this process in motion and can exist in many biologic situations. For example, tissues can develop hypoxia during periods of rapid growth as their metabolic demands exceed the supply of the inherent vasculature (as exhibited during embryonic development and tumorigenesis). Hypoxic tissues recruit new blood vessels by secreting growth factors that act specifically on vascular cells (described below), which leads to the breakdown of the vessel wall and concomitant migration/proliferation of endothelial cells towards the ischemic tissue. Once the endothelial circuit is re-established in the hypoxic tissue, oxygenation is increased, the hypoxic signal is abrogated, and the baseline vascular phenotype is resumed. Clearly, formation and maintenance of the vascular bed is a complex process that involves the coordinated actions of multiple cell types. However, the initial events of vascular development that involve endothelial differentiation, remodeling, and maturation, are the focus of this dissertation.

C. Genetic and Environmental Factors Influence Vasculogenesis

The anatomic basis for vascular development in the embryo has been clarified through studies that have been performed over the past century. In contrast, the molecular underpinnings for endothelial cell differentiation have only become clear in the last twelve years. In addition to cell-autonomous genetic determinants of endothelial differentiation, soluble factors are released that influence the fate of multiple cell lineages. Gain and loss-of-function studies have helped us identify some of the participants in vasculogenesis. Specifically, targeted gene inactivation studies have better-defined the molecular regulatory

mechanisms underlying these processes. In this section, the inter-relationships among some of the identified steps important to endothelial differentiation are reviewed.

Initiation. Key to any inductive process in the embryo is the elaboration of signals that are integrated to mark the initiation of a developmental cascade. In the case of early events in endothelial differentiation, these cues must determine the spatial and temporal appearance of hemangioblasts from undifferentiated mesoderm, and also the further maturation and assembly of these precursors into nascent blood vessels. While our understanding of these processes is still growing, the best data indicate that crucial roles exist for members of the Fibroblast Growth Factors (FGF) and Bone Morphogenetic Proteins (BMP) growth factor families as proximal inductive cues for the hematovascular lineage. Definitive endoderm and mesoderm specification is also thought to be mediated in part by *Brachyury*, a transcriptional target of the Wnt signaling pathway.[25] However, little is known about how these upstream factors interact to specify vascular identity of precursors and none of these signals has specificity for the vascular lineage.

The first secreted molecule with specificity for the endothelium during development is Vascular Endothelial Growth Factor (VEGF), and the role of VEGF family members in developmental and adult angiogenesis is now well-described. Mice lacking a single copy of the VEGF gene die early in development due to vascular defects, indicating that only slight perturbations of this signaling pathway can disrupt vascular development.[26] Since cells that respond to VEGF must first express its receptors (Flk1/VEGFR2 and Flt1/VEGFR1), VEGF itself cannot be the most proximal signal for endothelial differentiation.

VEGF/Flk1 Regulation. Several upstream factors have been shown to regulate VEGF and Flk1 expression. The transcription factor Hypoxia Inducible Factor-1 (HIF-1) and its

family members play crucial roles in sensing changes in tissue oxygen tension and in stimulating gene expression changes that enhance blood vessel growth into hypoxic tissues during post-gastrulation development[27] (described in next section). Evidence supports BMP signaling as a proximal stimulus for Flk1 expression, and at the transcriptional level there appears to be necessary roles for GATA family proteins as well as the homeodomain protein HoxB5 in upregulation of Flk1 during development.[28] Another layer of regulation in endothelial development is ascribed to the ETS family of transcriptional factors that direct downstream endothelial specific expression of Flk1, Flt1, the angiopoietin receptors, and MEF2C, a recently identified member of the MADS box superfamily of vascular developmental transcription factors.[29] A genetic mutation of an unknown gene, termed "cloche," leads to Flk1 deficiency with a subsequent failure in vasculogenesis.[30, 31] Further studies are needed to identify and characterize upstream cues that initiate Flk1 expression and concomitant endothelial differentiation.

Functional Markers of Endothelial Differentiation. Identified markers of endothelial cells are used to track their transition from the early stages of stem cell differentiation to the mature vessel, as well as to distinguish them from other lineages, such as hematopoietic and smooth muscle. Not surprisingly, many of the molecules used to track endothelial identity are also functionally important. The appearance of Flk1 expression is at the present time the earliest marker available for the endothelial lineage during development.[32, 33] Flk1 is expressed in early endothelial and hematopoietic precursor cells, but persists only in mature endothelium but not mature hematopoietic lineages. This pattern suggests that Flk1 may be a marker for hemangioblasts during early development, and this is born out by studies that indicate that the co-expression of Flk1 with mesoderm-derived transcription factors

Brachyury or Scl/Tal denotes hemangioblasts.[12, 34] As the endothelial lineage progresses, the expression of Brachyury followed by Scl/Tal are lost, whereas the Flk1 marker is retained.[35] Notably, *flk1*$^{-/-}$ mice are embryonic lethal due to drastically reduced levels of vascular precursors and failure in blood vessel formation, while elimination of *SCL/Tal* expression arrests hematopoiesis, but allows for endothelial cell progression.[36] The entire transcriptional program of the endothelial lineage is yet to be established, and this represents a major limitation in our understanding of how cell-autonomous mechanisms coordinate development of vascular cell lineages.

Combinations of endothelial-specific markers including VE-cadherin, PECAM-1, Tie-1, Tie-2, Flk1, and Flt1 are commonly used to trace endothelial differentiation. As might be expected, these markers also serve in functions vital to endothelial formation, remodeling, and maintenance. For example, VE-cadherin is expressed from the committed angioblast to the mature endothelial cell, but not in hematopoietic progenitor cells. VE-cadherin, an adhesive cell–cell recognition protein, participates in cell-migration during vascular morphogenesis. With the ability to anchor to the cortical actin cytoskeleton via catenin proteins and vinculin, VE-cadherin offers junctional strength between endothelial cells. Mice deficient for VE-cadherin fail to couple a VEGF–dependent endothelial survival signal to β-catenin and PI3-K, resulting in early embryonic death caused by severe vascular defects.[37] PECAM-1 and Tie also function during endothelial differentiation with roles in adhesion and vascular network formation, respectively.[38] PECAM-1 is commonly used to trace endothelial morphological development (i.e. sprouting vessels).[28, 39] Although the fine points of their signal transduction pathways remain poorly understood, the function of markers used to

identify endothelial cells and their progenitors have provided us a framework for reaching a more complete understanding of vasculogenesis.

Additional Regulatory Cues. Downstream of the early events, the cues required for blood vessel assembly become better known, if also more complex. Among paracrine factors, the Angiopoietin family members bind to the tyrosine kinase receptors Tie-1 and Tie-2, which play crucial roles in endothelial cell survival and remodeling of capillary plexi.[40] Several transcription factors are known to participate in vascular assembly including Vascular Endothelial Zinc Finger, GATA proteins, several members of the Kruppel-like family of zinc finger proteins, and Ets proteins.[41, 42] Finally, Platelet-derived Growth Factors (PDGFs) are required for recruitment of pericytes and VSMCs to invest developing arteries and establish vasomotor tone.[43] Obviously, blood vessel formation requires multiple pathways that talk among one another to coordinate the spatial and temporal differential gene expression pattern during blood vessel formation.

Vessel Fate. Interestingly, the fate of arterial, venous, and lymphatic vessels are in part genetically determined early during vascular development before circulation and during the amalgamation of blood islands in the yolk sac. Molecules such as Ephrins, Ephrin Receptors, neuropilin receptors, and type-A plexins serve as markers for arterial and venous identity, and appear well before the structuring of tube-like vessels.[44] Arterial cell fate appears to be determined by intersecting signaling cascades involving first sonic hedgehog (Shh), then VEGF, followed by the Notch pathway.[45] Loss of Shh or VEGF results in loss of arterial markers, whereas VEGF can rescue arterial differentiation in the absence of Shh signaling. The Notch pathway has also been reported to repress venous markers and to promote the expression of artery-specific genes.[46] Ephrins and their tyrosine kinase receptors

(Ephs) are required for arterio-venous communications.[47] Ephrin-B2, a membrane bound ligand with specificity toward the Eph-B4 receptor, marks arterial but not venous endothelial cells from the onset of vessel remodeling. In turn, Eph-B4 marks veins but not arteries.[48] The null phenotype for each of these genes is similar, suggesting reciprocal coordination between these molecules in the formation of capillary beds.

Environmental Factors. Clearly, genetics plays a crucial role in determining vascular development, especially in early stages such as endothelial specification and capillary plexus formation. However, the embryo doesn't develop in a vacuum. Rather, the developing embryo and its vasculature undergo drastic environmental changes that impact the development of the vasculature in fundamental ways (Fig. 1-2). First, following the initiation of the heart beat and completion of cardiac valvulogenesis, blood flow is initiated and both blood flow and blood pressure rise sharply. The endothelium is exposed to these changes and transduces a signal requiring KLF2 to remodel appropriately to these cues.[49] Second, shortly after the initiation of blood flow, the vasculature of the placenta forms and embryonic erythrocytes are juxtaposed to maternal blood for the first time. As maternal-fetal blood gas exchange proceeds, the oxygen levels of the developing embryo increase rapidly from hypoxia to relative normoxia, and all cells must transduce these environmental signals into appropriate developmental responses (discussed in next section).

Both the sudden increase in hemodynamics and the increase in blood gas oxygen levels are environmental cues that influence endothelial development around the remodeling stage. Perhaps these signals help to specify arterial/venous fate, and/or instruct the strengthening of major vessels (through VSMC recruitment) and the regression of minor

vessels.[50] Experiments to address these issues will require sophisticated models and approaches such as those described in Chapters 2 and 3.

D. Mechanisms of Oxygen Sensing in the Vascular Lineage

While it is well accepted that hypoxia drives angiogenesis in the adult vasculature, comparatively little is known about the behavior of the endothelium in the hypoxic environment of the early embryo, and how it responds to the drastic change in oxygen tensions following functional development of the placenta.

Cell-specific Oxygen Responses. The hypoxic response of cells of the vascular lineage is unique. Most non-vascular cells respond to hypoxia by switching to anaerobic respiration, blunting highly energetic processes such as protein translation and cell division, and halting differentiation.[51, 52] In addition, they secrete growth factors such as VEGF and Erythropoeitin (Epo) that act to re-establish perfusion and oxygenation, respectively. Each of these responses is mediated in large part by HIF transcription factors (described below). In contrast, in order to revascularize ischemic tissue, the cells of the vascular lineage must be able to undergo extensive morphologic and phenotypic changes such as migration, proliferation, and/or differentiation in the face of this hypoxic environment. Various data support this unique response of the vascular lineage to hypoxia, but the mechanisms behind this unique response, and the hypoxic response in general are limited.[53] Notably, HIF1α is nearly ubiquitously expressed, and therefore cannot account for this endothelial-specific responses to hypoxia.

Hypoxia Inducible Factor. The current understanding of the cellular response to oxygen tension centers around the Hypoxia Inducible Factor (HIF) family of transcription

factors whose steady-state levels and activity vary inversely with oxygen concentration (reviewed in [51, 54, 55]). HIF transcription factors are heterodimeric complexes composed of an α-subunit and a β-subunit. So far, 3 α-subunit genes and 3 β-subunit genes have been identified in eukaryotes. While β-subunits are nearly ubiquitously expressed at the protein level, α-subunits are constitutively transcribed and translated, but protein levels are tightly suppressed via post-translational modifications under normoxic conditions. This suppression (described below) is released under hypoxic conditions, allowing HIF dimerization and transcriptional activation. Once active, HIF heterodimers bind to hypoxia response elements (HREs) in the promoters of hypoxia responsive genes. These genes, in turn, are important for the hypoxic response of the whole organism, tissues, and individual cells. For example, in the face of global whole-animal hypoxia such as that found at high altitude, HIF induces the transcription of EPO which stimulates red blood cell formation and the oxygen-carrying capacity of the blood. In order to increase the oxygenation of ischemic tissues, HIF induces the transcription of a variety of angiogenic genes. Hypoxic stromal cells upregulate the HIF-dependent transcription and secretion of VEGF and FGF isoforms which act to stimulate the migration of endothelial cells into the hypoxic tissue. Furthermore, transcription of VEGF receptors Flk1 and Flt1 is activated in endothelial cells by HIF, further sensitizing them to the VEGF stimulation under hypoxic conditions. All of these signals function to revascularize the ischemic tissue, resulting in tissue reperfusion and return to normoxia. Finally, HIF activity orchestrates the immediate response of cells to hypoxia by allowing a short-term shift to oxygen-independent cellular metabolism. A variety of genes important in anaerobic respiration are upregulated to allow energy production in spite of decreased oxygen-dependent oxidative phosphorylation. These genes include glucose transporters such as Glut-

1), Phosphoglycerate Kinase (PGK1), and Lactate Dehydrogenase (LDH). HIF clearly plays an important role at all levels of the hypoxic response.

Mouse genetic models have shed considerable insight into the biologic importance of HIF family members during vascular development. *HIF1α*$^{-/-}$ mice die by day 11 of gestation due to extensive vascular defects and cardiovascular malformation.[56, 57] Vasculogenesis occurs normally in these mice, but the vascular endothelium fails to remodel properly and regresses by day 9.5.

HIF2α$^{-/-}$ mice have been generated by three separate groups with different results.[58-60] In the first group, *HIF2α*$^{-/-}$ mice died in mid-gestation due to bradycardia secondary to decreased catecholamine synthesis.[60] In the second group, some mice survived until birth, but died in the first day due to respiratory distress syndrome secondary to defects in lung surfactant production.[58] Finally, a third group also observed bradycardia with lethality between day 9.5 and day 13.5 that was accompanied by extensive defects in both intraembryonic and extraembryonic vascular remodeling.[59] Like the *HIF1α*$^{-/-}$ embryos, these animals exhibit normal vasculogenesis, but fail to normally remodel the capillary plexus, which produces improper vascular connections and results in vascular hemorrhage and pre-natal mortality.

Mice deficient for HIF1β/ARNT die by day 10.5 of embryonic development due to defective vascularization of the yolk sac, branchial arches, and placenta.[61-63] Again, similar to mice lacking HIFα subunits, initial development of the vascular beds proceeds normally, but vessel remodeling is abnormal. Taken together, these data support the hypothesis that HIF family members are crucial for proper vascular remodeling during embryonic development. Since remodeling of the primary capillary plexus into a mature vascular network occurs

during rapid changes in embryonic oxygen tension, it is intriguing to speculate that HIF family members are necessary for transducing environmental oxygen cues into appropriate vascular biologic responses and that this process is critical for proper vascular remodeling and maturation.

HIF Regulation. The mechanisms of oxygen sensing by the cell and by the HIF pathway specifically have been rigorously studied over the past eight years. While various organelles have been implicated as oxygen sensors, including mitochondria and endoplasmic reticulum, the exact mechanisms are still elusive.[64] On the other hand, the mechanisms of oxygen sensing by the HIF pathway are better understood (Fig. 1-3). HIFα subunits are hydroxylated on asparagine and proline residues by Factor Inhibiting HIF1α (FIH) and the Prolyl Hydroxylase enzymes (PHDs), respectively. These hydroxylation reactions are dependent upon oxygen concentration, and the K_D of these enzymes for oxygen lies in the range of physiologic oxygen concentrations. In this way, slight perturbations of oxygen concentrations result in drastic changes in enzymatic activity. Thus, these hydroxylase enzymes directly transduce oxygen environmental cues into biochemical signals: i.e. they act as biologic oxygen sensors. FIH-mediated HIF hydroxylation disrupts binding to the transcriptional co-activator p300 and results in decreased transcriptional activity, whereas PHD hydroxylation promotes binding of von Hippel Lindau (VHL) protein, a SOCS protein that mediates HIF polyubiquitination and proteasomal degradation through an Elongin-B/Elongin-C/Cul2/Roc1 complex. Since these hydroxylation reactions are oxygen-dependent, decreases in oxygen concentration (hypoxia) result in: 1) disruption of VHL binding to and degradation of HIF, leading to accumulation of HIF levels, and 2) promotion

of p300 binding, leading to an increase in HIF transcriptional activity. By these mechanisms, HIF is, according to its name, induced by hypoxia.

In conclusion, the oxygen-dependent effects of hydroxylase enzymes on HIF activity suggest that they act as cellular "oxygen sensors" that are important in the transduction of environmental hypoxic cues into appropriate cellular signals such as HIF-mediated upregulation of glycolytic and angiogenic genes. Nevertheless, the list of bona fide hydroxylation targets of FIH and the PHDs is limited. In fact, until recently, HIFα family members were the only known FIH and PHD substrates, and also the only identified cytosolic hydroxylated proteins. (Collagen and epidermal growth factor domain-containing proteins are extracellular hydroxylated proteins).[65] Thus, a major goal in the field has been to identify novel hydroxylation substrates in order to improve our understanding of the cellular response to changing oxygen tensions. Herein, I describe the endothelial-specific expression of ASB4, an FIH hydroxylation substrate that may provide a mechanistic explanation for the endothelial-specific response to hypoxia during development.

E. Embryonic Stem Cells as a Model System for Vasculogenesis

Embryonic stem (ES) cells, especially those of murine origin, have revolutionized our ability to investigate the process of blood vessel development. They have been used to study not only the earliest stages of endothelial specification,[10, 11, 28, 66] but also later morphogenetic processes in the primitive vasculature.[39] In fact, vascular differentiation in the embryoid body (EB) system so closely recapitulates that of early *in vivo* vascular development that it has been suggested that vascular phenotypes of genetic mutant animals can be predicted from the vascular phenotypes of their cognate ES cells in culture.[67-69] Furthermore, the accessibility of

this system has allowed genome-wide gene-trap analyses to identify novel genes whose expression is confined to the developing vasculature.[70] In addition to the EB model of ES cell differentiation, ES cells cultured under tightly defined conditions to promote the growth of specific lineages have been used to support and study the role of lineage-specific precursors, especially hemangioblasts, during development.[10] In fact, these same culture conditions have recently been used to determine that cells with potential to differentiate into both blood and endothelial cells exist in the posterior primitive streak of the early mouse embryo.[34] It is clear that the culture conditions (media, coated dishes, feeder cells, cystic embryoid body formation, etc.) play important roles not only in the kinetics but also in the extent of endothelial differentiation in these systems. It is also important to note that these stem cell systems are devoid of blood flow and lack the spatial organization and unique environmental conditions of the developing embryo, perhaps limiting their usefulness as physiologically relevant models for later developmental programs which exhibit greater degrees of cellular and environmental complexity. However, earlier processes such as vascular lineage specification, endothelial differentiation, and capillary plexus formation faithfully mimic the *in vivo* processes and ES cells can be cultured under hypoxic conditions to evaluate the effects of oxygen concentration on these biologic processes.

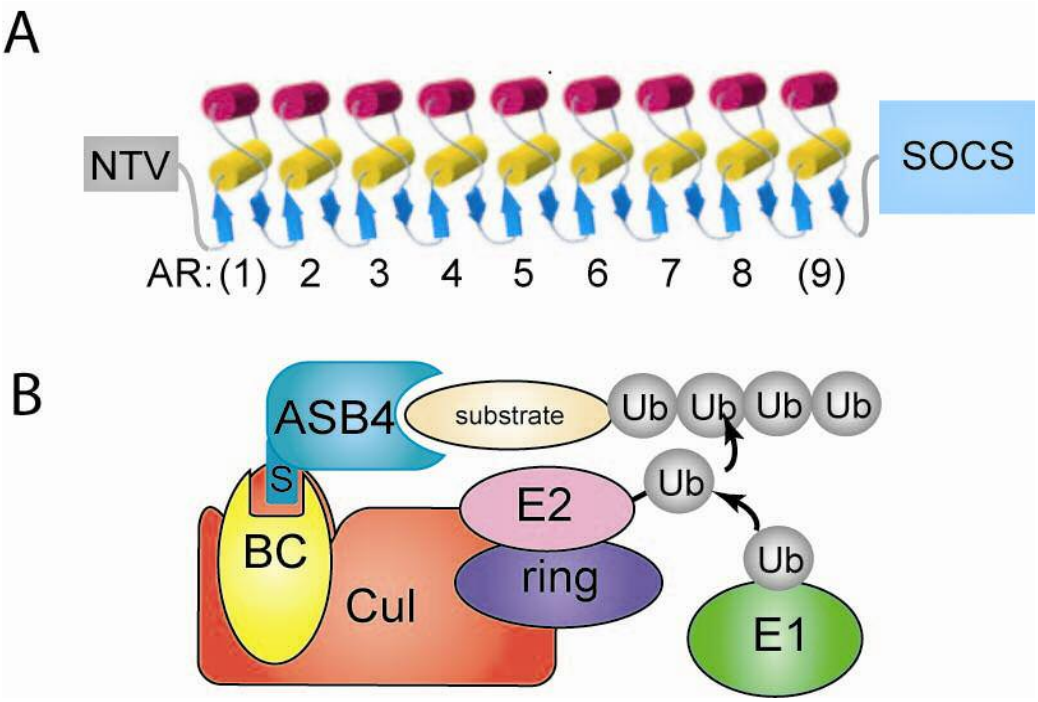

Figure 1-1 ASB4 Ubiquitin ligase complex.
(A) ASB4 structural schematic. ASB4's domain architecture consists of an N-terminal variable region (NTV), 9 tandem ankyrin repeats (AR) (poorly conserved ARs are denoted by parentheses), and a C-terminal SOCS box.

(B) Like other SOCS proteins, ASB4 binds an Elongin-B/Elongin-C/Cullin/Ring complex through its SOCS box, which contacts with both Elongins and Cullin. In this way, ASB4 confers substrate-specificity to the E3 ubiquitin ligase complex, mediating ubiquitination and subsequent degradation of substrate proteins. S, SOCS box. BC, elongins B/C. Cul, cullin. Ring, ring finger protein (also referred to as Roc). E2, ubiquitin conjugating enzyme. E1, ubiqutin activating enzyme. Ub, ubiquitin.

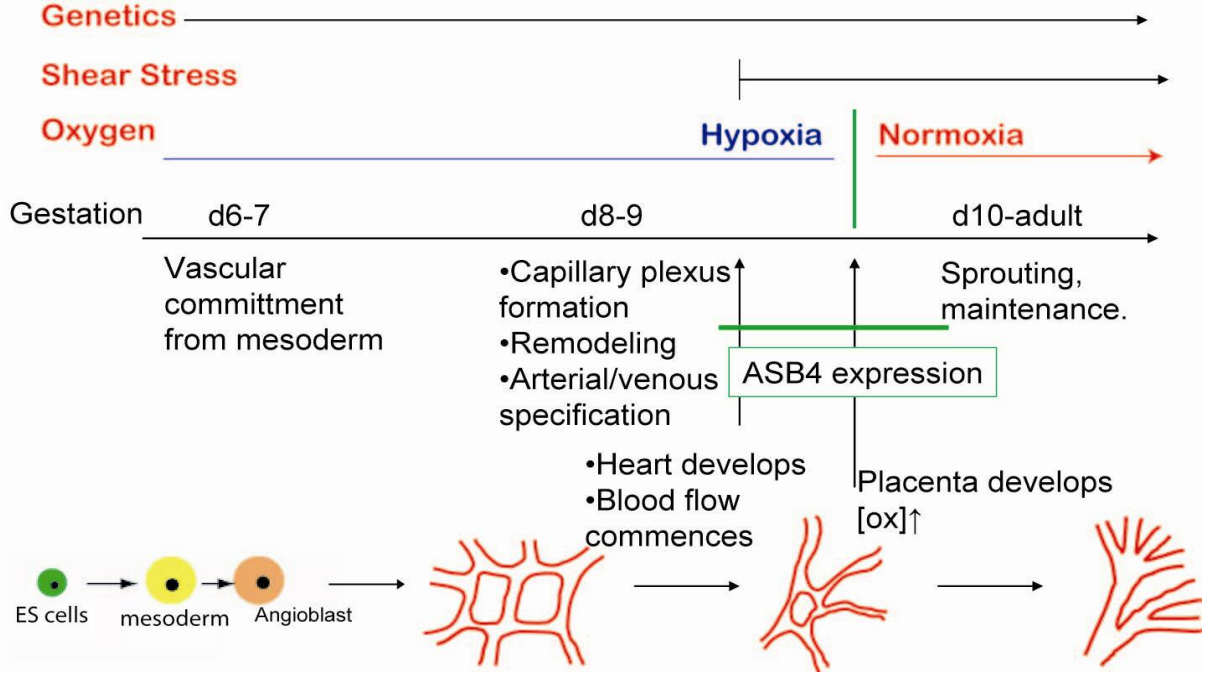

Figure 1-2 Endothelial development.
Early endothelial differentiation occurs under relatively constant environmental conditions, however later cardiovascular development including endothelial capillary plexus formation, and vascular sprouting and remodeling, occur alongside drastic changes in both blood flow (sensed by the endothelium in the form of shear stress), and oxygen levels. Shear stress increases drastically around day 8.5 as the heart tube begins to contract and endocardial cushions are remodeled into heart valves. Oxygen levels increase drastically around day 9.5 as the placental vasculature develops and maternal-fetal blood gas exchange is established. Notably, ASB4 is expressed in the endothelium during drastic changes in oxygen levels, suggesting that it might play a role in the response of the endothelium to changing oxygen tension.

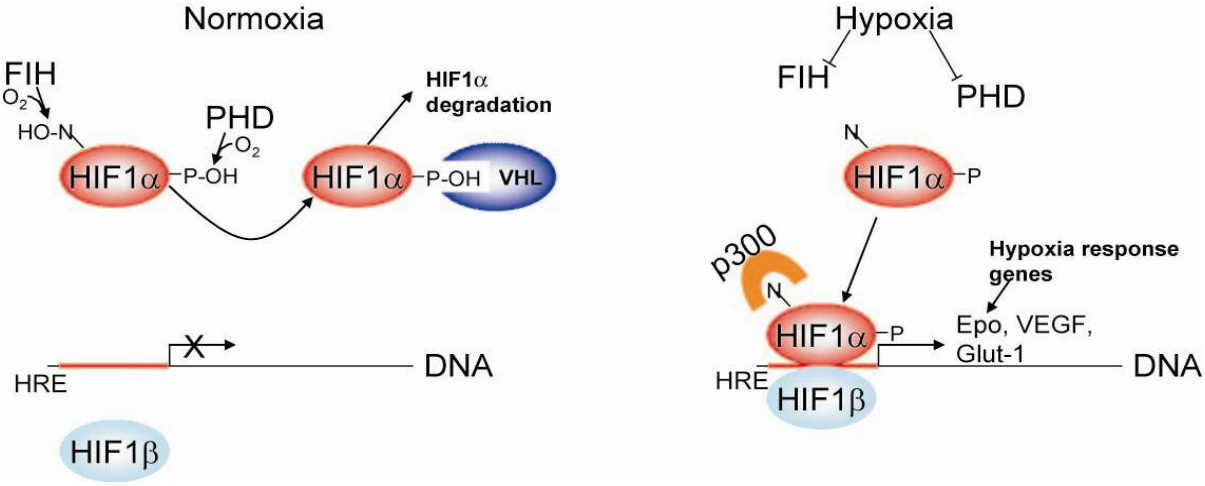

Figure 1-3 HIF schematic.
Under normoxic conditions, HIF1α subunits are hydroxylated on asparagine (N) and proline (P) residues by FIH and PHD enzymes, respectively. Asparagine hydroxylation prevents binding with the transcriptional co-activator p300, while proline hydroxylation promotes binding with the E3 ligase VHL, resulting in HIF1α degradation. Under hypoxic conditions, both hydroxylases are inhibited, HIF1α is stabilized and available for complex formation with HIF1β and p300. These complexes bind to hypoxia response elements (HRE) in the promoters of various hypoxia response genes to initiate the hypoxic response.

Chapter 2 ASB4 IS A HYDROXYLATION SUBSTRATE OF FIH AND PROMOTES VASCULAR DIFFERENTIATION VIA AN OXYGEN-DEPENDENT MECHANISM

A. INTRODUCTION

Protein members of the suppressor of cytokine signaling (SOCS) superfamily are E3 ubiquitin ligase components that contain a C-terminal SOCS box and an N-terminal protein-protein binding domain.[71,72] The SOCS box mediates interactions with an Elongin-B/Elongin-C/Cullin/Roc protein complex to constitute a functional E3 ubiquitin ligase complex,[73] while the N-terminal protein-protein binding domains recruit substrate proteins to mediate substrate polyubiquitination and proteasome-mediated degradation. In this way, SOCS proteins confer substrate specificity to the E3 ubiquitin ligase complex and are thus tightly regulated at both the transcriptional and post-translational levels in order to carefully control the steady state levels of substrate proteins.

Ankyrin repeat and SOCS box proteins (ASBs) constitute one subclass of the SOCS superfamily and are characterized by variable numbers of N-terminal ankyrin repeats as substrate-binding domains (reviewed in [1]). To date, at least 18 family members have been identified in mammals, and preliminary functional characterization is currently underway. So far, ASB proteins have been suggested to mediate the ubiquitination of a broad range of target proteins including TNF receptor II (ASB3)[2], Creatine Kinase B (ASB9)[3], and APS (ASB6)[4]. Since ankyrin repeats function as generic scaffolds creating modular binding sites that mediate interactions with an almost unlimited variety of binding motifs and domains,[5, 6]

it is not surprising that ASBs interact with and promote the degradation of a wide diversity of target substrate proteins.

Our previous data indicate that ASB4, a poorly characterized member of this family, is highly differentially expressed in the vascular lineage during development.[7] Vasculogenesis, or the *de novo* differentiation of pluripotent stem cells into the vascular lineage during development, is the first stage of blood vessel formation. Vasculogenesis begins shortly after gastrulation in the developing embryo, as cells with vasculogenic potential have been isolated from the primitive streak region in E6.5 mouse embryos.[34] These cells, termed hemangioblasts, derive from mesoderm, express Brachyury (T) and Flk1, and have both vascular and hematopoietic potential. Primitive capillary plexi of endothelial cells arise from these populations, and are then remodeled in a process similar to adult angiogenesis to yield mature lumenized vessels.

A complex combination of genetically pre-programmed molecular signals and external environmental cues are responsible for proper vascular development and remodeling, and an important role of oxygen tension in these processes has recently been discovered. The current understanding of the cellular response to oxygen tension centers around the Hypoxia Inducible Factor (HIF) family of transcription factors whose steady-state levels and activity vary inversely with oxygen concentration (reviewed in [51, 54, 55]). Factor Inhibiting HIF1 (FIH) and the Prolyl Hydroxylase enzymes (PHDs) catalyze the hydroxylation of HIFα on asparagine and proline residues, respectively. FIH-mediated HIF hydroxylation disrupts binding to the transcriptional co-activator p300 and results in decreased transcriptional activity, whereas PHD hydroxylation promotes binding of von Hippel Lindau (VHL) protein, a SOCS protein that mediates HIF polyubiquitination and

proteasomal degradation. Since atmospheric oxygen is utilized in these hydroxylation reactions, decreases in oxygen concentration (hypoxia) result in: 1) disruption of VHL binding to and degradation of HIF, leading to accumulation of HIF levels, and 2) promotion of p300 binding, leading to an increase in HIF transcriptional activity.

Because of its effects on HIF activity, FIH is considered, along with the PHDs, to be a cellular "oxygen sensor" that is central to the transduction of environmental hypoxic cues into appropriate cellular signals such as HIF-mediated upregulation of glycolytic and angiogenic genes. Nevertheless, the list of bona fide hydroxylation targets of FIH is limited. In the present report, we demonstrate that ASB4 is expressed during vascular development in a window of time during which oxygen tensions are rapidly changing, is a direct target for FIH-dependent hydroxylation, and promotes differentiation into the vascular lineage in an oxygen-dependent manner.

B. Materials and Methods

Plasmids— Flag-tagged mouse ASB4 in pEF1 and flag-tagged mouse ASB1 in pEF1 were generous gifts from W. Alexander. All PCR amplimers for cloning were TA-cloned, sequenced for accuracy, and (unless otherwise noted) subcloned into destination vectors as N-terminally tagged mouse sequences. All amino acid locations refer to accession #NP_075535. Myc-ASB4 was generated by PCR amplification using flag-ASB4 in pEF1 as a template and the primer pair: F: 5' ggatccttggacggcatcactgcccctatc, R: 5' gaattcttagtaaatgattccctctggctc, and subcloned into BamHI/EcoRI sites in pCMV3B. 3xflagASB4 in pCMV was generated by inserting a PCR amplimer using the following primers into EcoRI/BamHI digested p3xflag-CMV10 (Sigma): F:

5’gaattcagacggcatcactgcccctatc, R: 5’ggatccttagtaaatgattccctctggctc. The following flag-tagged constructs were amplified using flag-ASB4 in pEF1 as template with the following primers before sucloning into BamHI/EcoRI sites in pCMV2B (stratagene). Flag-ASB4 primers: F: 5’ctgggatccgacggcatcactgcccctatc, R: 5’ gaattcttagtaaatgattccctctggctc. Flag-ASB4ΔSOCS primers: F: 5’ctgggatccgacggcatcactgcccctatc, R: 5’ctggaattcttatctgggggtgttgcagcacac. Flag-ASB4ΔAR1 primers: F: 5’ctgggatcctgggccacaggcctgcatctg, R: 5’ gaattcttagtaaatgattccctctggctc. Flag-ASB4AR1-7 primers: F: 5’ctgggatccgacggcatcactgcccctatc, R: 5’ctggaattcttacagcacgtactggatagcggcaca. Flag-ASB4AR4-9 primers: F: 5’ctgggatccgggcacactgctttgcacttc, R: 5’ctggaattcttatctgggggtgttgcagcacac. Deletion mutants and point mutants were generated using the Quickchange mutagenesis kit (Stratagene) using flag-ASB4 in pCMV as a template with the following primers (since primers are reverse complements of each other, only one primer per pair is listed here): Flag-ASB4ΔAR4: 5’tactccctgagcgggcacacacccctgcacacggcagc. Flag-ASB4ΔAR5: 5’ccaacaaccaggatgaagagaccccactggccattgccacc. Flag-ASB4ΔAR6: 5’gcatgaacgcccacatggagaagtcccctctacac. Flag-ASB4ΔAR7: 5’cgctcgtgatgatgacttcaagacctccgtgcgtcctgctgc.

Human FIH was amplified from a yeast 2-hybrid prey clone described below using the following primers (F: 5’ggatccgcggcgacagcggcggaggctgtggcctctggctctg, R: 5’gaattcctagttgtatcggcccttgatcattg), digested with BamHI/EcoRI, and subcloned into pCMV3B (stratagene) to generate a myc-FIH mammalian expression construct.

An ASB4ΔSOCS fragment for use as a fusion bait protein in yeast 2-hybrid assays was amplified from flag-ASB4 in pEF1 template using the following primers (F: 5’

tatccatggtggacggcatcactgcccctatc, R: 5' ctgggatccttaatcccagtgtttctccaagtc), and was subcloned into pGBKT7 vector (Clontech) using NcoI-BamHI insertion sites. pGBKT7, pGADT7, pGBKT7-p53, and pGADT7-T were from Clontech.

To generate flag-ASB4 and flag-ASB4ΔSOCS encoding adenoviruses, inserts flanked by XbaI sites were excised with XbaI and subcloned into pAdTrack-CMV for adenoviral production. All adenoviruses co-expressed CMV promoter-driven GFP.

Reagents— MG-132 was dissolved in DMSO at a stock concentration of 20m*M* and diluted to 40μ*M* for use in experiments. Equal volumes of DMSO alone were used in all conditions not treated with MG-132. Dipyridyl, $CoCl_2$, and dimethyloxaloylglycine (DMOG) were obtained from Sigma dissolved sterile water. G418 (Gibco).

Antibodies— anti-FIH (Novus #NB 100-428). Anti-flag (Sigma), anti-myc (Santa Cruz, #sc-789), anti flag beads (Sigma #A2220), anti myc beads (Santa Cruz), cul2 (Xiong raised against: RSQASADEYSYVA)[74], cul5 (Xiong raised against SNLLKNKGSLQFEDK), elongin B (Santa Cruz #sc-1558), Roc1 has been described previously[74], anti-ub (Covance #MMS-258R).

Cell culture and Transfection Conditions— ES cell culture, differentiation, and FACS analysis have been described previously.[75] Hypoxia culture was performed at 1% O_2 and 5% CO2. HEK-293T cells were used in all transfection experiments. Unless otherwise noted, cells were transfected using Fugene 6 transfection reagent (Roche). Generally, 9μg DNA, 27 μl F6 per 10cm dish. Cells were incubated for 24 hrs before lysis. For generation of ASB4 overexpressing stable ES cells, 3X-flag ASB4 in p3xflag-CMV 10 (Sigma) was linearized and electroporated into R1 ES cells. Stable expressors were selected for with 250 μg/ml

G418 for 14 days. Positive clones were picked, amplified, and confirmed by anti-flag immunoblot. COS7 cells were used for adenoviral infection.

Microarray analysis— Purification and amplification of mRNA from ES cells, reverse transcription, and microarray techniques have been described previously.[7] For this analysis, the data set was subjected to supervised hierarchical clustering using only genes demonstrating ≥1.5 fold absolute Mean Fold Difference (MFD) in 84 h flk1+ cells from embryoid bodies. Median centered genes and arrays were clustered with Cluster (Version 2.11, http://rana.lbl.gov/EisenSoftware.htm) and heatmaps of cluster analyses were visualized with JavaTreeView (Version 1.0.12, release date 2005-3-14; http://sourceforge.net/projects/jtreeview/).

RT-PCR analysis—Briefly, total RNA was isolated using RNeasy kits (Qiagen). On-column DNase treatment was performed to prevent genomic DNA contamination. For semi-quantitative RT-PCR analysis, 2 μg of total RNA was reverse transcribed with SuperScript II RT (invitrogen) using oligo dT primers. One tenth of RT reaction volume was used as template for PCR analysis. ASB4 primers: F: 5'gagacacccctgcacacggcag, R: 5'ctcaggctgtgcagcaggacgc. GAPDH primers were: F: 5'accacagtccatgccatcac, R: 5'tccaccaccctgttgctgta.

For real-time PCR analysis, 250 ng of total RNA was reverse transcribed using iScript kit (Biorad) using a mixture of oligo dT and random hexamer primers. The reaction was diluted 1:10, and 2.5 μl of the resulting mixture was used as a template in 12.5 μl reactions for real-time PCR reactions performed using the ABI PRISM® 7900 sequence detection system, software, and reagents. The following pre-validated primer and probe sets based on Taqman® chemistry (Applied Biosystems) were used: flk1 mM01222419_m1,

PECAM: mM00476702_m1, VE-cadherin: Mm00486938_m1, tie2: mM01256892_m1, gata1: Mm00484678_m1, klf1: Mm00516096_m1, scl/tal: Mm00441665_m1, brachyury: Mm00436877_m1. Triplicate reactions from independent RNA samples were performed. RNA input was calibrated with 18S expression levels and relative mRNA levels were normalized to wild-type undifferentiated ES cells.

Whole-mount in situ hybridization— Whole mount in situ hybridization on mouse embryos has been previously described.[75] A 900bp template for ASB4 probe preparation was prepared using the primers (F: 5'tatccatggtggacggcatcactgcccctatc, R: 5'ctcaggctgtgcagcaggacgc) and TA cloned in pCRII topo vector (invitrogen). Digoxigenin labeled single-strand sense and antisense RNA probes were prepared using the DIG RNA labeling kit (Roche). Embryos were isolated from pregnant CD-1 mice from E6.5 to E11.5, fixed with 4% paraformaldehyde (PFA) in PBS with 0.1% tween-20 (PBT), dehydrated in an increasing methanol series, and rehydrated in a decreasing methanol series in PBT. Embryos were treated with 6% H_2O_2 for 1 hr, washed, treated with 10μg/ml Proteinase K for 5 mins, washed with 2mg/ml glycine, refixed for 20 mins in 0.2% glutaraldehyde/4% PFA in PBT, washed, and prehybridized at 70ºC for 1 hr in Formamide, 5X SSC pH 4.5, 0.5% Tween-20, 0.5μg/ml Heparin. Hybridization solution: 1/10 of probe reactions were added to prehybridization solution containing 10μg/ml herring sperm DNA, and 0.1% yeast tRNA, Embryos were hybridized overnight at 70ºC. Embryos were then extensively washed, incubated with 100μg/ml RNaseA for 30 mins, washed, blocked in 10% lamb serum in TBS-T, and incubated with anti-Digoxigenin antibody overnight at 4ºC. Embryos were then extensively washed, incubated with substrate (BCIP, NBT) until signal to noise ratio was optimal. The reaction was stopped and embryos were post-fixed in 4% PFA/PBT, washed,

and images were captured. To section, embryos were dehydrated in a methanol series, treated with ethanol/xylenes/paraffin mixtures, embedded in paraffin, cut into 10μM sections and mounted on superfrost microscope slides.

RNA isolation and Northern Blot analysis— Tissues of CD1 embryos and C57BL/6 adult mice were homogenized and used to prepare total RNA using RNeasy mini kit (Qiagen). Northern blot analysis has been described previously[75]. A 400bp ASB4 probe was amplified using primers: F: 5'gagacacccctgcacacggcag, R: 5'ctcaggctgtgcagcaggacgc. The amplimer was TA cloned into pCR2.1 vector (invitrogen), amplified, and digested with EcoRI to liberate insert, which was then gel purified (Qiagen Gel Purification Kit), and used as a template for radioactive double-stranded random-primed probe synthesis (stratagene).

Immunoprecipitation and immunoblot Analysis— Immunoprecipitation and immunoblot procedures have been described previously (reference). Unless otherwise noted, cells were lysed in protein buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton-X-100, 1X Complete protease inhibitor cocktail (Roche)): 1mg of protein lysate was incubated with 30ul of flag- or myc-conjugated beads O/N at 4ºC. Beads were washed four times with >20X volume lysis buffer, boiled in 30μl of 2X SDS loading buffer containing βME for 5 mins, and loaded on 10% protein gels. After electrophoresis, gels were transferred to PVDF membranes, and subjected to immunoblotting.

Yeast 2-hybrid assay— Yeast 2-hybrid screens for ASB4 binding partners were performed using the Matchmaker 2-Hybrid system 2 (Clontech). Briefly, ASB4ΔSOCS/GAL4 DNA binding domain (BD) fusion protein in pGBKT7 vector was transformed into AH109 yeast cells and selected for positive transformants on Trp-deficient agar. Positive transformants were amplified and mated with pretransformed

MATCHMAKER cDNA library-containing Y187 yeast (clontech) to produce co-transformants. The screen described in this manuscript used a library isolated from adult human heart that was primed using an oligo dT primers (Clontech #HY4042AH). Mating mixtures were plated on agar plates lacking trp, leu, his, and ade for high stringency selection. The library vector was rescued from selected colonies, and identified by sequencing. To reconfirm interactions, both ASB4ΔSOCS in pGBKT7 and resistant library constructs were co-transfected in various combinations with empty vectors as negative controls, and pGBKT7-p53/pGADT7-T vectors as positive interaction control. The prey clone representing human FIH contained a transcript encoding all but the 5 most N-terminal amino acids of FIH protein.

Molecular Model Development— A fragment of mouse ASB4 spanning ankyrin repeats (ARs) 6-7 (V^{201} through A^{288}) was used to query various fold recognition servers including FUGUE (http://tardis.nibio.go.jp/fugue/prfsearch.html), INUB (http://inub.cse.buffalo.edu/), and the successor to 3DPSSM, PHYRE (http://www.sbg.bio.ic.ac.uk/phyre/). Several ankyrin repeat containing protein crystal structures were identified as possessing similar folds to the mouse ASB4 fragment query. Homologous regions of 3 crystal structures were used as templates for model prediction. Model building was based on the ankyrin repeat domains from: 1) PYK2-associated protein beta (PDB ID 1DCQ), 2) Bcl-3 (PDB ID 1K1A) and the 3) Drosophila Notch receptor (PDB ID 1OT8). Models of the ASB4 ankyrin repeats were built, guided by the alignments returned from the fold-recognition servers, using the Modeler module of the InsightII molecular modeling system from Accelrys Inc. (www.accelrys.com).

Mass Spectrometry— Immunoprecipitates of flag-ASB4 infected COS7 cells were loaded on protein gels, stained with coomassie brilliant blue and the bands of interest were cut and processed according to the previously published protocols from the UNC/Duke Proteomic Center[76]. Peptide digests were analyzed on an ABI 4700 Proteomic Analyzer, MALDI-ToF/ToF (Applied Biosystems Inc., Foster City CA), and the identity of the protein was confirmed by searching the mass spectrometry results as outlined previously[76]. Following protein identification, the peptide mixtures were re-spotted and the peptide, m/z 1345.67, corresponding to the hydroxylated ASB4 peptide 1329.68, was analyzed by MS/MS along with the unhydroxylated peptide. For determination of peptide peak ratios, the curve areas of the 1329.68 and the 1345.66 peptide peaks relative to the curve area of the 1512.823 peptide peak (shown by MS/MS in all samples to be derived from ASB4) were determined.

C. RESULTS

ASB4 is expressed in the vascular lineage during mouse embryonic development— In order to identify genes important during vascular development, we performed a set of experiments that compared the gene expression profiles of Flk1+ *versus* Flk1- cells isolated during different stages of differentiating embryonic stem (ES) cells.[7] Out of 20,000 genes, ASB4 was the most highly differentially expressed gene in Flk1+ cells at early time points of differentiation (Fig. 2-1A). This differential expression was confirmed by RT-PCR analysis showing that ASB4 mRNA is highly enriched in the Flk1+ population at 84 h, 95 h, and 192 h of differentiation, but is undetectable at 72 h of differentiation (before Flk1 is expressed in this system) (Fig. 2-1B). Since Flk1 is expressed in early precursor cells of the endothelial, hematopoietic, vascular smooth muscle, and cardiomyocyte lineages,[77] we hypothesized that

ASB4 must be important during cardio- and/or hemato-vascular development. In support of this hypothesis, supervised hierarchical clustering analysis using only genes demonstrating ≥1.5 fold absolute Mean Fold Difference (MFD) in 84 h Flk1+ cells showed that ASB4 clusters most closely with other genes known to be important in cardiovascular development including Flk1, Fibronectin, Gata2, and Gata4 (Fig. 2-1A).

To confirm that ASB4 expression is high in anatomic locations known to harbor active vascular development and remodeling, and to further define exactly which tissue(s) ASB4 expression is confined to, we performed northern blots from embryonic and adult tissues. Global ASB4 mRNA expression was comparatively low in E7.5 embryos, but quickly increased until E9.5 (Fig. 2-2A). Highly vasculogenic tissues such as the allantois, yolk sac, and placenta all expressed high levels of ASB4. However, while ASB4 expression in the adult was highest in testis, ovary, and heart, it was undetectable in highly vascular organs such as lung, kidney, and liver (Fig. 2-2B), suggesting that ASB4 function may be critical to proper vascular development, but dispensable for maintenance of adult vessels in some tissues. Furthermore, tissues containing high numbers of hematopoietic cells such as spleen and bone marrow have undetectable levels of ASB4 mRNA expression.

To further define the anatomic location of ASB4 expression during embryogenesis, we performed whole-mount *in situ* hybridization analysis using DIG-labeled ASB4 antisense riboprobes on mouse embryos of varying gestational stages. In E9.5 embryos, ASB4 is expressed in the intersomitic vessels, dorsal aorta, forelimb buds, allantois/umbilical vessels, vitelline vessels, septum transversum, proepicardium, capillary plexi of the head and branchial arches, endocardium, and the yolk sac vasculature (Fig. 2-3A,B,D,G,H). In E10.5 embryos, areas of high ASB4 expression include the fore- and hind-limb buds, intersomitic

vessels, peripheral liver cells, and umbilical vessels (Fig. 2-3E,I). In E11.5 embryos, high levels of ASB4 expression are limited to the fore- and hind-limbs, and the most caudal (and most recently formed) intersomitic vessels (Fig. 2-3F). Analysis with sense probes confirmed the specificity of the antisense signal (Fig. 2-3C).

The most striking recurring pattern of ASB4 embryonic expression is its high expression in primitive capillary plexi, followed by downregulation as vessels mature. At E9.5, ASB4 is highly expressed in the capillary plexi of the head and branchial arches (Fig. 2-3B), but by E10.5 expression in these vascular beds is no longer detectable (Fig. 2-3E). Similarly, ASB4 expression is high in intersomitic vessels at E10.5, but by E11.5 is confined to only the most caudal (and thus recently formed) intersomitic vessels (Fig. 2-3F). This pattern is recapitulated in the placenta, with ASB4 expression decreasing between E9.5 and E.13.5. Finally, ASB4 expression is undetectable in highly vascularized adult organs such as kidney and lung. Taken together, these data indicate that ASB4 expression is high in the primitive vasculature during development, but is quickly downregulated in the mature vasculature.

The SOCS box containing protein ASB4 assembles with a ubiquitin ligase complex— Since other SOCS box-containing proteins function as substrate adaptor proteins for Elongin B/Elongin C/Cullin/Roc ubiquitin ligase complexes by binding the complex in a SOCS box-dependent manner, we investigated whether ASB4 exists in such complexes and can function as a ubiquitin ligase. In transient transfection assays using HEK-293T cells, flag-ASB4, but not empty vector (EV) or a mutant lacking the C-terminal SOCS box (ΔSOCS) co-precipitated Elongin B, Cul5, Roc1, and to a lesser extent Cul2 (Fig. 2-4A). Signal for co-precipitating Cul2 was greatly increased using lysates from COS7 cells infected with flag-

ASB4 adenovirus (Fig. 2-4B), indicating that ASB4 can utilize Cul2 or Cul5 complexes, and that this preference is highly dependent upon cellular context. Other ASBs have been shown to associate with Cul5/Roc1 and Cul5/Roc2 complexes, however Cul2 interactions in these reports were either not evaluated or evaluated only in HEK-293 cells.[3, 78-80] Further studies will be needed to determine if the interaction of ASB4 with Cul2 is unique to ASB4 or inherent to all SOCS family members in the appropriate cellular context, and if ASB4 function is affected by which Cullin complex is used.

To evaluate whether ASB4 functions as part of a ubiquitin ligase complex, *in vivo* ubiquitination assays were performed. HEK-293T cells were transfected with flag-ASB4 for 20hrs followed by treatment with the proteasome inhibitor MG-132 to allow polyubiquitin-tagged protein accumulation. Flag-immunoprecipitates were then subjected to anti-flag and anti-ubiquitin (ub) immunoblotting. Strong ubiquitin immunoreactivity was detected in flag immunoprecipitates of flag-ASB4 transfected cells after MG-132 treatment (Fig. 2-4C, panel 1). We reasoned that this signal could represent ubiquitinated ASB4-associated proteins, ubiquitinated ASB4 itself, or both. A 7kDa flag-immunoreactive laddering pattern was detected in flag-immunoprecipitates after treatment with MG-132, indicating that ASB4 itself is ubiquitinated (Fig. 2-4C, panel 2). Furthermore, flag-ASB4 co-precipitates with myc-ASB4 in co-transfected HEK-293T cells, indicating that ASB4 complexes with itself (Fig. 2-4D). Since additional co-IP experiments did not indicate that substrates are stably associated with ASB4 (data not shown), these data indicate that ASB4 is autoubiquitinated (which may represent a mechanism of self-regulation) and thus has intrinsic ubiquitin ligase activity similar to other SOCS proteins.

ASB4 binds to FIH through an EVNA motif— As a first step in the functional characterization of ASB4, we performed yeast 2-hybrid screens using an ASB4 mutant lacking the C-terminal SOCS box (ASB4ΔSOCS) as bait. This mutant was utilized to avoid reconfirmation of known interactions with SOCS-box binding partners Elongin-B and Elongin-C. Using a human heart oligo-dT primed pretransformed yeast library, we screened over 1.5 x 10^7 independent clones and identified Factor Inhibiting HIF1 (FIH) as an interacting protein under stringent conditions (positive clones grew on plates lacking tryptophan, leucine, histidine, and adenine). The FIH prey clone encoded all but the 5 most N-terminal amino acids of the human FIH protein. This interaction was confirmed in a wheel assay in which yeast were transformed with various combinations of ASB4ΔSOCS bait, FIH prey, empty vectors (V1, V2), or mismatched non-specific controls (Fig. 2-5A). p53 and T-antigen (T) encoding plasmids were used as a positive control for an interaction in this system. Flag-immunoprecipitates of HEK-293T cells overexpressing flag-ASB4 but not flag-ASB1 co-immunoprecipitated endogenous FIH, confirming the specificity of this interaction in mammalian cells (Fig. 2-5B).

To determine which domain of ASB4 is necessary for FIH binding, a variety of N-terminal flag-tagged mutants were generated that sequentially lack different domains of the ASB4 protein (Fig. 2-5C). Since the hydrophobic interactions between helices of adjacent ankyrin repeats are crucial for proper folding, we carefully positioned the borders of the deletions in order to best conserve the modular structure of the mutants. These mutants were transiently transfected into HEK-293T cells, flag immunoprecipitated, and immunoblotted with anti-FIH antibody to detect co-precipitating endogenous FIH protein (Fig. 2-5D). ASB4 ARs 6 and 7 are necessary for interaction with FIH since mutants lacking either ARs 6 or 7

were unable to co-precipitate FIH. Mutants lacking other ARs (1,4,5,8-9) or the SOCS box were still able to co-precipitate FIH, indicating that these domains are dispensable for FIH binding and that deletion of single ARs does not disrupt the tertiary structure of the FIH-interacting motif.

FIH is an asparagine hydroxylase that is known to hydroxylate at least four proteins: HIF1α, HIF2α,[81] IκBα, and the NF-κB precursor protein p105[82]. FIH has been shown to bind to and hydroxylate each of these proteins on the β-carbon of the asparagine residue that is found within leucine-flanked EVNA motifs, often in the context of ankyrin repeats (Fig. 2-5E). (The residues surrounding asparagine are not totally conserved in all FIH-substrate peptides, but for simplicity will be referred to herein as EVNA motifs). The flanking leucines of FIH substrates bind first to position the EVNA motif for optimal binding in the FIH catalytic cleft via a process called "induced fit".[83] Since ASB4 contains a leucine-flanked EVNA motif in AR 6, we generated a number of point mutants in and around this motif to test their involvement in the FIH interaction. Each flag-tagged point mutant was transiently transfected with N-terminal myc-FIH, immunoprecipitated with anti-flag-conjugated agarose beads, and immunoblotted with myc-antibody to detect co-precipitating myc-FIH. Consistent with the "induced fit" model, point mutation of any of the flanking leucines to aspartate residues (L238D, L239D, L256D) reduced co-precipitating myc-FIH to undetectable levels (Fig. 2-5F). Single point mutants of the EVNA motif disrupted binding to varying extents. Mutation of the asparagine residue (N246A) reduced binding to undetectable levels, while mutation of the adjacent glutamate (E244A) or valine (V245T) residues reduced, but did not abolish binding. Mutation of the EVN residues to ATA abolished binding to undetectable levels. Taken together, these data indicate that ASB4 binds to FIH through a conserved

EVNA hydroxylation motif in AR 6, and suggest that ASB4 may be a novel FIH hydroxylation substrate.

ASB4 is hydroxylated on asparagine 246 by an oxygen dependent mechanism—We initially hypothesized that the function of the ASB4-FIH interaction is to permit ASB4-mediated FIH ubiquitination and subsequent degradation. However, despite various experimental approaches, we have not detected ASB4-dependent FIH ubiquitination or degradation (data not shown). Since FIH hydroxylates other proteins on asparagine residues within EVNA motifs, we considered the alternative hypothesis that the function of the ASB4-FIH interaction may be to permit FIH-mediated ASB4 hydroxylation. To determine if the leucine-flanked EVNA motif is positioned in an appropriate conformation to allow binding to and hydroxylation by FIH, we generated a structural model of ASB4 ankyrin repeats 6 and 7 (Fig. 2-6A). This model shows that the EVNA motif and flanking leucine residues of ASB4 are optimally positioned to allow an "induced fit" of the EVNA motif into the catalytic cleft of FIH, and prompted further studies to examine the possibility of FIH-mediated ASB4 hydroxylation.

To test the hypothesis that ASB4 is an FIH hydroxylation substrate, adenovirally overexpressed flag-ASB4 was flag-immunoprecipitated from COS7 cells grown under normoxic culture conditions, in-gel trypsinized, and analyzed by matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry (MS). The predicted mass/charge ratio (m/z) of the EVNA-containing trypsin-digested peptide TLLDNNAEVNAR is 1329, and hydroxylation of this peptide is predicted to result in a 16 Da shift to 1345. By MALDI-TOF analysis, we detected the 1329 Da peptide, and a 1345 Da peptide that did not match the predicted m/z value for any of the peptides from trypsin-

digested flag-ASB4, suggesting that the m/z 1345 peak represents the hydroxylated form of the 1329 Da peptide (Fig. 2-6B). This was confirmed through MALDI-TOF-TOF (MS/MS) sequencing analysis of the 1329 and 1345 peptides, separately. Alignment of the MS/MS spectra of the unhydroxylated and hydroxylated peptides demonstrates that hydroxylation occurs only in peptides containing the asparagine residue of the EVNA motif, indicative of FIH-mediated asparagine hydroxylation of ASB4 (Fig. 2-6C).

Under normoxic conditions, the ratio of unhydroxylated to hydroxylated ASB4 peptide is 3:1, indicating that despite high oxygen concentrations, only 30% of ASB4 is hydroxylated (Fig. 2-6D). This is probably due to the supraphysiologic concentration of adenovirally-overexpressed ASB4 in this system, resulting in the saturation of endogenous FIH enzymatic activity. Further studies will be needed to determine the hydroxylation status of endogenous ASB4 in vivo during development.

Since FIH-mediated hydroxylation is O_2 dependent (and thus inhibited by hypoxia), we tested whether hypoxic conditions would decrease ASB4 hydroxylation. Importantly, when COS7 cells infected with flag-ASB4 adenovirus were treated with the chemical hypoxia mimetics dipyridyl (DP) and cobalt chloride ($CoCl_2$), or the hydroxylase inhibitor dimethyloxaloylglycine (DMOG), only 10% of ASB4 EVNA-peptides were hydroxylated, indicative of decreased levels of ASB4 hydroxylation during hypoxia. Together, these data indicate that FIH binds directly to ASB4 in order to hydroxylate asparagine 246 via an oxygen-dependent mechanism, which suggests that ASB4 function may be regulated in an oxygen-dependent manner.

ASB4 functions to promote ES cell differentiation into the vascular lineage via an oxygen-dependent mechanism— In order to investigate the biologic effects of ASB4, we

overexpressed flag-ASB4 in a variety of cell types including both endothelial and non-endothelial lineages. No overt effects on cell number or morphology were observed in these experiments (data not shown). We also attempted RNAi-mediated knockdown of ASB4 in endothelial cell lines using six different shRNA sequences from integrated lentivirus constructs, but were unable to attain greater than 50% silencing (data not shown), indicating that the ASB4 transcript may be inherently resistant to RNAi-mediated silencing.

Because ASB4 is expressed in vascular stem cells during development, and other SOCS family proteins function to regulate cell differentiation, we investigated the effects of ASB4 overexpression on ES cell differentiation. Mouse ES cells were electroporated with linearized 3X-flag tagged ASB4 or p3Xflag-CMV empty vector (EV) and placed on G418 selection to select for stable transfected clones. After 14 days, surviving colonies were picked and expanded, and expression was confirmed by immunoblotting (data not shown). Multiple clones were chosen for initial experiments to control for non-specific effects due to random integration. Upon differentiation, ASB4-expressing clones demonstrated a significant increase in Flk1+ cells at 96 h of differentiation compared to EV-clones (Fig. 2-7A-B), indicating that ASB4 expression leads to an expansion of the hematovascular lineage. To examine the causes of this Flk1+ cell increase, and to investigate the ramifications of this increase on downstream lineage commitment, real-time RT-PCR analysis was performed using stage- and lineage-restricted genes. Since Flk1+ cells arise from mesoderm cells during differentiation, we investigated the overall levels of Brachyury (T) expression as a marker for mesoderm. At 4d of differentiation, Brachyury levels were slightly, but significantly, increased in ASB4 expressing clones, suggesting increased mesodermal commitment (Fig. 2-7C). Since Flk1+ cells are progenitors for both hematopoietic and vascular cells in this

system, we investigated the global expression levels of a variety of hematopoietic (Gata1, Scl/Tal) and vascular markers (Tie2, VE-Cadherin). All of these markers were tested at time points representing the peak of their expression during embryoid body (EB) differentiation (day 6 for hematopoietic markers, day 8 for vascular makers). Interestingly, when compared to EV clones, ASB4 expressing clones exhibited increased expression of vascular markers and decreased expression of hematopoietic markers, suggesting that forced expression of ASB4 causes preferential commitment of stem cells to the vascular lineage (Fig. 2-7C). Together, these data suggest that ASB4 induces the formation of hemangioblasts from mesodermal precursors and promotes hemangioblast commitment to the vascular lineage.

As ASB4 is hydroxylated, and is thus likely to be regulated by oxygen concentration, we predicted that its effects on vascular lineage commitment are oxygen-dependent. To test this hypothesis, we differentiated the stably transfected ES cells described above under hypoxic conditions (1% atmospheric oxygen). Interestingly, the differences in the expression of hematopoietic and vascular lineage-specific markers observed with ASB4 overexpression under normoxic conditions were completely abrogated by hypoxic treatment (Fig. 2-7D). These results indicate that FIH-mediated hydroxylation of ASB4 under high oxygen concentrations is essential for ASB4's ability to promote differentiation/maturation of the endothelial lineage.

D. DISCUSSION

In the present study, we show for the first time that ASB4 is a substrate recognition molecule of an E3 ubiquitin ligase complex, is highly expressed in the vascular lineage during development, is hydroxylated by FIH, and functions to promote differentiation and

maturation of the vascular lineage by an oxygen-dependent mechanism. These findings suggest that ASB4 functions in an oxygen-dependent manner in order to promote endothelial differentiation and/or maturation in response to increasing oxygen levels during early vascular development.

ASB4 is a member of the SOCS superfamily and, like other family members, functions as the substrate-recognition molecule of a Cullin-based E3 ubiquitin ligase complex. The list of characterized E3 ubiquitin ligase proteins is rapidly expanding and recent studies have uncovered a novel role of ubiquitin ligases during endothelial differentiation and maturation. HIF and Notch family members are critical for proper vascular development, especially during endothelial remodeling, and both families are tightly regulated by ubiquitin-mediated proteasomal degradation. For example, von Hippel Lindau (VHL) protein binds to hydroxylated HIFα to mediate its degradation in an oxygen-dependent manner, while Fbw7, Numb, Itch, and Mind-bomb all modulate Notch signaling.[84-92] Deficiencies of these factors result in vascular abnormalities, indicating that signal regulation via the ubiquitin-proteasome system is critical for proper vascular development.[86, 93-95] The endothelial-restricted expression of ASB4 described in this report further emphasizes the important role of the ubiquitin-proteasome system in modulating endothelial biology during embryogenesis.

The vascular expression of ASB4 is not only spatially restricted to the primitive endothelium, but is also tightly temporally regulated. ASB4 is maximally expressed in both the embryonic vasculature and the developing placenta from E9.5-10.5, but is then quickly downregulated, demonstrating that ASB4 function in the vasculature is temporally confined. Notably, this brief period of high ASB4 expression coincides with rapid and drastic changes

in embryonic blood oxygen levels. As the placenta forms between E9.5 to E10.5, oxygen delivery to the embryo quickly changes from passive diffusion through the multiple cell layers of the decidua and the embryo to maternal-fetal blood gas exchange across thin placental membranes. Intraembryonic oxygen tension quickly rises and the endothelium must transduce these environmental cues into an appropriate biologic response. ASB4 is uniquely expressed temporally and spatially to play a role in the response of the endothelium to changing oxygen concentrations.

Our data indicate that one of the ramifications of ASB4 function is to increase the differentiation and maturation of the vascular lineage in an oxygen-dependent manner, suggesting that ASB4 may function to coordinate endothelial differentiation/maturation in the face of changing oxygen levels. This is especially intriguing considering the role of oxygen concentration on placental vascular development. The allantois, which has been shown to harbor mesoderm-derived vasculogenic cells,[15] makes contact with the chorion around E8.5 to initiate placental vascularization (reviewed in [96, 97]). During this process, a subset of trophoblast cells migrate into the uterine wall, proliferate, and differentiate into endothelial-like cytotrophoblast cells that line the maternal blood vessels to facilitate maternal-fetal blood-gas exchange. Oxygen tension is critical in this process. Early on, hypoxia activates cytotrophoblast proliferation and invasion into the uterine tissues, but as these cells reach the highly oxygenated maternal blood vessels, the high oxygen levels inhibit proliferation and promote their differentiation into an endothelial-like phenotype.[98, 99] It is interesting to note that overall ASB4 expression levels in whole allantoides and placentas are initially high and decrease concomitantly with vessel maturation and increasing oxygen

tensions, suggesting that ASB4 functions to modulate placental cellular phenotypes in response to changing oxygen microenvironments.

We identify that ASB4 is only the fourth in a limited list of known FIH hydroxylation substrates, and thus provide a mechanism for ASB4 modulation of cell phenotypes in an oxygen-dependent manner. FIH-mediated hydroxylation is known to be proportional to oxygen concentration, and in this way, ASB4 hydroxylation is the first step in the transduction of environmental oxygen cues to appropriate biological responses. Since we were unable to detect any differences in ASB4 expression, subcellular localization, or autoubiquitination under hypoxic conditions (data not shown), and since hydroxylation in other proteins has been shown to affect protein-protein interactions between the SOCS family member VHL and its ubiquitination substrate HIFα, we predict that the functional ramification of ASB4 hydroxylation is to modulate ASB4 binding to and degradation of substrate protein(s). Until recently, the only known intracellular hydroxylated proteins were HIFα subunits, and it was thus supposed that the cellular response to hypoxia was largely dependent upon HIF-mediated transcriptional upregulation of hypoxia response genes such as VEGF and GLUT-1.[52] However, our data indicate that ASB4 is a member of a currently limited number of non-HIF FIH hydroxylation substrates and suggest that the mechanisms of the cellular hypoxic response may be much broader and more complex than initially suspected.

We therefore propose that HIFα subunits are responsible for the *transcriptional* response to hypoxia, while ASB4 may represent a major component of the cellular *post-translational* response to changing oxygen levels. Compared to transcriptional upregulation, post-translational ubiquitination is much more rapid and enables a faster cellular response

without the lag-time associated with transcriptional activation and protein synthesis. For example, the ubiquitin-proteasome system extensively regulates the cell cycle, a situation in which rapid changes in protein levels are needed.[100] Thus, oxygen-dependent ubiquitin ligase activity of ASB4 allows the cell to respond rapidly to the quickly changing oxygen microenvironments that are found immediately following the initiation of placental blood gas exchange. Furthermore, unlike HIF1α, which is expressed in nearly all cell types, cell-specific expression of hydroxylated proteins such as ASB4 allows cell type-specific responses to changing oxygen levels. In this case, ASB4 functions to initiate a rapid endothelial-differentiation in response to quickly rising oxygen tensions during development. These data provide new insights into the mechanistic complexity of oxygen-sensing in mammalian cells and suggest that endothelial and other cell types may mount unique responses to changing oxygen tensions during development through oxygen-dependent modulation of the ubiquitin-proteasome system.

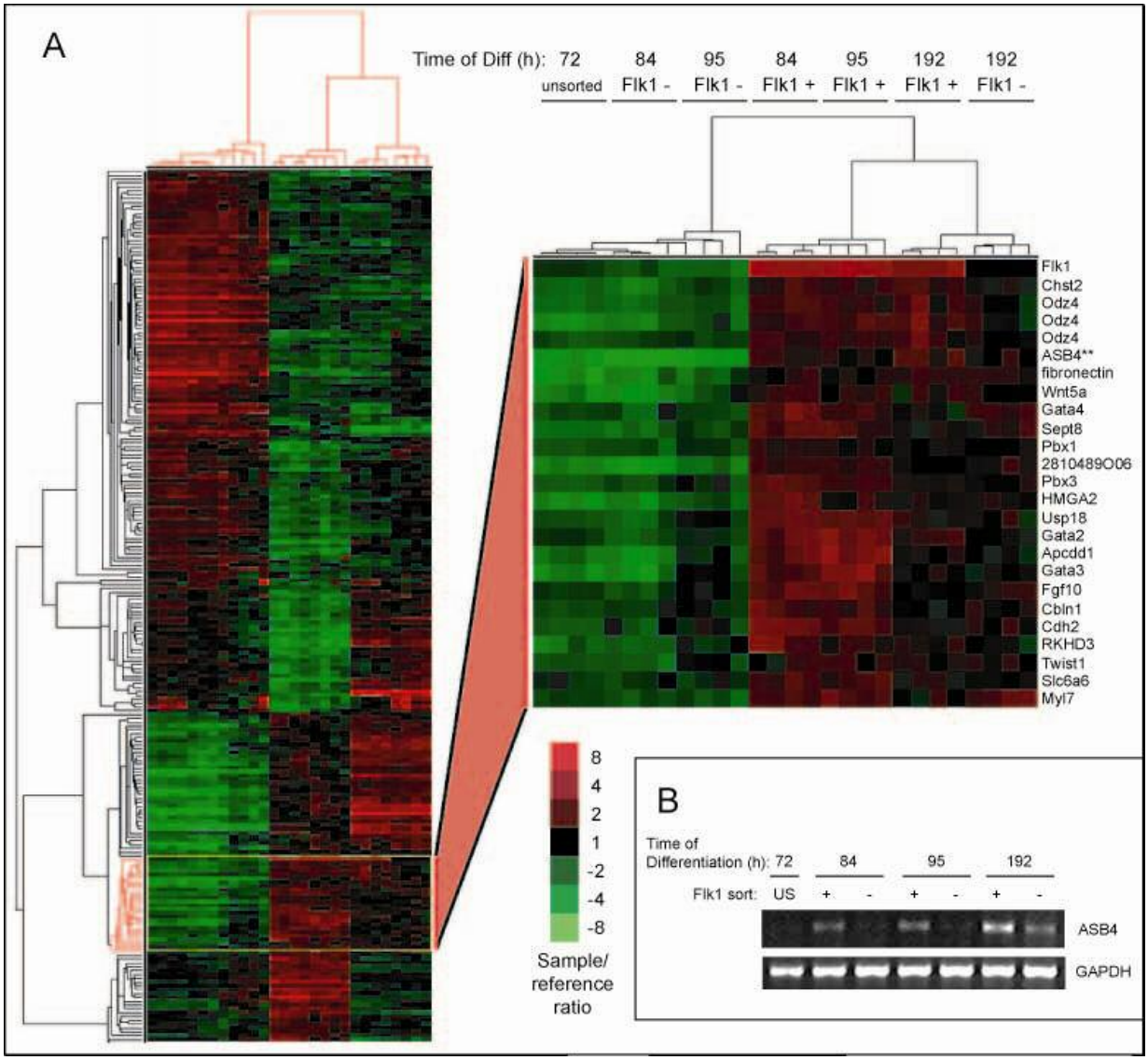

Figure 2-1 ASB4 expression in ES cells.

(A) Supervised hierarchical cluster of genes with ≥ 1.5 fold absolute mean fold difference (MFD) in 84 h Flk1+ cells from embryoid bodies (EBs). The Flk1 cluster containing ASB4 is shown at right. This experiment is described in detail in Wang H. et al,[7] and the complete data set is available online through the Gene Expression Omnibus (Series Record GSE3757, http://www.ncbi.nlm.nih.gov/geo) and through the University of North Carolina Microarray Database (http://genome.unc.edu).

(B) RT-PCR analysis of ASB4 expression levels in Flk1+ cells from differentiated EBs. GAPDH is used as a loading control. US: unsorted.

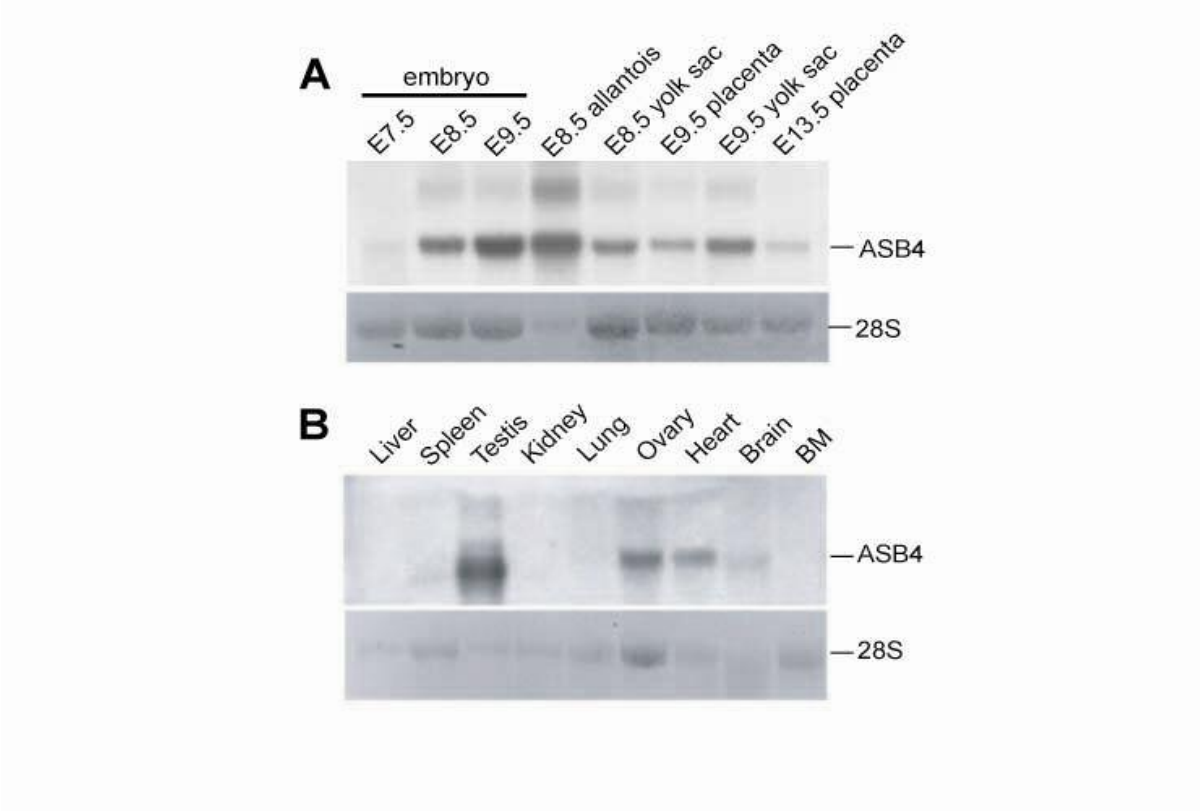

Figure 2-2 ASB4 mRNA expression in embryonic/adult tissues.
(A) Northern blot analysis of ASB4 expression levels in whole-embryos and dissected extra-embryonic tissues.

(B) Northern blot analysis of ASB4 expression levels in adult tissues. BM: bone marrow. Ethidium bromide staining of 28s rRNA was used for loading control.

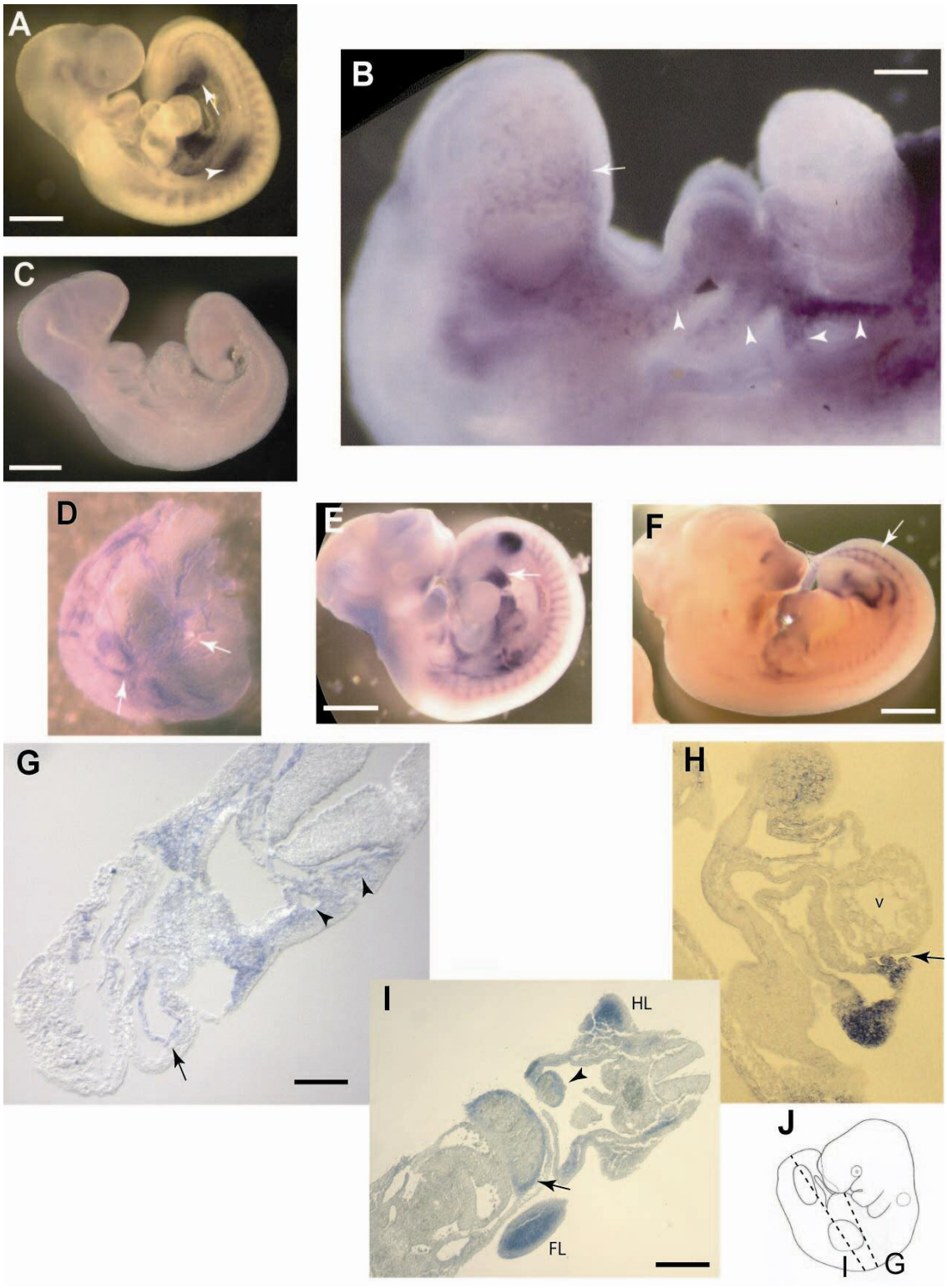

Figure 2-3 ASB4 Expression pattern during mouse embryogenesis.
Whole mount in situ hybridization using DIG-labeled RNA probes for ASB4 was performed on mouse embryos between days 9.5 and 11.5 post-coitum. All images used antisense probes except (C) (sense probe). Purple staining denotes positive signal. In some cases (G, H, I), stained whole embryos were paraffin-embedded and sectioned for microscopic examination. (Cont'd on following page).

(A) E9.5 embryo. Arrow: allantois. Arrowhead: forelimb. Bar: 500 μm.

(B) E9.5 embryo: high magnification. Arrow: rostral capillary plexus. Arrowheads: branchial arch capillary plexi. Bar: 50 μm.

(C) E9.5 embryo probed with sense probe as a negative control. Bar: 500 μm.

(D) E9.5 yolk sac. Arrows: yolk sac vessels. Bar: 750 μm.

(E) E10.5 embryo. Arrow: umbilical vessels. Bar: 800 μm.

(F) E11.5 embryo. Arrow: caudal intersomitic vessels. Bar: 1 mm.

(G) E9.5 embryo section. Transverse section of heart. Arrow: endocardium. Arrow heads: dorsal aorta and intersomitic vessel. Bar: 80 μm.

(H) E9.5 embryo section. Sagittal section of heart. v: ventricle. Arrow: pro-epicardium/septum transversum.

(I) E10.5 embryo section. Transverse section of liver. HL: hindlimb. FL: forelimb. Arrow: liver. Arrowhead: umbilical vessels. Bar: 150 μm.

(J) Schematic of section location in (G) and (I).

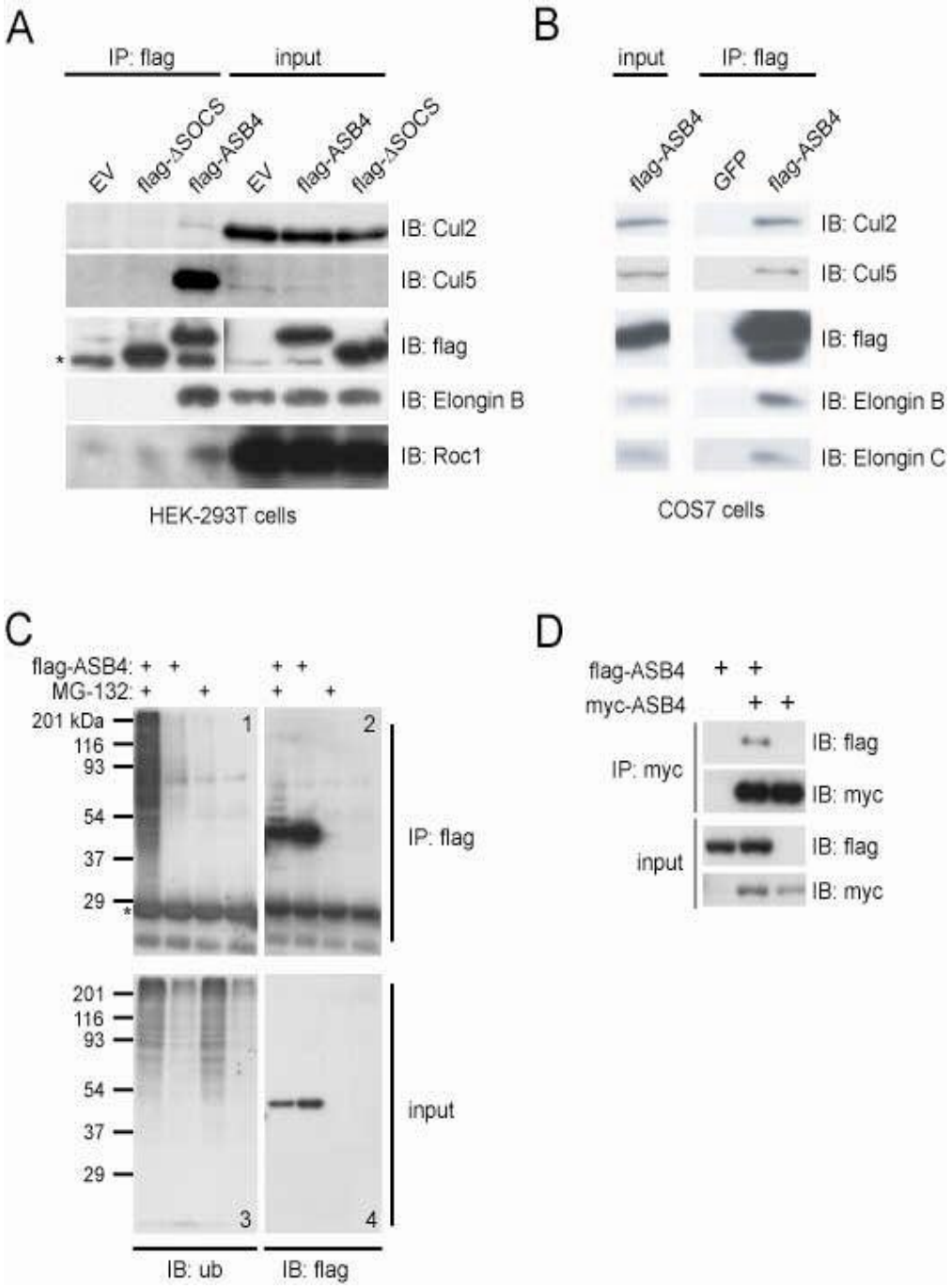

Figure 2-4 ASB4 associates with a ubiquitin ligase complex.

(A) HEK-293T cells were transfected with flag-ASB4, flag-ASB4ΔSOCS (flag-ΔSOCS), or empty pCMV vector (EV). Lysates were immunoprecipitated with anti-flag agarose beads and subjected to immunoblotting with anti-flag, Cul2, Cul5, Elongin B, and Roc1 antibodies. Asterices denote non-specific bands.

(B) COS7 cells were infected with flag-ASB4 adenovirus or GFP alone-adenovirus (GFP). Lysates were immunoprecipitated and immunoblotted as in (A).

(C) HEK-293T cells were transfected with flag-ASB4, cultured for 20 hrs, and then treated with 40μ*M* of the proteasome inhibitor MG-132 for 4 hrs before protein harvest. Lysates were immunoprecipitated as in (A) and immunoblotted with anti-Ubiquitin (ub) and anti-flag antibodies.

(D) HEK-293T cells were co-transfected with flag-ASB4 and myc-ASB4, immunoprecipitated with anti-myc agarose beads, and immunoblotted.

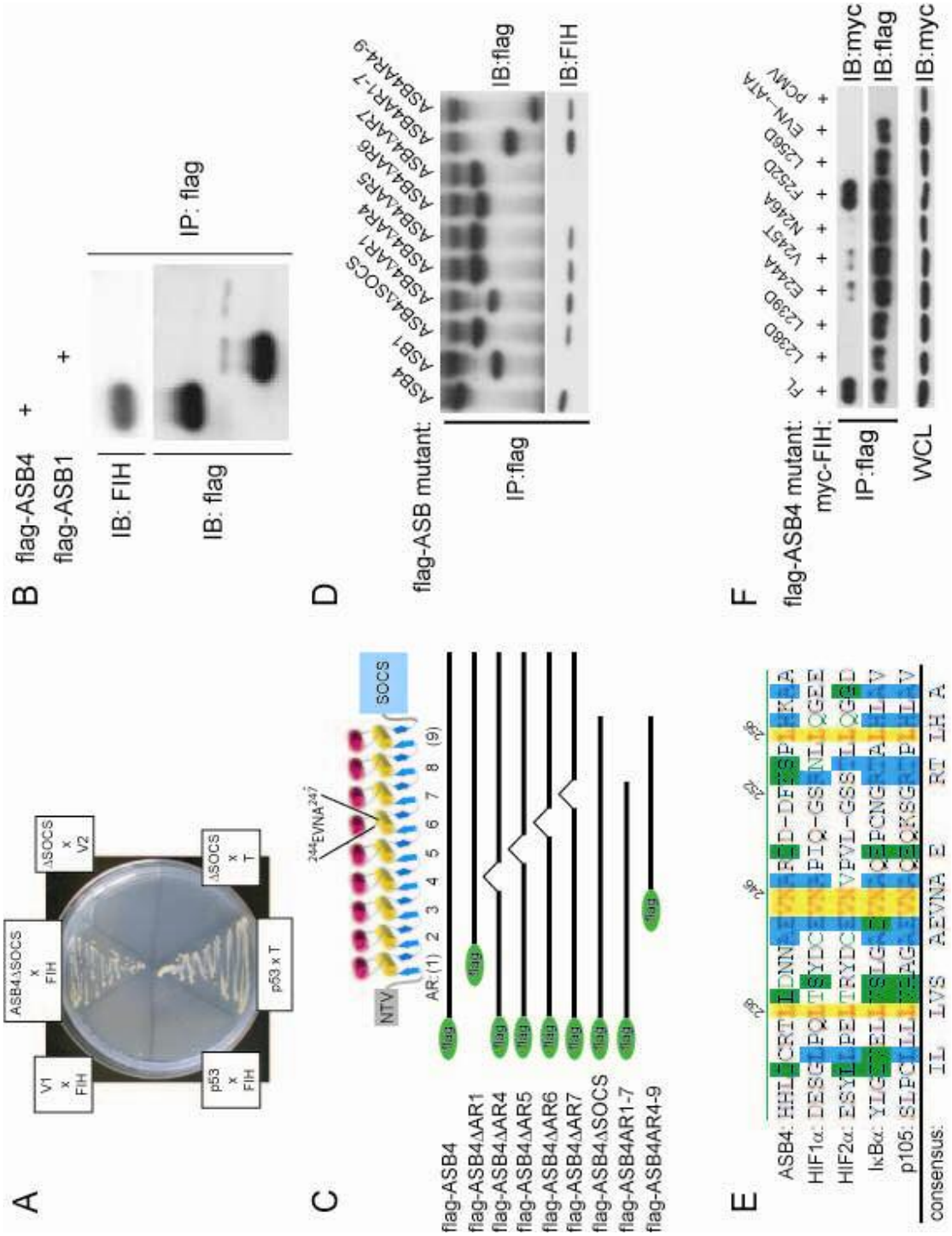

Figure 2-5 ASB4 binds to FIH through EVNA motif in ankyrin repeat #6

(A) Yeast wheel assay. Yeast transformed with different combinations of bait (ASB4ΔSOCS) and prey (FIH) constructs were spread on a high stringency selective plate lacking tryptophan, leucine, histidine, and adenine. p53 and T-antigen (T) were used as positive control. V1: empty bait vector (pGBKT7). V2: empty prey vector (pGADT7).

(B) HEK-293T cells were transfected with either flag-ASB4 or flag-ASB1. Lysates were immunoprecipitated with flag antibody-conjugated agarose beads and immunoblotted with anti-flag and anti-FIH antibodies.

(C) Schematic of ASB4 with N-terminal variable region (NTV), 9 tandem ankyrin repeats (poorly conserved repeats are indicated by parentheses), and a C-terminal SOCS box. The EVNA motif is contained in the loop of ankyrin repeat #6. Schematics of N-terminal flag-tagged constructs are denoted below.

(D) HEK-293T cells were transfected with flag-ASB4, flag-ASB1, and the flag-ASB4 deletion mutants described in (C). Protein lysates were immuoprecipitated with anti-flag agarose, and immunoblotted with anti-FIH antibody.

(E) Sequence alignment of mouse ASB4 and other known FIH hydroxylation substrates. Consensus sequence shows high conservation of EVNA motif and flanking leucine residues. Residue numbers are indicated above ASB4 sequence.

(F) HEK-293T cells were transfected with various flag-ASB4 point mutants and myc-FIH. Lysates were immunoprecipitated with flag antibody-conjugated agarose beads and immunoblotted with anti-flag and anti-myc antibodies.

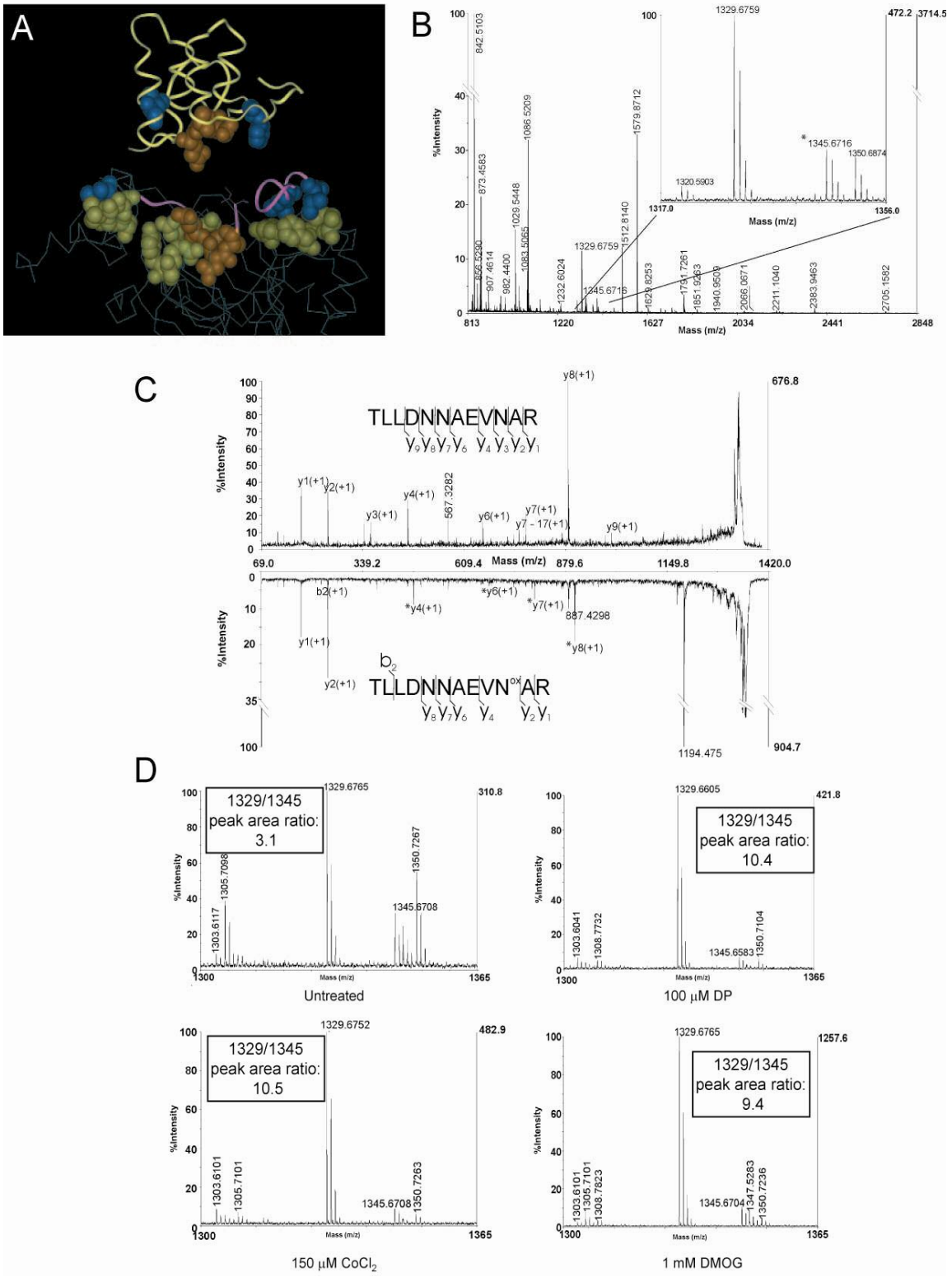

Figure 2-6 ASB4 is hydroxylated on asparagine 246.
(A) Structural model of ASB4 ARs 6-7 (yellow ribbon backbone) positioned above FIH (grey stick backbone)/HIF peptide (purple ribbon backbone) crystal structure (PDB ID 1H2K). Space filled EVNA residues are colored orange. Space filled FIH residues contributing to HIF binding are colored yellow. Leucines and other possible hydrophobic interacting residues are colored blue.

(B) COS7 cells were infected with flag-ASB4 adenovirus for 24 hours. Lysates were immunoprecipitated with flag antibody-conjugated agarose beads. Beads were eluted with flag-peptide and subjected to SDS-PAGE and coomassie staining. Flag-ASB4 bands were subjected to in-gel trypsin digest, and MALDI-TOF mass spectrometric analysis. The 1329 Da peak is the predicted molecular weight of the EVNA-motif containing peptide and the 1345 Da peak (asterisk) is the predicted molecular weight of the hydroxylated peptide.

(C) The 1329 and 1345 Da peptides were analyzed by MS/MS. A 16 Da shift was observed with the y4-y8 ions, but not with y2 ions, consistent with hydroxylation at the asparagine residue.

(D) COS7 cells infected with flag-ASB4 adenovirus were treated with various hypoxia mimetics/FIH inhibitors and flag-ASB4 protein was isolated and analyzed as in (B). The peak area ratio of unhydroxylated (1329 Da) to hydroxylated (1345 Da) peptide was compared after culturing cells in normoxia, 3hrs 100 μ*M* dipyridyl (DP), 24hrs 150 μ*M* $CoCl_2$, or 24 hrs 1m*M* dimethyloxalylglycine (DMOG).

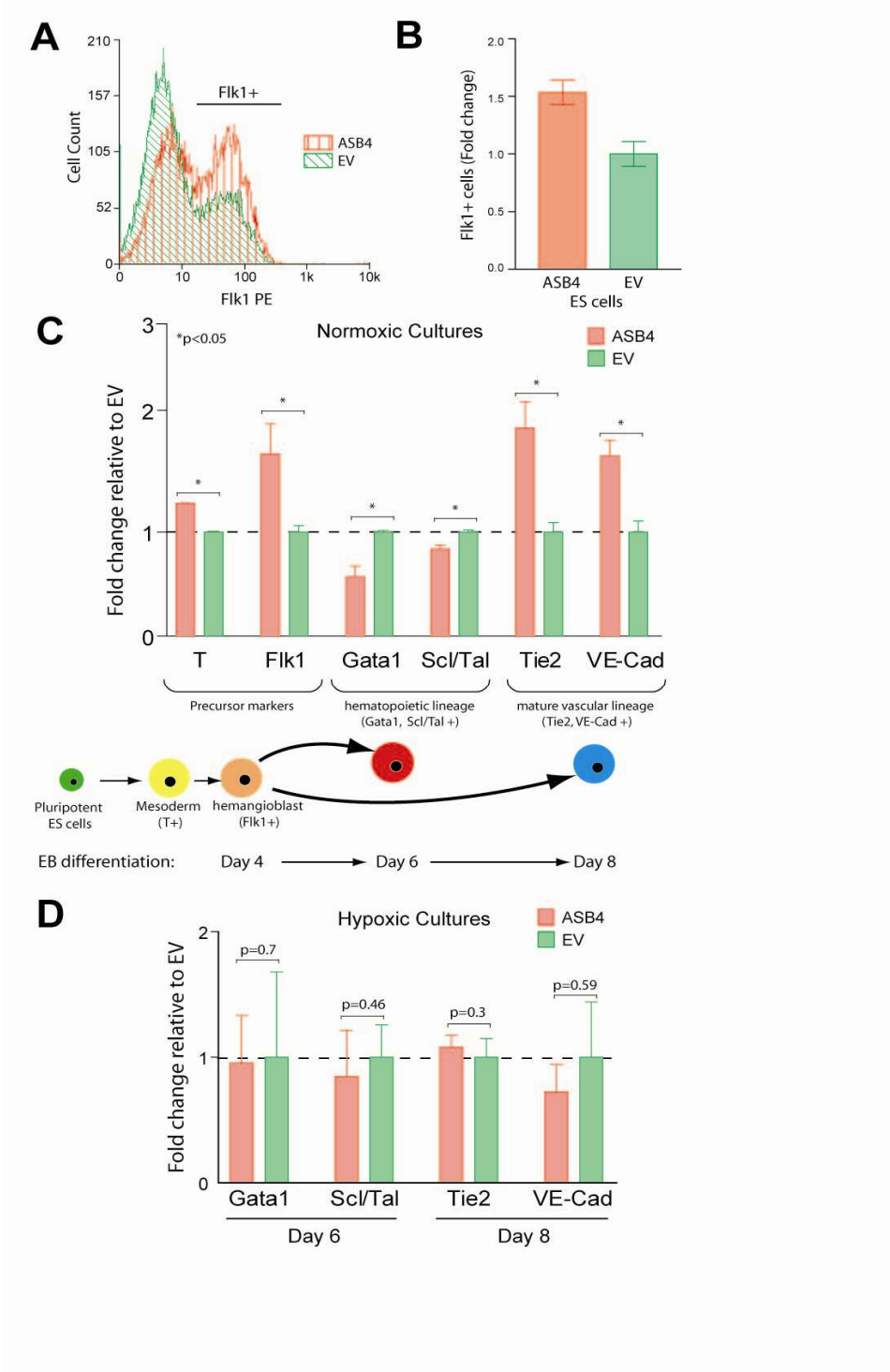

Figure 2-7 ASB4 oxygen-dependent effect on vascular differentiation.
ES cells were electroporated with constructs encoding CMV promoter-driven 3X-flag ASB4 or vector alone (empty vector, EV), and stably-expressing clones were selected for by culture in G418 for 14 days. Positive clones were isolated, expanded, and used for differentiation experiments as previously described.

(A,B) FACS analysis of 96 h differentiated embryoid bodies (EBs) shows drastic increase in Flk1+ cells in ASB4-expressing clones. Results in (A) are representative of three independent experiments. Results in (B)

represent an average of three independent clones in three independent differentiation experiments. Flk1+ cells in ASB4-expressing clones were normalized to EV clones in each experiment.

(C) Real-time RT-PCR analysis of differentiated EBs was used to determine the lineage commitment of Flk1+ cells. When compared with empty vector, ASB4 expressing ES cells show an increase in markers of mesoderm commitment (Brachyury (T)) and an increase in a hemangioblast marker (Flk1) at day 4 of differentiation, a decrease in hematopoietic lineage markers (Gata1, Scl/Tal) at day 6, and an increase in vascular markers (Tie2, VE-Cadherin) at day 8. Results represent averages of three independent biologic replicates.

(D) EBs stably expressing either ASB4 or EV were differentiated in hypoxic conditions (1% oxygen) and analyzed via real-time PCR as described in (C).

Chapter 3 *ASB4*$^{-/-}$ MOUSE GENERATION

A. INTRODUCTION

The generation of mice genetically engineered to lack specific genes ("knockout mice") has become a standard method of evaluating the biologic function and significance of specific genes. Knockout mice have been particularly useful in identifying genes with crucial roles during vascular development since vascular defects often lead to severe phenotypes and embryonic lethality. The studies described in Chapter 2 establish ASB4 as an oxygen-regulated SOCS-box E3 ubiquitin ligase that is highly expressed in the vasculature during development. Notably, mice lacking genes that are important in the cellular oxygen response (VHL, HIF1α, and HIF2α) die around E10 of development due to vascular defects.[56, 58-60, 101, 102] Since we have shown that ASB4 is involved in modulating the oxygen-response of the developing vasculature, we predicted that mice lacking ASB4 would die *in utero* due to vascular malformation. This chapter describes the production and very early stages of characterization of *ASB4*$^{-/-}$ mice.

Traditionally, knockout mice have been generated by "knock-in" of reporter genes such as *LacZ* or *eGFP* which, following homologous recombination, replace (or "knockout") one or more exons of the target gene. The presence of these reporter genes in the target gene locus allows for analysis of target gene expression in the generated mouse line, and also prevents transcriptional read-through of the locus due to the presence of strong transcriptional stop sites following the reporter gene sequences. However, if knockout mice

generated by this traditional approach are not viable (embryonic lethal), phenotypic characterization is limited to development, and adult characterization of the biologic role of the gene is impossible.

To avoid this problem, mice can be generated that contain exon(s) of the target gene that are flanked by loxP sites ("floxed"). LoxP sites are recognized by cre recombinase, which excises all genetic material between LoxP site pairs. Mice with floxed target gene exons can then be mated with mice which ubiquitously express cre recombinase to generate knockout mice similar to the traditional method described above. Importantly, if these knockout mice are embryonic lethal, the floxed mice can be mated with conditionally expressing *cre* mice to result in inducible or tissue-specific knockout mice. Thus, if the knockout is embryonic lethal, this approach allows considerable flexibility in further phenotypic characterization. For example, if floxed mice are crossed with tamoxifen-repressible promoter-driven *cre*, then pregnant mothers can be fed tamoxifen to repress *cre*, preventing target gene excision and allowing normal development. After birth, floxed cre^{+} mice can be fed with tamoxifen until the point at which the role of the target gene is going to be evaluated. Discontinuation of tamoxifen releases the tamoxifen-repressible promoter, thus activating *cre* expression and resulting in inducible cre-mediated excision of the floxed alleles. Alternatively, if floxed mice are crossed with tissue-specific promoter driven *cre*, then the cell-specific role of the target gene can be evaluated. Clearly, the conditional knockout approach has considerable advantages if (and only if) the phenotype is embryonic lethal.

There are also disadvantages to the conditional knockout approach when compared to the traditional method. First, no reporter gene is knocked-in, so further expression analysis of

the target gene is impossible. Second, it requires additional cloning steps during knockout construct production, and additional downstream mouse crosses (including the availability of *cre*-expressing lines). Finally, the risk of generating a hypomorphic but not null allele is greater using this approach. This is due to: 1) the lack of a knocked-in gene with its strong transcriptional stop site, 2) the inherent limitation in how much genetic material can be deleted (<1 kb compared to <25 kb with the traditional approach), and 3) the resultant possibility of transcriptional read-through and translation of truncated mutant proteins (with unpredictable cellular effects). However, all of these possibilities can be ruled out by testing for the presence of endogenous target gene protein product with appropriate antibodies. Since our expression data of ASB4 during development was considerable, since we predicted that mice lacking ASB4 would die *in utero*, and since we are able to confirm the absence of ASB4 protein by immunoblot, we decided to evaluate the biologic function of ASB4 by the conditional approach described above.

B. MATERIALS AND METHODS

Southern Blot. 5' external probe was amplified using the primers: SPF (cagttcccatccctgttcacattc), SPR (gagttaagggtgtccttgccagttc) and TA cloned into pCR2.1 topo vector (Invitrogen). Template for probe prep was excised using flanking EcoRI sites, and labeled with RT random Prime-It system (Stratagene) to produce cDNA labeled probe.

PCR primers for amplification of homology arms from 129 ES cell genomic DNA: LAF (long arm forward) (ggtaccgaaagatgcgccaggggcacttcgatggtc), LAR (gcggccgcggtgctggaaggcctaccacatcacctgag), TAF (target arm forward) (gtcgacagtttaaaggacctgagaactcagggagag), TAR

(ggtaccataacttcgtataatgtatgctatacgaagttatagcctgaccgagaaccacactgtcacac), SAF (caattgtctggggctaagttcaggcaagcctctc), SAR (atcgattaagcgggtcccttccgctctacgtg), PCR primer sequences for genotyping: TKR1 (tctagaaagtataggaacttcggcgcgcc), CAF (caattgtgtgaaggggatcagatccatgtaattgt), flpF2 (agtcagactaagatactgcggctccagccaggc), flpR2 (aagcggagcaatgatttatcaggagagcagc), LA5'R (gttcagcttttgttcctgcactg), TAFrc (tgagttctcaggtcctttaaactggag), cre1 (cctggaaaatgcttctgtccg), cre2 (cagggtgttataagcaatccc), chr11F (aacacacactggcaggactggctagg), chr11R (caatggtaggctcactctgggagatgata)

Rabbits were injected with the mouse ASB4 peptide ^{244}VNARDDDFKSPLHK to produce anti-ASB4 antibodies. Sera from these rabbits were immunopurified using the same peptide.

C. RESULTS

Since the conditional approach limits the amount of genetic material to be deleted to around 1 kb, we first needed to decide which exon(s) to flox. *ASB4* is composed of 5 exons spanning 41 kb which are relatively evenly distributed, thus preventing the floxing of multiple exons. At the time of production, it was not known which domain was important for ASB4 protein function, so we decided to flox the first exon with the start codon and upstream putative transcriptional start sites (Fig. 3-1). *ASB4* exon 1 is 197 bp long, and encodes the first 62 (out of 426) amino acids of the ASB4 protein. We also carefully placed the 5' loxP site to prevent any disruption of *ASB4* promoter structure to allow normal transcription from the *ASB4*^{flox} locus.

Genomic DNA of 129SvEv cells was used as a PCR template to generate the short, long, and target arms of homology. These arms were then subcloned into pAMC vector

(supplied by UNC Animal Models Core), which contains MC1 promoter-driven neomycin resistance gene between the arms of homology for positive selection, and PGK promoter-driven diphtheria toxin gene outside the arms of homology for negative selection in randomly-integrated clones. This ASB4 knockout construct was then linearized and electroporated into ES cells from male 129SvEv mice for homologous recombination. Cells were grown in G418 (a potent neomycin analog) for 14 days, and resistant clones were picked and expanded. Floxed recombinants were confirmed by PCR using the primer pair CAF/TKR1, producing a 2 kb product in positive clones, and no product in negative clones (Fig. 3-2A). CAF primer binds outside the short arm of homology, and thus anchors this amplimer to the *ASB4* locus and prevents amplification from random integration events. Out of 190 screened G418-resistant colonies, 5 were PCR-positive indicating a moderate rate of homologous recombination. PCR-positive recombinants were further confirmed using Southern blotting with a probe external to the arms of homology and HindIII digested tail DNA (Fig. 3-2B). Using this approach, the wild-type ASB4 locus shows a band of 5.7 kb while the targeted locus shows a band of 9.2 kb. All of the PCR-positive recombinants were reconfirmed using this approach.

Positive recombinants were then electroporated with constructs expressing flp-recombinase to excise the frt-flanked neomycin resistance cassette, since the *neo* gene in the ASB4 locus could have effects on ASB4 expression or general mouse development. Thymidine kinase (TK) is contained within the knockout construct in the *neo* cassette and allows for negative selection against non-flp-excised clones. TK integrates gancyclovir into the DNA of replicating cells, resulting in cell death. Thus, when flp-treated cells were grown in the presence of gancyclovir, only cells with flp-excised ("flipped") TK/*neo* cassettes could

survive. Positive flipped clones were confirmed by PCR using the primer pair flpF2/flpR2 (Fig. 3-2C). These primers both bind to the endogenous ASB4 locus, but flank ~100 bp of residual vector sequence (including one frt site and one loxP site) in the floxed allele. Thus, the *ASB4* WT locus results in an amplimer of 390 bp, while $ASB4^{flox}$ locus results in an amplimer of 504 bp. Wild-type ES cells show one band of 390 bp, while targeted floxed ASB4 allele-containing cells (denoted $ASB4^{flox/+}$) show one band of 390 bp (WT allele) and one band of 504 bp (floxed allele). This PCR reaction represents the genotyping strategy for $ASB4^{flox}$ mice.

Next, $ASB4^{flox/+}$ ES cells (129SvEv strain, male gender) were injected into C57BL/6 blastocysts and implanted into pseudopregnant female mice to produce $ASB4^{flox/+}$ chimeric mice (Fig. 3-3A). 24 male chimeras were produced which were then mated with WT C57BL/6 females to produce $ASB4^{flox/+}$ heterozygote containing litters. Germline transmission varied from 0% to near 100% resulting in >50 $ASB4^{flox/+}$ pups. (This $ASB4^{flox/+}$ line will be propagated in case of embryonic lethality of $ASB4^{-/-}$ mice.) To excise the floxed ASB4 exon 1, $ASB4^{flox/+}$ mice were crossed with C57BL/6 EIIa promoter-driven *cre* transgenic mice. EIIa promoter results in *cre* expression in all cell types beginning around the 4-cell stage of the embryo.[103] Thus, pups inheriting the *cre* allele will be predominantly $ASB4^{+/-}$, but might contain some level of $ASB4^{flox/+}$ chimerism. Cre-mediated excision of ASB4 exon 1 was confirmed by PCR using the primer set flpF2/LA5'R/TAFrc on tail clip genomic DNA (Fig. 3-3B). The flpF2/TAFrc primer pair results in amplimers of 234 bp only from the $ASB4^{+}$ allele since TAFrc is present in the WT locus, but absent from the cre-excised ($ASB4^{-}$ locus). The flpF2/LA5'R amplimer from the $ASB4^{-}$ locus is 390 bp, but this primer pair does not amplify from the $ASB4^{+}$ locus under the cycling conditions used

(extension time too short). To pass the *ASB4*$^{-}$ allele through the germline (thus eliminating chimeric effects) and to cross-out the *cre* allele (*cre* has been shown to have developmental effects, thus confounding phenotypic analysis of mice lacking *ASB4*), these mice were crossed with WT C57BL/6 mice to produce true *ASB4*$^{+/-}$ heterozygotes. *ASB4*$^{+/-}$ *cre*$^{-}$ mice were confirmed by PCR using the primer mix described above and a cre primer mix (Fig. 3-3B) (this PCR reaction represents the ASB4 knockout locus genotyping strategy).

At the time of dissertation preparation, these *ASB4*$^{+/-}$ mice had recently reached sexual maturity and the first *ASB4*$^{-/-}$ containing litters were born. If the *ASB4*$^{-/-}$ genotype is not embryonic lethal, then litters from *ASB4*$^{+/-}$ intercrosses should show the normal Mendelian ratios of inheritance: 25% *ASB4*$^{+/+}$, 50% *ASB4*$^{+/-}$, 25% *ASB4*$^{-/-}$. So far, out of 44 pups from *ASB4*$^{+/-}$ intercrosses, 7 out of 44 have been *ASB4*$^{-/-}$ (16%), 19 out of 44 have been *ASB4*$^{+/-}$ (43%), and 18 out of 44 have been *ASB4*$^{+/+}$ (41%) (Fig. 3-3C). While more litters will be needed to increase confidence in these ratios, these numbers indicate that some subset of *ASB4*$^{-/-}$ mice may die before genotyping (~14 days after birth) indicating that lack of ASB4 may result in an embryonic lethal phenotype with incomplete penetrance.

To verify that the *ASB4*$^{-/-}$ mice were in fact "true" knockouts (i.e. did not exhibit transcriptional read-through and translation of truncated ASB4 protein), we tested for ASB4 protein expression from testis lysates in one pair of *ASB4*$^{-/-}$ and *ASB4*$^{+/+}$ male mice by western blot (Fig. 3-4). The antibody used was raised against an ASB4 peptide in exon 3, and should recognize a truncated protein (if present) in the *ASB4*$^{-/-}$ mice. This immunoblot shows that *ASB4*$^{+/+}$, but not *ASB4*$^{-/-}$ mice show a single immunoreactive band in the appropriate molecular weight range for endogenous ASB4 (~48 kDa). No bands were identified of lower

molecular weight in the *ASB4*$^{-/-}$ lysates that might represent truncated protein products. These results indicate that *ASB4*$^{-/-}$ mice are indeed true "knockout" mice.

D. DISCUSSION

We describe here the successful production of mice with both an *ASB4* conditional floxed allele, and an *ASB4* null allele (*ASB4* knockout mice). These mice will be an important tool for investigating the biological role of *ASB4* in both embryonic development and adulthood.

Given the high levels of ASB4 expression in the developing vasculature, and the functional role of ASB4 in endothelial differentiation, we predicted that mice lacking ASB4 would die *in utero* due to vascular defects. Thus, the presence of any *ASB4*$^{-/-}$ mice from *ASB4*$^{+/-}$ intercrosses was surprising. However, the percentage of *ASB4*$^{-/-}$ mice in these litters is below the normal Mendelian ratio of 25%, suggesting that a subset of mice lacking ASB4 is dying prior to birth. Given this incompletely penetrant phenotype, it will be important to backcross this line onto an inbred C57BL/6 strain and re-evaluate genotype percentages. Furthermore, timed pregnancies will be required to identify the gestational timing and causes of lethality in *ASB4*$^{-/-}$ mice. Given the high level of vascular expression of ASB4 around E9.5 of development, I predict that a subset of *ASB4*$^{-/-}$ mice die around E10 due to vascular malformations and a subset of *ASB4*$^{-/-}$ mice survive due to so-far undefined compensatory mechanisms.

If significant numbers of *ASB4*$^{-/-}$ survive until birth, vascular phenotypes can be evaluated in newborns and adults. A few questions can be addressed: Do *ASB4*$^{-/-}$ mice vascularize the retina in response to hyperoxic/hypoxic challenge? Do *ASB4*$^{-/-}$ mice

vascularize tumors comparable to wild-type mice? Do *ASB4*$^{-/-}$ mice revascularize ischemic tissue similar to wild-type mice? All of these experiments will be particularly interesting given the involvement of hypoxia in retinal and tumor angiogenesis, combined with ASB4's role in modulating the cellular oxygen response.

ASB4 is also highly expressed in the heart, testis, and ovaries of adult mice. Therefore, if *ASB4*$^{-/-}$ mice survive until adulthood, phenotypic characterization should include structural and functional analysis of these organs. Do *ASB4*$^{-/-}$ mice have larger or smaller heart walls/defective valves/septal defects/etc.? Do *ASB4*$^{-/-}$ mice have a normal cardiac hypertrophic response? Do *ASB4*$^{-/-}$ mice have fertility phenotypes? These analyses will need to be accompanied by further analysis of the cellular expression pattern of ASB4 in these tissues (either by in situ hybridization or immunhistochemistry), but will be crucial to uncover novel biological roles of ASB4.

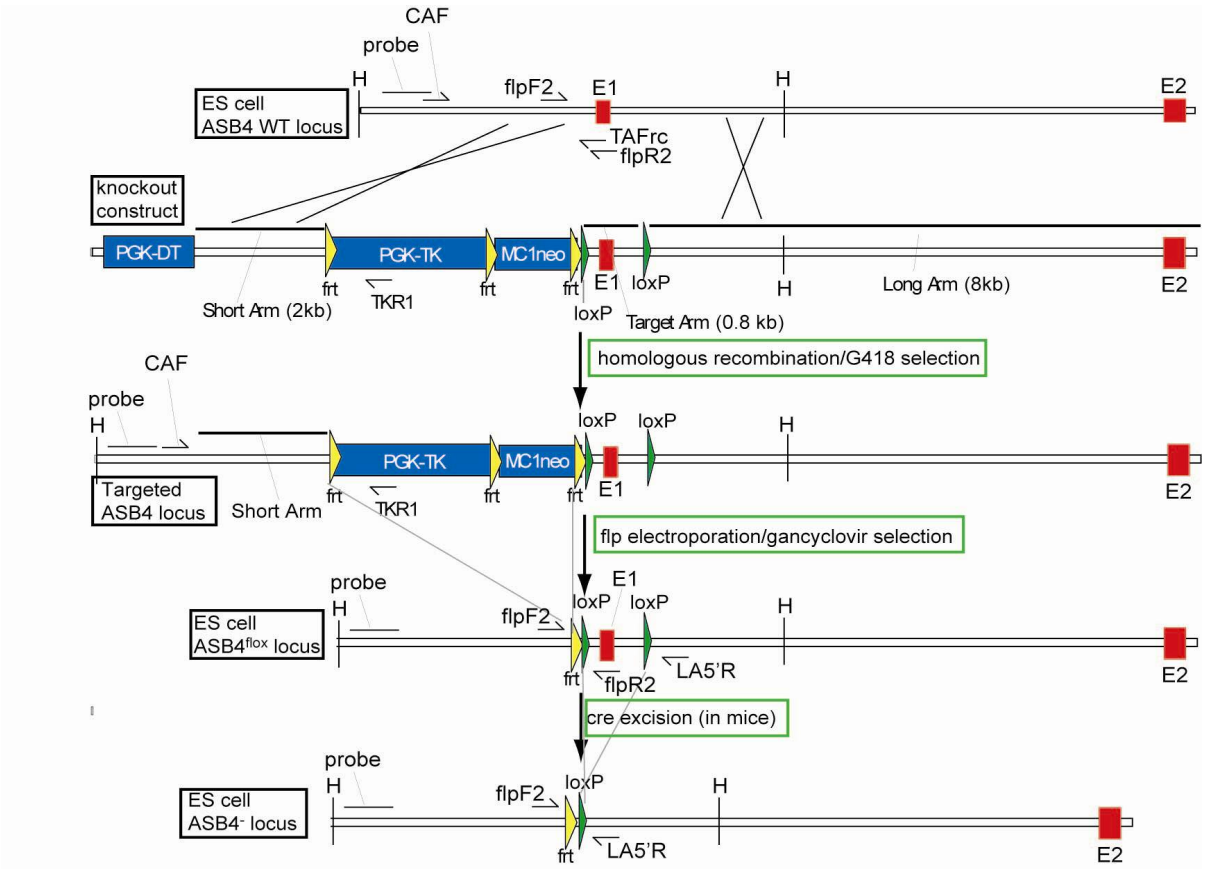

Figure 3-1 ASB4 knockout schematic
The ASB4 knockout construct was PCR-cloned from R1 mouse ES cell genomic DNA and contains a 2 kb short arm of homology, a 0.8 kb target arm that includes exon 1 flanked by loxP sites ("floxed") and thus susceptible to cre recombinase-mediated excision, and a 8 kb long arm of homology. The construct also includes a diphtheria toxin gene outside the homology arms to negatively select for random-insertion events, and an internal neomycin resistence gene for positive selection. ES cells were electroporated with linearized knockout construct and cultured in G418 (neomycin analog) for 14 days. G418 resistant clones were expanded and homologous recombination at the ASB4 locus was confirmed by PCR with CAF/TKR1 primers, and HindIII digested Southern blot using the denoted probe. Positive clones were then electroporated with flp recombinase expressing vector to excise the frt-flanked neomycin resistance cassette. Thymidine kinase in the original knockout construct kills cells in the presence of gancyclovir, and so provided negative selection for flp activity. Cells were grown in gancyclovir for 14 days, and surviving clones were expanded. "Flipped" clones were confirmed by PCR with flpF2/flpR2 primers. This locus is referred to as $ASB4^{flox}$. Cells containing this locus were used to generate $ASB4^{flox/+}$ mice, which were then crossed with cre-expressing mice to generate mice lacking exon 1 ($ASB4^{+/-}$ mice). Cre-excision was confirmed by PCR with flpF2/LA5'R primers (combined with flpF2/TAFrc primers as a PCR positive control. E1: Exon 1 (red squares), Frt: Flp recombinase recognitions sites (yellow triangles), loxP: cre recombinase recognitions sites (green triangles), H: HindIII restriction enzyme sites, probe: external southern probe binding site, PGK-TK: PGK promoter-driven thymidine kinase gene (blue squares), MC1neo: MC1 promoter-driven neomycin resistance gene (blue squares), PGK-DT: PGK promoter-driven diphtheria toxin gene (blue squares). PCR primer names:CAF, flpF2, flpR2, TAFrc, TKR1, LA5'R.

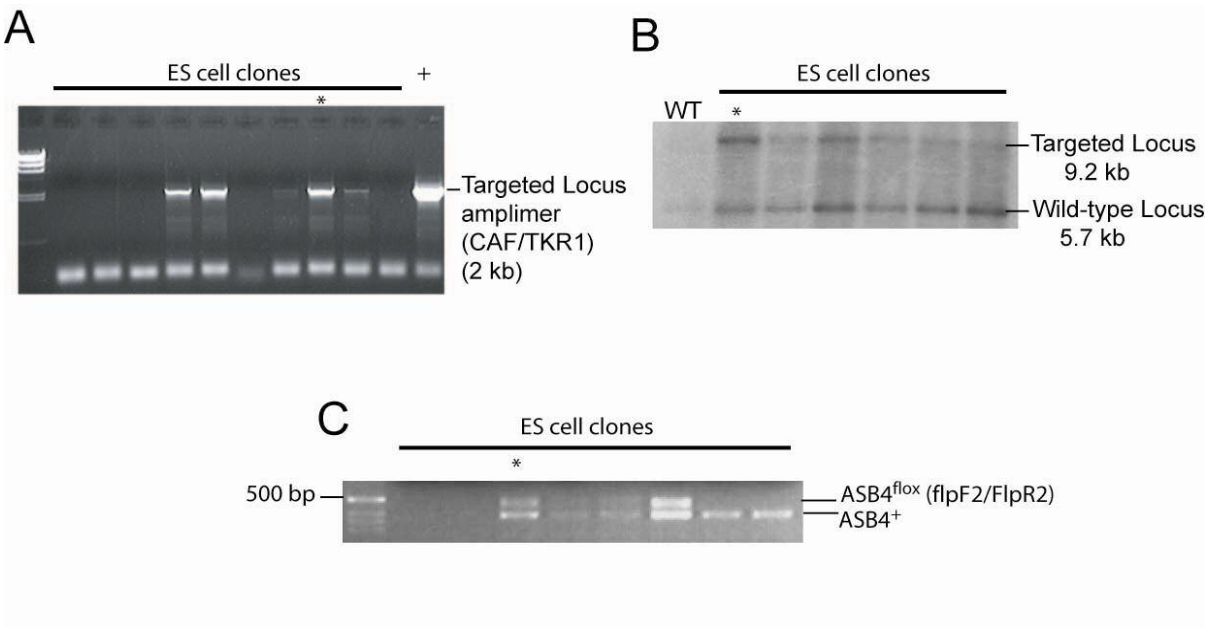

Figure 3-2 Confirmation of ASB4 locus manipulations in ES cells.
ES cells were electroporated with linearized ASB4 knockout construct (see fig. 3-1) and selected with G418 for 14 days.

(A) Lysates were used as a template for PCR using the primers CAF/TKR1 (see Fig. 3-1) to identify positive clones for a targeted ASB4 locus. (+) denotes PCR positive control. (*) denotes clone that was grown for downstream manipulations.

(B) Southern Blot using HindIII digested ES cell genomic DNA, and an external probe (as shown in Fig. 3-1) was performed on PCR positive clones. Wild-type locus showed a band of 5.7 kb, while the targeted locus showed a band of 9.2 kb (due to addition of neo/TK cassette).

(C) Targeted ES cells were treated with flp recombinase to excise the neo/TK cassette and grown in gancyclovir for 14 days to select against non-excised clones. Lysates were prepared and used as a template for PCR using flpF2/flpR2 primer pair (see Fig. 3-1). Amplification from the wild-type allele results in a 390 bp band while amplification from the "floxed" allele results in a 504 bp band. (*) denotes clone used for blastocyst injections.

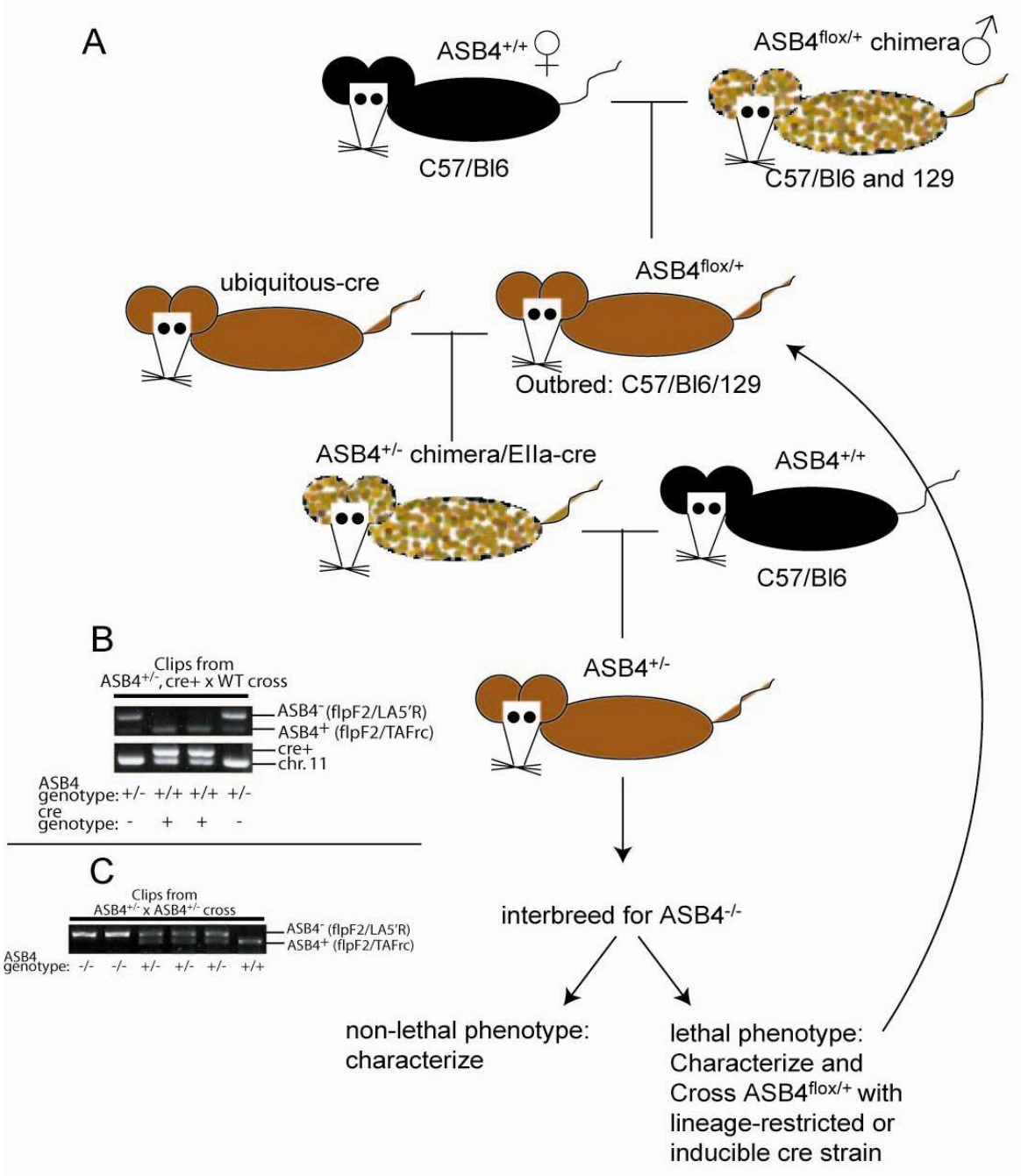

Figure 3-3 ASB4 knockout breeding diagram
Chimeric mice containing both 129-derived *ASB4*$^{flox/+}$ cells and C57BL/6-derived *ASB4*$^{+/+}$ cells were crossed with C57BL/6 *ASB4*$^{+/+}$ mice. Agouti pups contained 129-derived DNA, and half of these inherited the *ASB4*^{flox} allele. *ASB4*$^{flox/+}$ pups were confirmed by PCR with flpF2/flpR2 primers (see Fig. 3-1). *ASB4*$^{flox/+}$ mice were then crossed with transgenic mice expressing cre-recombinase in all tissues (ubiquitous *cre* mice) to produce *ASB4*$^{+/-}$ cre+ pups (confirmed by PCR with flpF2/TAFrc/LA5'R primers (see Fig. 3-1)). In order to cross out cre-recombinase, and to pass the *ASB4*$^{-}$ allele through the germline, these mice were crossed with *ASB4*$^{+/+}$ mice to result in true *ASB4*$^{+/-}$ heterozygotes (confirmed by PCR in inset B with primer pairs in parentheses (see Fig. 3-1)). These mice were interbred to generate *ASB4*$^{-/-}$ containing litters (genotyping shown in inset C (see Fig. 3-1)). If ASB4$^{-/-}$ mice are not viable, *ASB4*$^{flox/+}$ mice will be crossed with conditional-promoter driven *cre* mice to further evaluate timing and tissue-specific ramifications of *ASB4*$^{-/-}$ phenotype.

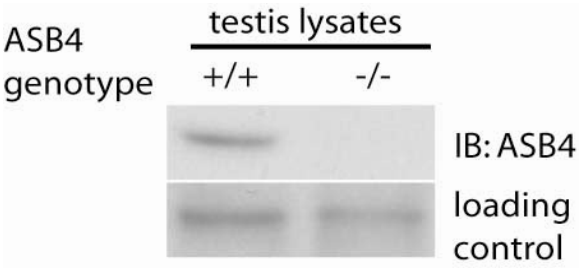

Figure 3-4 Immunoblot confirmation of ASB4 -/- genotype.
Protein lysates from whole testis from 2 month old male littermates were used to evaluate whether ASB4 protein is absent from ASB4-/- mice. A non-specific band is shown as a loading control.

Chapter 4 GENERAL DISCUSSION

A. ASB4 MOLECULAR FUNCTION

The experiments described herein attempt to fully characterize the role of ASB4 during vascular development by uncovering its expression, molecular function, biologic consequences, and molecular regulation. Data generated here reveal that 1) ASB4 expression is largely restricted to the vasculature during a narrow time window during development, 2) ASB4 binds to ubiquitination machinery and is associated with ubiquitination activity, 3) ASB4 promotes the differentiation of the vascular lineage, and 4) ASB4 is a hydroxylation substrate of FIH and is thus regulated by oxygen concentration (Chapter 2).

Like all E3 ubiquitin ligase proteins, the functional ramifications of ASB4 are dependent upon the function of the substrate protein(s) which it degrades (Fig. 4-1). Multiple attempts to identify binding partners of ASB4 have so far not yielded the identification of ASB4 ubiquitination substrate proteins. Yeast 2-hybrid screens were performed using libraries generated from E11.5 mouse embryo and adult human heart mRNA and over 12 million independent clones were screened overall. (The yeast 2-hybrid approach was used successfully in the identification of APS as an ASB6 substrate).[4] Using the embryo library a specific interaction with a protein called SET was identified, but this interaction could not be confirmed by co-IP in mammalian cells despite multiple attempts and approaches (data not shown). The screen using adult human heart library yielded the potential interacting protein FIH (one clone!) which was subsequently confirmed and functionally characterized in

mammalian cells (Chapter 2). This indicates that the yeast 2-hybrid approach can be used to identify binding partners of ASB4, but the fact that FIH was only represented by a single clone suggests that the sensitivity of this approach is low.

As an alternative approach, mammalian cells were transfected with flag-ASB4, flag-immunoprecipitated, and analyzed by coomassie staining of SDS-PAGE gels to identify co-purifying protein bands. (This approach was used successfully by other groups to identify ASB-9 binding partners).[3] Positive bands were then cut out and submitted for sequencing by mass spectrometry. Furthermore, ASB4ΔSOCS was used in these experiments to theoretically stabilize substrate proteins by a dominant negative mechanism. This experimental approach was performed using a variety of cell types that do and don't express ASB4 including HEK-293, COS7, HUVEC, EPCs, and rat neonatal cardiomyocytes. Specific bands were co-purified, but after sequencing they were eliminated as potential ASB4 substrates due to 1) known function of the proteins that did not match the known properties of ASB4, or 2) co-purification with other non-related flag-tagged proteins in other co-IP screens. Many of these proteins were chaperones or other proteins of high abundance and promiscuous binding potential (tubulins, myosins, dnaJ chaperones). Notably, despite many rounds of optimization, true interactors such as Elongin-B, Elongin-C, and FIH have not been visualized by coomassie stain, indicating that the sensitivity of the system for identifying binding partners under current conditions might be too low.

Major caveats exist with both of these approaches. First, yeast-2 hybrid libraries and cell lines used for overexpression must express substrate for these approaches to be fruitful. However, it is impossible to predict where substrate is expressed at high levels. As a first best guess, libraries and cell lines that harbor ASB4 expression were utilized since ASB4

substrate is most likely to be expressed where ASB4 is expressed. Second, low-abundance proteins are difficult to detect using these methods. If ASB4 substrate is expressed at low levels (either at transcript or protein level), then sensitivity using these approaches may not be high enough to detect an interaction.

The yeast 2-hybrid libraries that were used were generated commercially by an oligo-dT priming method. The advantage of this approach is the ability to directionally clone into prey vectors, but this approach by definition includes entire 3' untranslated regions (UTR) in prey constructs that don't encode proteins. Since the average cDNA size in the libraries was 1.9 kb (range: 0.4 - 3.8 kb) and the average 3' UTR is ~1 kb (depending on tissue source), almost half of the information in the cDNAs isn't translated into protein. In fact, the majority of positive clones in the screens turned out to be composed completely of 3' UTR sequence. Therefore, nearly all of the cDNAs in this library contain short stretches of C-terminal protein-encoding sequence followed by long 3' UTRs. Long stretches of protein-encoding regions are needed to preserve tertiary structure of potential interacting proteins. Interestingly, the FIH clone that was identified was quite long (>3kb) and encoded nearly the entire FIH protein. Because FIH folds in a jelly-roll manner, it can't be broken up into domains that retain secondary structure and thus requires the entirety of the coding sequence for proper folding and binding potential. This most likely explains why only 1 clone of FIH was identified out of 12 million screened clones despite relatively high and ubiquitous levels of FIH mRNA expression. This might also explain why I have not been able to identify co-purifying substrate proteins using this approach. As an alternative option, libraries generated using random primers can provide greater representation of N-terminal coding sequences,

although the ability to directionally clone would be lost and therefore half of all clones would be inserted in the antisense direction.

Further optimization using the mass-spec approach and more sophisticated biochemical manipulations (such as proteasome inhibition and anti-ubiquitination purification) are now underway in a last-ditch effort to identify ASB4 substrate. Several technical options could increase the sensitivity of this approach. 3x-flag tagged ASB4 could be used to increase the capture of bait protein. Silver stain could be used to increase the sensitivity of in-gel detection, although this approach also presents technical difficulties for downstream mass spectrometric analysis. Finally, lysates from difficult-to-transfect cell lines (HUVEC) could be flowed-over purified bead-bound protein produced from easily transfectable cells (HEK-293).

Identification of ubiquitin E3 ligase substrate proteins is a challenging endeavor since such interactions are often transient and result in destabilization of substrate protein. However, identification of substrate proteins for ASB4 will be an essential part of elucidating the biologic role of ASB4 during vascular development.

B. ASB4 AS A COMPONENT OF THE OXYGEN SENSING SYSTEM

The recent identification of the hydroxylase enzymes FIH and PHDs, whose activity is directly dependent on oxygen concentrations, provides a mechanism by which mammalian cells "sense" oxygen levels and transduce this environmental cue into an appropriate biologic response. However, until recently, the only known substrates of these hydroxylases, and indeed the only known intracellular hydroxylated proteins, were HIF transcription factors (Fig. 4-2). Thus, it was presumed that the hypoxic response was largely dependent on HIF-

mediated transcriptional upregulation of hypoxia response genes.

The identification of ASB4 as a non-HIF FIH substrate implies that the cellular response to changing oxygen levels is more mechanistically complex than initially thought, and involves both transcriptional and post-translational responses. Furthermore, the cell-specific expression of ASB4 suggests that the hypoxic response may differ among tissues, and this expression may provide insight into the endothelial-specific response to changing oxygen tensions during development.

How exactly hydroxylation of ASB4 affects its function at the molecular level is, at this point, not clear. Given that we have not observed any change in ASB4 expression, ubiquitination, or subcellular localization in response to hypoxia, we hypothesize that, similar to VHL, ASB4 binds to its substrate in a manner determined by oxygen concentration, and in this case, degrades substrate when hydroxylated under high oxygen conditions. Identification of substrate protein(s) (described above) will be necessary to test this hypothesis.

More generally, the identification of the leucine-flanked EVNA motif in the context of ankyrin repeats as a consensus hydroxylation site opens the door for the identification of many more FIH hydroxylation substrates. Ankyrin repeats are among the most common structural protein domains in all of nature and are present in a great diversity of proteins including secreted growth factors, receptors, transcription factors, scaffolding proteins, enzymes, etc. Furthermore, the leucine flanked EVNA motif is not rare among ankyrin repeats. For example, of 18 ASB family members, 14 contain motifs conserved enough to warrant further investigation (ASB1, ASB2, ASB3, ASB4, ASB5, ASB6, ASB8, ASB9, ASB11, ASB13, ASB14, ASB15, ASB16, ASB17), raising the possibility that hydroxylation of EVNA motifs specifically, and protein hydroxylation more generally, might be quite

common. In addition, Notch1, a protein shown to be involved in the hypoxic response, contains an EVNA motif within one of the ankyrin repeats that provide binding sites for transcriptional complex binding partners, perhaps providing a mechanism for the oxygen dependent activity of Notch1.[104-106] Investigating the tissue expression and functional ramifications of these hydroxylation events will be crucial to our understanding of the cellular oxygen response.

C. ASB4 BIOLOGIC FUNCTION

ASB4 is expressed in the developing endothelium during a time in which the vasculature is exposed to dynamic changes in environmental cues and undergoes drastic morphologic changes. For example, blood flow is initiated which exerts shear and mechanical stresses on the vasculature, and maternal-fetal blood-gas exchange in the placenta is initiated which drastically changes embryonic oxygen tension from hypoxia to relative normoxia. Furthermore, the vasculature undergoes extensive morphologic changes during this time including arterial-venous specification, capillary plexus remodeling, and vessel maturation. Links between these environmental cues and morphologic changes are just beginning to be identified, and ASB4 is spatio-temporally expressed and regulated in such a way as to represent a potential direct link between oxygen cues and vascular morphologic responses.

The data presented in Chapter 2 suggest that ASB4 promotes differentiation into the vascular lineage, but do not rule out the possibility that ASB4 may determine arterial-venous fate decisions in the developing vasculature. For example, ES cells overexpressing ASB4 show increased levels some vascular markers (Tie2 and VE-cadherin) but not others

(PECAM) following differentiation, suggesting that Tie2 and VE-cadherin may be markers for some subset of endothelium. Indeed, Tie2 has been shown to be highly restricted to venous endothelium in avians, although this pattern has not been described in mammals.[107, 108] In this light, it is intriguing to speculate that ASB4 may function to transduce an oxygen-signal to only well-perfused endothelium in order to specify an arterial fate to this subset of vessels. Alternatively, given that hypoxia is a potent activator of angiogenesis during adulthood, and that the endothelium reacts in a unique way to hypoxia (see Chapter 1), perhaps ASB4 functions to prevent disorganized vascular development in the global hypoxic environment of the early embryo. Or, perhaps ASB4 hydroxylation serves as an endothelial-specific signal to initiate a transition from an early-developmental phenotype, to a mature phenotype as blood oxygenation is initiated. The embryoid body system described in Chapter 2 could be used to evaluate further the effects of ASB4 on arterial-venous specification. *ASB4*$^{-/-}$ ES cells could be generated and used in these differentiation experiments (compared to *ASB4*$^{+/-}$ or WT cells) to determine if ASB4 serves as a genetic cue for arterial-venous specification. In these experiments, cells with or without ASB4 could be differentiated for four days with or without hypoxia, and sorted for flk1+ cells. These flk1+ cells could then be replated and further differentiated (with or without hypoxia) to analyze the relative expression patterns of arterial/venous markers (both by global and cell-specific experimental approaches). Furthermore, careful characterization of the vascular phenotype of *ASB4*$^{-/-}$ mice discussed in Chapter 3 should help elucidate the biologic role of ASB4 in the developing vasculature.

This dissertation so far has focused on the endothelial-specific functions of ASB4 both in the embryonic and placental vasculatures. However, ASB4 is expressed in other cell

types in the embryo and the adult that may contribute further evidence that ASB4 functions as an oxygen sensor for modulating differentiation. ASB4 expression in the testis is high in the spermatogenic lineage, and increases nearly two fold as type A spermatogonia differentiate into round spermatids (http://mrg.genetics.washington.edu/)[109]. It has been suggested that an oxygen gradient exists across the seminiferous tubule axis and serves as a signal to induce differentiation of sperm cells as they near the tubule lumen. Cells of the spermatogenic lineage are known to utilize highly anaerobic and glycolytic metabolism via the expression of a set of testes-specific glycolytic gene variants. Recently, a testes-specific HIF1α isoform, termed hHIF1αTe was identified and shown to function as a dominant negative to wild-type HIF1α.[110] Expression of this variant along with other genes shown to inhibit HIF activity,[112, 113] could downregulate HIF1α activity and the concomitant expression of the wild-type variants of glycolytic enzymes in order to allow the expression of testes-specific variants. Cells of the spermatogenic lineage clearly have a unique hypoxic response. Perhaps ASB4 functions to transduce the oxygen gradient signal into a differentiation signal to coordinate proper spermatogenic differentiation within the axis of the seminiferous tubule.

While it remains a distinct possibility that ASB4 could ubiquitinate different substrate proteins in different tissues based on substrate tissue expression patterns, it is intriguing to speculate that the single biologic activity of ASB4 could be to promote oxygen-dependent differentiation of multiple cell types in multiple tissues due to the degradation of a single unifying anti-differentiation substrate protein. Further analysis of the *ASB4*$^{-/-}$ phenotypes combined with identification of substrate proteins will be critical in the further elucidation of ASB4 function.

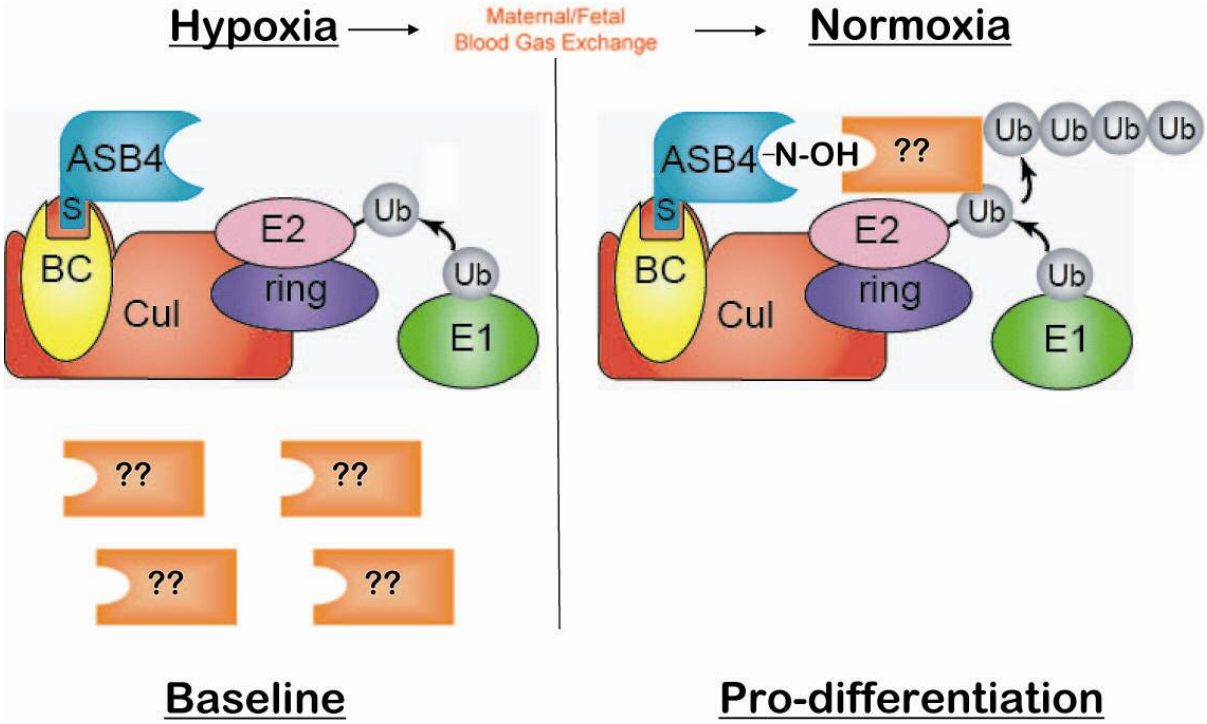

Figure 4-1 Model of ASB4 function.
ASB4 degradation of unknown substrate (denoted "??") occurs only when ASB4 is hydroxylated in a high oxygen environment. Based on ES cell differentiation studies, we hypothesize that this substrate must have anti-differentiation function. So, overexpression of ASB4 under normoxic conditions results in a pro-differentiation phenotype.

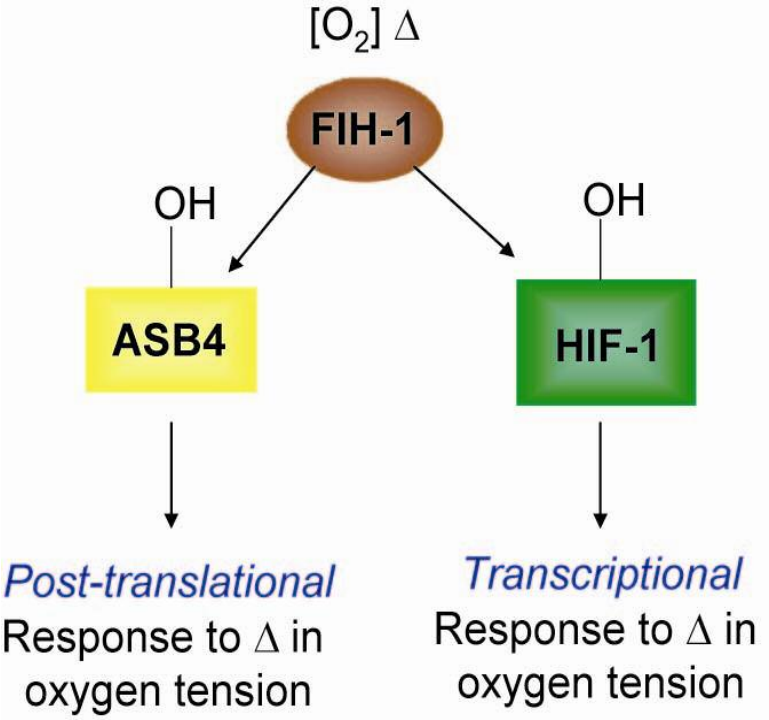

Figure 4-2 ASB4 represents a novel post-translational cellular oxygen response.

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